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JB Review

Ubiquitin-mediated modulation of the cytoplasmic viral RNA sensor RIG-I

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RIG-I-like receptors, including RIG-I, MDA5 and LGP2, recognize cytoplasmic viral RNA. The RIG-I protein consists of N-terminal CARDs, central RNA helicase and C-terminal domains. RIG-I activation is regulated by ubiquitination. Three ubiquitin ligases target the RIG-I protein. TRIM25 and Riplet ubiquitin ligases are positive regulators of RIG-I and deliver the K63-linked polyubiquitin moiety to RIG-I CARDs and the C-terminal domain. RNF125, another ubiquitin ligase, is a negative regulator of RIG-I and mediates K48-linked polyubiquitination of RIG-I, leading to the degradation of the RIG-I protein by proteasomes. The K63-linked polyubiquitin chains of RIG-I are removed by a deubiquitin enzyme, CYLD. Thus, CYLD is a negative regulator of RIG-I. Furthermore, TRIM25 itself is regulated by ubiquitination. HOIP and HOIL proteins are ubiquitin ligases and are also known as linear ubiquitin assembly complexes (LUBACs). The TRIM25 protein is ubiquitinated by LUBAC and then degraded by proteasomes. The splice variant of RIG-I encodes a protein that lacks the first CARD of RIG-I, and the variant RIG-I protein is not ubiquitinated by TRIM25. Therefore, ubiquitin is the key regulator of the cytoplasmic viral RNA sensor RIG-I.

Keywords: RIG-I/type I interferon/ubiquitin/virus.

Abbreviations: CARD, caspase activation and recruitment domain; CTD, C-terminal domain; dsRNA, double-stranded RNA; RLR, RIG-I-like receptor; pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; MEF, mouse embryonic fibroblast cell; BM, bone-marrow; Mf, macrophage; IFN, interferon; ISG, interferon-stimulated gene; TRIM, tripartite motif; RNF, RING finger.

Recognition of viral RNA

Type I interferons (IFNs) are inflammatory cytokines that possess strong anti-viral activity. During viral infection, type I IFNs are produced from dendritic cells (DC), macrophages (Mf) and fibroblast cells (Fig. 1A). Viral RNA is mainly recognized by Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). TLRs are type I transmembrane proteins. TLR3, 7 and 8, which are members of the TLR family, are localized to endosomes, and are responsible for the recognition of viral RNA (*1*). RLRs are DExD/H box RNA helicases and recognize viral RNA in the cytoplasmic region (Fig. 1B). There are three members of the RLR family: RIG-I, MDA5 and LGP2. RIG-I has the ability to recognize various types of viruses, and MDA5 mainly recognizes picornaviruses (*2*). LGP2 promotes RIG-I and MDA5-mediated signalling (*3*).

A cytoplasmic sensor for the detection of viral RNA

RIG-I, a cytoplasmic sensor for viral RNA, is induced by viral infection, polyIC and type I IFN stimulation (*4*). This protein is composed of two N-terminal caspase recruitment domains (CARDs), a central DExD/H box helicase/ATPase domain and a C-terminal regulatory domain (CTD) (Fig. 2). N-terminal CARDs are responsible for the binding to the adaptor molecule IPS-1/MAVS/VISA/Cardif, which is located on the outer membrane of the mitochondria (*5–8*). In the absence of viral RNA, RIG-I CTD represses the interaction between RIG-I CARDs and IPS-1 CARD (*9*). RIG-I CTD recognizes the 5′ triphosphate of short double-stranded RNA, leading to multimerization of RIG-I and IPS-1 (*10–13*). IPS-1 triggers signaling to induce type I IFN and other inflammatory cytokines through STING (also called MITA) protein, which is localized to the endoplasmic reticulum or the mitochondria (*14–17*). STING then activates transcription factors, such as IRF-3, IRF-7 and NF-κB (*15*, *18*).

