Supporting Information

Ketscher et al. 10.1073/pnas.1412881112

Viral Infections

Vaccinia Virus (VACV) Infections. VACV wild-type Western Reserve strain (WR) was grown on BSC-40 cells and purified as described (1). USP18$^{C61A/C61A}$ or WT mice were infected intranasally (i. n.) in 20 μL PBS with VACV at 10^5 pfu/mouse. Organs from inoculated animals were homogenized in DMEM (0.1 mg of tissue/mL), and assayed in triplicate for viral yield by standard plaque assay in BSC40 cells.

Influenza Virus Infections (Mice). For influenza B virus experiments 6 to 8 week old male mice were infected i.n. with 1×10^6 pfu of influenza B/Yamagata/88 diluted in 25 μL of PBS. Mice were monitored for weight loss and disease progression daily. For the determination of viral titers, mice were sacrificed on day 3 post infection, lungs were homogenized, and viral titers were determined as previously described (2).

Influenza Virus Infections (mTECs). For viral growth curves, mTECs were either left untreated or pre-treated with 30 U/mL of IFN-β in the basolateral chamber. After 24 h, the basolateral chamber was replaced by media lacking IFN-β before adding 9.0×10^5 pfu of influenza B/Yamagata/88 in 0.1 mL of DMEM with 1% penicillin and 1% streptomycin (1% P/S) to the apical chamber. After 1 h, the virus containing media was removed and the apical chamber was washed three times with 0.2 mL DMEM 1% P/S. Then 0.1 mL of DMEM 1% P/S was added back to the apical chamber. At various times apical media was assayed for viral titers, and replaced to allow infection to continue.

1. Guerra S, et al. (2003) Cellular gene expression survey of vaccinia virus infection of human HeLa cells. *J Virol* 77(11):6493–6506.

2. Lenschow DJ, et al. (2007) IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci USA* 104(4):1371–1376.

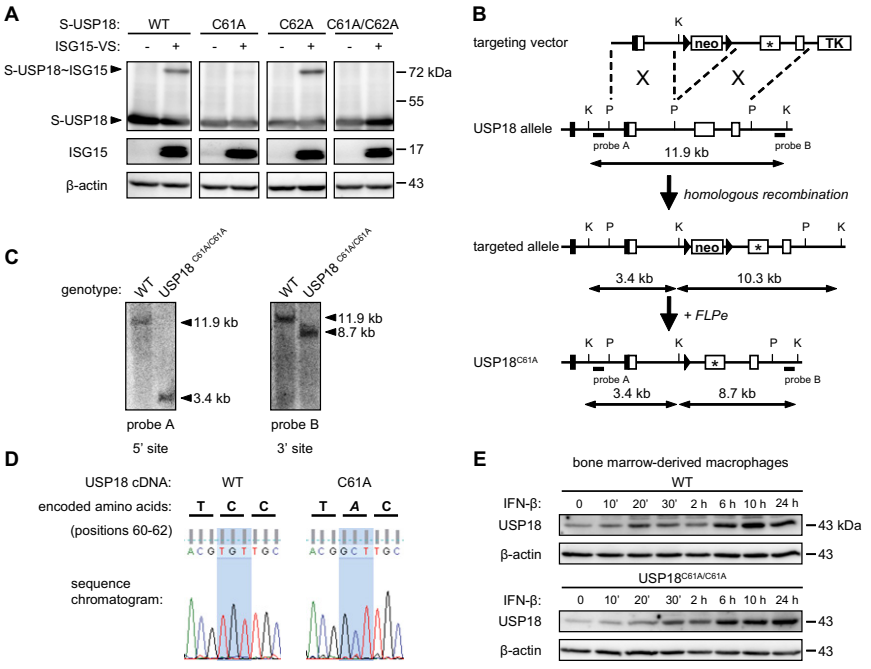
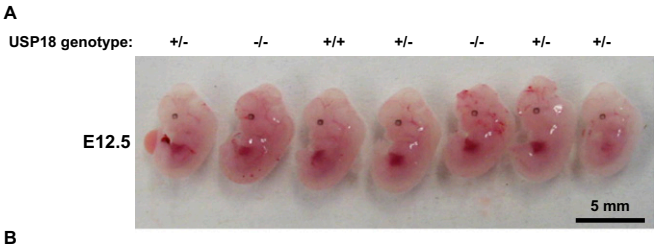
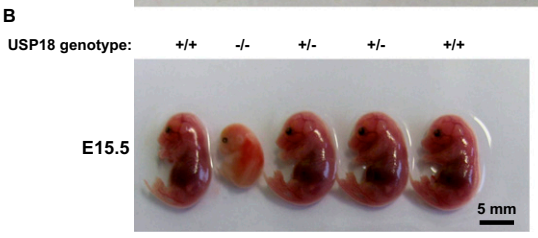


