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To TRIM or not to TRIM: the balance of host–virus interactions mediated by the ubiquitin system

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Abstract

The innate immune system responds rapidly to protect against viral infections, but an overactive response can cause harmful damage. To avoid this, the response is tightly regulated by post-translational modifications (PTMs). The ubiquitin system represents a powerful PTM machinery that allows for the reversible linkage of ubiquitin to activate and deactivate a target's function. A precise enzymatic cascade of ubiquitin-activating, conjugating and ligating enzymes facilitates ubiquitination. Viruses have evolved to take advantage of the ubiquitin pathway either by targeting factors to dampen the antiviral response or by hijacking the system to enhance their replication. The tripartite motif (TRIM) family of E3 ubiquitin ligases has garnered attention as a major contributor to innate immunity. Many TRIM family members limit viruses either indirectly as components in innate immune signalling, or directly by targeting viral proteins for degradation. In spite of this, TRIMs and other ubiquitin ligases can be appropriated by viruses and repurposed as valuable tools in viral replication. This duality of function suggests a new frontier of research for TRIMs and raises new challenges for discerning the subtleties of these pro-viral mechanisms. Here, we review current findings regarding the involvement of TRIMs in host–virus interactions. We examine ongoing developments in the field, including novel roles for unanchored ubiquitin in innate immunity, the direct involvement of ubiquitin ligases in promoting viral replication, recent controversies on the role of ubiquitin and TRIM25 in activation of the pattern recognition receptor RIG-I, and we discuss the implications these studies have on future research directions.

INTRODUCTION

Mammalian innate immunity functions as an early defence system to prevent the invasion of viruses and establishment of productive infections as well as guiding adaptive immunity [1]. Successful employment of the innate immune system relies on the optimal activation of numerous host factors that work in unison to create an antiviral state. However, some of these antiviral mechanisms can be detrimental to the host through induction of cellular apoptosis or by tissue damage if inflammatory responses are left unchecked. Post-translational modifications (PTMs) regulate these pathways through attachment or removal of molecules to proteins

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Abbreviations: aa, Amino acid; AEC, Alveolar epithelial cell; Arg, Arginine; B, B-boxes; BMDC, Bone marrow-derived dendritic cell; BMDM, Bone marrow-derived macrophage; CC, Coiled-coil; CIITA, Class II transactivator; CTD, C-terminal domain; CTL, Cytotoxic T lymphocyte; CVB3, Coxsackie virus B3; Cys, Cysteine; DENV, Dengue virus; DTMUV, Duck tembusu virus; DUB, Deubiquitinase; EBOV, Ebola virus; EBV, Epstein-Barr virus; ER, endoplasmic reticulum; EV71, Enterovirus 71; finTRIMs, Fish novel TRIMs; Gly, Glycine; HBV, Hepatitis B virus; HBx, Hepatitis B virus X; HCMV, Human cytomegalovirus; HCV, Hepatitis C virus; HECT, Homologous to the E6-AP Carboxyl Terminus; His, Histidine; hMPV, Human metapneumovirus; HPAIV, Highly pathogenic avian influenza virus; HSV, Herpes simplex virus; IAV, Influenza A virus; IBDV, Infectious bursal disease virus; IBV, Influenza B virus; IFN-I, Type-I IFN; IN, Integrase; ISG, IFN-stimulated gene; JEV, Japanese encephalitis virus; JUNV, Junin virus; K, Lysine; KFDV, Kyasanur Forest disease virus; KSHV, Kaposi's sarcoma-associated herpesvirus; L2, Long linker; LGTV, Langat virus; lncRNA, Long non-coding RNA; Lys, Lysine; Met, Methionine; N, Nucleocapsid; NBs, Nuclear bodies; NDV, Newcastle disease virus; NiV, Nipah virus; NoV, Norovirus; NP, Nucleoprotein; PAMP, Pathogen-associated molecular patterns; PCNT, Pericentrin; PML, Promyelocytic leukaemia; PPMO, Peptide-conjugated phosphorodiamidate morpholino oligomer; PRR, Pattern recognition receptor; PRRSV, Porcine reproductive and respiratory syndrome virus; PTM, post-translational modification; R, RING; RBR, RING-Between-RING; RGNNV, Red spotted grouper nervous necrosis virus; RING, Really Interesting New Gene; ROS, reactive oxygen species; Rta, Replication and transcription activator; SARS-CoV, Severe acute respiratory syndrome-related coronavirus; SeV, Sendai virus; SFTSV, Severe fever with thrombocytopenia syndrome virus; SGIV, Singapore grouper iridovirus; SINV, Sindbis virus; SIRPA, Signal regulatory protein alpha; Sp1, Specificity protein 1; TBEV, Tick-borne encephalitis virus; TRIM, tripartite motif; TRIM9s, TRIM9 short isoform; UPS, Ubiquitin-proteasome system; vRNP, Viral ribonucleoprotein; VSV-eGFP, Enhanced green fluorescent protein vesicular stomatitis virus; WNV, West Nile virus; WSSV, White spot syndrome virus; YFV, Yellow fever virus; ZAP, Zinc-finger antiviral protein; ZIKV, Zika virus.

