PHB1 Regulates mtDNA Release and Subsequent Inflammatory Responses

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Appendix

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Appendix Figure Legends

Appendix Figure S1. (A) Statistics analysis for the gene type of offspring from the intercross of *Phb1*$^{+/-}$ mice.

(B) *Phb1*$^{F/F}$ mice were generated by the knock-in of loxp fragments on both sides of *Phb1*. *Phb1*$^{F/F}$ mice crossed with *LysM-Cre* mice that Cre recombinase specifically express in marrow tissue to generate *Phb1*^{MyeKO} mice. *Phb1*^{MyeKO} mice lack *Phb1* in mature myeloid cells.

(C) After BMDMs were extracted from *Phb1*$^{F/F}$ mice and *Phb1*^{MyeKO} mice and incubated with 20% supernatant of L929 cells for 7 days for differentiation, the genome was extracted from differentiated BMDMs. PCR assays and agarose electrophoresis were used to confirm the deletion of *Phb1* gene in the genome of *Phb1*^{MyeKO} mice.

(D) After BMDMs were extracted from *Phb1*$^{F/F}$ mice and *Phb1*^{MyeKO} mice and incubated with 20% supernatant of L929 cells for 7 days for differentiation, the PHB1 levels were detected by WB assay to confirm PHB1 levels.

Appendix Figure S2. (A) HeLa cells, BMDMs, and J774A.1 cells were fixed and subjected to IF analysis for the co-localization between mitochondria (MitoTracker, red) and PHB1 (anti-PHB1, green or blue). Scale bar: 10μm.

(B) HeLa with or without stable PHB1 ablation was lysed. WB assays detected the protein levels in whole cell lysates.

(C) BMDMs extracted from *Phb1*$^{F/F}$ mice and *Phb1*^{MyeKO} mice and incubated with 20% supernatant of L929 cells for 7 days for differentiation. After were stimulated with LPS

(200ng/ml) for 6 hours, ATP (4 mM) for 45 minutes and H_2O_2 (8.8 mM) for 45 minutes, differentiated BMDM were incubated with PI. Flow cytometric analysis detected the fluorescence density of PI.

Appendix Figure S3. (A) J774A.1 cells with or without PHB1 ablation were fixed and subjected to IF analysis for the co-localization between mitochondria (MitoTracker, red) and mtDNA (anti-DNA antibody, green). Representative images were shown, and data are from at least three independent experiments. White boxed regions in the panels were enlarged. White arrow indicates mtDNA dots failed to co-localized with mitochondria. Scale Bar: 10μm.

(B) J774A.1 cells with or without PHB1 ablation were lysed, and DNA in the cytosol was isolated from that in the pellet. qPCR assays detected mtDNA levels in cytosol and pellet (Mean ± SEM). ** $p < 0.01$.

(C) BMDMs isolated from *Phb1*$^{F/F}$ mice and *Phb1*^{MyeKO} mice were fixed and subjected to IF analysis for the co-localization between mitochondria (MitoTracker, red) and mtDNA (SG-ALK, green). Representative images were shown, and data are from at least three independent experiments. White boxed regions in the panels were enlarged. The white arrows indicated mtDNA dots outside mitochondria. Scale Bar: 10μm.

(D-E) The co-localization between mtDNA and mitochondria in HeLa cells with or without PHB1 ablation were incubated with CsA 2 μM for 30 minutes and detected with confocal microscope. (MitoTracker, red) and mtDNA (SG-ALK or anti-DNA antibody, green). Representative images were shown, and data are from at least three

independent experiments. White boxed regions in the panels were enlarged. The white arrows indicated mtDNA dots outside mitochondria. Scale Bar: 10μm.

(F-H) HeLa cells with or without PHB1 ablation lysed, and DNA in the cytosol was isolated from that in the pellet. qPCR assays detected mtDNA levels in cytosol and whole cell lysis (Mean ± SEM). ** $p < 0.01$.

Appendix Figure S4.

(A) HeLa cells were treated with CsA 2 μM for 30 minutes and H_2O_2 5 mM for 20 minutes. Cells were fixed and subjected to IF analysis for the co-localization between mitochondria (MitoTracker, red) and mtDNA (PicoGreen, green). Representative images were shown, and data are from at least three independent experiments. Scale Bar: 10μm.

