Supporting Information

Franz et al. 10.1073/pnas.1716937115

SI Materials and Methods

Antibodies, Plasmids, and siRNAs. The antibodies used were anti-STING (Cell Signaling Technology), anti–β-actin (Sigma), anti-SeV (Meridian Life Science), anti-NS1 (from ref. 59), anti-T3D (gift from M. Nibert, Harvard Medical School, Boston), anti-puromycin (EMD Millipore), anti-eIF2α (Cell Signaling Technology), anti–phospho-eIF2α (Cell Signaling Technology), anti-VSV M (Kerafast), K1 [anti-poly(I:C)] (Scicons), and anti-HA (Sigma).

The shCTRL plasmid was a gift from D. Sabatini, Whitehead Institute for Biomedical Research, Cambridge, MA (Addgene plasmid no. 1864), and shSTING and mcGAS plasmid were gifts from Z. Chen, University of Texas Southwestern Medical Center, Dallas. In vitro-transcribed mRNA was cloned using sequences from pcDNA3 RLUC POLIRES FLUC and was a gift from N. Sonenberg, McGill University, Montreal (Addgene plasmid no. 45642). VSV-F_{IRES}R and in vitro-transcribed mRNA were cloned using sequences from pFR_CrPV_xb and were a gift from P. Sharp, Massachusetts Institute of Technology, Cambridge, MA (Addgene plasmid no. 11509). MSCV LC3-GFP, MSCV STING-HA, and pSTING were cloned within the laboratory.

The siRNAs targeting STING and the nontargeted control were purchased from Qiagen, and siRNA targeting MAVS was purchased from Life Technologies. RNA depletion was achieved by transfecting 20 nM siRNAs using Lipofectamine RNAiMAX (Invitrogen).

Cell Culture and Lentivirus Transduction. MEFs (spontaneously immortalized), 293T, and Veros were maintained in DMEM complete media supplemented with 10% FBS and cultured according to standard techniques. MAVS KO and matched control MEFs were a gift from Z. Chen, University of Texas Southwestern Medical Center, Dallas. Primary RIG-I KO and matched control MEFs were a gift from M. Gale, University of Washington, Seattle, and primary STING KO and matched control MEFs were a gift from A. Poltorak, Tufts University School of Medicine, Boston. RIG-I/MDA5 KO MEFs were a gift from S. Akira, Osaka University, Osaka. STING-HA allele and LC3-GFP were introduced into cells by retroviral vector transduction. shCTRL and shSTING were introduced into cells by lentiviral vector transduction.

Inhibitors and Ligands. Inhibitors and ligands used in this study were ISRIB (1 μM; Sigma), actinomycin D (5 μg/mL; Sigma), cycloheximide (10 μg/mL; Sigma), P6 (5 μM; Calbiochem), and DMSO (Sigma). High-molecular weight poly(I:C) (Invivogen) and herring testes DNA (Sigma) were transfected into cells with Lipofectamine 2000 (Thermo Fisher Scientific).

For senescence assays, shCTRL and shSTING MEFs were incubated with 10 μM BrdU for 12 h before cells were fixed. BrdU staining was performed using a BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol. Cells were analyzed via flow cytometry on a BD FACSCanto II (Becton Dickinson) and analyzed using FlowJo v10 (FlowJo, LLC).

Senescence β-galactosidase staining was performed according to the manufacturer's protocol (Cell Signaling Technology). Data were acquired using a Nikon TS100 Eclipse light microscope, and images were acquired using NIS Elements (Nikon Instruments) software.

cGAMP Activity Assay. To test for cGAMP activity, cells were homogenized in hypotonic buffer (10 mM Tris·HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$). Lysates were centrifuged at 100,000 × *g* for 20 min. Supernatants were incubated at 95 °C for 5 min and centrifuged at 12,000 × *g* for 5 min. Supernatants were recovered and treated with 1 U/uL of benzonase (EMD Millipore) for 30 min at 37 °C followed by 100 μg/mL of Proteinase K (Thermo Fisher Scientific) for 60 min at 50 °C. The supernatant was incubated at 95 °C for 5 min and then cooled to 25 °C (10). The supernatant was mixed with 10× digitonin permeabilization solution (50 mM Hepes, pH 7.0, 100 mM KCl, 3 mM MgCl2, 0.1 mM DTT, 85 mM Sucrose, 0.2% BSA, 1 mM ATP, 0.1 mM GTP, ±10 μg/mL Digitonin) to achieve a 1× digitonin permeabilization solution (60). The buffer was incubated with MEFs for 20 min at 37 °C. The digitonin permeabilization solution was then aspirated from the cells and replaced with normal growth media. RNA was isolated from the MEFs 8 h later, and *Ifnb1* transcript was analyzed.

