Supplementary Materials for

Nlrp6 regulates intestinal antiviral innate immunity

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Materials and Methods

Cell culture and viruses

Human embryonic kidney 293 cells transformed with T antigen of SV40 (HEK293T), *Homo sapien* lung carcinoma cells (A549), EMCV (VR-129B) and Sendai virus (SeV, Cantell strain, VR-907) were purchased from American Type Culture Collection (ATCC) (Manassas, VA20110 USA). Murine norovirus-1 (MNV-1) was a kind gift of Christiane Wobus at the Department of Microbiology and Immunology, University of Michigan. MNV-1 was grown in RAW264.7 cell culture, and purified by ultracentrifugation on a 30% sucrose cushion, titrated on RAW264.7 monolayer as described previously (*31*). HEK293T cells were grown in DMEM (Life Technologies, Grand Island, NY 14072 USA) supplemented with 10% FBS and antibiotics/antimycotics. A549 cells were cultured in F12K (Life Technologies) supplemented with 10% FBS, glutamine and antibiotics/antimycotics. For preparation of mouse embryonic fibroblasts, pregnant females were euthanized on day 14 of gestation. Heterologous and homogenous fibroblasts were isolated from the same littermates (pooled from 3 mice per genotype). Embryos were decapitated and eviscerated then digested with trypsin for 10 min at 37 °C rotating. Fibroblasts were filtered through 100 μM filters, cultured in MEM medium (Life Technologies, NY 14072 USA) supplemented with 10% fetal bovine serum and antibiotics/antimycotics, propagated for 2 passages and then frozen (*32*).

Animals and infection

All animals were housed in a state-of-the-art animal facility at Yale University. Age and sex-matched animals (6-12 weeks old C57BL/6 background) were infected with either 40 plaque forming unit (PFU) via intra-peritoneal injection or 1 x 10^5 PFU of EMCV (two doses on day 0 and day 1) via oral gavage. For cohousing experiments, 4-6 weeks-old wild type (C57BL/6) and *Nlrp6*$^{-/-}$ (*15*) mice were housed in the same cages at a 1:1 ratio for 2 weeks to a month. FLAG-Nlrp6 mice were generated in our facility by inserting FLAG or triple FLAG tag sequence right before the stop codon of Nlrp6. Animals were thoroughly perfused with 50 mL of 1x PBS prior to tissue analyses. All animal protocols were approved by the Yale University Institutional Animal Care & Use Committee (protocol 10404).

Isolation of intestinal epithelial cells (IECs)

Small intestines and colons were washed thoroughly with 1 x phosphate buffered saline (PBS) and cut longitudinally. The tissues were then incubated in 15 mL 1 mM EDTA in Hank's Balanced Salt Solution (HBSS) for 30 min at 37°C with shaking, and washed three times with 10 mL of 1 x PBS. IECs were collected by brief centrifugation.

Reagents and antibodies

The rabbit anti HA-tag (Cat # 3724), anti Myc-tag (Cat # 2278), anti GAPDH (Cat # 5174), rabbit anti-MAVS (Cat# 4983) antibodies were purchased from Cell Signaling Technology (Danvers, MA 01923 USA). The mouse anti-DHX15 (Cat # sc-271686), mouse anti-Oas1a (Cat # sc-365357) and rabbit anti-Isg56 (Cat# sc-134949)

were obtained from Santa Cruz Biotechnology (Santa Cruz, CA 95060). The Pierce Magnetic RNA-protein pull-down kit (Cat# 20164), In vitro translation kit (Cat# 88891), Lipofectamine 2000, DHX15 siRNA (ID# s4028, s4029) and GFP siRNA (AM4626), and HBSS buffer were from Thermo Fisher Scientific. Mouse Nlrp6 siRNA (Cat# SR414599) was purchased from OriGene (Rockville, MD20850, USA). M2 agarose beads (Cat# A2220), anti-FLAG antibody (Cat# F3165), 3x FLAG peptide (Cat# F4799), anti-Nlrp6 (Cat# SAB1302240) antibody, anti-HA (Cat# 3663) were available at Sigma-Aldrich (St. Louis, MO63103 USA). IFN ELISA kits and recombinant mouse IFN-α were from PBL Assay Science (Piscataway, NJ 08854 USA). Heavy/low molecule weight poly (I:C), poly d (A:T), Biotin-poly (I:C) and 5ppp dsRNA were purchased from Invivogen (San Diego, CA92121 USA). Recombinant mouse IFN-λ2 was purchased from R&D Systems (Cat# 4635), mouse IFN-λ2/3 (IL-28) ELISA kit from eBiosciences (Cat# BMS6028). Glutathione sepharose 4B beads (Cat# 17-0756-01) were from GE Health Life Sciences.

Construction of expression plasmids and transfection

Mouse Nlrp6 (NCBI accession #: NM_133946.2, NP_598707), human NLRP3 and mouse Dhx15 (NCBI accession #: NM_ NM_007839.3, NP_031865.2) were cloned into pcDNA3.1-FLAG vector. Myc-MAVS, FLAG-RIG-I, FLAG-MDA5 (*33*) and HA-STING (*34*) have been reported previously. For transfection of A549 or MEFs, 2-10 μg of plasmid DNA was electroporated into 2-4 x 10^6 cells using cell type specific transfection reagents (Lonza Inc., Walkersville, MD). About 80% transfection efficiency and 50% recovery of live cells were achieved. For transfection of HEK293T cells, Lipofectamine 2000 was used.

Biochemical assays

FLAG-immunoprecipitation was performed essentially according to the manufacturer's manual. Briefly, HEK293T cells were transfected with expression plasmids using Lipofectamine 2000. At 24 h post-transfection, HEK293T were lyzed in lysis buffer (50 mM Tris.HCl, pH 7.4, 0.5% NP-40, 2 mM EDTA, and 150 mM NaCl, with complete protease inhibitors). Lysates were cleared by centrifugation and then incubated for 2 h with M2 agarose beads (Sigma-Aldrich, St. Louis, MO). After 4 washes with lysis buffer and 2 washes with (Tris buffered saline (TBS), proteins bound to M2 beads were eluted using 3 x FLAG peptides (Sigma-Aldrich, St. Louis, MO). In the cases of protein-RNA binding, 24 h after plasmid DNA transfection, HEK293T cells were infected with EMCV or herpes simplex virus-1 (HSV-1) for 24 h. The cell lysates were then subject to FLAG-IP and the final 3 x FLAG elutes were used for RNA purification using QIAGEN RNeasy Mini Kit. The purified RNA was then reverse-transcribed and EMCV D3 or HSV-1 polymerase genes were quantified by SybrGreen PCR.

For co-immunoprecipitation of MAVS and Dhx15 from EMCV-infected IECs, WT and *Nlrp6*$^{-/-}$ mice were infected with 1000P.F.U of EMCV i.p, on day 3 after infection, IECs were purified and lyzed in lysis buffer (50 mM Tris.HCl, pH 7.4, 1% Triton X-100, 2 mM EDTA, and 150 mM NaCl, with complete protease inhibitors). Lysates were cleared by centrifugation, and 10 mg total proteins were incubated with a rabbit anti-mouse MAVS antibody (Cell Signaling, Cat# 4983, at 1:50 dilutions) at 4°C overnight. 20 μL of protein A/G beads was added to the reaction mix and incubated at

4°C for 1 hour. After stringency wash with lysis buffer, bound proteins were eluted from protein A/G beads in SDS-PAGE sample buffer. Bound Dhx15 was probed with a mouse anti-Dhx15 antibody (Santa Cruz Biotech, Cat # sc-271686).

For the GST pull-down assay, GST and GST-Dhx15 were expressed in *E. coli* and purified using glutathione sepharose beads. FLAG-Nlrp6 was expressed using a Thermo Scientific 1-Step *in vitro* Translation system. Sepharose-bound GST proteins were incubated with FLAG-Nlrp6 at 4°C overnight, and then washed with 1x PBS 4 times. GST-Dhx15 and its bound FLAG-Nlrp6 were then eluted with 50mM reduced glutathione.

RNA-protein pull-down was performed with a Pierce Magnetic RNA-protein pull-down kit (Cat# 20164). FLAG fusion proteins were expressed in HEK293T cells, purified using M2 beads and eluted with 3x FLAG. The following RNA labeling with biotin and RNA-protein binding steps were exactly the same as described in the product manual.

PCR array

Type I IFN response (Cat# PAMM-016Z) and antiviral response (Cat# PAMM-122Z) PCR arrays were purchased from QIAGEN (Valencia, CA 91355, USA). Intestinal epithelial cells (IECs) were isolated from WT and *Nlrp6*$^{-/-}$ intestines 3 days after EMCV infection (100 P.F.U) via the intraperitoneal route. RNA was extracted from IECs using a QIAGEN RNA easy kit, treated with DNase I and reversed-transcribed. PCR array was performed following the manufacturer's manual.

Quantitative RT-PCR (q-PCR) and plaque forming assays

q-PCR was performed using gene-specific primers and 6FAM-TAMRA probes or inventoried gene expression kits from Applied Biosystems (6FAM-MGB probes). q-PCR primers for human *IFNA1*, *IFNB1, IL6* have been reported previously (*32, 33, 35*).

Plaque forming assays with tissues, cell culture medium or plasma were performed as previously described (*35, 36*). Briefly, 100 µl of samples diluted with sterile PBS by 10-100 folds, or 30-100 µg (total proteins) of tissue lysates triturated in sterile PBS were applied to confluent Vero cells. Plaques were visualized using Neutral red (Sigma-Aldrich) after 1-3 days of incubation at 37 °C 5% CO_2.

