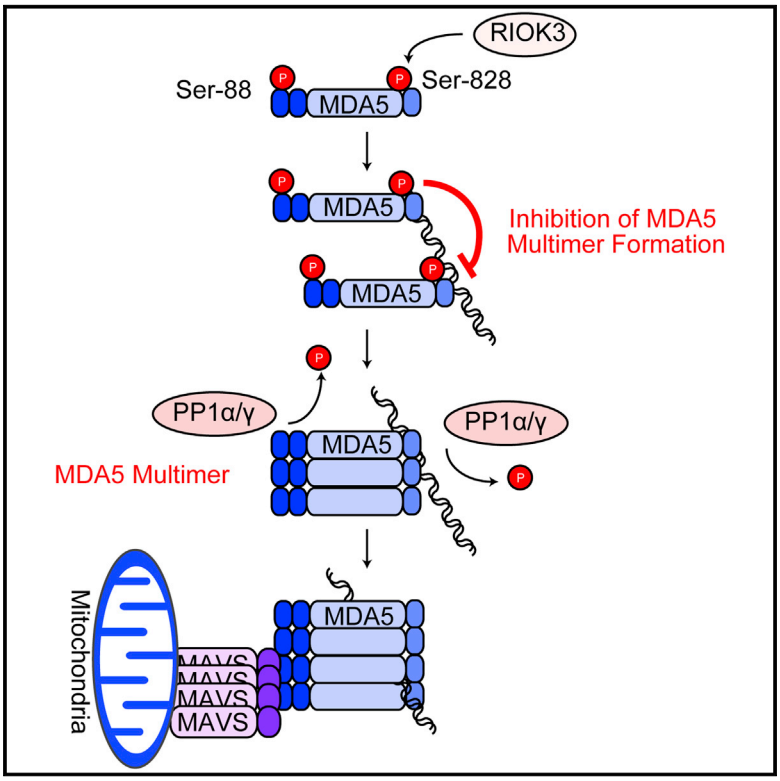
Report

Cell Reports

RIOK3-Mediated Phosphorylation of MDA5 Interferes with Its Assembly and Attenuates the Innate Immune Response

Graphical Abstract



Highlights

- RIOK3 attenuates MDA5-mediated innate immune response
- RIOK3 mediates phosphorylation of MDA5 Ser-828
- Phosphorylation of MDA5 Ser-828 disrupts its assembly and signaling

Authors

Ken Takashima, Hiroyuki Oshiumi, ..., Misako Matsumoto, Tsukasa Seya

Correspondence

oshiumi@med.hokudai.ac.jp (H.O.),
seya-tu@pop.med.hokudai.ac.jp (T.S.)

In Brief

The cytoplasmic viral RNA sensor MDA5 is essential for innate immune responses to viral infection. Takashima et al. now show that RIOK3 phosphorylates MDA5 at Ser-828 and that this phosphorylation disrupts MDA5 assembly in uninfected and virus-infected cells to attenuate the antiviral response.

Takashima et al., 2015, Cell Reports *11*, 192–200
April 14, 2015
http://dx.doi.org/10.1016/j.celrep.2015.03.027

CellPress

OPEN ACCESS
CellPress

Cell Reports
Report

RIOK3-Mediated Phosphorylation of MDA5 Interferes with Its Assembly and Attenuates the Innate Immune Response

Ken Takashima,[1] Hiroyuki Oshiumi,[1,*] Hiromi Takaki,[1] Misako Matsumoto,[1] and Tsukasa Seya[1,*]
[1]Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan
*Correspondence: oshiumi@med.hokudai.ac.jp (H.O.), seya-tu@pop.med.hokudai.ac.jp (T.S.)
http://dx.doi.org/10.1016/j.celrep.2015.03.027

SUMMARY

MDA5 is a cytoplasmic viral double-stranded RNA (dsRNA) sensor and triggers type I interferon (IFN) production. MDA5 assembles along viral dsRNA, leading to the formation of an MDA5 filament required for activating the MAVS adaptor. A recent study has revealed that PP1α and PP1γ phosphatases are responsible for dephosphorylating MDA5 and are essential for its activation. Here, we identified RIO kinase 3 (RIOK3) as a protein kinase that phosphorylates the MDA5 C-terminal region. *RIOK3* knockout strongly enhanced type I IFN and IFN-inducible gene expression following measles virus infection. Conversely, the ectopic expression of RIOK3 or a phosphomimetic MDA5-S828D mutation attenuated MDA5-mediated signaling. Moreover, RIOK3-mediated MDA5 phosphorylation impaired MDA5 multimer formation, indicating that MDA5 C-terminal phosphorylation interferes with MDA5 filament formation and suppresses its signaling. Our data revealed a regulatory mechanism underlying the activation of the cytoplasmic viral RNA sensor MDA5 in both uninfected and virus-infected cells.

INTRODUCTION

The innate immune response is essential for controlling viral infection. The RIG-I-like receptors (RLRs), MDA5 and RIG-I, are cytoplasmic viral RNA sensors and trigger the signal to induce innate immune responses following viral infection (Loo and Gale, 2011). MDA5 recognizes relatively long double-stranded RNA (dsRNA), whereas RIG-I recognizes relatively short dsRNA (Kato et al., 2008), and thus MDA5 and RIG-I recognizes different RNA viruses. Previous studies have revealed that MDA5 recognizes viral RNA from measles virus (MV), poliovirus, and encephalomyocarditis virus and is essential for the antiviral innate immune response (Abe et al., 2012; Loo and Gale, 2011; Takaki et al., 2011). The role of MDA5 in the development of autoimmune disorders has also been revealed (Funabiki et al., 2014).

The MDA5 protein comprises two N-terminal caspase recruitment domains (CARDs), an RNA helicase domain, and a C-terminal domain (CTD) (Yoneyama et al., 2005). The MDA5 CTD binds to viral dsRNA, following which the proteins stack along the RNA in a head-to-tail arrangement via direct protein-protein contacts, leading to MDA5 filament formation (Wu et al., 2013). The core MDA5 filament formation is essential for its CARDs oligomerization. The CARDs oligomer binds to the mitochondrial protein MAVS (also called IPS-1, Cardif, and MITA) and nucleates the MAVS filament, producing type I interferon (IFN) and other inflammatory cytokines (Wu et al., 2013). The paramyxovirus V protein disrupts this filament formation to escape the host innate immune responses (Davis et al., 2014; Motz et al., 2013).

