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# MDA5 Detects the Double-Stranded RNA Replicative Form in Picornavirus-Infected Cells

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## SUMMARY

RIG-I and MDA5 are cytosolic RNA sensors that play a critical role in innate antiviral responses. Major advances have been made in identifying RIG-I ligands, but our knowledge of the ligands for MDA5 remains restricted to data from transfection experiments mostly using poly(I:C), a synthetic dsRNA mimic. Here, we dissected the IFN- $\alpha/\beta$ -stimulatory activity of different viral RNA species produced during picornavirus infection, both by RNA transfection and in infected cells in which specific steps of viral RNA replication were inhibited. Our results show that the incoming genomic plus-strand RNA does not activate MDA5, but minus-strand RNA synthesis and production of the 7.5 kbp replicative form trigger a strong IFN- $\alpha/\beta$  response. IFN- $\alpha/\beta$  production does not rely on plus-strand RNA synthesis and thus generation of the partially double-stranded replicative intermediate. This study reports MDA5 activation by a natural RNA ligand under physiological conditions.

## INTRODUCTION

RIG-I-like receptors (RLRs) are ubiquitously expressed cytoplasmic pathogen recognition receptors. The RLR family of proteins are DExD/H box RNA helicases, and two of its members, namely retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), specialize in detecting viral RNAs in infected cells (reviewed in Kato et al., 2011). Upon ligand recognition, RIG-I and MDA5 interact with a mitochondrion-anchored adaptor molecule, MAVS, which activates kinase complexes eventually leading to the transcription of type I interferons (IFN- $\alpha/\beta$ ) and other proinflammatory cytokine genes (Kato et al., 2011).

RIG-I and MDA5 play differential roles in virus recognition. RIG-I recognizes most single-stranded (ss) RNA viruses investi-

gated to date, including all minus-strand RNA [(–)RNA] viruses (e.g., influenza virus, Sendai virus, and vesicular stomatitis virus) and some plus-strand RNA [(+)RNA] viruses (e.g., hepatitis C virus and Japanese encephalitis virus) (Kato et al., 2011). MDA5 recognizes some other (+)RNA viruses, namely encephalomyocarditis virus (EMCV) (Gitlin et al., 2006; Kato et al., 2006), Theiler's murine encephalomyelitis virus (TMEV) (Pichlmair et al., 2009), coxsackievirus B3 (CVB3) (Wang et al., 2010)—all of which are members of the Picornavirus family—and mouse norovirus (McCartney et al., 2008). Other ssRNA viruses, such as dengue virus, West Nile virus, mouse hepatitis virus, and several paramyxoviruses, are recognized by both RIG-I and MDA5 (Kato et al., 2011). Recognition of a double-stranded (ds) RNA virus, reovirus, has also been shown to involve both of these RLRs (Kato et al., 2008).

The differential recognition of viruses by RIG-I and MDA5 has been attributed to their distinct preferences for RNA ligands. RIG-I can be activated by 5'-triphosphate (ppp)-containing RNAs as well as short (<2 kbp) dsRNAs (Kato et al., 2011). The ligand specificity of RIG-I provides an explanation for how it differentiates viral RNAs from cellular RNAs. Many RNA viruses carry genomes that contain 5'ppp or produce 5'ppp-containing RNAs during their replication cycle in the cytoplasm of infected cells, whereas cytoplasmic cellular RNAs generally lack 5'ppp. The ligands of MDA5 are as yet poorly defined. MDA5 is activated by transfection of a long (>2 kbp), synthetic dsRNA analog of artificial sequence, namely polyinosinic:polycytidylic [poly(I:C)]. Additionally, transfection-based experiments showed that the L segments of the reovirus genome (~3.9 kbp dsRNAs) induce an IFN- $\beta$  response that is partially dependent on MDA5 (Kato et al., 2006). Based on these findings, it is believed that MDA5 recognizes long dsRNAs, which would be intrinsically “nonself.” However, little is known about the identities and characteristics of physiological ligands of MDA5 in infected cells.

Many (+)RNA viruses, including picornaviruses, produce dsRNAs during infection. *Picornaviridae* is a large, highly diverse family of human and animal viruses, including many pathogens of great medical and/or economical significance. The picornavirus genome is a 7.5–8.5 kb ssRNA molecule that harbors a single open reading frame flanked by structured 5' and 3' nontranslated

regions and a poly(A) tail at the 3' end. The 5' terminus contains a small (20–24 aa) viral peptide, VPg, linked via an unusual tyrosine-RNA phosphodiester bond. Upon virus entry and uncoating, the virion RNA (vRNA) is released into the cytoplasm where it is directly translated. Viral RNA templates used for protein synthesis no longer contain VPg due to the activity of a yet unknown cellular enzyme (further referred to as unlinkase) (Rozovics et al., 2011). The viral polyprotein is proteolytically processed to release the viral proteins that engage in genomic RNA replication. During this complex process, several species of viral RNAs with unique features are generated (Figure 2A). First, the viral genomic ssRNA is transcribed into a complementary (–)RNA by the virally encoded RNA-dependent RNA polymerase using VPg as primer, yielding a 7.5 kbp dsRNA named the replicative form (RF). The (–)RNA is subsequently used as a template for VPg-primed synthesis of large amounts of new (+)RNAs, which are identical to vRNA. Newly synthesized (+)RNAs either enter a new cycle of translation and RNA replication or are encapsidated to form new virion particles. During the process of (+)RNA synthesis, a partially ds replicative intermediate (RI)—consisting of multiple incomplete (+)RNAs undergoing active transcription along the full-length (–)RNA—is formed.

It is generally assumed that viral dsRNAs produced in cells infected with picornaviruses, as well as other (+)RNA viruses, activate MDA5. However, experimental evidence supporting such a hypothesis is lacking. In this study, we sought to identify physiological ligand(s) of MDA5 by studying the recognition of distinct picornavirus RNA species in transfected cells as well as during infection. Here, we identify a naturally occurring RNA from infected cells, the picornavirus RF, as a potent and specific activator of MDA5 during infection.

## RESULTS

### MDA5 Recognizes Viruses from Various Picornavirus Genera

The Picornavirus family contains hundreds of highly diverse members that are subcategorized in 12 genera. Based on the observation that MDA5 recognizes EMCV and TMEV, both members of the *Cardiovirus* genus, and CVB3, an *Enterovirus* (Kato et al., 2011), it is generally assumed that all picornaviruses are recognized by MDA5, but experimental proof for this is lacking. Here, we set out to investigate the role of RLRs in the recognition of representative picornaviruses from various genera using wild-type (WT) and RIG-I<sup>−/−</sup>, MDA5<sup>−/−</sup>, or MAVS<sup>−/−</sup> mouse embryonic fibroblasts (MEFs). To bypass the requirement for specific entry receptors, we introduced vRNAs into the cytoplasm by transfection rather than infection. Figure 1A shows IFN-β responses in WT and knockout MEFs triggered by transfection of vRNA of WT mengovirus (a strain of EMCV) or infection of a mutant mengovirus (mengo-Zn). This virus generates high levels of IFN-β because its IFN antagonist has been compromised (Hato et al., 2007). Both methods resulted in a MDA5- and MAVS-dependent, but RIG-I-independent, IFN-β induction, demonstrating the suitability of the transfection method to identify the RLR(s) responsible for detecting picornaviruses.