For quantification of mouse stool bacterial loads, stool DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN, Germantown, MD). Primers for Prevotellaceae detection were 5'--CCAGCCAAGTAGCGTGCA-3' and 5'-TG GACCTTCCGTATTACC-3'; for TM7 were 5'-GCAACTCTTTACGCCCAGT-3' and 5'-GAGAGGATGATCAGCCAG-3'. Results were normalized against mouse beta-actin and expressed as fold change over WT levels.

For quantification of stool MNV-1, stool was suspended in 0.5mL 0.9%NaCl; suspension was cleared by centrifugation, filtered using a 0.22µM filter. 0.14mL filtrate was used as starting material following the Viral RNA Mini Spin Protocol (QIAGEN, Germantown, MD). The primers for MNV-1 quantification were sense primer 5'-GTGCGCAACACAGAGAAACG-3', antisense primer 5'-CGGGCTGAGCTTCCTGC-3', and probe 5'-FAM-CTAGTGTCTCCTTTGGAGCACCTA-3'-TAMRA-FAM (*37*).

Graphs and Statistics

Survival curves, charts and statistical analysis were performed using PRISM 4 software (Graphpad Software, San Diego, CA). The significance of results was analyzed using unpaired two-tailed Students' t-test or nonparametric Mann-Whitney test, with a cut-off p value of 0.05.

Author notes

P.W. and S.Z. performed the majority of the experimental procedures. L.Y., S.C., W.P., R.J., Y.Z., A.R., Q.S., G.Y. and S.G. contributed to some of the experiments. R.L. provided technical support. P.W., S.Z., R.A.F., and E.F. conceived, designed and/or analyzed the data. P.W., S.Z., R.A.F., and E.F. wrote the paper and all the authors reviewed and/or modified the manuscript.

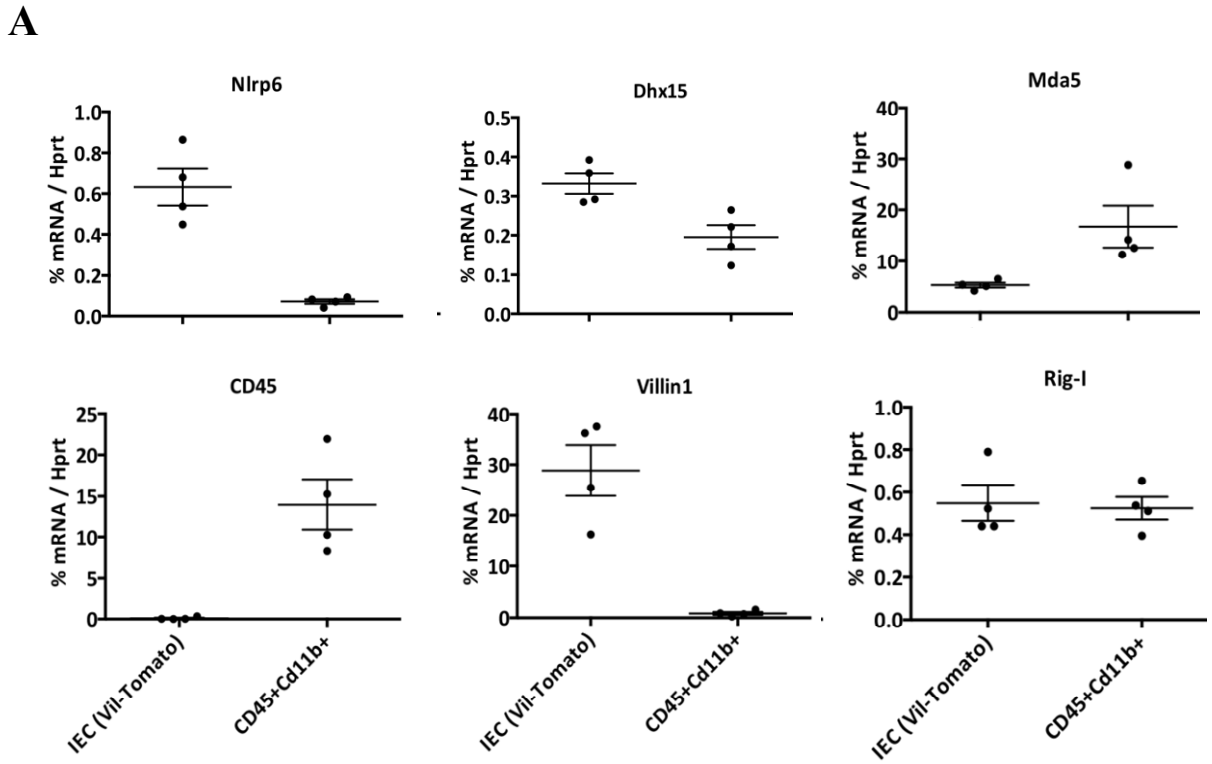


Fig. S1. Nlrp6 is exclusively expressed in the intestinal tissues. **(A)** Quantitative PCR analyses of indicated gene transcripts in purified intestinal epithelial cells (IECs) and intestinal $CD45^{+}CD11b^{+}$ cells. **(B)** Immunoblotting analysis of FLAG-Nlrp6 expression in various tissues of FLAG-Nlrp6 knock-in mice (see Methods for details). The data are representative of 2-3 independent experiments

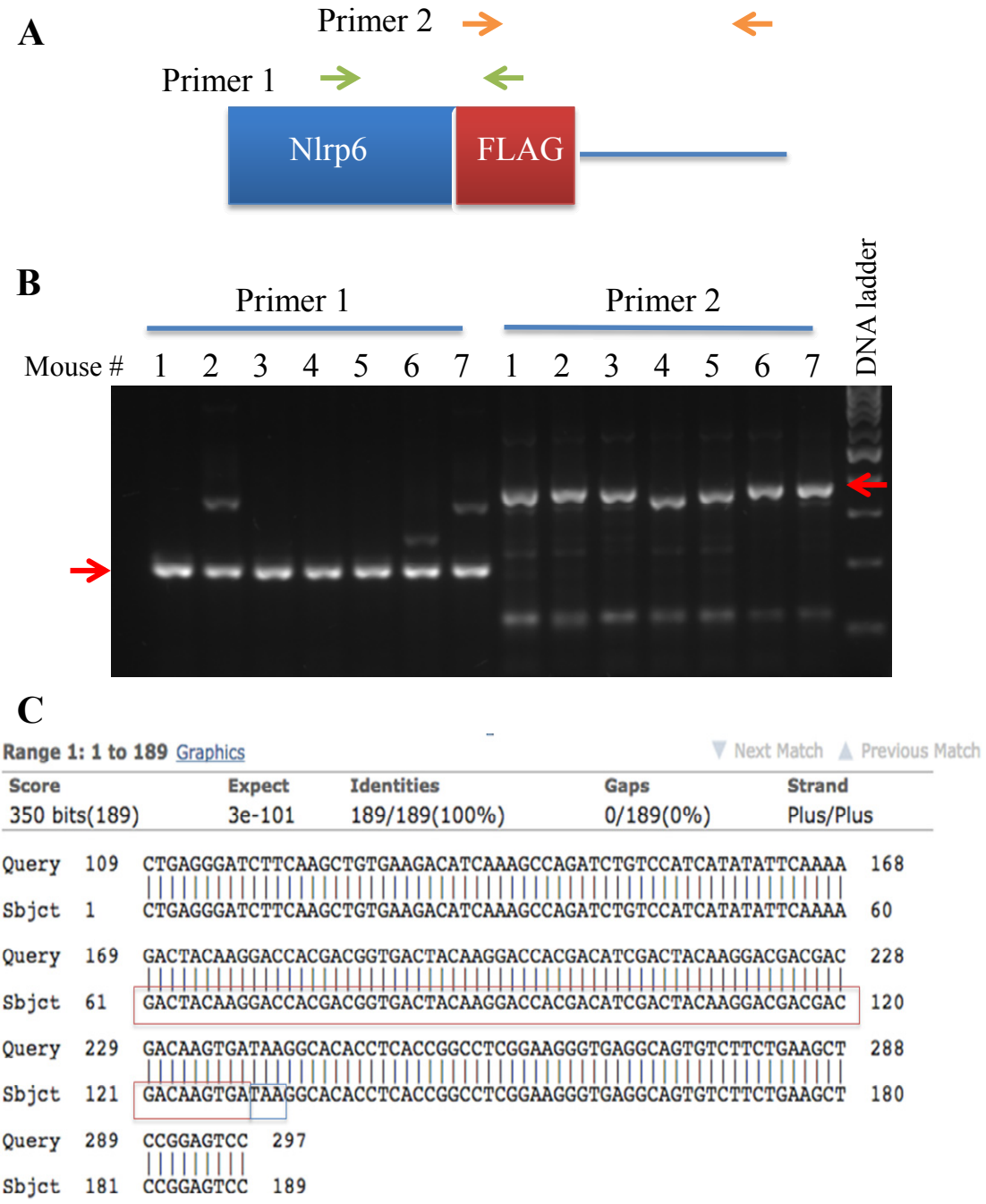


Fig. S2 Genotyping and sequencing of FLAG-Nlrp6 knock-in mice. FLAG-Nlrp6 mice were generated in our facility by inserting the FLAG sequence right before the stop codon of Nlrp6. **(A)** Primer design. Primer 1: the forward is a part of Nlrp6 gene, the reverse is the FLAG sequence. Primer 2: the forward is the FLAG sequence, the reverse is a part of genome. **(B)** An agarose DNA gel showing the PCR products of Primer 1 and 2. Red arrows indicate the PCR DNA bands. **(C)** The representative sequencing result (mouse # 1) showing the correct insertion of 3copies of FLAG (in the red box) right before the stop codon (in the blue box) of Nlrp6 gene.

