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Supplemental Data

Key Role of Ubc5 and Lysine-63 Polyubiquitination in Viral Activation of IRF3

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Supplemental Experimental Procedures

Plasmids

cDNA encoding human TRAF3 or IRF3 was subcloned into pFastBac 1 (Invitrogen) in-frame with an N-terminal His_8 tag for expression in Sf9 cells using the baculovirus system. pcDNA3-Flag-IRF3 (2A) in which Ser 385 and Ser 386 were substituted with alanine was constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). cDNA encoding human TRAF3 and TANK were cloned into pEF-IRES-puro vector with an N-terminal Flag and C-terminal HA tag, respectively. Human NEMO expression constructs were prepared as follows: coding sequences of full-length NEMO WT (aa 1-419), ΔKBD (aa 86-419), ΔZF (aa 1-365) and ΔLZ-ZF (aa 1-302) were subcloned into pcDNA3 vector in-frame with an N-terminal Flag tag. Y308S (aa 1-419, Y308S) and Y308S-ΔZF (aa 1-365, Y308S) were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) from full-length NEMO WT and ΔZF, respectively. Human MAVS (No-Lys) was constructed by mutagenesis of all 15 lysines with arginine using the QuikChange kit. This cDNA and wild type MAVS were cloned into a modified retroviral vector (LZRS-pBMNZ) to produce retroviruses, which were used to infect *Mavs*$^{-/-}$ MEF cells (Sun et al., 2006).

RT-PCR

Total RNA from MEF cells was isolated using TRIzol (Invitrogen). 2 μg total RNA was reverse transcribed into cDNA using random hexamer (Applied Biosystems). The resulting cDNA was used as the template for PCR amplification with the following primers specific for mouse β-actin and TRAF3:

Mouse β-actin, 5'-TGACGTTGACATCCGTAAAGACC-3', and 5'-AAGGGTGTAAAACGCAGCTCA-3';

Mouse TRAF3, 5'-CCTAAGATGGAGTCAAGC-3' and 5'-ACCGTCTTCACAAACTTC-3'.

Cell Culture, siRNA Transfection, Luciferase Reporter Assay and Interferon-β ELISA

All cells were cultured at 37°C in an atmosphere of 5% CO_2. HEK293T and HEK293T-MAVS (stable cells with Flag-MAVS expression) were cultured in DMEM with 10% (v/v) cosmic calf serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin. U2OS and MEF cells were also growing in DMEM with the antibiotics, except that 10% (v/v) FBS (tetracycline-free, Invitrogen) and 10% (v/v) FBS (Atlanta) were used, respectively.

For luciferase reporter assays in HEK293T cells, the cells were seeded in 12-well plates on day 0. On day 1, siRNA were transfected in duplicate at a final concentration of 20 nM using calcium phosphate precipitation method. On day 2, siRNA was transfected again with 50 ng of luciferase reporters (pISRE-Luc or IFN-β-Luc) together with 100 ng of pCMV-LacZ plasmid as an internal control. On day 4, the cells were infected with Sendai virus for 16 hours before luciferase and β-galactosidase activities were measured as previously described (Seth et al., 2005).

siRNA oligo sequences are as follows (only sense strand is shown): Ubc5, CAGUAAUGGCAGCAUUUGUTT; MAVS, CCACCUUGAUGCCUGUGAATT; RIG-I, CGAUUCCAUCACUAUCCAUTT; TBK1, UCAAGAACUUAUCUACGAATT; GFP (as a control), ACUUGUACAGCUCGUCCAUTT.

Luciferase Reporter Assay in NEMO-Deficient MEF Cells

For luciferase reporter assays in NEMO-deficient and reconstituted MEF cells, the cells were seeded in 12-well plates on day 0. On day 1, 20 ng of NEMO expression plasmids were transfected together with 250 ng of pISRE-luc and 250 ng of pRL-CMV as an internal control in duplicate using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). On day 2, cells were infected with VSV(ΔM51)-GFP at an MOI of 1 for 14 hours before the luciferase activity was measured. For measurement of IFN-β production, culture media was harvested from cells infected with Sendai virus for 16 hours and then assayed using human or mouse IFNβ ELISA Kit (PBL Biomedical Laboratories).

Immunoprecipitation

For co-immunoprecipitation of TANK, TBK1 and various versions of NEMO, HEK293T cells were seeded in 100-mm dishes on day 0. On day 1, each dish of cells was transfected with 2 μg of pcDNA3-TANK-HA, 3 μg of pcDNA3-Flag-NEMO or indicated version of NEMO mutants, and 3 μg of pcDNA3-Flag-TBK1 by calcium phosphate precipitation method. In some experiments, siRNA oligos against Ubc5b/c were transfected into the cells one day before the expression plasmids were transfected. Cells were harvested two days after the plasmid transfection, and lysed in buffer A [20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (w/v) TritonX-100, 20 mM β-glycerol phosphate, 1 mM Na_3VO_4, 0.1 mM NaF, 0.1 mM PMSF, and EDTA-free protease inhibitors cocktail]. The cell lysates were incubated with rat monoclonal anti-HA agarose (Roche) at 4^{o}C for 5 hours. After washing the beads four times with buffer A, bound proteins were analyzed by immunoblotting with indicated antibodies.

For immunoprecipitation of TRAF3, NEMO, TANK, TBK1, IKKε and IRF3, expression vectors for the indicated genes and MAVS were co-transfected to HEK293T cells for 2 days. Cells were lysed in buffer A plus 10 mM N-ethylmaleimide and 4 M urea. The lysates were diluted 10 fold in phosphate buffered saline (PBS) before immunoprecipitation.

To detect ubiquitination of endogenous MAVS, HEK293T cells were infected with Sendai virus for up to 7 hours, then the mitochondrial fraction (P5) was prepared and lysed in PBS containing 1% SDS. The lysate was diluted 10 fold with PBS before immunoprecipitation with a MAVS antibody and subsequent immunoblotting with a ubiquitin antibody.

Ubiquitination of TRAF3 and Its Association with NEMO

Purified His_8-TRAF3 (0.5 μg) was incubated with 0.1 μg of E1, 0.2 μg of Ubc5c and 5 μg of ubiquitin in the presence of 2 mM ATP and 5 mM $MgCl_2$ for 1 hour at 30°C. The reaction mixture was then incubated with 0.1 μg Flag-NEMO at 4°C for 2 hours before 10 μl Ni-NTA-agarose beads were added. After rotating at 4°C for 1 hour, the beads were washed with a buffer containing 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 10% (v/v) glycerol, 0.1% TritonX-100 and 40 mM imidazole. Bound Flag-NEMO was eluted with the same buffer containing 250 mM imidazole and analyzed by immunoblotting with the anti-Flag antibody.

