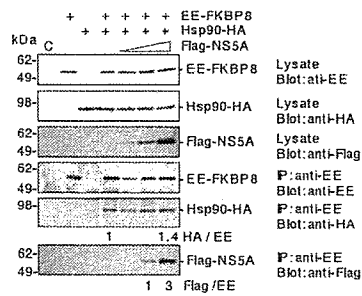
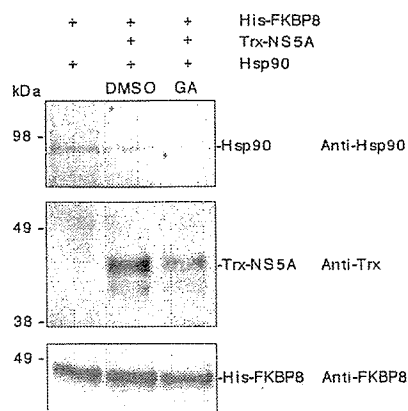


Supplementary Figure 2. Lack of apoptosis in FKBP8-knockdown cells

Huh7 9-13 cells were transfected with siRNA for the non-target control or FKBP8-Target 1 at a concentration of 80 nM, which was able to suppress the expression of FKBP8 (Fig. 5). Some cells were treated with 0.5 μg/ml staurosporin as a control for apoptosis. The cells were stained using the Vybrant apoptosis assay kit 1 (Molecular Probes, Eugene, OR).



Supplementary Figure 3. Interaction of NS5A, FKBP8 and Hsp90
 EE-FKBP8 was co-expressed with Hsp90-HA and Flag-NS5A in 293T cells and immunoprecipitated with anti-EE antibody. Precipitates were analyzed by Western blotting by anti-EE, -HA or -Flag antibody. Effect of increase of Flag-NS5A expression on the association of FKBP8 with Hsp90 was examined by transfection with 0.1, 0.2 or 0.4 μ g of Flag-NS5A expression plasmid.



Supplementary Figure 4. Disruption of NS5A/FKBP8/Hsp90 complexes by geldanamycin

Purified His-FKBP8, Hsp90 and/or Trx-NS5A were mixed with DMSO or geldanamycin (GA) (100 nM) and subjected to immunoprecipitation with anti-FKBP8 antibody. Precipitates were immunoblotted with antibody to Hsp90, thioredoxin, or FKBP8.

Supplementary materials and methods

Preparation of monoclonal antibody to FKBP8

Glutathione-S-transferase-fused human FKBP8 (GST-FKBP8) was expressed in *Escherichia coli* strain JM109 transformed with pGEX-4T3 containing FKBP8 gene. GST-FKBP8 was purified with Glutathione-conjugated Sepharose Affinity Matrix (Amersham Pharmacia Biotech, Franklin Lakes, NJ). Purified GST- FKBP8 was immunized to *Balb/c* mouse. Lymphonodus cells were obtained after 5 boost immunizations and were fused to mouse myeloma PAI cells. The resulting hybridomas were screened by enzyme-linked immuno-sorbent assay using GST and GST-FKBP8. The selected clones were further screened by flow cytometry using 293T cells expressing HA-FKBP8 (O'Reilly *et al.*, 1998). Among several positive clones, two clones strongly reactive to human FKBP8 were designated as KDM-11 and 19 (IgG2b). Antibodies were purified from supernatants of cell culture by Protein G Sepharose 4B beads (Amersham).

Preparation of recombinant proteins

His₆-tagged FKBP8 (His-FKBP8) and thioredoxin-fused NS5A (aa 25-213, domain I) (Trx-NS5A) were generated from recombinant *Escherichia coli*. Either pET30a encoding FKBP8 or pET32a encoding NS5A (aa 25-213) was introduced into *E. coli* strain BL21(DE3). Ten milliliter of overnight culture was added into 1 L of 2 x YT medium and was incubated at 37°C. When the absorbance of culture supernatant indicated 0.4 OD₆₀₀, isopropyl beta-thiogalactoside (IPTG) was added at final concentration of 0.4 mM and was then incubated at 20°C overnight. After centrifugation, the cell pellet was washed once with 10 ml phosphate buffered saline (PBS). The washed cell pellet was suspended in 40 ml lysis buffer (50mM phosphate buffer [pH 8.0] containing 150mM NaCl, 1% Triton X-100 and 0.2 µg/ml lysozyme) and was incubated at 4°C for 2h. After freezing and thawing, the mixture was sonicated at 4°C for 5 min and was treated with 0.02 mg/ml of DNase at room temperature for 5 min. The cell lysates were centrifuged at 10,000 x g for 5 min. The resulting supernatant

was mixed with 0.5 ml of Nickel agarose beads (Sigma, St. Louis, MO) and was rotated at 4°C for 60 min. The Nickel beads were washed twice with PBS containing 10 mM imidazole. The recombinant protein was eluted from Nickel beads with PBS containing 0.25 M imidazole. Bovine Hsp90 was purchased from Sigma. Bovine Hsp90- α shares 99.5% amino acid identity to human Hsp90- α .

Reference

- O'Reilly, L.A., Cullen, L., Moriishi, K., O'Connor, L., Huang, D.C. and Strasser, A. (1998) Rapid hybridoma screening method for the identification of monoclonal antibodies to low-abundance cytoplasmic proteins. *Biotechniques*, **25**, 824-830.

Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses

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The innate immune system senses viral infection by recognizing a variety of viral components (including double-stranded (ds)RNA) and triggers antiviral responses^{1,2}. The cytoplasmic helicase proteins RIG-I (retinoic-acid-inducible protein I, also known as Ddx58) and MDA5 (melanoma-differentiation-associated gene 5, also known as Ifih1 or Helicard) have been implicated in viral dsRNA recognition³⁻⁷. *In vitro* studies suggest that both RIG-I and MDA5 detect RNA viruses and polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue³. Although a critical role for RIG-I in the recognition of several RNA viruses has been clarified⁸, the functional role of MDA5 and the relationship between these dsRNA detectors *in vivo* are yet to be determined. Here we use mice deficient in MDA5 (*MDA5*^{-/-}) to show that MDA5 and RIG-I recognize different types of dsRNAs: MDA5 recognizes poly(I:C), and RIG-I detects *in vitro* transcribed dsRNAs. RNA viruses are also differentially recognized by RIG-I and MDA5. We find that RIG-I is essential for the production of interferons in response to RNA viruses including paramyxoviruses, influenza virus and Japanese encephalitis virus, whereas MDA5 is critical for picornavirus detection. Furthermore, *RIG-I*^{-/-} and *MDA5*^{-/-} mice are highly susceptible to infection with these respective RNA viruses compared to control mice. Together, our data show that RIG-I and MDA5 distinguish different RNA viruses and are critical for host antiviral responses.