Knockout of RIG-I abrogates the production of type I IFNs and inflammatory cytokines from mouse embryonic fibroblasts (MEFs), conventional DC and Mfs in response to viral infections, including infections caused by vesicular stomatitis virus (VSV), Sendai virus (SeV), influenza A virus, Newcastle disease virus, hepatitis C virus and Japanese encephalitis virus (*2*, *19*). However, RIG-I is not necessary for the production of type I IFNs by plasmacytoid dendritic cells (pDCs), which are strong inducers of type I IFNs *in vivo* (*19*). In pDCs, TLR7 is responsible for the detection of viral RNA (*20*). In addition, knockout of IPS-1 and STING inhibits the production of type I IFNs from MEFs, Mfs and cDCs, but not from pDCs (*15–18*). Once type I IFNs are produced from these cells, IFN production is secondly amplified via the IFNAR (*21*). The deficiency of the RIG-I-dependent pathway causes a reduction in early type I IFN production *in vivo* but shows only a marginal effect on late type I IFN production (*15–18*). Knockout of RIG-I increases the

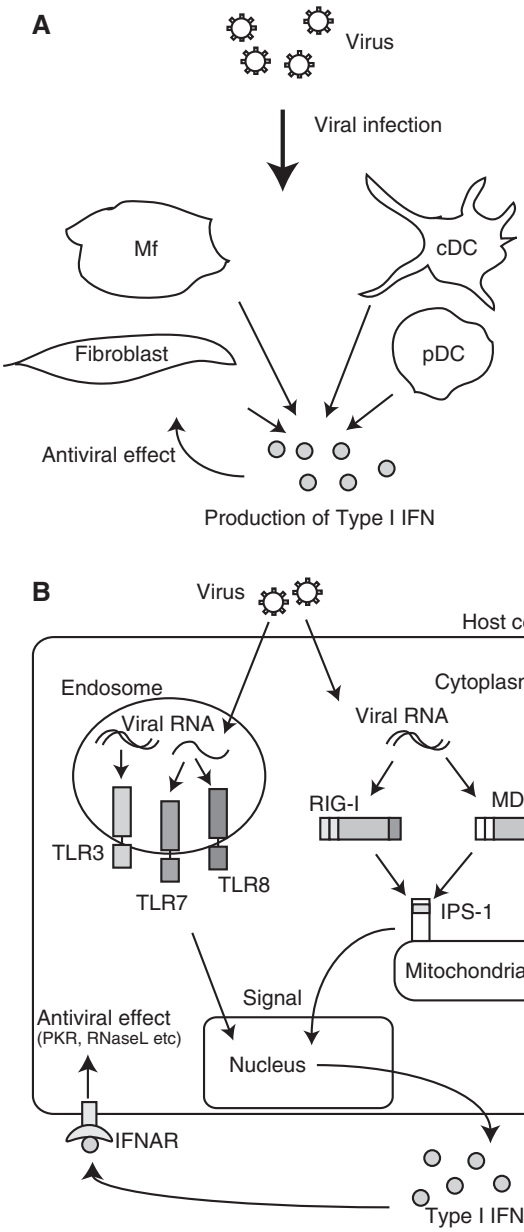

Fig. 1 Production of type I IFN in response to viral infection. (A) Type I IFN is a cytokine that possesses strong anti-viral activity. Type I IFN is produced from fibroblast cells, cDC, pDC and Mf in response to viral infection. (B) TLR3, 7 and 8 are localized to endosomes and are responsible for the recognition of viral RNA. Viral RNA in the cytoplasmic region is recognized by RIG-I and MDA5, leading to the activation of the adaptor molecule IPS-1. IPS-1 triggers the signal to induce type I IFNs. Type I IFNs binds to an IFN receptor, IFNAR, leading to the activation of anti-viral factors, such as PKR and RNaseL.

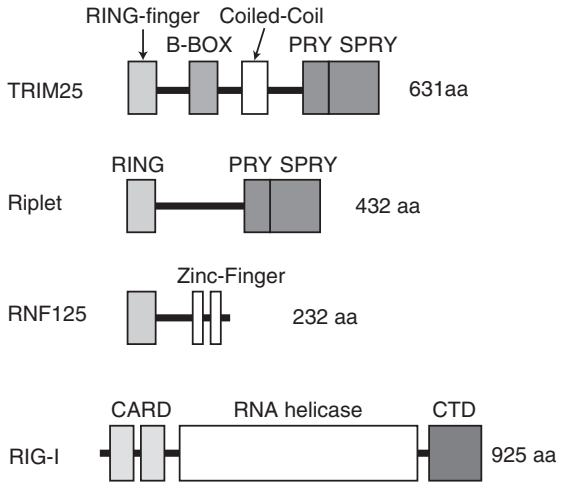

Fig. 2 Domain structures of TRIM25, Riplet, RNF125 and RIG-I. TRIM25 consists of RING finger, B-box, coiled-coil, PRY and SPRY domains. Riplet is similar to TRIM25 and consists of RING-finger, PRY and SPRY domains. RNF125 consists of RING-finger and two zinc-finger domains. Three proteins mediate the polyubiquitination of RIG-I. RIG-I consists of two N-terminal CARDs, central RNA helicase and CTDs.

mortality due to viral infections (*2*, *19*). Thus, RIG-I-dependent pathways are necessary for efficient early type I IFN production and are required for protection against viral infections (*18*).

