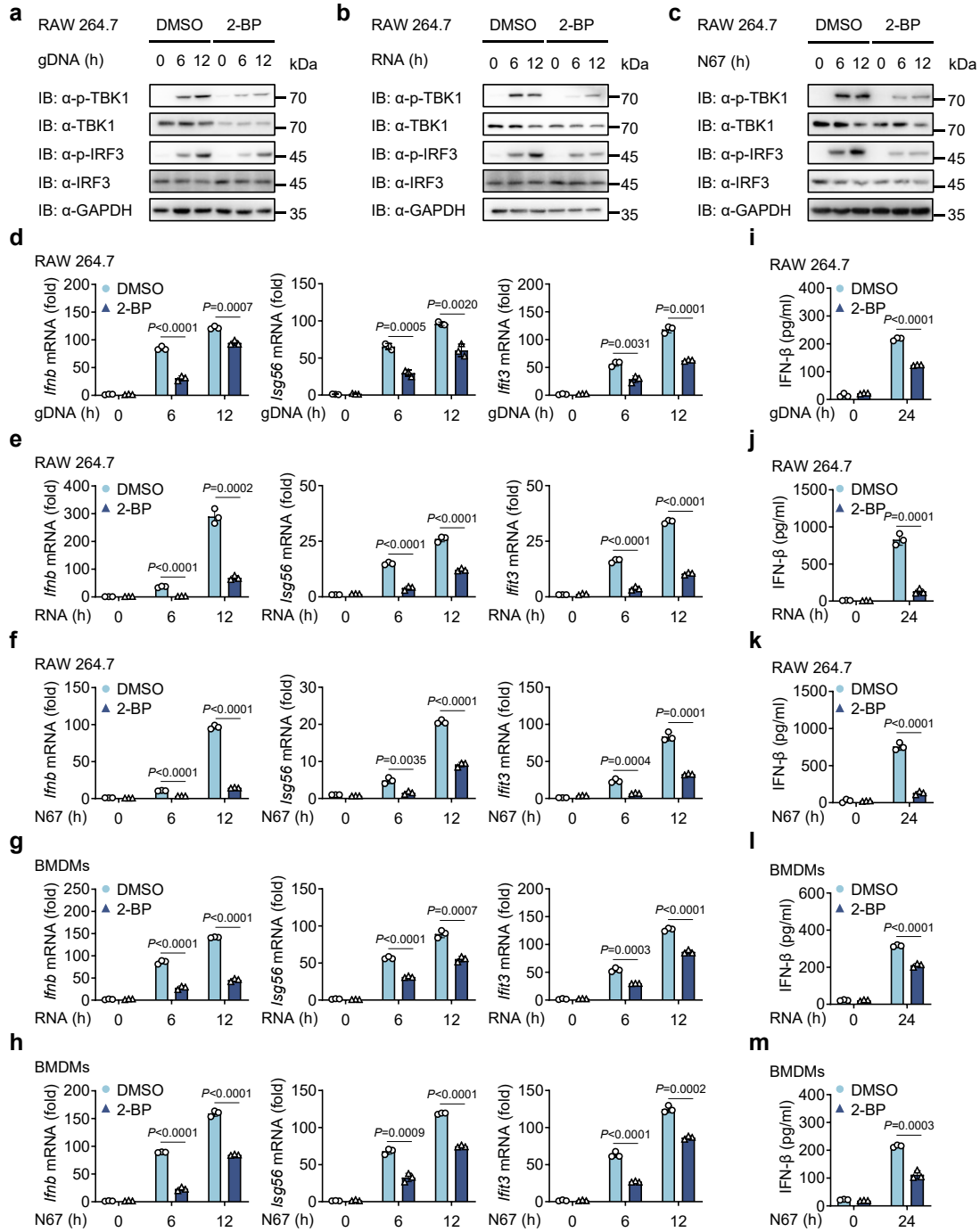


Supplementary Information

Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity in mice

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Supplementary Fig. 1 Palmitoylation enhances the IFN-I immune response during malarial infection

(a-f) RAW 264.7 cells were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with N67 gDNA, RNA or freeze-thawed iRBCs at indicated time points. Cell lysates