Supplemental Reference

Seth, R.B., Sun, L., Ea, C.K., and Chen, Z.J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell *122*, 669-682.

Sun, Q., Sun, L., Liu, H.H., Chen, X., Seth, R.B., Forman, J., and Chen, Z.J. (2006). The specific and essential role of MAVS in antiviral innate immune responses. Immunity *24*, 633-642.

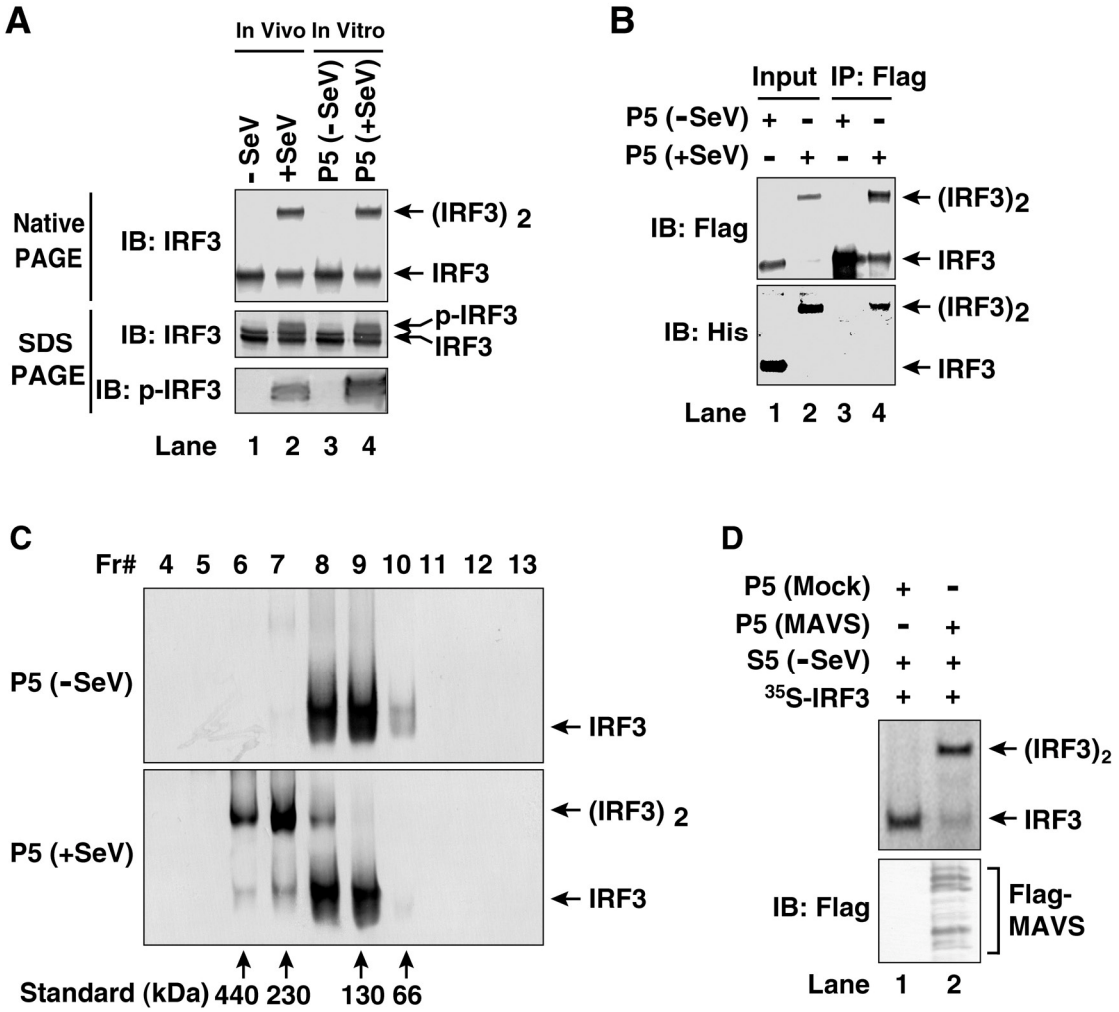

Figure S1. Characterization of the Cell-Free IRF3 Activation Assay
(**A**) Comparison of the active IRF3 dimer *in vitro* and *in vivo*. Cytosolic extract (S5) was incubated with the mitochondrial fractions (P5) from HEK293T cells infected with or without Sendai virus (+/- SeV). Dimerization of IRF3 in the reactions (lanes 3 & 4) was analyzed by native gel electrophoresis along with extracts from mock-treated or virus-infected cells (lanes 1 & 2; upper panel). Aliquots of the reaction mixtures and cell lysates were resolved by SDS-PAGE followed by immunoblotting with an antibody against IRF3 or phospho-IRF3 (p-IRF3 at Ser 396; lower panels). (**B**) Recombinant His_8-IRF3 protein was mixed with cytosolic extract (S5) from HEK293T cells expressing Flag-IRF3, and the mixture was incubated with P5 (+/- SeV). Aliquots of the reaction mixtures were immunoprecipitated with a Flag antibody, followed by native gel electrophoresis and immunoblotting with an antibody against Flag or His_5. Input: reaction mixtures before immunoprecipitation. (**C**) Cytosolic extract (S5) from HEK293T cells stably expressing Flag-IRF3 was incubated with P5 (+/- SeV), then IRF3 was purified using anti-Flag agarose beads. The purified IRF3 was loaded onto a Superdex-200 column, and aliquots of the fractions were analyzed by immunoblotting with a Flag antibody. (**D**) Mitochondria containing overexpressed MAVS activate IRF3 *in vitro*. HEK293T cells were transfected with 10 μg of pcDNA3-Flag-MAVS or the vector (mock). Two days after transfection, the mitochondrial fractions (P5) were prepared and incubated with cytosolic extract (S5) from uninfected cells in the presence of ATP and ^{35}S-IRF3. Dimerization of IRF3 was analyzed by native gel electrophoresis and autoradiography.