Fig. S1. Generation of mice expressing catalytically inactive USP18. (*A*) Either WT or mutants of S-tagged mUSP18 protein where cysteine (Cys) at position 61 (C61A), Cys62 (C62A), or Cys61 and Cys62 (C61A/C62A) was mutated to alanine, were overexpressed in HEK293T cells and protein lysates were incubated with ISG15-VS probes. ISG15-VS adduct formation, indicating isopeptidase activity toward ISG15, was detected by a shift upon immunoblotting with anti–S-Tag antibody. (*B*) Targeting strategy of the USP18C61A mutation. A KpnI fragment with the Cys61 coding region was subcloned from USP18 clone (RPCI-21 332G10) and the codon for Cys61 5′-TGT-3′ was mutated to 5-GCT-3′ encoding alanine. A 5′-homology arm and the 3′-homology with the mutation were inserted in pPNT-frt3 up- and downstream of the neomycin resistance gene (neo), respectively. The 5′-homology of the targeting vector includes exon (boxes) two with untranslated (black) and translated region (white). The 3′-homology harbors exons 3 and 4. The vector contained a neomycin resistance gene (neo) flanked by flippase (flp) recognition target (frt) sites (black triangles) and a thymidine kinase gene (TK). The codon for cysteine (C61) 5′-TGT-3′ in exon 3 of USP18 replaced by the alanine (Ala) encoding triplett 5′-GCT-3′ is indicated by an asterisk. Homologous recombination and excision of neo by frt-flp recombination can be detected by Southern analysis of KpnI (K)-digested genomic DNA as indicated. The resulting target vector was linearized and transfected into E14.1 ES cells. (*C*) Southern analysis of the mutated USP18 gene locus in mice. Successful mutation of the USP18 gene was verified upon KpnI restriction digest of genomic DNA using a 5′-probe A and a 3′-probe B for hybridization as indicated in Fig. 1*B*. Positive ES cells were injected into C57BL/6 morulae and germ-line chimeras were interbred with a FLP deleter strain to eliminate neo. Mice homozygous for the USP18C61A mutation exhibited the expected 3.4-kb and 8.7-kb KpnI fragments detected by probes A and B, respectively. (*D*) Cys61 to Ala (C61A) mutation of USP18 in mutant mice was confirmed by sequencing of cDNA derived from IFN-stimulated BMMs. (*E*) Normal induction of USP18C61A protein in IFN-β–treated bone marrow derived macrophages (BMMs). Cells were stimulated with 250 units/mL IFN-β and USP18 protein expression was analyzed by immunoblotting.





C

genotype	quantity	percentage
$USP18^{+/+}$	38	28.8 %
$USP18^{+/-}$	87	65.9 %
$USP18^{-/-}$	4	3.0 %
n. d.	3	2.3 %

Fig. S2. USP18 deficiency leads to embryonic lethality between E15.5 and birth on a C57BL/6 background. To generate $USP18^{-/-}$ mice on a homogenous C57BL/6 background $USP18^{+/-}$ mice were backcrossed to C57BL/6 for 10 generations and then used to set up intercrosses. (*A*) Embryos derived from $USP18^{+/-} \times USP18^{+/-}$ matings at E12.5 were genotyped as depicted and showed no obvious abnormalities, indicating that lack of USP18 does not interfere with early embryonic development. (*B*) Embryos at E15.5 of indicated genotypes were derived from heterozygous matings. Severe growth retardation was observed in embryos lacking USP18 (no. 2). (*C*) Genotype distribution in the offspring of $USP18^{-/+}$ intercrosses on a C57BL/6 background at birth. Only 3% of all pups examined exhibited a $USP18^{-/-}$ genotype indicating embryonic lethality. n. d., not determined.