(B-C) Confocal microscopy analyses for the fluorescence images in time-series mode. HeLa cells stably expressing 4mt-RCaMPh with or without PHB1 ablation were treated with H_2O_2 5 mM for 20 minutes. During images acquisition, cells were treated with histamine 200 μM. Traces of mitochondrial Ca^{2+} (4mt-RCaMPh) dynamics are shown on the upper (Mean ± SEM). $n \geq 3$. Scale bar: 10 μm. * $p < 0.05$ relative to Scramble group.

Appendix Figure S5.

(A) J774A.1 cells with or without *Phb1* knockdown were stimulated with poly (dA:dT) (1 μg/ml) for 24 hours, and subsequently were lysed. Proteins were detected by WB assay.

(B) J774A.1 cells with *Phb1* knockdown, *Aim2* knockdown, or combined *Phb1* and

Aim2 knockdowns were stimulated by LPS (200 ng/ml) for 6 hours and ATP (4 mM) for 45 minutes. Protein levels in whole cell lysates and in the culture medium were detected by WB assay.

(C) J774A.1 cells with or without *Phb1* knockdown were stimulated with LPS (200 ng/ml) for 6 hours and ATP (4 mM) for 45 minutes. Cells were fixed and subjected to IF analysis to detect the co-localization between AIM2 (anti-AIM2, red) and mtDNA (PicoGreen, green). White boxed regions in the panels are enlarged. The white arrows indicate mtDNA co-localized with AIM2. Scale bar: 10 μm.

(D) Quantitative analysis of data from (C) (mean ± SEM). n.s. no significance.

(E) J774A.1 cells with or without *Phb1* knockdown were stimulated with poly (dA:dT) (1 μg/ml) for 24 hours. After cells were lysed, AIM2 was immunoprecipitated by anti-AIM2 antibody. mtDNA levels (*D-loop*) from immunoprecipitants were detected by qPCR in J774A.1 cells with or without *Phb1* knockdown (mean ± SEM). n.s. no significance, ** $p < 0.01$, *** $p < 0.001$.

Appendix Figure S6. (A) J774A.1 cells were incubated with EtBr (150 ng/ml or 450 ng/ml) for 3 days. qPCR was used to detect the mtDNA level in the whole cell lysates (mean ± SEM). n.s., no significance, **** $p < 0.0001$.

(B) J774A.1 cells with or without *Phb1* knockdown were incubated with EtBr (150 ng/ml or 450 ng/ml) for 4 days, followed by IF analysis for intracellular mtDNA level. The white arrows indicate mtDNA dots. Scale bar: 10 μm.

(C) After being treated with H_2O_2 (8.8 mM) for 45 minutes and NAC (2 mM) for 8

hours, J774A.1 cells were incubated with DHE. Flow cytometric analysis detected the fluorescence density of DHE.

Appendix Figure S7. (A-B) BMDMs from *Phb1*$^{F/F}$ mice and *Phb1*^{MyeKO} mice were stimulated by H_2O_2 (500 μM) for 18 hours. RT-qPCR assays detected mRNA levels in whole cell lysis (Mean ± SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Appendix Figure S8. (A) HeLa cells overexpressing FLAG and PHB1-FLAG were lysed. The whole cell lysates were immunoprecipitated by anti-FLAG antibody. WB was used to detect the protein levels in the precipitated products.