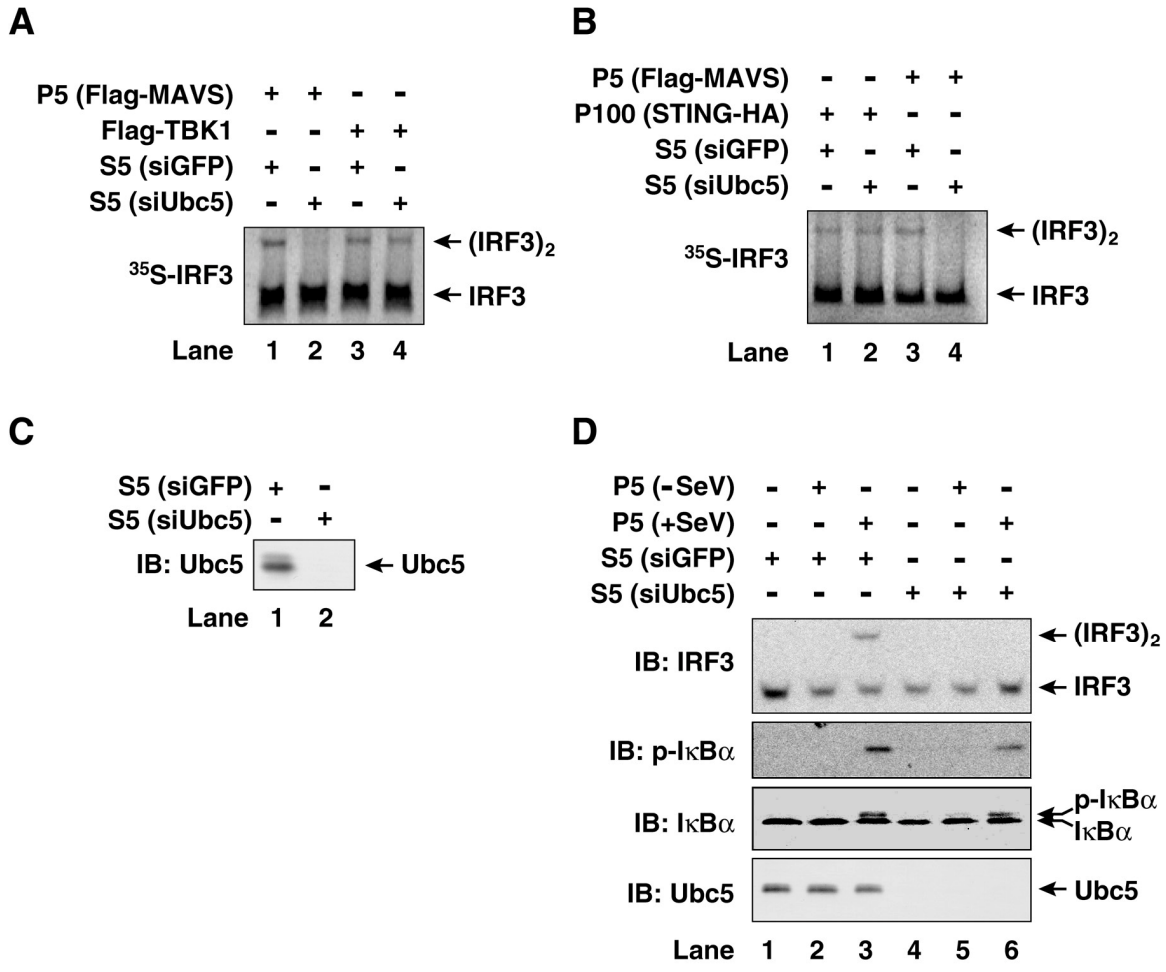

Figure S2. **Role of Ubc5 in IRF3 and IKK Activation In Vitro**
(**A - C**) Ubc5 is required for IRF3 activation by MAVS but not TBK1 or STING. HEK293T cells were transfected with siRNA against Ubc5b/c or GFP (control), then cytosolic extract was prepared and incubated with Flag-TBK1 protein (**A**) or the membrane fractions (P5 and P100) from HEK293T cells overexpressing MAVS (**A & B**) or STING (**B**). Dimerization of ^{35}S-IRF3 was analyzed by native gel electrophoresis. Knockdown of Ubc5 was confirmed by immunoblot analysis (**C**). (**D**) *In vitro* phosphorylation of IκBα by virus-activated mitochondria. Reactions contained P5 (+/- SeV) and S5 (-SeV) of HEK293T cells transfected with siRNA against Ubc5 or GFP. Dimerization of endogenous IRF3 in S5 was analyzed by native gel electrophoresis (upper panel). Aliquots of the reaction mixtures were analyzed by SDS-PAGE followed by immunoblotting with an antibody against phospho-IκBα, IκBα or Ubc5.