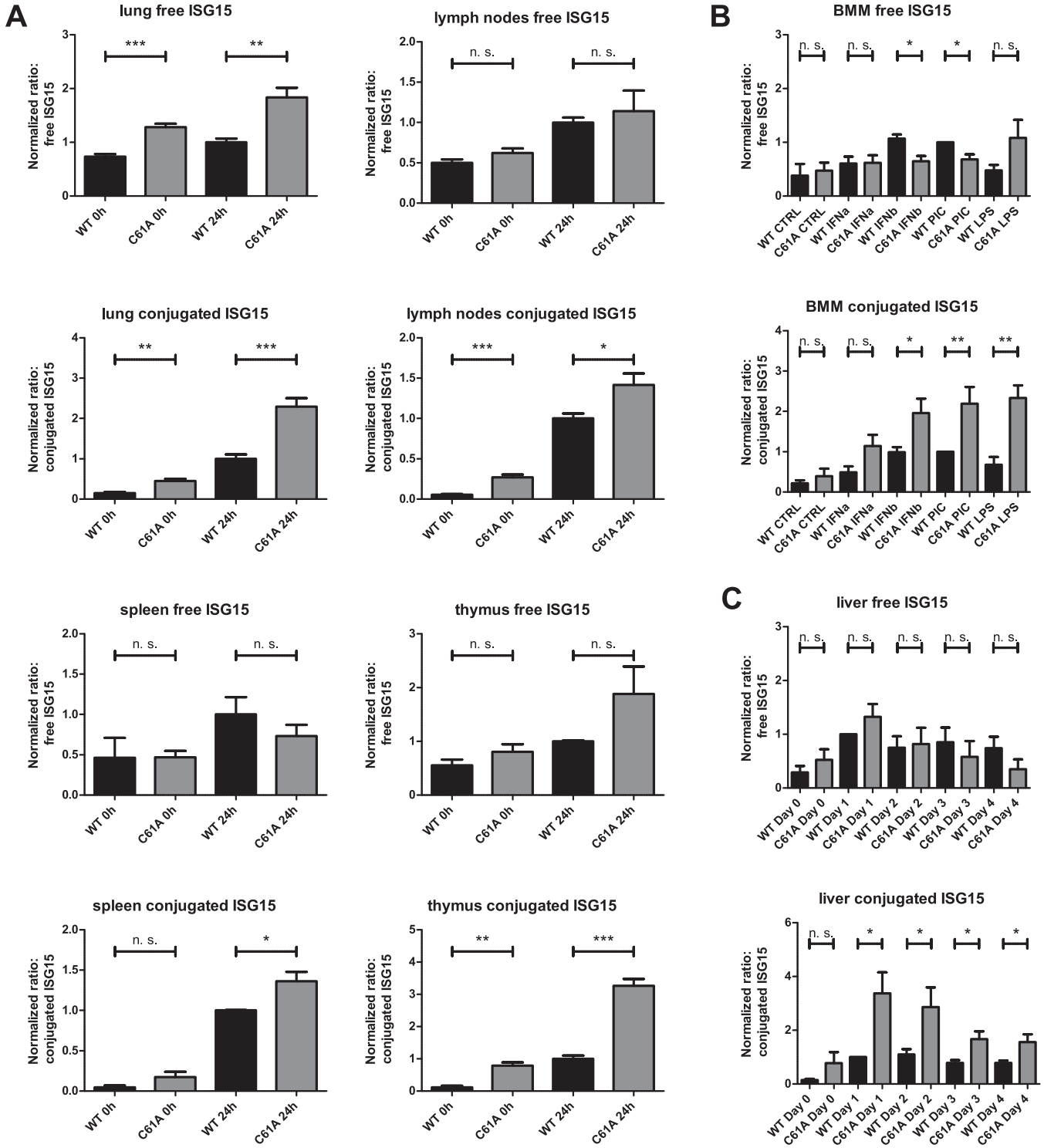


Fig. S3. Densitometric and statistical analysis of Western blots from Fig. 1. Results were obtained from three to five independent experiments. Densitometric analysis was performed using ImageJ. Quantification of ISGylation was performed by normalizing the ratio of ISG15 over loading control relative to the sample of WT 24 h after stimulation with poly(I:C). Mean values are depicted +SEM (standard error of mean). Statistical analysis was performed with an unpaired two-tailed Students *t* test with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (*A*) Analysis in indicated organs upon treatment with poly(I:C) for 24 h. (*B*) Analysis in bone marrow derived macrophages upon treatment with indicated stimuli for 24 h. (*C*) Analysis in liver upon treatment with poly (I:C) for indicated time periods. n. s., not significant.

