Expanded View Figures

Figure EV1. RNF34 negatively regulates RLR signaling pathways.

A Immunoblot showing RNF34 levels in the PMA-differentiated THP-1 cells transfected with siCtrl, siRNF34-1, siRNF34-2, or siRNF34-3 oligos shown in Fig 1A and B.

B Immunoblot showing Flag-RNF34 and Flag-RNF34 H342A levels in the cells presented in Fig 1C and D. α-Tubulin was used as a loading control.

C Immunoblot showing RNF34 levels in the control (shCtrl) and three stable RNF34-knockdown (shRNF34-1, shRNF34-2, and shRNF34-3) HEK293T cell lines shown in Fig 1E and F.

D NDV-GFP replication was defined as the product of the mean fluorescence intensity of GFP-positive cells in shRNF34-1, shRNF34-2, or shRNF34-3 cells and the mean fluorescence intensity of shCtr cells.

E-I (E, F, H) Luciferase activity driven by the IFN-β promoter in HEK293T cells transfected with Flag-MAVS (E), Flag-N-RIG-I (F), or poly(I:C) (10 μg/ml; H) together with increasing amounts of the HA-RNF34 expression vector. Luciferase assays were performed 24 h after transfection, and luciferase activity was reported as the fold induction. (G, I) Luciferase activity driven by the IFN-β promoter in HEK293T cells transfected with Flag-STING and Myc-cGAS (G), or (dAT:dT) (10 μg/ml; I) together with increasing amounts of the HA-RNF34 expression vector. Luciferase assays were performed 24 h after transfection, and luciferase activity was reported as the fold induction.

J Luciferase activity driven by the NF-κB promoter in HEK293T cells transfected with poly(I:C) together with increasing amounts of the HA-RNF34 expression vector. Luciferase assays were performed 24 h after transfection, and luciferase activity was reported as the fold induction.

K Immunoblot showing the levels of the phosphorylated (-p) and total IRF3, TBK1, and RNF34 proteins in PBMCs transfected with siCtrl or siRNF34-1 oligos and infected with VSV for the indicated times.

Data information: Cell-based studies were performed independently at least three times with comparable results. The luciferase reporter and ELISA data are presented as means ± SEM. Two-tailed Student's *t*-tests were used for statistical analyses: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

Source data are available online for this figure.

