Supplementary Materials for

Targeting RNA exonuclease XRN1 potentiates efficacy of cancer immunotherapy

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SUPPLEMENTARY METHODS

Reverse transcriptional quantitative PCR (RT-qPCR). RNAs were extracted using QIAamp RNA kit (Qiagen) and transcribed to cDNA using EvoScript Universal cDNA Master (Roche). Quantitative PCR was performed using KAPA SYBR FAST qPCR Master Mix (Roche). Sequence of RT-qPCR primers are listed on **Table S2**. Relative expression of each gene was normalized to either human *GAPDH* or murine *β-Actin.* All qPCR reactions were performed in duplicates on Applied Biosystems 7300 system.

siRNA silencing. Twenty-five picomole of siRNA (siCtrl, siXrn1, siSting or siMavs) were transfected into B16/F10 cells in 6-cm dishes for 48 h (unless otherwise mentioned) using the RNAiMAX reagents (Invitrogen, cat#13778150). Detailed information for siRNA is listed on **Table S4**.

MTT assay. One thousand murine melanoma cells (B16/F10, D4M.3A) or 2,000 of human melanoma cells (SKMEL-30, WM88, Melho) were suspended in 100 μL culture medium and seeded in 96-well plates. Thirty microliters of MTT dye was added at indicated time point and incubated for 1 h in 37 °C before carefully removing all liquid. One hundred microliters of MTT stop solution was added and incubate at room temperature with shaking (200 rpm, 2 h). Absorbance at 570 nm was recorded to indicate cell growth.

Transfection of *in vitro* transcribed mRNA. Luciferase mRNA (250 ng each) with differnet 5' terminus were transfected into B16/F10 cells [Cas9 control and XRN1 depleted cells (Xrn1_KO#7 and Xrn1_KO#9)] using RNAiMax. Total RNA were extracted 6 h later. B16/F10 cells were FBS starved for 12 hours before RNA transfection to synchronize the cell status. 5'ppp firefly luciferase mRNA (mRNA with 5' triphosphate moiety, positive control) was produced by in vitro transcription (IVT) using T7 RNA polymerase (HiScribe® T7 High Yield RNA Synthesis Kit, NEB) according to users' instruction. "ARCA-cap RNA" was firstly synthesized using a similar kit (HiScribe® T7 ARCA mRNA Kit, NEB) with standard NTP mix replaced with ARCA (Anti-Reverse Cap Analog, NEB)/NTP mixture; the generated RNA

was then succesively digested with 5' polyphosphatase (Lucigen) and recombinant Xrn1 protein (NEB) to eliminate any 5'ppp RNA by-product. 5'ppp RNA was further treated with 5' polyphosphatase to produce 5'p RNA (RNA with 5' monophosphate moiety). The RNAs were purified using Trizol plus Direct-zol RNA Kit (Zymo) in each step.