Host pattern recognition receptors, such as Toll-like receptors (TLRs) and helicase family members, have an essential role in the recognition of molecular patterns specific for different viruses, including DNA, single-stranded (ss)RNA, dsRNA and glycoproteins^{9,10}. dsRNA can be generated during viral infection as a replication intermediate for RNA viruses. TLR3, which localizes in the endosomal membrane, has been shown to recognize viral dsRNA as well as the synthetic dsRNA analogue poly(I:C) (refs 11, 12). The cytoplasmic proteins RIG-I and MDA5 have also been identified as dsRNA detectors^{3-5,7,13}. RIG-I and MDA5 contain two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain. RIG-I recruits a CARD-containing adaptor, IPS-1 (also known as MAVS, VISA or Cardif)¹⁴⁻¹⁷. IPS-1 relays the signal to the kinases TBK1 and IKK-i, which phosphorylate interferon-regulatory factor-3 (IRF-3) and IRF-7, transcription factors essential for the expression of type-I

interferons¹⁸⁻²². In contrast, TLR3 activates TBK1 and IKK-i through the TIR-domain-containing adaptor TRIF (also known as Ticam1)¹².

In vitro studies have shown that both RIG-I and MDA5 can bind to poly(I:C) and respond to poly(I:C) and RNA viruses⁶. We have generated *RIG-I*^{-/-} mice, and show that RIG-I is essential for eliciting the immune responses against several RNA viruses, including Newcastle disease virus (NDV), Sendai virus (SeV) and vesicular stomatitis virus (VSV), in various cells except for plasmacytoid dendritic cells (pDCs)⁸. Hepatitis C virus and Japanese encephalitis virus are also reported to be recognized by RIG-I *in vitro*^{23,24}.

The *in vivo* functional relationship between RIG-I and MDA5 remains to be determined. To investigate a functional role for MDA5 *in vivo*, we generated *MDA5*^{-/-} mice and investigated viral recognition (Supplementary Fig. 1). In contrast to *RIG-I*^{-/-} mice, which are mostly embryonic lethal, *MDA5*^{-/-} mice are born in a mendelian ratio, grow healthily and do not show gross developmental abnormalities until 24 weeks of age. Flow cytometric analysis of leukocytes from the spleen and lymph nodes (staining for CD3, B220 and CD11c) revealed that the composition of lymphocytes and dendritic cells is similar in wild-type and *MDA5*^{-/-} mice (data not shown).

TLR3, RIG-I and MDA5 have been implicated in the recognition of poly(I:C) and the subsequent induction of antiviral responses. However, their exact contribution to *in vivo* responses against dsRNA has yet to be clarified. We therefore examined the *in vivo* responses to poly(I:C) in mice lacking RIG-I, MDA5 or TRIF, or both MDA5 and TRIF. Administration of poly(I:C) led to rapid induction of the cytokines interferon- α (IFN- α), IFN- β , interleukin-6 (IL-6) and IL-12 in sera of both wild-type and *RIG-I*^{-/-} mice (Fig. 1a and Supplementary Fig. 2a). In contrast, *MDA5*^{-/-} mice failed to produce IFN- α and IFN- β in response to poly(I:C), and production of IL-6 and IL-12p40 was also significantly impaired (Fig. 1b). Although *Trif*^{-/-} mice produced normal amounts of IFN- α , they also showed severely impaired production of IL-12p40 and partial impairment in IL-6 production. *MDA5*^{-/-}; *Trif*^{-/-} double-knock-out mice failed to induce IFN- α , IL-6 and IL-12p40 in response to poly(I:C). These results indicate that MDA5 is essential for poly(I:C)-induced IFN- α production and TLR3 signalling is critical for IL-12 production, whereas both MDA5 and TLR3 regulate IL-6 production.

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When bone-marrow-derived dendritic cells generated by granulocyte-macrophage colony-stimulating factor (GM-CSF) were incubated in the presence of poly(I:C), production of IFN- α and IFN- β was severely impaired in *MDA5*^{-/-}, but not in *RIG-I*^{-/-} or *Trif*^{-/-}, GM-CSF-DCs (Fig. 1c and Supplementary Fig. 2b). Even when poly(I:C) was transfected into GM-CSF-DCs using lipofectamine, poly(I:C) induced IFN- β production in an MDA5-dependent, but not a RIG-I- or TRIF-dependent, manner (Fig. 1d). IFN- β production in response to poly(I:C) was also impaired in *MDA5*^{-/-} mouse embryonic fibroblasts (MEFs) (Fig. 1e), indicating that poly(I:C) is primarily recognized by MDA5, not RIG-I and TLR3, in these cells.

dsRNAs transcribed *in vitro* (Supplementary Fig. 2c) also stimulated MEFs to produce IFN- β . Unlike for poly(I:C), wild-type and *MDA5*^{-/-} MEFs produced comparable amounts of IFN- β (Fig. 1e) in response to *in vitro* transcribed dsRNAs. In contrast, *RIG-I*^{-/-} MEFs did not produce detectable amounts of IFN- β , indicating that RIG-I is essential for the detection of *in vitro* transcribed dsRNAs. As RIG-I, but not MDA5, is responsible for IFN- β production in response to dsRNAs of various lengths, these helicases probably distinguish nucleotide structure or sequence, but not length. Together, these results indicate that MDA5 and RIG-I are involved

in the detection of poly(I:C) and *in vitro* transcribed dsRNAs, respectively.

This finding led us to hypothesize that RIG-I and MDA5 are involved in the detection of different RNA viruses. We have previously shown that a set of negative-sense RNA viruses are recognized by RIG-I⁸. We first examined IFN- β and IFN- α production in *MDA5*^{-/-} MEFs in response to a set of negative-sense ssRNA viruses, including NDV, ScV, VSV and influenza virus. As infection with most of the wild-type viruses (except NDV) failed to induce type-I interferons in MEFs, owing to suppression of interferon responses by viral proteins (data not shown), we also used mutant viruses lacking viral interferon-inhibitory proteins. As shown in Fig. 2a and Supplementary Fig. 4b, wild-type MEFs produce IFN- β and IFN- α in response to these mutant viruses. Production of type-I interferons was severely impaired in *RIG-I*^{-/-} MEFs compared to wild-type cells, but MDA5 was dispensable for the production of type-I interferons. Japanese encephalitis virus (JEV), a positive-sense ssRNA virus belonging to the flavivirus family, also required RIG-I, but not MDA5, for IFN- β production (Fig. 2b).