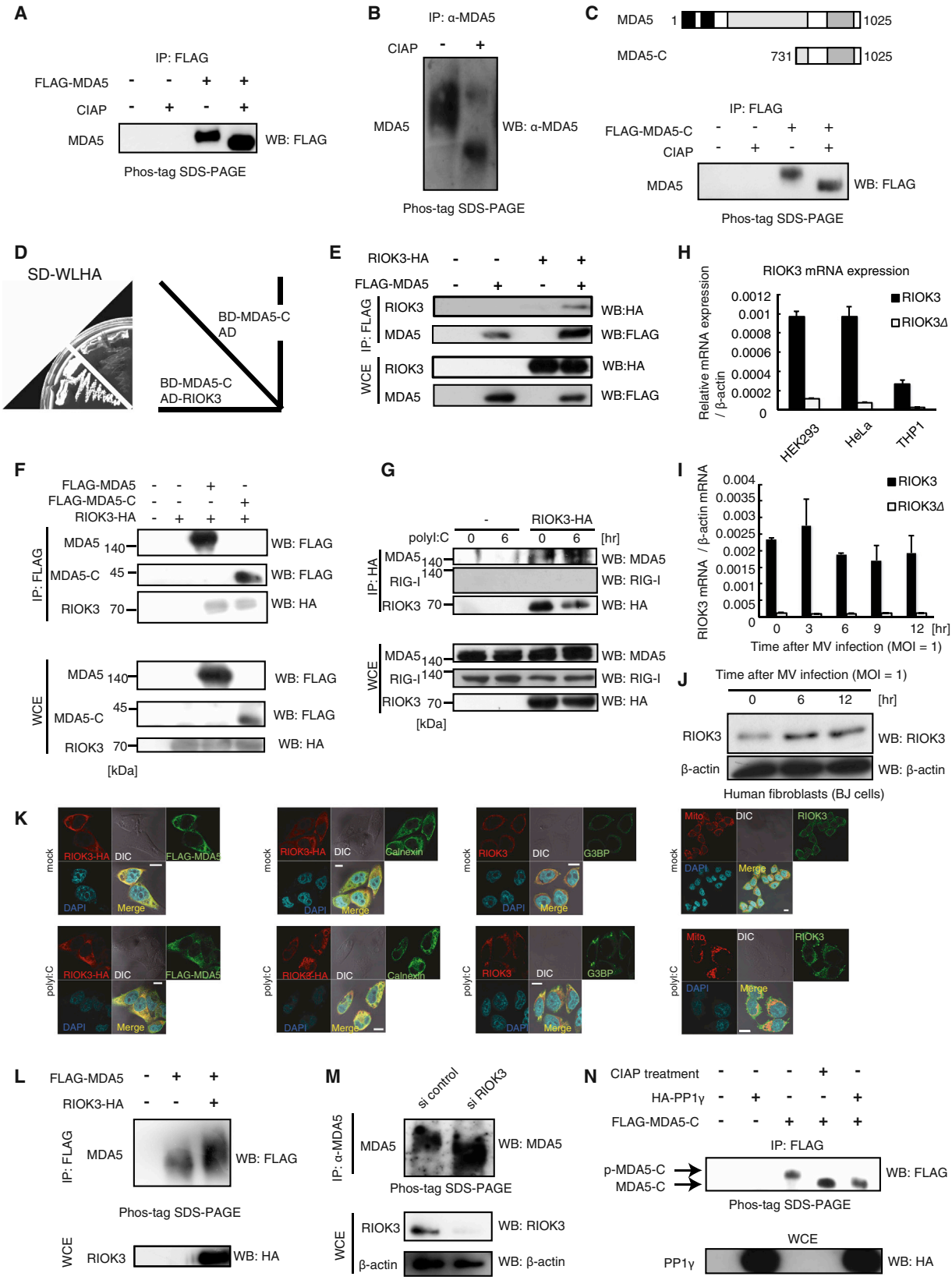
Gack and colleagues have shown that MDA5-signaling activity is regulated by a dynamic balance between the phosphorylation and dephosphorylation of the MDA5 CARDs (Wies et al., 2013). They have revealed that Ser-88 within MDA5 CARDs undergoes phosphorylation under normal conditions and that this inhibits its signaling (Davis et al., 2014; Wies et al., 2013). However, the underlying mechanism has not been fully elucidated. It is also unknown whether the MDA5 C-terminal region is phosphorylated. Here, we demonstrated that not only N-terminal CARDs but also C-terminal region of MDA5 are phosphorylated. We identified RIOK3 as a protein kinase required for phosphorylation of the MDA5 C-terminal region and revealed that phosphorylation regulates MDA5 multimer formation and attenuates the innate immune response.

RESULTS

A Protein Kinase, RIOK3, Is Involved in MDA5 C-Terminal Phosphorylation

The MDA5 protein undergoes phosphorylation, which suppresses its activation (Wies et al., 2013). To detect MDA5 phosphorylation, we performed phosphate-affinity SDS-PAGE using a Phos-tag gel, wherein phosphorylated proteins migrate more slowly than non-phosphorylated proteins (Kinoshita et al., 2009). The FLAG-tagged MDA5 protein migrated more slowly than the CIAP-treated protein (Figure 1A). The CIAP treatment also increased the mobility of the endogenous MDA5 protein band (Figure 1B). Interestingly, the CIAP treatment increased the mobility of an MDA5 C-terminal fragment (731–1,025 aa) (Figure 1C), suggesting phosphorylation of the MDA5 C-terminal region.

To isolate the protein kinase targeting the C-terminal region, we conducted yeast two-hybrid screening using an MDA5



(legend on next page)

C-terminal fragment and isolated cDNA clones encoding RIOK3, which contains a RIO kinase domain (Figure 1D). One of the isolated cDNA clones encoded the protein that lacks a part of the RIO kinase domain (RIOK3Δ), which is thought to be an alternatively spliced form lacking kinase activity (Figures S1A and S1B). Furthermore, to corroborate the physical interaction, we conducted immunoprecipitation assays. Ectopically expressed HA-tagged RIOK3 was co-immunoprecipitated with the full-length and C-terminal MDA5 fragments, even in the absence of the MDA5 ligand (Figures 1E and 1F). The RIOK3Δ protein also bound to MDA5 (Figure S1C). Endogenous MDA5, but not RIG-I, co-immunoprecipitated with HA-tagged RIOK3 before and after polyI:C stimulation (Figure 1G). These data indicate that the RIOK3 protein binds to the MDA5 C-terminal region (731–1,025 aa; Figure S1D).