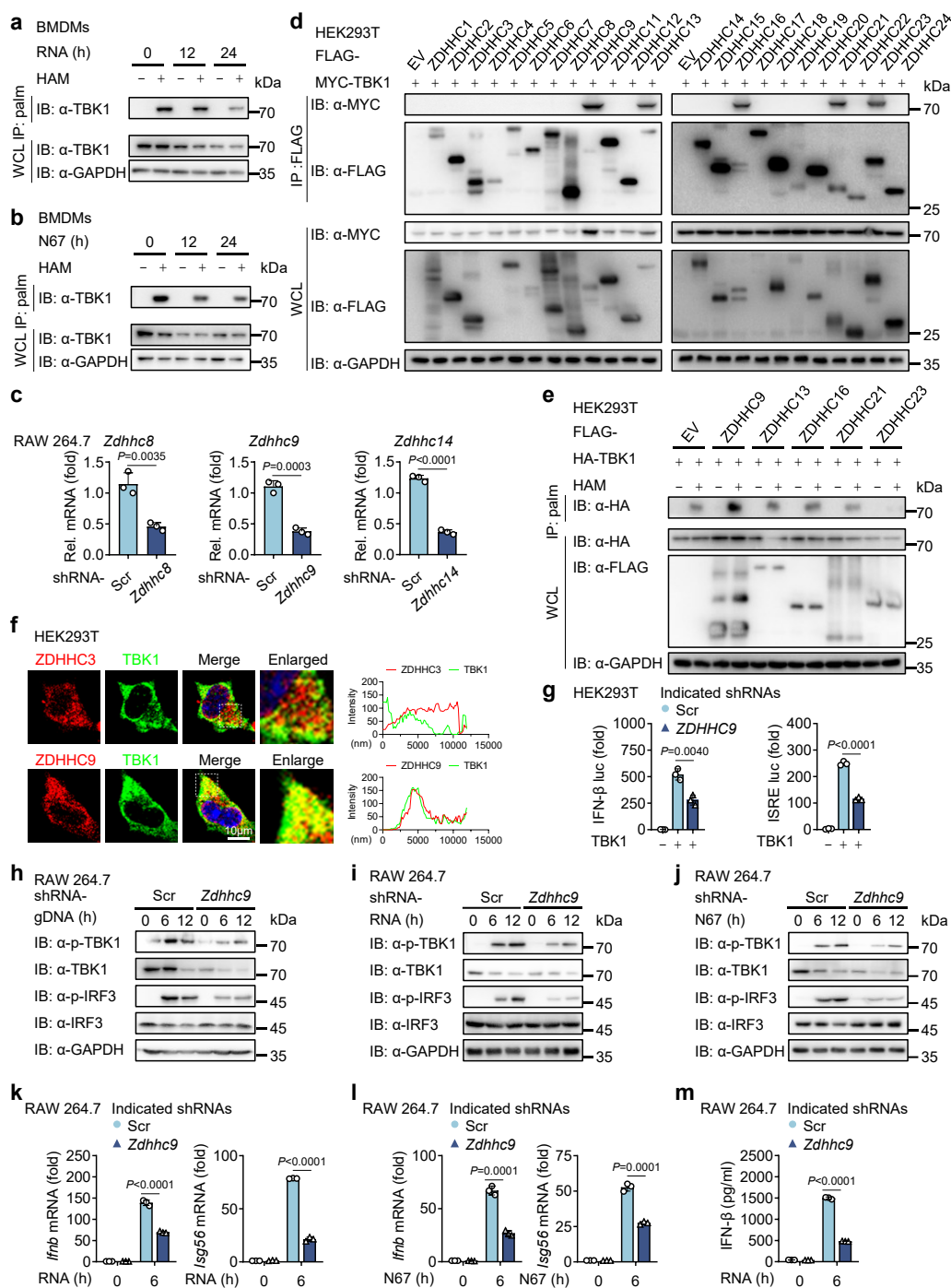
were collected for immunoblot, this was repeated $n=3$ independent times with similar results (**a-c**) and RT-qPCR analysis ($n=3$ biological replicates) (**d-f**).

(**g,h**) BMDMs were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for RT-qPCR analysis ($n=3$ biological replicates).

(**i-k**) RAW 264.7 cells were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with gDNA, RNA or freeze-thawed iRBCs at indicated time points. IFN- β release in the supernatants was determined by ELISA ($n=3$ biological replicates).

(**l,m**) BMDMs were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with RNA or freeze-thawed iRBCs at indicated time points. IFN- β release in the supernatants was determined by ELISA ($n=3$ biological replicates).