We then examined the interferon responses of MEFs to encephalomyocarditis virus (EMCV), a positive-sense ssRNA virus belonging to the picornavirus family. EMCV-induced IFN- β production was abrogated in *MDA5*^{-/-} MEFs (Fig. 2c). In contrast, wild-type and *RIG-I*^{-/-} MEFs produced comparable amounts of IFN- β , indicating that EMCV is specifically recognized by MDA5. The induction of genes encoding IFN- β , IP-10 and IL-6 in response to EMCV was abrogated in *MDA5*^{-/-} macrophages (Supplementary Fig. 3d). The synthesis of cellular proteins in *MDA5*^{-/-} MEFs was progressively inhibited during EMCV infection, to an extent and with kinetics similar to wild-type MEFs (Supplementary Fig. 5), indicating that the EMCV infection was established in wild-type and *MDA5*^{-/-} MEFs in a similar manner. Moreover, other viruses belonging to the picornavirus family (Theiler's and Mengo viruses) also induced IFN- α through MDA5 (Supplementary Fig. 4d). Furthermore, the production of IFN- β in response to SeV and EMCV was impaired in *RIG-I*^{-/-} and *MDA5*^{-/-} GM-CSF-DCs, respectively (Fig. 2d, e), indicating that conventional dendritic cells (cDCs) also use these helicases for the differential recognition of viruses. EMCV-induced production of IL-6 was also abrogated in *MDA5*^{-/-}, but not *RIG-I*^{-/-}, cDCs (Supplementary Fig. 4c). Therefore, MDA5 is critical for the regulation of pro-inflammatory cytokines as well as type-I interferons in response to EMCV.

We next examined whether viral RNAs derived from VSV and EMCV recapitulate the production of interferons through MDA5 and RIG-I. When transfected into GM-CSF-DCs by lipofection, RNAs prepared from VSV or EMCV induced production of IFN- α in a RIG-I- or MDA5-dependent manner, respectively (Fig. 2f). We also performed reconstitution experiments by transfecting RIG-I or MDA5 expression vectors into *RIG-I*^{-/-}; *MDA5*^{-/-} MEFs, in which IFN- β induction was completely abrogated in response to infection with EMCV or ScV Cm (ScV with a mutated C protein) (Fig. 2g). The ectopic expression of human RIG-I, but not MDA5, activated the *Irfn* promoter in response to SeV Cm. Reciprocally, cells expressing human MDA5, but not RIG-I, activated the *Irfn* promoter in response to EMCV in a dose-dependent manner (Fig. 2h). These results indicate that human RIG-I and MDA5 recognize different RNA viruses by recognizing viral RNAs.

Previous studies have shown that pDCs use mainly the TLR system instead of RIG-I in the recognition of several RNA viruses⁸. MyD88 is an adaptor protein essential for TLR signalling (except through TLR3). We purified B220⁺ pDCs from Flt3L-generated bone-marrow-derived dendritic cells (Flt3L-DCs) and infected them with EMCV. pDCs from *Myd88*^{-/-}, but not *MDA5*^{-/-}, mice showed a profound defect in IFN- α production (Supplementary Fig. 6). Reciprocally, MDA5, but not MyD88, is required for the production of IFN- α in B220⁺ pDCs purified from Flt3L-DCs (Supplementary Fig. 6). These results indicate that both MDA5 and RIG-I are

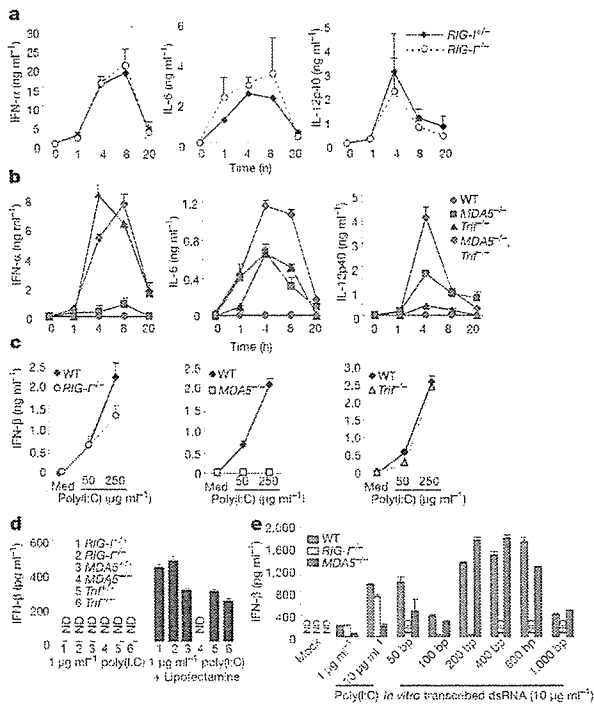


Figure 1 | Roles of MDA5, RIG-I and TRIF in the recognition of synthesized dsRNAs and dsRNA analogues. **a**, **b**, *RIG-I*^{-/-} and littermate *RIG-I*^{+/-} mice (**a**) or wild-type (WT), *MDA5*^{-/-}, *Trif*^{-/-} or *MDA5*^{-/-}; *Trif*^{-/-} double-knockout mice (**b**) were injected intravenously with 200 μ g poly(I:C) for the indicated periods, and IFN- α , IL-6 and IL-12p40 production was measured in serum by ELISA. Data show mean \pm s.d. **c**, GM-CSF-DCs from *RIG-I*^{-/-}, *MDA5*^{-/-}, *TRIF*^{-/-} and littermate control mice were incubated in the presence of 50 or 250 μ g ml⁻¹ poly(I:C) for 24 h. IFN- β production in the cell culture supernatants was measured by ELISA. Med, medium only. **d**, GM-CSF-DCs were treated with 1 μ g ml⁻¹ poly(I:C) complexed with or without lipofectamine 2000 for 24 h, and IFN- β production was measured. **e**, Wild-type, *RIG-I*^{-/-} and *MDA5*^{-/-} MEFs were treated with poly(I:C) or *in vitro* transcribed dsRNAs of indicated lengths complexed with lipofectamine 2000 for 12 h, and IFN- β production was measured. Error bars indicate s.d. of triplicate wells in a single experiment; data are representative of three independent experiments. ND, not detected.

