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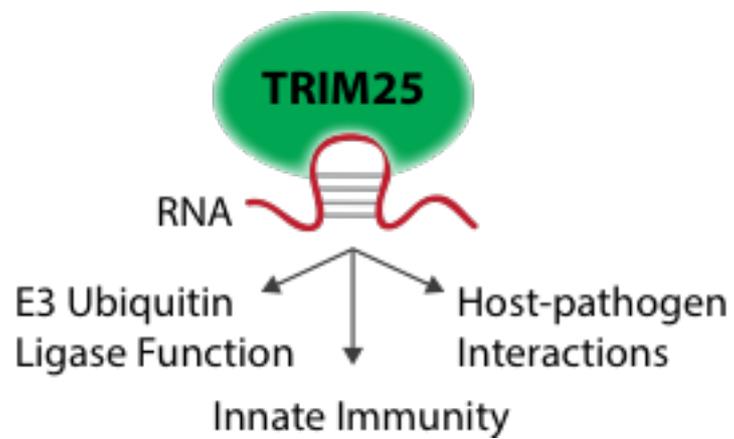
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ABSTRACT

The innate immune system is the body's first line of defence against viruses, with pattern recognition receptors recognising molecules unique to viruses and triggering the expression of interferons and other anti-viral cytokines, leading to the formation of an anti-viral state. The Tripartite Motif Containing 25 (TRIM25) is an E3 ubiquitin ligase thought to be a key component in the activation of signalling by the pattern recognition receptor Retinoic Acid-Inducible Gene I Protein (RIG-I). TRIM25 has recently been identified as an RNA-binding protein, raising the question of whether its RNA-binding activity is important for its role in innate immunity. Here, we review TRIM25's mechanisms and pathways in non-infected and infected cells. We also introduce models that explain how TRIM25 binding to RNA could modulate its functions and play part in the antiviral response. These findings have opened new lines of investigations into functional and molecular roles of TRIM25 and other E3 ubiquitin ligases in cell biology and control of pathogenic infections.

GRAPHICAL ABSTRACT/CAPTION



E3 ubiquitin ligase TRIM25 is newly identified RNA-binding protein which is emerging as a key factor in the innate immune response to RNA viruses.

INTRODUCTION

Humans, as well as other organisms, must protect themselves from infection by viruses and other pathogens. To do this, they developed a robust means of distinguishing self from non-self and responding accordingly. Their primary means of doing so is through the immune system. The immune system is divided into two parts, the adaptive and innate immune systems. Adaptive immunity consists of antigen-specific receptor-mediated responses to specific pathogens, while innate immunity consists of pathways for the detection of factors common to many pathogens as well as physical barriers such as the skin. In general, the innate immune system is fast-acting, involving elements that are ubiquitously expressed in somatic cells and acts in early infection. In contrast, the adaptive immune response is slower, acting in late infection, as there are only small numbers of each antigen-specific receptor and the cells expressing these must undergo clonal expansion before an effective response can be mounted¹.

The innate immune system involves non-antigen-specific pattern recognition receptors (PRRs) that recognise pathogen associated molecular patterns (PAMPs) that are common to many pathogens, but not found in host cells. Upon detection of PAMPs by PRRs, a signalling cascade is initiated that results in the expression of various anti-pathogenic molecules such as interferons (IFNs) and other cytokines, resulting in activation of host defences such as inflammation and recruitment of the adaptive immune system. Importantly, no single pathogen is recognised by a single PRR and biologically unrelated pathogens can be recognised by the same PRR, allowing for a fast and efficient response to any pathogen². Different classes of pathogens are recognised by different PAMPs, for example viruses are recognised through glycoproteins and various DNA and RNA species³. Bacteria through lipoproteins⁴, peptidoglycan and derivatives⁵, CpG DNA⁶, lipopolysaccharides (LPS)⁷ and

proteins such as flagellin⁸. Fungi are generally recognised through cell wall or cell surface components such as phospholipomannan⁹ or β -glycan¹⁰.

There are a wide variety of PRRs found in humans that recognise different classes of PAMPs. PRRs are generally classed into the membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as well as the cytoplasmic RIG-I like receptors (RLRs), Nod-like receptors (NLRs) and cytosolic DNA sensors such as cGAS. The RLR family consists of three members; Retinoic Acid-Inducible Gene I (RIG-I), Melanoma Differentiation-Associated gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2). The RLRs primarily recognise viral RNAs, with RIG-I recognising RNAs with a 5'-triphosphate (5'ppp) moiety and MDA5 recognising long dsRNAs². Signalling through PRR pathways generally results in the activation of transcriptional activators such as IRF-3, IRF-7 and NF- κ B in the cytoplasm. These transcription factors translocate to the nucleus where they induce expression of various proteins that contribute to the establishment of an anti-pathogenic state such as IFNs and pro-inflammatory cytokines. This leads to robust host defences against virus infection, resulting in large-scale gene expression changes and the formation of an antiviral state².

Here we will focus on the Tri-partite Motif containing protein 25 (TRIM25), which was thought to be involved in the RIG-I/INF pathway. Recent findings showed that TRIM25 is an RNA-binding protein^{11, 12} and put into question its major role in the RIG-I activation^{13, 14}. We will elaborate on what is known about TRIM25 and its RNA-binding activity and speculate how it can affect its antiviral properties. This protein represents a large group of proteins with newly identified RNA-binding potential and can serve as an exemplar towards critical evaluation of protein functions in biological systems.

TRIM25'S ROLE IN INNATE IMMUNITY

TRIM25 is an E3 ubiquitin ligase

The Tri-partite Motif (TRIM) family is a large (>80 members in humans) group of E3 ubiquitin ligase proteins that share a common domain structure. Human TRIM25 is a 630 amino acid, 71kDa E3 ubiquitin ligase that is widely expressed across human cell types and is conserved among vertebrates including fish, birds and mammals¹⁵⁻¹⁹. Like other TRIM family proteins, it consists of an N-terminal zinc-finger Really Interesting New Gene (RING) domain, responsible for its E3 ubiquitin ligase activity, two B-box zinc finger domains of unknown function, a coiled-coil domain (CCD), responsible for homo and heterodimerization, with a linker domain leading to a C-terminal associated with SPRY/SPIA and the RYanodine Receptor (PRY/SPRY) domain, responsible for protein-protein interactions.