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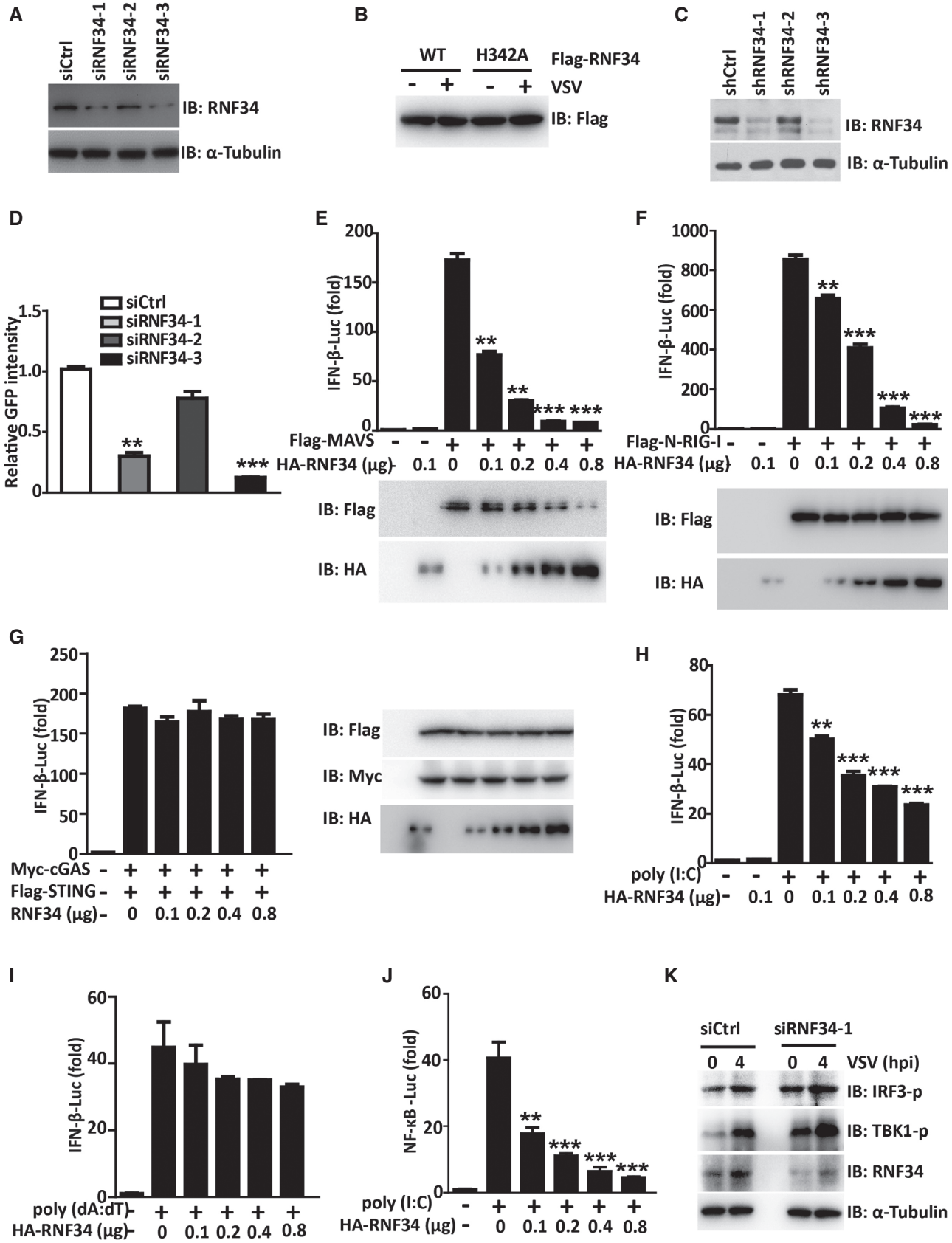

Figure EV1.

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Figure EV2. RNF34 interacts with MAVS and enhances the K27-/K29-linked ubiquitination of MAVS.

A Immunoblot showing the levels of HA-RNF34 and Flag-N-RIG-I, Flag-MAVS, Flag-STING, and Flag-TBK1 in the cells shown in Fig 2A.
B Immunoprecipitation analysis of HEK293T cells transfected with Myc-RNF34 together with Flag-V, Flag-MAVS, Flag-RIG-I, Flag-STING, or Flag-TBK1. Anti-Flag or IgG agarose immunoprecipitates were analyzed using immunoblotting with an anti-Myc or anti-Flag antibody.
C Immunoblot showing the levels of MAVS, RIG-I, STING, and TBK1 in HEK293T cells transfected with the Myc-vector (Myc-V) or Myc-RNF34.
D Quantification of MAVS colocalization with RNF34 in Fig 2F.
E Schematic of MAVS along with predicted translation product mMAVS with the start site corresponding to Met 142.
F Immunoprecipitation analysis of HEK293T cells expressing Flag-MAVS and Myc-RNF34 together with HA-Ub (WT; K27R; K29R; K27/29R). Anti-Flag immunoprecipitates were analyzed using immunoblotting with an anti-HA or anti-Flag antibody. Levels of the transfected proteins were analyzed using immunoblotting with an anti-HA, anti-Myc, or anti-Flag antibody.
G Immunoblot showing levels of the RNF34 protein in cells transfected with the control siRNA and RNF34 siRNA-1 shown in Fig 3F. α-Tubulin was used as a loading control.