Data are presented as the mean $\pm$ SD. *P* values were determined by unpaired two tailed Student's *t*-tests (**d-m**). Source data are provided as a Source Data file.



Supplementary Fig. 2 ZDHHC9 mediates TBK1 palmitoylation

(a,b) BMDMs were stimulated with RNA (a) or freeze-thawed iRBCs (b) at indicated time points.

TBK1 palmitoylation levels were detected by ABE assay and immunoblot analysis. This was

repeated n=3 independent times with similar results.

(c) RAW 264.7 cells were transfected with Scr shRNA or *Zdhhc* shRNAs for 24 h, and the silencing effect was assessed using RT-qPCR analysis (n=3 biological replicates).

(d) HEK293T cells were transfected with plasmids encoding FLAG-ZDHHC family members and MYC-TBK1. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis. This was repeated n=3 independent times with similar results.

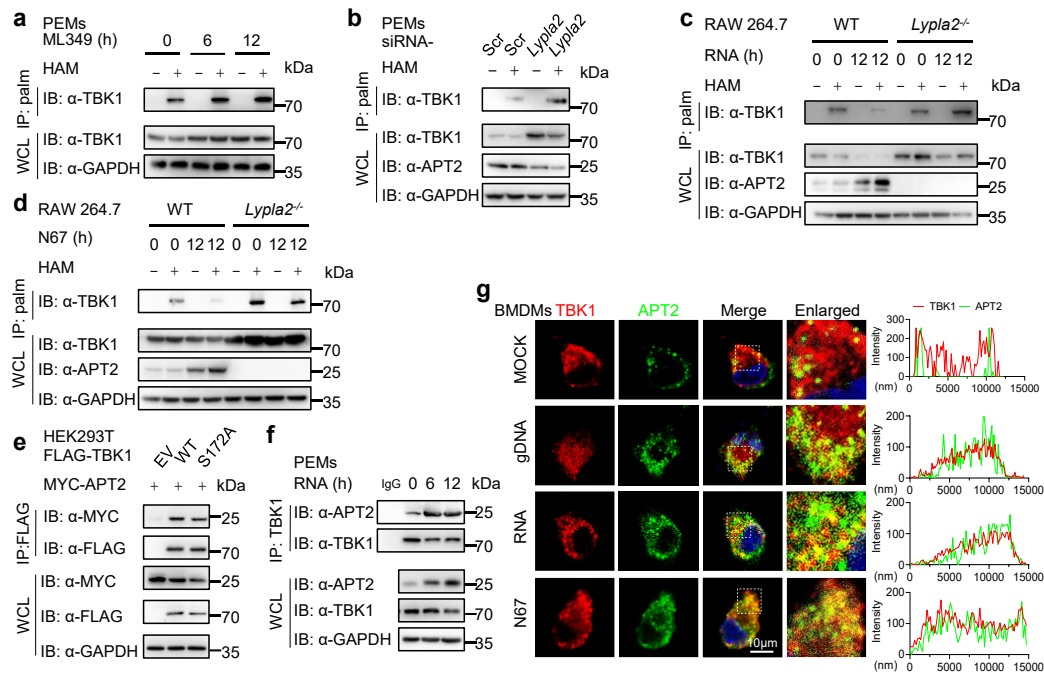
(e) HEK293T cells were transfected with HA-TBK1 and either FLAG-tagged empty vector (EV), ZDHHC9, ZDHHC13, ZDHHC16, ZDHHC21, or ZDHHC23 for 24 h. TBK1 palmitoylation levels were detected by ABE assay and immunoblot analysis. This was repeated n=3 independent times with similar results.

(f) HEK293T cells were transfected with FLAG-ZDHHC3 or FLAG-ZDHHC9. The colocalization between FLAG-ZDHHC3/9 (red) and TBK1 (green) was examined by confocal microscopy. The nucleus was stained with DAPI (blue). Scale, 10 μ m. The intensity analysis is next to the image. The interaction between FLAG-ZDHHC3 and TBK1 was used as a negative control. This was repeated n=3 independent times with similar results.

(g) HEK293T cells were transfected with Scr shRNA or *ZDHHC9* shRNA for 24 h, followed by transfection with a luciferase reporter for IFN- β - or ISRE-luc and FLAG-EV or FLAG-TBK1 for 24 h. Cell lysates were collected for luciferase reporter assays (n=3 biological replicates).