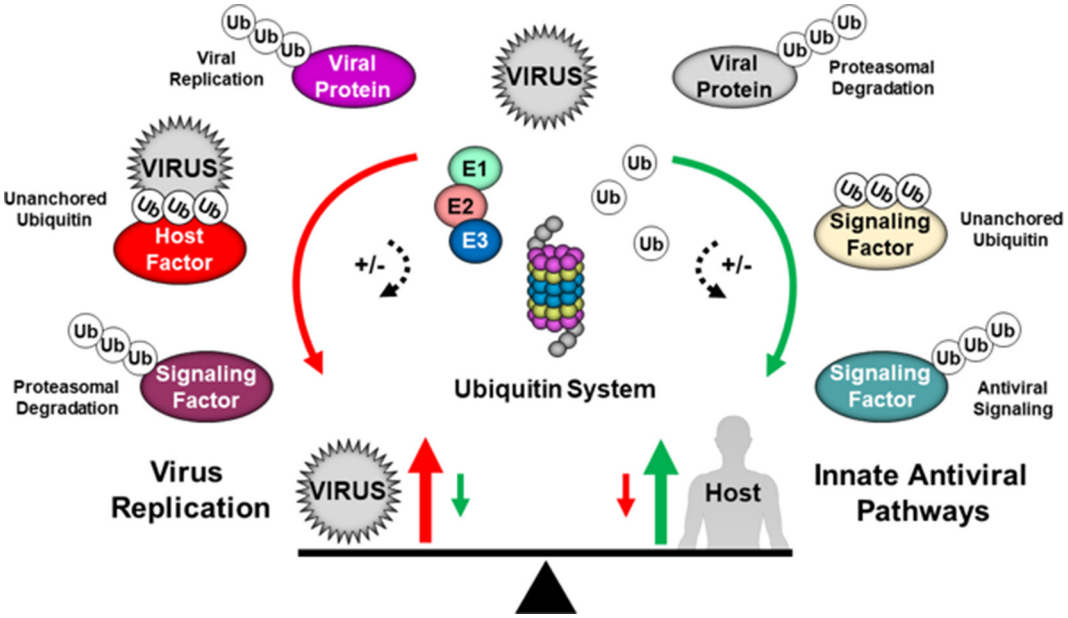

Fig. 1. The ubiquitin system: a balance between enhancement and inhibition of viral replication. Ubiquitin post-translational modifications (anchored or unanchored) play an important role in protecting the host from viral challenge by activating critical antiviral signalling factors or degrading harmful pathogen proteins. However, viruses have evolved to hijack this system to promote their own replication through enhancement of viral replication, through elimination of host signalling factors, or through interactions with host factors that promote an aspect of the viral life cycle.

that act as on and off switches to regulate functions, thereby expanding the capabilities of the host proteome. PTMs play such a vital role in regulating host functions that viruses have developed fascinating mechanisms to take advantage of PTMs to establish productive infections while also circumventing antiviral responses. These virus strategies have evolved from an ongoing arms race with PTM systems that may act in antiviral pathways. Here, we discuss recent studies related to PTMs by ubiquitination and its function in both enhancing and inhibiting virus replication (Fig. 1). Although we focus on current developments within the tripartite motif (TRIM) field, other excellent reviews that focus more on the antiviral aspects of TRIMs and that highlight older studies are also available [2–10].

UBIQUITIN POST-TRANSLATIONAL MODIFICATIONS

PTMs alter the functionality of targeted proteins in the cell by regulating the activity, stability, cellular compartmentalization, conformation and future interactions with additional factors. Rapidly altering and expanding the capabilities of cellular factors confers the ability to respond to countless environmental stimuli including pathogen sensing and immune responses [5, 11, 12]. One of the most widely used and ubiquitous forms of PTMs is protein ubiquitination [13].

Ubiquitin is a 76 amino acid (aa) protein and is able to interact with targets through covalent bonds (anchored ubiquitin) or non-covalent associations (unanchored ubiquitin) [4, 5, 14].

Ubiquitination of a target protein occurs through a series of reactions involving three distinct enzymes. In the first stage, an E1 ubiquitin-activating enzyme will form a thioester bond between its own active site cysteine (Cys) and the C-terminal glycine (Gly) of its ubiquitin target in an ATP-dependent manner [4, 5, 15]. This ubiquitin is then transferred to the Cys active site of one of approximately 40 E2 ubiquitin-conjugating enzymes through a transthiolation reaction to complete the second stage [4, 5, 16]. In the final stage, interaction between the E2 and one of over 600 E3 ubiquitin-ligating enzymes allows for the transfer of ubiquitin from the E2 onto the target protein (Fig. 2) [4, 5, 15, 17]. The E2 and E3 enzymes can be expressed in a tissue- or cell-type-specific manner, and this may also depend on stimulus, including virus infection. The differential expression of these enzymes and their activation conditions makes the study of the ubiquitin process a challenging effort.

During ubiquitin conjugation, ubiquitin can become covalently bound usually to the ε-amino group of a lysine (Lys, K) on the target protein (Fig. 2) [5, 17]. Conjugation of a single ubiquitin moiety is referred to as monoubiquitination while multi-monoubiquitination describes conjugation of two or more monoubiquitins to different residues on the same target [5, 17]. Conjugation of ubiquitin to Lys groups can also occur between ubiquitin molecules to form polyubiquitin chains. Formation of polyubiquitin is made possible by covalent interactions between the C-terminal Gly of one ubiquitin and one of seven Lys residues on another ubiquitin (K6-, K11-, K27-, K29-, K33-, K48- and K63-linked polyubiquitin).