Immunofluorescence. For immunofluorescence analysis, cells were fixed with 2% paraformaldehyde in PBS for 15 min at 25 °C. Cells were permeabilized with 0.1% Triton X in PBS for 10 min before incubating overnight in block buffer (2% goat serum, 50 mM ammonium chloride in PBS) at 4 °C. The indicated antibodies were diluted in block buffer and incubated with the cells overnight at 4 °C. Antibody binding was detected by secondary antibodies conjugated to AlexaFluor 488 or 568 (ThermoFisher Scientific). Nuclei were stained with DRAQ5 (Thermo Scientific). Confocal images were acquired using a spinning disk confocal head (CSU-X1; Perkin-Elmer Co.) coupled to a fully motorized inverted Zeiss Axiovert 200M microscope equipped with a 63× lens (Pan Apochromat, 1.4 N.A.). The imaging system operates under control of SlideBook 6 (Intelligent Imaging Innovations Inc.).

Statistical Analysis. Statistical analysis of viral growth curves was performed as described (61). All statistical analysis was done in Prism 7 (GraphPad Software, Inc.). Except where noted, all statistical analysis was carried out by unpaired Student's *t* test, with $P < 0.05$ considered statistically significant. When performing statistical analysis on viral titers, statistical analysis was done on the log transform of the data.

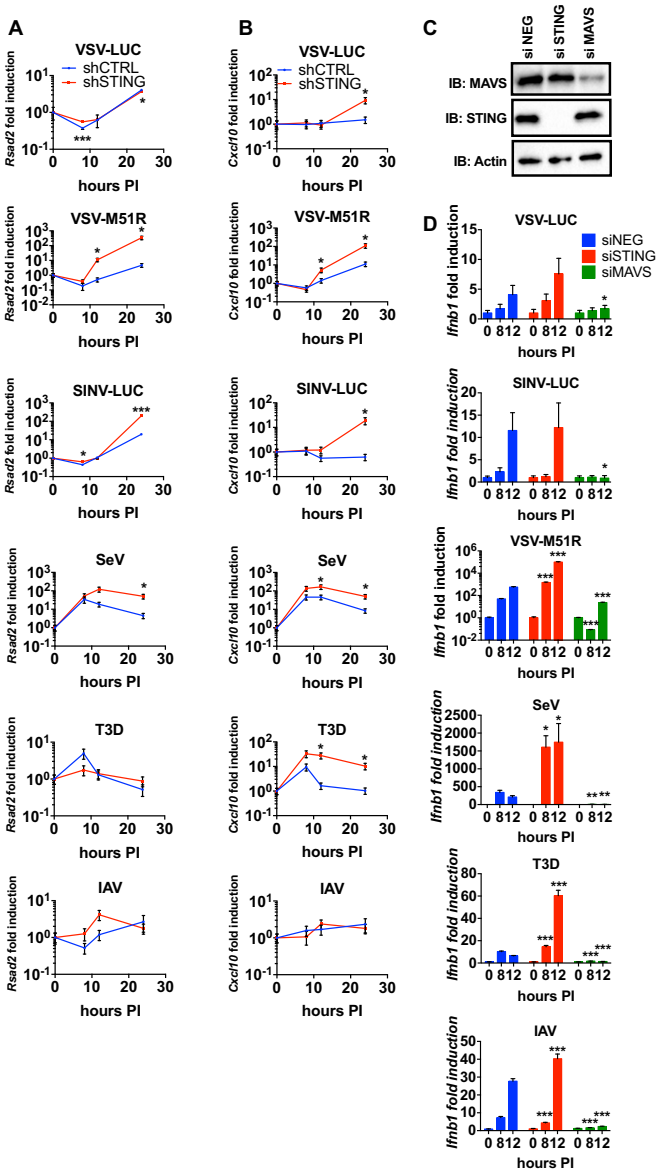

Fig. S1. IFN-stimulated gene expression is MAVS- not STING-dependent. (*A* and *B*) Induction of the ISGs *Rsad2*, and *Cxcl10* during the indicated RNA virus infection. Samples were the same as those analyzed in Fig. 1 *E* and *G*. Data are displayed as fold induction of the indicated gene compared with uninfected cells. Data are represented as mean ± SEM. (*C*) WT MEFs were transfected with siRNAs targeting the indicated genes. Lysates were separated by SDS/PAGE and endogenous protein expression was detected with STING-, MAVS-, and actin-specific antibodies. (*D*) MEFs transfected with the indicated siRNAs were infected with the indicated virus. RNA was isolated at the indicated time, and *Ifnb1* transcripts were analyzed with qRT-PCR. Data are displayed as fold induction of *Ifnb1* compared with uninfected cells. Data are represented as mean ± SEM. Statistics are calculated from comparison of siNEG to siSTING or siMAVS at the indicated time point. $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$ (Student's *t* test).