Data information: Cell-based studies were performed independently at least three times with comparable results. The luciferase reporter and ELISA data are presented as means ± SEM. Two-tailed Student's *t*-tests were used for statistical analyses: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.
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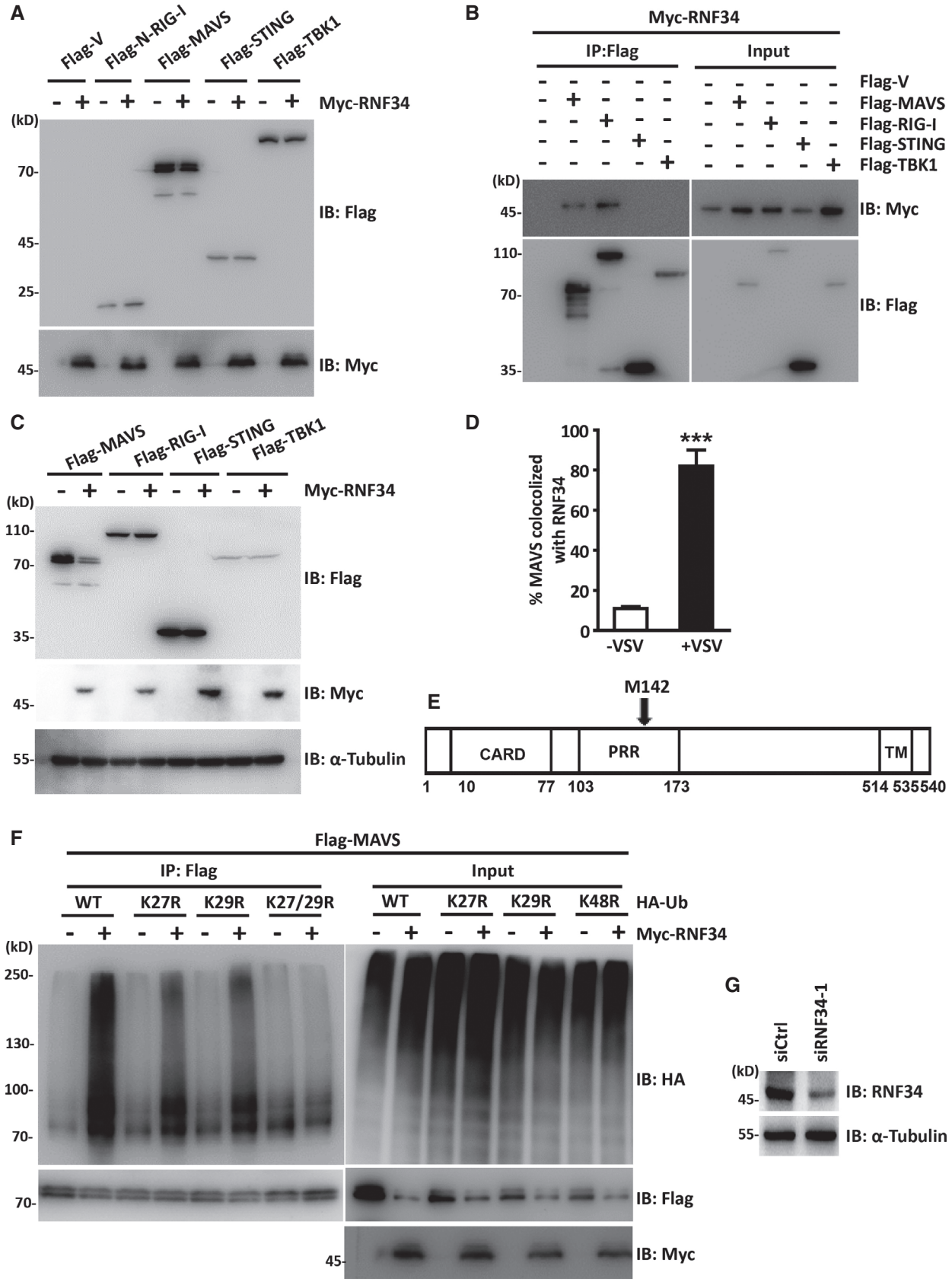

Figure EV2.