The primary role of TRIM E3 ubiquitin ligases is to catalyse the addition of polyubiquitin chains or single ubiquitin monomers (monoubiquitination) to lysine residues on their target proteins. Ubiquitin is a 76aa, 8.5kDa protein and polyubiquitin chains are made by the formation of isopeptide bonds between the ubiquitin C-terminal glycine and one of the 7 lysine residues present in the protein^{20, 21}. It has also been shown that polyubiquitin chains can form in a 'head-to-tail' manner via the N-terminal methionine residue²². The addition of ubiquitin monomers to a target protein or extension of a polyubiquitin chain involves three types of proteins; E1 activating enzyme, E2 conjugating and E3 ubiquitin ligase²³. The multiple lysines present in ubiquitin allow the formation of several different types of polyubiquitin chains, each of which has different functionality. The most well studied are straight, homogenous K48-linked polyubiquitination, which leads to targeting of proteins for degradation via the

proteasome, and K63-linked polyubiquitination which is used in many intracellular signalling pathways, such as activation of NF- κ B and induction of RIG-I/IKK/Interferon type I²⁴.

TRIM25 forms an antiparallel dimer mediated by its CCD, with the RING domain of each monomer at opposite ends of the dimer and the PRY/SPRY domains positioned at the centre via the CCD-PRY/SPRY linker²⁵ (Figure 1). Further work indicated that the RING domain of TRIM25 must dimerize in order to catalyse polyubiquitin chain formation, implying that higher-order assembly of TRIM25 dimers is required for its activity²⁶. Two separate mechanisms of higher-order assembly were proposed. Firstly, an 'end-to-end' model in which RING domains on each end of the dimer interact with RING domains from separate dimers. Secondly, a 'tetramer' model in which TRIM25 dimers effectively stack on top of each other with RING domains on either end of one dimer interacting with both RING domains from another dimer²⁶. When human TRIM25 RING domain was crystallised with an ubiquitin-charged E2 conjugating enzyme, UBE2D1, it was shown to form a dimer with both RING monomers contacting the ubiquitin molecule²⁷. Crystal structures have also been generated for the PRY/SPRY domain of mouse TRIM25, showing that its overall structure is that of two anti-parallel β -sheets in a sandwich type conformation, similarly to PRY/SPRY domains found in other proteins²⁸.

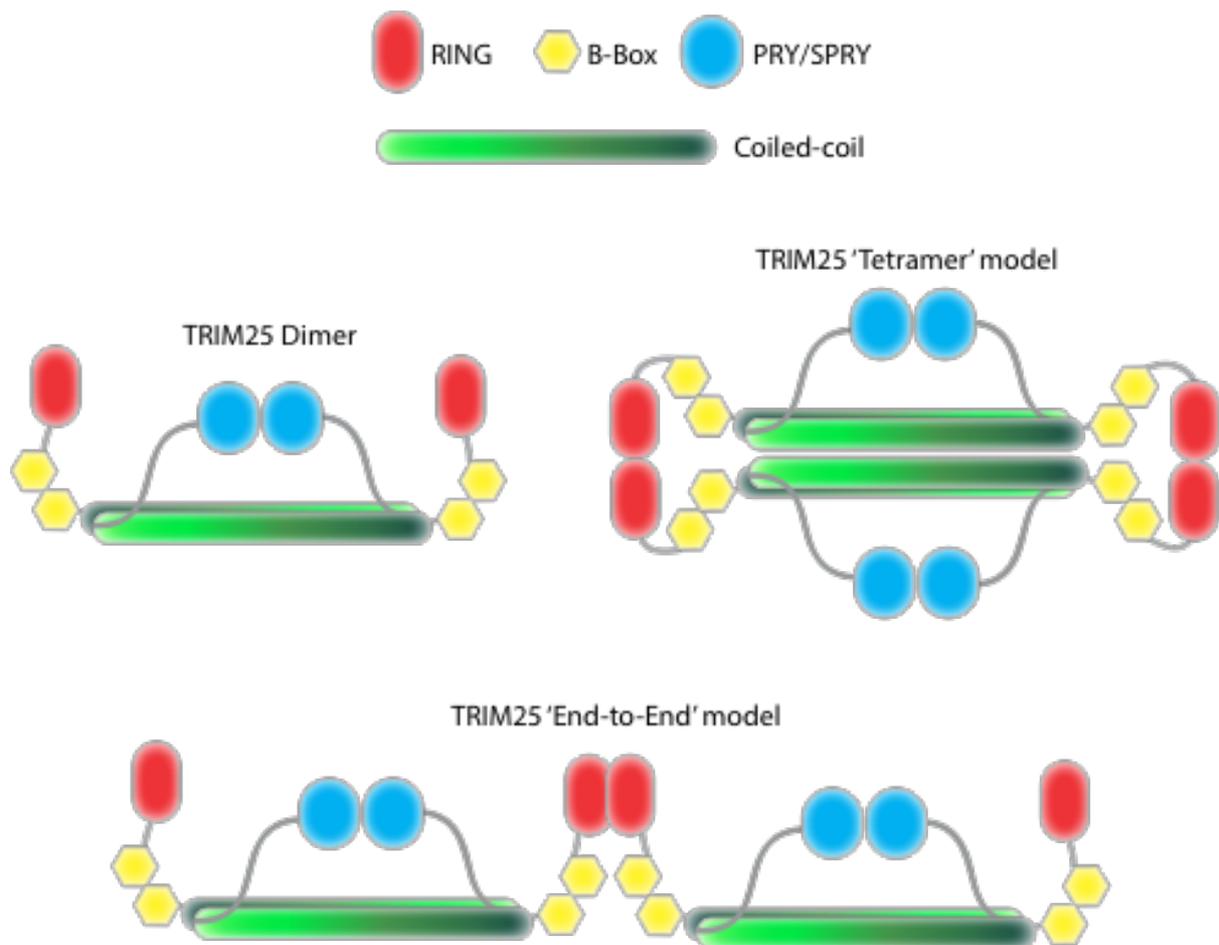


Figure 1. Possible models of dimerization of the RING domains of TRIM25 dimers as proposed by Sanchez et al. Due to the anti-parallel structure of the TRIM25 dimer, RING domains from TRIM25 molecules found in the same dimer cannot dimerize themselves. This implies that higher order oligomerisation is required for TRIM25's catalytic activity.