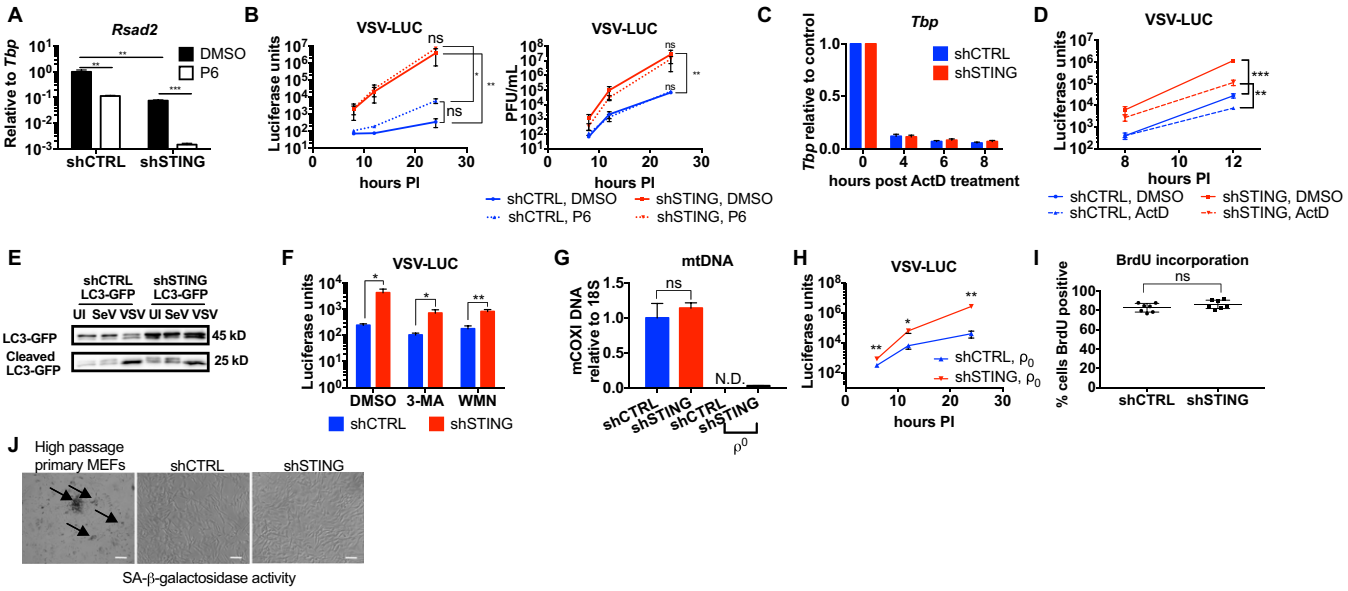

Fig. S2. Mechanistic analysis of STING-dependent restriction of VSV replication. (*A*) The MEFs indicated were incubated with P6 or DMSO for 24 h. Rsad2 transcripts were analyzed by qRT-PCR. Data are represented as mean ± SEM. (*B*) P6- and DMSO-treated cells were infected with VSV-LUC (MOI 0.1) and monitored by luciferase assay at the indicated time after infection (*Left*). VSV-LUC titer was determined by pfu assay (pfu/mL) at the indicated time post-infection (*Right*). Data are represented as mean ± SEM. (*C*) The MEFs indicated were treated with DMSO or actinomycin D (ActD). *Tbp* transcripts were analyzed by qRT-PCR at the times indicated. Level of *Tbp* is represented as the ratio between *Tbp* in ActD-treated cells at the indicated time and *Tbp* in DMSO-treated cells at time 0. Data are represented as mean ± SEM. (*D*) The MEFs indicated were treated with DMSO or ActD for 30 min and then infected in the presence of drug with VSV-LUC at MOI 0.1. Virus replication was monitored by luciferase assay at the indicated time after infection. Data are represented as mean ± SEM. (*E*) The MEFs indicated were transduced with a vector expressing LC3-GFP. Cells were infected with WT VSV at MOI 1.0 or SeV at 250 HAU/mL for 8 h. Lysates were separated by SDS/PAGE. LC3-GFP was detected with a GFP antibody. (*F*) The MEFs indicated were treated with DMSO, 3-MA, or WMN for 30 min and then infected in the presence of drug with VSV-LUC at MOI 0.1. Virus replication was monitored by luciferase activity assay 8 h after infection. Data are represented as mean ± SEM. (*G*) The mitochondrial DNA gene mCOX1 was analyzed by qPCR for the indicated cell lines and is represented relative to 18S DNA. Data are represented as mean ± SEM. (*H*) ρ^0- and mock-treated cells were infected with VSV-LUC at MOI 0.1. N.D., not detected. (*H*) Virus replication was monitored by luciferase assay at the indicated time postinfection. Data are represented as mean ± SEM. (*I*) The MEFs indicated were incubated with BrdU for 12 h. BrdU incorporation was measured by flow cytometry. Data represent independently cultured replicates. (*J*) Images of shCTRL, shSTING, and primary MEFs 30 d in culture, stained for SA–β-gal activity. (Scale bar, 100 μm.) Arrows highlight positive stained cells. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.0001 (Student's *t* test).

