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A Distinct Role of Riplet-Mediated K63-Linked Polyubiquitination of the RIG-I Repressor Domain in Human Antiviral Innate Immune Responses

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Abstract

The innate immune system is essential for controlling viral infections, but several viruses have evolved strategies to escape innate immunity. RIG-I is a cytoplasmic viral RNA sensor that triggers the signal to induce type I interferon production in response to viral infection. RIG-I activation is regulated by the K63-linked polyubiquitin chain mediated by Riplet and TRIM25 ubiquitin ligases. TRIM25 is required for RIG-I oligomerization and interaction with the IPS-1 adaptor molecule. A knockout study revealed that Riplet was essential for RIG-I activation. However, the molecular mechanism underlying RIG-I activation by Riplet remains unclear, and the functional differences between Riplet and TRIM25 are also unknown. A genetic study and a pull-down assay indicated that Riplet was dispensable for RIG-I RNA binding activity but required for TRIM25 to activate RIG-I. Mutational analysis demonstrated that Lys-788 within the RIG-I repressor domain was critical for Riplet-mediated K63-linked polyubiquitination and that Riplet was required for the release of RIG-I autorepression of its N-terminal CARDs, which leads to the association of RIG-I with TRIM25 ubiquitin ligase and TBK1 protein kinase. Our data indicate that Riplet is a prerequisite for TRIM25 to activate RIG-I signaling. We investigated the biological importance of this mechanism in human cells and found that hepatitis C virus (HCV) abrogated this mechanism. Interestingly, HCV NS3-4A proteases targeted the Riplet protein and abrogated endogenous RIG-I polyubiquitination and association with TRIM25 and TBK1, emphasizing the biological importance of this mechanism in human antiviral innate immunity. In conclusion, our results establish that Riplet-mediated K63-linked polyubiquitination released RIG-I RD autorepression, which allowed the access of positive factors to the RIG-I protein.
mediated type I IFN production in response to vesicular stomatitis virus (VSV), Flu, and Sendai virus (SeV) infections [23]. However, the functional difference between Riplet and TRIM25 remains unclear, and the molecular mechanism of how Riplet-mediated RIG-I ubiquitination activates RIG-I signaling remains unresolved.

Hepatitis C virus (HCV) is a major cause of hepatocellular carcinoma (HCC) worldwide. HCV RNA is primarily recognized by RIG-I in vitro and in vivo [24]. The HCV protease NS3-4A can suppress type I IFN production [25]. NS3-4A cleaves IPS-1 to suppress RIG-I-mediated innate immune responses [7,26]. Human monocyte-derived dendritic cells recognize HCV RNA through Toll-like receptor 3, and NS3-4A has the ability to cleave TICAM-1, which is a solo adaptor molecule of Toll-like receptor 3 [27,28]. In this study, we found that Riplet was another target of those proteins (Figure 2D and 2F). Recently, it was reported that RIG-I and TRIM25 in perinuclear region in VSV infected cells (Figure 2A–2C and 2E). This is consistent with a previous observation that Riplet and TRIM25 co-localized extensively at cytoplasmic perinuclear bodies [15]. Pearson’s correlation coefficient values also suggested the correlation of the colocalizations of those proteins (Figure 2D and 2F). Recently, it was reported that Riplet recognized short polyIC in stress granules [30]. G3BP is a marker of the stress granule [30]. In resting cells, G3BP dispersed in cytoplasm [30], and thus barely detectable (Figure 2G and 2H), whereas G3BP speckles were detected in cells stimulated with short polyIC in the cytoplasm (Figure 2G and 2H). Riplet and TRIM25 localizations within G3BP speckles were detected in the stimulated cells (Figure 2G and 2H). Taken together, these data indicated that both Riplet and TRIM25 associated with RIG-I after stimulation.

Next, we assessed the RIG-I regions that bind to the two ubiquitin ligases using RIG-I fragments. As previously reported, Riplet bound to RIG-I CARDs fragment (Figure 2I). However, Riplet bound to RIG-I RD (735–925 aa), but not to CARDs fragment (Figure 2J). Deleting the Riplet binding region (RIG-I ΔCTD) abrogated Riplet effect on RIG-I signaling (Figure 2K). This was contrast to TRIM25, which affects RIG-I CARDs. Taken together, our genetic and biochemical data indicated that the two ubiquitin ligases associated with RIG-I after stimulation but showed different effects on RIG-I activation. Thus, we next focused on Riplet specific role in RIG-I activation.

Riplet-mediated RIG-I polyubiquitination is dispensable for RIG-I RNA binding activity

RIG-I CARDs harbor K63-linked polyubiquitination [15]. As RIG-I CARDs, RIG-I RD harbored K63-linked polyubiquitination (Supplemental Figure S1). Riplet expression increased the polyubiquitination of RIG-I RD but not that of CARDs (Figure 3A). RIG-I RD has two functions. One is RNA binding activity and the other is autorepression of its CARDs signaling. Firstly, we tested whether Riplet affects RIG-I RNA binding activity. In a pull-down assay using biotin-conjugated dsRNA and streptavidin beads, we found that both polyubiquitinated and non-ubiquitinated RIG-I were recovered (Figure 3B), which suggested that Riplet-mediated polyubiquitination was dispensable for RIG-I