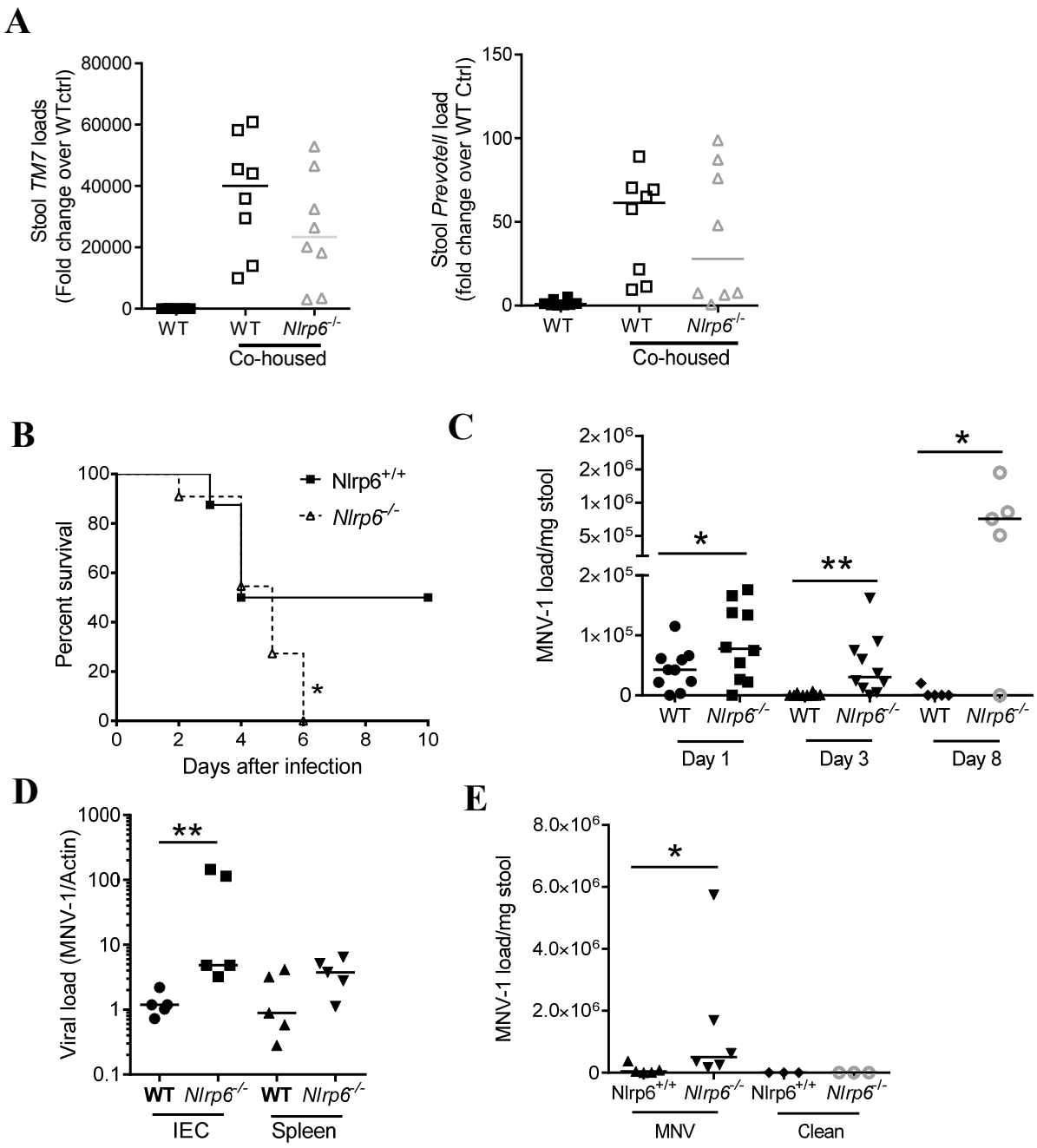
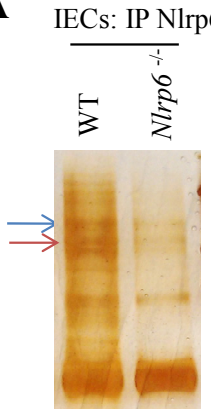


Fig. S3 Nlrp6 limits enteric virus infection of the intestine. (A) Quantitative PCR analyses of mouse stool bacteria. The data are normalized against mouse beta actin DNA and presented as fold change over WT. **(B)** The survival curves of *Nlrp6*$^{+/+}$ and *Nlrp6*$^{-/-}$ littermates after oral infection with EMCV. N=8-11, P<0.05 (Log-Rank test). qPCR quantification of murine norovirus-1(MNV-1) loads in **(C)** the intestinal epithelial cells (IEC) and spleen, and **(D)** stool of WT and *Nlrp6*$^{-/-}$ mice after oral infection with MNV-1. **(E)** qPCR quantification of murine norovirus-1 loads in the stool of *Nlrp6*$^{+/+}$ and *Nlrp6*$^{-/-}$ littermates day 3 after oral infection. Each dot represents an animal. The horizontal lines in the figures indicate the median of the results. *, p<0.05; **, p<0.01 (non-parametric Mann Whitney test). In **(C)** and **(E)**, the results are expressed as MNV copies per mg of stool. The data are representative of 2-3 independent experiments.

A



>Dhx15 NP_031865 length=795

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KDRFTDILVRHQSFVLVGETGSGKTTQIPQWCVEYMRSLPGPKRGVACTQPRRVAAMSVAQRVADEMDV
MLGQEVGYSIRFEDCSSAKTILKYMTDGMLLREAMNDPLLERYGVIILDEAHERTLATDILMGVLKEVVRQ
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TGQEEIDEACKRIKREVDDLGPEVGDIKIIPLYSTLPPQQQQRIFEPPPPKKQNGAIGRKVVVSTNIAETSLTID
GVVFVIDPGFAKQKVYNPRIRVESLLVTAISKASAQQRAGRAGRTRPGKCFRLYTEKAYKTEMQDNTYPEI
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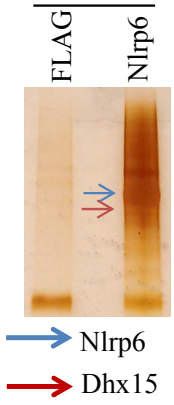
B



>DHX15 NP_001349 length=795

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KDRFTDILVRHQSFVLVGETGSGKTTQIPQWCVEYMRSLPGPKRGVACTQPRRVAAMSVAQRVADEMDV
MLGQEVGYSIRFEDCSSAKTILKYMTDGMLLREAMNDPLLERYGVIILDEAHERTLATDILMGVLKEVVRQ
RSDLKVIVMSATLDAGKFQIYFDNCPLLTIPGRTHPVEIFYTPEPERDYLEAAIRTVIQIHMCEEEEGDLLLFL
TGQEEIDEACKRIKREVDDLGPEVGDIKIIPLYSTLPPQQQQRIFEPPPPKKQNGAIGRKVVVSTNIAETSLTID
GVVFVIDPGFAKQKVYNPRIRVESLLVTAISKASAQQRAGRAGRTRPGKCFRLYTEKAYKTEMQDNTYPEI
LRSNLGSVVLQLKKLGIDDLVHFDFMDPPAPETLMRALELLNYLAALNDDGDLTELGSMMAEFPLDPQLA
KMVIASCDYNCSNEVLSITAMLSVPQCFVRPTEAKKAADEAKMRFAHIDGDHLTLLNVYHAFKQNHESVQ
WCYDNFINYRSLMSADNVRQQLSRIMDRFNLPRRSTDFTSRDYYINIRKALVTGYFMQVAHLERTGHYLTV
KDNQVVQLHPSTVLDHKPEWVLYNEFVLTTKNYIRTCTDIKPEWLVKIAPQYYDMSNFPQCEAKRQLDRII
AKLQSKEYSQY

Red: identified peptides by MS

Fig. S4 Nlrp6 binds Dhx15. Mass spectrometry analyses of Nlrp6-bound proteins from **(A)** mouse intestinal epithelial cells (IECs) using an anti-Nlrp6 antibody, and from **(B)** HEK293T cells transiently overexpressing FLAG-Nlrp6using an anti-FLAG antibody. Left panels are silver-stained SDS-PAGE gels. The blue and red arrows indicate Nlrp6 and Dhx15 respectively. Right panels are the sequences that were identified by mass spectrometry (highlighted). IP: immunoprecipitation.