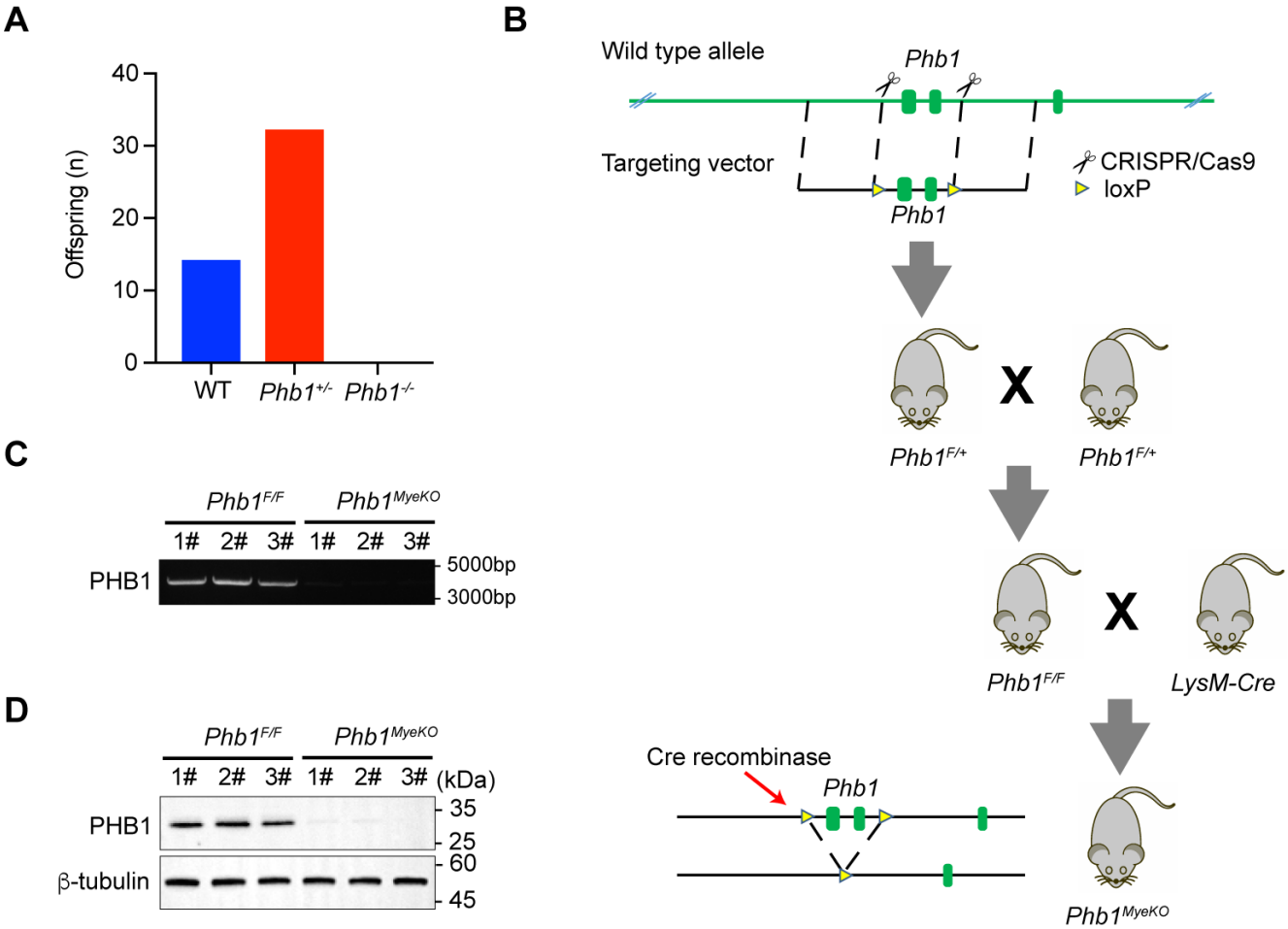
Appendix Figure S9. (A) HeLa cells were treated with CsA (2 μM) for 30 minutes and H_2O_2 (5 mM) for 20 minutes. WB assay was used to detect the protein levels in the whole cell lysates.

(B-C) Confocal microscopy analyses of mitochondrial Ca^{2+} in time-series mode. HeLa cells stably expressing 4mt-RCaMPh with or without *Phb1* knockdown or *Spg7* knockdown were monitored. During acquisition of fluorescence images (B), cells were treated with histamine (200 μM). Traces of mitochondrial Ca^{2+} (4mt-RCaMPh) dynamics are shown in (C) (mean ± SEM). $n \geq 5$. Scale bar: 10 μm.

(D-E) Confocal microscopy analyses of mitochondrial Ca^{2+} in time-series mode. HeLa cells stably expressing 4mt-RCaMPh with or without *Phb1* knockdown or *Afg3l2* knockdown were monitored. During acquisition of fluorescence images (D), cells were treated with histamine (200 μM). Traces of mitochondrial Ca^{2+} (4mt-RCaMPh) dynamics are shown in (E) (mean ± SEM). $n \geq 8$. Scale bar: 10 μm.

(F) The co-localization between mtDNA (SG-ALK, green) and mitochondria (MitoTracker, red) in HeLa cells with or without *Phb1* knockdown, *Spg7* knockdown and *Afg3l2* knockdown was detected with live-cell imaging. Images shown are representative of at least three independent experiments. White boxed regions in the panels are enlarged. The white arrows indicate mtDNA outside mitochondria. Scale bar: 10 μm.