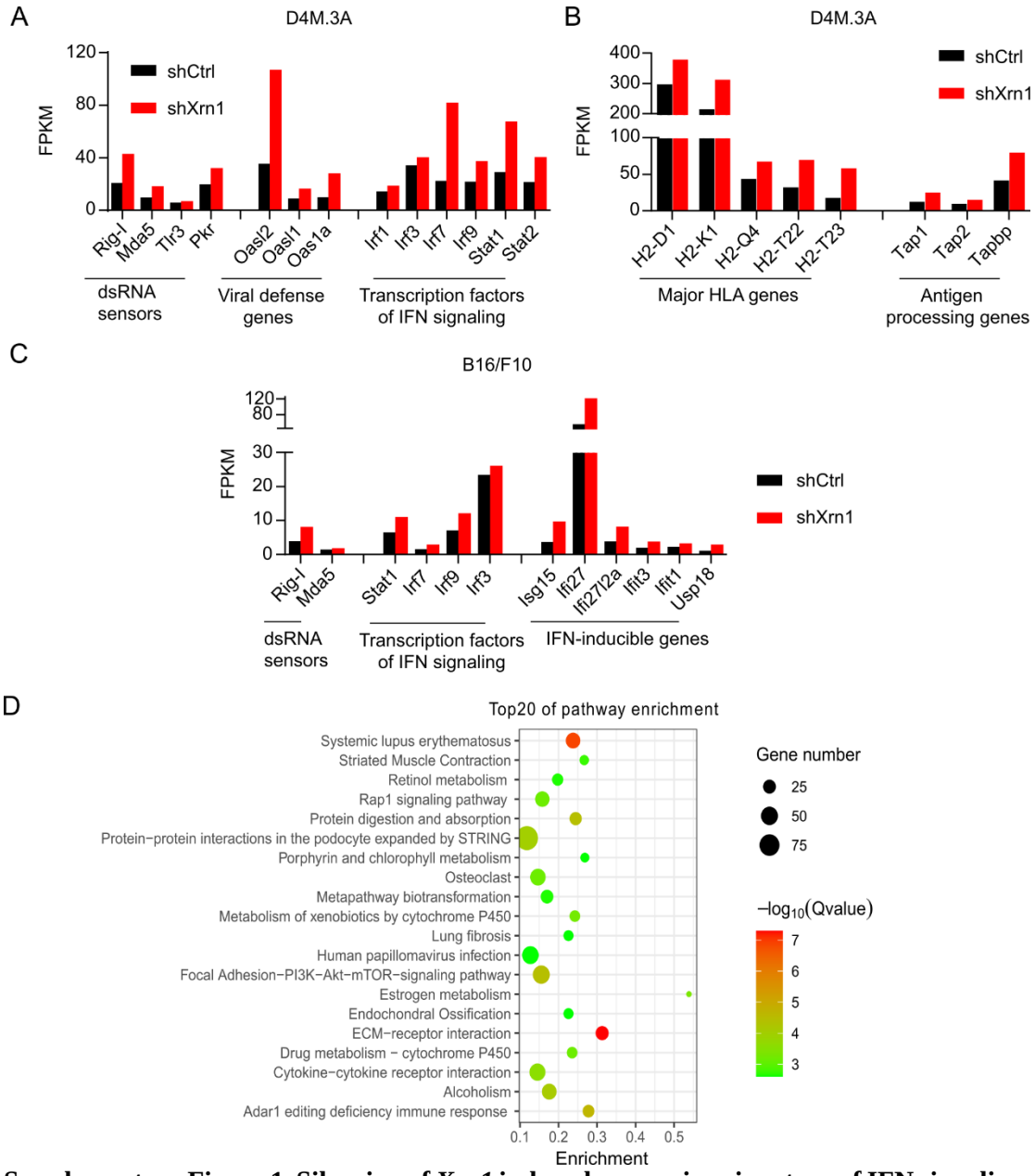
Dual-luciferase reporter assay. The luciferase reporter plasmids were constructed as follows: pGL3 promoter vector was inserted upstream of *luciferase* promoter with either XRN1-WT fragment (pGL3-WT, cloned with primer XRN1-WT-F and XRN1-WT-R) or XRN1-Ctrl fragment (pGL3-Ctrl, cloned with primer XRN1-Ctrl-F and XRN1-Ctrl-R). The mutation of STAT1 binding motif in XRN1-WT fragment was introduced by PCR using primer XRN1-Mut-F and XRN1-Mut-R to construct pGL3-Mut plasmid. The reporter plasmids were transfected into 293T cells and the cells were subsequently treated with human recombinant IFN for 24 h. CMV-Renilla plasmid was used as normalization control. The luciferase activity was measured by Dual-Luciferase® Reporter Assay System (Progmega). Primers used are listed on **Table S5**.

RNA-sequencing (RNA-seq). RNA expression was profiled using either Illumina Hiseq4000 or BGISEQ-500 high-throughput sequencing platform. For sequencing results of cell lines, reads were mapped to *Mus musculus* genome (GRCm38.p6) by STAR (2.4.0i, RRID: SCR_004463) with default parameters for paired end. Gene expression was analyzed using featureCounts (v1.5.0-p1). The expression of selected genes was further validated using real-time q-PCR. Differentially expressed genes [threshold log2 (fold change) $\geq$ 0.5] in Xrn1-silenced cells were analyzed on ConsensusPathDB (RRID: SCR_002231) for pathway enrichment analysis. For sequencing results of tumor tissue derived RNA, reads were mapped to murine genome (GRCm39) and quantified using Salmon (1). The transcript expression was normalized with "DESeq2" package in R (2). The results generated was further subjected to Principal component analysis (PCA) to visualize the variation between different samples using "DESeq2" package.