**Author Summary**

The cytoplasmic viral RNA sensor RIG-I recognizes various types of pathogenic viruses and evokes innate immune responses, whereas several viruses have evolved strategies to escape the host innate immune responses. RIG-I triggers a signal to induce type I interferon and inflammatory cytokines. RIG-I activation is regulated by K63-linked polyubiquitin chains mediated by the ubiquitin ligases TRIM25 and Riplet; however, the functional difference between the two ubiquitin ligases remains unclear, and the molecular mechanism underlying Riplet-mediated RIG-I activation is unknown. We revealed sequential roles of the two ubiquitin ligases during RIG-I activation and found that Riplet-mediated polyubiquitination of the RIG-I repressor domain released RIG-I autorepression of its N-terminal CARDs responsible for triggering the signal, which resulted in an association with TRIM25 ubiquitin ligase and Tbk1 protein kinase. Interestingly, we found that this mechanism was targeted by hepatitis C virus, which is a major cause of hepatocellular carcinoma. This result emphasizes the vital role of Riplet-mediated release of RIG-I RD autorepression in antiviral responses. Our results establish that Riplet releases RIG-I RD autorepression and demonstrated the biological significance of this mechanism in human innate immune responses.
RNA binding activity. As RIG-I is known to form homo-oligomers [3], it is possible that non-ubiquitinated RIG-I was recovered through ubiquitinated RIG-I by the pull-down assay. To exclude this possibility, we used RIG-I mutants, RIG-I 5KR and RIG-I K788R, which were barely ubiquitinated by Riplet (described below). We compared the binding abilities of the RIG-I mutants to that of wild-type RIG-I by pull-down assay using cell lysate isolated from cells without stimulation with RIG-I ligand to avoid any ubiquitination. The results showed that the RIG-I 5KR and RIG-I K788R mutant proteins were recovered by pull down assay as wild-type RIG-I (Figure 3C). This data also indicated that the polyubiquitination is dispensable for RIG-I RNA binding activity.

Riplet releases RIG-I RD autorepression of CARDs signaling

Secondly, we investigated whether Riplet expression affects RIG-I RD autorepression of CARDs signaling. To test this possibility, we constructed RIG-I mutant proteins (Figure 4A). We previously showed that Lys to Ala amino acid substitutions at Lys-849, 851, 888, 907, and 909 of RIG-I (RIG-I 5KA) severely reduced RIG-I polyubiquitination and activation [21]. However, it is possible that the substitutions of Lys with Ala affect other function of RIG-I because the substitution abolishes positive charge of Lys residues. Thus, we constructed the RIG-I 5KR mutant protein, in which the five Lys residues were substituted with Arg, and examined the signal activation ability. The results showed that the 5KR mutation reduced RIG-I signaling, however residual activation of RIG-I 5KR was still detected (Figure 4B–4D). Thus, we assessed other Lys residues within RIG-I RD. Because Riplet is essential for RIG-I activation, it is expected that the Lys residues targeted by Riplet are conserved during evolution. Thus, we tested Lys residues within RIG-I RD conserved among vertebrate, and found that an RIG-I K788R mutation reduced RIG-I signaling at a level comparable to that by a K172R mutation, which abrogates TRIM25-mediated RIG-I activation [15,19] (Figure 4B–4D). Interestingly, the 5KR and K788R
Riplet Releases RIG-I Autorepression

Several viruses have evolved strategies to escape the innate immunity. For instance, NS1 of influenza A virus inhibits TRIM25 function. This emphasizes the vital role of TRIM25 in preventing RIG-I autorepression. Our data indicate that Riplet releases RIG-I from autorepression, thereby promoting RIG-I-mediated innate immune responses.

If Riplet is essential for the release of RIG-I RD autorepression, it is expected that Riplet expression will increase the interaction between RIG-I and TRIM25. However, we found that ectopically expressed IKK-ε, TBK1, and NEMO ubiquitin binding region co-immunoprecipitated with RIG-I RD (Figure 6A–6D), and RIG-I expression increased the interaction of RIG-I with TBK1, IKK-ε, and the NEMO ubiquitin binding region (Figure 6D–6G). The physical interactions between these proteins were not through IPS-1, as IPS-1 did not co-immunoprecipitate with RIG-I RD (Figure 6B).

Microscopy analysis showed that the Riplet-RIG-I complexes were exclusively localized on mitochondria (Figure 6J). This was consistent with our immunoprecipitation results that RIG-I RD was associated with mitochondrial antigens. However, co-immunoprecipitation of RIG-I with TBK1 was also detected in the region where there is no mitochondria (yellow stained region in Figure 6J). This was consistent with our immunoprecipitation results that RIG-I RD was associated with mitochondrial antigens.

Hepatitis C virus protease NS3-4A targets the Riplet protein

Hepatitis C virus protease NS3-4A targets the Riplet protein.
modulating antiviral response [34]. To assess the biological significance of Riplet-mediated release of RIG-I RD autorepression in antiviral innate immune response, we investigated whether viral protein suppresses this mechanism.

The endogenous Riplet protein level was not affected by poly(I:C), HCV dsRNA stimulations, or by VSV infection (Figure 7A–7C), however the Riplet protein level was severely reduced in a human hepatocyte cell line with HCV 1b full-length replicons (O cells) compared with hepatocyte cell line without these replicons (O control cells: Oc cells; Figure 7D), suggesting that viral protein reduced the Riplet protein level. Riplet knockout abolished the expression of type I IFN, IP-10, and type III IFN in response to HCV RNA (Figure 7E), indicating that Riplet was essential for type I IFNs expression in response to HCV RNA.

Because HCV protease NS3-4A suppresses type I IFN expression in response to viral infection [7,25], we examined whether NS3-4A could cleave the Riplet protein. N-terminal FLAG-tagged Riplet or C-terminal HA-tagged Riplet was expressed with or without NS3-4A, after which the Riplet protein levels were compared. NS3-4A expression severely reduced the FLAG-tagged and HA-tagged Riplet protein level but not that of FLAG-tagged RIG-I, whereas catalytically inactive NS3-4A* (S139A) failed to reduce the Riplet protein level (Figure 7F, 7G, and S3A). This suggested that this protease’s activity reduced the Riplet protein level. Although NS3-4A reduced IPS-1 protein level as previously reported, NS3-4A did not reduce the TRIM25 and IKK-epsilon protein levels (Figure 7H, 7I, S3B–D).

Within the Riplet RING-finger domain is a sequence that is similar to the NS3-4A target consensus sequence (D/E-x-x-x-x-C/T-S/A; Figure 7J) [35]. NS3-4A cleaves the target just after C/T within this consensus sequence. Acidic amino acids before the C/T site are conserved among the NS3-4A cleavage site within HCV polypeptide. The acidic amino acids from 16 to 18 aa within the Riplet sequence were substituted with Ala, and a Riplet-3A mutant protein was constructed (Figure 7J). The Riplet-3A mutant protein was resistant to NS3-4A (Figure 7I and 7K). Moreover, the Riplet-3A protein co-localized with NS3-4A in cytoplasm (Figure 7L). Interestingly, recombinant NS3-4A that was purified from E. coli cleaved the immunoprecipitated FLAG-tagged Riplet protein and recombinant GST-fused Riplet protein purified from E. coli, and the cleaved fragments were detected at expected size (Figure 7M and 7N). These data indicated that NS3-4A directly targeted the Riplet protein.