(h-m) RAW 264.7 cells were transfected with Scr shRNA or *Zdhhc9* shRNA for 24 h, then stimulated with gDNA, RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for immunoblot, this was repeated n=3 independent times with similar results **(h-j)** and RT-qPCR analysis (n=3 biological replicates) **(k,l)**. IFN- β release in the supernatants was determined by ELISA (n=3 biological replicates) **(m)**.

Data are presented as the mean $\pm$ SD. *P* values were determined by unpaired two tailed Student's *t*-tests **(c, g, k-m)**. Source data are provided as a Source Data file.



Supplementary Fig. 3 APT2 mediates the depalmitoylation of TBK1 during malarial infection

(a) PEMs were treated with ML349 (10 μ M) at indicated time points. Cell lysates were collected for ABE assay and immunoblot analysis. This was repeated n=3 independent times with similar results.

(b) PEMs were transfected with Scr-siRNA or *Lypla2*-siRNA for 36 h. Cell lysates were collected for ABE assay and immunoblot analysis. This was repeated n=3 independent times with similar results.

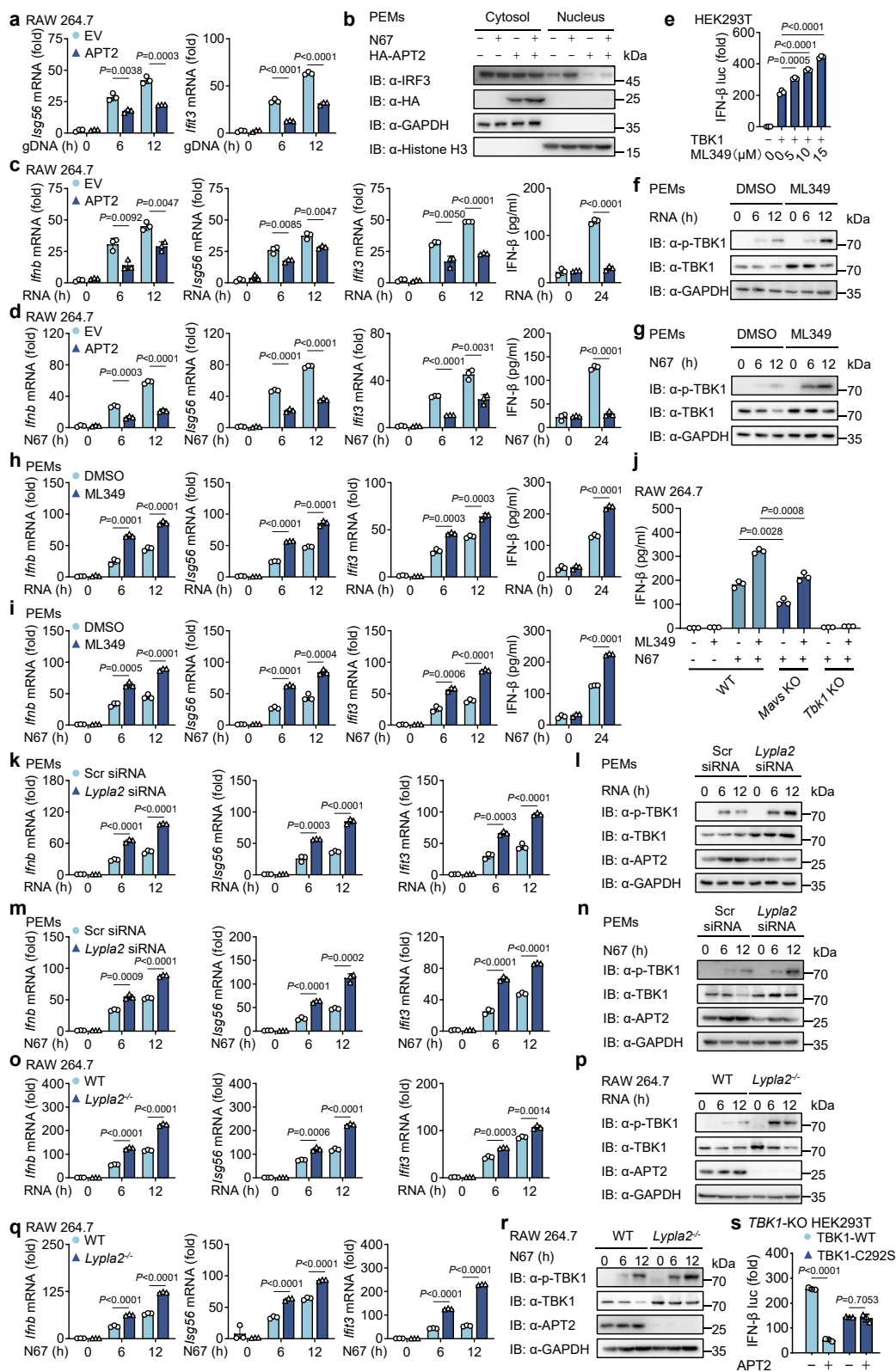
(c,d) WT or *Lypla2*-KO RAW 264.7 cells were stimulated with RNA or freeze-thawed iRBCs for 12 h. Cell lysates were collected for ABE assay and immunoblot analysis. This was repeated n=3 independent times with similar results.