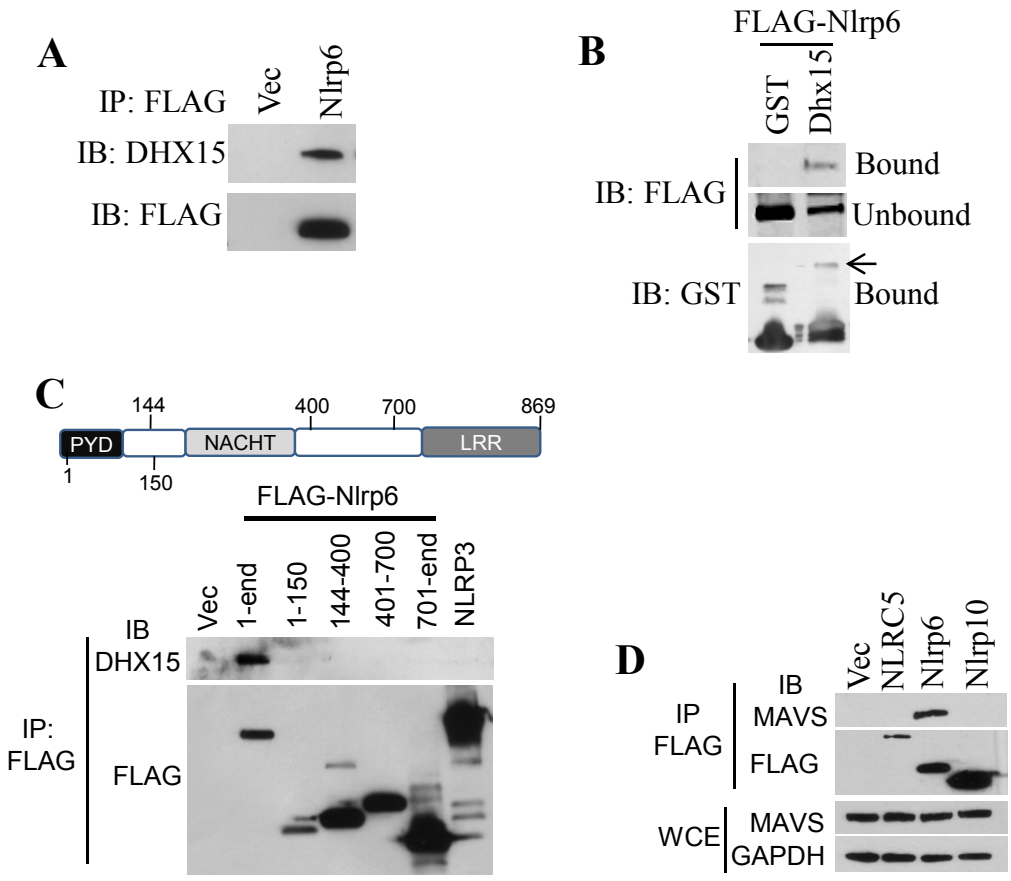

Fig. S5 Nlrp6 binds Dhx15 and MAVS. **(A)** Co- immunoprecipitation (IP) of FLAG-Nlrp6 with endogenous DHX15 from HEK293T cells overexpressing FLAG-tagged proteins using an anti-FLAG monoclonal antibody, followed by immunoblotting (IB). **(B)** Immunoblots of GST-precipitated FLAG-Nlrp6 (expressed from an *in vitro* mammalian translation system). GST proteins were expressed from *E. coli*. Arrow points GST-DHX15. **(C)** Co-IP of FLAG-Nlrp6 domains with endogenous DHX15 from HEK293T cells overexpressing FLAG-tagged proteins using an anti-FLAG monoclonal antibody, followed by IB. Upper panel, a schematic diagram showing the functional domains of Nlrp6. **(D)** Co-IP of FLAG-proteins with endogenous MAVS as in **(A)**. WCE: whole cell extract. The data are representative of 2-3 independent experiments.

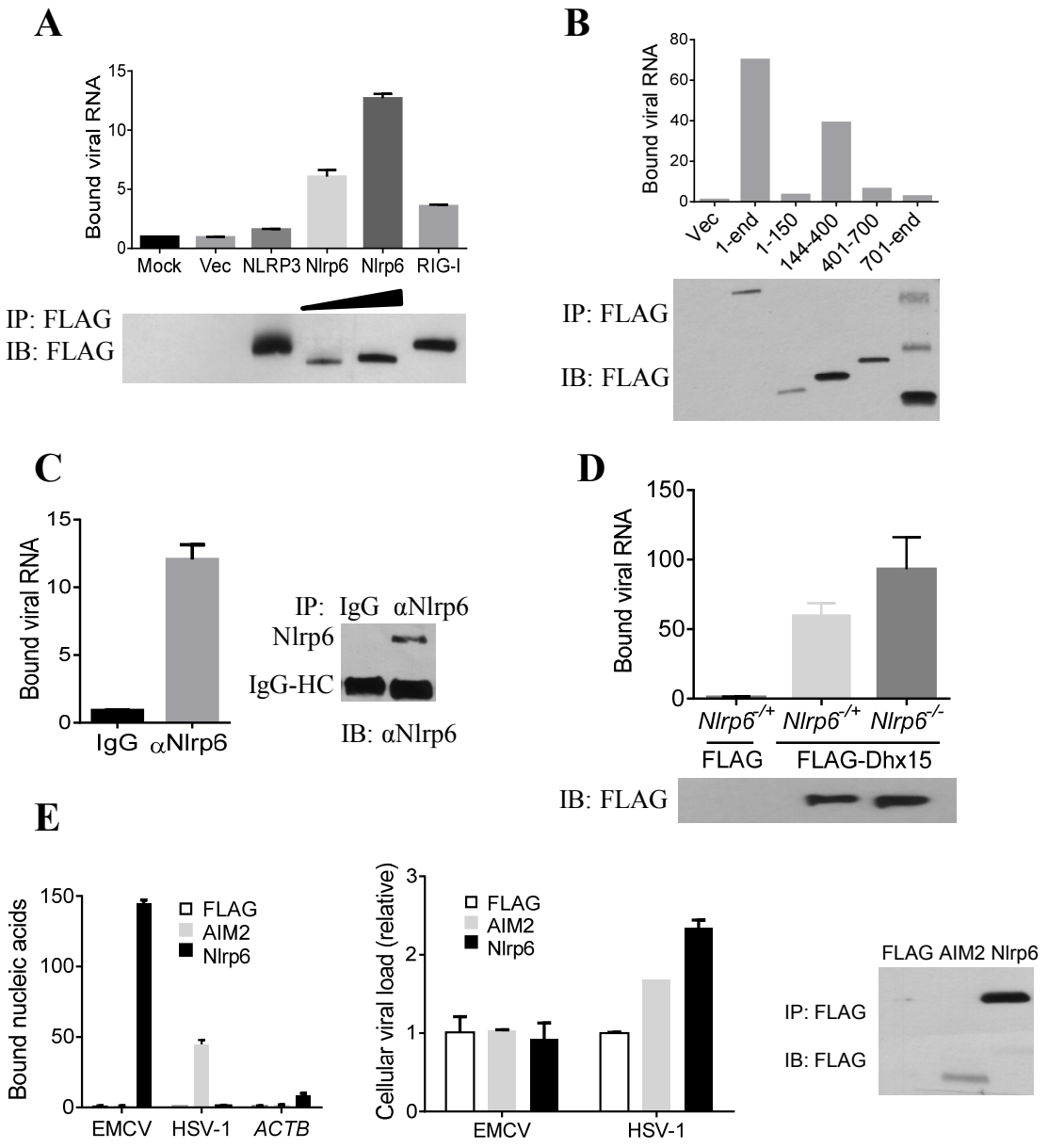

Fig. S6 Nlrp6 binds viral RNA not viral DNA. **(A)** and **(B)** Quantitative PCR analyses of viral RNA bound by FLAG-tagged proteins from EMCV infected FLAG-expressing HEK293T cells. Mock, M2 agarose beads only. Lower panel: immunoblots (IB) of FLAG-proteins immunoprecipitates (IP). **(C)** Binding of endogenous Nlrp6 to viral RNA. Left chart, quantitative PCR analyses of viral RNA bound by endogenous Nlrp6 in IECs. Right panel: immunoblots of Nlrp6 immunoprecipitates. **(D)** Quantitative PCR analyses of viral RNA bound by FLAG-DHX15 from EMCV-infected, FLAG- DHX15 transfected MEFs. Lower panel: immunoblots of FLAG-DHX15 immunoprecipitates. **(E)** Left panel shows quantitative PCR analyses of EMCV RNA, herpes simplex virus-1 (HSV-1) DNA

or beta actin (*ACTB*) mRNA bound by FLAG fusion proteins. The data are presented as fold increase over FLAG control. The middle chart shows the quantitative PCR analyses of intracellular EMCV or HSV-1 loads in HEK293T cells expressing FLAG fusion proteins. The right panel indicates FLAG immunoprecipitates. AIM2: absent in melanoma 2. Intracellular EMCV loads are normalized with human *ACTB* and presented as fold increase over FLAG control. n=3.

A

Gene	Fold	Gene	Fold	Gene	Fold
Anxa11	-1.4	*H2-M3*	-11.57	*Mnt*	-4.05
Arl5b	-8.77	*H2-Q1*	-9.09	*Mx1*	-1.55
Atf5	-1.06	*H2-Q7*	-1.16	***Mx2***	**-2.95**
B2m	1.59	*H2-T10*	-3.75	*Myd88*	2.72
Bag3	1.25	*Hoxb2*	-6.32	*Nmi*	-6.93
Bst2	-1.46	*Hspa1l*	3.6	*Npepps*	-1.97
Casp1	-1.19	*Ifi204*	5.54	*Nrg1*	-8.58
Cav1	1.54	*Ifi30*	-1.75	***Oas1a***	**-5.91**
Cbfb	N/A	*Ifit1*	-1.67	***Oas1b***	**-3.2**
Cd70	N/A	*Ifit3*	N/A	*Oas2*	-1.12
Cdkn1b	3.6	*Ifitm1*	N/A	*Pml*	3.6
Cnp	-1.5	*Ifitm2*	N/A	*Prkcz*	1.04
Col16a1	3.6	*Ifna2*	3	*Prkra*	1.34
Cxcl10	N/A	*Ifna4*	1.69	*Psme2*	-3.74
Dad1	-1.46	*Ifnar1*	N/A	*Pttg1*	-1.6
Diablo	-3.09	*Ifnar2*	1.81	*Rcbtb1*	-1.03
Dnajb2	-2.92	***Ifnb1***	**-2.31**	*Samsn1*	-2.44
Eif2ak2	-1.06	*Irf1*	1.16	*Sh2d1a*	-1.11
Gbp2b	3.22	*Irf2*	-1.06	*Shb*	3.6
Gbp2	-1.66	***Irf3***	**-5.48**	*Shfm1*	-3.01
Gch1	-1.94	***Irf5***	**-2.16**	*Slc1a2*	N/A
H2-Bl	N/A	***Irf7***	**-7.04**	*Stat1*	N/A
H2-D1	-1.15	***Isg15***	**-6.37**	*Stat2*	3.6
H2-K1	-2.2	*Isg20*	-1.73	*Tap1*	-2.77
H2-M10.1	-4.03	*Irf9*	1.33	*Tnfsf10*	-1.94
H2-M10.2	1.07	*Itih2*	2.45	*Traf3*	1.4
H2-M10.4	-1.42	*Mal*	1.01	*Vegfa*	1.66