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Figure EV3. RNF34 transfers ubiquitin to Lys 297, 311, 348, and 362 Arg in MAVS.

A MS analyses were performed on MAVS recovered from an *in vitro* ubiquitination assay.

B Schematic showing the distribution of the four Lys residues in MAVS that are ubiquitinated by RNF34. MS analyses were performed on MAVS recovered from an ubiquitination assay.

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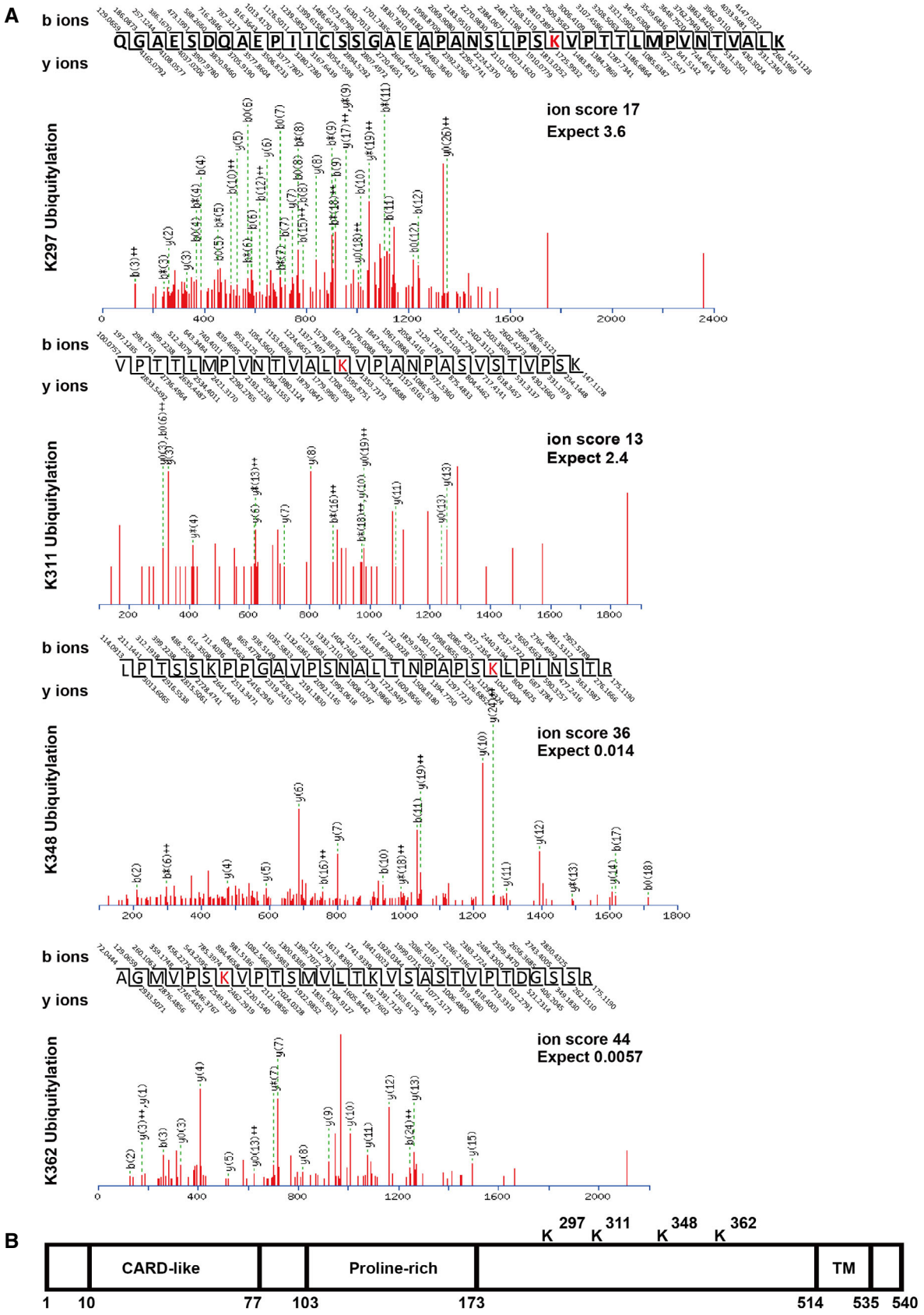

Figure EV3.