Analysis of immune cell infiltration and T cell receptor (TCR) repertoire. Immune cell infiltration was estimated using RNA sequencing data of tumor tissues and analyzed with TIMER2.0 (3). mMCP-counter scores were used for heatmap plotting. TCR rearrangement repertoire in tumor tissues was analyzed using MiXCR v3.0.13 (4). The number of TCR clonotypes of each sample and the expression level of each clonotype were assessed.

Immunohistochemistry (IHC) staining. Tumor tissues were merged and fixed in 10% formalin solution, neutral buffered (Sigma Aldrich) for 24 hours. Fixed tissues were dehydrated through graded ethanol from 70%, 80%, 95% and lastly 100% ethanol, followed by clearing with Xylene to allow infiltration of paraffin wax. The tissue sections with a thickness of 5 µm were prepared on charged slides for following IHC staining, which was did on a Leica BondMax instrument and BOND Polymer Refine Red Detection kit (#DS9390). Antibodies used for IHC staining are listed in **Table S3**.

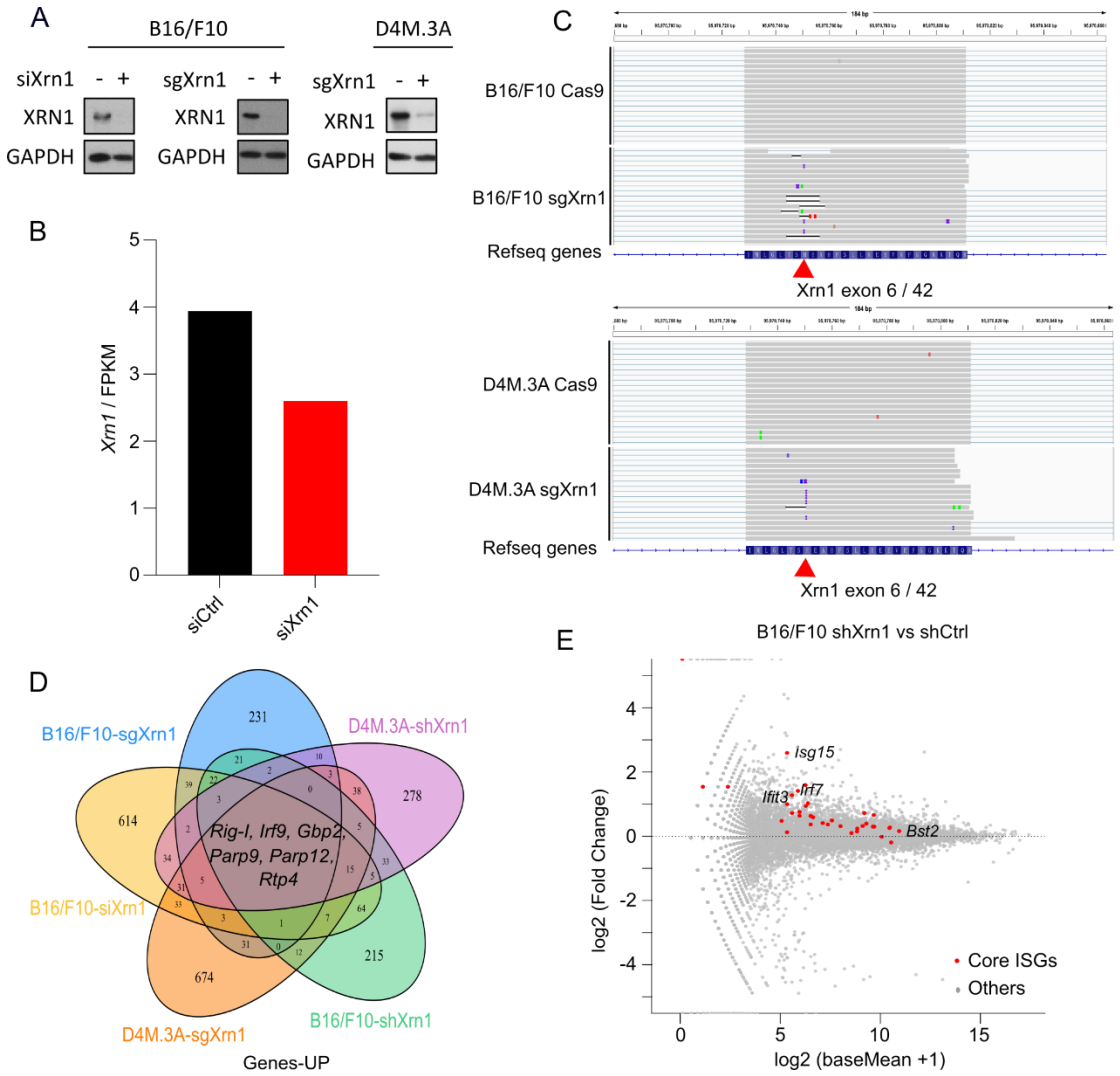
Fig. S1.