B

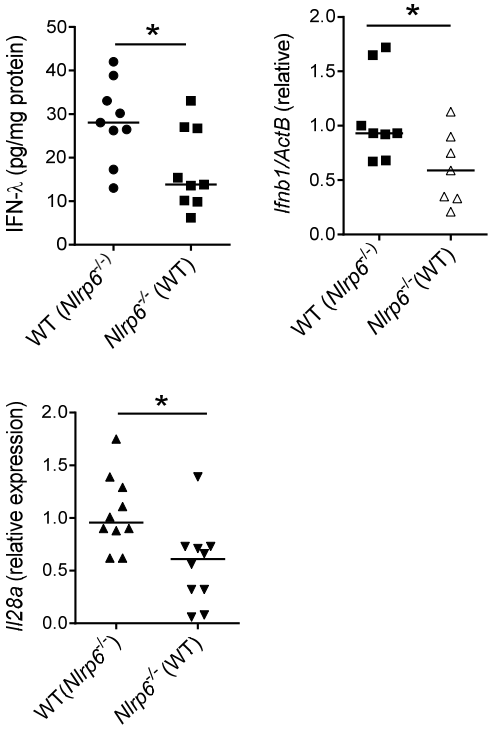

Fig. S7 Nlrp6 regulates type I/III IFN and ISG expression. **(A)** PCR array of antiviral genes in the primary intestinal epithelial cells of WT and *Nlrp6*$^{-/-}$ mice 3 days post EMCV infection via the intra-peritoneal route (i.p.). Results are presented as fold changes in cells from *Nlrp6*$^{-/-}$ over WT mice. A negative value indicates down-regulation; a positive means up-regulation. N.A, expression below detection in either *Nlrp6*$^{-/-}$ or WT. **(B)** Quantitative PCR analyses of *Ifnb1* and *Il28a* mRNA, and IL-28 protein expression in whole intestine of co-housed animals 3 days post EMCV infection i.p. Each symbol represents one mouse; small horizontal lines indicate the median of the result. *P < 0.05 (Students' t-Test). The data are representative of two independent experiments.

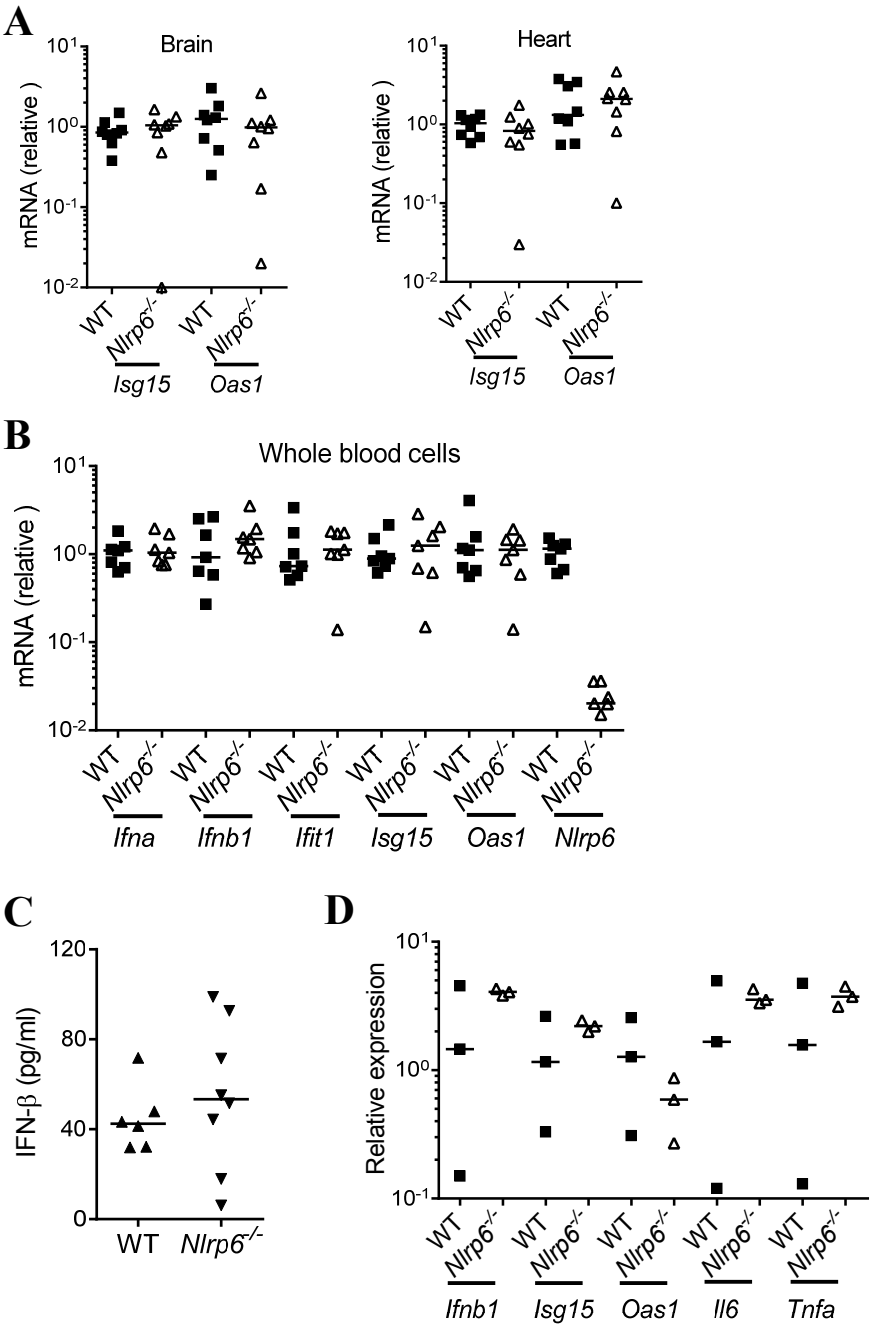

Fig. S8 Nlrp6 is dispensable for type I/III IFN and ISG expression in non-intestinal tissues. Quantitative PCR analyses of selected ISG mRNA expression in **(A)** brain and heart, **(B)** blood cells from WT and *Nlrp6*$^{-/-}$ mice 3 days after EMCV infection via the intra-peritoneal route (*i.p.*). Data are normalized with mouse beta actin and are presented as fold changes over the mean of the results of WT mice. **(C)** ELISA of IFN-β concentrations in the sera of WT and *Nlrp6*$^{-/-}$ mice 3 days after EMCV infection via *i.p.* **(D)** Quantitative PCR analyses of cytokine expression in the intestine of clean WT and *Nlrp6*$^{-/-}$ mice. Each dot represents an animal. The horizontal lines in the figures indicate the median of the results. The data are representative of 2 independent experiments.