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Figure EV4. RNF34-mediated autophagic degradation of MAVS is facilitated by NDP52.

A Immunoblot showing levels of MAVS, RNF34, and LC3 in THP-1 cells infected with VSV for 9 h in the presence or absence of 3-MA (1 mM). α-Tubulin was used as a loading control.

B The efficiency of ATG5, ATG7, or tetherin knockdown was assayed using RT–PCR. The target gene was normalized to the ACTB gene.

C, D Immunoblot showing MAVS levels in ATG5- (C) or ATG7-knockdown (D) cells transfected with Myc-RNF34. α-Tubulin was used as a loading control.

E Immunoblot showing Flag-MAVS levels in HEK293T cells expressing Flag-MAVS together with increasing amounts of Myc-RNF34. α-Tubulin was used as a loading control.

F Immunoblot showing Flag-MAVS levels in siCtrl or siTetherin cells infected with or without VSV. α-Tubulin was used as a loading control.

G Immunoblot showing levels of the MAVS protein in HEK293T cells expressing Flag-MAVS or its KR mutants together with Myc-RNF34. α-Tubulin was used as a loading control.

H, I Luciferase activity driven by the IFN-β (I) or ISRE (J) promoter in HEK293T cells transfected with Myc-RNF34 together with Flag-MAVS or 4KR and subsequently infected with VSV for 12 h.

J Immunoprecipitation analysis of HEK293T cells transfected with Flag-NBR1, Flag-OPTN,Flag-SQSTM1, and Flag-OPTN together with HA-MAVS. Anti-Flag immunoprecipitates were analyzed using immunoblotting with an anti-HA or anti-Flag antibody.

K Luciferase activity driven by the IFN-β promoter in HEK293T cells transfected with HA-MAVS together with Flag-NBR1, Flag-OPTN, Flag-SQSTM1 or Flag-NDP52 (top panel). Immunoblotting analysis of loading controls (lower panel).

L Immunoblotting analysis of loading controls for the experiments shown in Fig 6B.

M Immunoblotting analysis of loading controls for the experiments shown in Fig 6D.

N Immunoblot showing levels of the MAVS protein in MEFs expressing Flag-NDP52 together with Myc-RNF34. α-Tubulin was used as a loading control.

Data information: Cell-based studies were performed independently at least three times with comparable results. The luciferase reporter and ELISA data are presented as means ± SEM. Two-tailed Student's *t*-tests were used for statistical analyses: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

