RIG-I detects mRNA of intracellular Salmonella enterica serovar Typhimurium during bacterial infection

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Abstract

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RIG-I Detects mRNA of Intracellular Salmonella enterica Serovar Typhimurium During Bacterial Infection

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ABSTRACT

The cytoplasmic helicase RIG-I is an established sensor for viral 5′-triphosphorylated RNA species. Recently, RIG-I was also implicated in the detection of intracellular bacteria. However, little is known about the host cell specificity of this process and the bacterial pathogen-associated molecular pattern (PAMP) that activates RIG-I. Here we show that RNA of Salmonella enterica serovar Typhimurium activates production of beta interferon in a RIG-I-dependent fashion only in nonphagocytic cells. In phagocytic cells, RIG-I is obsolete for detection of Salmonella infection. We further demonstrate that Salmonella mRNA reaches the cytoplasm during infection and is thus accessible for RIG-I. The results from next-generation sequencing analysis of RIG-I-associated RNA suggest that coding bacterial mRNAs represent the activating PAMP.

IMPORTANCE S. Typhimurium is a major food-borne pathogen. After fecal-oral transmission, it can infect epithelial cells in the gut as well as immune cells (mainly macrophages, dendritic cells, and M cells). The innate host immune system relies on a growing number of sensors that detect pathogen-associated molecular patterns (PAMPs) to launch a first broad-spectrum response to invading pathogens. Successful detection of a given pathogen depends on colocalization of host sensors and PAMPs as well as potential countermeasures of the pathogen during infection. RIG-I-like helicases were mainly associated with detection of RNA viruses. Our work shows that S. Typhimurium is detected by RIG-I during infection specifically in nonimmune cells.

Pattern recognition receptors (PRRs) recognize broadly shared molecular structures known as pathogen-associated molecular patterns (PAMPs). Five main families of PRRs are known: Toll-like receptors (TLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR), cytoplasmic DNA receptors, and C-type lectin-like receptors (reviewed in references 1, 2, 3, 4, and 5). Recognition of a microbial PAMPs results in the activation of PRR-specific downstream-signaling cascades and expression of a variety of antimicrobial and proinflammatory cytokines and chemokines.

In contrast to the use of purified artificial PAMPs, successful induction of an immune response during infection depends on a number of factors, including expression of the PRR in the infected cell type, colocalization of the PRR and PAMP in the same subcellular compartment, and the ability of the cell to overcome pathogen evasion strategies that serve to block innate immune recognition and signaling. Depending on the cell type and the pathogen studied, these factors may differ.

RIG-I is a member of the RLR family (4). Upon binding of 5′-triphosphorylated RNA, RIG-I undergoes conformational changes and posttranslational modifications that allow multimerization and interaction with the mitochondrial antiviral signaling protein (MAVS) (6). Subsequent signaling events ultimately result in formation of the beta interferon (IFN-β) enhanceosome and IFN-β expression. IFN-β is a key cytokine in innate antiviral immune responses, mediating expression of hundreds of IFN-stimulated genes (ISGs) that are responsible for the establishment of an antimicrobial state in the infected tissue.

A role for RIG-I in bacterial sensing has recently been described (reviewed in reference 7). However, the underlying mechanisms of detection and the nature of the activating PAMP, especially under infection conditions, remain less well characterized. Here we show that RIG-I-dependent recognition of intracellular bacteria is pathogen and cell type dependent, using Salmonella Typhimurium as a model pathogen. We further demonstrate that bacterial mRNA is recognized by RIG-I during infection, leading to expression of IFN-β.