TRIM25 ubiquitin ligase is a positive factor for the RIG-I activation

During viral infection, the RIG-I protein has a modified form of ubiquitin. TRIM25 (also called Efp) is a ubiquitin ligase (*22*, *23*), and its domain structure is described in Fig. 2. This protein interacts with the first CARD of RIG-I (*22*, *24*). T55I mutation of the first CARD of RIG-I is found in RIG-I-deficient HuH7.5 cells. T55 of RIG-I is critical for the interaction between TRIM25 and RIG-I (*9*, *24*, *25*). Gack et al. detected the polyubiquitination of the K99, K169, K172, K181, K190 and K193 residues of RIG-I CARDs by mass spectrometry analysis (*22*), and the K172R mutation alone causes a near-complete loss of the polyubiquitination of RIG-I CARDs (*22*). TRIM25 delivers the K63-linked polyubiquitin moiety to the K172 residue of the second CARD of RIG-I, leading to efficient interaction with IPS-1/MAVS/VISA/Cardif (*22*, *24*). On the other hand, Zeng *et al.* reported another mechanism of the activation of RIG-I by ubiquitin. They reconstituted RIG-I pathway *in vitro* and showed that RIG-I CARDs sense unanchored polyubiquitin chains mediated by TRIM25, and the binding of RIG-I CARDs to the unanchored polyubiquitin chains leads to the activation of RIG-I (*26*). Knockout of TRIM25 abrogates IFN-β production from MEF in response to viral infection (*22*). Thus, ubiquitination or polyubiquitin binding is essential for the activation of RIG-I (Fig. 2).

The expression of a splice variant of RIG-I mRNA is robustly up-regulated upon viral infection (*24*). This splice variant encodes a protein that lacks the first 36–80 amino acid region within the first CARD of RIG-I; therefore, the RIG-I splice variant (RIG-I SV) protein loses TRIM25 binding, CARD ubiquitination and downstream signalling ability (Fig. 3) (*24*). RIG-I SV inhibits the multimerization of the wild-type RIG-I protein and IPS-1 interaction and shows a dominant negative effect on the RIG-I-mediated anti-viral IFN response (*24*). Thus, RIG-I SV acts as the off switch regulator of its own signalling pathway (*24*).

In addition to the IPS-1 adaptor molecule, RIG-I also binds to the inflammasome adaptor apoptosis-associated speck-like protein containing a CARD domain (ASC), also known as Pycard, in response to viral infection (*27*). ASC activates caspase-1, leading to the proteolytic processing of pro-IL-1β into mature, bioactive IL-1β (*28*). TRIM25 activity is dispensable for caspase-1 activation through ASC (*27*). Thus, RIG-I polyubiquitination by TRIM25 is dispensable for ASC inflammasome adaptor activation (*27*).