Miscellaneous roles of TRIM25 in the cell

TRIM25 has been found to perform many roles in the cell and has been implicated in several cancers²⁹⁻³⁸. TRIM25 was initially identified as a protein responsive to oestrogen in a screen for regions of DNA bound by the oestrogen receptor and was shown to be upregulated in oestrogen receptor-positive mammary cells³⁹. Since then, TRIM25 has been found to have an

important role in the defence against viruses and has been implicated in the activation of the RIG-I/INF pathway, which will be discussed further in the next section.

TRIM25 has been shown to play other functions in innate immunity. It was shown to positively regulate Melanoma Differentiation-Associated gene 5 (MDA5)-mediated signalling through tumour Necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin protein ligase, leading to activation of NF- κ B⁴⁰. TRIM25 has also been shown to enhance the activity of Zinc-finger Antiviral Protein (ZAP), which is a known antiviral protein, this interaction will be addressed later in this review^{41, 42}. In contrast, TRIM25 can also be involved in the dampening of RIG-I signalling. The ubiquitin-like FAT10 (also known as Ubiquitin D) forms a complex with TRIM25/RIG-I that sequesters RIG-I away from the mitochondria and causes it to form insoluble aggregates to prevent further signal transduction⁴³. TRIM25 stabilises FAT10, which is usually unstable, by preventing its proteasome-mediated degradation⁴³.

TRIM25's role in the RIG-I/INF pathway

RIG-I recognises RNAs with a 5'- di- or triphosphate (5'pp/5'ppp) moiety triggering downstream signalling pathways via the adaptor Mitochondrial Antiviral Signalling protein (MAVS, also known as Interferon Beta Promoter Stimulator Protein 1 (IPS1), CARD Adapter Inducing Interferon Beta (CARDIF) or Virus-Induced-Signalling Adapter (VISA)), leading to activation of IRF-3, IRF-7, NF- κ B and INF type 1 expression⁴⁴. To prevent inappropriate activation of innate immune responses, RIG-I signalling must be repressed in the absence of 5'ppp-RNA. This is achieved by several auto-repression mechanisms⁴⁵⁻⁴⁸. Upon binding to 5'ppp-RNA, RIG-I translocates down the dsRNA stem, allowing multiple RIG-I molecules to form a 'beads-on-a-string' complex on the RNA^{49, 50}. This oligomerisation activity is ATP hydrolysis-dependent and increases the strength of the resulting type I IFN response⁵¹.

Oligomerisation of RIG-I is also important for the formation of tetramers of the 2CARD (two Caspase Activation and Recruitment Domains), which is required for activation of downstream signalling via MAVS through interaction with MAVS' own CARD⁵². A critical process in the formation of the 2CARD tetramer and the activation of MAVS is the TRIM25-mediated K63-linked polyubiquitination of the 2CARD, which has been shown to stabilise the 2CARD tetramer and enhance the formation of MAVS filaments that are required for signalling^{53, 54} (Figure 2). It has been showed that another E3 ubiquitin ligase Riplet is also necessary for release of the 2CARD and RIG-I activation^{55, 56}.

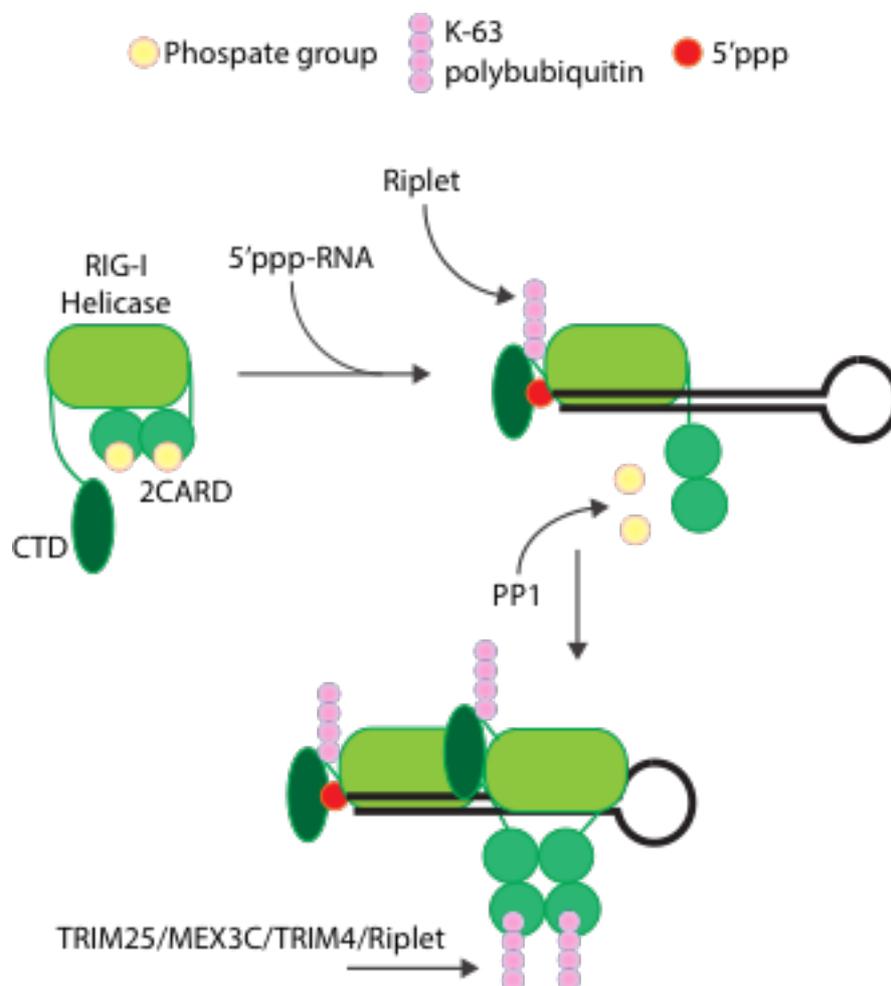


Figure 2. Model of activation of RIG-I based on the sequential ubiquitination model proposed by Okamoto et al. Upon recognition of 5'ppp-dsRNA, RIG-I undergoes a conformational