Appendix Figure S1



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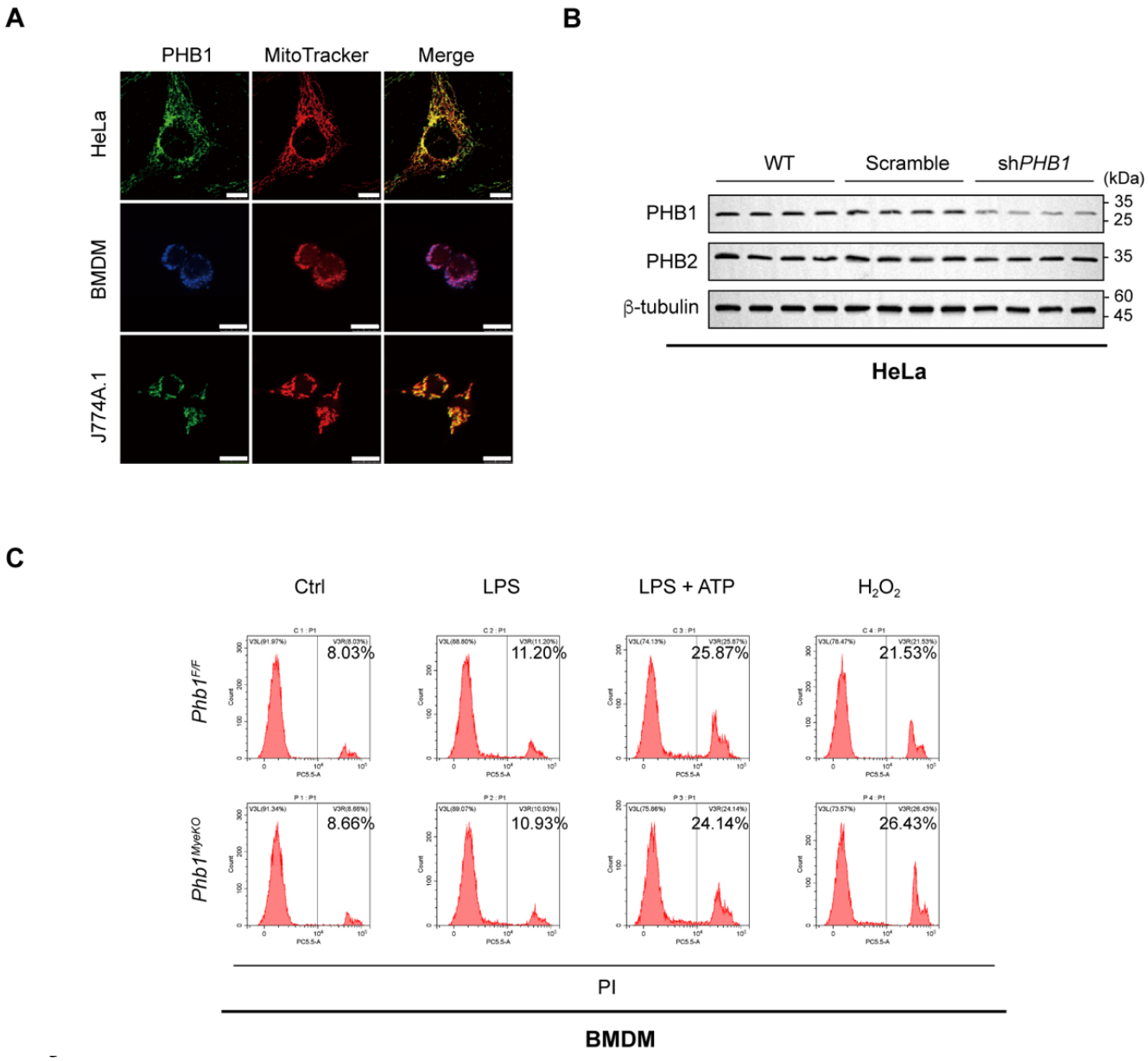