RESULTS

Salmonella enterica consists of a group of flagellated, Gram-negative, facultative intracellular bacteria (reviewed in reference 8). They are the leading cause for gastroenteric disease in animals, including humans. S. enterica serovar Typhimurium causes self-limiting gastroenteritis in humans. To test if RIG-I is involved in
recognition of *S. Typhimurium*, we compared the type I interferon response in wild-type (WT) and RIG-I−/− fibroblasts upon infection. Infection of WT fibroblasts with *S. Typhimurium* induced expression of IFN-β mRNA (Fig. 1A). IFN-β expression was abolished in RIG-I−/− murine embryonic fibroblasts (MEFs), suggesting that RIG-I was the primary PRR leading to IFN-β expression. Reconstitution of RIG-I−/− MEFs with RIG-I restored IFN-β expression, albeit at levels lower than those seen with WT MEFs (see Fig. S1A and B in the supplemental material). We observed similar titers of intracellular bacteria in WT, RIG-I−/−, and RIG-I-reconstituted MEFs, suggesting that differences in replication did not account for the lack of IFN-β induction in RIG-I−/− MEFs (see Fig. S1C).

RIG-I- and melanoma differentiation-associated protein 5 (MDA5)-dependent induction of IFN-β requires adaptor MAVS. In accordance with our data from RIG-I−/− MEFs, we detected reduced IFN-β mRNA levels in *S. Typhimurium*-infected MAVS−/− MEFs compared to MAVS+/+ MEFs (Fig. 1B). As expected, both Sendai virus (SeV) and encephalomyocarditis virus (EMCV), which are recognized specifically through the activity of RIG-I and MDA5, respectively, also show defective IFN-β induction in MAVS−/− MEFs. In support of the idea of primary detection of *S. Typhimurium* by RIG-I, fibroblasts deficient in Myd88 and Trif showed no defect in the induction of IFN-β upon *S. Typhimurium* infection (Fig. 1C). We confirmed RIG-I-dependent recognition of *S. Typhimurium* infection in two human RIG-I knockdown epithelial cells. Human A549 and HT-29 cells constitutively expressing small hairpin RNAs (shRNAs) against RIG-I showed reduced IFN-β mRNA levels (see Fig. S1D and E in the supplemental material). Taken together, these data implicate primary detection of *Salmonella* by the RIG-I/MAVS pathway in fibroblasts in the induction of IFN-β in response to bacterial infection.

*S. Typhimurium* infects both phagocytic and nonphagocytic cell types in *vivo*. To investigate the role of RIG-I in phagocytic cells, we chose macrophages as a model system. In contrast to our results in nonphagocytic cells, *S. Typhimurium* infection of RIG-I−/− bone marrow-derived macrophages (BMDM) induced...
IFN-β responses equivalent to those seen with WT BMDM under the chosen experimental conditions (Fig. 1D), despite a significant reduction in response to Sendai virus, confirming RIG-I deficiency. In accordance, IFN-β production was also independent of the presence of MAVS in BMDM (Fig. 1E).

The lack of a role for RIG-I in IFN-β induction in BMDM suggested that other PRRs were responsible for the detection of S. Typhimurium in these cells. To test this, we infected BMDM deficient in MyD88 and TIR-domain-containing adapter-inducing IFN-β (TRIF) with S. Typhimurium. In contrast to our results with RIG-I−/− BMDM, MyD88/Trif−/− macrophages showed a significant reduction in IFN-β expression in response to S. Typhimurium and Salmonella lipopolysaccharide (LPS) but normal responses to Sendai virus (Fig. 1F). This suggests that RIG-I acts as the primary sensor for S. Typhimurium in nonphagocytic cells, whereas TLR are the primary sensors in phagocytic cells.