RIOK3 mRNA was highly expressed in HEK293 and HeLa cells compared with THP1 cells (Figure 1H), which were not induced by polyI:C stimulation or MV infection (Figures 1I and S1E). MV infection moderately increased the endogenous RIOK3 protein level in BJ cells, which are human primary fibroblasts (Figure 1J). The alternatively spliced form of RIOK3Δ was expressed at a lower level in all cell lines compared with that of full-length RIOK3 (Figures 1H and 1I), and the endogenous RIOK3Δ protein was barely detected (Figure S1F). A confocal microscopic analysis demonstrated that the RIOK3 protein was localized in the cytoplasm before and after polyI:C stimulation and was partially colocalized with MDA5, ER, and stress granules, but not with mitochondria (Figure 1K). We confirmed that polyI:C was detected in the cytoplasm after polyI:C transfection under our experimental conditions (Figure S1G).

Next, we investigated whether RIOK3 is involved in MDA5 phosphorylation. Ectopically expressed RIOK3 delayed mobility of the MDA5 protein (Figure 1L), and *RIOK3* knockdown increased MDA5 mobility (Figure 1M). We also found that RIOK3 expression induced phosphorylation of the MDA5 C-terminal fragment (as described below). PP1γ phosphatase expression increased the migration of the MDA5-C-terminal fragment (Figure 1N) and augmented MDA5-S88A signaling (Figure S1H), suggesting that PP1γ mediates dephosphorylation of MDA5 not only at Ser-88 but also at other Ser/Thr residues. Taken together, our data indicated that RIOK3 phosphorylates the MDA5 C-terminal region.

RIOK3 Is a Negative Regulator of MDA5

Next, we investigated the role of RIOK3 in MDA5 signaling. We found that RIOK3 overexpression reduced the activation of the MDA5-mediated IFN-β promoter, but not that of the MAVS-mediated IFN-β promoter (Figures 2A and 2B). RIOK3Δ failed to reduce the activation of the MDA5-mediated promoter (Figure 2C), suggesting the importance of the kinase domain. Conversely, *RIOK3* knockdown increased polyI:C-induced IFN-β mRNA expression (Figure 2D). To further corroborate the role of RIOK3 in MDA5 signaling, we generated *RIOK3* knockout (KO) cells using the CRISPR method, wherein endogenous *RIOK3* harbored a frame-shift mutation (Figures S2A and S2B). Western blotting confirmed that the endogenous RIOK3 protein was not detected in *RIOK3* KO cells (Figure 2E). As expected, *RIOK3* KO increased the activation of the polyI:C-induced IFN-β promoter (Figure 2F), which was suppressed by RIOK3 ectopic expression, suggesting complementation of the defect

Figure 1. RIOK3, a Protein Kinase, Phosphorylates the MDA5 C-Terminal Fragment

(A) Cell lysates were prepared from HEK293FT cells expressing FLAG-tagged MDA5. The MDA5 protein was immunoprecipitated with an anti-FLAG antibody and, subsequently, was either left untreated or treated with CIAP. The proteins were subjected to Phos-tag SDS-PAGE and detected by western blotting.

(B) Endogenous MDA5 protein from unstimulated HEK293FT cells was immunoprecipitated with an anti-MDA5 antibody and treated with or without CIAP. The proteins were subsequently subjected to Phos-tag SDS-PAGE and detected by western blotting.

(C) Schematic representation of the MDA5 C-terminal fragment (MDA5-C) (upper panel). FLAG-tagged MDA5-C from HEK293FT cells was immunoprecipitated with an anti-FLAG antibody and treated with or without CIAP. The proteins were subsequently subjected to Phos-tag SDS-PAGE and detected by western blotting (lower panel).

(D) Yeast two-hybrid analysis of the association between RIOK3 and MDA5. Yeast cells with MDA5-C and RIOK3 grew on a selective plate (SD-WLHA), whereas cells with MDA5-C alone did not.

(E and F) FLAG-tagged MDA5-, FLAG-tagged MDA5-C-, and/or HA-tagged RIOK3-expressing vectors were transfected into HEK293FT cells for 24 hr, and cell lysates were then prepared. The proteins were immunoprecipitated with an anti-FLAG antibody and subjected to SDS-PAGE. The proteins were detected by western blotting.

(G) The RIOK3-HA-expressing vector was transfected into HEK293FT cells. 24 hr after transfection, cells were stimulated with polyI:C for 0 or 6 hr. The immunoprecipitation assay was performed with an anti-HA antibody, and the proteins were detected by western blotting with anti-RIG-I, anti-MDA5, and anti-HA antibodies.

(H) Total RNA was extracted from HEK293, HeLa, and THP-1 cells, and RIOK3 and RIOK3Δ expression were determined by qRT-PCR. Data are presented as mean ± SD.

(I and J) BJ cells were infected with MV at MOI = 1. RIOK3 and RIOK3Δ mRNA levels were determined by qRT-PCR (I). Data are presented as mean ± SD. Cell lysates were prepared from MV-infected BJ cells, and the endogenous RIOK3 protein level was detected by western blotting (J).

(K) FLAG-MDA5 and/or RIOK3-HA were transiently expressed in HeLa cells. The cells were either mock or stimulated with polyI:C for 4 hr and were subsequently fixed and stained with anti-HA, anti-FLAG, anti-calnexin, anti-RIOK3, antibodies, and/or MitoTracker Red. "RIOK3-HA" represents ectopically expressed RIOK3-HA, and "RIOK3" represents endogenous RIOK3. The scale bar represents 10 μm.

(L) FLAG-MDA5 and RIOK3-HA were transiently expressed in HEK293FT cells and immunoprecipitated with anti-FLAG antibody. The proteins were subjected to Phos-tag SDS-PAGE and detected by western blotting.

(M) siRNA targeted to *RIOK3* (in addition to a negative control) was transfected into HEK293 cells for 48 hr, and cell lysates were prepared. Endogenous MDA5 protein was immunoprecipitated with anti-MDA5 antibody, subjected to Phos-tag SDS-PAGE, and then detected by western blotting.

(N) FLAG-MDA5-C and PP1γ were transiently expressed in HEK293FT cells and immunoprecipitated with anti-FLAG antibody. The CIAP-treated and untreated proteins were analyzed by Phos-tag SDS-PAGE and detected by western blotting.

See also Figure S1.