Supplementary Figure 1. Silencing of *Xrn1* induced expression signature of IFN signaling.

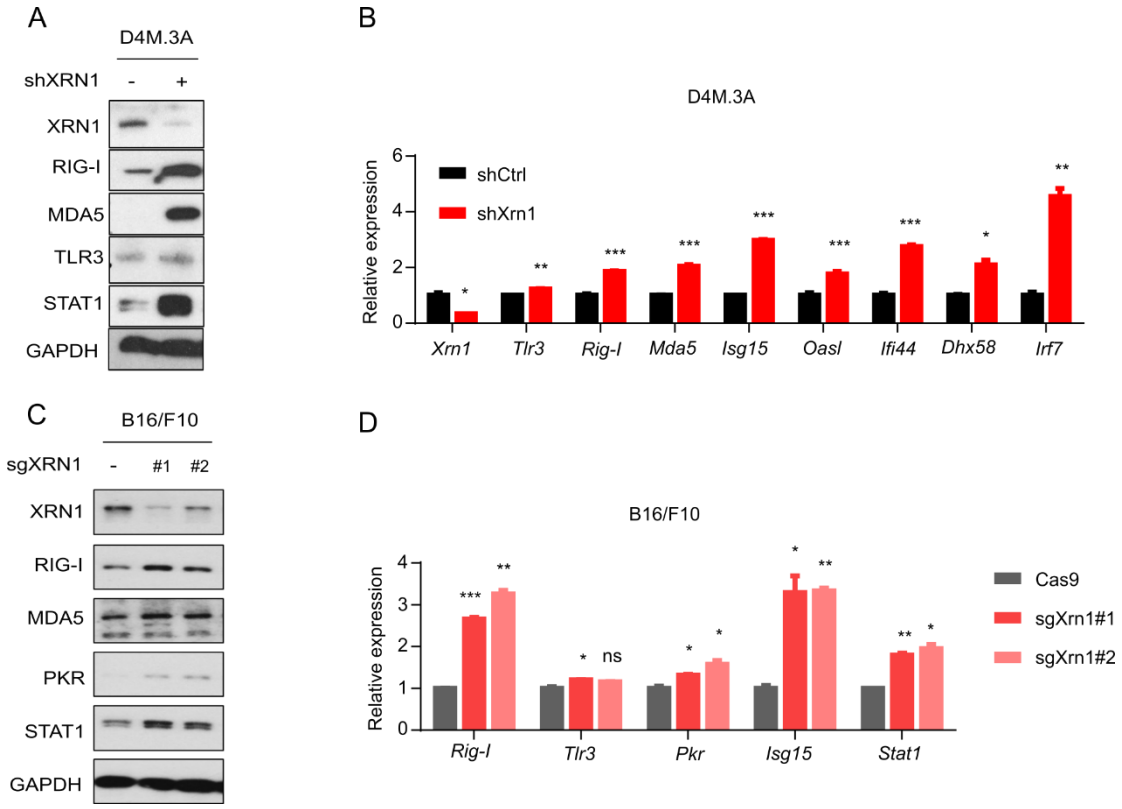
A, B, C, Expression levels of representative interferon-stimulated/viral-defense genes (**A, C**) and antigen presentation-related genes (**B**) in *Xrn1* silenced cells. Data were plotted using FPKM values (**A**, **B**, D4M.3A cells; **C**, B16/F10 cells). **D,** Bubble plot showed the top 20 pathways significantly upregulated in B16/F10-shXrn1 compared with B16/F10-shCtrl. Enrichment stands for the ratio of upregulated genes to whole pathway gene sets. Color and bubble size represent the significance [-log10 (Q value)] and gene numbers upregulated in each gene set, respectively.