In contrast to the natural infection route, transfection of BMDM with Salmonella RNA led to IFN-β induction in a RIG-I-dependent and MyD88/Trif-independent fashion, confirming that BMDM are capable of recognizing bacterial RNA through RIG-I (Fig. 1G). This suggests either that bacterial RNA does not reach RIG-I in the cytoplasm during natural infection or that TLR responses mask the effect of RIG-I activation. Two recent studies showed RIG-I-dependent recognition of Listeria monocytogenes (9, 10). Abdullah and colleagues showed a 3- to 5-fold reduction in responses that shows defective invasiveness. We confirmed that the invG mutant strain showed 100-fold-reduced invasiveness in nonphagocytic cells (Fig. 3A). Interestingly, MEFS infected with WT Salmonella at a multiplicity of infection (MOI) of 1 provoked robust induction of IFN-β (Fig. 3B). The attenuated strain required 100- to 1,000-fold more bacteria to induce similar IFN-β

![Graph](graph.png)
mRNA levels, indicating that similar amounts of intracellular bacteria are required for induction of IFN-β for WT and ΔinvG Salmonella.

Next, we tested if RIG-I has access to SCVs, a possibility which could explain the RIG-I-dependent detection of bacterial RNA. We examined the localization of RIG-I in HeLa cells due to their large cytoplasm. By confocal laser scanning microscopy (LSM), we analyzed the localization of RIG-I-mCherry in Salmonella-infected cells. RIG-I did not colocalize with SCV (Fig. 3C), suggesting that bacterial RNA must be transferred from SCV to the cytoplasm for detection by RIG-I. To investigate whether Salmonella RNA reaches the cytoplasm during infection, we used HeLa cells constitutively expressing MS2-green fluorescent protein (MS2-GFP) with a nuclear localization signal (Fig. 3D and E). In this system, newly translated MS2-GFP translocates into the nucleus, keeping the cytoplasm free from background fluorescence. In contrast, MS2-GFP forms foci in the cytoplasm in the presence of cytoplasmic RNA containing M2 binding sites. HeLa MS2-GFP cells were infected with either S. Typhimurium SL1344 or SL1344 expressing an RNA with 24 repeats of MS2 binding sites (SL1344-MS2aptamer). SL1344-MS2aptamer-infected HeLa MS2-GFP cells showed cytoplasmic foci containing GFP at a high MOI (Fig. 3G). We did not detect these GFP accumulations in mock-infected cells (Fig. 3E). In the samples infected with WT SL1344,
we found only 1 cell with a GFP complex in the cytoplasm, despite clear accumulation of intracellular bacteria (Fig. 3F). Quantification in 40 randomly chosen, SL1344-positive cells revealed an average of 4.025 MS2-GFP complexes per infected cell (Fig. 3H). Taking these findings together, this suggests that intracellular replication of Salmonella leads to RNA transfer from SCVs into the cytoplasm where RIG-I activation occurs.

RIG-I is activated by a variety of RNA species in vitro (4). It was shown that 5′-triphosphorylated RNA is a very potent ligand of RIG-I (13–16). In addition, double-stranded RNA motifs and poly(U/UC) stretches also contribute to recognition by RIG-I (17). To understand the characteristics of the bacterial immunostimulatory RNA, we first fractionated total RNA from S. Typhimurium using size-specific-cutoff centrifugation columns (Fig. 4A; see also Fig. S2A in the supplemental material). A significant proportion of the total bacterial RNA was shorter than 500 bp (Fig. 4B). Nevertheless, removal of this 80% fraction of RNA did not affect the immunostimulatory capacity of the remaining RNA (Fig. 4A). This suggests that small bacterial RNAs do not play a major role in activating RIG-I during bacterial infection.