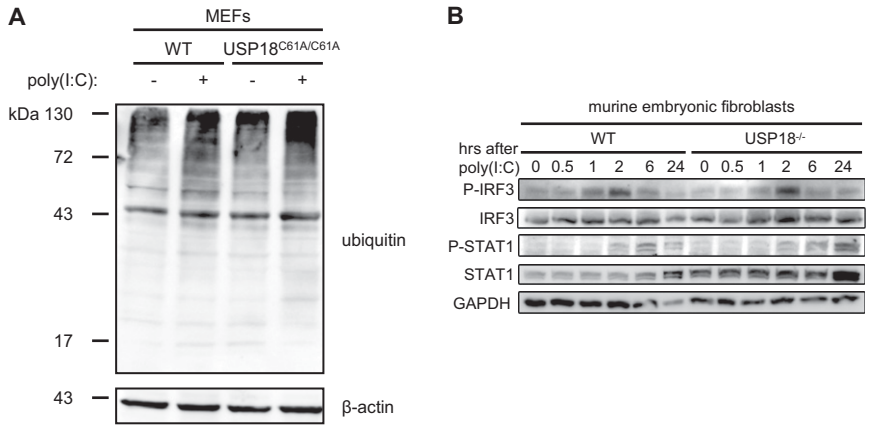


Fig. S4. Unaltered polyubiquitination in USP18$^{C61A/C61A}$ MEFs. Unaltered IRF3-P in USP18$^{-/-}$ MEFs. (*A*) Wild-type and USP18$^{C61A/C61A}$ MEFs were stimulated with 25 μg/mL poly(I:C) for 24 h or left untreated. Protein lysates were analyzed for ubiquitin modified substrates by immunoblotting using a ubiquitin-specific antibody (P4D1). (*B*) Wild-type and USP18$^{-/-}$ MEFs were stimulated with 25 μg/mL poly(I:C) for the indicated times and protein lysates were subsequently analyzed by Western blotting using the indicated antibodies.