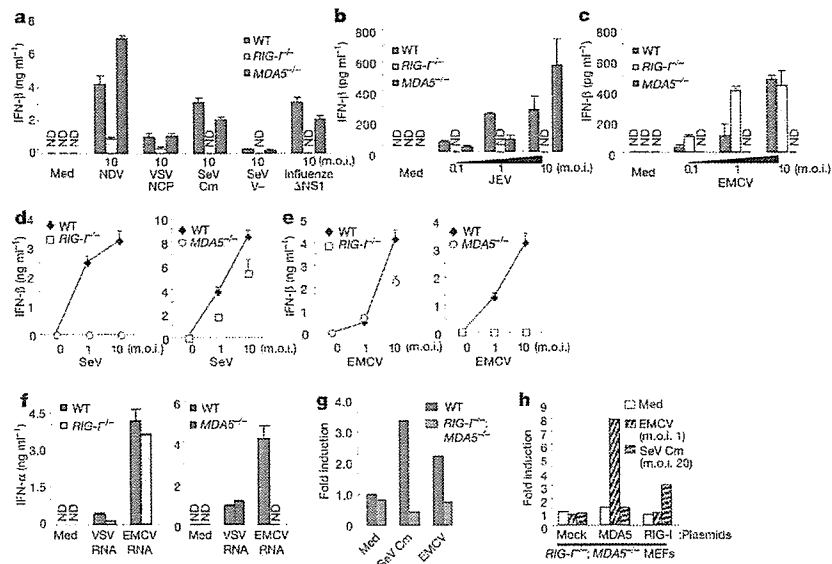


Figure 2 | Differential viral recognition by RIG-I and MDA5. **a**, Wild-type, *RIG-I*^{-/-} and *MDA5*^{-/-} MEFs were exposed to negative-sense ssRNA viruses, including NDV, VSV lacking a variant of M protein (NCP), SeV with a mutated C protein (Cm), SeV lacking V protein (V⁻), and influenza virus lacking the NS1 protein (Δ NS1) for 24 h. IFN- β production in the culture supernatants was measured by ELISA. **b, c**, Wild-type, *RIG-I*^{-/-} and *MDA5*^{-/-} MEFs were exposed to the positive-sense ssRNA viruses JEV (**b**) and EMCV (**c**), and IFN- β production was measured. **d, e**, GMCSF-DCs from *RIG-I*^{-/-} and *MDA5*^{-/-} mice and their littermate wild-type mice were infected with an increasing m.o.i. of SeV V⁻ (**d**) or EMCV (**e**) for 24 h, and IFN- β production was measured. **f**, Wild-type, *RIG-I*^{-/-} and *MDA5*^{-/-}

GMCSF-DCs were treated with RNAs directly prepared from VSV and EMCV (complexed with lipofectamine 2000) for 24 h, and IFN- α production was measured. **g**, Wild-type and *RIG-I*^{-/-}; *MDA5*^{-/-} MEFs were transiently transfected with a reporter construct containing the *Irfn* promoter and exposed to SeV Cm or EMCV for 24 h. Cell lysates were then prepared and subjected to a luciferase assay. **h**, *RIG-I*^{-/-}; *MDA5*^{-/-} MEFs were transiently transfected with the *Irfn* promoter construct together with expression plasmids encoding human RIG-I or MDA5. The cells were then infected with EMCV or SeV Cm for 24 h and were subjected to a luciferase assay. Error bars in **a-g** indicate s.d. of triplicate wells in a single experiment; data are representative of three independent experiments. ND, not detected.

dispensable for the viral induction of IFN- α in pDCs.

We next examined the *in vivo* roles of MDA5 and RIG-I in host defence against viral infection. Although most *RIG-I*^{-/-} mice are embryonic lethal⁶, we could efficiently obtain live adult mice by intercrossing the *RIG-I*^{+/-} mice obtained after *RIG-I*^{+/-} \times ICR crosses (Supplementary Table 1). When the mice were infected with JEV, serum IFN- α levels were markedly decreased in *RIG-I*^{-/-} mice compared to littermate *RIG-I*^{+/-} mice. In contrast, *MDA5*^{-/-} mice did not show a defect in JEV-induced systemic IFN- α production (Fig. 3a). IFN- α production was partially impaired in *Myd88*^{-/-} mice compared to wild-type mice, but the extent of this impairment was far less than in *RIG-I*^{-/-} mice (Fig. 3a). These data suggest that the TLR system is not critical for the induction of serum IFN- α *in vivo* in response to JEV. Consistent with this finding, *RIG-I*^{-/-} mice, but not *MDA5*^{-/-} or *Myd88*^{-/-} mice, were more susceptible to JEV infection than control mice (Fig. 3b). Furthermore, *RIG-I*^{-/-} mice, but not *MDA5*^{-/-} mice, succumbed to VSV infection, consistent with abrogated interferon responses (Supplementary Fig. 7). Thus, RIG-I-mediated recognition of a specific set of viruses has a critical role in antiviral host defence *in vivo*.

We next challenged the mice with EMCV as a model virus that is recognized by MDA5. Induction of IFN- β , IFN- α , RANTES and IL-6 was severely impaired in the sera of *MDA5*^{-/-} mice (Fig. 4a and Supplementary Fig. 8). *MDA5*^{-/-} mice and mice null for the IFN- α/β receptor (*Irfn1*^{-/-}) were highly susceptible to EMCV infection (viral titre of 1×10^2 plaque-forming units (p.f.u.)) compared to littermate controls ($P < 0.01$) (Fig. 4b). In contrast, deficiency of neither RIG-I nor TLR3 affected the survival of mice infected with EMCV. Consistent with a previous report²², *Myd88*^{-/-} mice were modestly susceptible to EMCV infection compared to wild-type mice, implying that pDC-mediated responses are not critical for eliminating EMCV (Fig. 4b).