To identify the class of bacterial RNA responsible for RIG-I activation, lysates of epithelial cells were incubated with total RNA from S. Typhimurium. RIG-I complexed with RNA was immunoprecipitated. Next, we sequenced the RIG-I-complexed RNA by next-generation Illumina sequencing (Fig. 4C). The majority (95.4%) of the total Salmonella RNA from bacterial lysates corresponded to ribosomal and other noncoding RNAs. Only a small fraction (2.3%) of the total RNA represented coding RNAs (Fig. 4C; input). However, the RNA specifically immunoprecipitated by RIG-I was highly (64.2%) enriched for coding bacterial RNAs without specific preference for a subset of coding RNAs (Fig. 4C; see also Table S1 in the supplemental material). Nonspecific RNA that was immunoprecipitated with control IgG had ratios of coding versus noncoding bacterial RNA similar to those seen with the input RNA, confirming the specificity of the pulldown. In order to confirm enrichment of immunostimulatory RNAs among the RIG-I-bound RNAs, we transfected input and pulldown RNAs into A549 cells and tested IFN-β mRNA expression by quantitative PCR (qPCR) (Fig. 4D). RNA pulled down with RIG-I resulted in increased IFN-β mRNA levels compared to the results seen with identical amounts of input RNA (40 ng). Transfection of the pulldown RNA into WT, RIG-I−/−, and reconstituted RIG-I−/− MEFs confirmed the immunostimulatory capacity of the RNA in a RIG-I-dependent fashion (see Fig. S2B).

Eubacteria do not encode the enzymatic machinery for synthesis of RNA caps (18); hence, intact bacterial mRNAs expose a 5′-triphosphorylated end. Removal of the first two phosphate groups initiates degradation of bacterial mRNA (19). To demonstrate that primary transcripts of coding bacterial RNAs represent a RIG-I-detectable PAMP, we utilized purified Escherichia coli RNA-polymerase holoenzyme. We generated a bacterial mRNA transcript (E. coli CLPb) in vitro (see Fig. S3A in the supplemental material). Transfection of this RNA into MEFs induces robust, RIG-I-dependent IFN-β mRNA expression in amounts as low as 10 ng/5 × 10⁴E4 cells (Fig. 4E). To test if this immunostimulatory capacity is sequence specific to ClpB mRNA or a feature of all primary products of bacterial polymerase, we tested mCherry mRNA, a nonbacterial mRNA, generated with E. coli holoenzyme in vitro. As shown before for RNA generated from a bacterial template, this RNA activates IFN-β expression (data not shown). In order to validate the binding of RIG-I to coding RNAs under infection conditions, we used Salmonella expressing an exogenous marker mRNA (MS2tapamer). We infected A549 cells with these bacteria for 8 h and pulled down RIG-I-associated RNA as described before. We compared the relative amounts of MS2tapamer mRNA in isotype control and RIG-I-specific pulldowns by qPCR using identical amounts of RNA (Fig. 4F). RIG-I-specific pulldown results in a 10-fold enrichment of marker mRNA over control IgG pulldown.

To determine if RIG-I activation by S. Typhimurium RNA is dependent on a 5′-triphosphate, we treated total RNA of S. Typhimurium, in vitro-transcribed (IVT) bacterial RNA, influenza virus A (IAV) RNA, and long poly(I·C) with calf intestinal phosphatase (CIP) for 5 to 60 min and transfected these RNAs in A549 cells (see Fig. S3B in the supplemental material). Long poly(I·C) is recognized in a 5′-triphosphate- and RIG-I-independent manner by MDA-5, resulting in induction of IFN. CIP treatment of poly(I·C) did not affect IFN-β induction, confirming the absence of RNase contamination of the CIP enzyme. Dephosphorylation abolished the IFN-β-inducing potential of IAV RNA within 5 min. In contrast, the immunostimulatory capacity of S. Typhimurium RNA and IVT bacterial RNA decreased more slowly, reaching ~1% of the initial IFN-β-inducing capacity after 40 to 60 min of CIP treatment (see Fig. S3B in the supplemental material).

Our data suggest that RIG-I preferentially binds to coding RNA from S. Typhimurium during bacterial infection. Recognition occurs through the RD of RIG-I, and immunostimulatory activity depends on 5′ triphosphorylation.

DISCUSSION

Recently, multiple studies investigated the role of RLR signaling in antibacterial responses (reviewed in reference 7). However, the underlying mechanisms of detection and the nature of the activating PAMP remain less well understood.