change and the Helicase-CTD linker can be ubiquitinated by Riplet. This releases the 2CARDs from auto-repression and allows their dephosphorylation by PP1. Ubiquitination of the linker also promotes the assembly of other RIG-I molecules along the dsRNA, although the CTD of only one molecule can bind the 5'ppp moiety. The 2CARDs from RIG-I molecules assembled along the dsRNA can form 'tetramer' structures that are stabilised by K63-linked polyubiquitination of the 2CARD. This ubiquitination is performed by several E3 ubiquitin ligases including TRIM25, MEX3C, TRIM4 and Riplet. This tetramer structure interacts with the CARDs of MAVS and promotes its oligomerisation, leading to further downstream signalling and expression of type I interferon.

Initially, TRIM25 was identified as the key E3 ubiquitin ligase for the ubiquitination of the RIG-I 2CARD in both mice and humans by Gack *et al.*⁵⁷. In this report, 6 lysine residues on the RIG-I 2CARD that underwent K63-linked polyubiquitination were identified by mass spectrometry (MS); K99, K169, K172, K181, K190 and K193. Of these, only mutation of K172 to arginine resulted in a reduction in polyubiquitination of the 2CARD and a concomitant reduction in activation of the NF- κ B and IFN β promoters when the 2CARD was transfected into HEK293 cells. This suggested that polyubiquitination of this residue is key for RIG-I 2CARD-mediated signalling. However, later work suggested that a RIG-I K172R mutant was fully functional and could efficiently trigger innate immune signalling in response to Sendai virus infection, suggesting that K172 may not be required for signalling⁵⁸. Gack *et al.* also identified TRIM25 as a binding partner of the 2CARD in co-immunoprecipitation (Co-IP) experiments and demonstrated that this interaction was mediated by the C-terminal SPRY domain of TRIM25. Furthermore, knockdown of TRIM25 using RNA interference (RNAi) resulted in a reduction of

RIG-I 2CARD polyubiquitination and IFN β promoter activity in response to 2CARD transfection. Finally, production of IFN β in TRIM25 knockout (KO) mouse embryonic fibroblast (MEF) cells upon Sendai virus infection was reduced compared to WT cells, while replication of VSV was increased in the TRIM25 KO cells compared to WT⁵⁷.

Further work has underlined the role that TRIM25 plays in ubiquitination of the RIG-I 2CARD. Mutation of T55 in the first RIG-I CARD was found to abolish the TRIM25-2CARD interaction and this was required for TRIM25-mediated polyubiquitination of the 2CARD⁵⁹. *In vitro* reconstitution of the human RIG-I pathway suggested that direct conjugation of K63-linked polyubiquitin chains to the 2CARD was not necessary for activation of signalling as unanchored K63-linked chains generated by TRIM25 can be bound by the 2CARD and this is sufficient for activation of signalling⁶⁰. Transfection of TRIM25 into HEK293T KO cells enhanced IFN β promoter activity by about 2-fold compared to TRIM25 KO cells alone in response to transfection of the RIG-I 2CARD. However, a roughly 20-fold induction of IFN β promoter activity when compared to cells not transfected with the RIG-I 2CARD was seen in the TRIM25 KO HEK293T cells, suggesting that there may be some redundancy in the role of TRIM25 in RIG-I signalling²⁶.

Importantly, other E3 ubiquitin ligases have also been implicated in the ubiquitination of the RIG-I 2CARD. In addition to its role in ubiquitinating the RIG-I helicase-CTD linker, Riplet (also known as RIG-I E3 Ubiquitin Ligase (REUL)) was found to polyubiquitinate the RIG-I 2CARD at lysines 154, 164 and 172. Knockdown of Riplet was shown to inhibit IFN β expression in response to Sendai virus infection and resulted in increased replication of VSV⁶¹. Knockdown of TRIM4 was found to inhibit activation of the IFN β promoter in response to overexpression of RIG-I or infection with Sendai virus⁶². This study also found that TRIM4 primarily targeted

K164 and K172 of the RIG-I 2CARD for polyubiquitination. A systems biology approach combined with experimental validation identified the ubiquitination of K164 and K172 by TRIM25 and TRIM4 as being key for RIG-I signalling activation, with TRIM25 and TRIM4 working synergistically to optimise activation⁶³. Another E3 ubiquitin ligase, Mex-3 RNA Binding Family Member C (MEX3C), was found to co-localise with RIG-I in antiviral stress granules and ubiquitinate the RIG-I 2CARD at K48, K99 and K169. Cells derived from MEX3C KO mice were shown to have impaired activation of the IFN β promoter in response to infection with VSV and Newcastle Disease Virus (NDV) while activation after infection with Encephalomyocarditis Virus (EMCV, recognised by MDA5) was unaffected⁶⁴. Shi *et al.* showed that in a HEK293T cell-free system knockout of Riplet abrogates RIG-I mediated aggregation of MAVS in response to VSV genomic RNA while knockout of TRIM25, MEX3C or TRIM4 did not abrogate aggregation. However, MAVS aggregation could still be triggered in response to addition of the RIG-I 2CARD even in the absence of Riplet⁶⁵. This may suggest that activation of full-length RIG-I signalling in response to viral RNA requires Riplet activity, but Riplet is redundant with the other E3 ubiquitin ligases in the ubiquitination of the RIG-I 2CARD alone.