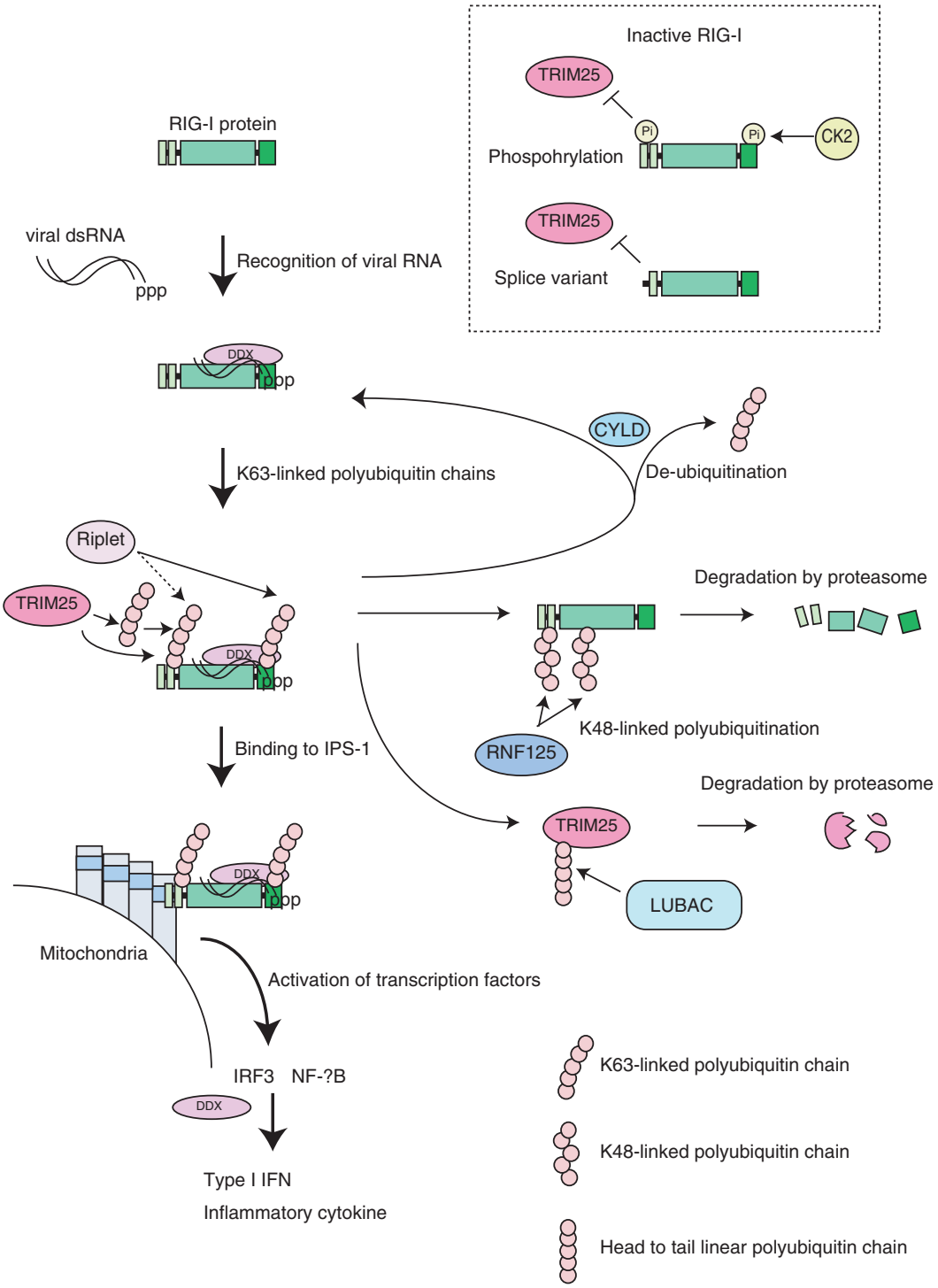

Fig. 3 Regulation of RIG-I by the ubiquitin chain. RIG-I binds to viral RNA together with other cofactors, such as DDX3. After the recognition of viral RNA, RIG-I changes its conformation and harbours K63-linked polyubiquitination by TRIM25 and Riplet. Polyubiquitination causes the activation of IPS-1, leading to the production of type I IFN. CYLD, a deubiquitin enzyme, removes the polyubiquitin chain of RIG-I. CK2 and other unknown kinase phosphorylate RIG-I, and the phosphorylated RIG-I protein is not polyubiquitinated by TRIM25. In addition, splice variant RIG-I (SV RIG-I) is not polyubiquitinated by TRIM25, and the SV RIG-I protein acts as a dominant negative form. RNF125 mediates the K48-linked polyubiquitinatioin of RIG-I, which causes the degradation of RIG-I by proteasomes. The LUBAC protein complex suppresses TRIM25 function by mediating the head-to-tail polyubiquitination of TRIM25.

However, RIG-I polyubiquitination is essential for NF-κB activation by RIG-I, which is required for IL-1β mRNA expression; thus, knockout of TRIM25 reduces the production of mature IL-1β (*4*, *19*, *27*).

Riplet ubiquitin ligase is essential for the activation of RIG-I

Riplet (also called Reul or RNF135) was isolated by yeast two-hybrid screening to isolate RIG-I CTD binding proteins (*29*). The Riplet protein is composed of N-terminal RING finger, C-terminal SPRY and PRY domains, and is similar to TRIM25 (Fig. 2). However, this protein lacks B-box, which is a typical feature of TRIM family proteins. Thus, the protein does not belong to the TRIM family. Riplet expression is observed in various tissues and cells such as DC, Mfs and MEF (*29*, *30*). Hu *et al*. (*31*) detected endogenous Riplet protein in human DC lysates. Riplet expression is induced in mouse bone marrow-derived DCs (BM-DCs) by polyIC stimulation, which is a double-stranded RNA analog; however, its expression is not changed in human fibroblast and HeLa cells (*29*).

