Supplementary Fig. 1

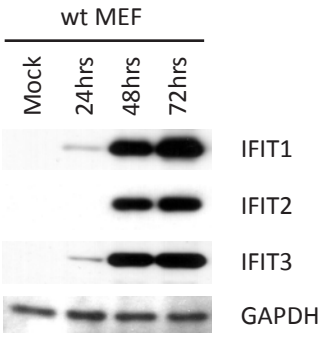

Fig. S1. IFIT1, IFIT2, and IFIT3 protein expression in WNV-infected wt MEF. Wt Bl6 MEF was mock-infected or infected with WNV-MAD at an MOI of 1. Protein lysate was collected at indicated times and immunoblotted using IFIT1, IFIT2, and IFIT3 antibodies. GAPDH was used as loading control.

Supplementary Fig. 2

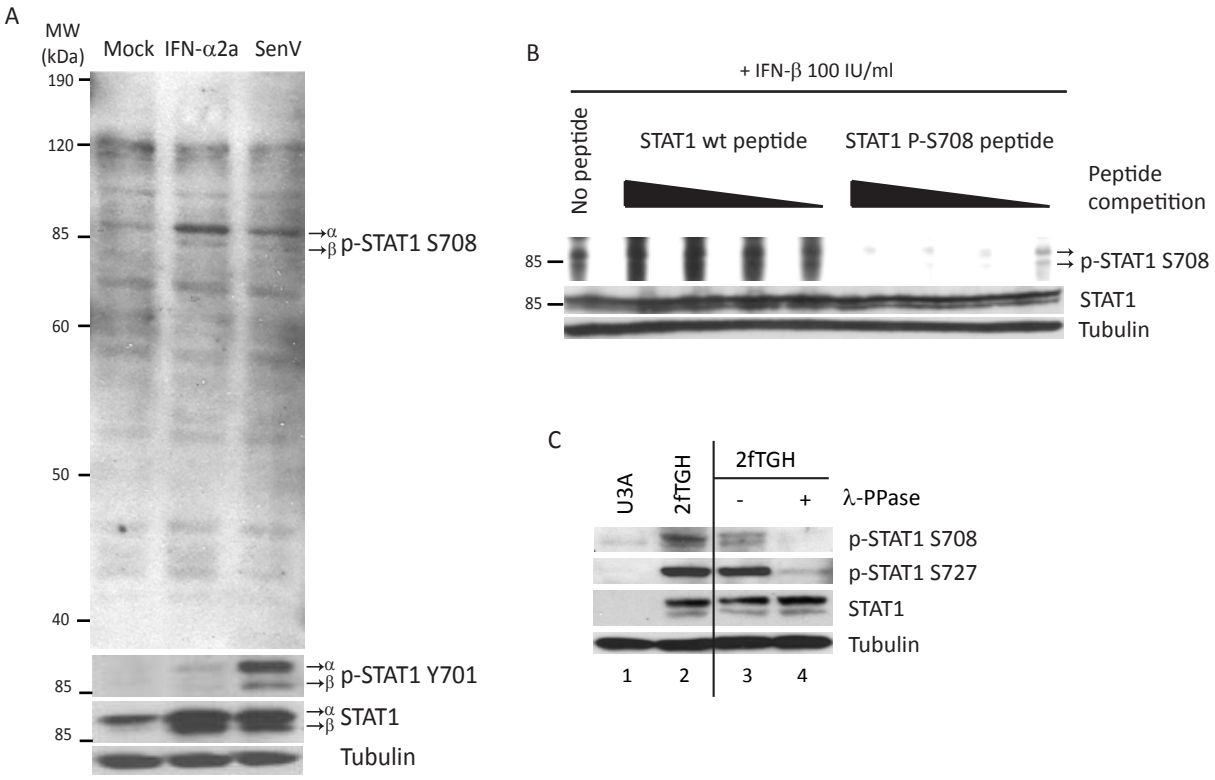

Fig. S2. Characterization of phospho-specific STAT1 S708 antibody. **(A)** Phosphospecific rabbit polyclonal antibody was generated against STAT1 peptide YIKTELI{pS}VSEVHP. The antibody was tested using immunoblotting against lysate from HEK293 cells treated with 100 IU/ml of IFN-α2a or infected with 100 HA U/ml SenV for 16 hours. Total and p-STAT1 Y701 antibodies were also used for immunoblotting. **(B)** A peptide competition assay was performed to determine specificity of the p-STAT S708 antibody. The p-STAT1 S708 antibody was pre-incubated with titrated dose of wt STAT1(701-714) peptide or S708-phosphorylated peptide (left-to-right: 0.5 µM, 0.05 µM, 0.005 µM, 0.0005 µM) overnight at 4°C. Antibody-peptide solutions were used in an immunoblot of IFN-β-stimulated HEK293 cells. The membrane was