Although Riplet digestion products were not observed in HEK293 cell lysate (Figure S3E and S3F), it is known that the digestion products of TICAM-1 (TRIF) obtained by NS3-4A are not detectable because these products are unstable [28]. Cys-21 of Riplet corresponds to the C/T site in the NS3-4A target consensus sequence. The Cys-21 residue is the first Cys in the RING-finger motif; thus a C21A substitution causes the disruption of RING finger domain structure [36]. The Riplet-C21A mutant protein was unstable and barely detectable (Figure S3G), which suggested that the loss of Cys-21 destabilized the Riplet protein.

HCV protease NS3-4A abolishes an early step of RIG-I activation

To determine if NS3-4A abolishes RIG-I activation by disrupting Riplet, we examined RIG-I ubiquitination and

Figure 3. Riplet function is dispensable for RIG-I RNA binding activity. (A) Expression vectors encoding Riplet, FLAG-tagged RIG-I CARDs, and/or FLAG-tagged RIG-I RD were transfected into HEK293FT cells together with an HA-tagged ubiquitin expression vector. Cell lysate was prepared at 24 hours after transfection, followed by immunoprecipitation with an anti-FLAG (RIG-I) antibody. (B) HEK293FT cells were transfected with expression vectors encoding FLAG-tagged RIG-I, Riplet, and HA-tagged ubiquitin. Cell lysate was prepared at 24 hours after transfection, and then incubated with biotin-conjugated (Biotin-dsRNA) or non-conjugated (dsRNA) double-stranded RNA. Biotin-dsRNA was pull-downed with streptavidin beads. Samples were subjected to SDS-PAGE, and proteins were detected by western blotting. (C) HEK293FT cells were transfected with FLAG-tagged wild-type RIG-I, RIG-I 5KR, or RIG-I K788R expression vector. 24 hours after the transfection, the cell lysate was prepared. The pull down assay with biotin-dsRNA was performed as described above.
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interaction between RIG-I and TRIM25. Riplet-mediated ubiquitination of full-length RIG-I or RIG-I RD was abolished by NS3-4A expression (Figure 8A and 8B). K63-linked polyubiquitination of RIG-I RD in SeV infected cells were also reduced by NS3-4A expression (Figure 8C). Moreover, NS3-4A expression reduced the interaction between TRIM25 and full-length RIG-I (Figure 8D).

An IPS-1-C508A mutant protein is resistant to NS3-4A cleavage [7]. A reporter gene assay showed that NS3-4A expression severely reduced IFN-β promoter activation induced by RIG-I and Riplet expression even in the presence of the IPS-1 C508A mutant protein in HEK293 cell (Figure 9A), and a catalytically inactive NS3-4A mutant failed to reduce this signal (Figure 9B). These data were consistent with our observation that NS3-4A reduced the Riplet protein level. We could not test whether NS3-4A failed to impair IFN-β promoter activity in the presence of the Riplet-3A and Riplet C21A mutants because the mutant proteins were not functional and failed to activate RIG-I (Supplemental Figure S3H and S3I). Thus, we could not exclude the possibility that NS3-4A targeted another protein in addition to Riplet and IPS-1.

Hepatitis C virus abrogates endogenous Riplet function

Endogenous RIG-I exhibited punctate staining in the human hepatocyte cell line HuH7 and HepG2 cells after simulation with...
HCV RNA (Figure 9E) but not in HuH7.5 cell line (Figure 9E). In HuH7.5 cells, there is a T55I mutation within endogenous RIG-I gene that disrupts the interaction between RIG-I and TRIM25 [38]. We investigated whether Riplet is required for RIG-I to exhibit punctate staining, and found that knockdown of Riplet decreased RIG-I punctate staining induced by HCV RNA (Supplemental Figure S4). We next investigated whether HCV abrogated Riplet-dependent RIG-I punctate pattern in the cytoplasm. As expected, RIG-I failed to exhibit punctate staining in O cells with HCV replicons in NS3 positive cells and HuH7

Figure 5. Riplet affects RIG-I RD autorepression of CARDs signaling. (A, B) IFN-β promoter activation was determined using a p125luc IFN-β reporter gene. Expression vectors encoding RIG-I CARDs, full-length RIG-I, RIG-I K788R, RD, RD-K788, and/or Riplet were transfected into HEK293 cells as indicated. Reporter activation was determined after transfection. Data are presented as mean ± SD (n = 3). *p<0.05. (C) IFN-β reporter activation was determined using a p125luc IFN-β reporter gene. RIG-I and Riplet expression vectors were transfected into HEK293 cells as indicated. 24 hours after transfection, cells were infected with SeV at MOI = 1 for 24 hours, and the reporter activities were determined. (D–F) HEK293FT cells were transfected with expression vectors encoding FLAG-tagged RIG-I, HA-tagged TRIM25, HA-tagged Riplet, FLAG-tagged RIG-I K788R as indicated. Cell lysate was prepared at 24 hours after transfection, followed by immunoprecipitation with an anti-FLAG antibody (RIG-I). Relative band intensity of immunoprecipitated TRIM25 in panel D was determined (E). (G) Mouse splenocyte was isolated from wild-type and Riplet KO mice spleen. The cells were infected with SeV, and then cell lysate was prepared. Immunoprecipitation was carried out with anti-RIG-I rabbit mAb (D14G6), and subjected to SDS-PAGE. Endogenous K63-linked polyubiquitin chain was detected using K63-linked polyubiquitin chain specific antibody.

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cells infected with HCV JFH1 (Figure 9F–9H). We confirmed that HCV disrupted IPS-1 in our experimental condition (Figure 9G and 9H).