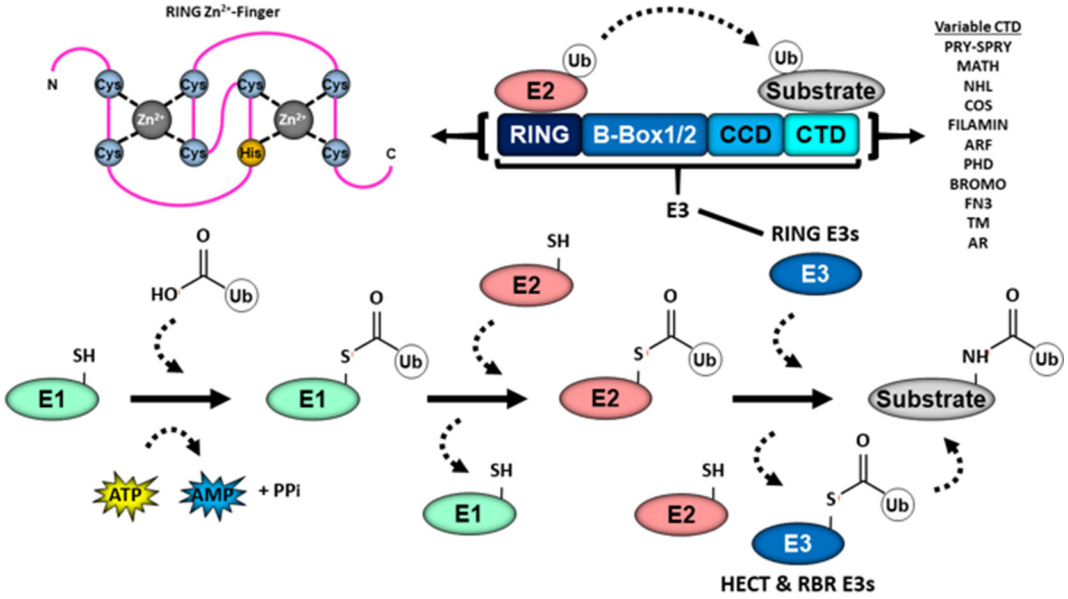

Fig. 2. The ubiquitin enzymatic reaction. Ubiquitination is a stepwise series of enzymatic reactions involving an E1-activating, E2-conjugating and E3-ligating enzyme. Free ubiquitin is bound through its C-terminal Gly to the E1 active site through a thioester bond in an ATP-dependent reaction. Ubiquitin is then transferred to the E2 active site before being moved to the substrate with help from one of the three E3 ligase families. HECT and RBR E3 ligases first require transfer of ubiquitin from the E2 to their catalytic domain before final transfer to a substrate while RING family E3 ligases facilitate direct movement of ubiquitin from the E2 to the substrate. RING E3 ligases achieve this through an interaction between the ubiquitin-loaded E2 and the conserved Zn^{2+}-finger array in their RING domain. High-specificity interactions between an E3 and its substrate occur through the variable C-terminal domain.

Alternatively, the C-terminal Gly of one ubiquitin can covalently bind the N-terminal methionine (Met) of another ubiquitin to form 'linear' polyubiquitin (M1-linked polyubiquitin) [4, 5, 11, 17]. Furthermore, the ubiquitin machinery is capable of synthesizing polyubiquitin chains that are not covalently bound to a substrate (unanchored polyubiquitin). These unanchored polyubiquitin chains are unique as they retain their free C-terminal Gly, typically conjugated to a substrate's Lys. This absence of direct conjugation allows unanchored ubiquitin to function as a three-dimensional signal that can be amplified by multiple non-covalent interactions with specific signalling factors [4, 5, 7, 17, 18].

Ubiquitin PTMs serve multiple purposes in the cell with the exact function determined by the particular linkage involved. The variety observed in ubiquitin chain formation therefore acts as a 'code' with distinct instruction for the cell to act upon [15]. Functionality of the different chain topologies can vary. For instance, K48-linked polyubiquitin is most commonly associated with targeting substrates to the proteasome for degradation while K63-linked polyubiquitin is generally regarded as an important component in signalling complex formation, although there are examples of all types of ubiquitin linkages being involved in multiple functions. K11- and K29-linked polyubiquitin have been attributed with roles in protein turnover, while K33-linked polyubiquitin is believed to regulate trans-Golgi network trafficking. M1-linked polyubiquitin are involved in immune processes like type-I IFN (IFN-I) production, and K6- and K27-linked polyubiquitin have been implicated to play roles in mitophagy and autoimmunity respectively [17, 19]. Involvement of monoubiquitination and multi-monoubiquitination in protein localization can be observed through the nuclear and cytoplasmic translocation of factors after ubiquitin labelling [15]. Finally, both K48- and K63-linked unanchored polyubiquitin chains have been proposed to function as secondary messengers in innate immunity. Unanchored K63-linked polyubiquitin potently activates both TAK1 and RIG-I signalling *in vitro* and unanchored K48-linked polyubiquitin facilitates activation of the IKKε complex for downstream antiviral signalling [20–22]. Whether or not unanchored polyubiquitin of other linkages have specific functions in immunity has yet to be explored.

TRIM E3 UBIQUITIN LIGASES

Determining the target to receive ubiquitin is the responsibility of the E3 ubiquitin-ligating enzyme. E3 ubiquitin ligases are the most abundant of the three enzymes that comprise the ubiquitin pathway out of necessity as this great quantity facilitates a high degree of substrate specificity for the more limited number of E2 enzymes [23]. E3 enzymes can be grouped into three subfamilies consisting of the Really Interesting New Gene (RING), Homologous to the E6-AP Carboxyl Terminus (HECT), and RING-Between-RING (RBR) families with a majority (~600) belonging to the RING family [23]. Whereas HECT and RBR E3s facilitate ubiquitination via a two-step mechanism, in which ubiquitin is first added from the E2 to

the E3 catalytic Cys residue before transfer from the E3 to the actual substrate, RING E3s allow for direct movement of ubiquitin from the E2 to the substrate (Fig. 2) [23]. Further interpretations on the importance of the RING family as a whole in coordinating antiviral immune defences can be found in additional reviews [2, 5, 24, 25]. Of particular noteworthiness from within the RING family is a grouping of over 70 members encoded in the human genome known as TRIM proteins [5, 23, 26–28]. What makes TRIMs so interesting in the context of antiviral innate immunity is due not only to the high proportion of members directly involved in aspects related to regulation of immune pathways, but also to the rapid expansion of the group at the evolutionary onset of our modern innate and adaptive immune systems [7, 18, 29–31]. This has raised the theory that TRIMs evolved in this manner as a way to directly regulate increasingly complex immune systems, using the ubiquitin code as a result of positive evolutionary pressure exerted by constant interactions with viruses, especially retroviruses [18, 32, 33].