Okamoto *et al.* proposed a 'sequential ubiquitination' mechanism to explain the activation of RIG-I by polyubiquitination by different E3 ubiquitin ligases (Figure 2)⁶⁶. In this model, Riplet first ubiquitinates the helicase-CTD linker at K788 upon RIG-I RNA binding to release the 2CARD from auto-repression and this step is required for the E3 ligases to access the 2CARD. This is followed by ubiquitination at various sites on the 2CARD by Riplet, TRIM25, TRIM4 and MEX3C. This would fit with several recent observations^{13, 14, 65} that Riplet is absolutely required for efficient RIG-I signalling activation, while TRIM25 is not, as well as explaining why there is seemingly redundancy between the other E3 ligases. In particular, recent work by Cadena *et al.* showed that deletion of TRIM25 from HEK293T and MEF cells results in an

increase, not a reduction in RIG-I activation in response to 5'ppp-RNA. In contrast, deletion of Riplet from the same cell line results in complete abolition of RIG-I signalling, to the same level as deletion of RIG-I itself¹⁴. The same was found to be true for expression of IFN β mRNA in response to infection with Sendai virus, with deletion of TRIM25 in this case resulting in a significant increase in IFN β expression¹⁴. Interestingly this study also found that TRIM25 was not capable of ubiquitinating RIG-I *in vitro*, even in the presence of 5'ppp-dsRNA. This goes against previous work which has shown TRIM25 to be capable of ubiquitinating the RIG-I 2CARD *in vitro*, although these studies used purified 2CARD and not full-length RIG-I^{26, 67}

In order to be able to replicate efficiently, most human viruses have evolved mechanisms for avoiding the triggering of innate immune signalling. Many RNA viruses that can be recognised by RIG-I have developed mechanisms of inhibiting RIG-I signalling at different stages of the pathway. The NS1 protein of IAV is known to block ubiquitination of RIG-I through interactions with TRIM25, Riplet and RIG-I itself⁶⁸⁻⁷⁰. IAV NS1 binds to the coiled-coil domain of TRIM25, and this was thought to prevent its multimerisation which it requires for its catalytic activity⁶⁸. However, more recent structural data indicates that the inhibitory effect of NS1 on TRIM25 activity is due to disruption of interactions between the TRIM25 PRY/SPRY and coiled-coil domains that are required for RIG-I ubiquitination activity, not a lack of TRIM25 multimerisation⁷¹. IAV NS1 is capable of inhibiting Riplet activity in both mouse and human cells and this is likely important for the overall inhibitory effect of NS1 on RIG-I signalling⁷⁰.

Other viruses also target TRIM25-mediated RIG-I ubiquitination for inhibition in order to dampen the innate immune response. The V proteins of several paramyxoviruses (Nipah, measles, Sendai and parainfluenza viruses) were found to interact with both the RIG-I 2CARD and the SPRY domain of TRIM25, preventing TRIM25-mediated ubiquitination of RIG-I⁷².

Similarly, RSV NS1 and N protein from SARS-CoV were also found to interfere with TRIM25 activity^{73, 74}.

TRIM25 can be targeted for inhibition by the host cell in order to downregulate RIG-I signalling to prevent excessive inflammation and IFN responses. The Linear Ubiquitin Assembly Complex (LUBAC), composed of Heme-Oxidized IRP2 Ubiquitin Ligase 1 (HOIL-1) and HOIL-1 Interacting Protein (HOIP), competes with TRIM25 for binding to RIG-I and also targets TRIM25 for degradation via the proteasome⁷⁵.

The partnership between TRIM25 and ZAP

ZAP is a well know IFN induced antiviral protein that has been shown to inhibit the replication of many different types of viruses, such as SINV⁷⁶, Ebola⁷⁷, HIV⁷⁸ and Hepatitis B⁷⁹. However, this inhibition is specific as some viruses such as Herpes simplex 1 and vesicular stomatitis virus are not repressed by ZAP⁷⁶. ZAP was found to bind CG dinucleotides in viral RNAs and inhibit virus replication⁸⁰. The frequency of CG dinucleotides is suppressed in host RNAs allowing the distinction of self from non-self. However, RNA viruses have evolved to suppress their CG dinucleotide content, thereby evading the host response. ZAP's ability to suppress a virus was shown to correlate with the viral RNA CG content⁸⁰. This inhibition seems to be mainly through reducing the levels of viral mRNAs, however the mechanisms are still unknown, that said ZAP has been shown to bind viral RNAs directly⁸¹ and to promote translational repression⁸². Additionally, ZAP can degrade viral RNAs by recruiting proteins to remove the poly-A tail and trigger de-capping of the mRNA and finally recruit the mRNA degradation machinery^{78, 83, 84}. ZAP is targeted to stress granules during viral infection, which is important for its antiviral activity^{85, 86}. Tang *et al.* found that NS1 binds to and inhibits ZAP's ability to block IAV mRNA translation and degradation, by interfering with its RNA binding