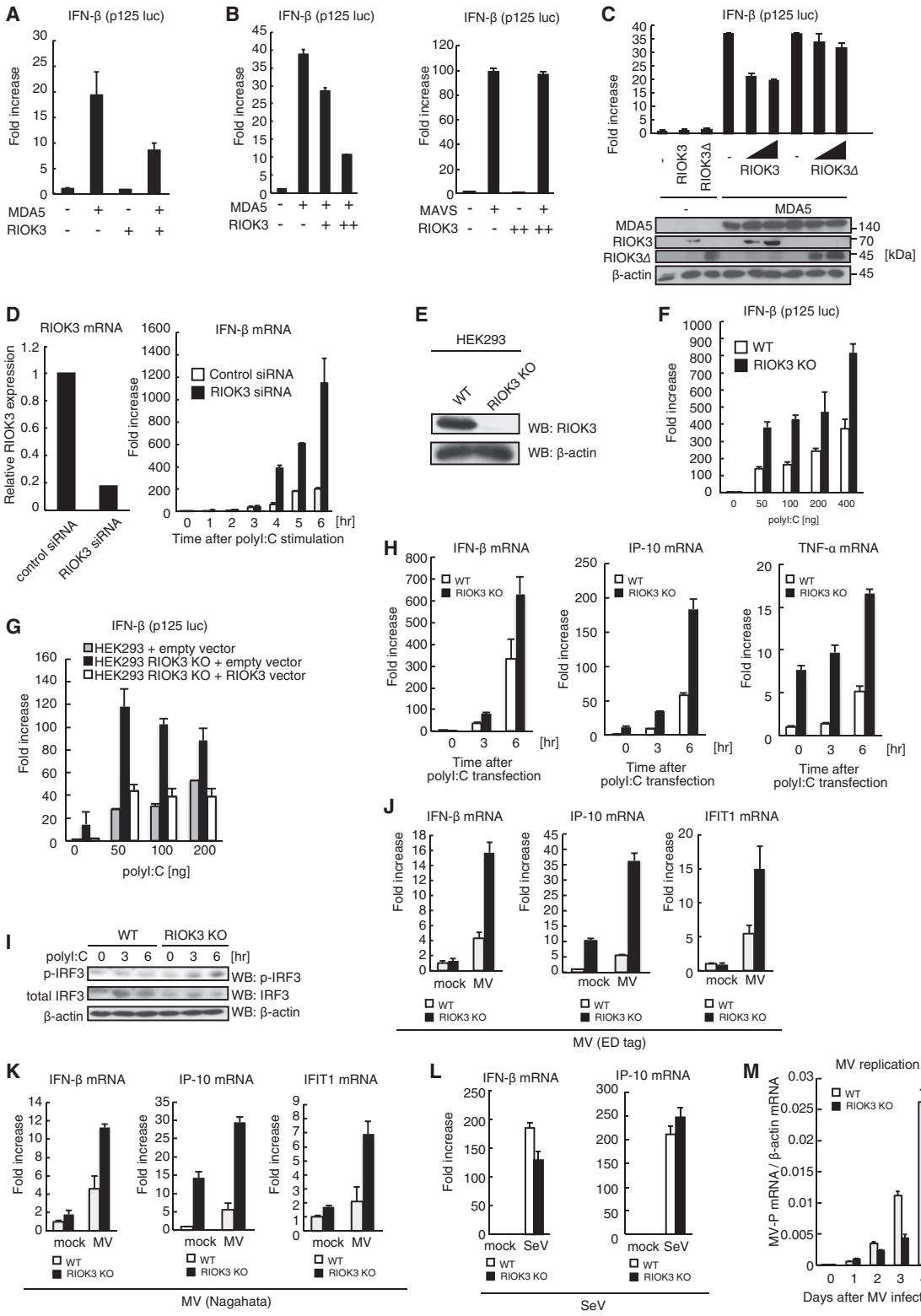


Figure 2. RIOK3 Attenuates MDA5 Signaling

(A–C) MDA5-, MAVS-, RIOK3-, and/or RIOK3Δ-expressing vectors were transfected into HEK293 cells together with p125 luc and *Renilla* luciferase vectors for 24 hr, and the luciferase activities were determined. Immunoblots of cell lysates are shown in the lower (C).

(legend continued on next page)

(Figure 2G). *RIOK3* KO increased the expression of IP-10 and TNF-α mRNAs, even in the resting state (Figure 2H), and polyI:C-induced expression of IFN-β, IP-10, and TNF-α mRNAs was augmented by *RIOK3* KO (Figures 2H and S2C). Moreover, *RIOK3* KO enhanced IRF-3 phosphorylation induced by polyI:C stimulation (Figure 2I). These data indicate that RIOK3 negatively regulates MDA5 activation.

Next, we investigated the virus-induced type I IFN and IFN-inducible gene expression. MV is a virus recognized by MDA5. Although wild-type MV minimally induces the production of type I IFN, several laboratory-adapted or vaccine strains facilitate its induction (Naniche et al., 2000; Shingai et al., 2007). *RIOK3* KO increased MV EDtag- and Nagahata-induced expression of IFN-β, IFIT1, and IP-10 mRNAs (Figures 2J and 2K) but failed to increase IFN-β and IP-10 expression by SeV infection, which is recognized by RIG-I, but not MDA5 (Figure 2L). Conversely, the replication of MV was suppressed by *RIOK3* KO (Figure 2M). Taken together, these data indicate that RIOK3 negatively regulates MDA5 signaling before and after viral infection.

RIOK3 Phosphorylates MDA5 Ser-828

RIOK3 contains a Ser/Thr kinase domain. Thus, to determine the phosphorylated Ser/Thr residue that is essential for RIOK3-mediated suppression of MDA5 signaling, we replaced all Ser/Thr residues with Ala within the MDA5 C-terminal region (821–1,025 aa; Figures 3A and 3B). Substitution at the RIOK3 phosphorylation site was expected to increase MDA5-signaling activity, because RIOK3-mediated phosphorylation attenuated MDA5 signaling. A S828A amino acid substitution robustly enhanced MDA5 signaling, and a T829A mutation moderately increased MDA5 signaling (Figure 3A). No other substitutions enhanced MDA5 signaling (Figure 3A). Next, we investigated whether a phosphomimetic mutation would attenuate MDA5 signaling. The phosphomimetic S828D mutation severely reduced MDA5-mediated signaling (Figures 3B–3D), whereas T829D mutation caused only a marginal effect (Figure 3C). Furthermore, MDA5-S828A-mediated signaling was not attenuated by the RIOK3 expression (Figure 3E) and RIOK3 expression failed to increase mobility of the RIOK3-S828A protein on a Phos-tag gel (Figure S3A). These data suggested that RIOK3 mediates phosphorylation of MDA5 at Ser-828.