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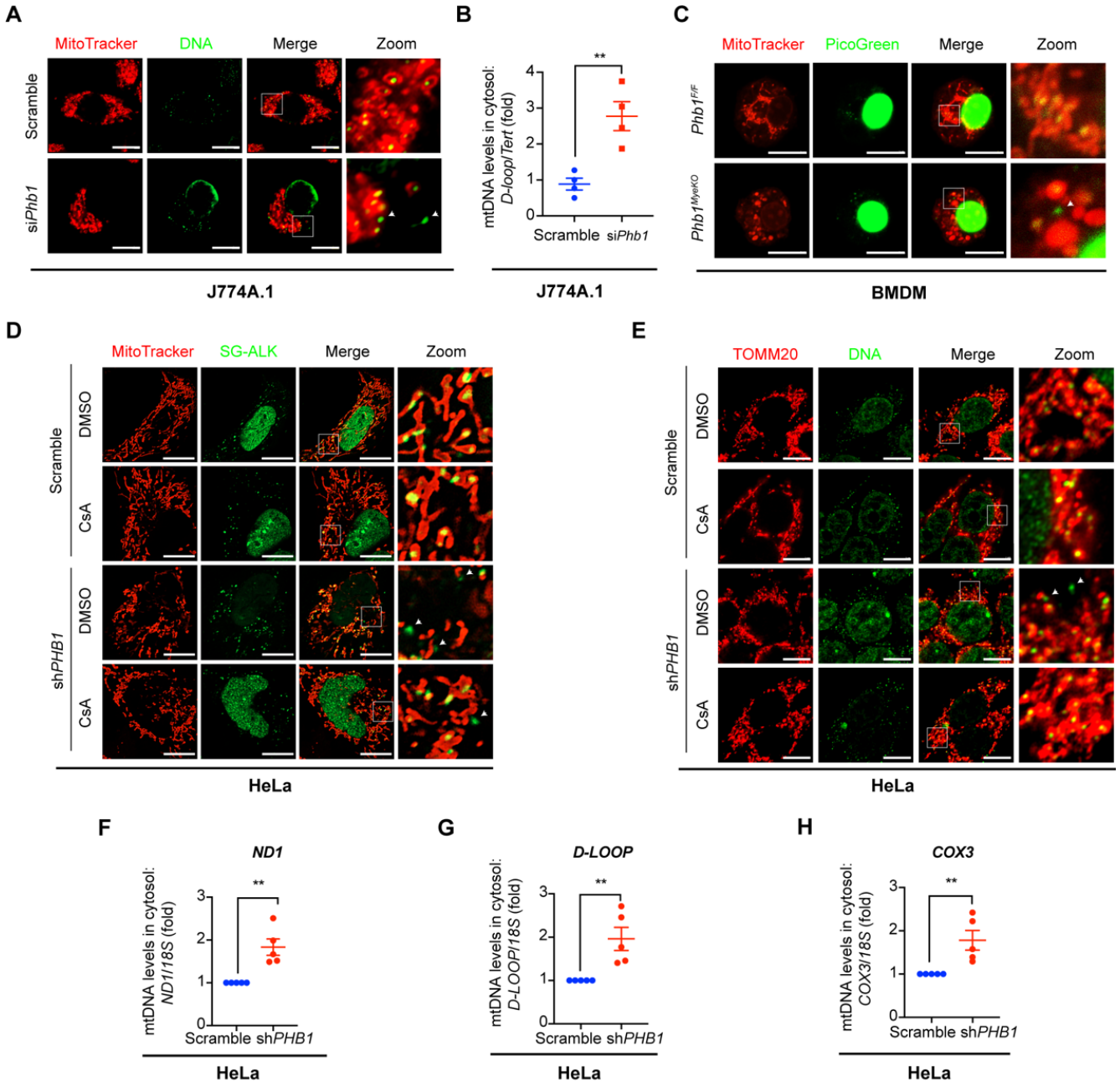