Using this vRNA transfection assay, we studied recognition of members of three human *Enterovirus* species. In addition, we

included Saffold virus (a recently identified human *Cardiovirus*), human parechovirus (*Parechovirus* genus), and equine rhinitis A virus (a member of the *Aphthovirus* genus that also includes foot-and-mouth disease virus). Infectious viruses were produced upon transfection of all vRNAs, indicating efficient RNA replication in MEFs (data not shown). Replication of all viruses induced an MDA5- and MAVS-dependent, but RIG-I-independent, IFN-β response (Figures 1B and 1C). These data provide experimental evidence that a broad spectrum of picornaviruses is indeed recognized by MDA5.

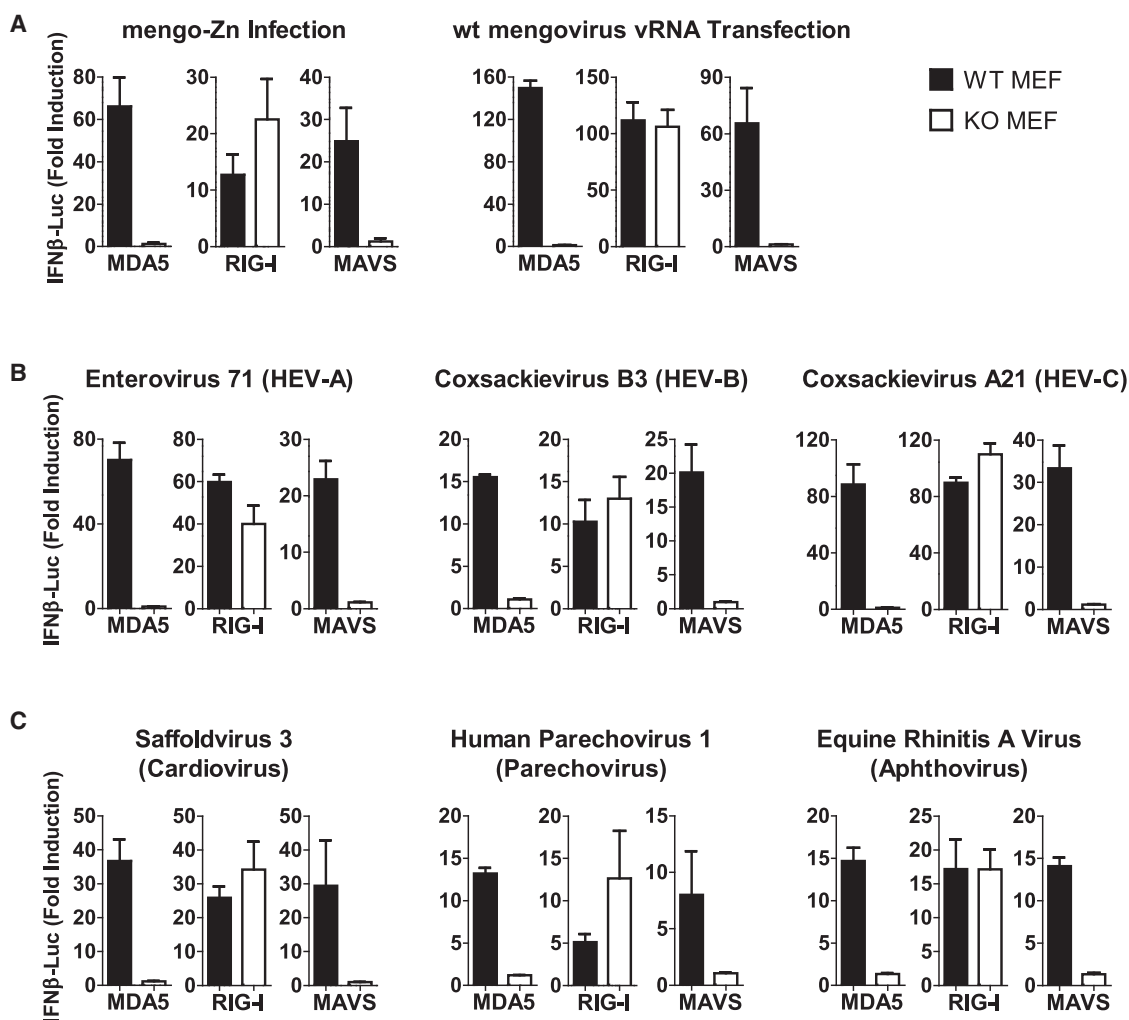
Two other cellular proteins, namely PKR and RNaseL, have also been implicated in recognition of some viruses (Kato et al., 2011). However, we found no difference in IFN-α/β responses to mengo-Zn in cells deficient in RNaseL or PKR (Figure S1), arguing against a major role of these proteins in detecting picornavirus RNA (see Extended Results and Discussion).

### Enrichment of ssRNA and dsRNA Fractions from Picornavirus-Infected Cells

To gain more insight into the identity of the RNA species detected by MDA5 in picornavirus-infected cells, we separately enriched for ssRNAs and dsRNAs by LiCl differential precipitation from mock- and CVB3-infected HeLa cells (Figure 2B). The ssRNA pool of infected cells contained one additional RNA species of the same electrophoretic mobility as purified CVB3 vRNA (Figure 2C), suggesting that it represents viral genomic ssRNA. Although partially ds, the viral RI is known to precipitate by 2M LiCl (Richards et al., 1984), the same condition used here to prepare ssRNA fractions. However, the amount of RI is most likely too low to be detected on gel. The dsRNA pool of CVB3-infected cells contained an RNA species of approximately 7.5 kbp that was absent in the dsRNA pool of mock-infected cells (Figure 2B). Both the viral ssRNA and dsRNA bands were absent in HeLa cells infected with CVB3 in the presence of GuHCl (Figure 2B), a well-known inhibitor of enterovirus (–)RNA synthesis (Barton and Flanagan, 1997) (Figures 2D and S2), demonstrating that these RNAs were of viral origin.

### The Picornavirus 7.5 kbp RF Is a Potent MDA5 Ligand upon Transfection

The dsRNA fraction from CVB3-infected cells (Figure 2B) was transfected into MEFs and IFN-β mRNA levels were measured at 2 hr posttransfection (hr.p.t.), a time point preceding RNA replication (Figure S2), thereby excluding the possible role of other, newly produced viral RNA species. The dsRNA fraction triggered a MDA5- and MAVS-dependent IFN-β response (Figure 3A). We next set out to identify the IFN-stimulatory RNA species in this fraction. In the dsRNA fractions, we observed two bands on gel; an infection-specific, 7.5 kbp species, and a larger fragment that was present in both mock- and CVB3-infected samples. To determine the nature of the higher band, we treated the dsRNA fractions with nucleases specific for ssRNA (RNase A), dsRNA (RNase III), and DNA. The nonspecific larger band was completely digested upon DNase treatment, indicating that it was DNA (Figure 3B). The 7.5 kbp CVB3 RF was specifically digested by RNase III, confirming that this is a dsRNA molecule (Figure 3B).