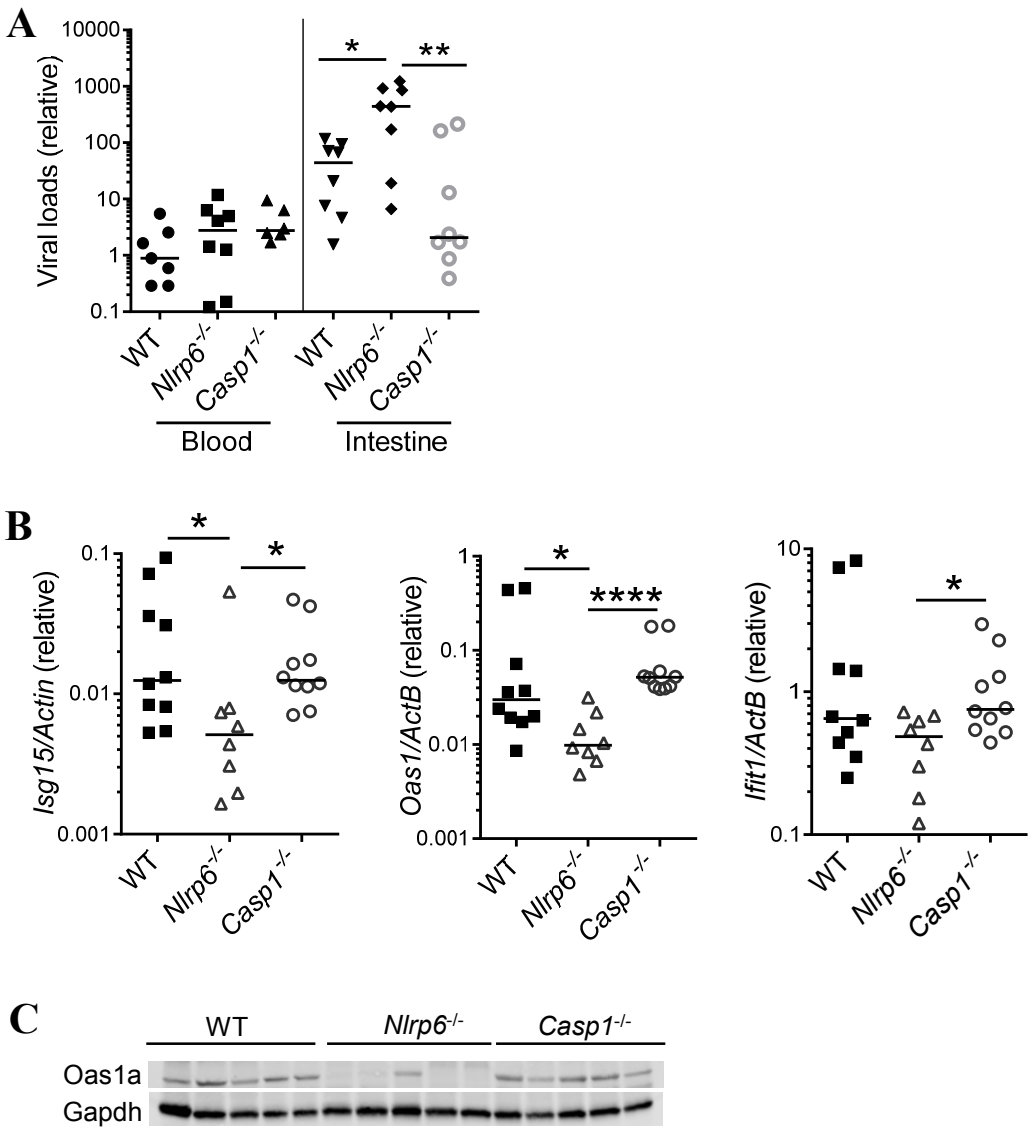

Fig. S9 Nlrp6 regulates type I/III IFN and ISG expression in the intestine. Quantitative PCR analyses of **(A)** viral loads in the blood and intestines and **(B)** *ISG*s in the intestines of WT, *Nlrp6*$^{-/-}$ and *Casp1*$^{-/-}$ mice. **(C)** Immunoblotting analyses of Oas1a protein abundance in whole intestine. In **(A)**, the data are normalized with mouse beta actin and are presented as fold change over the mean of the results of WT blood. Each band/dot represents an animal. The horizontal lines in the figures indicate the median of the results. *P < 0.05, **P < 0.01 and ***P<0.001 (nonparametric Mann-Whitney analysis).

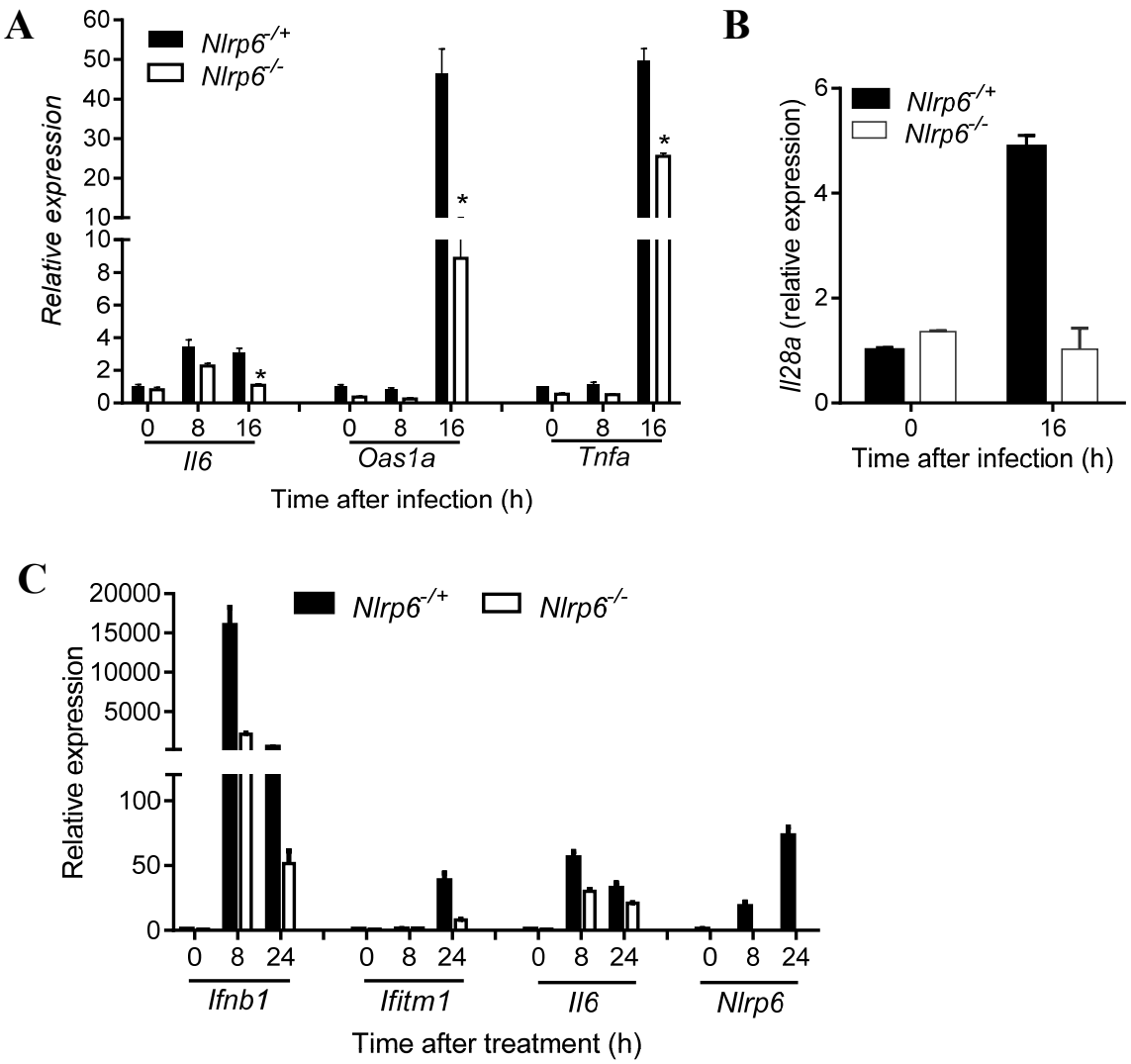

Fig. S10 Nlrp6 regulates type I/III IFN and ISG expression in MEFs. Quantitative PCR analyses of immune gene expression in MEFs **(A-B)** infected with EMCV (MOI=0.1), or **(C)** transfected with 10 μg/ml high molecular weight poly I:C. The data are normalized with mouse beta actin and are presented as fold changes over 0 h. The data are representative of 2-3 independent experiments. Bars: mean+S.E.M.

A

Gene	Fold	Gene	Fold	Gene	Fold
AIM2	-1.27	IFIH1	2.15	MEFV	4.2
APOBEC3G	1.04	IFNA1	3.16	MX1	2.26
ATG5	1.83	IFNA2	3.89	MYD88	1.75
AZI2	2.22	IFNAR1	1.18	NFKB1	-1.71
CARD8	2.38	IFNB1	3.48	NFKBIA	-1.06
CASP1	2.64	IKBKB	-1.29	NLRP3	1.18
CASP10	1.76	IL12A	2.41	NOD2	2.17
CASP8	1.39	IL12B	3.22	OAS2	3.57
CCL3	3.57	IL15	2.23	PIN1	-2.37
CCL5	2.46	IL18	2.03	PSTPIP1	3.38
CD40	-1.09	IL1B	3.81	PYCARD	3
CD80	2.7	IL6	1.32	PYDC1	4.15
CD86	3	IL8	2.05	RELA	-1.29
CHUK	1.09	IRAK1	-1.9	RIPK1	1.15
CTSB	1.33	IRF3	1.46	SPP1	3.49
CTSL1	-1.02	IRF5	1.71	STAT1	1.47
CTSS	2.03	IRF7	1.59	SUGT1	2.28
CXCL10	3.11	ISG15	1.39	TBK1	2.25
CXCL11	3.69	JUN	-2.11	TICAM1	1.24
CXCL9	3.2	MAP2K1	-1.52	TLR3	2.45
CYLD	1.99	MAP2K3	1.74	TLR7	3.63
DAK	-1.56	MAP3K1	-1.24	TLR8	3.07
DDX3X	1.35	MAP3K7	1.85	TLR9	5.31
DDX58	1.41	MAPK1	1.16	TNF	2.99
DHX58	1.68	MAPK14	-1.1	TRADD	1.1
FADD	-1.74	MAPK3	-1.62	TRAF3	-1.25
FOS	-1.02	MAPK8	1.19	TRIM25	-1.45
HSP90AA1	1.68	MAVS	1.51	B2M	-1.99