(e) HEK293T cells were transfected with MYC-APT2 and FLAG-EV, -WT TBK1, or -S172A TBK1 for 24 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis. This was repeated n=3 independent times with similar results.

(f) PEMs were stimulated with RNA at indicated time points. Cell lysates were collected for IP and immunoblot analysis. This was repeated n=3 independent times with similar results.

(g) BMDMs were stimulated with gDNA, RNA, or repeated freeze-thaw cycles of N67 iRBCs for 12 h. The colocalization between TBK1 (red) and APT2 (green) was examined by confocal microscopy. The nucleus was stained with DAPI. Scale, 10 μ m. The intensity analysis is next to the image. This was repeated n=3 independent times with similar results.

Source data are provided as a Source Data file.



Supplementary Fig. 4 APT2 inhibits TBK1-mediated IFN-I responses

(a) RAW 264.7 cells were transfected with HA-EV or HA-APT2 for 24 h, then stimulated with gDNA at indicated time points. Cell lysates were collected for RT-qPCR analysis (n=3 biological replicates).

(b) Cellular fractionation and immunoblot analysis of IRF3 in PEMs were transfected with HA-EV or HA-APT2 for 24 h, then stimulated with freeze-thawed iRBCs for 12 h. This was repeated n=3 independent times with similar results.

(c,d) RAW 264.7 cells were transfected with HA-EV or HA-APT2 for 24 h, then stimulated with RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for RT-qPCR analysis (n=3 biological replicates). IFN- β release in the supernatants was determined by ELISA (n=3 biological replicates).

(e) HEK293T cells were transfected with a luciferase reporter for IFN- β -luc, and FLAG-TBK1 for 24 h, followed by treatment with different concentrations of ML349. Cell lysates were collected for luciferase reporter assays (n=3 biological replicates).

(f-i) PEMs were incubated with ML349 (10 μ M) for 12 h and then stimulated with RNA or freeze-thawed iRBCs at the indicated time points. Cell lysates were prepared for immunoblot, this was repeated n=3 independent times with similar results **(f,g)** and RT-qPCR analysis (n=3 biological replicates) **(h,i)**. IFN- β release in the supernatants was measured by ELISA (n=3 biological replicates) **(h,i)**.

(j) WT, *Mavs*-KO, or *Tbk1*-KO RAW 264.7 cells were treated with or without ML349 (10 μ M) for 12 h and subsequently stimulated with freeze-thawed iRBCs for 6 h. IFN- β release in the supernatants was determined by ELISA (n=3 biological replicates).