**Figure 1. MDA5 Recognizes Picornaviruses across Various Genera**

(A) MEFs of indicated genotypes were either infected with mengo-Zn (MOI 50) or transfected with virion RNA (vRNA) from WT mengovirus. IFN- $\beta$  promoter activation at 8 hr.p.t. was determined by an IFN reporter assay.

(B) MEFs of indicated genotypes were transfected with vRNAs from three human enteroviruses (HEVs), namely enterovirus 71, coxsackievirus B3, or coxsackievirus A21, a close relative to poliovirus. IFN- $\beta$  promoter activation at 8 hr.p.t. was determined by IFN reporter assay.

(C) Same assay as (B) using vRNAs from Saffold virus 3, human parechovirus 1, and equine rhinitis A virus. Data presented as mean  $\pm$  SD.

See also Figure S1.

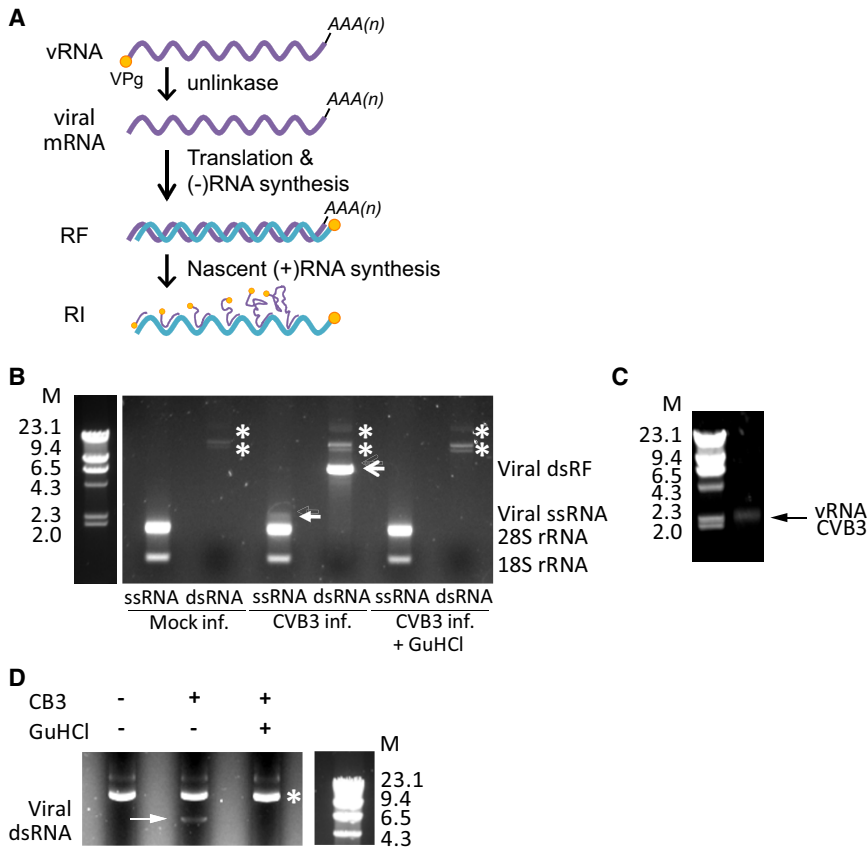
Next, we examined the ability of purified CVB3 RF to activate MDA5. When gel-extracted and transfected into MEFs, the RF, but not the DNA molecule, induced high levels of IFN- $\beta$  transcription in an MDA5- and MAVS-specific manner (Figure 3C). Similar experiments were performed with nucleic acids extracted from cells infected with mengovirus. The dsRNA fraction from mengovirus-infected cells appeared indistinguishable on gel from that from CVB3-infected cells (Figure 3D), and the gel-purified mengovirus RF also proved to be a potent MDA5 agonist (Figure 3E). These data show that the RF of prototype members of at least two genera of picornaviruses can specifically and potently induce an MDA5-dependent IFN- $\alpha/\beta$  response.

To investigate whether the RF can directly activate MDA5, we performed *in vitro* ATPase assays where recombinant MDA5

was incubated with gel-purified RF or the nonspecific DNA band in the presence of ATP. ATP hydrolysis was followed at different time points by measuring free phosphate. CVB3 RF induced high levels of MDA5 ATPase activity—hydrolyzing nearly 150  $\mu$ M ATP in 60 min—while the DNA band did not induce any ATP hydrolysis during this time period (Figure 3F). These results are in line with our transfection data (Figures 3C and 3E) and confirm that RF can be directly recognized by MDA5 and potently induce its activation.

#### Picornavirus ssRNAs Do Not Activate MDA5

We also tested the ssRNA fraction from infected cells (Figure 2B) for its IFN- $\beta$ -stimulatory activity. To our surprise, transfection of this fraction also led to an MDA5- and MAVS-dependent IFN- $\beta$



**Figure 2. Enrichment of ssRNA and dsRNA Fractions of CVB3-Infected Cells**

(A) Viral RNA species produced during picornavirus infection. RF, replicative form. RI, replication intermediate. Purple line, (+)RNA; blue line, (-)RNA; orange circle, VPg.

(B) HeLa cells were infected with CVB3 in the absence or presence of replication inhibitor GuHCl (2 mM) for 5 hr and total RNA was extracted. ssRNA and dsRNA fractions were separated by LiCl differential precipitation and examined on agarose gel. Note that the ssRNA fraction was diluted 400-fold relative to the dsRNA fraction to load comparable amounts of RNAs. M, dsDNA marker with indicated size in kbp; closed arrow, viral ssRNA; arrowhead, viral dsRNA; asterisk, unknown bands.

(C) vRNA purified from pelleted virus particles was visualized on agarose gel next to the same DNA marker as used in (A).

(D) HeLa cells were infected with CVB3 (MOI 100) in the absence or presence of GuHCl (2 mM) for 2.5 hr and the dsRNA fraction was purified from total RNA extract by LiCl precipitation and examined on gel. M, dsDNA marker; arrow, viral dsRNA; asterisk, unknown bands.

See also Figure S2.