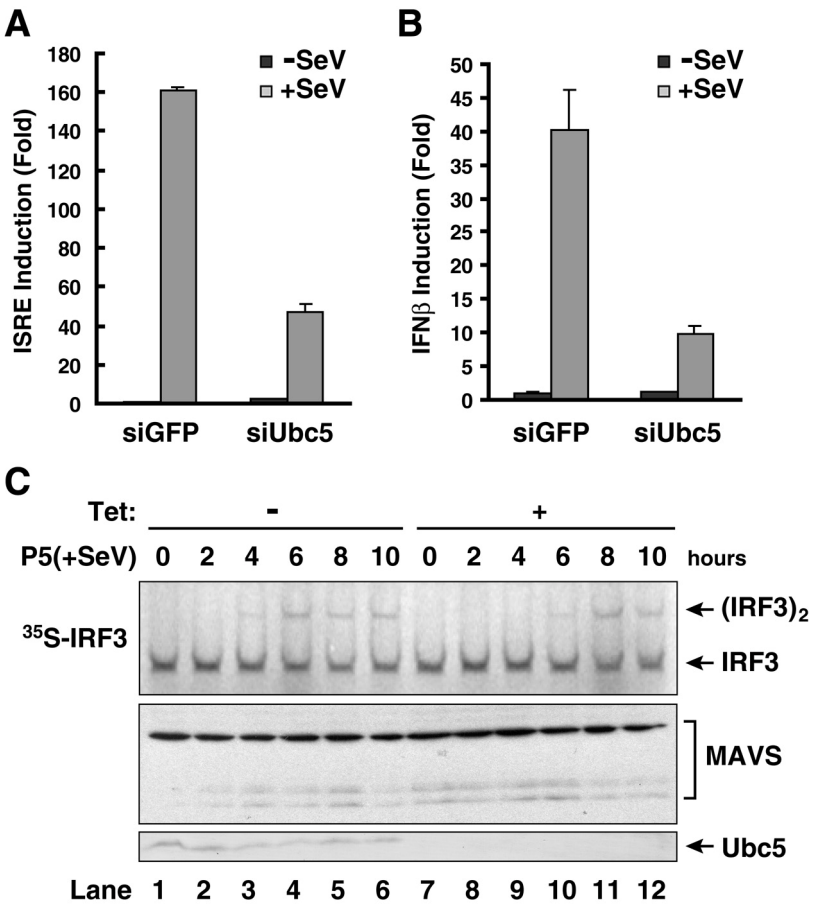

Figure S3. **Role of Ubc5 in IFNβ Induction and MAVS Activation by Sendai Virus**
HEK293T cells were transfected with siRNA against Ubc5 or GFP together with ISRE-luciferase (**A**) or IFNβ-luciferase (**B**) reporter plasmid. Three days after transfection, the cells were infected with Sendai virus for 16 hours before the luciferase activity was measured. Error bars represent variation ranges of duplicate experiments. (**C**) Ubc5 knockdown modestly delayed viral activation of MAVS. U2OS cells stably integrated with tetracycline-inducible shRNA against Ubc5b/c were treated with or without tetracycline for 7 days, then infected with Sendai virus for the indicated time. The mitochondrial fractions (P5) from these cells were incubated with cytosolic extract (S5) from HEK293T cells to measure IRF3 dimerization (upper panel). Expression levels of MAVS in P5 and Ubc5 in cell lysates were examined by immunoblotting.

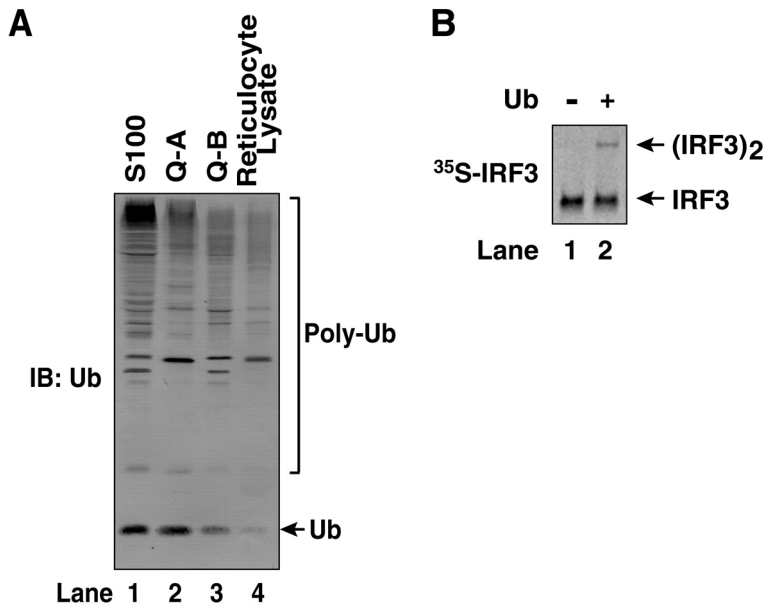

Figure S4. **Ubiquitin-Dependent Activation of IRF3 In Vitro**
(**A**) Presence of ubiquitin in the fractions used in Fig. 2B was examined by immunoblot analysis. (**B**) Ubiquitin-dependent activation of IRF3. The fraction Q-B as described in Fig. 2A was further fractionated by gel filtration. High molecular mass fractions were pooled and incubated with a mitochondrial fraction (P5; +SeV), affinity-purified ^{35}S-IRF3 and Ubc5c in the presence or absence of ubiquitin. Dimerization of IRF3 was analyzed by native gel electrophoresis.

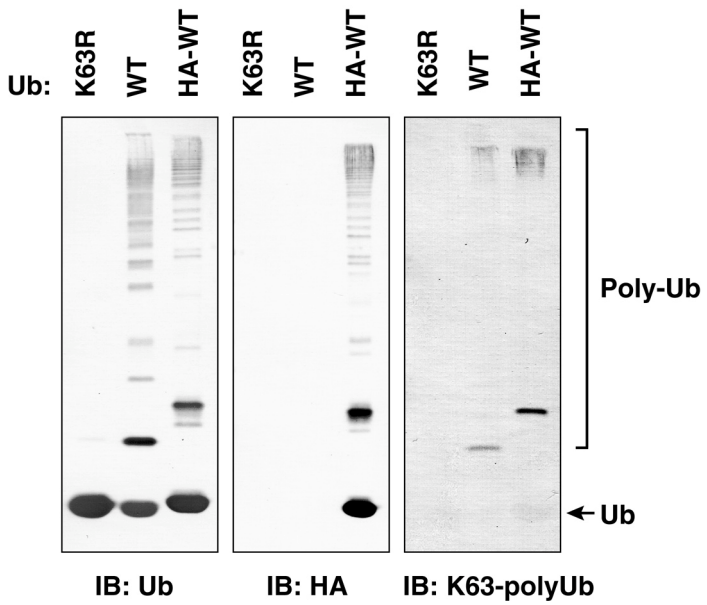

Figure S5. **Synthesis of K63-Linked HA-Ubiquitin Chain Catalyzed by TRAF6 and Ubc13/Uev1A**

Enzymatic synthesis of K63-linked HA-ubiquitin chain was carried out as described in Experimental Procedures. The ubiquitin chains were analyzed by immunoblotting with an antibody specific for ubiquitin, HA, or K63-linked polyubiquitin.

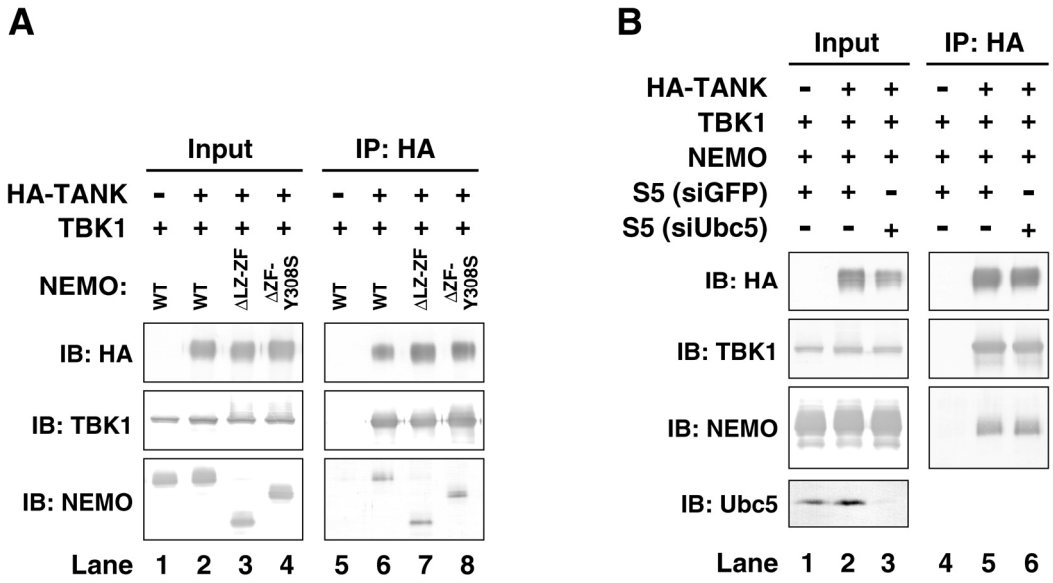

Figure S6. Ubiquitination Is Not required for NEMO to Interact with TANK and TBK1