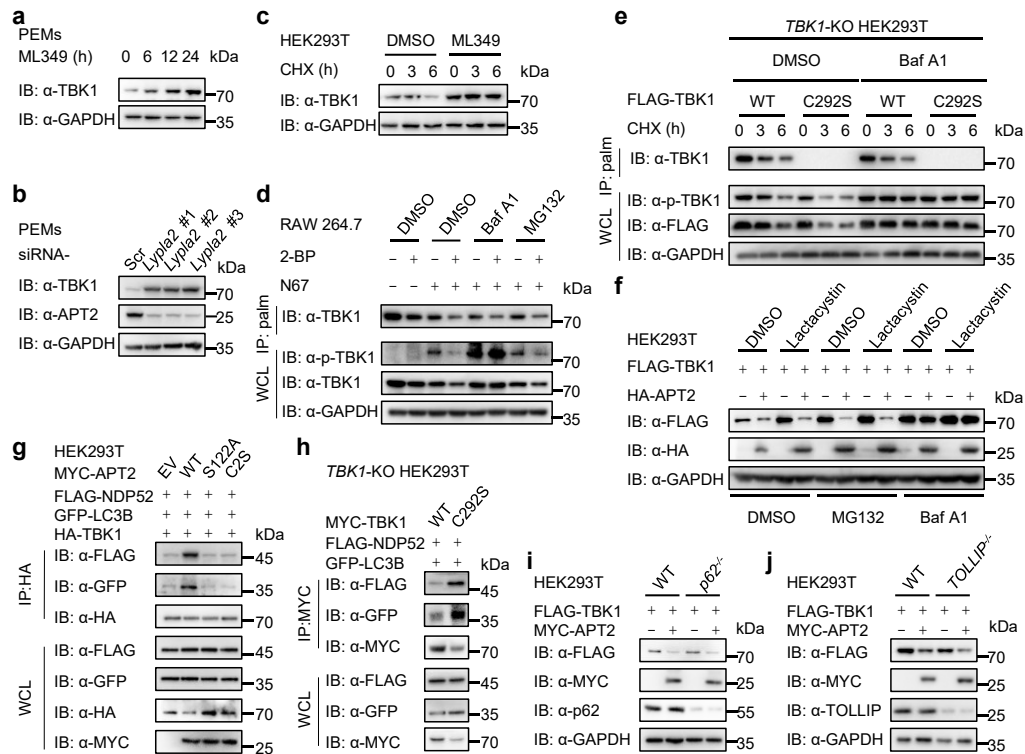
(k-n) PEMs were transfected with Scr-siRNA or *Lypla2*-siRNA for 36 h, then stimulated with RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for RT-qPCR analysis (n=3 biological replicates) and immunoblotting (this was repeated n=3 independent times with similar results).

(o-r) WT or *Lypla2*-KO RAW 264.7 cells were stimulated with RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for RT-qPCR analysis (n=3 biological replicates)

and immunoblotting (this was repeated n=3 independent times with similar results).

(s) *TBK1*-KO HEK293T cells were transfected with a luciferase reporter for IFN- β -luc, WT FLAG-TBK1 or FLAG-TBK1 C292S mutant with or without APT2 for 24 h. Cell lysates were collected for luciferase reporter assays (n=3 biological replicates).

Data are presented as the mean $\pm$ SD. *P* values were determined by unpaired two tailed Student's *t*-tests (**a, c-e, h-k, m, o, q, s**). Source data are provided as a Source Data file.



Supplementary Fig. 5 APT2-mediated depalmitoylation promotes TBK1 autophagic degradation

- (a) PEMs were treated with ML349 (10 μ M) at the indicated time points. Cell lysates were collected for immunoblot analysis. This was repeated n=3 independent times with similar results.
- (b) PEMs were transfected with Scr-siRNA, *Lypla2* siRNA #1, #2 or #3 for 36 h, Cell lysates were collected for immunoblot analysis. This was repeated n=3 independent times with similar results.
- (c) HEK293T cells were treated with ML349 (10 μ M) for 12 h, then treated with CHX (100 mg/mL) at the indicated time points. Cell lysates were collected for immunoblot analysis. This was repeated n=3 independent times with similar results.
- (d) RAW 264.7 cells were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with freeze-thawed iRBCs for 12 h and then DMSO (control), Baf A1 (0.2 μ M), or MG132 (10 μ M) treatment for 6 h. Cell lysates were collected for ABE assay and immunoblot analysis. This was repeated n=3 independent times with similar results.
- (e) *TBK1*-KO HEK293T cells were reconstituted with plasmids encoding wild-type FLAG-TBK1