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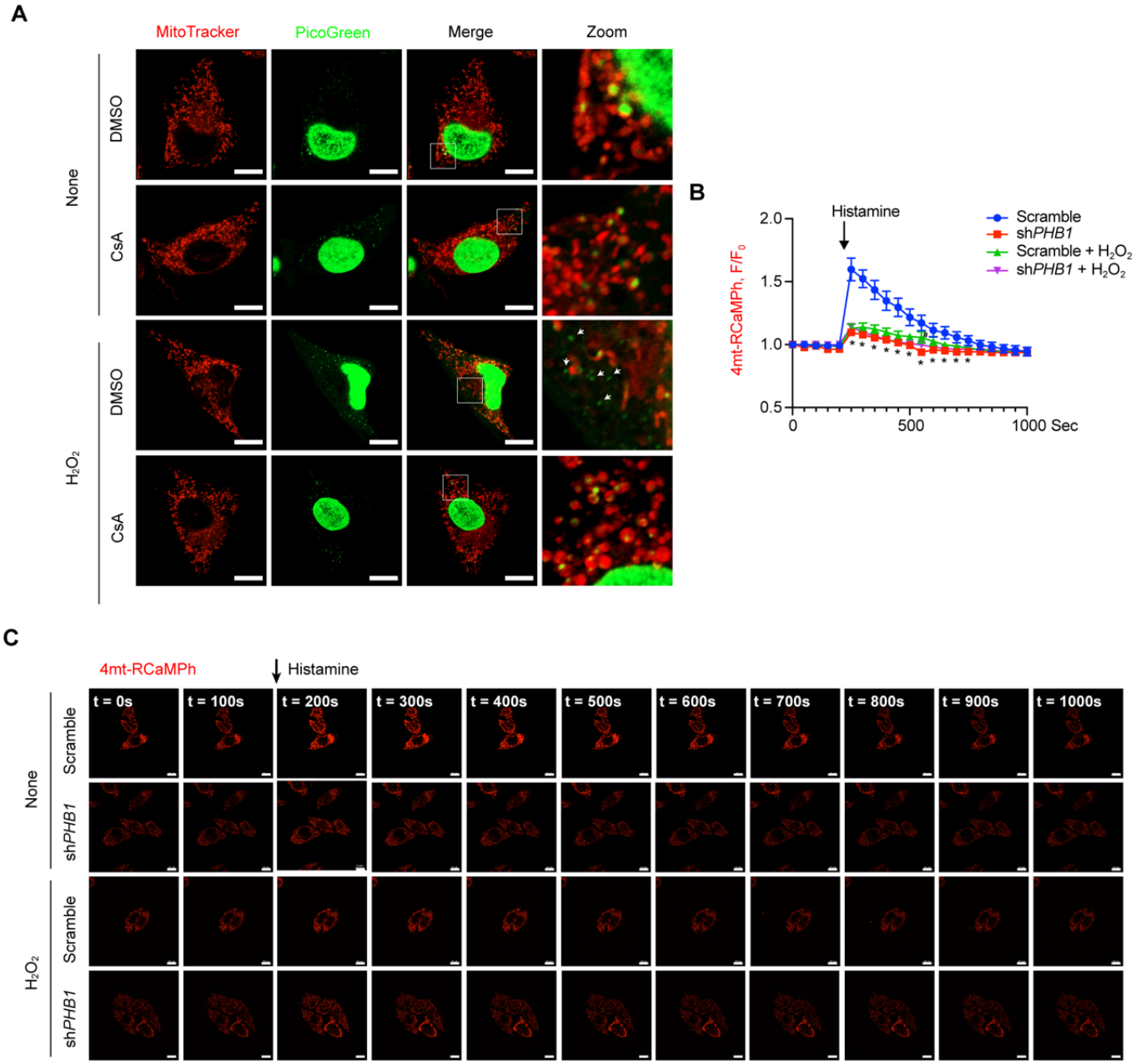