Fig. S2.



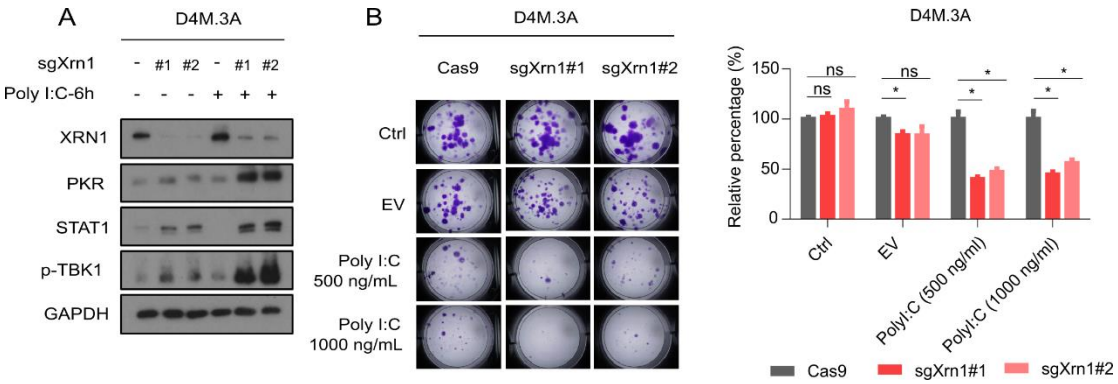
Supplementary Figure 2. Depletion of XRN1 triggered expression of ISG signature. A, Western blotting confirmed successful knockdown of XRN1 protein**. B,** *Xrn1* mRNA expression level (FPKM) confirmed successful knockdown of *Xrn1* by siRNA. **C,** Small deletion (black) and insertion (purple) on *Xrn1* transcripts introduced by CRISPR sgXrn1 in both B16/F10 and D4M.3A cells as shown in IGV viewer. sgXrn1 targeting site (the 6th of 42 exons) is indicated with a red triangle. **D,** Venn diagram analysis identified common upregulated genes in the 5 *Xrn1* silenced cells. **E,** Bland-Altman plot analyzed RNA-seq results of shXrn1 and shCtrl of B16/F10 cells. Fold change of FPKM value of each gene after *Xrn1* silencing was plotted according to the mean expression level ("baseMean", average FPKM of Ctrl and shXrn1). Core interferon-stimulated genes (ISGs) are highlighted in red.

Fig. S3.



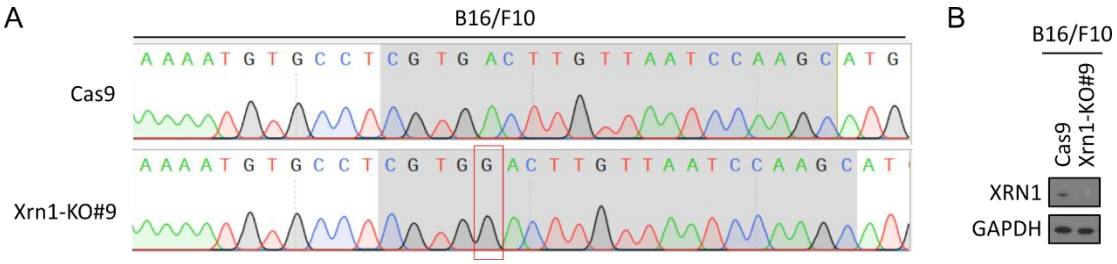
Supplementary Figure 3. Elevation of ISG expression in *Xrn1* silenced cells. **A, B,** Western blot (**A**) and RT-qPCR (**B**) confirmed the upregulated of IFN inducible genes in D4M.3A cells after silencing of *Xrn1* using shRNA. **C, D,** Western blot (**C**) and RT-qPCR (**D**) validation of IFN inducible genes in *Xrn1* silenced B16/F10 cells using CRISPR sgRNAs. RT-qPCR data are means ± SEM. Student T-test measured the significance of difference. ns, no significant difference. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. S4.