TRIM family members are characterized by their conserved RBCC domain that comprises the RING (R), one or two B-boxes (B), and the coiled-coil (CC) domains (Fig. 2) [5, 34]. The function of the RING domain, a conserved region enriched in Cys and histidine (His) residues that coordinate two zinc ions, is to recruit the ubiquitin-loaded E2 and is usually considered as the E3 ligase domain (Fig. 2) [23, 34]. TRIM B-box domains have been implicated in facilitating higher-order multimerization (e.g. TRIM5α) and have been suggested to also interact with RING domains to facilitate quaternary arrangements [35, 36]. The coiled-coil domain allows for dimerization and oligomerization of TRIMs, and for some TRIMs it has been shown to be important for E3 ligase function [5, 34, 37]. Structural analysis of this domain from several TRIMs reveals that the coiled-coil forms an antiparallel dimer that is probably conserved amongst TRIM family members due to conservation in coiled-coil sequences [5, 34]. The C-terminal domain of TRIMs allows for interaction with target substrates with high specificity [5, 25]. The most prevalent C-terminal domain is the PRY-SPRY domain (or B30.2) which is only present in vertebrates [2, 38].

DIRECT ANTIVIRAL ROLES OF TRIMs

TRIM family members have garnered increased attention due to their essential roles in regulating numerous cellular processes through ubiquitin PTMs [4, 7, 18, 39]. The mammalian innate immune system in particular appears to be closely tied to TRIM-mediated regulation of cellular components that identify the presence of microbial products, thereby allowing TRIMs to indirectly limit viral replication [5, 7, 18]. TRIMs can also take direct action against foreign organisms through both canonical proteasome-mediated destruction and a variety of non-canonical means. The importance of TRIMs in innate immunity is made clear upon examination of the number of approaches employed by TRIMs to undermine pathogen functions.

Proteasome-independent antiviral mechanisms

Although common, labelling of factors with polyubiquitin (typically K48-linked polyubiquitin) for proteasomal degradation is not the only means of viral restriction available to TRIMs [4]. TRIM79α, an IFN-stimulated gene (ISG), plays a significant role in the suppression of tick-borne encephalitis virus (TBEV) replication through a direct interaction with the viral NS5 protein [40]. Taylor and colleagues identified a role for TRIM79α as a TBEV restriction factor by identifying an interaction between TRIM79α and the NS5 protein from the TBEV serogroup member Langat virus (LGTV). Not only is this interaction specific for the viral NS5 protein and not C or NS4A, it also appears exclusive to tick-borne flaviviruses as neither the West Nile virus (WNV) nor the Japanese encephalitis virus (JEV) NS5 proteins bound TRIM79α [40]. The nature of this interaction revealed that TRIM79α degrades NS5 not through the proteasome or ubiquitination, but rather through lysosomes. Lysosomes function in the breakdown of macromolecules, making them ideal for the removal of the approximately 900 aa large NS5. Indeed, not only NS5, but the entire replication complex containing NS5, NS3 and NS2B was shown to be re-directed for lysosomal degradation by TRIM79α [40, 41].

An example of a TRIM displaying antiviral function by a non-degradative mechanism was observed with TRIM56. Initial studies on TRIM56 demonstrated its ability to inhibit a range of pathogens including members of the pestivirus, flavivirus and coronavirus families [42, 43]. In all of the aforementioned studies, the E3 ligase function of TRIM56 was required for successful viral inhibition. This is in contrast to what was observed with members of the negative sense family of RNA viruses. TRIM56 can effectively target both influenza A and B viruses (IAV, IBV) but not Sendai (SeV) or human metapneumovirus (hMPV), indicating a preference for human respiratory pathogens belonging to *Orthomyxoviridae* [44]. Furthermore, the ability to restrict IAV and IBV relied not on any of the conserved domains (RBCC), but instead depended on a 63 aa portion of the C terminus [44]. The presence of TRIM56 correlated with a reduction in both viral mRNA transcription and cRNA synthesis. Interestingly, a portion of TRIM56 was shown to relocate from the cytoplasm to the nucleus upon IAV infection, suggesting TRIM56 may target a component of the viral ribonucleoprotein (vRNP) or the vRNA itself [44]. Although the exact mechanism of restriction is unclear, this hypothesis is plausible as the C terminus of TRIM56 was found to have high sequence homology to the positively charged surfaces in NHL repeats, which would afford a means to interact with the negatively charged sugar phosphate backbone of vRNA (Fig. 3) [44]. In the case of Zika virus (ZIKV) infection, TRIM56 can indeed identify and capture vRNA [45]. Restriction of ZIKV by TRIM56 depended on an intact C-terminal region as well as its E3 ligase activity signifying, in contrast to what was observed with IAV, that TRIM56 may recognize vRNA and utilize its E3 ligase activity to prevent ZIKV replication through an as yet unresolved mechanism [45].