To further corroborate phosphorylation of Ser-828, we constructed a MDA5 C-terminal fragment (MDA5-C-A18), wherein all Ser/Thr residues within 821–1,025 aa were replaced with Ala (Figure S3B). Phosphorylation of MDA5-C-A18 fragment was hardly detected (Figure 3F), indicating that the Ser/Thr residues within 821–1,025 aa are phosphorylated. Next, we constructed another MDA5 C-terminal fragment (MDA5-C-S828-A17), wherein all Ser/Thr residues except Ser-828 were replaced with Ala (Figure S3B). RIOK3 expression induced phosphorylation of the MDA5-C-S828-A17 fragment (Figures 3G and 3H), and phosphorylation of the fragment was reduced by polyI:C stimulation (Figure 3I). The in vitro kinase assay revealed that immunoprecipitated RIOK3, but not RIOK3Δ, induced phosphorylation of the in-vitro-translated MDA5-C-S828-A17 fragment (Figure S3C). Collectively, our data indicate that RIOK3 phosphorylates MDA5 Ser-828, leading to attenuated MDA5 signaling. Unlike the MDA5-C fragment, only part of the MDA5-C-S828-A17 fragments was phosphorylated; and thus, we do not exclude the possibility that other Ser/Thr residues within the 821–1,025 aa region were also phosphorylated by RIOK3 or other kinases.

C-Terminal Phosphorylation Interferes with MDA5 Assembly

The molecular mechanism for enhanced MDA5-mediated signaling by LGP2 has been reported (Bruns et al., 2014). We investigated whether an amino acid substitution at MDA5 Ser-828 would affect the association between MDA5 and LGP2; however, the S828A and S828D mutations caused only a marginal effect on the association (Figure 4A).

RIG-I forms homomultimers following stimulation with a RIG-I ligand as detected by native-PAGE (Saito et al., 2007). MDA5 also forms homomultimers along dsRNA, which is essential for creating MDA5-dsRNA filament (Wu et al., 2013). We investigated MDA5 multimer formation by native PAGE. Multimerization of ectopically expressed FLAG-MDA5 was detected in resting and polyI:C-stimulated cells (Figure 4B). The S828A mutation promoted FLAG-MDA5 multimer formation (Figures 4B and 4C). Conversely, the S828D mutation severely reduced the MDA5 multimer before and after polyI:C stimulation (Figures 4B–4E). Moreover, similar to the S828A mutation, *RIOK3* KO increased FLAG-MDA5 multimerization in both polyI:C-stimulated and unstimulated cells (Figures 4F and 4G).

Next, we investigated endogenous MDA5 multimerization. The endogenous MDA5 multimer was barely detected in resting cells but was detected after polyI:C stimulation (Figure 4H). *RIOK3* KO increased multimer formation of the endogenous MDA5 in mock and polyI:C-stimulated cells (Figures 4H and 4I). Taken together, our data indicate that RIOK3-mediated phosphorylation impairs MDA5 multimer formation, resulting in attenuated MDA5 signaling.

(D) siRNA for negative control or *RIOK3* was transfected into HEK293 cells for 48 hr. Cells were stimulated by polyI:C transfection, and total RNA was extracted at the indicated times following stimulation. RIOK3 and IFN-β mRNA expression levels were determined by qRT-PCR and normalized to β-actin.
(E) Immunoblots of cell lysates of WT and *RIOK3* KO HEK293 cells.
(F) WT and *RIOK3* KO HEK293 cells were transfected with p125 luc and *Renilla* luciferase vector for 24 hr and then stimulated by polyI:C transfection.
(G) An empty or a RIOK3-expressing vector was transfected into WT and *RIOK3* KO HEK293 cells together with p125 luc and *Renilla* luciferase vectors for 24 hr and then stimulated by polyI:C transfection.
(H and I) WT and *RIOK3* KO HEK293 cells were stimulated with polyI:C. IFN-β, IP-10, and TNF-α mRNA expression levels were determined by qRT-PCR and normalized to β-actin (H). Cell lysates were prepared and subjected to SDS-PAGE, and the proteins were detected by western blotting (I).
(J–L) WT and *RIOK3* KO HEK293 cells were infected with MV Ed-tag or Nagahata strain for 24 hr at an MOI = 1 (Ed-tag) or 0.1 (Nagahata) (J and K) or infected with SeV (L). IFN-β, IP-10, and IFIT1 mRNA expression levels were determined by qRT-PCR and normalized to β-actin.
(M) Total RNAs of WT and RIOK3 KO HEK293 cells were extracted on the indicated day after MV infection at MOI = 0.001. MV RNA levels were determined by qRT-PCR using PCR primers for the MV P-encoding region and normalized to β-actin.
Data are presented as mean ± SD. See also Figure S2.