### Formation of Viral RF, but Not RI, Is Required for IFN- $\beta$ Induction in Infected Cells

Our results indicated that the picornavirus RF, and possibly also the RI, is a potent

response (Figure 4A). However, subsequent nuclease digestion experiment revealed that the IFN- $\beta$ -stimulatory activity is sensitive to RNase III (dsRNA-specific) treatment, but not RNase A (ssRNA-specific) (Figures 4B and 4C). Indeed, after removal of rRNAs—the most abundant RNA species present in the ssRNA fractions—we observed a RNA species on gel that is specifically present in infected cells and has the same electrophoretic mobility as the RF band found in the dsRNA fraction from infected cells (Figure S3A). These results suggest that residue amounts of RF are still present in our ssRNA fraction preparations, and the RF, but not viral ssRNAs, is responsible for the observed IFN- $\beta$  induction upon transfection of this fraction. In line with this conclusion, we found that VPg-containing vRNA (Figures 4D and 4E), VPg-unlinked viral mRNAs (Figure 4E), and in vitro transcribed ssRNAs of viral sequence (Figures S3B and S3C) all failed to induce any IFN- $\beta$  response when purified and transfected into cells.

The viral RI was also reported to segregate in the ssRNA fraction according to the LiCl precipitation method (Richards et al., 1984). To study the effect of RI on IFN- $\alpha/\beta$  induction without complications from RF, we attempted to remove the remaining RF from CVB3 ssRNA fraction by repeating the 2M LiCl precipitation procedure. However, even after two additional rounds of precipitation, we did not observe significant loss of RF from the CVB3 ssRNA fraction, nor did we observe a reduced IFN- $\beta$  response upon transfection (data not shown). Hence, we were unable to determine whether RI also exerts IFN-inducing activity.

MDA5 activator when delivered into the cytoplasm by lipid-based transfection. However, the amount of RF/RI and their accessibility to MDA5 may differ greatly from the situation in infected cells, where viral RNA is covered with replication enzymes and localized in virus-induced, membrane-associated replication organelles (reviewed in Belov and van Kuppeveld, 2012). Therefore, we set out to dissect the IFN-stimulatory abilities of different viral RNAs produced in cells infected with mengo-Zn, which induces strong IFN- $\beta$  responses.

We first generated a comprehensive profile of the kinetics of viral RNA replication as well as host IFN- $\beta$  response to mengo-Zn in HeLa cells by measuring viral RNA and IFN- $\beta$  mRNA levels by real-time quantitative PCR (qPCR) every hour up until 10 hr postinfection (hr.p.i.) (Figure 5A). Viral RNA level remained at input levels in the first 3 hr of infection, then rapidly increased (i.e., active viral RNA replication) to reach a maximum at 6 hr.p.i., and stabilized at this level through 10 hr p.i. The host IFN- $\beta$  response showed a slight delay as IFN- $\beta$  mRNA levels in infected cells remained at background levels through the first 6 hr, then started to increase from 7 hr.p.i., and reached their maximum at 8–9 hr p.i.

To study the separate contribution of viral ssRNA and dsRNA in inducing IFN- $\alpha/\beta$  response during infection, we employed inhibitors of mengovirus replication, cycloheximide (CHX) and di-pyridamole (DIP) (Figure 5B), which affect viral replication at different stages. CHX prevents protein synthesis and thereby RNA replication. DIP has little effect on (-)RNA synthesis but

strongly inhibits (+)RNA synthesis in cell-free extract (Fata-Hartley and Palmenberg, 2005). Indeed, (–)RNAs were detected in cells infected in the presence of DIP, but not CHX, using a strand-specific PCR (Figure 5C). The finding that the signal obtained from DIP-treated cells was lower than that from no-drug-treated cells likely reflects a reduced amount of (–)RNA as DIP only allows (–)RNA synthesis from incoming genomic (+)RNA, whereas in the absence of DIP newly synthesized (+)RNAs can enter a new round of RNA replication, and therefore lead to the production of more RF.

To determine the IFN- $\beta$  response to different viral RNA species formed in infected cells, we used DIP and CHX in a time-of-drug-addition experiment. HeLa cells were infected with mengo-Zn, and DIP or CHX was added to cells at the indicated times (Figure 5D). Cells were incubated until 12 hr.p.i., harvested, and viral RNA and IFN- $\beta$  mRNA levels were determined by real-time qPCR. As shown in Figure 5D, both DIP and CHX were effective in inhibiting viral RNA replication when added at early stages (0–2 hr.p.i.) of infection. Later addition of the drugs resulted in partial, and eventually loss, of effect on RNA replication.

Under conditions where CHX and DIP completely inhibited viral RNA replication (i.e., added at 0–2 hr.p.i., Figure 5D), no IFN- $\beta$  induction was detected in CHX-treated cells, whereas a 100-fold, MDA5-dependent (Figure S4) induction of IFN- $\beta$  mRNA level was observed in DIP-treated cells. Additionally, when CHX or DIP was added at 3 hr.p.i., a maximal IFN- $\beta$  response was observed in DIP-treated cells, but virtually no IFN- $\beta$  was induced in the CHX-treated cells (Figure 5D). Analysis of viral RNA levels indicate that only a few new (+)RNAs had been made because addition of either CHX or DIP at this time point led to only a slight increase in viral RNA copy number at 12 hr.p.i. These newly synthesized (+)RNAs could serve as template to make additional RFs in the DIP-treated cells, but not in the CHX-treated cells, and result in the increase of IFN- $\beta$  induction observed at 3 hr p.i. It is theoretically possible that the observed differences in IFN- $\beta$  levels in CHX- and DIP-treated cells were due to nonspecific blockade or stimulation of the RLR signaling pathway by CHX or DIP, respectively. However, this is unlikely because these drugs had no such effects on poly(I:C)-induced IFN- $\beta$  responses (Figure S5).

In the experiment described above, the incoming vRNA alone (i.e., in the presence of CHX) induced no IFN- $\beta$  response. To exclude that the amount of incoming vRNA at the used MOI was too low to induce a detectable IFN- $\beta$  mRNA upregulation, we increased the infection dose from MOI 10 to 1,000. This resulted in increased levels of IFN- $\beta$  in no-drug- and DIP-treated samples, but in CHX-treated cells the IFN- $\beta$  level remained undetectable (Figure 5E). This result clearly shows that the incoming vRNA, even when present at very high quantities, does not activate MDA5. Taken together, our results demonstrate that (–)RNA synthesis (i.e., formation of RF), but not (+) RNA synthesis (i.e., formation of RI), is required for achieving high levels of IFN- $\alpha/\beta$  induction.