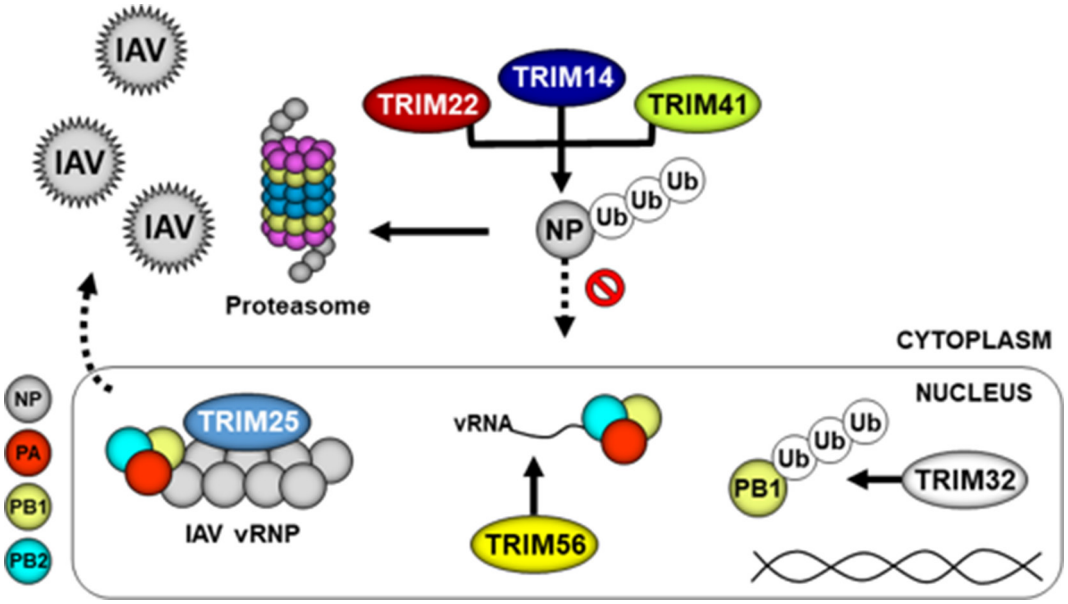

Fig. 3. TRIM restriction of influenza virus. The ubiquitination of influenza NP by TRIM22, TRIM14 and TRIM41 targets the viral protein for the proteasome, preventing nuclear translocation and formation of vRNPs. TRIM56 and TRIM25 inhibit influenza replication by inhibiting vRNA synthesis and blocking vRNA chain elongation respectively. TRIM32 targets the viral PB1 component of the polymerase for ubiquitin-mediated degradation.

Viral restriction that is independent of a TRIM's E3 ubiquitin ligase function was recently described for TRIM25 [46]. Elimination of IAV-induced RIG-I signalling through CRISPR-Cas9 deletion of RIG-I revealed TRIM25 was still capable of limiting IAV replication in the absence of IFN signalling. The addition of RING domain mutations at positions C13/16A that abrogate TRIM25 autoubiquitination still did not impair its ability to inhibit IAV, hinting at an as yet undefined role for TRIM25 in IAV restriction [46]. TRIM25 was shown to directly bind IAV vRNPs *in vitro* as well as in the nucleus of infected cells and this interaction required vRNA as RNA-free oligomeric nucleoprotein (NP) complexes could not associate with TRIM25 [46]. Recognition of vRNPs by TRIM25 did not alter the endonuclease activity or cap-binding functions of the viral PA or PB2 respectively, indicating that TRIM25 utilizes its RNA-binding activity to halt the onset of viral mRNA chain elongation. This hypothesis was supported by the lack of full-length viral mRNAs without an increase in short vRNAs that are characteristic of premature termination of elongation [46]. Conservation of this novel TRIM25 function was also evident in gibbon TRIM25 in which the non-human primate version displayed a strong affinity to vRNPs from the human strains of IAV tested (Fig. 3) [46].

TRIM proteins have also been shown to limit viral replication by recognizing and blocking viral-induced cellular changes. TRIM19, also known as promyelocytic leukaemia (PML) protein, was identified as a restriction factor for enterovirus 71 (EV71) through an inhibition of autophagy [47]. Loss of TRIM19 with siRNA or TRIM19$^{-/-}$ cell lines resulted in an increase of EV71 replication while overexpression of two TRIM19 isoforms (TRIM19-III and TRIM19-IV) efficiently reduced EV71 titres by several logs [47]. EV71-induced autophagy can facilitate replication and absence of TRIM19 during EV71 infection correlated with a loss in the expression of p62 and an increase in the presence of LC3-II, two markers for autophagy [47]. Recent studies on TRIM19 have identified it as a cellular stress sensor that recognizes the accumulation of reactive oxygen species (ROS) [48]. Production of ROS as a result of EV71 infection may activate TRIM19's function as an autophagy repressor through a mechanism that has yet to be determined. Additional studies highlighting the important roles for TRIM19, SUMOylation, and nuclear bodies in innate immune signalling and antiviral function have been presented [49–52]. TRIM19's effect on autophagy highlights a developing field of TRIM research [53]. Initiation of autophagy upon viral infection has also been shown for TRIM5α and TRIM23 [53–55]. Autophagy-mediated degradation of HIV-1 p24 was found to occur via trafficking to the autophagosome by TRIM5α, leading to recruitment of both Beclin-1 and ULK1 [54]. Degradation of microbial components via autophagy involves recognition by phosphorylated p62 through TBK1 [56] and this activation of p62 was recently found to involve TRIM23 [55]. Viral infection triggered TRIM23 autoubiquitination and downstream interactions with TBK1, ultimately leading to autophagy-mediated clearance of several pathogens [55].

Mobilization of the host cell's cytoskeletal components to limit viral invasion has been described for TRIMs [57, 58]. TRIM11 was identified as an HIV-1 binding protein that recognizes the viral capsid, leading to early uncoating and restriction of reverse transcription [57]. This antiviral function was linked to the host microtubule system and not to other more

common degradation pathways involving proteasomes or lysosomes [57]. Similar to what was described for microtubule network restriction of retroviruses by TRIM5α, TRIM11 utilizes host microtubules for uncoating of the viral capsid [57, 58]. However, unlike TRIM5α, TRIM11-microtubule restriction is not broad spectrum for other retroviruses such as MLV [57].