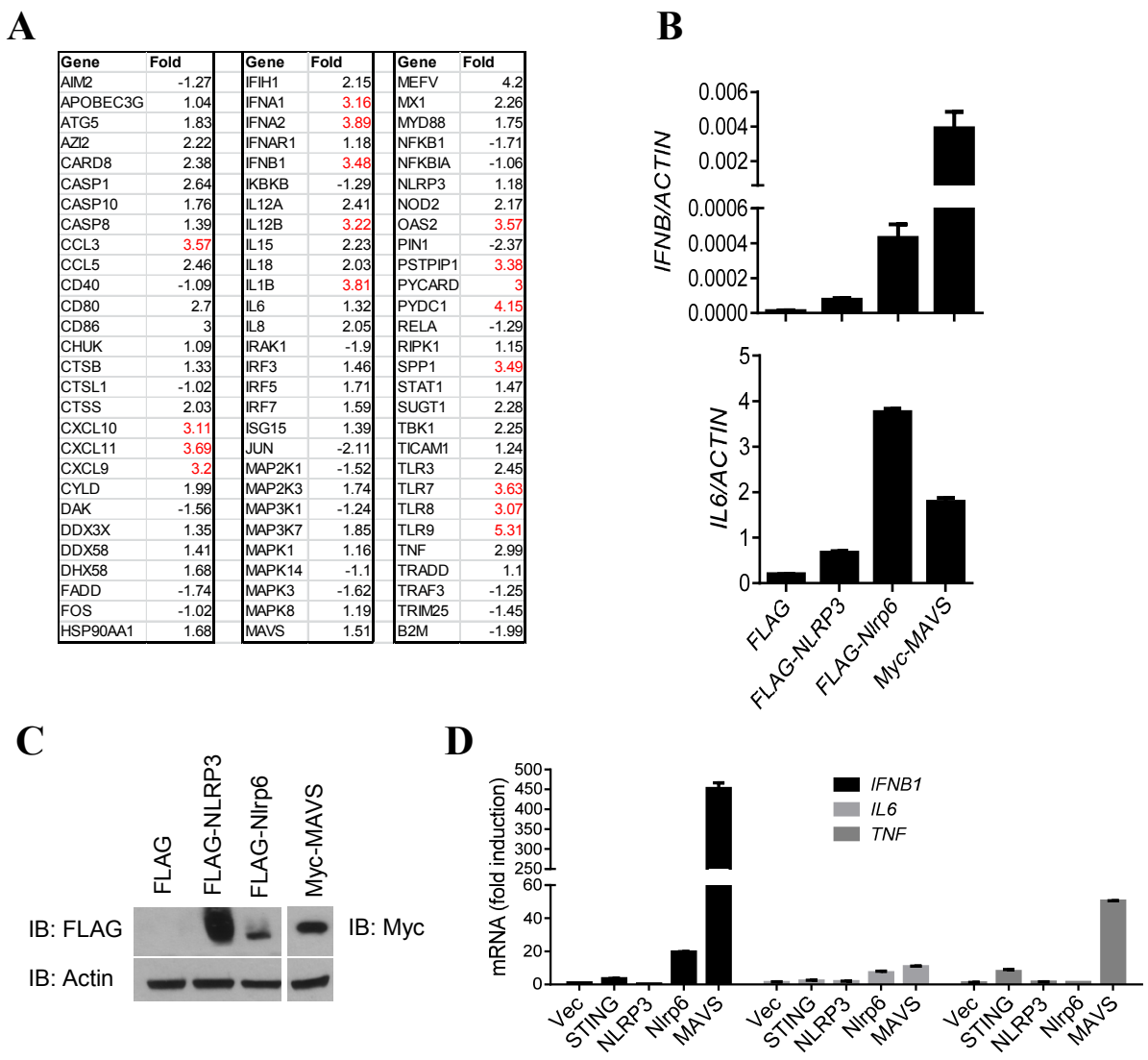

Fig. S11 Overexpression of Nlrp6 induces immune responses. **(A)** PCR array of antiviral genes in A549 cells transiently overexpressing FLAG-Nlrp6. Results are presented as fold changes of FLAG-Nlrp6 over vector. A negative value indicates down-regulation; a positive means up-regulation. Fold changes of 3 or higher are considered significant. **(B)** Quantitative PCR analyses of *IFNB1* and *IL6* mRNA expression in human A549 cells transiently over-expressing indicated genes. **(C)** Immunoblots of overexpressed proteins in the whole cell lysates of A549 cells. **(D)** Quantitative PCR analyses of cytokine mRNA expression in HEK293T cells overexpressing indicated genes (X-axis). The data are normalized with human beta actin and are presented as fold changes over vector (Vec). Bars: mean+S.E.M. n=3. Thedata are representative of 2-3 independent experiments.

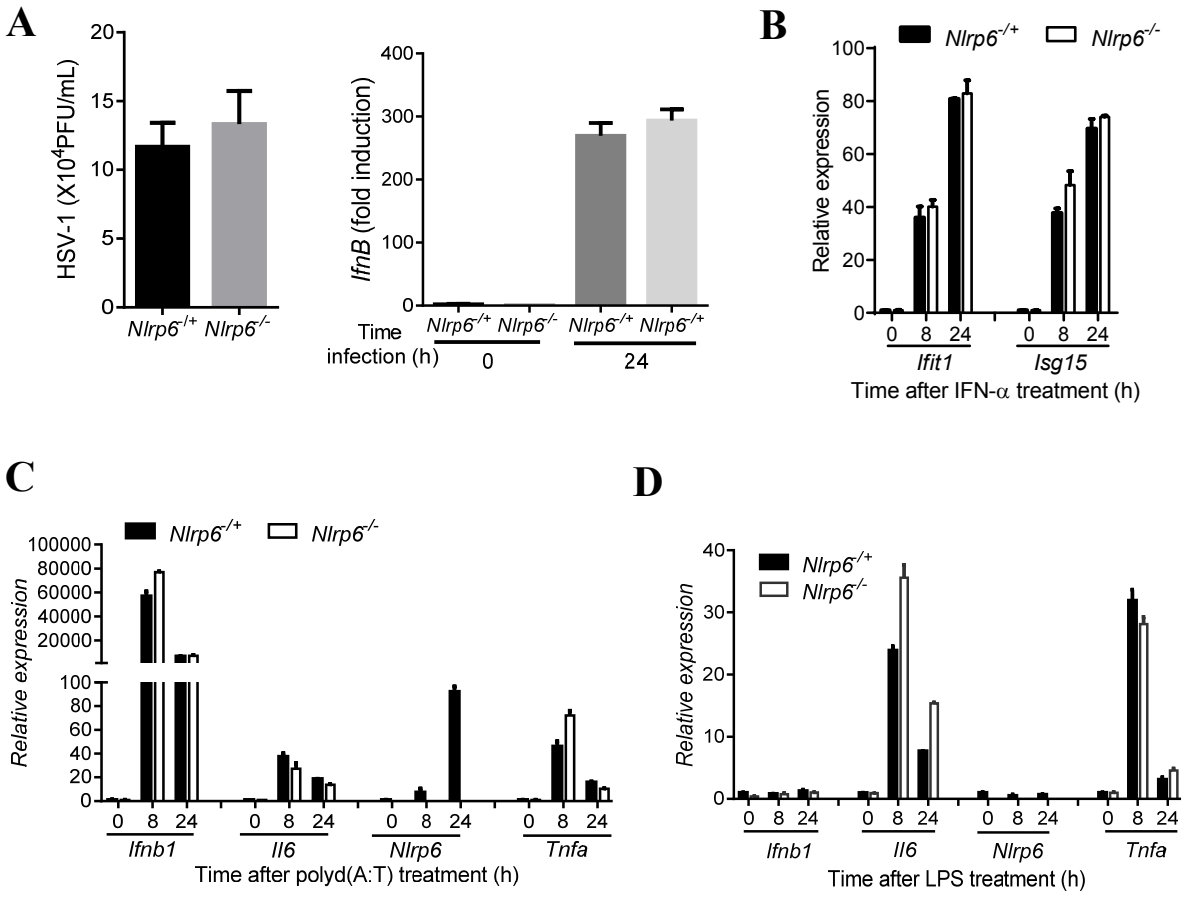

Fig. S12 Nlrp6 is not required for DNA virus, IFN-α, poly d(A:T) or lipopolysaccharide (LPS)-induced immune responses. (A) Numbers of infectious viral particles (presented as plaque forming units per ml of cell culture medium) of mouse embryonic fibroblasts 24 hours after herpes simplex virus 1(HSV-1) infection, and quantitative PCR analyses of *Ifnb1*. Quantitative PCR analyses of cytokine and ISG mRNA levels in MEFs after treatment with **(B)** 0.25 μg/ml IFN-α, **(C)** 10 μg/ml polyd(A:T), **(D)** 10 μg/ml LPS. The data are normalized with mouse beta-actin and are resented as fold changes over 0 h *Nlrp6*$^{-/+}$. Bars: mean + S.E.M. n=3.

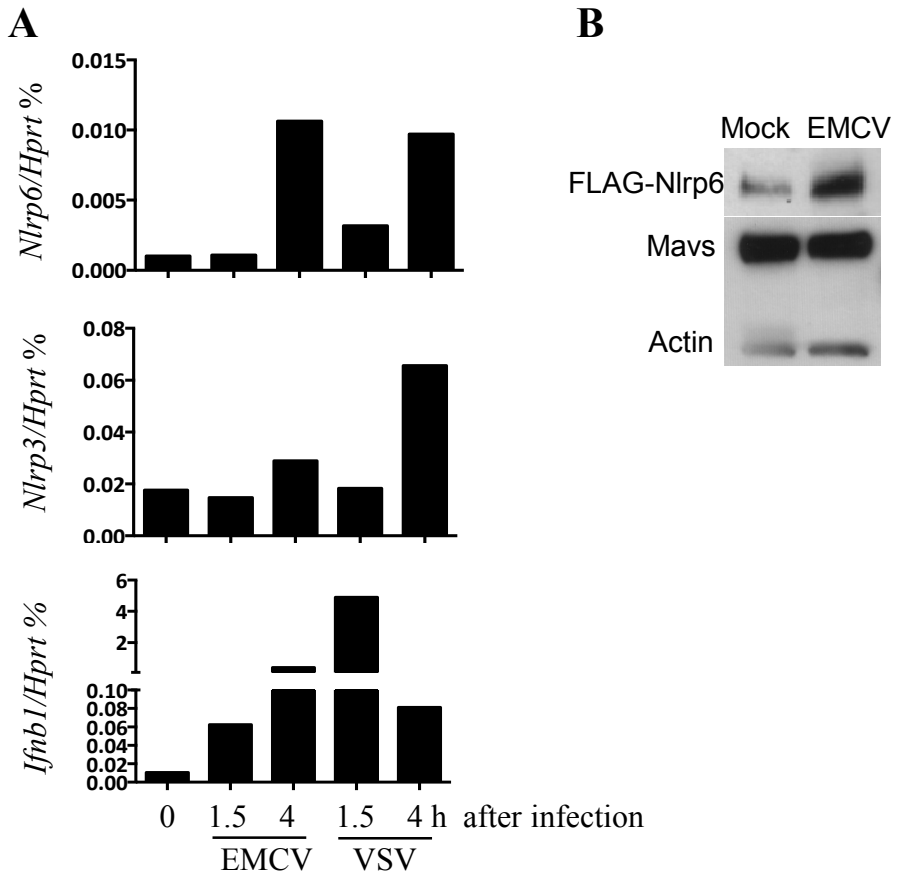

Fig. S13 Viral infection induces Nlrp6 expression. (A) Quantitative PCR analyses of *Nlrp6*, *Nlrp3* and *Ifnb1* mRNA in Mode-K intestinal epithelial cell line after EMCV or vascular stomatitis virus (VSV) infection. Data are normalized with a mouse housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (Hprt). **(B)** Immunobots of FLAG-Nlrp6 expression in mock or EMCV-infected (16 h) primary mouse embryonic fibroblasts (MEFs) (from FLAG-Nlrp6 knockin mice) using an anti-FLAG antibody.