### Termini and Length Dependency of MDA5 Activation by Long dsRNAs

We next investigated whether the termini of picornavirus RF are important for MDA5 activation. The viral RF contains a VPg

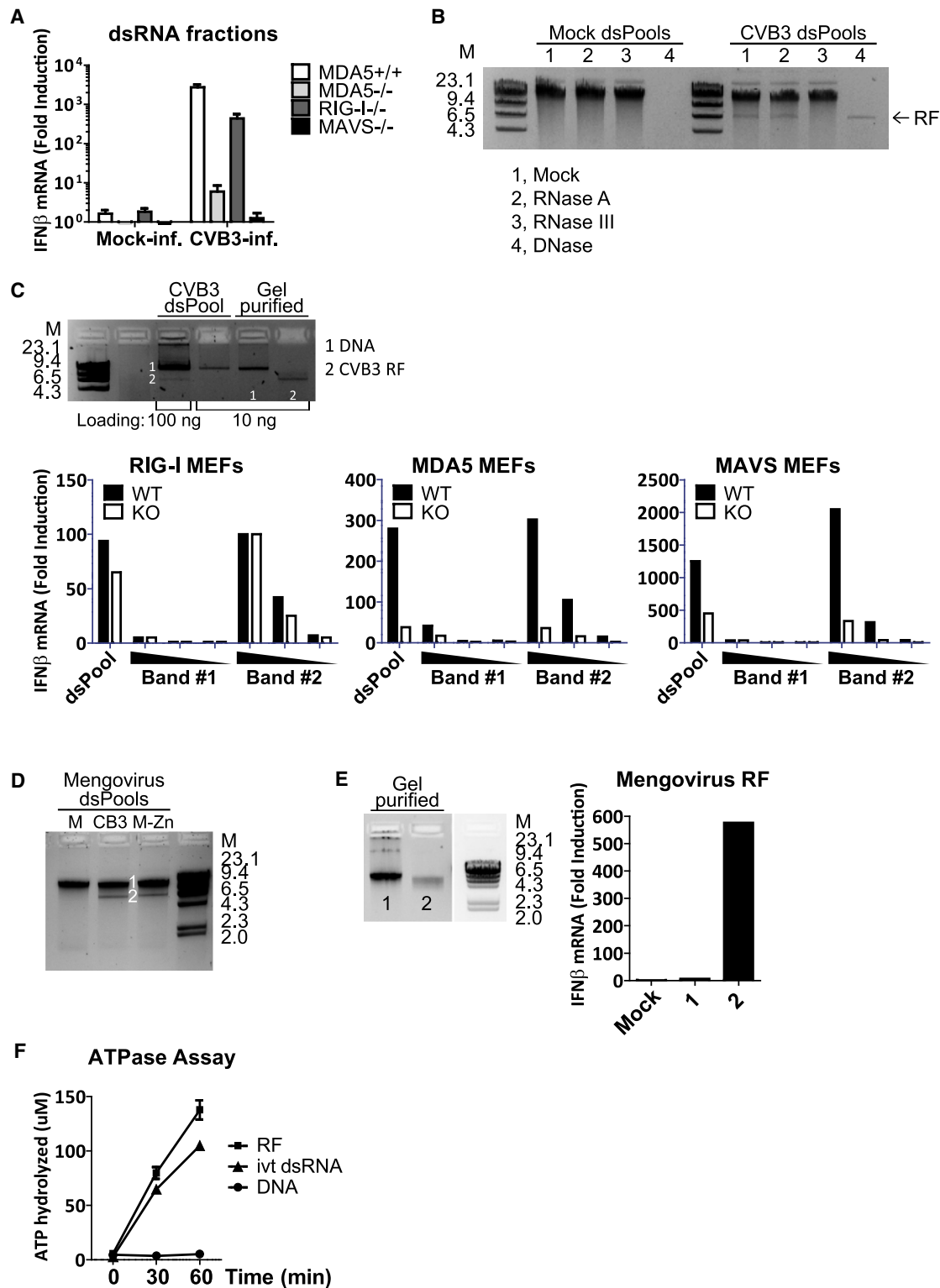
peptide at the 5' end of the (–)RNA, but not at the 5' end of the (+)RNA, because of the activity of the unlinking enzyme. Furthermore, the RF contains a large unpaired poly(A) tail at the 3' end of the (+)RNA (van Ooij et al., 2006). To investigate whether these features are important for MDA5 activation, we produced CVB3 and mengovirus dsRNAs with 5'ppp and perfectly paired termini by in vitro transcription (ivt dsRNA) and compared them with viral RF purified from infected cells for their ability to activate MDA5. Strikingly, all dsRNAs triggered a similar IFN- $\beta$  response upon transfection into RIG-I<sup>–/–</sup> MEFs (Figure 5F). Additionally, CVB3 ivt dsRNA activated MDA5 to a similar extent as CVB3 RF in our in vitro ATPase assay (Figure 3F). Thus, the recognition of long dsRNAs by MDA5 is likely independent of the termini of the RNA molecule.

Lastly, we investigated whether MDA5 activation by long dsRNAs is length dependent as proposed by Kato et al., who showed that only long polyIC molecules (>2 kb) are efficiently recognized by MDA5 (Kato et al., 2008). To this end we generated ivt dsRNAs of various lengths (0.1–7.4 kbp) and determined IFN- $\beta$  activation levels by real-time qPCR upon transfection of different amounts of these fragments. At relatively low quantities (10 ng), ivt dsRNAs shorter than 2.4 kbp induced only low levels of IFN- $\beta$  mRNA. However, when larger amounts of dsRNA (100 ng) were transfected, smaller dsRNAs were also able to trigger an IFN- $\beta$  response (Figure 5G), indicating that there may not be a strict length cut-off value for MDA5 ligands.

## DISCUSSION

Since the initial discovery of RLRs, significant progress has been made in identifying ligands for RIG-I, but the identity and molecular characteristics of viral MDA5 ligands remain largely unknown. Based on transfection experiments using poly(I:C), MDA5 is believed to recognize long dsRNAs. However, viral ssRNAs should not be excluded because base-paired regions in ssRNAs, as those in the picornavirus genomic RNA, could potentially be sufficient for MDA5 recognition. Furthermore, a virally encoded peptide, VPg, is covalently attached to the 5' terminus of picornavirus RNAs via a unique tyrosine-RNA bond, providing a potential “nonself” signature at the 5' end. Bearing this information in mind, we followed an unbiased approach to determine the IFN-stimulatory activities of different viral RNA species produced in picornavirus-infected cells.

The viral dsRNA, RF, has been assumed to be the MDA5 ligand produced by picornaviruses based on the fact that all known activators of MDA5 are dsRNAs (mimics). Here, we provide experimental evidence that purified picornavirus RF can not only induce an MDA5-dependent IFN- $\alpha/\beta$  response upon transfection, but also directly bind to, and activate, MDA5 in vitro. Viral ssRNAs, on the other hand, proved poor MDA5 stimuli. Neither vRNA (VPg-containing) nor viral mRNA (VPg-lacking) induced an IFN- $\beta$  response when transfected into cells. This is in line with the observation that the full-length EMCV RNA failed to activate MDA5 ATPase activity in vitro (Peisley et al., 2011). Although we could not test IFN-stimulatory activity of purified RI, this molecule does not seem to play a crucial role in inducing IFN- $\alpha/\beta$  because treatment of the CVB3 ssRNA fraction with RNase A did not affect its ability to



**Figure 3. Picornavirus RF Is a Potent MDA5 Agonist**

(A) dsRNA fractions from mock- or CVB3-infected HeLa cells were transfected-into MEFs of indicated genotypes, and IFN- $\beta$  mRNA level at 2 hr.p.t. was measured by real-time qPCR. Data presented as mean  $\pm$  SD.