It is known that EMCV preferentially infects cardiomyocytes and causes myocarditis. Consistent with increased susceptibility to EMCV, viral titre in the heart was much higher in *MDA5*^{-/-} mice compared to control mice (Fig. 4c). Histological analysis of hearts two days after EMCV infection revealed that focal necrosis of

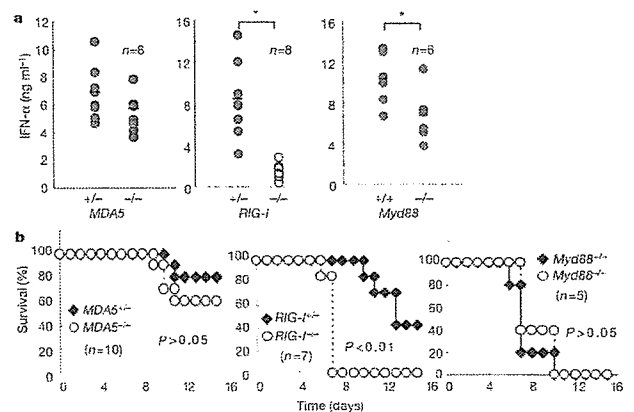


Figure 3 | Susceptibility of *RIG-I*^{-/-} and *MDA5*^{-/-} mice to JEV infection. **a**, *RIG-I*^{+/-}, *RIG-I*^{-/-}, *MDA5*^{+/-} and *MDA5*^{-/-} mice ($n = 8$), and *Myd88*^{+/-} or *Myd88*^{-/-} mice ($n = 6$), were injected intravenously with 2×10^7 p.f.u. JEV. Sera were collected 24 h after injection, and IFN- α production levels measured by ELISA. Circles represent individual mice, bars indicate mean values. Asterisk, $P < 0.05$ versus controls (*t*-test). **b**, The survival of 6-week-old mice (genotypes as indicated) infected intravenously with 2×10^7 p.f.u. JEV. Mice were monitored for 15 days ($P < 0.01$ between *RIG-I*^{-/-} mice and their littermate controls, generalized Wilcoxon test).

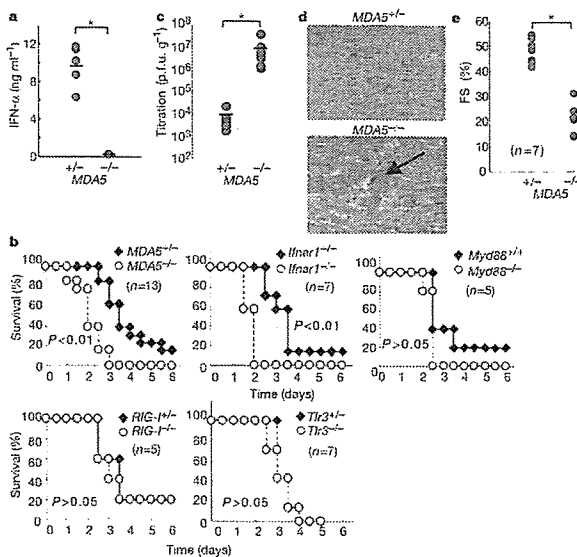


Figure 4 | Role of MDA5 in host defence against EMCV infection. **a**, $MDA5^{+/-}$ and $MDA5^{-/-}$ mice ($n = 5$) were inoculated intravenously with 1×10^7 p.f.u. EMCV. Sera were prepared 4 h after injection and IFN- α production levels determined by ELISA. **b**, The survival of 6-week-old mice (genotypes as indicated) infected with 1×10^2 p.f.u. EMCV intraperitoneally was monitored every 12 h for six days ($P < 0.01$ between $MDA5^{-/-}$ or $Ifnar1^{-/-}$ mice and their littermate controls, generalized Wilcoxon test). **c**, $MDA5^{+/-}$ and $MDA5^{-/-}$ mice were infected intraperitoneally with 1×10^2 p.f.u. EMCV. After 48 h, mice were killed and virus titres in hearts were determined by plaque assay. **d**, Heart sections of $MDA5^{+/-}$ and $MDA5^{-/-}$ mice, two days after infection, were assessed for histological changes using haematoxylin and eosin staining. Arrow indicates the focal necrosis of cardiomyocytes. **e**, Cardiac function of mice 48 h after EMCV infection was assessed by echocardiography (see Supplementary Fig. 8b). The fractional shortening (FS) after infection determined by transthoracic M-mode echocardiographic tracings is shown. Asterisk, $P < 0.05$ versus $MDA5^{+/-}$ mice (t -test).

cardiomyocytes had developed in $MDA5^{-/-}$ mice, but wild-type hearts showed no histological abnormalities at this time point (Fig. 4d). Notably, no infiltration of immune cells was observed in either wild-type or $MDA5^{-/-}$ heart sections at this time point. However, when cardiac performance was analysed by echocardiography two days after infection (Fig. 4e), cardiac contractility was severely depressed in $MDA5^{-/-}$ mice (fractional shortening $48.2 \pm 4.9\%$ in $MDA5^{+/-}$ mice, $21.2 \pm 5.8\%$ in $MDA5^{-/-}$ mice), indicating that $MDA5^{-/-}$ mice developed severe heart failure due to virus-induced cardiomyopathy. Thus, MDA5-mediated recognition of EMCV is a prerequisite for triggering antiviral responses as well as for prevention of myocardial dysfunction.

Together, our results demonstrate that RIG-I and MDA5 have essential roles in the recognition of different groups of RNA viruses, as well as in the subsequent production of type-I interferons and pro-inflammatory cytokines. We have found that poly(I:C) and *in vitro* transcribed dsRNA are recognized by MDA5 and RIG-I, respectively; this is in contrast to results from previous *in vitro* studies. RIG-I probably recognizes dsRNA generated over the course of RNA virus replication, as all *in vitro* transcribed dsRNAs tested except for poly(I:C) induced type-I interferons through RIG-I. In contrast, the endogenous ligand of MDA5 remains enigmatic. Moreover, how RIG-I and MDA5 differentially recognize natural dsRNAs is undetermined. Given that the helicase domains of RIG-I and MDA5 bind to dsRNA, analyses of the crystal structures of these domains should help achieve a better understanding of the molecular mechanisms underlying this differential recognition.

Furthermore, it is still possible that unknown dsRNA-binding proteins also function as direct receptors for viral RNAs.

Finally, the picornavirus family contains several viruses that are pathogenic for humans, including poliovirus, rhinovirus and the virus causing foot-and-mouth-disease. Our studies suggest that human MDA5 and RIG-I also recognize RNA viruses. Thus, identification of therapeutic agents that modify RIG-I or MDA5 may lead to antiviral strategies against selected viruses.

METHODS

Mice, cells and reagents. The generation of $MDA5^{-/-}$ mice is described in the Supplementary Information. $Myd88^{-/-}$, $Tlr3^{-/-}$ and $Trif^{-/-}$ mice have been described previously¹². $Ifnar1^{-/-}$ mice have also been described previously²⁵. $RIG-I^{+/+}$ mice in a 129Sv \times C57BL/6 background were crossed with ICR mice, and the resulting $RIG-I^{-/-}$ mice were further intercrossed. Interbreeding of these $RIG-I^{-/-}$ mice produced healthy and fertile $RIG-I^{-/-}$ offspring, although their number was less than half that of $RIG-I^{+/+}$ progeny (Supplementary Table 1). $RIG-I^{-/-}$ and $RIG-I^{+/+}$ littermate mice were used for *in vivo* experiments. $RIG-I^{-/-}$; $MDA5^{-/-}$ mice in a 129Sv \times C57BL/6 background were lethal at embryonic day 12.5. Additional details regarding cells, reagents and the preparation of *in vitro* transcribed dsRNA are provided in the Supplementary Information.