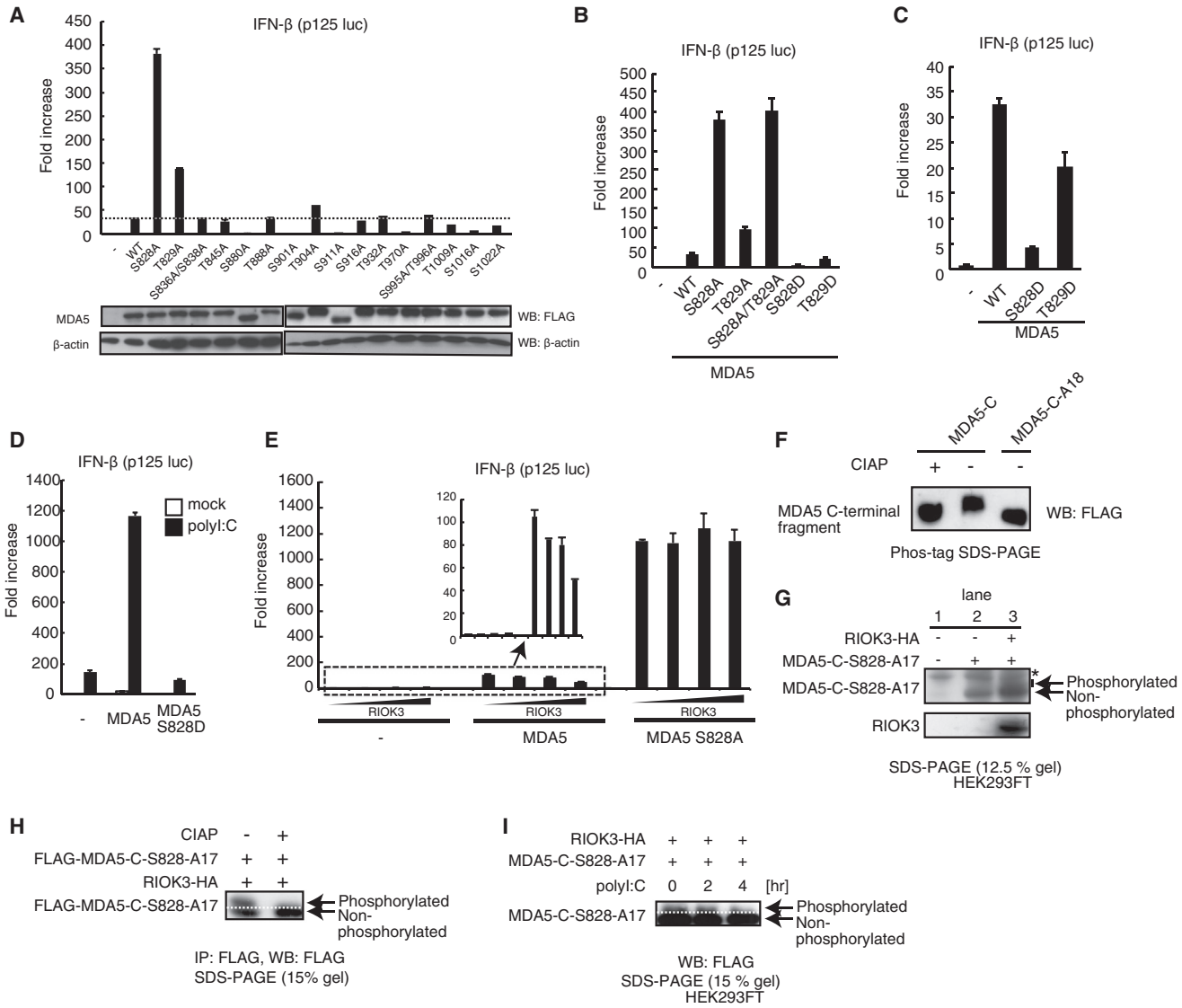


Figure 3. RIOK3 Phosphorylates Ser-828 within the MDA5 C-Terminal Region

(A–C) The mutant MDA5-protein-expressing vectors were transfected into HEK293 cells together with the p125luc and *Renilla* luciferase vectors for 24 hr, and luciferase activities were determined (right panel). Immunoblots of cell lysates are shown in the lower (A).

(D) MDA5 (WT) or the MDA5-S828D-mutant-expressing vector was transfected with p125 luc and *Renilla* luciferase vector for 24 hr and then stimulated with mock or 100 ng of polyI:C transfection for 24 hr, and the luciferase activities were measured.

(E) MDA5-, MDA5-S828A-, and/or RIOK3-expressing vectors were transfected into HEK293 cells together with the p125 luc and *Renilla* luciferase vectors for 24 hr, and then luciferase activities were determined.

(F) FLAG-tagged MDA5-C or MDA5-C-A18 was transiently expressed in HEK293FT cells and immunoprecipitated with anti-FLAG antibodies. The proteins were treated with or without CIAP and subjected to Phos-tag SDS-PAGE.

(G–I) FLAG-MDA5-C-S828-A17 and/or RIOK3 were transiently expressed in HEK293FT cells. Cells were unstimulated (G and H) or stimulated with polyI:C (I). Lane 3 sample in (G) was immunoprecipitated with anti-FLAG antibody and subsequently treated with or without CIAP (H). The immunoprecipitates and cell lysates were subjected to normal SDS-PAGE, and the proteins were detected by western blotting.

Data are presented as mean ± SD. See also Figure S3.

DISCUSSION

Gack and colleagues first reported that the MDA5 protein is tightly regulated by phosphorylation and that dephosphorylation at Ser-88 is critical for activating MDA5 signaling (Wies et al., 2013). Recent studies have revealed that the MV inhibits dephosphorylation of Ser-88 to escape the host innate immune response (Davis et al., 2014; Mesman et al., 2014). Here, we demonstrated that RIOK3 mediates phosphorylation of MDA5 at Ser-828, resulting in attenuated MDA5 signaling. These