To further assess whether HCV abrogates endogenous Riplet function, we observed endogenous K63-linked polyubiquitination of RIG-I in cells with HCV replicons. Although SeV infection induced endogenous K63-linked polyubiquitination of RIG-I in HuH7 cells, HCV replicons failed to increase the polyubiquitination (Figure 9E). Next, we investigated the association of endogenous RIG-I with TRIM25 and TBK1, which is promoted by Riplet as shown in Figure 5 and 6. SeV infection induced the association of endogenous RIG-I with TRIM25 and TBK1, whereas HCV replicons failed to induce the association (Figure 9F). Taken together, these data indicated that HCV abrogated endogenous Riplet function.

Although NS3-4A cleaves IPS-1, a mutation within endogenous RIG-I gene increased the permissiveness to HCV infection in HuH7-derived cells [3], indicating that RIG-I is required for antiviral response to HCV infection before NS3-4A cleaves IPS-1. We used siRNA to knockdown endogenous Riplet in HuH7 cells, and then the cells were infected with HCV JFH1. Interestingly, Riplet knockdown increased the permissiveness to HCV JFH1 infection (Figure 9K), indicating that endogenous Riplet is required for antiviral response to HCV infection.

**Discussion**

RIG-I activation is regulated by two ubiquitin ligases Riplet and TRIM25 [15,21]. The two ubiquitin ligases are essential for RIG-I activation [15,23], however the functional difference had been unclear. It is known that TRIM25 is essential for RIG-I oligomerization and association with IPS-1 adaptor molecule [15,19]. Here, we demonstrated that Riplet was essential for the release of RIG-I RD autorepression of its CARDS, which resulted in the association with TRIM25. This functional difference explained the reason why RIG-I requires the two ubiquitin ligases for triggering the signal.

It has been reported that TRIM25 activates RIG-I signaling [15,19]. We confirmed that ectopic expression of TRIM25 increases RIG-I CARDS-mediated signaling. However, most previous studies used a RIG-I CARDS fragment but not full-length RIG-I [15,19,30]. Unexpectedly, we found that the increase of full-length RIG-I-mediated signaling by TRIM25 expression was much less than that of the CARDS-mediated signaling. It is intriguing that Riplet helped TRIM25 to activate full-length RIG-I. Riplet expression promoted the interaction between TRIM25 and full-length RIG-I, and this interaction was abrogated by an RIG-I K788R mutation, which reduced Riplet-mediated RIG-I ubiquitination. Thus, we propose that Riplet-mediated polyubiquitination of RIG-I RD is a prerequisite for TRIM25 to activate RIG-I (Figure 10).

Ectopic expression of Riplet activated RIG-I without stimulation with RIG-I ligand. This is not surprising because ectopically expressed Riplet bound to RIG-I without stimulation with RIG-I ligand, whereas endogenous Riplet bound to endogenous RIG-I after stimulation with RIG-I ligand. RIG-I undergoes its conformational change after binding to a ligand [3,39]. The conformational change would allow the access of endogenous Riplet to RIG-I, which resulted in Riplet-mediated K63-linked polyubiquitination leading to the release of RD autorepression. This model is consistent with the observation that TRIM25 ectopic expression did not activate full-length RIG-I without Riplet expression, because TRIM25 hardly bound to full-length RIG-I without Riplet.

Previously, we reported that the five Lys residues within RIG-I RD were important for Riplet-mediated RIG-I ubiquitination. We constructed the RIG-I 5KR mutant and indicated that the 5KR mutation reduced RIG-I ubiquitination and activation without loss of RNA binding activity. This is consistent with our previous conclusion. However, there is residual ubiquitination of RIG-I 5KR mutation, and we found that K788R mutation showed more severe phenotype. These data indicated that Riplet targeted the several Lys residues within RIG-I RD. This is not surprising, because TRIM25 targets not only Lys-172 but also other Lys residues within mouse RIG-I CARDS [32].

TBK1 and IKK-ε are downstream factors of IPS-1. We found that TBK1 and IKK-ε could bind RIG-I RD. It is possible that RIG-I associates with TBK1 through IPS-1. However, Hiscott J and colleagues demonstrated that IKK-ε could bind IPS-1 and that TBK1 did not bind IPS-1 [40]. Moreover, RIG-I RD did not bind IPS-1, and RIG-I and TBK1 co-localization was detected in the cytoplasmic region where there are no mitochondria. These observations weaken this possibility. Our results indicated that RIG-I RD bound to the NEMO ubiquitin binding region. IRF-3 activation requires the ubiquitin binding domain of NEMO, and an endogenous K63-linked polyubiquitin chain plays a key role in IRF3 activation [41]. Thus, we prefer a model in which TBK1 associates with RIG-I RD-anchored polyubiquitin chain through NEMO (Figure 10). Although Riplet knockout reduced the binding of RIG-I to TBK1, residual binding was still detectable. Thus, there appears to be Riplet-dependent and independent associations between RIG-I and TBK1. TRAF3 is an E3 ubiquitin ligase, and is involved in the RIG-I-mediated type I IFN production pathway [42]. Because there is residual activation of the type I IFN production pathway even in TRAF3 knockout cells [41], it is possible that the RIG-I polyubiquitin chain may compensate for the TRAF3 defect in recruiting TBK1 to mitochondria. Further studies will be needed to determine the precise molecular mechanisms. Although TBK1 dispersed in the cytoplasm, p-TBK1 was exclusively localized on mitochondria. Considering that TBK1 is phosphorylated in its activation loop [33], these results suggested that RIG-I RD associated with...
Figure 7. NS3-4A of HCV targets the Riplet protein. (A–D) Endogenous RIG-I and Riplet protein levels were observed by western blotting. HeLa cells were stimulated with polyIC transfection (A), HCV dsRNA transfection (B) or infected with VSV (C). HCV replicon positive (HCV) and negative (-) cell lysates were prepared from a HuH7-derived cell line O cell that contains HCV 1b full-length replicons and O cured cell (Oc cell) in which HCV replicons were removed by IFN treatment (D). (E) The response to HCV RNA in wild-type and Riplet KO MEFs was examined by RT-qPCR. Wild type (WT) and Riplet knockout (KO) MEF cells were transfected with 100 ng of HCV ssRNA and dsRNA. Six hours after stimulation, mRNA expressions of IFN-α2, IP10, and IFN-λ2/3 were measured by RT-qPCR. Data are presented as mean ± SD (n = 3). *p < 0.05. (F–H) FLAG-tagged Riplet and RIG-I (F), HA-tagged Riplet (G), or HA-tagged TRIM25 (H) expression vectors were transfected into HEK293FT cells together with NS3-4A or NS3-4A* expression vectors. NS3-4A* mutant protein harbors an amino acids substitution at its catalytic site Ser-139 with Ala. 24 hours after transfection, cell lysate was prepared and subjected to SDS-PAGE. (I) Band intensity ratio of IPS-1, Riplet, TRIM25, IKK-e, and Riplet-3A with/without NS3-4A expression (mean ± sd, n = 3). (J) NS3-4A cleavage sites within an HCV polypeptide are compared with a candidate site in the Riplet RING-finger domain. Homologous amino acids are shown in bold, and identical amino acids are underlined. In Riplet-3A mutant protein, three acidic amino acids, Glu-16, Asp-17, Asp-18, were substituted with Ala. An expression vector encoding wild-type Riplet or Ripled-3A mutant protein was transfected into HEK293 cells together with NS3-4A or NS3-4A* expression vectors. Cell lysate was prepared 24 hours after transfection, and subjected to SDS-PAGE. (L) HA-tagged Riplet-3A and NS3-4A expression vectors were transfected into HepG2 cell. 24 hours after transfection, the cells were fixed and stained with anti-HA monoclonal antibody (mouse) and anti-NS3-4A polyclonal antibody (goat). (M) N-terminal FLAG-tagged Riplet was expressed in HEK293FT cells, and immunoprecipitation was carried out with anti-FLAG antibody. Immunoprecipitates were subjected to SDS-PAGE analysis. The proteins were detected by western blotting. (N) Purified GST fused Riplet (1–210 aa) was incubated with or without recombinant NS3-4A (NS3-4A) at 37 °C for 30 min. The proteins were subjected to SDS-PAGE and detected by western blotting.