Viruses. NDV (ref. 3), VSV, VSV lacking a variant of M protein (NCP) (ref. 8), influenza virus lacking the NS1 protein (Δ NS1) (ref. 26), JEV (ref. 27) and EMCV (ref. 3) have been described previously. SeV and SeV lacking the V protein (V^{-}) or with mutated C proteins (Cm) were provided by A. Kato²⁶.

Luciferase assay. Wild-type or $RIG-I^{-/-}$; $MDA5^{-/-}$ MEFs were transiently transfected with a reporter construct containing the *Irf3* promoter together with an empty vector (mock), or $RIG-I$ or $MDA5$ expression vectors. As an internal control, a *Renilla* luciferase construct was transfected. Transfected cells were untreated or infected with EMCV or SeV Cm (m.o.i. 20) for 24 h. The cells were lysed and subjected to a luciferase assay using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Analysis of mice after EMCV infection. Methods for plaque assays, histological analysis and echocardiography are described in the Supplementary Information.

Measurement of cytokine production. Cell culture supernatants were collected and analysed for IFN- β , IFN- α , IL-6 or IL-12p40 production using enzyme-linked immunosorbent assays (ELISAs). ELISA kits for mouse IFN- α and IFN- β were purchased from PBL Biomedical Laboratories, and those for IL-6, IL-12p40 and RANTES were obtained from R&D Systems.

Statistical analysis. Kaplan–Meier plots were constructed and a generalized Wilcoxon test was used to test for differences in survival between control and mutant mice after viral infection. Statistical significance of any differences in cytokine concentration and EMCV titres was determined using Student's t -tests.

Received 30 January; accepted 20 March 2006.

Published online 9 April 2006.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank all colleagues in our laboratory, K. Takeda, T. Shioda, E. Nakayama and K. Kiyotani for helpful discussions, A. Kato, T. Abe, Y. Mori, B. S. Kim and A. Palmenberg for viruses and plasmids, M. Hashimoto for secretarial assistance, and Y. Fujiwara, M. Shiokawa, N. Kitagaki and A. Shibano for technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan, and from the 21st Century Center of Excellence Program of Japan.

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Supplementary Information

I. Supplementary Materials and Methods

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I. Supplementary Materials and Methods

Generation of MDA5^{-/-} mice

The MDA5 gene was isolated from genomic DNA extracted from ES cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 4.3-kb fragment encoding the MDA5 ORF (including DExH box) with a neomycin-resistance gene cassette (*neo*), and a herpes simplex virus thymidine kinase (HSV-TK) driven by PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into ES cells, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and further confirmed by Southern blotting. Homologous recombinants were micro-injected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed in order to obtain MDA5^{-/-} mice. MDA5^{-/-} and littermate control mice were used throughout the experiments.

Cells and Reagents

RIG-I^{-/-} or MDA5^{-/-} MEFs were prepared from embryos under 129Sv and C57BL/6 background derived at 12.5 days postcoitum. RIG-I^{-/-}MDA5^{-/-} MEFs were prepared from embryos (129Sv X C57BL/6 background) at 11.5 days postcoitum. Bone marrow derived DCs were generated in RPMI 1640 medium containing 10% FCS, 50 mM 2-mercaptoethanol, and 10 ng/ml GM-CSF or 10 ng/ml Flt3L. pDCs and cDCs were isolated from Flt3L-DCs by MACS using anti-B220 and CD11c microbeads from

Miltenyi Biotech as described. Poly I:C was purchased from Amersham Biosciences. For the synthesis of poly I:C, poly I (152-539 bases) and poly C (319-1305 bases) have been separately synthesized and then annealed. Therefore, the expected length of poly I:C is 319-539 bps (Amersham Biosciences). Poly I:C was complexed with cationic lipids, Lipofectamin 2000 reagents (Invitrogen), and added to MEFs. DCs were incubated with or without Lipofectamine 2000 for stimulation.

Northern blot

PECs were treated with or without 1000 U/ml mouse IFN- β (Calbiochem) for 8 h, and total RNA was extracted using TRIzol reagent (Invitrogen). RNA was electrophoresed, transferred to nylon membranes and then hybridized with indicated cDNA probes. To detect the expression of MDA5 mRNA, a 308 bp fragment (777-1084) was used as a probe. The same membrane was rehybridized with a β -actin probe.

Western blot analysis and an antibody

MEF were treated with 1000 U/ml IFN- β for 8 h. Cells were then lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-Cl (pH7.5), 1 mM EDTA and protease inhibitor cocktail (Roche). Cell lysates were dissolved by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blotted with the specific antibody to MDA5 protein, and visualized with an enhanced chemiluminescence system (Perkinermer). Polyclonal anti-MDA5 antibody was raised against corresponding to amino acids 1005-1019 of mouse MDA5.

Preparation of in vitro transcribed dsRNA

The mouse Lamin A/C cDNA sequence was amplified by PCR and cloned into the pT7 blue T vector (Novagen) and sequenced. Various lengths of dsRNAs corresponding to the sequence of mouse Lamin A/C were generated using a T7 RiboMAXTM Express RNAi

System (Promega) according to the manufacturer's instruction. In brief, DNA fragments tagged with T7 RNA polymerase promoters corresponding to parts of Lamin A/C (50, 100, 200, 400, 600, 1000 bps) were amplified by a PCR reaction using Lamin A/C cDNA as a template, and with following primers;

T7 Lamin. Forward, TAATACGACTCACTATAGGacttggtggctgcgcagge

T7 Lamin.50 reverse, TAATACGACTCACTATAGGtgagaagagcctcgaggctctt

T7 Lamin.100 reverse, TAATACGACTCACTATAGGcaatgtgcgcttctcactgagagcag

T7 Lamin.200 reverse, TAATACGACTCACTATAGGccactcgcctcagcatctcat

T7 Lamin.400 reverse, TAATACGACTCACTATAGGctgtccacctggtcctcatg

T7 Lamin.600 reverse, TAATACGACTCACTATAGGtcctccaggtcagcagcttt

T7 Lamin.1000 reverse, TAATACGACTCACTATAGGggacttggtgcgcagccgcacgaac

The PCR products were purified and used as templates for in vitro transcription with T7 RNA polymerase. The product was annealed to form dsRNA followed by treatment with DNase and RNase to digest ssRNA and DNA. The dsRNA was further purified by isopropanol precipitation and resuspended in Nuclease-free water. The generation of dsRNAs was visualized by Agarose gel electrophoresis (Supplementary Fig. 2b). To stimulate MEFs, the dsRNA was complexed with Lipofectamine 2000, then added to the cells, and incubated for 24 hours.