ability⁸⁷. ZAP has also been shown to interact with and activate RIG-I by promoting its oligomerisation in human cells⁸⁸. However, the RIG-I-dependent type I interferon response does not seem to be important in the antiviral defence against all viruses and in all cell types such as primary mouse cells⁸⁵, HBV infection of human liver cancer cells⁷⁹, and Xenotropic murine leukemia virus-related virus (XMRV) infection of human cells⁸⁹. ZAP exists as four isoforms (L, XL, M, S) with ZAPL being the most abundant in normal cells, while ZAPS is upregulated the most following IFN- α treatment. ZAPL and ZAPXL show higher antiviral activity against some viruses, but not all. All isoforms induce type I IFN expression similarly⁹⁰. ZAP does not have enzymatic activity⁹¹ and is thought to require co-factors. TRIM25 has been shown to be required for ZAP's antiviral function^{41,42}. TRIM25 was found to interact with ZAP through its SPRY domain, with both the ubiquitin ligase activity and multimerisation of TRIM25 being important in enhancing ZAP's antiviral activity (Figure 3). TRIM25 was also shown to be dependent on ZAP for inhibiting SINV replication⁴². TRIM25 was shown to mediate K48- and K63-linked polyubiquitination of ZAP, but only K68-linked ubiquitination enhanced its antiviral activity^{41, 42}. TRIM25 was found to do this by aiding the RNA binding ability of ZAP, leading to inhibition of viral translation^{41, 42}. However, although TRIM25's E3 ligase activity was needed to enhance ZAP-mediated inhibition of Sindbis virus RNA translation, ubiquitination of ZAP itself did not seem to directly affect antiviral activity⁴². TRIM25 was also found to be required for ZAP's antiviral activity against a HIV-1 virus with clustered CpGs⁸⁰. Another co-factor KHNYN (a cytoplasmic protein of previous unknown function) is thought to form a complex with ZAP and TRIM25 to inhibit the replication of HIV-1 with clustered CpGs. KHNYN required both TRIM25 and ZAP for its antiviral activity. However, KHNYN was not needed in the inhibition of SINV replication⁹². The authors

speculated that KHNYN could be needed for ZAP mediated RNA degradation, but not translation inhibition.

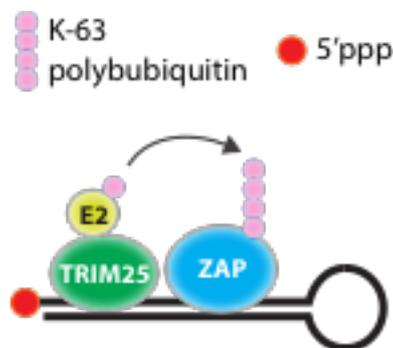


Figure 3. RNA-binding is necessary for TRIM25's E3 ligase ubiquitin activity and activation of its partner – RNA-binding protein ZAP.

RNA-BINDING ROLES OF TRIM25

TRIM25 is an RNA-binding protein

TRIM25 was initially discovered to be an RNA-binding protein (RBP) in a screen of mRNA binding proteins in HeLa cells¹¹. Proteins were cross-linked to RNA via UV irradiation and mRNAs were isolated from the cell lysate with oligo(dT) probes before bound proteins were analysed by mass spectrometry¹¹. A similar strategy also identified mouse Trim25 as an RBP in mouse embryonic stem cells⁹³. Binding of human TRIM25 to RNA was further validated by immunoprecipitation (IP) of TRIM25 followed by radiolabelling of RNA. Signal from radiolabelled RNA after TRIM25 IP was reduced in cells in which TRIM25 had been knocked-down by RNAi, indicating that TRIM25 was binding to RNA⁹³. This study also tested several truncation mutants of TRIM25 for their RNA binding activity. Truncations in which the N-terminal RING and B-box domains or the C-terminal PRY/SPRY domain were deleted were capable of binding RNA in this assay as seen by signal from radiolabelled RNA corresponding

to the size of the constructs as visualised by western blot. Conversely, any mutants in which the CCD was deleted were incapable of binding RNA, suggesting that the CCD could play a role in TRIM25 RNA binding activity⁹³.

We independently discovered TRIM25's RNA binding potential and showed the first RNA-dependent function for TRIM25 in the Lin28-mediated degradation of pre-let-7a-1⁹⁴. TRIM25 was found to bind to the conserved terminal loop (CTL) of pre-let-7 and activate LIN28a, which leads to more efficient TuT4-mediated uridylation and thereby degradation. Subsequently, we showed that TRIM25 binds hundreds of coding and non-coding RNAs and that the PRY/SPRY domain of TRIM25, specifically amino acids 470-508 (VALSECYTVASVAEMPQNYRPHPQRFTYCSQVLGLHCYK) of human TRIM25, is required for binding to RNA¹² (Figure 4). TRIM25 Δ RBD (delta RNA binding domain), in which these amino acids were deleted, was unable to bind to pre-let-7a-1 in RNA pulldown or EMSA and exhibited loss of binding to target mRNAs and miRNAs in RIP experiments. Interestingly, TRIM25 Δ RBD was capable of associating with TRIM25 WT, suggesting that dimerization with two intact PRY/SPRY domains is necessary for RNA binding (Figure 4). Aligning amino acids 470-508 with the crystal structure of the PRY/SPRY domain from mouse Trim25 (the composition of which is highly similar to humans) shows that this region comprises β sheet 1, 2 and 3 as well as loops 2, 3 and 4 of the PRY/SPRY domain²⁸.

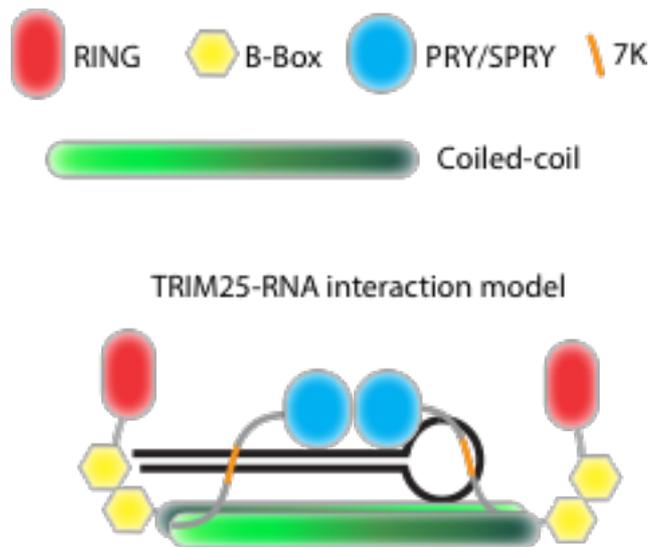


Figure 4. Model of TRIM25 binding to RNA. The major point of contact between RNA and TRIM25 is the PRY/SPRY domain. Additional RNA-binding activity has been attributed to a 7 Lysine peptide (7K) and the Coiled-coil domain. TRIM25 has been shown to bind both single and double-stranded RNAs.