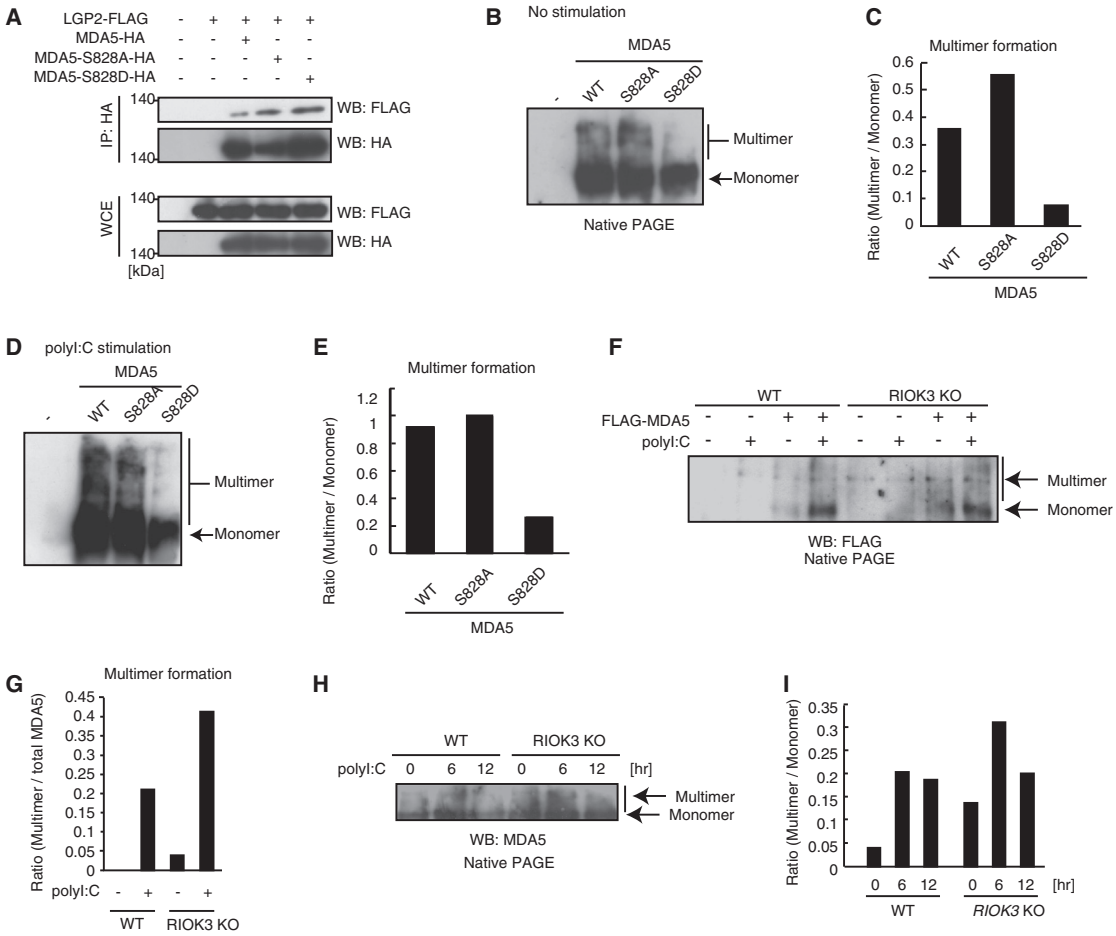


Figure 4. RIOK3 Disrupts MDA5 Multimer Formation

(A) MDA5-S828A-HA, MDA5-S828D-HA, MDA5-HA, and/or LGP2-FLAG were expressed in HEK293FT cells, and immunoprecipitation was conducted with anti-HA antibodies. Proteins were subjected to SDS-PAGE and detected by western blotting.

(B–E) HEK293FT cells were transfected with FLAG-tagged MDA5-, MDA5-S828A-, and MDA5-S828D-expressing vectors for 24 hr. The cells were unstimulated (B) or stimulated with 400 ng of polyI:C (D) for 12 hr. Cell lysates were prepared and analyzed by native-PAGE, and the proteins were subsequently detected by western blotting with an anti-FLAG antibody. Band intensities of (B) and (D) were semi-quantified and are shown in (C) and (E), respectively.

(F and G) WT and *RIOK3* KO HEK293 cells were transfected with FLAG-tagged MDA5 for 24 hr and then stimulated with polyI:C for 12 hr. Cell lysates were prepared and subsequently analyzed by native-PAGE. The proteins were detected by western blotting with an anti-FLAG antibody (F). Band intensities were semi-quantified (G).

(H and I) WT and *RIOK3* KO HEK293 cells were stimulated with polyI:C. Cell lysates were prepared and subjected to native-PAGE. Proteins were detected by western blotting with anti-MDA5 antibody (H). Band intensities were semi-quantified (I).

See also Figure S4.

observations indicate that both N- and C-terminal phosphorylation events are critical for regulating MDA5 activity. Another RLRs member, RIG-I, is also regulated by both N- and C-terminal modifications (Loo and Gale, 2011; Oshiumi et al., 2013).

RIOK3 or a phosphomimetic mutation at Ser-828 abolished MDA5 multimer formation and attenuated type I IFN expression. MDA5 assembles tightly packed filaments on dsRNA, wherein contact with adjacent MDA5 monomers is required for stabilizing the RNA-bound monomers and for cooperative filament assembly (Wu et al., 2013). Therefore, we propose that RIOK3-mediated MDA5 Ser-828 phosphorylation interferes with MDA5 assembly on dsRNA, thus disrupting MDA5-dsRNA filament formation, which attenuates MDA5 signaling (Figure S4C). MDA5 Ser-88 phosphorylation suppresses MDA5-MAVS binding and downstream signaling (Davis et al., 2014). MDA5 Ser-88 phosphorylation may interfere with MDA5 2CARDs oligomerization or the MDA5 CARDs-MAVS association.

Ectopic MDA5 expression activates its signaling without the ligand, and endogenous MDA5 multimerization in resting cells was increased by *RIOK3* KO. These observations suggest that RIOK3 suppresses abnormal activation of MDA5 in resting cells. MDA5 G821S mutation also activates its signaling, even in the absence of the ligand, and causes lupus-like autoimmune symptoms (Funabiki et al., 2014). Considering that the G821S mutation is close to Ser-828, this mutation may inhibit RIOK3-mediated MDA5 phosphorylation required for

inhibiting abnormal multimer formation without dsRNA in resting cells.

In general, a protein kinase targets several proteins. We revealed a crucial role of RIOK3 for regulating MDA5 activation; however, previous studies have shown that RIOK3 also associates with caspase-10, TBK1, and non-ribosomal pre-40S particle components (Baumas et al., 2012; Feng et al., 2014; Shan et al., 2009). Our microscopic study showed diffuse cytoplasmic localization of RIOK3 before and after stimulation. Therefore, RIOK3 appears to target several cytoplasmic proteins; thus, it is likely to be involved in several cytoplasmic phenomena.

Robust cytokine production can be harmful. TLR3-mediated production of inflammatory cytokines leads to the breakdown of the blood-brain barrier, resulting in an increased mortality rate upon West Nile virus infection (Wang et al., 2004), and the abnormal activation of MDA5 signaling in a resting state can develop an autoimmune disease (Funabiki et al., 2014). KO mouse studies are required to reveal the in vivo role of RIOK3 in host protection against viral infections and autoimmune diseases.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Reagents

HEK293 cells were cultured in DMEM low-glucose medium with 10% heat-inactivated fetal calf serum (FCS). HEK293FT and HeLa cells were cultured in DMEM high-glucose medium with 10% FCS and minimum Eagle's medium with 2 mM L-Gln and 10% FCS, respectively. The MV Edmonston (ED tag) and Nagahata strains were amplified in Vero cells expression SLAM, and viral titers were determined by plaque assay. The yeast AH109 strain was purchased from Clontech.

Phosphate-Affinity SDS-PAGE

Phos-tag SDS-PAGE was performed with 5.0%–7.5% of polyacrylamide gels containing 50–75 μM of Phos-tag acrylamide (Wako) and 100–150 μM $MnCl_2$. Following electrophoresis, Phos-tag acrylamide gels were washed with 10 mM EDTA-blotting buffer (25 mM Tris-HCl [pH 7.5], 192 mM glycine, 20% methanol, and 10 mM EDTA) with shaking for 10 min three times and then with blotting buffer (25 mM Tris-HCl [pH 7.5], 192 mM glycine, and 20% methanol) without EDTA for 10 min. The proteins were subsequently detected by western blotting.