Histological analysis

Hearts were taken from EMCV infected mice, and fixed with 3.7% formaldehyde. Transverse sections through the heart (5 μ m) were cut and stained with hematoxylin and eosin.

Plaque assay

Forty-eight hours after EMCV infection, Hearts were prepared and homogenized in PBS. Virus titration in the virus containing PBS was determined by standard plaque assay as

described previously⁸. After centrifugation, supernatants were serially diluted, and added to plates containing HeLa cells. The cells were overlaid with DMEM containing 1% low melting agarose and incubated for 48 h. Then plaques were counted.

Echocardiography

Two days after EMCV infection, echocardiography was performed on mice anesthetized with 2.5% avertin (8 µl/g) using ultra-sonography (SONOS-5500, equipped with a 15-MHz linear transducer, Philips Medical Systems). Hearts were imaged in a two-dimensional parasternal short-axis view, and an M-mode echocardiogram of the midventricle was recorded at the level of the papillary muscles*. Heart rate, anterior and posterior wall thickness, and end-diastolic and end-systolic internal dimensions of the LV were obtained from the M-mode image.

Viruses

Mengo virus¹ was kindly provided by A. Palmenberg. Theiler's virus² have been described previously.

Preparation of viral RNA.

BHK cells and L cell plated on 10 X 15 cm dishes were infected with moi= 0.01 of wt VSV and EMCV, respectively. At 1h after infection, medium was removed and replaced with DMEM containing 10 % FCS and the cells were incubated for 2 days at 37 °C. Then the supernatants were collected and centrifuged at 3,000 rpm for 15 min to remove cells for avoiding cellular RNA contamination. Then the supernatants were harvested and centrifuged at 25,000 rpm for 90 min in an SW28 rotor at 4 °C. The viral pellet was suspended in TRIzol reagent (Invitrogen) and RNA was extracted. 5-10 µg/ml VSV RNA and 0.5-3 µg/ml EMCV RNA were obtained from single preparation.

Analysis of total protein synthesis.

Cultures of wild-type and *MDA5*^{-/-} MEFs were infected with EMCV. At various time of labeled by incorporation of 50 μ Ci of [³⁵S]Met-Cys (GE Healthcare) for 1 h. Then the cells were lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-Cl (pH7.5), 1 mM EDTA and protease inhibitor cocktail (Roche). Total cell extracts were separated by polyacrylamide gel electrophoresis, and the proteins were visualized by autoradiography.

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II. Supplemental Figure Legends

Supplementary Fig. 1: Targeted disruption of the murine *MDA5* gene.

(a) Structure of the mouse *MDA5* gene, the targeting vector and the predicted disrupted gene. Closed boxes denote the coding exon. B; BamH I (b) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with BamHI, separated by electrophoresis and hybridized with the radiolabelled probe indicated in (a). Southern blot gave a single 9.4 kb band for wild-type (+/+), a 4.6 kb band for homozygous (-/-) and both bands for heterozygous (+/-) mice. (c) Northern blot analysis of peritoneal exudates cells (PECs). Total RNA from wild-type (WT) and *MDA5*^{-/-} PECs treated with 1000 U/ml IFN- β for 8 h was extracted and subjected to Northern blot analysis for the expression of *MDA5* mRNA. The same

membrane was rehybridized with a *β-actin* probe. (d) Western blot analysis of MDA5 expression. WT and MDA5^{-/-} MEFs were treated with 1000 U/ml IFN-β for 8 h, and whole cell lysates were immunoblotted with antibody against MDA5. N.S. non specific.

Supplementary Fig. 2. Involvement of MDA5 or RIG-I in the recognition of dsRNAs.

- (a) RIG-I^{-/-} and MDA5^{-/-}, and their littermate WT mice were injected intravenously with 200 μg of poly I:C for the indicated periods and the production of IFN-β in the sera was measured by ELISA. The data are means ± S.D. of sera samples.
- (b) GMCSF-DCs from RIG-I^{-/-} and MDA5^{-/-}, TRIF^{-/-} and their littermate control mice were incubated in the presence of 50, 250 μg/ml poly I:C for 24 h. The production of IFN-α in the culture supernatants was measured by ELISA.
- (c) Generation of different lengths of dsRNAs corresponding to mouse lamin A/C. Different lengths of dsRNAs corresponding to mouse lamin A/C were synthesized as described in Methods section. 1 μg of dsRNAs were separated on 1% Agarose gel and visualized by staining with ethidium bromide. All dsRNAs synthesized appear with the estimated size.

Supplementary Fig. 3: Contribution of RIG-I and MDA5 in the induction of genes encoding type I IFNs and IFN-inducible proteins in response to viral infection.

- (a) WT, RIG-I^{-/-} or MDA5^{-/-} MEFs were treated with 5 μg/ml poly I:C complexed with lipofectamine 2000 for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β*, *IP10* and *β-actin* mRNA.
- (b) WT, RIG-I^{-/-} and MDA5^{-/-} MEFs were treated with 5 μg/ml dsRNA corresponding to Lamin A/C (600 bps) complexed with lipofectamine 2000 for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of

IFN-β and *IP10* mRNA. 28S and 18S ribosomal RNA bands on ethidium bromide-stained gel were used to control the RNA loading (lower panel).

(c) WT, RIG-I^{-/-} and MDA5^{-/-} MEFs were infected with moi=10 of SeV V(-) for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β* and *IP10* mRNA. 28S and 18S ribosomal RNA bands on ethidium bromide-stained gel were used to control the RNA loading (lower panel).

(d) WT and MDA5^{-/-} PECs were exposed to moi=10 of EMCV for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β*, *IP10*, *IL-6* and *β-actin* mRNA.

Supplementary Fig. 4: Role of MDA5 and RIG-I in the IFN-α responses against various viruses.

(a) WT, RIG-I^{-/-} and MDA5^{-/-} MEFs were exposed to negative-sense ssRNA viruses, including NDV, VSV NCP, SeV Cm, SeV V- and influenza ΔNS1. IFN-α production in the culture supernatants was measured by ELISA.