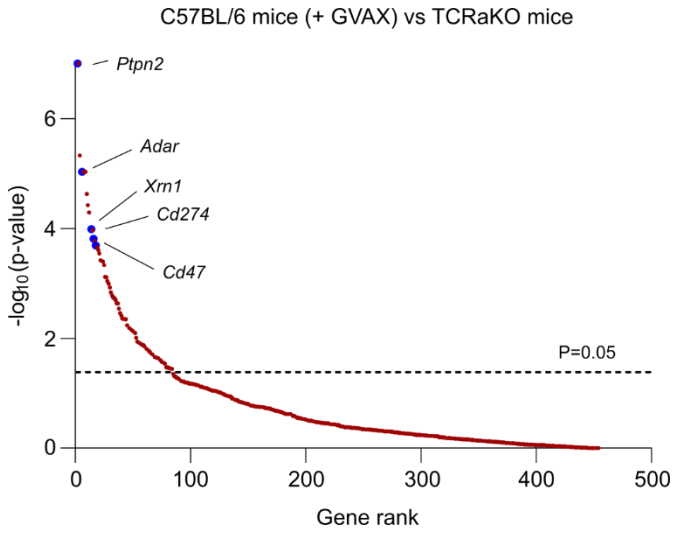
Supplementary Figure 4. Knockdown of *Xrn1* augmented IFN signaling induced b poly I:C. A, Poly I:C was transfected into control (sgCtrl) and Xrn1-knockdown (sgXrn1) D4M.3A cells (1000 ng/mL, 6 h) using lipofectamine; empty vesicle (EV) used as control. Western blot was conducted to examine the expression levels of indicated proteins. **B,** Control and Xrn1-knockdown D4M.3A cells were seeded in 6-well plates (500 cells per well). Either empty vesicles (EV) or indicated concentration of poly I:C was added into each well at day 7; one more week later, cells were fixed and stained using crystal violet. Results were recorded by both photographing (left panel) and absorbance (after dissolving with MTT stop solution, measured at 570 nm) of the stained plates (right panel). Data are means ± SEM. Student T-test was conducted to examine the significance of difference. ns, no significant difference. * $P < 0.05$.

Fig. S5.



Supplementary Figure 5. Generation of monoclonal XRN1 depleted B16/F10 cell line. A, Sanger sequencing validation of *Xrn1* single clonal knockout using "*Xrn1-KO#9-validation*" oligo. Grey regions indicate the DNA sequences targeted by CRISPR single guide RNA. Alteration of sequence is highlighted by a red rectangle. **B,** Western blotting confirmed the knockout of XRN1 in B16/F10.

Fig. S6.



Supplementary Figure 6. *Xrn1* depletion enhanced antitumor immunity in *in vivo* CRISPR library screening. sgRNA targeting *Xrn1* was significantly depleted in CRISPR library containing tumors grown in immunocompetent mice with GVAX treatment vs the same tumors growth in T cell receptor knockout mice (TCRaKO) (5). The *P* value of *Xrn1* is comparable to other well appreciated immunotherapy regulating genes, such as *Adar*, *Cd274* (*Pd-L1*) and *Cd47* (don't eat me signal).

Table S1. Oligos used to generate shRNA and sgRNA vectors.