(**A**) Mutations of ubiquitin-binding domains of NEMO do not interfere with its binding to TANK and TBK1. HEK293T cells were transiently transfected with expression vectors for HA-TANK, Flag-TBK1, and Flag-NEMO or its mutants. Cell lysates were immunoprecipitated with an antibody against HA, then immunoblotted with an antibody against TBK1 or NEMO. (**B**) Ubc5 is dispensable for the formation of NEMO-TANK-TBK1 complex. HEK293T cells were transfected with a siRNA against Ubc5b/c or GFP, and subsequently transfected with expression vectors for HA-TANK, Flag-TBK1 and Flag-NEMO. Formation of the NEMO-TANK-TBK1 complex was examined by immunoprecipitation followed by immunoblotting as described in (**A**).

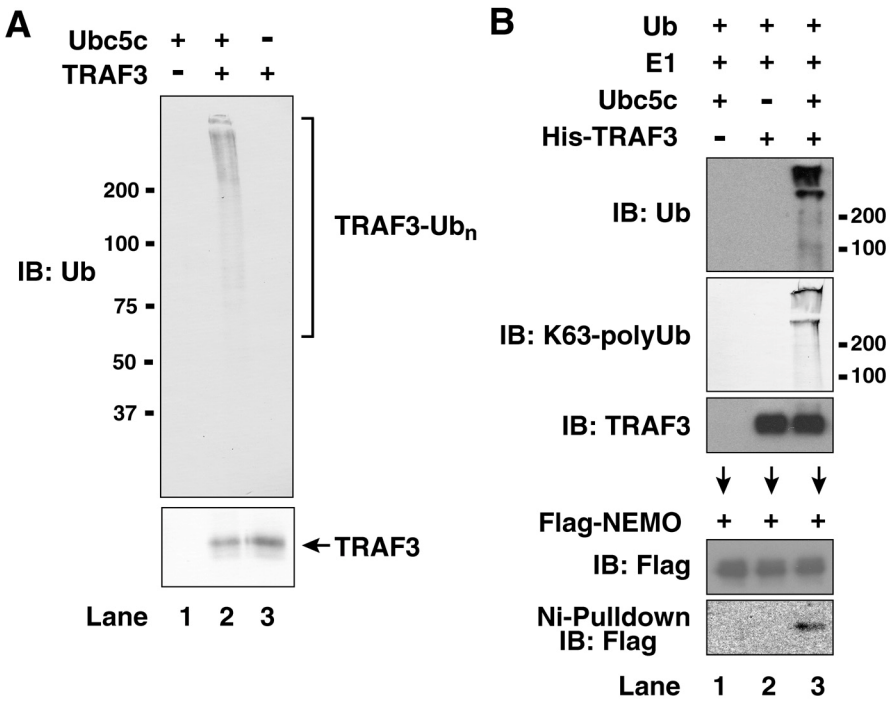

Figure S7. Ubiquitination of TRAF3 Enhances Its Binding to NEMO
(**A**) Ubc5 supports polyubiquitination of TRAF3. His_8-TRAF3 was incubated with E1, Ubc5c and ubiquitin in the presence of ATP. After incubation at 30°C for 1 hour, His_8-TRAF3 was absorbed to Ni-NTA-agarose beads in the presence of 8 M urea, then immunoblotted with an antibody against ubiquitin or TRAF3. (**B**) Ubiquitination-dependent binding of NEMO to TRAF3. His_8-TRAF3 was polyubiquitinated by Ubc5c as described (**A**), then incubated with Flag-NEMO immunopurified from HEK293T cells. After absorption on Ni-NTA beads, the bound proteins were analyzed by immunoblotting with a Flag antibody.