S. Typhimurium invades nonphagocytic as well as phagocytic cells in vivo. The composition of the intracellular compartment that Salmonella establishes for replication depends on the host cell type (reviewed in reference 20) and the mechanism of bacterial entry (21). It was therefore unclear whether the innate immune system provides PRR in nonphagocytic cells to detect this invasion and whether the mechanisms of detection are similar in phagocytic and nonphagocytic cells.

Here we demonstrate that recognition of S. Typhimurium requires different subsets of PRR in phagocytic and nonphagocytic cells. The reasons for this are most likely multifactorial. Macrophages rely highly on TLR- and NLR-dependent recognition of phagocytosed bacterial pathogens (22). This broad panel of PRRs allows the detection of multiple easily accessible bacterial PAMPs, such as components of the bacterial cell wall or flagella. Moreover,
Salmonella coding RNAs bind to RIG-I and activate IFN-β expression in a 5’-triphosphate-dependent fashion (A) Column fractionation was used to eliminate RNAs smaller than 30 or smaller than 500 bp. A549 cells were transfected with 1 μg or 4 μg of total RNA and equal volumes of fractionated RNAs. Average n-fold expression levels of IFN-β mRNA over 18S rRNA levels of three independent biological samples each measured in technical triplicate experiments ± SD are depicted. n.s., not significant. (B) Concentrations of RNAs after column fractionation were compared to total RNA as determined by spectrometry. (C) Relative representations (%) of reads of coding (black) and noncoding (white) RNAs from total bacterial RNA (Total), GFP pulldown RNA (GFP), and RIG-I pulldown RNA groups. n.d. (gray) represents RNAs that could not be classified as coding or noncoding. (D) A549 cells were transfected with 40 ng of pulldown RNA for 16 h. Average n-fold expression levels of IFN-β mRNA over 18S rRNA levels of three independent biological samples each measured in technical triplicate experiments ± SD are depicted. (E) In vitro-transcribed RNA was transfected into RIG-1+/−, RIG-1−/−, or reconstituted RIG-1−/− MEF. Average n-fold expression levels of IFN-β mRNA over 18S rRNA levels of three independent biological samples each measured in technical triplicate experiments ± SD are depicted. Significance is indicated in reference to no-enzyme control samples. (F) Enrichment of marker mRNA from Salmonella-infected A549 cells (MOI of 10, 8 h) after pull down with RIG-1-specific antibody over pull down with control IgG antibody. Equal amounts of pulldown RNA were reverse transcribed using gene-specific primers for marker mRNA. Average n-fold levels over control IgG pull down levels from three independent biological samples each measured in technical triplicate experiments ±SD are depicted.
the potential routes of infection of S. Typhimurium differ between phagocytic and nonphagocytic cells, allowing the presented PAMPs to be located in different subcellular compartments, with different sets of PRRs.

We show that Salmonella RNA is transferred into cytoplasm in nonphagocytic cells during infection. It binds to RIG-I and activates IFN-β production. These data are in accordance with an earlier study showing that transfection of Helicobacter pylori RNA into RIG-I-overexpressing 293T cells induces production of IFN-β mRNA (23). Our RIG-I pulldown experiments implicate bacterial mRNA as the immunostimulatory species among the total RNA. By column fractionation, we could rule out an impact of small RNA molecules on the immunostimulatory capacity of total bacterial RNA. A recent study showed that bacterial tRNA can activate TLR7, when lacking a posttranscriptional 2'-O-methylated G(m) 18 modification (24). It is well established that RIG-I preferentially binds to 5'-triphosphorylated RNA species (4, 13). Bacterial mRNA is uncapped and 5'-triphosphorylated. Accordingly, dephosphorylation of bacterial RNA abolished its immunostimulatory capacity in our hands. For in vitro-synthesized bacterial mRNA, it was previously shown that the addition of a 3' poly(A) tail reduces the stimulatory capacity of this RNA in dendritic cells, suggesting the presence of immunostimulatory structures at the 3' end of bacterial mRNA (25). However, detection of bacterial RNAs through RIG-I might play a minor role due to the dominance of TLR signaling in these cells.