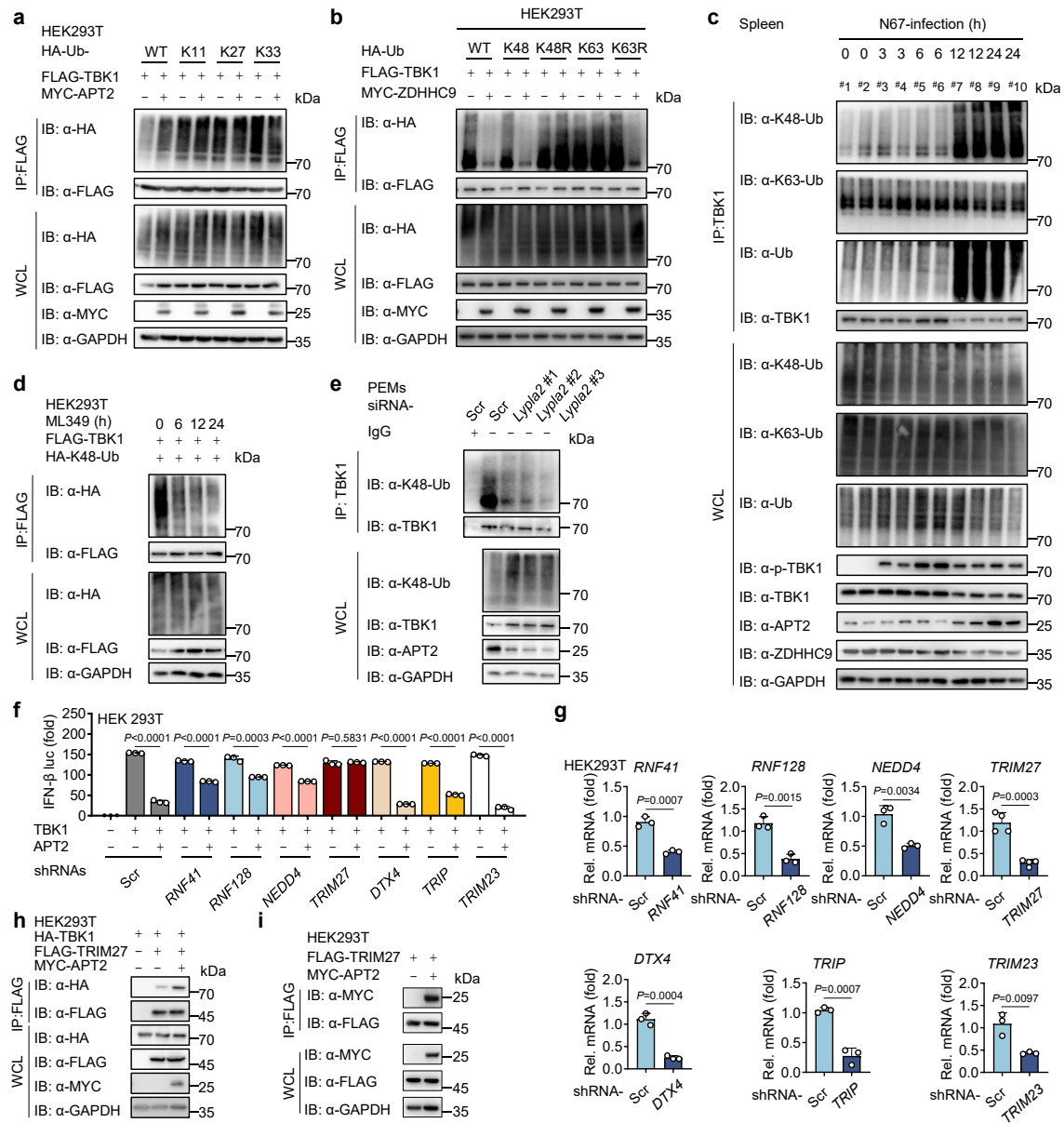
or the FLAG-TBK1 C292S mutant for 24 h. Subsequently, the cells were treated with CHX (100 mg/mL) at specified time points, along with DMSO (as a control) and Baf A1 (0.2 μ M) treatment for 6 h. Cell lysates were then collected for ABE assay and immunoblot analysis. This was repeated n=3 independent times with similar results.

(f) HEK293T cells were transfected with plasmids for FLAG-TBK1, together with HA-EV or HA-APT2 for 24 h. Then, cells were treated with DMSO (control) or Lactacystin (10 μ M) for 6 h, followed by treatment with DMSO (control), MG132 (10 μ M), or Baf A1 (0.2 μ M) for an additional 6 h. Cell lysates were collected for immunoblot analysis. This was repeated n=3 independent times with similar results.

(g,h) WT **(g)** and *TBK1*-KO **(h)** HEK293T cells were transfected with indicated plasmids. Cell lysates were collected for IP and immunoblot analysis. This was repeated n=3 independent times with similar results.