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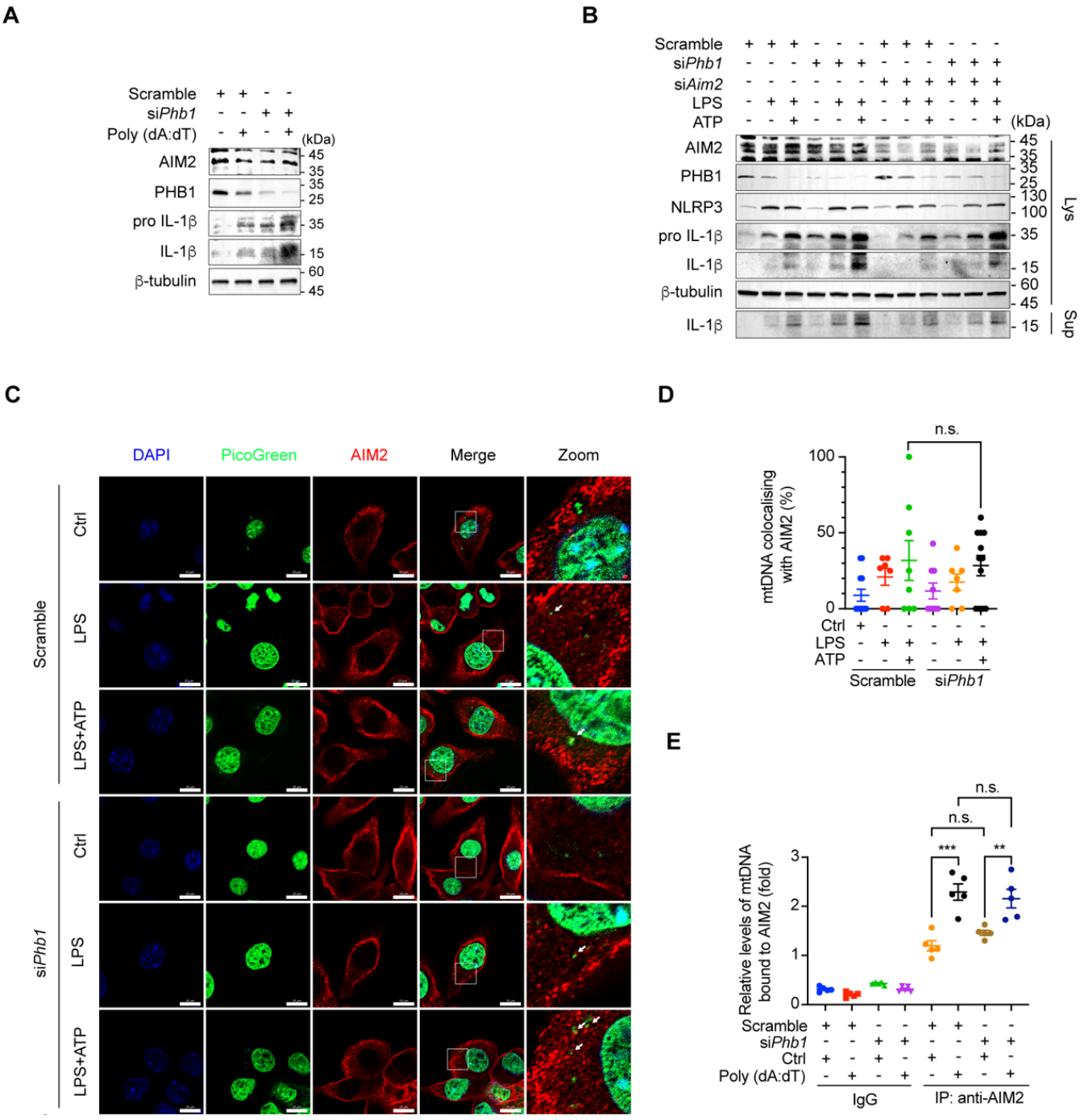

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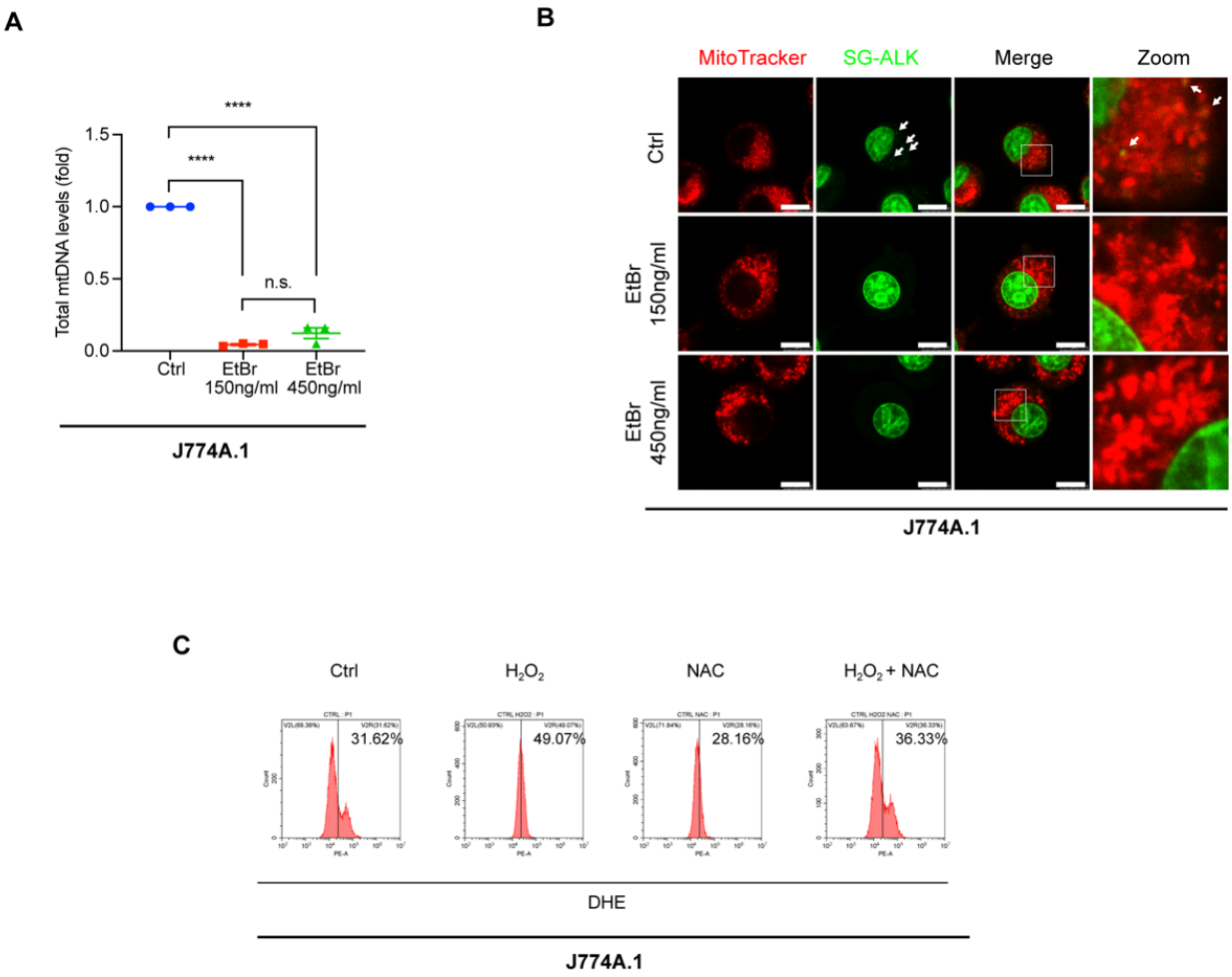