In a recent study, Abdullah and colleagues demonstrated that macrophages partially depend on RIG-I for production of interferon upon L. monocytogenes infection (9). The same group very recently published data revealing the dependence on RIG-I of the type I interferon response to Listeria infection in nonimmune cells (10). In conjunction with our data from S. Typhimurium infections, this implies that different intracellular bacterial pathogens are sensed by different mechanisms, depending on the biology of the invading pathogen. This highlights the importance of accessibility of bacterial PAMPs to cellular PRRs for the innate host response during infection.

In summary, we demonstrate RIG-I-mediated innate immune recognition of S. Typhimurium mRNA in nonphagocytic cells under infection conditions. This is in contrast to IFN-β production in phagocytic cells in response to S. Typhimurium infection, which relies on TLR signaling. Our data implicate a role for RIG-I in detection of mRNA of invading bacterial pathogens that complements the orchestra of well-studied PRRs known to detect other bacterial PAMPs.

MATERIALS AND METHODS

Reagents. Sendai virus RNA was isolated from SeV Cantell-infected embryonated chicken eggs. TLR-grade LPS solution from Salmonella Minnesota R594 was purchased from Alexis.

Bacteria. Salmonella Typhimurium WT SL1344 (ATCC), SL1344 transformed with pM973, encoding GFP under the control of the SPI-2 promoter PsaAG (WT-GFP) (26), and SB161 (ΔinvG) (27) cells were grown in standard LB liquid culture. For infection of cell cultures, Salmonella was freshly inoculated from glycerol stocks and grown in LB medium for 16 h at 37°C and 300 rpm. Cultures were diluted 1:500 with fresh medium and grown to an optical density at 420 nm (OD_{420}) of 0.6 to 0.8. CFU levels were estimated by optical density at 420 nm and confirmed by plating serial dilutions on MacConkey’s agar. Intracellular bacterial titers were determined by permeabilization with phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Triton X-100 for 15 min. Bacterial supernatants were diluted and spotted on MacConkey’s agar containing suitable antibiotics. MS2-aptamer RNAs expressing S. Typhimurium SL1344 were generated by electroporation of S. Typhimurium SL1344 with pcCR-24XMS2SL-stable (Addgene plasmid 31865 [28]) and selection with 100 µg/ml ampicillin.

Listeria monocytogenes was kindly provided by Thomas Moran (Icahn School of Medicine at Mount Sinai). L. monocytogenes was grown in brain heart infusion broth.

Cells. Stimulation of 293T-FF cells was described previously (16). RI5-/-/+, RI5-/-/-, reconstituted RI5-/-/- MEF, MAVS-/-/- MEF, and TRIF/MdD88 double-knockout (DKO) fibroblasts were described earlier (29–31). All MEFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM)—10% fetal calf serum (FCS) containing penicillin-streptomycin. Mouse bone marrow-derived macrophages (BMDM) were generated and cultured as described earlier (32).

HeLa, HT29, and A549 cells were purchased from ATCC and cultured in DMEM—10% FCS containing penicillin-streptomycin. MEFs were kindly provided by Shizuo Akira’s group (30, 33, 34). BMDM were isolated as described before (32).

HeLa cells stably expressing MS2-GFP were generated by lentiviral transduction and subsequent puromycin selection. The MS2-GFP open reading frame (ORF) was cloned from pMS2-GFP (Addgene plasmid 27121 [35]) into pLVX-IREs (internal ribosome entry site)-puro (Clontech). Cells were selected with 2 µg/ml puromycin for 2 weeks.

A549 and HT-29 cell lines stably expressing shRNAs (GAAGTGCAG TATATCCAGGTCTAAGAGACCTGAATATACTGCACCTCTTTTT) against RIG-I were generated by lentiviral transduction using pLKO.shRNA RIG-I (kindly provided by R. E. Randall, University of St. Andrews, United Kingdom). Cells were selected with 2 µg/ml puromycin for 2 weeks.