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Figure 8. NS3-4A inhibits Riplet-mediated RIG-I polyubiquitination. (A, B) Riplet, NS3-4A, and/or HA-tagged ubiquitin (HA-Ub) expression vectors were transfected into HEK293FT cells along with either full-length RIG-I (A) or RIG-I RD (B). Cell lysate was prepared 24 hours after transfection, and subjected to SDS-PAGE. The proteins were detected by western blotting. (C) HEK293FT cells were transfected with Myc-tagged K63-only ubiquitin, FLAG-tagged RIG-I RD, and/or NS3-4A expression vectors 24 hours after transfection, cells were infected with SeV for six hours, and then cell lysate was prepared. Immunoprecipitation was carried out with anti-FLAG antibody, and the samples were subjected to SDS-PAGE. (D) HA-tagged TRIM25, Riplet and/or FLAG-tagged RIG-I expression vectors were transfected into HEK293FT cells with or without NS3-4A expression vector. Cell lysate was prepared 24 hours after the transfection, and immunoprecipitation assay was performed with anti-FLAG antibody. The precipitates were subjected to SDS-PAGE.

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Riplet Releases RIG-I Autorepression

Figure A: HEK293 p125Luc (IFN-β) Fold increase

Figure B: HEK293 p125Luc (IFN-β) Fold increase

Figure C: IPS-1 KO hepatocyte p125Luc (IFN-β) Fold increase

Figure D: IPS-1 KO hepatocyte p125Luc (IFN-β) Fold increase

Figure E: HCV RNA stimulation (6 hr)

Figure F: HCV(0) HCV replicon

Figure G: HuH7.5 HCV JFH1 infection (7 days)

Figure H: HuH7 HCV JFH1 infection (7 days)

Figure I: HuH7 IP anti-RIG-I

Figure J: HuH7 IP anti-RIG-I

Figure K: HCV JFH1 RNA (HuH7) Riplet mRNA
Figure 9. HCV abrogated Riplet-mediated RIG-I activation. (A and B) The inhibition of IFN-β promoter activation by NS3-4A was assessed by reporter gene assays. IPS-1-C508A mutant protein harbors an amino acid substitution at Cys-508 with Ala. 100 ng of IPS-1, IPS-1-C508A, RIG-I, Riplet, NS3-4A, and/or NS3-4A* expression vectors were transfected into HEK293 cells in 24-well plates with p125Luc reporter plasmid. The total amount of transfected DNA (800 ng/well) was kept constant by adding empty vector (pEF-BOS). 24 hours after the transfection, the reporter activities were measured. Data are presented as mean ± SD (n = 3). *p<0.05. (C and D) IPS-1 KO mouse hepatocyte was transfected with IPS-1 C508A, RIG-I, Riplet, and/or NS3-4A expression vectors together with p125Luc and Renilla luciferase plasmids. Transfected cells were stimulated with 50 ng of HCV dsRNA for 24 hours by transfection (D). Data are presented as mean SD (n = 3). *p<0.05. (E and F) Intracellular localizations of endogenous TBK1 and RIG-I were determined by confocal microscopy. HepG2, HuH7, and HuH7.5 cells were stimulated with 100 ng of HCV dsRNA for six hours by transfection (E). Stimulated cells (E) and O cells with HCV replicons (F) were stained with anti-RIG-I, TBK1, and/or NS3 antibodies. (G and H) HuH7 cells were infected with mock or SeV infected HuH7 or HuH7 cells with HCV replicons (O cell). Immunoprecipitation using high salt buffer was performed with anti-RIG-I (Alme-1) antibody. The samples were subjected to SDS-PAGE. Endogenous K63-linked polyubiquitin chain was detected using ubiquitin K63-linkage specific antibody. (I) HuH7 cells were infected with SeV at MOI = 1 for 24 hours. Cell lysates were prepared from mock or SeV infected HuH7 or HuH7 cells with HCV replicons (O cell). Immunoprecipitation using high salt buffer was performed with anti-RIG-I (Alme-1) antibody. The samples were subjected to SDS-PAGE. Endogenous K63-linked polyubiquitin chain was detected using ubiquitin K63-linkage specific antibody. (J) HuH7 cells were infected with SeV at MOI = 1 for 24 hours. Cell lysates were prepared from mock or SeV infected HuH7 or HuH7 cells with HCV replicons (O cell). Immunoprecipitation using high salt buffer was performed with anti-RIG-I (Alme-1) antibody. The samples were subjected to SDS-PAGE. (K) HuH7 cells were transfected with siRNA for mock or Riplet. 48 hours after the transfection, cells were infected with HCV JFH1 for 2 days. RT-qPCR was performed to determine HCV genome RNA, GAPDH, and Riplet expression. doi:10.1371/journal.ppat.1003533.g009

inactive TBK1 and that TBK1 was activated after loading on to mitochondria (Figure 10).