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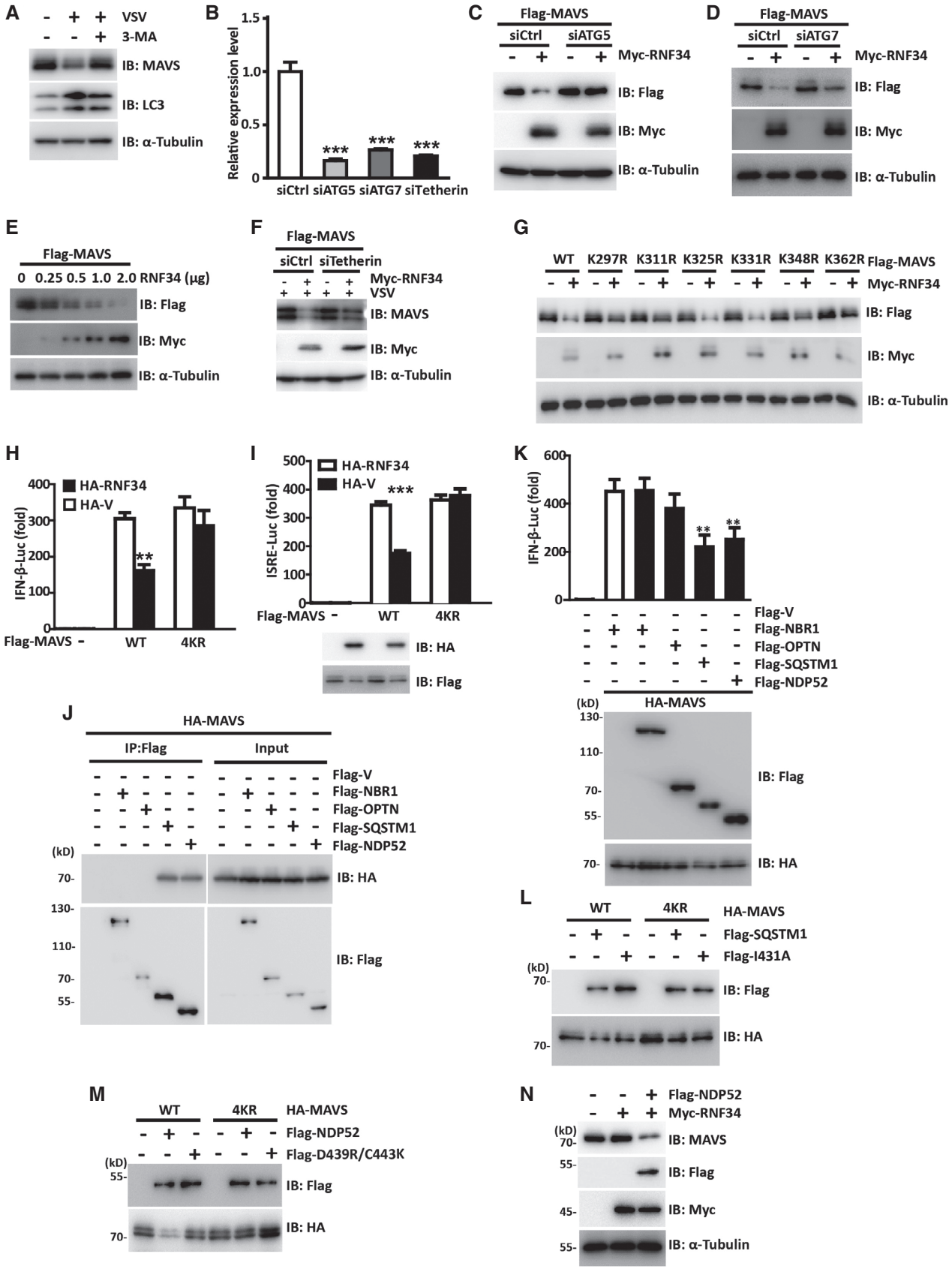

Figure EV4.

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Figure EV5. RNF34 is required for the clearance of damaged mitochondria upon viral infection.

A Immunoblot showing MAVS, IRF3-p, LC3, TOM20, and HSP60 levels in siCtrl- or siRNF34-1-transfected cells infected with VSV for the indicated times. α-Tubulin was used as a loading control.

B Immunoblot showing the levels of activated caspase-3 in 293T cells infected with VSV for the indicated times and cultured in the presence or absence of Z-VAD (50 μM). α-Tubulin was used as a loading control.

C Representative confocal images of immunofluorescence staining for TOM20 clearance in shCtrl- or shRNF34-1 cells infected with VSV and stained with anti-T0M20 and anti-LAMP1 antibodies. Red represents TOM20; green represents LAMP1. Scale bar, 10 μm.

D Quantification of TOM20 colocalization with LAMP1 in the cells shown in (C).

E The product of the mean fluorescence intensity of TOM20 in GFP-RNF34-transfected cells and the mean fluorescence intensity of GFP-transfected cells.

F Representative confocal images of TOM20 immunofluorescence staining in cells transfected with GFP or GFP-RNF34. Scale bar, 10 μm.