TRIM-dependent blockades against the formation of host–viral protein complexes has been observed to counter hepatitis B virus (HBV) replication [59]. TRIM14 is an ISG induced as a result of HBV infection [59]. The presence of TRIM14 correlated with a loss in HBV replication and a screen for TRIM14 binding against HBV proteins revealed an interaction between TRIM14 and the hepatitis B virus X (HBx) protein through the TRIM14 SPRY domain and the HBx C-terminal domain [59]. HBx is known to facilitate HBV replication by targeting and degrading the host Smc5/6 restriction factor with help from the host DDB1 protein [59, 60]. The presence of TRIM14 obstructed the HBx–DDB1 interaction and as a result rescued Smc5/6 expression [59]. Curiously, the authors did not investigate whether the TRIM14 interaction via the SPRY domain resulted in the ubiquitination of HBx. Since the SPRY domain of TRIMs is involved with identifying a target to receive ubiquitination and the C-terminal domain of HBx is the transactivaton/co-activation domain that interacts with host factors, it is conceivable that TRIM14 may either target HBx for proteasomal degradation or sequester HBx from its binding partner DDB1.

The prominence of TRIM structure as a means for viral restriction has grown in appreciation thanks to over a decade of work on characterizing the anti-retroviral capabilities of TRIM5α. TRIM5α is thought to directly restrict incoming retroviruses through recognition, binding, hexagonal lattice formation around the viral capsid, and ubiquitination, as well as indirectly by inducing innate immune signalling (Fig. 4) [61–65]. The inhibition of retroviral reverse transcription has been shown to require all domains of TRIM5α, each providing a unique tool for host defence.

The SPRY domain of TRIM5α is able to bind the capsid of HIV, albeit weakly, indicating a need for the higher-order TRIM5α structures. Multivalent interactions between proteins that afford stronger interactions with a target when individual units have limited avidity is a key component of TRIM5α recognition. In the TRIM5α dimer, the SPRY domains have been shown to couple and form sturdier bonds with the HIV capsid [66]. This increase in avidity is achieved through the formation of α-helical folds in the long linker (L2) region that connects the RBCC and SPRY domains [66, 67]. The presence of this α-helix allows for packing of the CC/L2/SPRY regions to create distinct arrangements of SPRY dimers that more closely match retroviral capsids [66]. This stiffer formation of the SPRY dimer was found to promote TRIM5α avidity, and mutations that interrupted this packing arrangement without disturbing the SPRY dimer (I193A, E201A) reduced the restriction efficacy [66].

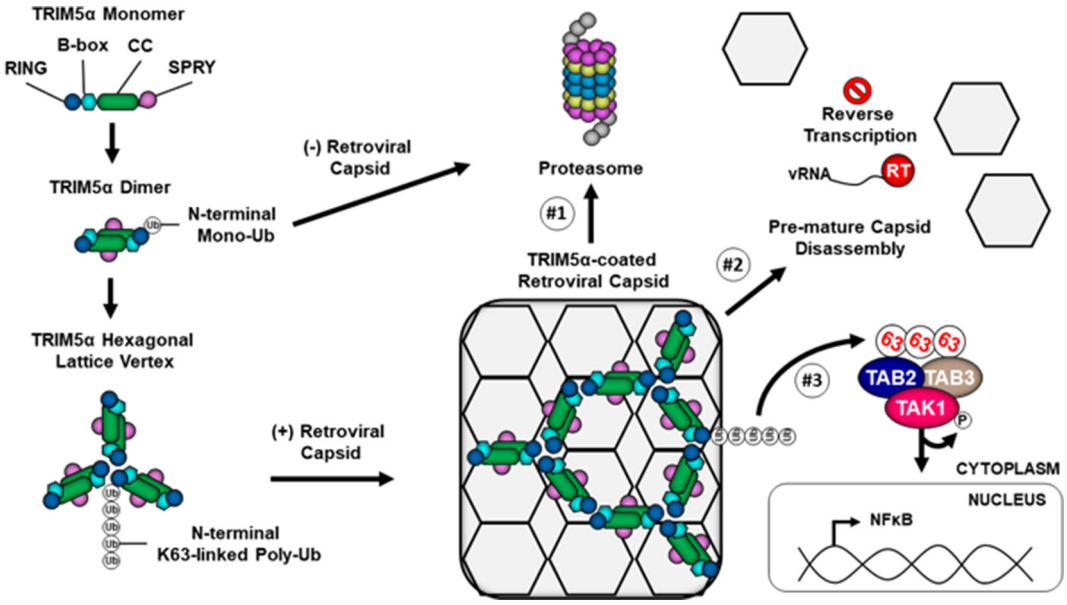

Fig. 4. TRIM5α restriction of retroviruses. Rapid turnover of TRIM5α is achieved through the N-terminal monoubiquitination and proteasomal degradation of TRIM5α dimers in the absence of retroviral capsid substrates. Upon recognition of retroviral capsids, TRIM5α forms higher-order hexagonal lattice structures on the capsid surface and extends its N-terminal K63-linked polyubiquitin chain. Potential mechanisms of TRIM5α retroviral restriction include: (#1) proteasomal-mediated degradation, (#2) pre-mature capsid disassembly that prevents reverse transcription, and (#3) production of unanchored K63-linked polyubiquitin that activates TAK1-mediated innate immune signalling.