(B) dsRNA fractions (dsPool) of mock- and CVB3-infected cells were treated with 10 ng/ $\mu$ l RNase A, 10 mU RNase III, or 10 mU DNase I at 37°C for 15 min, and analyzed on agarose gel.

induce IFN- $\alpha/\beta$ . This observation also speaks against the hypothesis that other (viral) ssRNAs—which might have been produced by the action of cellular RNases like RNaseL—may be recognized by MDA5.

Collectively, our transfection experiments clearly identify the RF as an MDA5 agonist. Previously, Pichlmair and colleagues identified two virus-induced RNA species in their total RNA extract from EMCV-infected cells: one dsRNA of approximately 12 kbp and one high-molecular-weight RNA (HMW-RNA) that consisted of both ssRNAs and dsRNAs (Pichlmair et al., 2009). The HMW-RNA, but not the 12 kbp dsRNA, was found to be IFN- $\alpha/\beta$  stimulatory upon transfection. We never observed either of these RNAs in our total RNA extracts or the fractionated RNA preparations. The identity of the HMW-RNA and the IFN-stimulatory element(s) within their mixed RNA population remains unclear.

Although transfection may be a convenient method to study aspects of MDA5 ligand recognition, it may not faithfully recapitulate physiological conditions for several reasons. First, transfection delivers large amounts of viral RNA directly into the cytoplasm at once. This is different from the RNA release process during infectious entry as well as the gradual accumulation of viral RNAs during virus replication. Second, purified, and therefore “naked,” RNAs are transfected into cells whereas viral RNAs are heavily coated with viral and host factors during infection. Third, (+)RNA viruses induce the formation of membranous replication organelles, which may restrict access of MDA5 to replication intermediates such as RF and RI. To gain insight into MDA5 activation in a more physiologically relevant situation, we studied MDA5 activation in infected cells. Our data show that the incoming genomic ssRNA alone cannot induce any detectable IFN- $\beta$  response even when large amounts were delivered by high MOI infection. When (–)RNA synthesis (i.e., formation of RF) was permitted but (+)RNA synthesis (i.e., formation of RI) was inhibited, a significant upregulation of IFN- $\beta$  mRNA was observed. These data further support the idea that picornavirus RF is the essential MDA5 agonist in the course of infection. Of note, a much higher IFN- $\beta$  response was observed when RNA replication was allowed to proceed fully (i.e., in the absence of inhibitor). Although this finding may be interpreted as that RIs also activate MDA5, this increase can be attributed, at least partly, to the increased amount of RF produced from newly synthesized (+)RNAs that enter new rounds of RNA replication.

Recently, Züst et al. suggested that MDA5 may also recognize RNA molecules with a “nonself” 5' termini, as a mutant coronavirus deficient in fully capping its mRNAs activated MDA5 in infected cells (Züst et al., 2011). Because picornavirus RF harbors distinctive termini, we investigated whether this terminal

composition of the RF is essential for MDA5 activation. Changing the physiological terminal groups of RF into 5'ppp and fully complemented 3' end did not change its ability to induce IFN- $\beta$  upregulation. Other features such as secondary structures are common requirements for ligand recognition; however, the picornavirus RF is not known to contain distinct higher structures. Thus, it is likely that the long ds nature of the RF, and not its unique termini, renders this RNA a potent MDA5 activator.

We also investigated the length dependency of MDA5 ligand recognition because it has been suggested that only dsRNAs longer than 2 kbp can induce MDA5 activation (Kato et al., 2008). We tested dsRNAs of viral sequence in vitro that span a wide range of sizes (0.1–7.4 kbp), and found that although longer dsRNAs activate MDA5 more efficiently when provided at equal mass, short dsRNAs could also induce some levels of MDA5 activation when present in large quantities. These data suggest that MDA5 may not require its substrate to be longer than a strict cut-off size. In line with this, others have demonstrated efficient activation of MDA5 ATPase activity by dsRNAs as short as 112 bp (Peisley et al., 2011). Authors of this paper also showed that MDA5 ATPase activity, which is triggered by ligand binding, induces disassembly of MDA5 from the RNA ligand, the rate of which is inversely related to the length of the bound RNA molecule (Peisley et al., 2011). This provides some molecular explanation why longer dsRNAs are more efficient in activating MDA5 than shorter ones.

While this paper was in preparation, another study reported that enterovirus dsRNAs, but not ssRNAs, can activate MDA5-dependent IFN- $\beta$  responses upon transfection (Triantafilou et al., 2012). Also shown in this study is colocalization of MDA5 with dsRNA in enterovirus-infected cells. Although not providing evidence of direct binding or functional activation, these data nicely show that MDA5 and viral dsRNA can be found in physical vicinity, and therefore support our results that the RF can activate MDA5 in infected cells.

Collectively, our data suggest that the 7.5 kbp RF of picornaviruses is a highly potent MDA5 activator and is directly recognized by MDA5. Moreover, this study shows that manipulating specific steps of virus replication is a powerful approach to study recognition of specific viral RNA species by RLRs under physiological conditions.

## EXPERIMENTAL PROCEDURES

### Cells, Viruses, Infectious cDNA Clones, and Reagents

MEFs and HeLa cells were maintained in DMEM supplemented with 10% FBS and cyproxin (1 mg/ml). Viruses are described in [Extended Experimental](#)