Mice. Generation of RI5-/-/- and RI5-/-/- mice was described earlier (33). MAVS-/-/- mice were described earlier (30). TRIF/MdD88 DKO MEFs were described earlier (34). Bacterial infection was performed similarly to a previously published protocol (36).

Ethics statement. All research studies involving the use of animals were reviewed and approved by the Institutional Review Boards (IRB) of Mount Sinai School of Medicine (MSSM). All human cell lines used were purchased from ATCC. All animal procedures performed in this study were in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and have been approved by the IACUC of Mount Sinai School of Medicine (protocol 020274).

RNA extraction and qPCR. Total RNAs from cultured cells and bacteria were isolated with a Qiagen RNeasy Minikit (Qiagen) and TRizol (Invitrogen), respectively.

Mean n-fold expression levels of cDNA from three individual biological samples, each measured in triplicate, were normalized to 18S RNA levels and calibrated to mock-treated samples according to the 2^(-ΔΔCT) method (37). The primer sequences were as follows: for the murine gene, the IFN-β forward primer was 5'-CAGCTCCAAGAAAGGACGAAG-3', the IFN-β reverse primer was 5'-GGCGTGTAACTCTTCTGATG-3'; the 18S forward primer was 5'-GTAACCCGTTGAACCCCATT-3'; and the 18S reverse primer was 5'-GAGAAGCACAACAGGAGGCAA-3'; for the human gene, the IFN-β forward primer was 5'-TCTGGCAACACAGGTAATGGCC-3' and the reverse primer was 5'-CCATCAATCGGTAGTACCCG-3'; and for the other primers, the MSZap forward primer was 5'-ACGGTACTTATTGGCAAAAGACG-3' and the MSZap reverse primer was 5'-GATGAAACCCTTGATACCTTGAGC-3'.

Western blot analyses. SDS-PAGE and Western blot analyses were performed as described previously (38).

RNA. For fractionation, total RNAs of S. Typhimurium were depleted of small RNA fractions using ChromaSpin columns (Clontech).

For dephosphorylation, RNAs were treated with 10 U of RNase A/µg of RNA for 1 h at 37°C or with 10 U of calf intestinal alkaline phosphatase.
(CIAP)/μg of RNA for 5 min to 1 h at 37°C. Treated RNAs were purified using Qiagen RNeasy Mini columns.

For in vitro transcription, in vitro-transcribed bacterial RNA was generated using purified, sigma-saturated E. coli holoenzyme (EpiBio) and linearized pCpB (Addgene plasmid 1235; kindly provided by Susan Lindquist [39]) or pFPVmCherry (Addgene plasmid 20956; kindly provided by Olivia Steele-Mortimer, NIH, Washington, DC [40]) as a template. RNAs were purified using Qiagen RNeasyMini columns. Template DNA was removed by on-column digestion with RNase-free DNase (Qiagen).

Denaturing RNA gel electrophoresis. Denaturing agarose gels (1% agarose, 6.5% formaldehyde, 2.5 mM sodium acetate, 1.25 mM EDTA, 20 mM MOPS [morpholinopropanesulphonic acid], pH 7.0) were prepared under RNase-free conditions. RNA was heated to 65°C for 5 min in 5 mM EDTA–50% formamide–0.0125% bromophenol blue–0.0125% xylene cyanol. Gels were run in 2.5 mM sodium acetate–1.25 mM EDTA–20 mM MOPS (pH 7.0) containing 8.7% formaldehyde.

RIG-I pulldown and deep sequencing. Immunoprecipitation of RIG-I and recovery of associated RNA were described earlier [16]. For RNA sequencing, cDNA libraries for RNA samples were prepared using an Illumina sample preparation kit (Illumina) and RNA sequencing was performed on an Illumina platform at the Genomics Sequencing Core at Stanford University, Philadelphia, PA) for providing reagents.

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