TRIM5α's B-box 2 domain is necessary for the formation of higher-order complexes that direct the characteristic retroviral capsid binding and restriction [68]. The B-box 2 domain itself can form dimers and trimers but it is the trimeric form that cooperatively functions to form the characteristic hexagonal nets observed on retroviral capsids (Fig. 4) [36, 69]. Retroviral capsids are not two-dimensional, requiring a certain degree of flexibility from the TRIM5α hexagonal lattice. This prerequisite is fulfilled by trimeric B-box 2 through rigid 'swinging' movements between the B-box 2 and the CC domains that are hypothesized to function as a 'scanning' mode for identifying ideal capsid interaction points for SPRY domains [36]. These higher-order structures can also impose restrictions within TRIM5α domains. TRIM5α assembly onto retroviral capsids has been shown to promote its E3 ligase activity and the interactions between B-box 2 trimers is thought to obstruct the RING domains such that they face away from the capsid [36, 63].

In the TRIM5α dimer, antiparallel interactions in the CC domain result in the placement of the RING and B-box domains at opposing ends [37, 70]. Dimerization between two TRIM5α RING domains is not energetically favourable, implying that the interactions between RING domains observed in TRIM5α hexagonal lattices on retroviral capsids are a result of prerequisite higher-order assemblages of the other TRIM5α domains that orientate the RINGs in proximity to one another [71]. The TRIM5α RING dimer mutant (I77R) has a reduced ability to restrict HIV that correlated with an inability to synthesize K63-linked polyubiquitin chains, thus identifying a link between higher-order self-assembly, ubiquitination and viral restriction [71].

Proteasome-dependent antiviral mechanisms

Shortly after labelling with K48-linked polyubiquitin, proteins are degraded by the proteasome. Ubiquitin receptors present in the proteasome allow for high-affinity binding to K48-linked ubiquitin chain topologies. Cooperation with deubiquitinases (DUBs) allows for the recycling of ubiquitin chains, making the ubiquitin–proteasome system (UPS) a powerful tool for both protein turnover as well as removal of unwanted cellular and viral components [72]. In addition to its known roles in retroviral restriction, TRIM5α has now been described suppressing flavivirus replication with the help of the proteasome [73]. In a recent screen against several members of both tick- and mosquito-borne viruses, TRIM5α showed restriction against specific members of the TBEV serogroup including TBEV (Sofjin strain), Kyasanur Forest disease virus (KFDV), and LGTV but not against WNV, dengue virus (DENV) or ZIKV [73]. By targeting the viral NS2B/3 protease, TRIM5α is able to hamper viral replication through conjugation of K48-linked polyubiquitin that targets the viral protease for proteasomal degradation [73]. Herpesviruses are also susceptible to TRIM5α, with the replication and transcription activator (Rta) from Epstein-Barr virus (EBV) as an identified target [74]. MS screening of host factors that interact with Rta identified TRIM5α and further characterization revealed that both factors interact in the nucleus [74]. Rta functions during the immediate-early stage of EBV infection and facilitates the transcription of EBV lytic genes that initiate progeny virion formation and release [74]. This process is subdued by TRIM5α through Rta targeting for ubiquitination and proteasomal degradation, suggesting nuclear TRIM5α maintains EBV latency in infected cells [74].

TRIM-mediated degradation of flavivirus replication components has also been established for JEV [75]. TRIM52 targets the viral NS2A protein for ubiquitin-mediated proteasomal degradation resulting in a loss of JEV replication [75]. The NS2A protein of flaviviruses is involved in vRNA replication, countering host innate immune responses, and the formation and release of nascent viral particles [76]. Although proteasome-mediated degradation of NS2A is the end result of this TRIM52 interaction, it is unknown whether the loss in JEV replication is the result of a recovered innate immune factor that NS2A was inhibiting or a loss in JEV RNA replication due to an absent NS2A. Flavivirus non-structural proteins are a target for TRIMs as TRIM69 can identify the DENV NS3 and destroy it via the proteasome through ubiquitin labelling [77]. As part of the protease complex, NS3 combines with NS2B to form NS2B/3 which functions in cleavage of the DENV precursor polyproteins as well as escape from host immunity [77]. The importance of TRIM69 in countering DENV can not only be attributed to the loss of NS3, but also to the recovery of innate immune factors targeted by NS2B/3. Indeed, expression of TRIM69 can reduce the NS2B/3-mediated cleavage of STING [77].

TRIM14 and TRIM22 are two TRIMs that function as ISGs during hepatitis C virus (HCV) infection [78, 79]. The presence of either TRIM14 or TRIM22 conferred restriction against HCV replication that depended on an interaction with the viral NS5A protein in both cases [78, 79]. This interaction resulted in the destruction of the viral NS5A by ubiquitination and subsequent proteasome targeting [78, 79]. Although TRIM14 is known to function as a mitochondrial adaptor for IFN and NF-κB signalling, usage of the TRIM14 mutant that fails to mediate innate immune signalling (K365R) revealed restriction against HCV was unaltered [78]. Another ISG TRIM, TRIM21, promotes destruction of viral capsids via the proteasome, which in turn reveals the viral genome for awaiting PRRs such as cGAS and RIG-I to further fuel a productive innate response [80]. This ability to recognize and degrade antibody-bound components found within the cell has made TRIM21 a desirable target for development of novel protein degradation methods [81].