stripped and re-blotted; total STAT1 and Tubulin antibodies were included as controls. **(C)** Lambda-protein phosphatase (λ-PPase; New England BioLabs) was used to remove phosphate groups from serine residues, to further assess the crossreactivity the p-STAT1 S708 antibody with non-phosphorylated STAT1 protein. U3A (lane 1) and 2fTGH cells (lane 2-4) were treated with 100 IU/ml IFN-β for 24-hours. Following harvest, 2fTGH cell lysate was treated with buffer only (lane 3) or with 400U of λ-PPase (lane 4) for 1-hour at 30°C. p-STAT1 S708, p-STAT1 S727, and total STAT1 antibodies were used for immunoblotting.

Suplementary Fig. 3

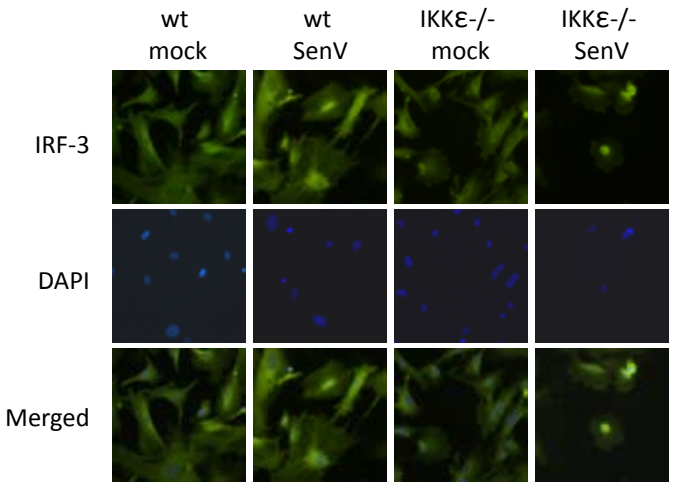

Fig. S3. Loss of IKKε does not affect IRF-3 activation. Wt Bl6 or IKKε-/- MEFs were mock-infected or infected with 100 HA U/ml SenV. At 16 hpi, cells were fixed and visualized by immunofluorescent staining with IRF-3.

Supplementary Fig. 4

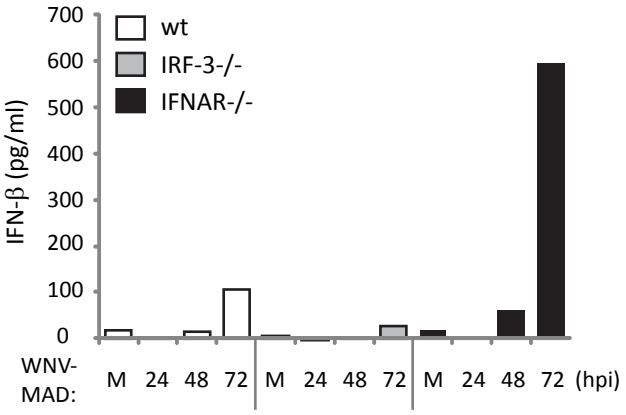

Fig. S4. IFN-β secretion of wt, IRF-3-/-, and IFNAR-/- MEFs following WNV-MAD infection. MEFs were mock-infected or infected with WNV-MAD at MOI of 1. Culture supernatant was collected at 24-, 48-, and 72-hours post infection (hpi) and subjected to IFN-β ELISA.