HCV is a major cause of HCC and has the ability to evade host innate immune response [7,43]. HCV RNA is primarily recognized by the cytoplasmic viral RNA sensor RIG-I. Previous studies showed that the protease NS3-4A cleaves IPS-1 to shut off RIG-I signaling. However, our results indicated that there was another target of NS3-4A in RIG-I signaling. First, RIG-I failed to exhibit punctate staining in cells infected with HCV. Second, NS3-4A reduced RIG-I signaling even in the presence of an IPS-1-C508A mutant, which is resistant to the cleavage by NS3-4A. Third, the endogenous Riplet protein level was severely reduced in cells with HCV replicons. Fourth, NS3-4A targeted Riplet and abrogated Riplet-dependent RIG-I ubiquitination and complex formation with TRIM25 and TBK1. These data support our model that NS3-4A targets not only IPS-1 but also Riplet to escape host innate immune responses (Figure 10). Recently it was reported that NS1 proteins of Influenza A virus inhibited Riplet function [32]. These findings indicated biological importance of Riplet in RIG-I activation during viral infection.

In general, a ubiquitin ligase has several targets. We have performed yeast two-hybrid screening using Riplet as bait and found a candidate clone that encodes a tumor suppressor gene. Our pilot study showed that Riplet mediated K63-linked polyubiquitination of this tumor suppressor and suppressed retinoblastoma (Rb) activity. Thus, Riplet disruption by NS3-4A might be a cause of liver disease induced by HCV infection.

Materials and Methods

Ethics statement

All animal studies were carried out in strict accordance with Guidelines for Animal Experimentation of the Japanese Associations for Laboratory Animal Science. The protocols were approved by the Animal Care and Use Committee of Hokkaido University (Permit Number: 08-0245 and 09-0215).

Cell

HEK293, Vero, and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium low glucose medium (D-MEM) with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen). HeLa cells were cultured in minimum Eagle’s medium with 2 mM L-glutamine and 10% heat-inactivated FCS. HEK293FT cells were maintained in D-MEM high glucose medium containing 10% of heat-inactivated FCS (Invitrogen). Human hepatocyte cell line with HCV 1b full-length replicons (O cells) and O curred cells (Oc cells) were kindly gifted from Katoh N [44]. O cells were cultured in D-MEM high glucose with 10% of heat-inactivated FCS, G418, NEAA, and L-Gln.

Viruses

VSV Indiana strain and SeV HVJ strain were amplified using Vero cells. To determine the virus titer, we performed plaque assay using Vero cells. HCV JFH1 was amplified using HuH7.5 cells.

Mice

Generation of IPS-1 KO and Riplet KO mice was described previously [23,45]. Splenocyte was isolated from C57BL/6 wild-type and Riplet KO mice. Isolated cells were cultured in RPMI1640 containing 10% of heat-inactivated FCS. The preparations of wild-type and Riplet KO MEFs were described previously [23]. Preparation of IPS-1 KO mouse hepatocyte was described previously [37]. All mice were maintained under specific-pathogen free conditions in the animal facility of the Hokkaido University Graduate School of Science (Japan).

Plasmids

Expression vectors encoding for N-terminal FLAG-tagged RIG-I, N-terminal FLAG-tagged RIG-I CARDS (dRIG-4), FLAG-tagged RIG-I ΔRD (RIG-I-dRD), FLAG-tagged RIG-I RD, C-terminal HA-tagged TRIM25, C-terminal HA-tagged Riplet, and RipletΔRING (Riplet-DN) plasmids were described previously [21,23]. The amino acids substitutions from 16 to 18 with Ala was carried out by PCR-mediated mutagenesis using primers, Ripelt-AF and Riplet-A-R and pEF-BOS/Riplet plasmid as a template. The primer sequence is Riplet-AF: TTC CCC TGT GCC TGG CCG CCG CCG CCC TCG GCT GCA TCA TCT GCC, and Riplet-A-R: GCC AGA TGA TGC AGC CGA GGG CCG CCG CCG CCA GCC AGA CGG GAA. RIG-I K172R and RIG-I K788R expression vectors was constructed by PCR-mediated mutagenesis using primers, RIG-I K172R-F and RIG-I K172R-R, and RIG-I K788R-F and RIG-I K788R-R, and pEF-BOS/FLAG-RIG-I plasmid as a template. The primer sequences are RIG-I K172R-F: GGA AAA CTG GCCCAA AAc ATT GAG ACT TGC TTT GGA GAA AG, RIG-I K172R-R: CTT TCT CTA CCA AAG CAA GTC TCG AAG TTT TGG GCC AGT TTT CC, RIG-I K788R-F: TGG ATA TAC AGA CTC ATG AAA GAT TCA TCA GAG ATC AAG AA, and RIG-I K788R-R: CTT GAC TAT CTC TGA TCA TTC CAT GAG TCT GTA TAT GCA G. RIG-I 5KR expression vectors were constructed by PCR-mediated mutagenesis using primers, RIG-I 849 851 RR-F and RIG-I 849 851 RR-R, and pEF-BOS/FLAG-RIG-I plasmid as a template. The primer sequences are RIG-I 849 851 RR-F: GGA AAA CTG GCCCAA AAc ATT GAG ACT TGC TTT GGA GAA AG, RIG-I 849 851 RR-R: CTT TCT CTA CCA AAG CAA GTC TCG AAG TTT TGG GCC AGT TTT CC, RIG-I 849 851 RR-F: TGG ATA TAC AGA CTC ATG AAA GAT TCA TCA GAG ATC AAG AA, and RIG-I 849 851 RR-R: CTT GAC TAT CTC TGA TCA TTC CAT GAG TCT GTA TAT GCA G. RIG-I 888R-F and RIG-I 888R-R, RIG-I 907 909 RR-F, and RIG-I 907 909 RR-R, and
pEF-BOS/FLAG-RIG-I plasmid as a template. The primer sequences are RIG-I 849 851 RR-F: AGT AGA CCA CAT CCC AGG CCA AGG CAG TTT TCA AGT TTT G, RIG-I 849 851 RR-R: CAA AAC TTG AAA ACT GCC TTG GCC TGG GAT GTG GTC TAC T, RIG-I 888R-F: GAC ATT TGA GAT TCC AGT TAT AAG AAT TGA AAG TTT TGT GGT GGA GG, RIG-I 888R: CCT CCA CCA CAA AAC TTT CAA TTC TTA TAA CTG GAA TCT CAA ATG TC, RIG-I 907 909RR-F: GTT CAG ACA CTG TAC TCG AGG AGG GAC TTT CAT TTT GAG AAG, RIG-I 907 909RR-R: CTT CTC AAA ATG AAA GTC CCT CCA CCT CGA GTA CAG TGT CTG AAC. HCV cDNA fragment encoding NS3-4A of JFH1 strain was cloned into pCDNA3.1 (-) vector. The mutation on catalytic site of NS3-4A S139A was constructed by PCR-mediated mutagenesis using primers, NS3-4A S139A-F and NS3-4A S139A-R, and pCDNA3.1 (-)/NS3-4A plasmid as a template. The primer sequences are NS3-4A S139A-F: TTC GAC CTT GAA GGG GTC CGC GGG GGG ACC GGT GCT TTG C and NS3-4A S139A-R: AAG CAC CGG TCC CCC CGC GGA CCC CTT CAA GGT CGA AAG G.