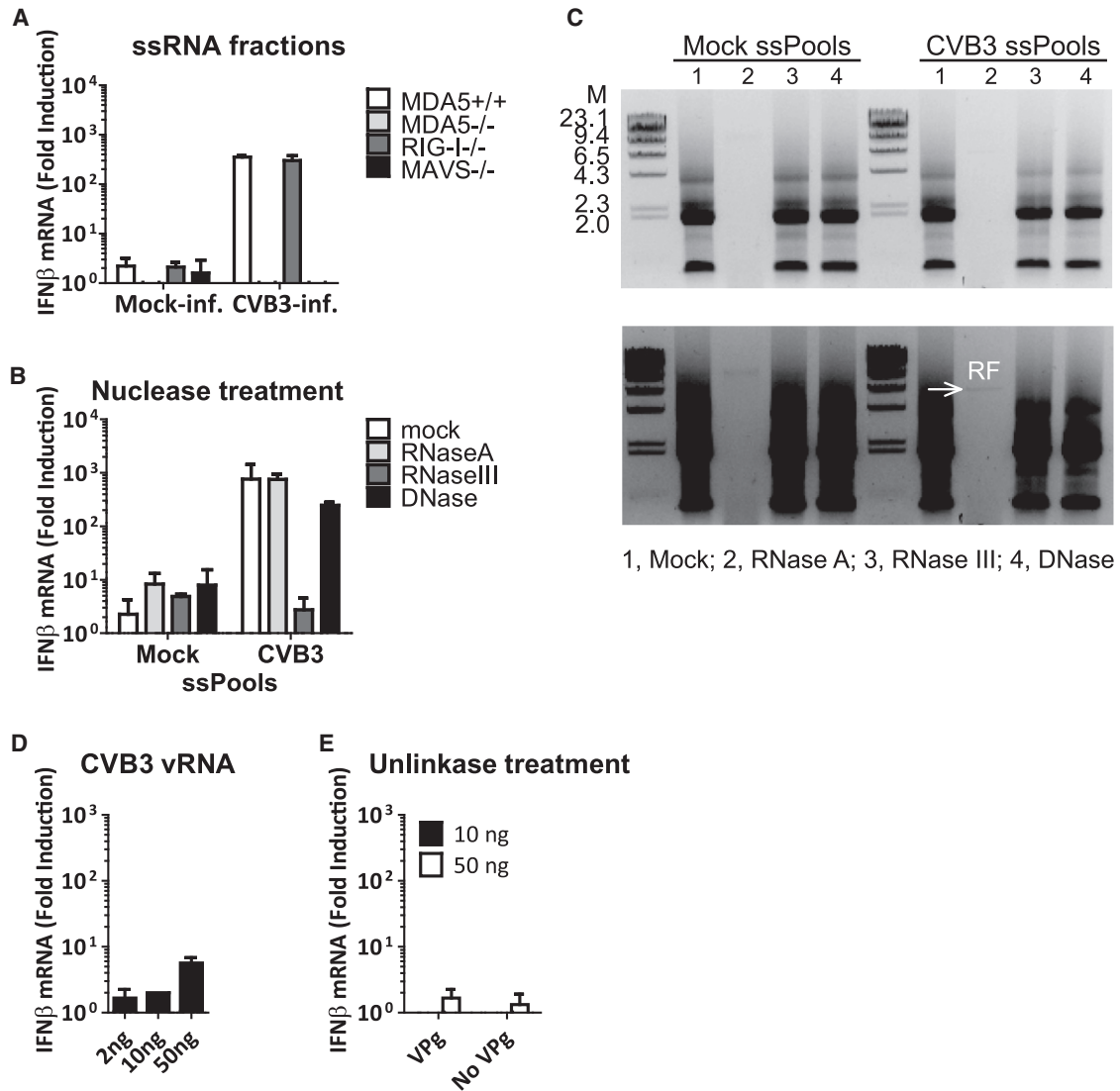
(C) DNA and RF bands observed in CVB3 dsRNA fraction were gel purified and analyzed on agarose gel. CVB3 dsRNA fraction (10 ng per well in 24-well format), and the gel-purified RNAs (10, 2, or 0.4 ng/well) were transfected into WT, RIG-I<sup>-/-</sup>, MDA5<sup>-/-</sup>, or MAVS<sup>-/-</sup> MEFs in the presence of CHX (10  $\mu$ g/ml). IFN- $\beta$  mRNA induction was determined by real-time qPCR at 8 hr.p.t.

(D) dsRNA fractions of mock-, CVB3-, or mengovirus-infected cells were analyzed on agarose gel.

(E) DNA and RF bands from mengovirus dsRNA fraction were gel purified and transfected into RIG-I<sup>-/-</sup> MEFs in the presence of CHX (10  $\mu$ g/ml). IFN- $\beta$  response at 8 hr.p.t. was measured by real-time qPCR.

(F) Gel-purified RF and DNA bands from CVB3 dsRNA fraction, as well as a *in vitro* transcribed dsRNA of CVB3 sequence (ivt dsRNA) (0.3  $\mu$ g/ml), were incubated with recombinant MDA5 (0.3  $\mu$ M) at 37°C in the presence of 2mM ATP, and free Pi was measured using Green Reagent at 0, 30, and 60 min after reaction was started. M, dsDNA marker with indicated size in kbp. Bands number 1 and 2 on gel corresponds to the samples used in RNA transfection. Data presented as mean  $\pm$  SD.





**Figure 4. ssRNAs from CVB3-Infected Cells Do Not Activate MDA5**

(A) ssRNA fraction from mock- or CVB3-infected HeLa cells were transfected into MEFs of indicated genotypes, and IFN- $\beta$  mRNA level at 2 hr.p.t. was measured by real-time qPCR. Data presented as mean  $\pm$  SD.

(B) ssRNA fractions (ssPools) of mock- and CVB3-infected cells were treated with 10 ng/ $\mu$ l RNase A, 10 mU RNase III, or 10 mU DNase I at 37°C for 15 min. Resulting RNA samples equivalent to 500 ng starting material were transfected into approximately 200,000 MAVS<sup>+/+</sup> MEFs and IFN- $\beta$  mRNA induction was determined by real-time qPCR at 8 hr.p.t. Data presented as mean  $\pm$  SD.

(C) The same samples as in (B) were analyzed on agarose gel.

(D) CVB3 vRNA was isolated from pelleted viral particles. Indicated amounts of vRNA were transfected into RIG-I<sup>-/-</sup> MEFs in the presence of CHX (10  $\mu$ g/ml), and IFN- $\beta$  mRNA induction was determined by real-time qPCR at 8 hr.p.t.. Data presented as mean  $\pm$  SD.

(E) Indicated amounts of mock-treated or unlinkase-treated poliovirus vRNAs were transfected into RIG-I<sup>-/-</sup> MEFs in the presence of CHX (10  $\mu$ g/ml). Total RNA was extracted at 8 hr.p.t. and IFN- $\beta$  mRNA induction was determined by real-time qPCR. Data presented as mean  $\pm$  SD.

See also Figure S3.

**Procedures.** Infectious cDNA clones p53CB3/T7 (Wessels et al., 2005) and pM16.1 (Hato et al., 2007) were used to produce in vitro transcribed RNAs with CVB3 or mengovirus sequences, respectively. CHX and DIP were purchased from Sigma-Aldrich, and RNase A, RNase III, and DNase I from Ambion.