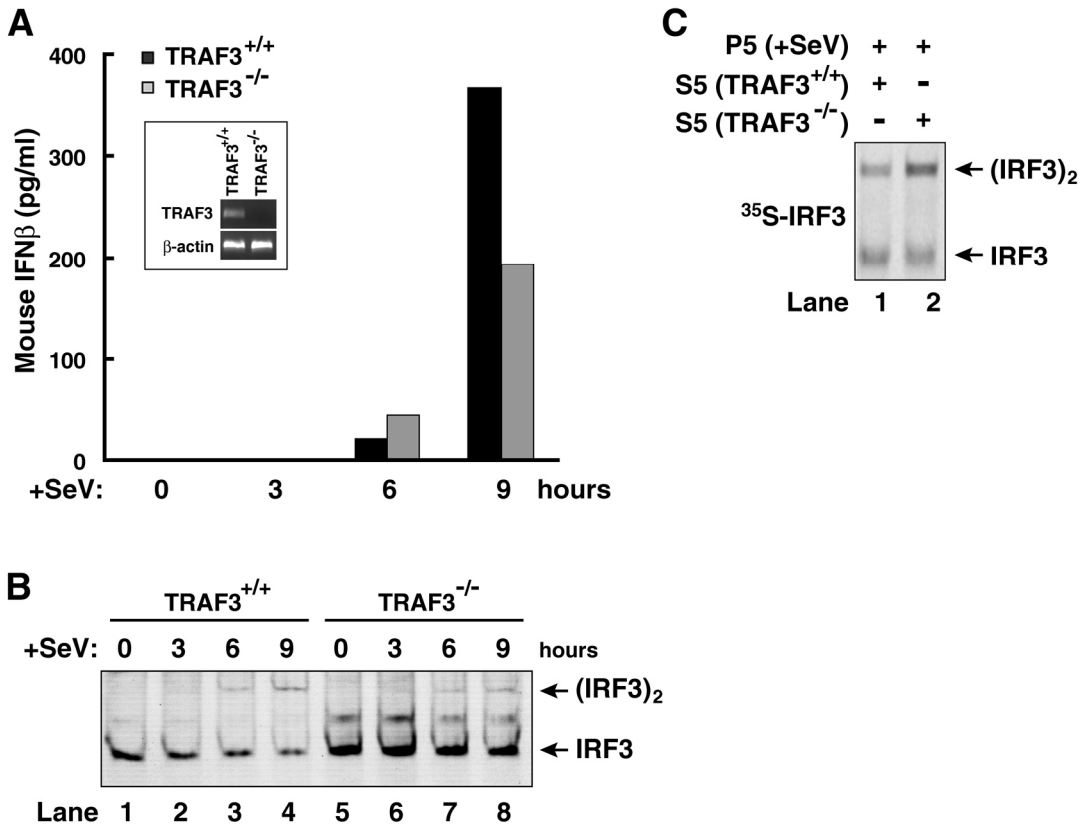

Figure S8. **TRAF3 Deficiency Does Not Completely Block IFNβ Induction or IRF3 Activation by Sendai Virus**

(**A & B**) Viral-induced IFNβ production and IRF3 activation in TRAF3$^{-/-}$ MEF cells. TRAF3$^{+/+}$ and TRAF3$^{-/-}$ MEF cells were infected with Sendai virus for the indicated time before IFNβ production and IRF3 dimerization were measured by ELISA (**A**) and native gel electrophoresis (**B**), respectively. Deficiency of TRAF3 in the MEF cells was confirmed by RT-PCR (inset in **A**). The ELISA measurement is representative of three independent experiments. (**C**) Activation of IRF3 in TRAF3-deficient cytosol *in vitro*. Cytosolic extract (S5) was prepared from TRAF3$^{+/+}$ and TRAF3$^{-/-}$ MEF cells and incubated with Sendai virus-activated mitochondria (P5). Dimerization of ^{35}S-IRF3 was analyzed by native gel electrophoresis..

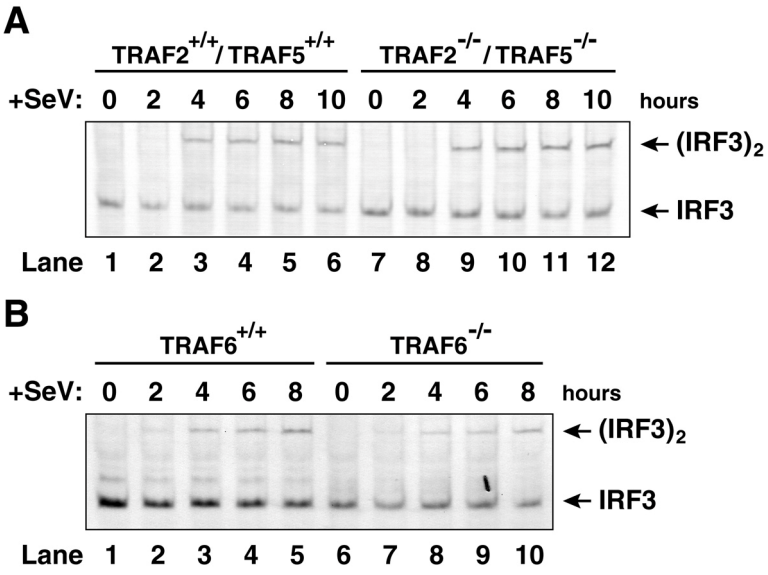

Figure S9. **TRAF2, 5, and 6 Are Dispensable for IRF3 Activation by Sendai Virus**
MEF cells lacking both TRAF2 and TRAF5 (**A**) or TRAF6 (**B**) and their wild-type counterparts were infected with Sendai virus for the indicated time, then cell lysates were analyzed for IRF3 dimerization by native gel electrophoresis and immunoblotting.