The requirement for the PRY/SPRY domain was confirmed by a recent study by Sanchez *et al.* that found a TRIM25 construct consisting of only the RING, B-box and CCD did not co-purify with nucleic acids, while full-length TRIM25 did⁶⁷. These and our results contradict the earlier finding by Kwon *et al.* that TRIM25 without the PRY/SPRY domain precipitated with RNA⁹³. It is worth noting that the latter construct did include the CCD-PRY/SPRY ‘linker’, while the former did not. Sanchez *et al.* also identified a motif containing 7 lysine residues (amino acids 381-392, KKVSKEEKSKK, termed 7K) in the linker region that seemingly contributed to RNA binding⁶⁷ (Figure 4). Mutating all the lysines in 7K led to a significant decrease, although not a complete abolition, of RNA binding in EMSA experiments. A CCD-7K construct was not sufficient for RNA binding whereas a CCD-SPRY construct was (with an even higher affinity for RNA than full-length TRIM25) underlining the requirement for the PRY/SPRY domain⁶⁷. Taken

together, these results indicate that the PRY/SPRY domain is essential for TRIM25's RNA binding and that binding is enhanced by the 7K motif in the CCD-PRY/SPRY linker and by CCD-mediated dimerization. It remains unclear exactly which amino acids are involved in direct contact with the RNA and how many other PRY/SPRY domains harbour RNA-binding activity.

TRIM25 inhibits the IAV RNA polymerase

Recent work has identified a role for the RNA binding of TRIM25 in the restriction of IAV⁹⁵. TRIM25 proteins from human and gibbon were overexpressed in Crandell Reese Feline Kidney (CRFK) cells that were subsequently infected with IAV. Levels of viral proteins were reduced in cells overexpressing TRIM25, with gibbon TRIM25 having a greater effect than human TRIM25, and this was rescued by expression of WT NS1 protein. Virus titres and levels of viral RNAs were also reduced upon overexpression of TRIM25, again with gibbon TRIM25 showing a larger effect and this was also seen for TRIM25 mutants lacking ubiquitin ligase activity (TRIM25 C13A/C16A)⁹⁵. Deletion of RIG-I and TRIM25 from human A549 lung cells resulted in increased viral titres, viral protein, and vRNA levels, which could be rescued by expression of human or gibbon TRIM25. Interestingly, deletion of RIG-I alone had no effect, suggesting this function of TRIM25 is RIG-I-independent⁹⁵. Further evidence that RIG-I is not required for this activity was shown by the use of a viral minigenome assay in HEK293T Δ RIG-I cells. Overexpression of both human and gibbon TRIM25 in these cells reduced expression of a luciferase reporter that could only be expressed in the context of IAV RNA polymerase activity, indicating that TRIM25 was inhibiting the activity of the IAV polymerase⁹⁵. TRIM25 was subsequently shown to bind to IAV viral RNPs (vRNPs) in an RNA-dependent manner and that gibbon TRIM25 binds to vRNPs more efficiently than human TRIM25. In addition to this,

purified human or gibbon TRIM25 was able to inhibit viral mRNA chain elongation *in vitro*, again with gibbon TRIM25 doing this more efficiently, reflecting its higher ability to restrict viral replication and protein production as well as its stronger binding to vRNPs⁹⁵. The authors of this study proposed a mechanism whereby TRIM25 blocks the IAV RNA polymerase from moving down the vRNA template and prevents the onset of chain elongation, thus restricting the virus.

Dengue virus RNA inhibits TRIM25

Dengue virus has been shown to take advantage of TRIM25's RNA-binding by expressing a subgenomic flavivirus RNA (sfRNA) that binds to and inhibits TRIM25 preventing RIG-I activation and type I IFN induction. This led to an increase in the epidemiological fitness of a new Dengue virus clade (PR-2B) compared to the previous one (PR-1)⁹⁶. The authors found that the sfRNA from PR-2B bound more than that from PR-1 by EMSA and showed that TRIM25 bound to PR-2B sfRNA was more ubiquitinated than that bound by PR-1 sfRNA. The sfRNA did not affect TRIM25's ability to co-immunoprecipitate with RIG-I, but TRIM25 bound to RIG-I was still ubiquitinated more in the presence of PR-2B sfRNA than PR1 sfRNA. However, it was unclear if this difference was because of the increased level of TRIM25 pulled down. It was concluded that the increased ubiquitination of TRIM25 was due to the inability of USP15 to de-ubiquitinate TRIM25 thereby preventing RIG-I activation and type I IFN induction. As mentioned previously, LUBAC binds to and ubiquitinates TRIM25 targeting it for degradation by the proteasome, preventing it from ubiquitinating and activating RIG-I⁷⁵. However, Manokaran *et al.* did not test the nature of TRIM25 ubiquitination. In fact, more TRIM25 was co-immunoprecipitated with RIG-I in the presence of PR-2B sfRNA. This

ubiquitination band could be the reported auto-ubiquitination of TRIM25, which has as yet to be found a function for^{12, 26, 75}.

TRIM25 requires RNA binding for its E3 ubiquitin ligase activity

We recently found that TRIM25 requires RNA binding to be able to efficiently auto-ubiquitinate itself and to ubiquitinate ZAP, one of its targets¹². TRIM25 showed efficient auto-ubiquitination in *in vitro* ubiquitination assays, while TRIM25 Δ RBD did not. The same results were shown in *in vitro* ubiquitination experiments with ZAP. The importance of RNA binding was confirmed by RNase treatment of the protein extracts, which led to complete abolition of poly-ubiquitination both on TRIM25 and ZAP (Figure 3). Similar results were obtained by Sanchez *et al.* who found that TRIM25-mediated ubiquitination of the RIG-I 2CARD was severely reduced in the TRIM25 7K mutant compared to TRIM25 WT⁶⁷. Overexpression of GST-2CARD in HEK293T cells resulted in robust ubiquitination of the 2CARD and induction of IFN β promoter activity in cells expressing WT TRIM25 but not in cells expressing the 7K mutant⁶⁷. These results support the hypothesis that TRIM25 requires RNA binding to ubiquitinate its target proteins and further suggest that this is a general property of TRIM25 activity that is not restricted to a single target protein. Sanchez *et al.* also found that RNA binding mediated the localisation of TRIM25 to stress granules, and that RNA binding was required for Trim25's antiviral activity. Virus replication was suppressed by WT TRIM25 but not TRIM25 7K mutant during infection with Dengue virus, vesicular stomatitis virus and IAV⁶⁷.