Data information: Cell-based studies were performed independently at least three times with comparable results.

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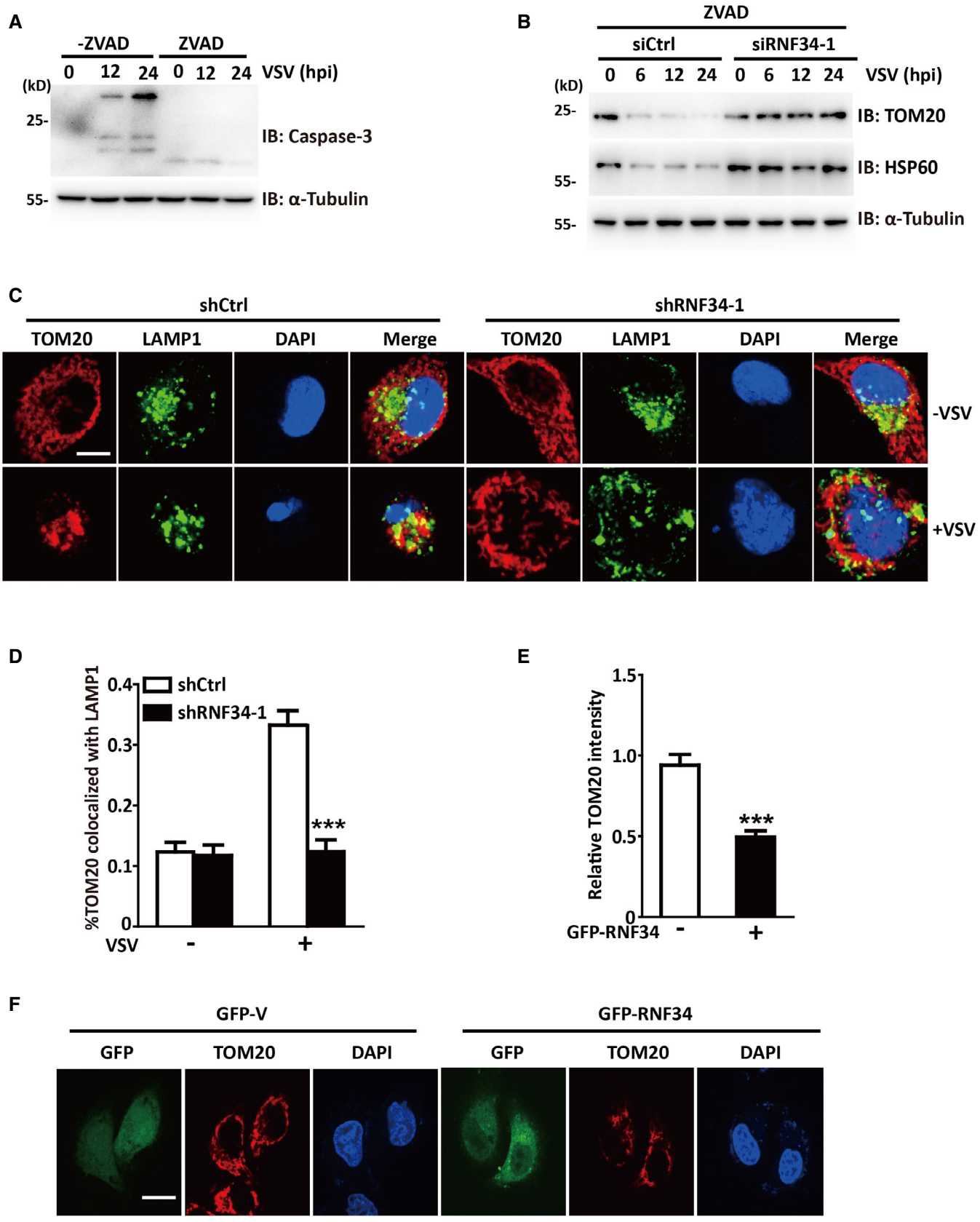

Figure EV5.

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