Name	Sequence forward (5' to 3')	Sequence reverse (5' to 3')
Ctrl	CCGGCCTAAGGTTAAGTCGCCCTC GCTCGAGCGAGGGCGACTTAACCT TAGGTTTTTG	AATTCAAAAACCTAAGGTTAAGTC GCCCTCGCTCGAGCGAGGGCGACT TAACCTTAGG
shXRN1#1	CCGGCGATGTTGTACAAGGTATAA ACTCGAGTTTATACCTTGTACAAC ATCGTTTTTG	AATTCAAAAACGATGTTGTACAAG GTATAAACTCGAGTTTATACCTTGT ACAACATCG
shXRN1#2	CCGGGTTACTCACAGGTCGTAAAT ACTCGAGTATTTACGACCTGTGAG TAACTTTTTG	AATTCAAAAAGTTACTCACAGGTC GTAAATACTCGAGTATTTACGACCT GTGAGTAAC
shXrn1#1	CCGGCGATGTTGTACAAGGTATAA ACTCGAGTTTATACCTTGTACAAC ATCGTTTTTG	AATTCAAAAACGATGTTGTACAAG GTATAAACTCGAGTTTATACCTTGT ACAACATCG
shXrn#2	CCGGGTGGTATTAATACCGTTTATT CTCGAGAATAAACGGTATTAATACC ACTTTTTG	AATTCAAAAAGTGGTATTAATACCG TTTATTCTCGAGAATAAACGGTATT AATACCAC
shXrn1#3	CCGGTGATATCTATGTGTCAGAAAT CTCGAGATTTCTGACACATAGATAT CATTTTTG	AATTCAAAAATGATATCTATGTGTC AGAAATCTCGAGATTTCTGACACA TAGATATCA
sgXrn1#1	caccgGCTTGGATTAACAAGTCACG	aaacCGTGACTTGTTAATCCAAGCc
sgXrn1#2	caccgTTTACCGATGGATCTCGGAG	aaacCTCCGAGATCCATCGGTAAAc
sgMda5	caccgCGTAGACGACATATTACCAG	aaacCTGGTAATATGTCGTCTACGc
sgPkr	caccgTGTAGATGTGACAACGCTAG	aaacCTAGCGTTGTCACATCTACAc
sgMavs	caccgTCTCTCGTAAGCCATGGTCA	aaacTGACCATGGCTTACGAGAGAc
sgSting	caccgCAGTAGTCCAAGTTCGTGCG	aaacCGCACGAACTTGGACTACTGc
sgRig-I	caccgGATATCATTTGGATCAACTG	aaacCAGTTGATCCAAATGATATCc

Table S2. Primers used for real time qPCR and Sanger sequencing.