Another recent report also suggested that TRIM25 RNA binding is important for its ubiquitination of RIG-I 2 CARD. TRIM25 binding to the lncRNA Lnczc3h7a was shown to enhance its interaction with and ubiquitination of RIG-I upon VSV infection and the presence of Lnczc3h7a increased the type I IFN response to RNA virus infection⁹⁷. In addition to this,

both RIG-I and TRIM25 co-purified with Lnczc3h7a from cells that had been infected with VSV and Lnczc3h7a interacted with the RIG-I helicase domain and TRIM25 in RNA pull-downs. The authors of this study proposed a model whereby Lnczc3h7a acts as a scaffold, binding to both RIG-I and TRIM25 in order to bring them closer together. However, it is unlikely that this would be sufficient to explain the differences in type I IFN induction seen due to the apparent redundancy of TRIM25 in RIG-I signalling with other E3 ubiquitin ligases such as Riplet, TRIM4 and MEX3C⁶⁶. It is possible, for example, that the action of Lnczc3h7a is not restricted to TRIM25 and it promotes the association of RIG-I with the other E3 ligases as well.

CONCLUSION AND FUTURE PERSPECTIVE

TRIM25 plays an important role in innate immunity and has proven importance in inhibiting virus replication, however the precise mechanisms are still unclear. Previous reports of TRIM25's significance in RIG-I activation has recently come under scrutiny with several studies also implicating RIPLET, MEX3C and TRIM4 as potential activators of RIG-I, of which RIPLET seemingly the most important^{13, 14, 61-66}. This discrepancy in TRIM25 ubiquitination of RIG-I could be a result of earlier studies using isolated GST-2CARD and not full-length RIG-I. It is possible that TRIM25 is not capable of ubiquitinating full-length RIG-I in cells as there may be other factors in the cell that are required for this activity that are not present *in vitro*. Cadena *et al.* showed that deletion of TRIM25 did result in lower RIG-I signalling activity in response to transfection of GST-tagged 2CARD alone, suggesting that there may be different mechanisms of activation for isolated 2CARD and 2CARD in the context of full-length RIG-I. This study also further explored the interaction between Riplet and RIG-I. It found that Riplet only binds to RIG-I in the presence of RNA and that it can bind to full-length RIG-I or a mutant in which the 2CARD has been deleted but not to the isolated 2CARD¹⁴. Interestingly, the Riplet

PRY/SPRY domain was required for its interaction with RIG-I and Riplet dimerization was also required for this interaction. This bears striking similarities to the requirements for TRIM25 binding to RNA, which also requires dimerization and the PRY/SPRY domain^{12, 98}. Due to the similar domain structures of TRIM25 and Riplet, and the RNA-dependence of the Riplet-RIG-I interaction, this raises the question of whether Riplet is binding directly to RNA in this instance and, if it is, whether this RNA binding is necessary for the interaction with RIG-I.

It is likely that TRIM25 is dispensable for RIG-I activation in human cells while Riplet is essential. It is, however, still possible that requirements for different E3 ligases vary between different cell and tissue types as expression levels may be different and there could be other differences in the environments of different cells. As such it would be prudent to perform a large-scale screen of Riplet and TRIM25 (as well as TRIM4 and MEX3C) knock outs of many different cell types. The idea that TRIM25 was the key E3 ligase for RIG-I activation was mainly based off experiments in which the RIG-I 2CARD was overexpressed in cells or *in vitro* experiments using the 2CARD. The experiments performed with full length RIG-I exemplify the fact that full-length proteins may function differently to their domains in isolation and this can lead to confusion about how biological processes work.

RIG-I-independent functions of TRIM25 in innate immunity have been described, and these seem to be RNA-binding dependent. Meyerson *et al.* showed that TRIM25 binds vRNPs and inhibits the Influenza A polymerase⁹⁵. While TRIM25 is required for the antiviral activity of ZAP^{41, 42}, with TRIM25 ubiquitination of ZAP being RNA dependent¹². Sanchez *et al.* further showed that RNA binding was required for TRIM25's antiviral response to several viruses⁶⁷. Additionally, they showed that TRIM25 localises to stress granules in an RNA-binding

dependent way. ZAP also localises to stress granules^{85, 86} and it is possible this helps these proteins to co-localise.

The mechanism by which TRIM25 RNA-binding facilitates its ubiquitination activity is unknown. It is known that the RING domain of TRIM25 is active as a dimer and it is likely that this requires higher order oligomerisation of TRIM25 dimers. It is therefore possible that RNA binding is important for this higher order organisation, for example by clustering the PRY/SPRY domains from separate dimers together to facilitate formation of a 'tetramer'-like structure. This is rendered less likely by our data showing that purified His-TRIM25 Δ RBD and His-TRIM25 WT both form tetramers *in vitro*, although it remains possible that the proteins behave differently *in vivo*. It is also possible that binding to RNA causes TRIM25 to undergo a conformational change that allows ubiquitin ligase activity.

Although TRIM25 has been established as an RNA-binding protein there are many outstanding questions. How does the emerging RNA-binding roles of TRIM25 contribute to innate immunity? Which host and viral RNAs do TRIM25 bind during viral infection? Do viral proteins, such as IAV NS1, interfere with TRIM25 binding to host RNAs? Is the ubiquitination and PRY-SPRY mediated RNA-binding activity of TRIM25 necessary for antiviral defence? Which other E3 ubiquitin ligases bind to RNA and are involved in the innate immune response? Answering these questions will result in a paradigm shift in our understanding of TRIM25-mediated control of RNA viruses and will pave the way towards novel, targeted, antiviral therapies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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