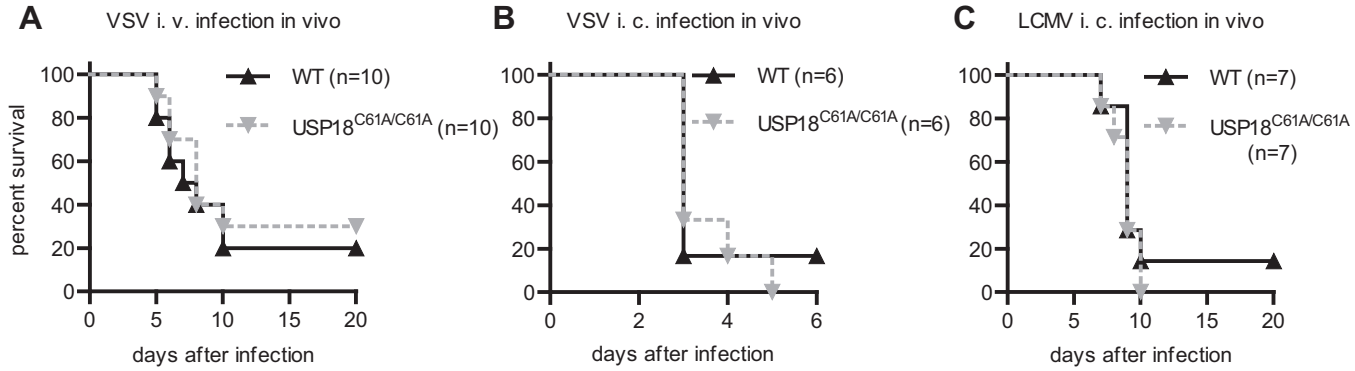


Fig. S5. VSV and LCMV infections in vivo. (*A* and *B*) VSV infection in vivo showed no significant differences between WT and USP18$^{C61A/C61A}$ animals. Indicated numbers of WT or USP18$^{C61A/C61A}$ mice were i.v. (10^8 pfu) or i.c. (10 pfu) injected with VSV (strain Indiana) in 300 μL PBS. Survival was monitored daily. (*C*) Survival of LCMV-infected USP18$^{C61A/C61A}$ mice was not altered in comparison with WT animals. Groups of seven WT or USP18$^{C61A/C61A}$ mice were infected intracerebrally with 10^5 IU of LCMV, strain WE, and monitored daily for survival.

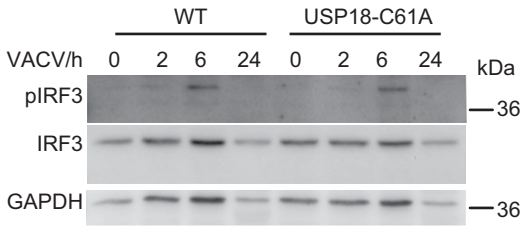


Fig. S6. IRF3 phosphorylation in USP18$^{C61A/C61A}$ MEFs is unaltered upon VACV infection. WT and USP18$^{C61A/C61A}$ MEFs were infected with 1 unit per cell VACV for indicated times and IRF3 phosphorylation was monitored by immunoblotting.

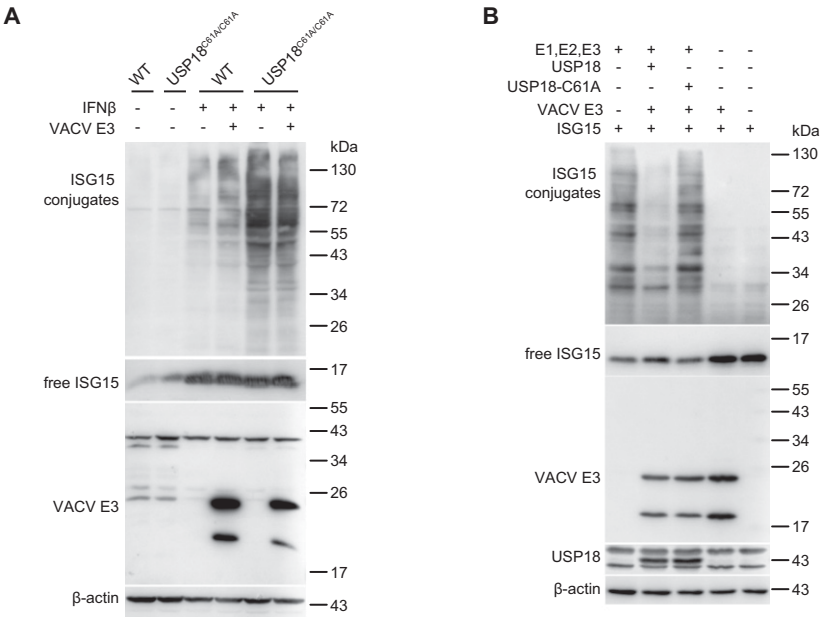


Fig. S7. Vaccinia virus E3 is not modified by ISG15 upon overexpression in USP18$^{C61A/C61A}$ cells. (*A*) MEFs isolated from wild-type and USP18$^{C61A/C61A}$ mice were transfected with a vector encoding VACV E3 protein or with empty vector as a control using Xtreme Gene (Roche). Twenty-four hours after transfection, the cells were stimulated with IFNβ (250 units/mL) for an additional 24 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (*B*) HEK293T cells were transfected with expression vectors for Ube1L (E1), UbcH8 (E2), Herc6 (E3), ISG15, and VACV E3 to induce ISGylation. Where indicated vectors encoding for USP18 or USP18-C61A were added. Forty-eight hours after transfection, cells were lysed and analyzed by immunoblotting with the indicated antibodies.