Target	Sequence forward (5' to 3')	Sequence reverse (5' to 3')
XRN1	CTGTCTCAGCGAAGTGGTGA	CTGCCTCCTTTGCTGACCTAA
GAPDH	AACGGGAAGCTTGTCATCAA	TGGACTCCACGACGTACTCA
Xrn1	GAAGCTGACATGCCATATGAACAA	GCTCTCTCCTTGGCTTCACT
Isg15	GGTGTCCGTGACTAACTCCAT	TGGAAAGGGTAAGACCGTCCT
Ifna1	CTGCCTGAAGGACAGGAAGG	AGCTCACTCAGGACAGGGAT
Ifna4	CCCAGAGAGTGACCAGCATC	TGTCAAGGCCCTCTTGTTCC
Ifnb1	CGTGGGAGATGTCCTCAACT	CTGAAGATCTCTGCTCGGACC
Ifng1	CGGCACAGTCATTGAAAGCC	TGTCACCATCCTTTTGCCAGT
Ifnl2	CTGAGCCACATTCACTCCCA	AGGCGAAACAGGTTGGAGG
Rig-I	GAGACCGAGCGAGAGCTTAC	TTCAGAGCATCCACGAGTGC
Oasl	CAGGAGCTGTACGGCTTCC	CCTACCTTGAGTACCTTGAGCAC
Ifi44	TACCCATGACCCACTGCTGA	CCAAATGCAGAATGCCATGTTT
Dhx58	CTCTAGTTAAGCGGGCAGCA	GTAGACCGAGAAGTTGGGGT
Irf1	CAGCATCTCGGGCATCTTTC	AGTGATTGGCATGGTGGCTTT
Irf7	CTCTGCCCACACAGGTTCTG	ATGCTGCATAGGGTTCCTCG
Pkr	GAAACAGAAGAGAACCGGCCA	AATGGCTACTCCGTGCATCT
Stat1	CGTGCAGTGAGTGAGTGAGA	ACCACTGTGACATCCTTGAGAT
Cxcl10	GAGAGACATCCCGAGCCAAC	CGTGGCAATGATCTCAACAC
Actin	AGTGTGACGTTGACATCCGT	TCAGTAACAGTCCGCCTAGA
Mda5	GTGCAGCTGTCAGACTTCTCT	GACTGCTTTTTGGCTGCTCC
Tlr3	GTGAGATACAACGTAGCTGACTG	TCCTGCATCCAAGATAGCAAGT
Iftt1	CCGTAGGAAACATCGCGTAGA	TGGCCTGTTGTGCCAATTCT
Ifit3	TGTGGAGTGCTGCTTATGGG	TCAAAAGGTGCTCTGTCTGC
Ifit3b	CACTGTAGTTGCTTAACCCTGA	CCACCACACACAGGACTCTAT
Ifitm3	GGGATCGGAAGATGGTGGGT	CCGGAAGTCGGAATCCTCTATT
Cd3d	GAAGATGGAACACAGCGGGA	AACCATCCTTCCACCGTTCC
Cd3e	TTCAGAAATGAAGTAATGAGCTGGC	TGTCTAGAGGGCACGTCAAC
Cd19	CGTGGAGGATAGTGGGGAGAT	GATTCAAACTGCTCCCCCGA
Cd274	CGCTGAAAGTCAATGCCCCA	TTCTCTTCCCACTCACGGGT
U6	GACTATCATATGCTTACCGT	
Xrn1-KO#9-validation	CGGTGCTAAGGGACTACCA	

Table S3. Antibodies used in this study.

Target antigen	Vendor	Catalogue No.
Human & mouse XRN1	Santa Cruz	sc-165985, RRID: AB_2304774
Mouse RIG-I	Santa Cruz	sc-376845, RRID: AB_2732794
Mouse MDA5	Protein Tech	21775-1-AP
Mouse STAT1	Cell signaling technology	9172
Mouse p-STAT1	Cell signaling technology	7649
Mouse p-IRF3	Cell signaling technology	4947S
Mouse p-IRF7	Thermo Fisher	PA5-64834
Mouse p-TBK1	Cell signaling technology	5483
Human & mouse GAPDH	Cell signaling technology	2118
Human β-ACTIN	Sigma Aldrich	A5441
Mouse PKR	Santa Cruz	sc-6282
Mouse TLR3	Santa Cruz	sc-32232
Mouse ISG15	Santa Cruz	sc-166755
Mouse MAVS	Cell signaling technology	4983
Mouse STING	Cell signaling technology	13647
Mouse CD3e	Abcam	ab16669, RRID: AB_443425
Mouse CD4	Abcam	ab183685, RRID: AB_2686917
Mouse CD8a	Cell Signaling Technology	98941

Table S4. siRNAs used in this study.

Target	**Vendor**	**Sequence/ Catalogue No.**
Ctrl	Sigma Aldrich	UCGAAGUAUUCCGCGUACG
Mouse *Xrn1*	Sigma Aldrich	SASI_Mm01_00154570
Mouse *Sting*	Sigma Aldrich	SASI_Mm02_00429135
Mouse *Mavs*	Sigma Aldrich	SASI_Mm01_00042222

Table S5. Primers used to generate luciferase reporter constructs.

Primer name	Sequence (5' to 3')
XRN1-WT-F	cccggggtaccCCAGTACATTGCAGAAATGGAGAC
XRN1-WT-R	cccggacgcgtGTTCACAAAGCTGTTTCTGGGT
XRN1-Ctrl-F	cccggggtaccttggattataggcatgagccacc
XRN1-Ctrl-R	cccggacgcgtccggccCAATccctgtatc
XRN1-Mut-F	AGGTTTTCAGTccCTCTacgTCATCTTGTAAAAACAGAAA
XRN1-Mut-R	CAAGATGAcgtAGAGggACTGAAAACCTATGCTACTGAGG

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