RT-PCR and Real-Time PCR
Total RNA was extracted with TRIZOL (Invitrogen), after which the samples were treated with DNaseI to remove DNA.
contamination. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (ABI). Quantitative PCR analysis was performed using Step One software ver2.0 (ABI) with SYBER Green Master Mix (ABI). HCV ss and dsRNA was in vitro synthesized with SP6 and/or T7 RNA polymerase using 3' UTR of HCV cDNA as template as described previously [46].

Confocal microscopy

Cells were plated onto microscope cover glasses (matsunami) in a 24-well plate. The cells were fixed for 30 min using 3% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 for 15 min. Fixed cells were blocked with 1% bovine serum albumin in PBS for 10 min and labeled with the indicated primary Abs for 60 min at room temperature. Alexa-conjugated secondary Abs were incubated for 30 min at room temperature to visualize staining of the primary Ab staining. Samples were mounted on glass slides using Prolong Gold (Invitrogen). Cells were visualized at a magnification of ×63 with an LSM510 META microscope (Zeiss). Data collected with confocal microscopy were analyzed with ZEISS LSM Image Examiner software. NS3, RIG-I, TBK1, IPS-1, and p-TBK1 were stained with anti-NS3 goat pAb (abcam), anti-RIG-I mouse mAb (Alme-1, ALEXIS BIOCHEMICALS), anti-NK- (TBK1) rabbit mAb (EP611Y, abcam), anti-MAVS (IPS-1) rabbit pAb (Bethyl Laboratories Inc), and anti-p-TBK1 rabbit mAb (Cell Signaling Technology).

Reporter gene analysis

HEK293 cells were transiently transfected in 24-well plates using FuGene HD (Promega) or lipofectamine 2000 (Invitrogen) with expression vectors, reporter plasmids (IFN-β; p125luc), and an internal control plasmid coding Renilla luciferase. The total amounts of plasmids were normalized using an empty vector. Cells were lysed in a lysis buffer (Promega), and luciferase and Renilla luciferase activities were determined using a dual luciferase assay kit (Promega). Relative luciferase activities were calculated by normalizing the luciferase activity by control. HCV dsRNA (3’ UTR polyU/UC region) was synthesized using T7 and SP6 RNA polymerase as described previously [46].

Pull-down assay

RNA used for the assay was purchased from JBioS. The RNA sequences are as follows: (sense strand) AAA CUG AAA GGG AGA AGU GAA G; and (antisense strand) CAC UUC UCC CUU UCA GUU GU. Biotin was conjugated at 3’ end of HCV dsRNA in a 6-well plate. HCV dsRNA (HCV 3’ UTR polyU/UC region) was synthesized using T7 and SP6 RNA polymerase as previously described [46]. Cell lysates were prepared at the indicated times. Immunoprecipitation was performed with an anti-RIG-I mouse monoclonal antibody (Alme-1). An anti-FLAG M2 monoclonal antibody (Sigma) was used for the immunoprecipitation of FLAG-tagged protein. An anti-TRIM25 rabbit polyclonal antibody (abcam), an anti-p-TBK1 rabbit mAb (Cell Signaling Technology), an anti-NK- (TBK1) rabbit mAb (EP611Y), and an anti-RNF135 (Riplet) pAb (SIGMA), were used for western blotting. For ubiquitination assay, immunoprecipitates were washed three times with high salt lysis buffer (20 mM Tris-HCl pH 7.5, 1M NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM Na3VO4, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride). Immunoprecipitation used an anti-RIG-I Rabbit monoclonal antibody (D14G6, Cell Signaling Technology). To detect endogenous K63-linked polyubiquitin chain that is ligated to RIG-I, 6×10^7 of mouse splenocytes were infected with SeV at MOI = 0.2 for 24 hours. Immunoprecipitation was performed with anti-RIG-I mAb (D14G6). Anti-K63-linkage specific poly-ubiquitin (D7A11) Rabbit mAb (Cell Signaling) was used for western blotting. HEK293FT cells were transfected with or without 0.8 μg of HCV dsRNA in a 6-well plate. HCV dsRNA (HCV 3’ UTR polyU/UC region) was synthesized using T7 and SP6 RNA polymerase as previously described [46]. Cell lysates were prepared at the indicated times. Immunoprecipitation was performed with an anti-RIG-I mouse monoclonal antibody (Alme-1). An anti-FLAG M2 monoclonal antibody (Sigma) was used for the immunoprecipitation of FLAG-tagged protein. An anti-TRIM25 rabbit polyclonal antibody (abcam), an anti-p-TBK1 rabbit mAb (Cell Signaling Technology), an anti-NK- (TBK1) rabbit mAb (EP611Y), and an anti-RNF135 (Riplet) pAb (SIGMA), were used for western blotting. For ubiquitination assay, immunoprecipitates were washed three times with high salt lysis buffer (20 mM Tris-HCl pH 7.5, 1M NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM Na3VO4, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride) to dissociate unanchored polyubiquitin chain [21], and then washed once with normal lysis buffer described above for SDS-PAGE analysis. Band intensity was semi-quantified using Photoshop software.