(i,j) WT, *p62*-KO or *TOLLIP*-KO HEK293T cells were transfected with FLAG-TBK1 in the presence of MYC-APT2 or not for 24 h. Cell lysates were collected for immunoblot analysis. This was repeated n=3 independent times with similar results.

Source data are provided as a Source Data file.



Supplementary Fig. 6 APT2 modulates K48-linked polyubiquitination of TBK1 through TRIM27

(a) HEK293T cells were transfected with FLAG-TBK1 and HA-tagged WT ubiquitin (HA-Ub) or its mutants, together with MYC-EV or MYC-APT2 for 24 h, followed by treatment with Baf A1 (0.2 μM) for 6 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis. This was repeated n=3 independent times with similar results.

(b) HEK293T cells were transfected with FLAG-TBK1 and HA-tagged WT ubiquitin (HA-Ub) or

its mutants, together with MYC-EV or MYC-ZDHHC9 for 24 h, followed by treatment with Baf A1 (0.2 μ M) for 6 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis. This was repeated n=3 independent times with similar results.

(c) C57BL/6J female mice were infected with N67 for various time points. Spleen tissue lysates were collected for IP (with anti-TBK1) and immunoblot analysis. This was repeated n=3 independent times with similar results.

(d) HEK293T cells were transfected with FLAG-TBK1 and HA-K48-linked ubiquitin for 24 h, followed by treatment with ML349 (10 μ M) at the indicated time points. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis. This was repeated n=3 independent times with similar results.

(e) PEMs were transfected with Scr-siRNA, *Lypla2* siRNA #1, #2 or #3 for 36 h. Cell lysates were collected for IP (with anti-TBK1) and immunoblot analysis. This was repeated n=3 independent times with similar results.

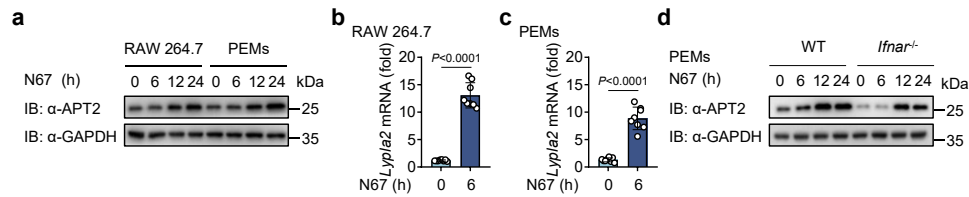
(f) HEK293T cells were transfected with Scr shRNA or other E3 ligase-specific shRNAs for 24 h, followed by transfected with FLAG-TBK1, together with a luciferase reporter for IFN- β -luc and MYC-EV or MYC-APT2 for 24 h. Cell lysates were collected for luciferase reporter assays (n=3 biological replicates).

(g) HEK293T cells were transfected with Scr shRNA or other E3 ligase-specific shRNAs for 24 h. The silencing effect was assessed using RT-qPCR analysis (n=3 biological replicates).

(h) HEK293T cells were transfected with HA-TBK1, FLAG-TRIM27 and MYC-APT2 for 24 h, followed by treatment with Baf A1 (0.2 μ M) for 6 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis. This was repeated n=3 independent times with similar results.

(i) HEK293T cells were transfected with FLAG-TRIM27 and MYC-EV, -APT2 for 24 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis. This was repeated n=3 independent times with similar results.

Data are presented as the mean $\pm$ SD. *P* values were determined by unpaired two tailed Student's *t*-tests (f, g). Source data are provided as a Source Data file.



Supplementary Fig. 7 ML349 Enhances Mice Resistance to N67 Infection

(a-c) RAW 264.7 cells or PEMs were stimulated with freeze-thawed iRBCs at indicated time points. Cell lysates were collected for immunoblot and RT-qPCR analysis. In (a), this was repeated n=3 independent times with similar results. In (b, c), the data shown are from one representative experiment (n=8 per group). Consistent results were observed across three biological replicates.

(d) WT and *Ifnar*^{-/-} PEMs were stimulated with freeze-thawed iRBCs at the indicated time points. Cell lysates were collected for immunoblot analysis. This was repeated n=3 independent times with similar results.

Data are presented as the mean $\pm$ SD. *P* values were determined by unpaired two tailed Student's *t*-tests (b, c). Source data are provided as a Source Data file.