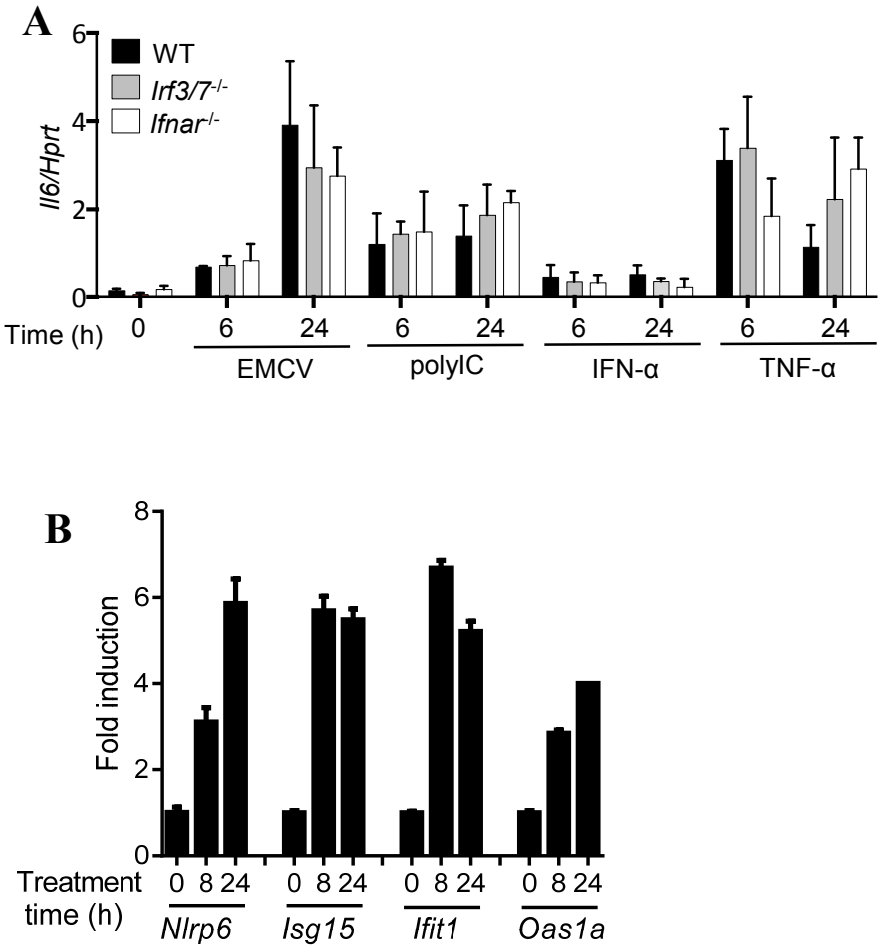

Fig. S14 Nlrp6 is an ISG. Quantitative PCR analyses of the transcripts of **(A)** *Il6* in WT, *Irf3/7*$^{-/-}$ and *Ifnar*$^{-/-}$ MEFs treated with EMCV, polyI:C, recombinant IFN-α or TNF-α. The data are expressed as percentage of a house keeping gene *Hprt*. **(B)** Quantitative PCR analyses of *Nlrp6* and ISG mRNA expression (x-axis) in MEFs treated with 2 ng/ml of recombinant IFN-λ2. The data are presented as fold induction relative to untreated (0 hour). Bars: mean + S.D. The data are representative of at least two independent experiments.

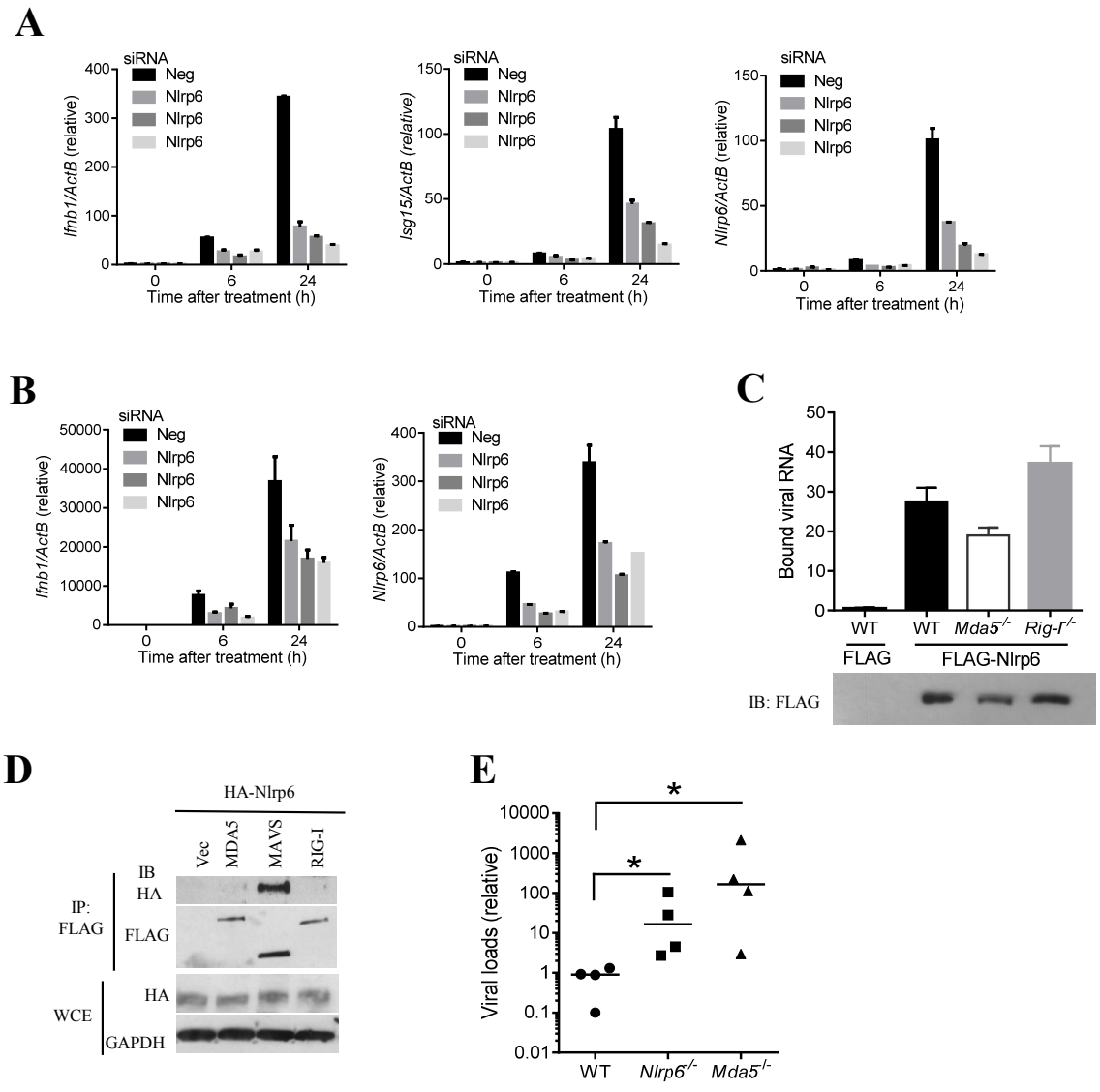

Fig. S15 Nlrp6 regulates IFN-I response independently of Mda5 and Rig-I. Quantitative PCR analyses of *Ifnb1*, *Isg15* and Nlrp6 mRNA levels in **(A)** *Mda5*$^{-/-}$ or **(B)** *RigI*$^{-/-}$ MEFs transfected with siRNA and 40 h later with 50 µg/ml of heavy molecular weight polyI:C. The data are presented as fold induction relative to untreated (0 h). The data are normalized with mouse beta-actin and are presented as fold changes over negative control siRNA 0 h. Bars: mean + S.D. **(C)** Quantitative PCR analyses of viral RNA bound by FLAG-Nlrp6 from EMCV-infected, FLAG-Nlrp6 transfected MEFs. Lower panel: immunoblots (IB) of FLAG-Nlrp6 immunoprecipitates. **(D)** Co-IP of FLAG-proteins with HA-Nlrp6 from HEK293T cells co-transfected with FLAG and HA-Nlrp6 plasmids, using an anti-FLAG monoclonal antibody, followed by immunoblotting (IB). WCE: whole cell extract. **(E)** Viral loads in the intestinal epithelial cells of WT, *Nlrp6*$^{-/-}$ and *Mda5*$^{-/-}$ mice 3 days post EMCV infection via oral gavage. Each dot represent ones mouse. *, P<0.05 (non-parametric Mann-Whitney test).