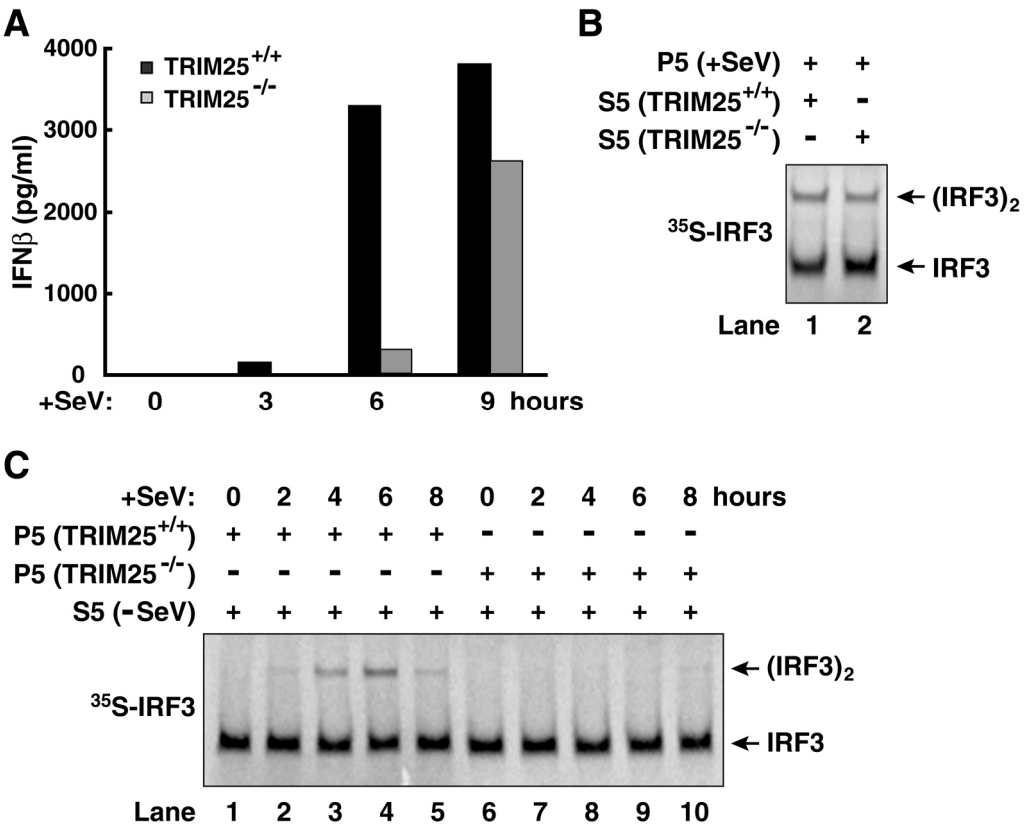

Figure S10. **TRIM25 Functions Upstream of MAVS in IRF3 Activation**
(**A**) Impaired IFNβ production in TRIM25$^{-/-}$ MEFs. TRIM25$^{+/+}$ and TRIM25$^{-/-}$ MEFs were infected with Sendai virus for the indicated time before IFNβ in the medium was measured by ELISA. The measurement is representative of two independent experiments. (**B**) TRIM25 is not required for IRF3 activation downstream of MAVS. Cytosolic extract (S5) from TRIM25$^{+/+}$ or TRIM25$^{-/-}$ MEFs was incubated with Sendai virus-activated mitochondria (P5) and then dimerization of ^{35}S-IRF3 was analyzed by native gel electrophoresis. (**C**) TRIM25 is required for the activation of MAVS by virus. TRIM25$^{+/+}$ and TRIM25$^{-/-}$ MEFs were infected with Sendai virus for the indicated time and then the mitochondrial fractions (P5) were prepared. The P5 fractions were incubated with cytosolic extract (S5) from uninfected cells to induce dimerization of IRF3.

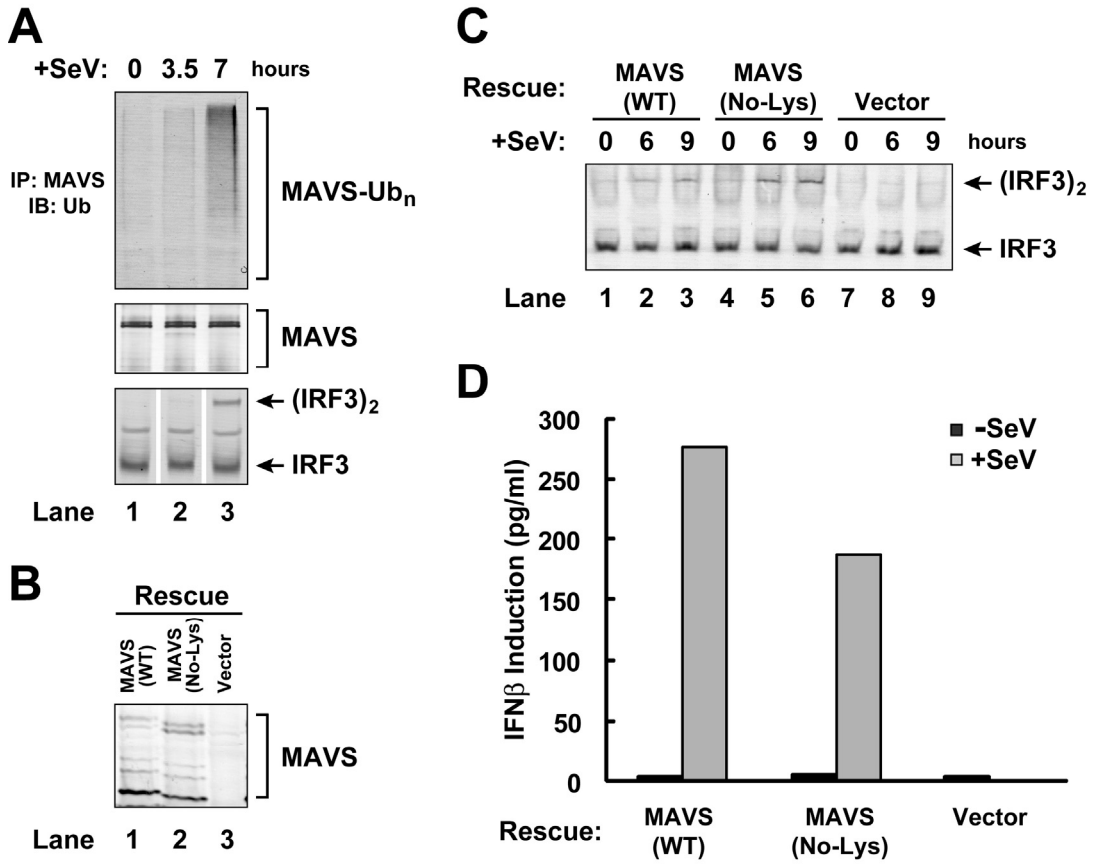

Figure S11. Ubiquitination of MAVS Is Dispensable for IFNβ Induction and IRF3 Activation

(**A**) HEK293T cells were infected with Sendai virus for the indicated time, then endogenous MAVS was immunoprecipitated with a MAVS antibody under a denaturing condition followed by immunoblotting with a ubiquitin antibody. Dimerization of IRF3 was examined by native gel electrophoresis. (**B-D**) A MAVS lysine-less mutant (No-Lys) or its wild type counterpart was stably expressed in *Mavs*$^{-/-}$ MEF cells. The cells were infected with Sendai virus, then cell lysates were analyzed for IRF3 dimerization by native gel electrophoresis (**C**) and IFNβ secretion was measured by ELISA (**D**). Aliquots of the cell lysates were analyzed by immunoblotting with a MAVS antibody (**B**). The ELISA measurement is representative of two independent experiments.