The Riplet protein physically interacts with RIG-I CTD, and in some experimental conditions, it binds to RIG-I CARDs (*29*, *32*). The Riplet C-terminal region is responsible for this binding. Riplet mediates K63-linked polyubiquitination of RIG-I CTD, leading to the activation of RIG-I (Fig. 3) (*29*). The five CTD lysine residues at 849, 851, 888, 907 and 909 are important for the polyubiquitination and activation of RIG-I (*29*, *30*). In contrast, Gao *et al*. (*32*) reported that Riplet mediates K63-linked polyubiquitination of K154, K164 and K172 of RIG-I CARDs in their experimental conditions (Fig. 3).

In some strain backgrounds, RIG-I-deficient mice are embryonic lethal, but Riplet knockout mice are born at expected Mendelian ratios from Riplet$^{+/-}$ mice (*19*, *30*, *33*). Moreover, the development of DCs and Mfs is also normal in Riplet-deficient mice (*30*). Douglas *et al*. (*30*, *34*) reported that Riplet/RNF135 haploinsufficiency causes an overgrowth syndrome and learning disabilities in human: however, knockout of the Riplet gene in mice does not cause any apparent defects with regard to development. Knockout of Riplet severely reduces the production of type I IFN and abrogates the activation of RIG-I and RIG-I CTD polyubiquitination (*30*). Riplet knockout mice are more susceptible to VSV infection than wild-type mice. As IPS-1 and STING, Riplet is necessary for efficient, early type I IFN production *in vivo*, but it is dispensable for late type I IFN productions (*30*). This indicates the essential role that Riplet plays in the RIG-I-dependent innate immune response against RNA virus infection. Genetic evidence shows that knockout of either Riplet or TRIM25 destroyed the RIG-I-dependent innate immune response; therefore, both ubiquitin ligases are required for the activation of RIG-I in response to RNA virus infection (*22*, *30*). RLR pathways contribute to type I IFN expression in response to cytoplasmic DNA (*35*–*37*). However, Riplet-independent type I IFN expression pathway in response to cytoplasmic DNA exists in MEF (*30*).

Ubiquitin ligases target several proteins. For example, TRIM25 targets the proteolysis of 14-3-3 σ, a negative cell cycle regulator that causes G2 arrest, and thus, promotes breast tumour growth (*23*). Proteome analysis reveals that Riplet binds to the TRK-fused gene (TFG), which is a target of chromosome translocation in lymphoma (*38*–*40*). Pasmant *et al*. (*41*) reported that the Riplet/RNF135 gene is down-regulated in tumour Schwann cells from malignant peripheral nerve sheath tumours, and their study suggested the involvement of Riplet/RNF135 in an increased risk of malignancy observed in NF1 microdeletion patients. Thus, it is possible that Riplet targets not only RIG-I but also other proteins.

Negative regulators of RIG-I

The RNF125 (also called TRAC1) protein possesses a RING finger domain and functions as a ubiquitin ligase (*42*). Arimoto *et al*. (*43*) isolated RNF125 by yeast two-hybrid screening to obtain the protein that binds to UbcH8, which is an E2 ubiquitin-conjugating enzyme, and found that RNF125 also binds to RIG-I. Unlike Riplet and TRIM25, RNF125 ubiquitin ligase mediates K48-, but not K63-linked polyubiquitination of RIG-I, leading to the degradation of RIG-I by proteasomes (Fig. 3) (*43*). UbcH5c is possibly an E2 enzyme, which cooperates with RNF125, and UbcH8 acts as a negative factor in the RNF125-mediated polyubiquitination of RIG-I (*43*, *44*). Furthermore, RNF125 ubiquitinates MDA5, a member of RLRs, and the expression of RNF125 impairs MDA5-mediated signalling (*43*). RNF125 expression is induced by type I IFN and polyIC treatment. The increase in RNF125 mRNA expression correlates temporally with the decrease in RIG-I expression (*43*). Knockdown of RNF125 increases the type I IFN expression in response to viral infection (*43*). Since RNF125 is enhanced by type I IFN, the function of RNF125 constitutes a negative regulatory loop circuit for type I IFN production.