(b and c) GMCSF-DCs from RIG-I^{-/-} and MDA5^{-/-} mice and their littermate WT mice were infected with indicated moi of EMCV for 24 h. The production of IFN-α (b) and IL-6 (c) in the culture supernatants was measured by ELISA.

(d) GMCSF-DCs from wild-type and MDA5^{-/-} mice were infected with indicated moi of Theiler's virus or Mengovirus for 24 h. IFN-α production in the culture supernatants was measured by ELISA. The data are means ± S.D. of triplicates.

Supplementary Fig. 5: EMCV-mediated protein synthesis shutoff was not altered between wild-type and MDA5^{-/-} MEFs.

Cultures of wild-type and MDA5^{-/-} MEFs were infected with EMCV and labeled by incorporation of [³⁵S]Met-Cys for 1 h at various times after infection. Total cell extracts

were separated by polyacrylamide gel electrophoresis, and the proteins were visualized by autoradiography.

Supplementary Fig. 6: Differential involvement of MDA5 and MyD88 in EMCV-mediated IFN production in cDCs and pDCs.

DCs were induced from bone marrow cells obtained from MyD88^{+/-}, MyD88^{-/-}, MDA5^{+/-} and MDA5^{-/-} mice by cultivating in the presence of Flt3L. At day 7, B220⁺CD11c⁺ pDCs and B220⁻CD11c⁺ cDCs were purified by MACS, and infected with EMCV for 24h. IFN- α production in the culture supernatants was measured by ELISA. Error bars indicate \pm S.D. of triplicates.

Supplementary Fig. 7: The survival of MDA5^{-/-}, RIG-I^{-/-} or IFN α / β R^{-/-} mice in response to VSV infection.

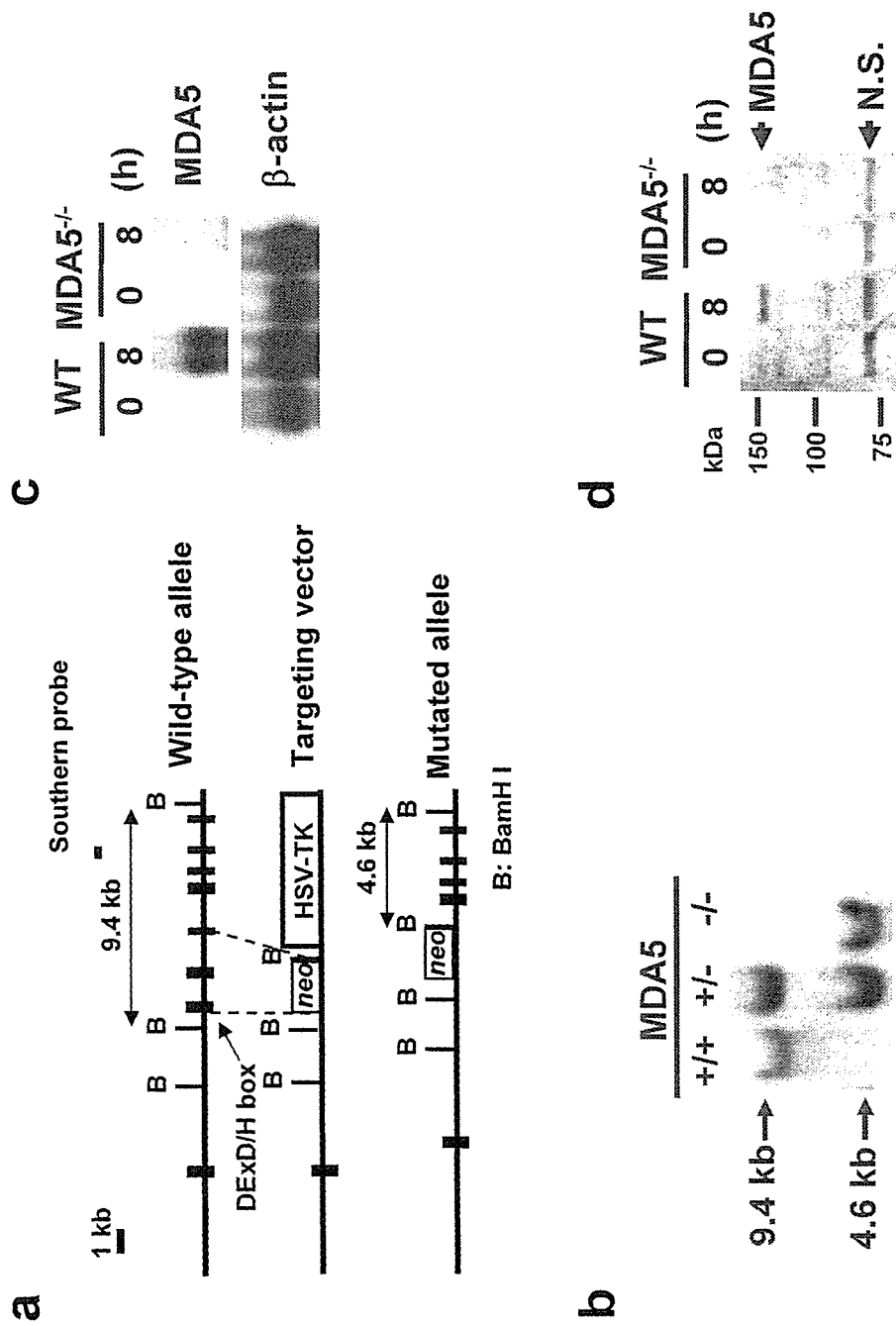
The survival of the mice (3-weeks old) infected with 4×10^6 pfu VSV intranasally was monitored for 9 days ($p < 0.01$ by the generalized wilcoxon test between RIG-I^{-/-} mice and their littermate controls).

Supplementary Fig. 8: Responses of MDA5^{-/-} mice against EMCV infection

- (a) MDA5^{+/-} and MDA5^{-/-} mice (n=5) were intravenously inoculated with 1×10^7 pfu EMCV. Sera were taken at 4 h after injection, and IFN- β , Rantes and IL-6 production levels were determined by ELISA. *, $P < 0.05$ versus controls by the student's t-test.
- (b) Cardiac function of the mice 48 h after EMCV infection was assessed by echocardiography. Transthoracic M-mode echocardiographic tracings from MDA5^{+/-} and MDA5^{-/-} mice 48 h after EMCV infection.

Supplementary Table I: Genotypes of mice derived from RIG-I^{+/-} intercrosses or crosses between RIG-I^{+/-} and RIG-I^{-/-} mice.

Supplemental Figure 1.



Supplemental Figure 2.