Native-PAGE

Cell lysates were prepared from approximately 1×10^6 cells using lysis buffer (50 mM Tris-HCl [pH 8.0], 1% NP40, 150 mM NaCl, 1 mM PMSF, and 5 mM orthovanadate). The lysate was suspended in 2× native PAGE sample buffer (125 mM Tris-HCl [pH 6.8], 30% glycerol, and BPB). The proteins were separated using native-polyacrylamide gels (Bio-Rad) and immunoblotted with an anti-FLAG or MDA5 antibody. The band intensities were semi-quantitated using the Photoshop histogram tool.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.027.

AUTHOR CONTRIBUTIONS

H.O. and H.T. amplified MV Edtag and Nagahata strains. K.T. and H.O. performed the experiments. H.O., K.T., and T.S. designed the experiments. M.M and T.S. supervised the research. H.O. wrote the manuscript.

ACKNOWLEDGMENTS

We thank our laboratory members for helpful discussions. This work was supported in part by Kato Memorial Bioscience Foundation and Grants-in-Aid from Ministry of Education, Science, and Culture of Japan and Ministry of Health, Labor, and Welfare of Japan.

Received: September 25, 2014
Revised: January 23, 2015
Accepted: March 11, 2015
Published: April 9, 2015

REFERENCES

Abe, Y., Fujii, K., Nagata, N., Takeuchi, O., Akira, S., Oshiumi, H., Matsumoto, M., Seya, T., and Koike, S. (2012). The toll-like receptor 3-mediated antiviral response is important for protection against poliovirus infection in poliovirus receptor transgenic mice. J. Virol. *86*, 185–194.

Baumas, K., Soudet, J., Caizergues-Ferrer, M., Faubladier, M., Henry, Y., and Mougin, A. (2012). Human RioK3 is a novel component of cytoplasmic pre-40S pre-ribosomal particles. RNA Biol. *9*, 162–174.

Bruns, A.M., Leser, G.P., Lamb, R.A., and Horvath, C.M. (2014). The innate immune sensor LGP2 activates antiviral signaling by regulating MDA5-RNA interaction and filament assembly. Mol. Cell *55*, 771–781.

Davis, M.E., Wang, M.K., Rennick, L.J., Full, F., Gableske, S., Mesman, A.W., Gringhuis, S.I., Geijtenbeek, T.B., Duprex, W.P., and Gack, M.U. (2014). Antagonism of the phosphatase PP1 by the measles virus V protein is required for innate immune escape of MDA5. Cell Host Microbe *16*, 19–30.

Feng, J., De Jesus, P.D., Su, V., Han, S., Gong, D., Wu, N.C., Tian, Y., Li, X., Wu, T.T., Chanda, S.K., and Sun, R. (2014). RIOK3 is an adaptor protein required for IRF3-mediated antiviral type I interferon production. J. Virol. *88*, 7987–7997.

Funabiki, M., Kato, H., Miyachi, Y., Toki, H., Motegi, H., Inoue, M., Minowa, O., Yoshida, A., Deguchi, K., Sato, H., et al. (2014). Autoimmune disorders associated with gain of function of the intracellular sensor MDA5. Immunity *40*, 199–212.

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T.S., Fujita, T., and Akira, S. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J. Exp. Med. *205*, 1601–1610.

Kinoshita, E., Kinoshita-Kikuta, E., and Koike, T. (2009). Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. Nat. Protoc. *4*, 1513–1521.

Loo, Y.M., and Gale, M., Jr. (2011). Immune signaling by RIG-I-like receptors. Immunity *34*, 680–692.

Mesman, A.W., Zijlstra-Willems, E.M., Kaptein, T.M., de Swart, R.L., Davis, M.E., Ludlow, M., Duprex, W.P., Gack, M.U., Gringhuis, S.I., and Geijtenbeek, T.B. (2014). Measles virus suppresses RIG-I-like receptor activation in dendritic cells via DC-SIGN-mediated inhibition of PP1 phosphatases. Cell Host Microbe *16*, 31–42.

Motz, C., Schuhmann, K.M., Kirchhofer, A., Moldt, M., Witte, G., Conzelmann, K.K., and Hopfner, K.P. (2013). Paramyxovirus V proteins disrupt the fold of the RNA sensor MDA5 to inhibit antiviral signaling. Science *339*, 690–693.

Naniche, D., Yeh, A., Eto, D., Manchester, M., Friedman, R.M., and Oldstone, M.B. (2000). Evasion of host defenses by measles virus: wild-type measles virus infection interferes with induction of Alpha/Beta interferon production. J. Virol. *74*, 7478–7484.

Oshiumi, H., Miyashita, M., Matsumoto, M., and Seya, T. (2013). A distinct role of Riplet-mediated K63-Linked polyubiquitination of the RIG-I repressor domain in human antiviral innate immune responses. PLoS Pathog. *9*, e1003533.

Saito, T., Hirai, R., Loo, Y.M., Owen, D., Johnson, C.L., Sinha, S.C., Akira, S., Fujita, T., and Gale, M., Jr. (2007). Regulation of innate antiviral defenses

through a shared repressor domain in RIG-I and LGP2. Proc. Natl. Acad. Sci. USA *104*, 582–587.

Shan, J., Wang, P., Zhou, J., Wu, D., Shi, H., and Huo, K. (2009). RIOK3 interacts with caspase-10 and negatively regulates the NF-kappaB signaling pathway. Mol. Cell. Biochem. *332*, 113–120.

Shingai, M., Ebihara, T., Begum, N.A., Kato, A., Honma, T., Matsumoto, K., Saito, H., Ogura, H., Matsumoto, M., and Seya, T. (2007). Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. J. Immunol. *179*, 6123–6133.

Takaki, H., Watanabe, Y., Shingai, M., Oshiumi, H., Matsumoto, M., and Seya, T. (2011). Strain-to-strain difference of V protein of measles virus affects MDA5-mediated IFN-β-inducing potential. Mol. Immunol. *48*, 497–504.

Wang, T., Town, T., Alexopoulou, L., Anderson, J.F., Fikrig, E., and Flavell, R.A. (2004). Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat. Med. *10*, 1366–1373.

Wies, E., Wang, M.K., Maharaj, N.P., Chen, K., Zhou, S., Finberg, R.W., and Gack, M.U. (2013). Dephosphorylation of the RNA sensors RIG-I and MDA5 by the phosphatase PP1 is essential for innate immune signaling. Immunity *38*, 437–449.

Wu, B., Peisley, A., Richards, C., Yao, H., Zeng, X., Lin, C., Chu, F., Walz, T., and Hur, S. (2013). Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. Cell *152*, 276–289.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.M., Gale, M., Jr., Akira, S., et al. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. *175*, 2851–2858.