CYLD is a deubiquitinase that cleaves the K63-linked polyubiquitin chain. This protein acts as a negative regulator of NF-κB and Jun N-terminal kinase signalling pathways by cleaving the K63-linked polyubiquitin chains of NEMO, TRAF2 and BCL3 (*45*–*48*). Friedman *et al*. (*49*) performed a microarray analysis and found that the expression profile of RIG-I is correlated with that of CYLD. Moreover, they found that the CYLD protein physically interacts with RIG-I, TBK1 and IKKε, and deubiquitinates these proteins. CYLD inhibits SeV-induced type I IFN production. Thus, it is expected that CYLD attenuates the establishment of an anti-viral state (Fig. 3).

There are host and viral negative regulators for TRIM25. HOIL-1L and HOIP are members of the RING-IBR-RING (RBR) E3 ubiquitin ligase family and form complexes (*50*). HOIL-1L and HOIP form ubiquitin polymers through the linkage between the C- and N-termini of the ubiquitin molecules in order to assemble a head-to-tail linear polyubiquitin chain; thus,

the protein complex is designated as LUBAC (linear ubiquitin assembly complex) (*50*). LUBAC has the ability to induce polyubiquitination of TRIM25; it specifically suppresses TRIM25-mediated RIG-I ubiquitination by inducing TRIM25 degradation and inhibiting TRIM25 interaction with RIG-I (Fig. 3) (*51*). Excessive production of IFNs or inflammatory cytokines is destructive rather than protective; thus, an absolute regulation of the immune signalling pathway is essential for a successful immune response against viral infections. HOIL-1L- and HOIP-mediated suppression of TRIM25 would be important for the absolute regulation of an immune response (*51*).

Viruses have evolved sophisticated mechanisms to evade the host IFN system. There are several virus-encoded IFN antagonists that inhibit host innate anti-viral responses. NS1 of the influenza A virus is one of the IFN antagonists (*52*, *53*). It sequestrates viral dsRNA from cellular sensors including RIG-I (*52*). In addition, it interacts with the coiled-coil region of TRIM25 and blocks TRIM25 multimerization and RIG-I CARD polyubiquitination (*54*).

Perspectives

Several ubiquitin-like proteins (UBLs) exist. ISG15 is a UBL and is induced in response to viral infection (*55*). Several anti-viral proteins are modified by ISG15, including RIG-I (*44*, *55*). UbcH8 is an E2 enzyme that promotes ISG15 conjugation to RIG-I (*44*). However, ISG15 knockout mice do not either reduce immunological functions or decrease anti-viral activity (*56*). Thus, the physiological role of ISG15 conjugation to RIG-I remains unknown.

In addition, the RIG-I protein is modified by phosphorylation. The T170 residue of RIG-I is phosphorylated under normal conditions, and phosphorylation is reduced after SeV infection (*24*). Phosphorylation of RIG-I CARDs inhibits the TRIM25-mediated polyubiquitination (Fig. 3). Thus, Gack *et al*. suggested that dephosphorylation of RIG-I permits the TRIM25 binding and TRIM25-mediated polyubiquitination of RIG-I, allowing RIG-I to form a stable complex with IPS-1 in order to trigger an IFN-mediated anti-viral innate immune response. However, the kinase and phosphatase that target RIG-I N-terminal CARDs are still unknown. In addition to RIG-I CARDs, RIG-I CTD is regulated by phosphorylation. In resting cells, casein kinase II (CK2) phosphorylates T770, and S854 and S855 (*57*). The phosphorylation of RIG-I CTD suppresses the RIG-I-mediated signalling (Fig. 3) (*57*). Following viral infection, phosphatases cause dephosphorylation of the RIG-I CTD, leading to the activation of RIG-I-mediated signalling (*57*).

RIG-I requires several cofactors. High mobility group box proteins are required for the RIG-I to recognize viral RNA (*58*). DDX3 and DDX60 are non-RLR helicases that are involved in RLR signallings, and play pivotal roles in RIG-I-mediated signalling (Fig. 3) (*59*–*62*). It remains to be determined whether the post-translational modification of RIG-I affects the interaction with those co-factors.

Riplet ubiquitinates RIG-I CTD. The molecular mechanism of how the Riplet-dependent polyubiquitination of RIG-I CTD triggers the downstream signalling remains to be determined yet. RIG-I CTD has two functions. In the absence of viral RNA, RIG-I CTD suppresses the activation of RIG-I CARDs. Following viral infection, RIG-I CTD binds to viral RNA, leading to the conformational changes and ultimately removal of the suppression. It is possible that CTD polyubiquitination affects both functions of RIG-I CTD.

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Conflict of Interest
None declared.

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