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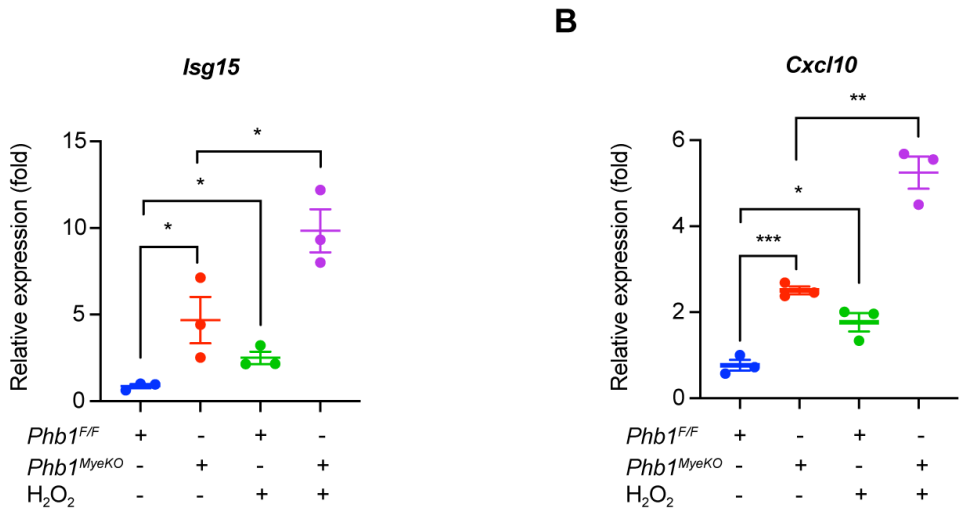

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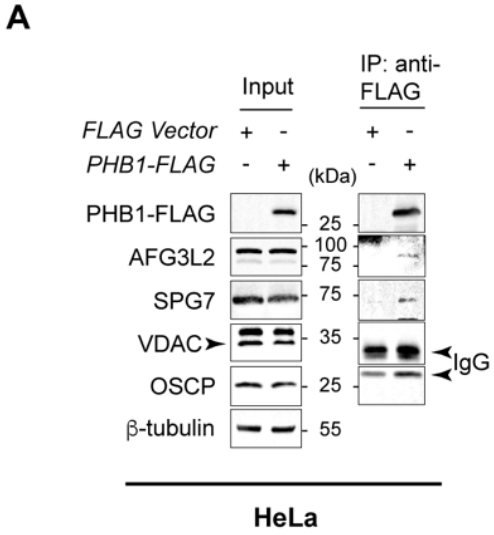
Appendix Figure S8



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Appendix Figure S9