Supplementary Fig. 5

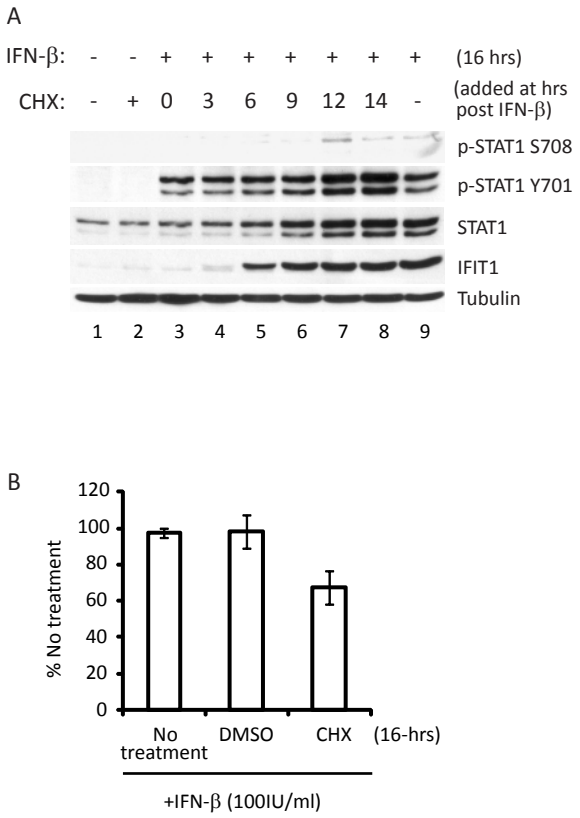

Fig. S5. STAT1 S708 phosphorylation requires *de novo* protein synthesis. **(A)** 2fTGH cells were mock-stimulated (lane 1-2) or stimulated with 100IU/ml IFN-β (lane 3-9). At various points following addition of IFN-β to culture supernatant, cells were chased with CHX (0 hr: CHX was added at time of IFN-β addition; –: no CHX). Cells were harvested at 16 hours post-mock or IFN stimulation and immunoblotted using p-STAT1 S708, p-STAT1 Y701, total STAT1, and IFIT1 antibodies. **(B)** 2fTGH cells were cultured in the presence of IFN-β only (No treatment), IFN-β and DMSO (DMSO), or IFN-β and CHX (CHX) for 16-hours. CHX toxicity in cells treated with IFN-β was assessed using mitochondrial toxicity test (MTT; Sigma). Absorbance at 570nm was read and normalized to background absorbance at 630nm. Average value from DMSO and CHX-treated cells were compared to those of non-treated cells (set as 100%) and graphed. Error bars represent standard deviations of triplicate experiments.

Supplementary Fig. 6

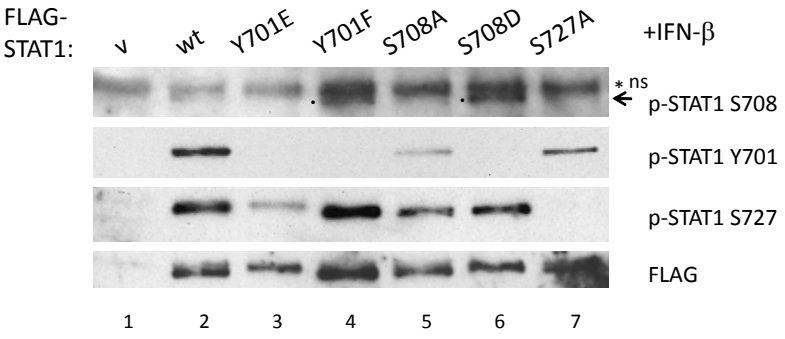

Fig. S6. STAT1 phosphorylation at Y701 and S708 are mutually exclusive. U3A cells lacking STAT1 were vector transfected, or reconstituted by transient transfection of FLAG-STAT1 wt, Y701E, Y701F, S708A, S708D, or S727A. At 16-hours post-transfection, cells were stimulated with IFN-β and then harvested 16 hours later. Reconstituted STAT1 was immunoprecipitated using FLAG-M2 agarose beads and eluates were immunoblotted using antibodies against p-STAT1 S708, p-STAT1 Y701, p-STAT1 S727, and FLAG (*, non-specific band; •, specific STAT1 S708P band).