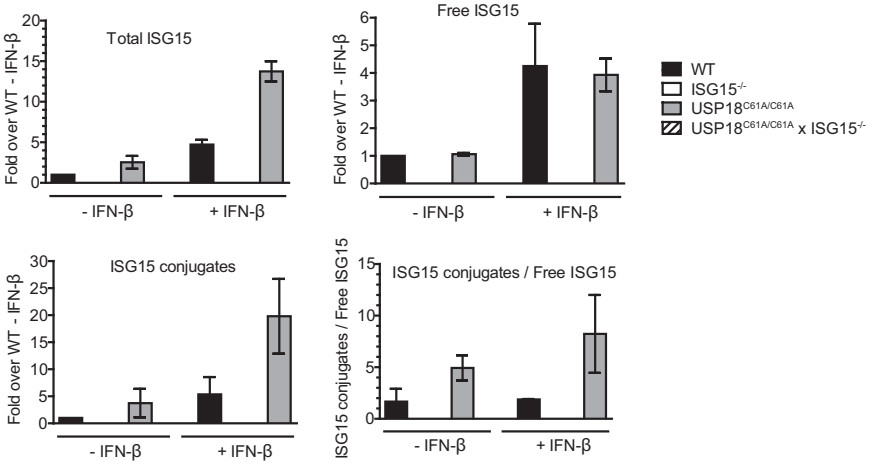


Fig. S8. Densitometric analysis of Western blot analysis from Fig. 5*E*. Data were pooled from two independent IFN stimulations. Densitometry analysis was performed using Adobe Photoshop CS4 histogram analysis.

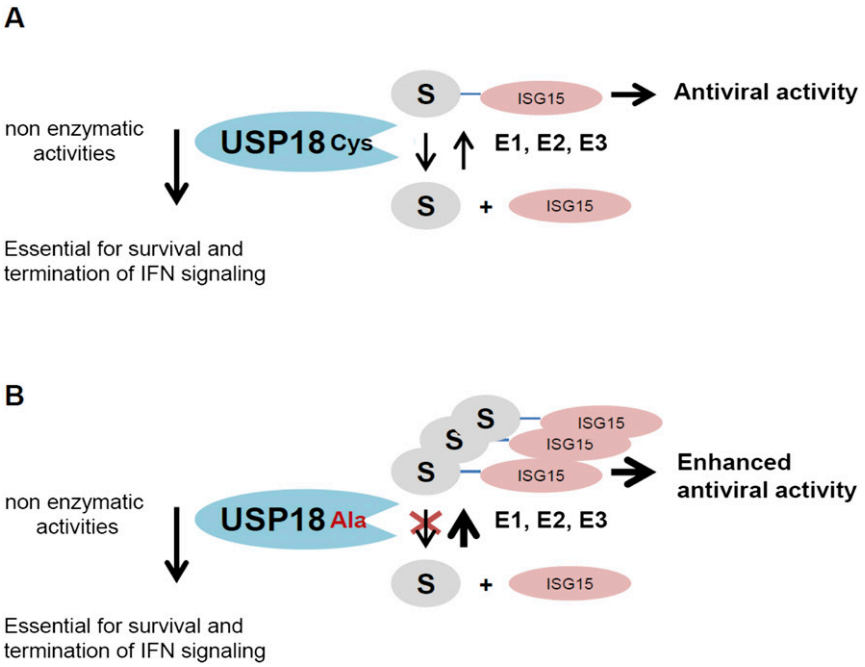


Fig. S9. Model for protease-dependent and -independent functions of USP18. (*A*) USP18 exerts enzymatic and nonenzymatic functions in vivo. The isopeptidase function is essential for ISG15 deconjugation and thus counteracts ISG15 conjugation to target substrates (S) mediated by E1, E2, and E3 conjugation. In contrast, nonenzymatic functions are necessary to prevent lethality and ensure proper termination of IFN signaling in vivo. (*B*) Loss of USP18 isopeptidase function by specific mutation of the active site cysteine (Cys) 61 to alanine (Ala) inactivates the ISG15-deconjugating activity but does not interfere with nonenzymatic functions of USP18, which are essential to prevent the dramatic phenotypic alterations seen in USP18$^{-/-}$ mice. Consequently ISGylation is selectively enhanced, resulting in increased viral resistance without affecting viability and/or poly(I:C) sensitivity.