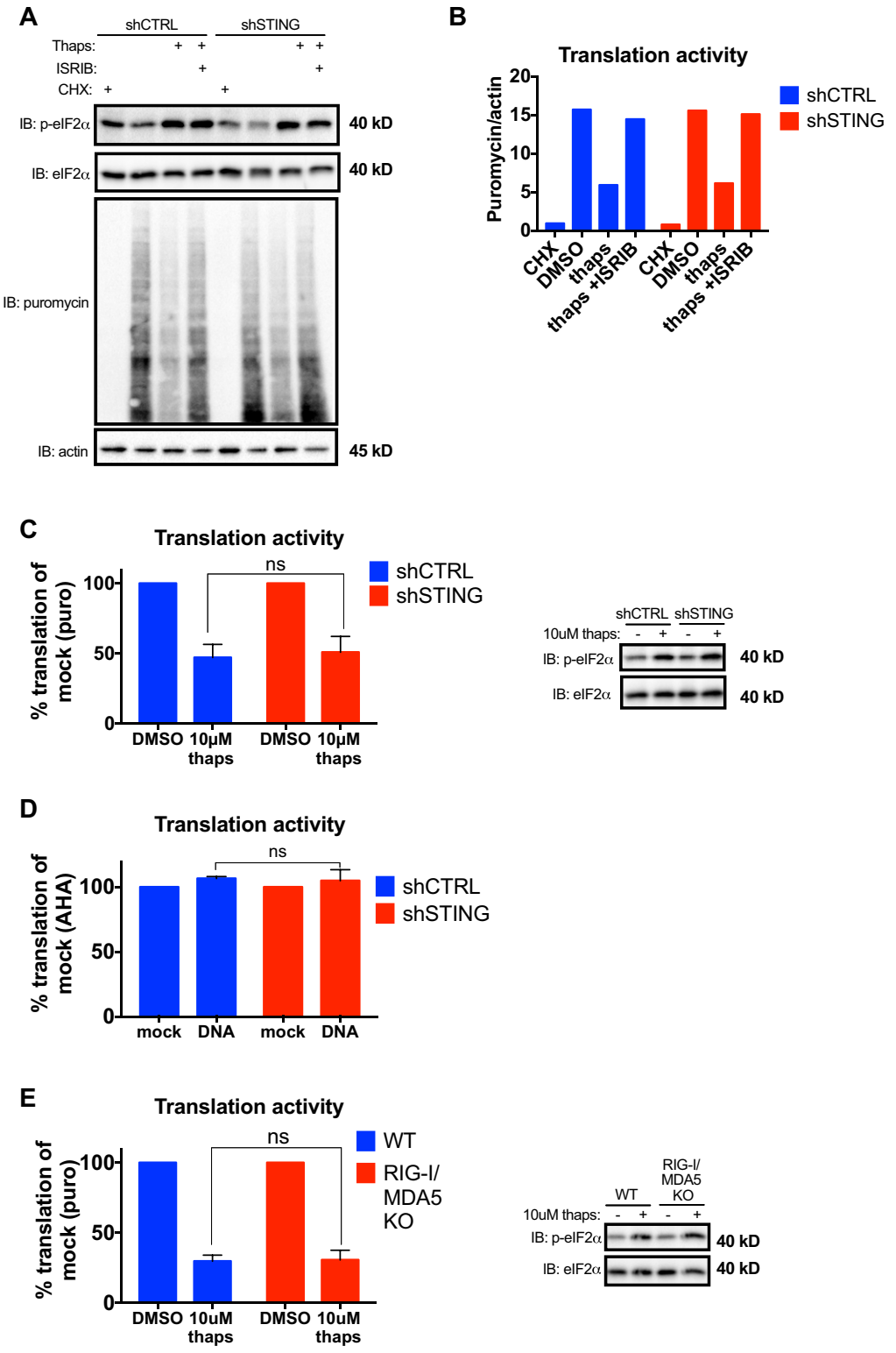

Fig. S3. STING does not regulate translation in response to non-RNA ligands. (*A*) shCTRL and shSTING MEFs were treated with DMSO or 10 μM thapsigargin for 6 h. Indicated cells were treated with 1 μM ISRIB or 10 μg/mL cycloheximide for 10 min before incubating cells with 15 μg/mL puromycin for 15 min. Lysates were separated by SDS/PAGE. Phospho-eIF2α, total eIF2α, actin, and puromycin were detected by specific antibodies. (*B*) Puromycin and actin signal was quantified with ImageJ. Data represent puromycin signal normalized to actin. (*C*, *Left*) shCTRL and shSTING MEFs were treated with DMSO or thapsigargin for 6 h. Cells were incubated with 15 μg/mL puromycin for 15 min. Lysates were separated by SDS/PAGE, and actin and puromycin were detected by specific antibodies. Puromycin signal was quantified and normalized to the actin signal. Data are represented as percent puromycin signal of mock-treated. (*C*, *Right*) Lysates were separated by SDS/PAGE, and phospho-eIF2α and total eIF2α were detected by specific antibodies. (*D*) shCTRL and shSTING MEFs were transfected with 5 μg/mL of herring testes DNA and then labeled with AHA. Six hours after transfection, cells were fixed and AHA incorporation was measured with flow cytometry.