RNAi

siRNAs for human Riplet (Silencer Select Validated siRNA) and negative control were purchased from Ambion. siRNA sequences for Riplet are: (sense) GGA ACA UCU UGU AGA CAU Uut and (anti-sense) AAU GUC UAC AAG AUG UUC CCac. siRNA was transfected into cells using RNAiMax Reagent (Invitrogen) according to the manufacturer’s instructions.

In vitro NS3/4A cleavage assay

FLAG-tagged Riplet was expressed in HEK293FT cells, and cell lysate was prepared with the lysis buffer described above. The protein was immunoprecipitated with anti-FLAG antibody and protein G sepharose beads, and washed with Buffer B (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40). The samples were suspended in 50 μl of Buffer B, and incubated with 400 ng of recombinant NS3-4A (rNS3-4A) protein at 37°C for one hour, and then subjected to SDS-PAGE analysis. The NS3-4A protein was purchased from AnaSpec Inc (CA). N-terminal GST-fused Riplet (1-210 aa) (rRiplet) was purchased from Abnova. 500 ng of rRiplet was incubated with or without 500 ng of rNS3-4A in 10 μl of reaction buffer (20 mM Tris-HCl (7.5), 4% Glycerol, 5 mM DTT, 150 mM NaCl, 0.1% of Triton-X100, 0.9% polyvinyl alcohol) at 37°C for 30 min.

Accession numbers

The accession numbers are Riplet (BAG4604), TRIM25 (NP_050707), TBK1 (NP_037386), IKK-ε (AAF45307), IPS-1 (BAE79738), RIG-I (NP_055129), and G3BP (CAG38772).

Supporting Information

Figure S1 K63-linked polyubiquitination of RIG-I RD. HA-tagged ubiquitin and FLAG-tagged RIG-I RD expression vectors were transfected into HEK293FT cells. 24 hours after transfection, the cells were infected with VSV at MOI = 1 for six
hours. Then, cell lysate was prepared. Immunoprecipitation was carried out using anti-FLAG antibody. The samples were subjected to SDS-PAGE, and the proteins were detected by western blotting using anti-HA, FLAG, and K63-linked poly-ubiquitin specific antibodies. (TIF)

Figure S2 Intracellular localization of RIG-I, NEMO, and p-TBK1 proteins. (A) HeLa cells were transfected with HCV dsRNA using lipofectamine 2000 reagent. The cells were fixed six hours after transfection. The microscopic analysis was performed using anti-RIG-I mAb (Alme-1) and anti-NEMO pAb. (B) HeLa cells were transfected with HCV dsRNA using lipofectamine 2000 reagent (Invitrogen). The cells were fixed at indicated hour. The microscopic analysis was performed using anti-RIG-I mAb (Alme-1), (C) HepG2 cells were transfected with HCV dsRNA using lipofectamine 200 reagent. The cells were fixed six hours after the transfection. The microscopic analysis was performed using anti-RIG-I (Alme-1) mAb and anti-p-TBK1 antibody. (TIF)

Figure S3 NS3-4A of HCV cleaves IPS-1 and Riplet but not IKK-ε. (A) HA-tagged Riplet was transfected into HEK293 cells together with NS3-4A. 24 hours after transfection, cell lysate was prepared and subjected to SDS-PAGE. The proteins were detected by western blotting and CBB staining. (B, C) HA-tagged cells together with NS3-4A. 24 hours after transfection, cell lysate was analyzed by SDS-PAGE. (G) HA-tagged wild-type Riplet or mutant Riplet-C21A expression vector were transfected into HEK293FT cells with NS3-4A or NS3-4A*. 24 hours after the transfection, the cell lysate was prepared, and analyzed by SDS-PAGE. The proteins were detected by western blotting using anti-HA or anti-β actin antibodies. (H) RIP-1 or HA-tagged expression vector was transfected into HEK293FT cells with or without NS3-4A expression vectors. 24 hours after transfection, cell lysate was prepared and subjected to SDS-PAGE. The proteins were detected by western blotting using anti-HA antibody. (E, F) N-terminal FLAG-tagged Riplet (E) or C-terminal HA-tagged Riplet (F) expression vector was transfected into HEK293FT cells with NS3-4A or NS3-4A*. 24 hours after the transfection, the cell lysates were analyzed by SDS-PAGE. (G) HA-tagged wild-type Riplet or mutant Riplet-C21A expression vector were transfected into HEK293FT cells with NS3-4A or NS3-4A*. 24 hours after the transfection, the cell lysate was prepared, and analyzed by SDS-PAGE. The proteins were detected by western blotting using anti-HA or anti-β actin antibodies. (H, I) RIP-1, Riplet-3A (H), and/or Riplet C21A (I) mutant expression vectors were transfected into HEK293 cells together with p125Luc reporter and Renilla luciferase. 24 hours after transfection, luciferase activity was measured. (TIF)

Figure S4 siRNA for Riplet or control was transfected into HeLa cells in 24-well plate using RNAi MAX (Invitrogen) according to manufacturer’s protocol. 48 hours after transfection, the cells were transfected with 100 ng of HCV dsRNA. Six hours after transfection, the cells were fixed and stained with anti-RIG-I mAb (Alme-1) and anti-mouse Alexa-488 Ab. (TIF)

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Author Contributions
Conceived and designed the experiments: HO MMi MMa TS. Performed the experiments: HO MMi. Analyzed the data: HO MMi. Contributed reagents/materials/analysis tools: HO MMi. Wrote the paper: HO MMi TS.

References