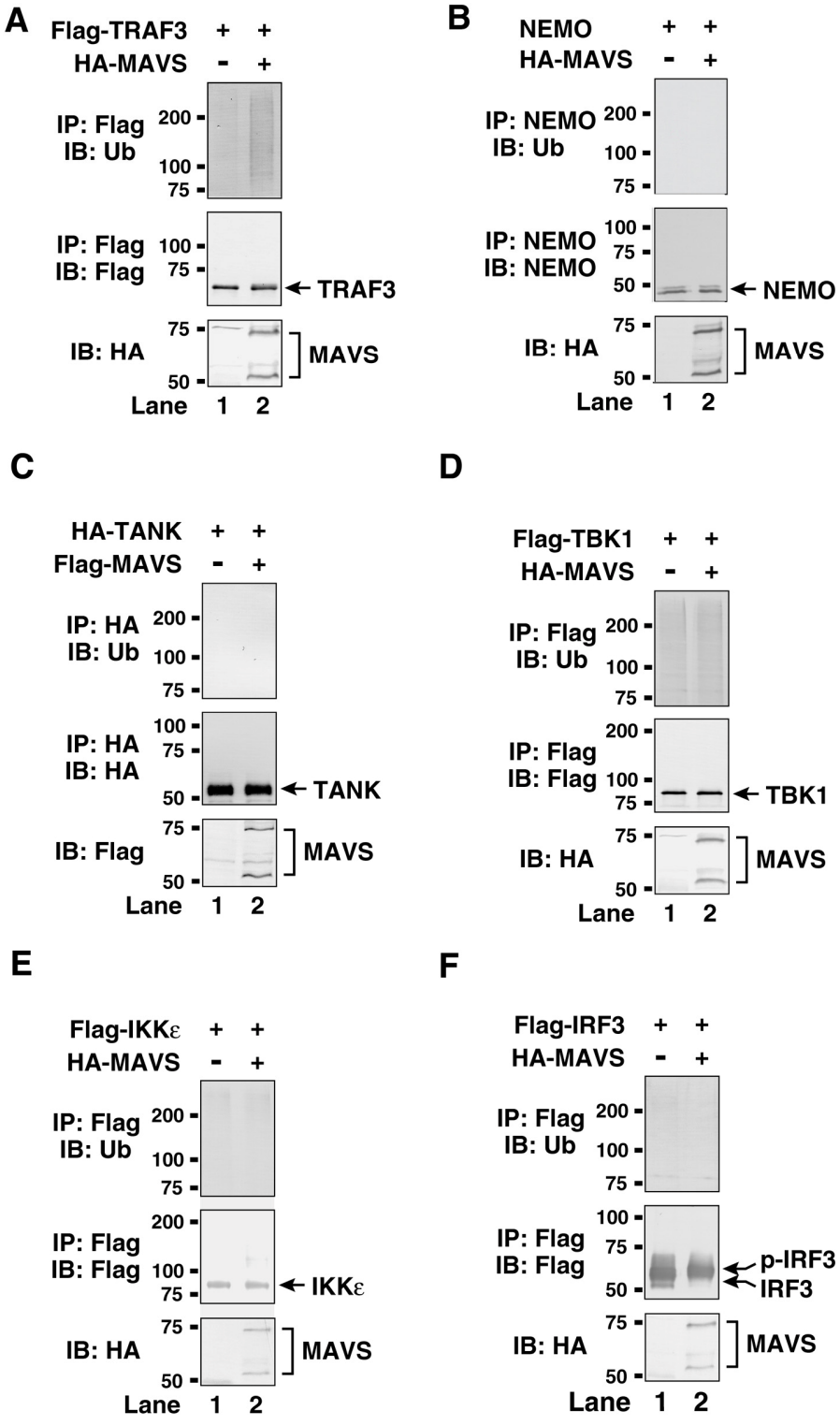

Figure S12. MAVS-Dependent Ubiquitination of TRAF3, but Not Other Signaling Proteins
HEK293T cells were transfected with expression vectors for TRAF3 (**A**), NEMO (**B**), TANK (**C**), TBK1 (**D**), IKKε (**E**) or IRF3 (**F**) together with MAVS as indicated. The expressed proteins were immunoprecipitated under a denaturing condition as described in the Experimental Procedures, then immunoblotted with the indicated antibodies.

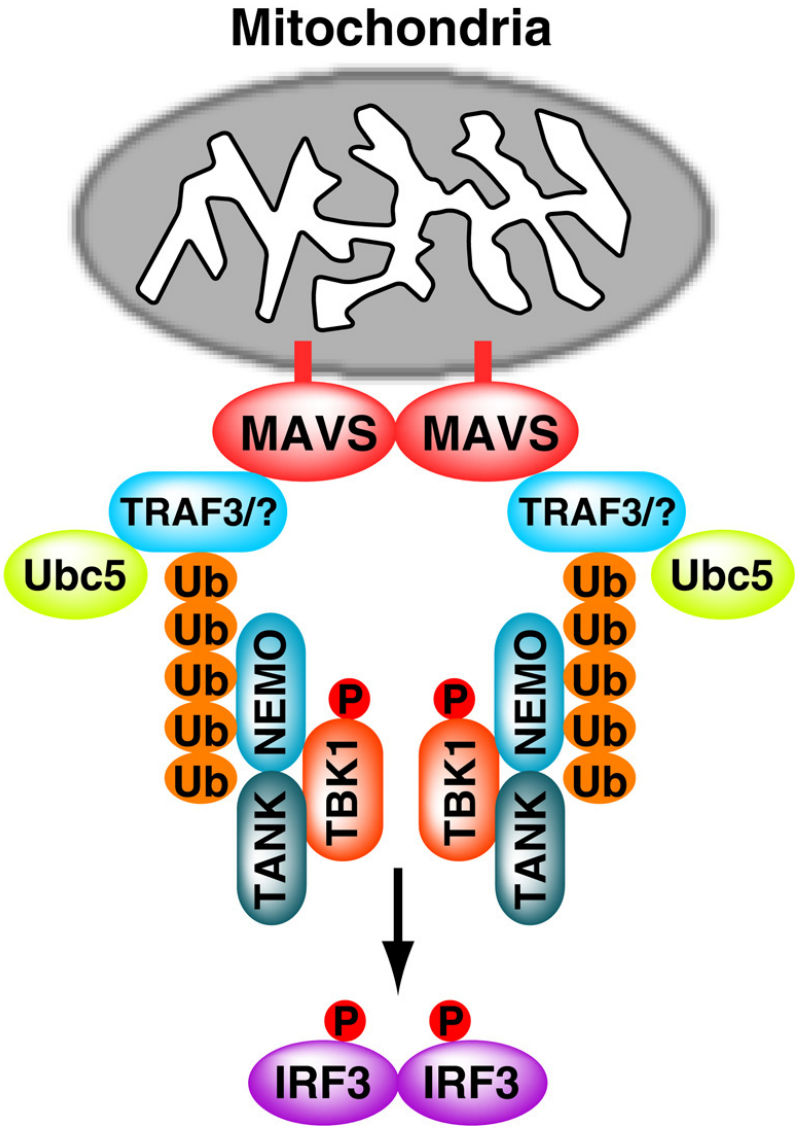

Figure S13. A Model of MAVS-Mediated Activation of IRF3 through K63 Polyubiquitination

Viral infection leads to the oligomerization of MAVS on the mitochondrial outer membrane. MAVS recruits TRAF3 and other ubiquitin ligases, which function together with Ubc5 to catalyze K63 polyubiquitination of target proteins including TRAF3 itself. The polyubiquitin chains recruit NEMO, which in turn binds to TANK and TBK1. TBK1 is then activated to phosphorylate IRF3, leading to the induction of type-I interferons.