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AHA MFI of all populations was determined. Data are shown as percent MFI of mock-transfected cells. Data are represented as mean ± SEM. (*E*, *Left*) WT and RIG-I/MDA5 KO MEFs were treated with DMSO or thapsigargin for 6 h. Cells were incubated with 15 μg/mL puromycin for 15 min. Lysates were separated by SDS/PAGE, and actin and puromycin were detected by specific antibodies. Puromycin signal was quantified as in *C*. (*E*, *Right*) Lysates were separated by SDS/PAGE, and phospho-eIF2α and total eIF2α were detected by specific antibodies. ns, not significant.

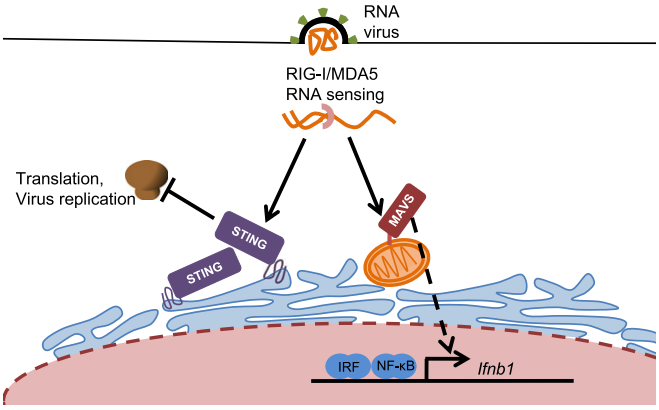

Fig. S4. STING restricts translation to control RNA virus replication. During infections of WT fibroblasts, RIG-I and/or MDA5 activate IFN through MAVS and inhibit translation through STING. In the absence of STING, IFN responses remain high, but viruses gain access to translation machinery and replicate efficiently.

Table S1. Viruses used in this study

Virus name	Genome type	Family	Viral replication subcellular location
VSV	(–) ssRNA	*Rhabdoviradae*	Cytoplasm
SINV	(+) ssRNA	*Togaviridae*	Cytoplasm, on endosomes
SeV	(–) ssRNA	*Paramyxoviridae*	Cytoplasm
Influenza A PR8 (IAV)	(–) ssRNA, segmented	*Orthomyxoviridae*	Nucleus
Reovirus T3D (Reo)	dsRNA, segmented	*Reoviridae*	Cytoplasm

ssRNA denotes single-stranded RNA genome; dsRNA denotes dsRNA genome. (+) denotes genome is positive-sense; (–) denotes genome is negative-sense. Unless noted, genomes are nonsegmented.