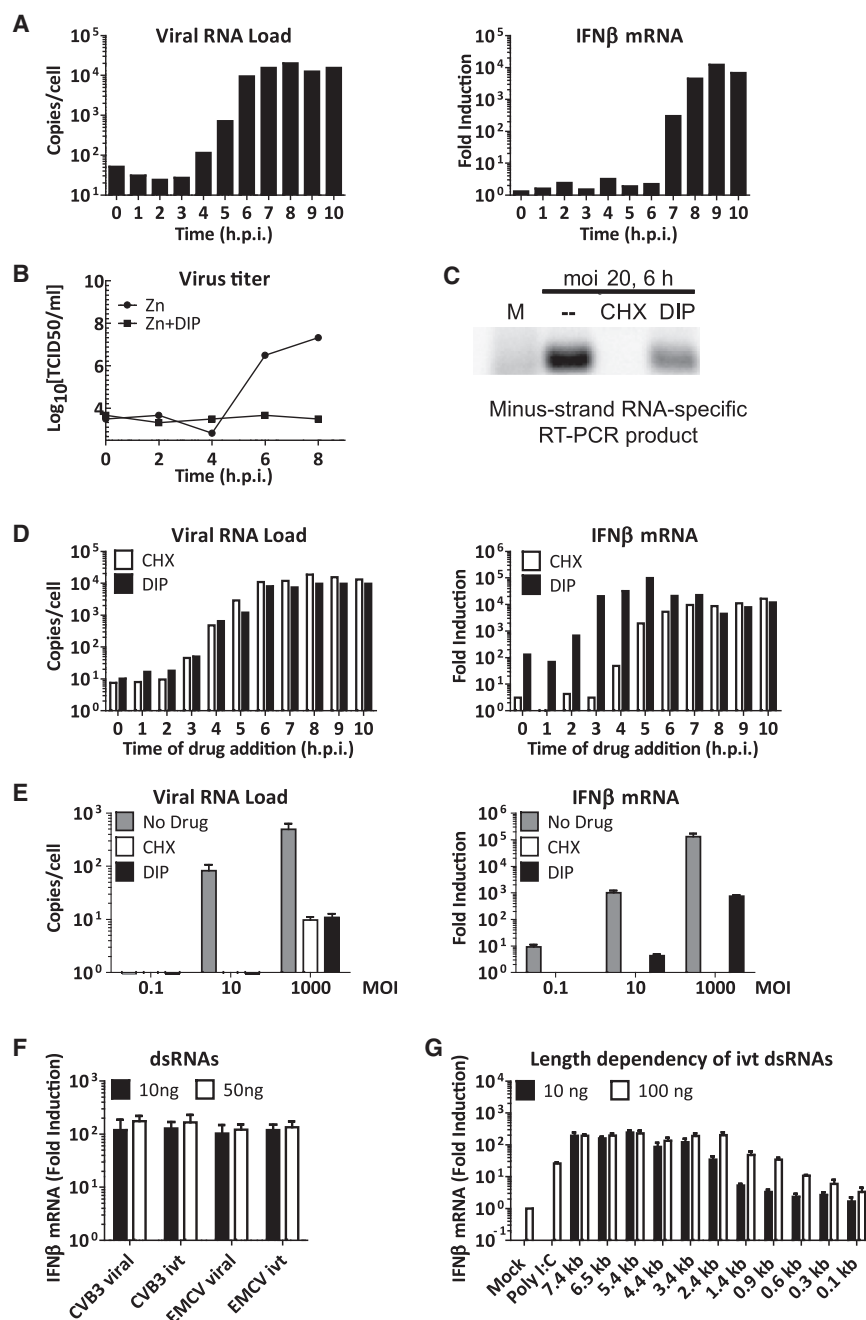
#### Isolation of Viral RNA from Virions

Cells were infected with virus and grown until cytopathic effect was complete. This lysate was subjected to three freeze-thaw cycles, cleared, and centri-

fuged at 25,000 rpm for 6 hr in an SW28 rotor at 4°C. The virus pellet was suspended in either TRIzol reagent (Invitrogen) or the lysis buffer of GenElute mammalian total RNA Miniprep Kit (Sigma-Aldrich), and RNA was isolated according to the manufacturers' instructions.

#### Isolation of ssRNA and dsRNA Fractions from Control and Infected Cells

Total RNA was isolated using TRIzol (Invitrogen) according to manufacturer's protocol. LiCl was added to the total RNA extract to an end concentration of



**Figure 5. Viral RNA Recognition in Infected Cells and Characterization of Determinant for MDA5 Activation in RF**

(A) HeLa cells were infected with mengo-Zn (MOI 10) and samples were taken every hour post-infection. Total RNA was extracted and viral RNA and host IFN- $\beta$  mRNA levels were assayed by real-time qPCR.

(B) Cells were infected with mengo-Zn in the absence or presence of DIP (100  $\mu$ M). Production of infectious particles was determined by end-point titration on BHK-21 cells.

(C) HeLa cells were mock-infected or infected with mengo-Zn (MOI 20) in the absence or presence of CHX (10  $\mu$ g/ml) or DIP (100  $\mu$ M) and total RNA was extracted at 6 hr.p.i. dsRNA fraction was purified by LiCl precipitation and subjected to RT-PCR using primers specific to viral (-) RNA.

(D) HeLa cells were infected with mengo-Zn at MOI 10. CHX (10  $\mu$ g/ml) or DIP (100  $\mu$ M) was added to infected cells either at time of infection or at indicated times postinfection. All samples were harvested at 12 hr.p.i. Total RNA was extracted and viral RNA and host IFN- $\beta$  mRNA levels were analyzed by real-time qPCR.

(E) HeLa cells were infected with mengo-Zn at indicated MOI's in the absence or presence of CHX (10  $\mu$ g/ml) or DIP (100  $\mu$ M). Total RNA was extracted at 10 hr.p.i. and viral RNA and host IFN- $\beta$  mRNA levels were measured by real-time qPCR. Data presented as mean  $\pm$  SD.

(F) RFs of CVB3 and mengovirus as well as in vitro transcribed dsRNAs of viral sequences were transfected into RIG-I<sup>-/-</sup> MEFs in the presence of CHX (10  $\mu$ g/ml) at indicated amounts per well in 24-well format. IFN- $\beta$  mRNA levels were determined at 8 hr.p.t. by real-time qPCR. Data presented as mean  $\pm$  SD.

(G) polyIC or in vitro transcribed dsRNAs of indicated length were transfected into RIG-I<sup>-/-</sup> MEFs in the presence of CHX (10  $\mu$ g/ml) at indicated amounts. IFN- $\beta$  mRNA levels were determined at 8 hr.p.t. by real-time qPCR. Data presented as mean  $\pm$  SD. See also Figures S4 and S5.

2 M. This was incubated at 4°C for 2–16 hr, and ssRNAs were precipitated by centrifugation at 16,000  $\times$  g for 30 min at 4°C. The supernatant was subjected to ethanol precipitate in the presence of 0.8M LiCl to precipitate dsRNAs. RNA pellets were washed with 75% ethanol, air-dried, and dissolved in RNase-free water.

IFN reporter assay and real-time qPCR analysis were carried out as described previously (Hato et al., 2007).

#### Preparation of Full-Length IVT RNAs

ssRNAs and dsRNAs were transcribed using Riboprobe in vitro transcription systems (Promega) and Replicator RNAi Kit (Finnzyme), respectively, following the manufacturers' instructions. RNAs were treated with DNaseI and purified prior to transfection.

Ribosomal RNA removal was performed using Ribo-Zero rRNA Removal Kit (Human.Mouse.Rat) (Epicenter RZH1046) according to the manufacturer's protocol.

#### Additional Information

More detailed descriptions of experimental procedures and materials are provided in Extended Experimental Procedures.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Results and Discussion, Extended Experimental Procedures, and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.10.005>.

**LICENSING INFORMATION**

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