The IAV NP has been shown to be a major target of TRIM-mediated degradation in recent years beginning with TRIM22. TRIM22 is an ISG that is significantly upregulated upon IFN treatment or IAV infection [82, 83]. TRIM22 displayed restriction capabilities against a range of IAV strains including H1N1 and H3N2 that relied on TRIM22's ability to target the viral NP for polyubiquitination and subsequent proteasomal degradation [82]. Recent evidence suggests that the ability of TRIM22 to ubiquitinate NP arose from arginine (Arg, R) to Lys mutations that appeared on the NP of seasonal strains of

IAV after the 1918 pandemic [84]. Although it is unclear what advantage these NP R-to-K mutations provided the virus, the authors suggest they may allow for evasion of T-cell-mediated immunity because some of the mutations reside in known cytotoxic T lymphocyte (CTL) epitopes [84]. Another ISG identified as an IAV restriction factor was TRIM14 [85]. Contrary to its role as a mediator in IFN signalling, TRIM14's ability to inhibit IAV replication was independent of IFN production and signalling pathways as both TBK1$^{-/-}$ and IFNAR1$^{-/-}$ cell lines failed to prevent TRIM14's restriction of IAV [85]. TRIM14 was shown to interact with IAV NP through its PRY-SPRY domain, resulting in K48-linked polyubiquitination and proteasomal degradation of NP [85]. Surprisingly, TRIM14 was also shown to prevent translocation of NP from the cytoplasm to the nucleus, suggesting that, in addition to proteasome degradation of NP, TRIM14 prevents vRNP formation by sequestering NP from forming NP–vRNA complexes [85]. Although the production of TRIM22 and TRIM14 in response to a stimulus is a potent means of IAV restriction, other TRIMs that operate independently of IFN stimulation offer the host a more rapid means of defence. In a recent proteomics screen of host–IAV interactions, TRIM41 was identified as another NP binding protein [86]. The constitutively expressed TRIM41 was found to identify and target NP through its SPRY domain without prior IFN induction [86]. This association showed TRIM41 to be a host restriction factor against IAV through polyubiquitin-mediated degradation, revealing a possible hierarchy of TRIMs that operate in a redundant fashion to seek and destroy incoming NP at various points during IAV infection (Fig. 3).

Aside from NP, additional IAV components are known to be substrates for TRIMs. Affinity-purified MS detailing IAV PB1–host interactions found TRIM32 as an interacting partner for PB1 that was conserved in multiple IAV strains including H1N1, H3N2, H5N1, and H7N9 [87]. This binding required at minimum the CC domain and part of the linker region of TRIM32 as well as the C-terminal domain of PB1 [87]. PB1's N- and C-terminal domains contain regions for interaction with the viral PA and PB2 subunits respectively, making it the core component for formation of the vRNP. Despite a clear interaction with the PB1 C terminus, TRIM32 did not compete with PB2 for binding to PB1 [87]. Instead, TRIM32 selects PB1 for K48-linked polyubiquitination and proteasomal degradation, leading to a loss in polymerase activity and viral replication [87]. Of note is the means by which TRIM32 interacts with PB1. TRIM32 is ubiquitously expressed and does not require prior IFN stimulation, allowing for early detection of PB1 from uncoated viral particles [87]. This initial interaction in the cytoplasm allows for TRIM32 to translocate with PB1 to the nucleus where it accumulates, prompting the authors to hypothesize a 'trap' mechanism for concentrating TRIM32 in the cellular compartment where its substrate is most abundant (Fig. 3) [87].

Besides TRIM5α, other TRIMs have been identified directly targeting HIV components. TRIM33 was recognized in an RNAi screen of all host ubiquitin machineries as a direct antiretroviral factor that inhibits the HIV-1 integrase (IN) [88]. Although the half-life of the HIV-I IN is ~1 hr, this timeframe is sufficient for successful incorporation of the proviral DNA into the host genome, thereby necessitating a rapid and specific response from host factors for successful termination [88]. Indeed, TRIM33 targets HIV-I IN for ubiquitin-mediated proteasomal degradation, thus impeding HIV-I [88]. Notably, TRIM33 localization is primarily nuclear, which is consistent with the presence of a bromodomain that can interact with acetylated Lys residues on histones, raising the possibility that nuclear TRIM33 can identify the viral IN immediately after it enters the nucleus [88, 89].

INDIRECT ANTIVIRAL ROLES OF TRIMs

Innate immune signalling programmes are initiated by the host upon recognition of pathogen materials that possess intrinsic characteristics identifying themselves as foreign. These pathogen-associated molecular patterns (PAMPs) trigger the activation of innate immune signalling through various specialized sensors that recognize alien substances. These pattern recognition receptors (PRRs) are evolutionarily conserved sentinels that, upon interaction with pathogen components, initiate powerful antimicrobial defences through a cascade of intracellular signalling pathways [11]. Proper regulation of these mechanisms through PTMs is therefore essential for appropriately responding to attacks. As E3 ubiquitin ligases, TRIMs have indirectly found themselves at the forefront of PTM control of innate immune signalling [7, 9]. Aside from direct restriction of viral factors, TRIM-mediated ubiquitination of immune components affords other host factors the opportunity to exercise their own antiviral traits.

Antiviral immune signalling by TRIMs

TRIMs can be involved in the inhibition of viral invasion indirectly by promoting induction of antiviral cytokines, including IFNs [39]. An example of the importance of TRIMs in facilitating the precise cascades of immune pathways was shown with the short isoform version of TRIM9 (TRIM9s) [90]. An enhanced green fluorescent protein vesicular stomatitis virus (VSV-eGFP) screen to identify TRIMs important in immune responses identified TRIM9s [90]. TRIM9s expression promoted IFN-I and ISG responses while simultaneously obstructing pro-inflammatory cytokine production. This skewed activation of innate immunity favoring the IFN-I pathway over NF-κB was the result of a direct interaction with TBK1 and GSK3β [90]. GSK3β itself can promote the oligomerization of TBK1 and the interaction between GSK3β and TBK1 was reinforced in the presence of TRIM9s [90]. In this way, TRIM9s functions as a type of molecular bridge linking host factors together that was dependent on TRIM9s K63-linked autoubiquitination [90].

TRIM56 is also capable of enhancing the performance of PRRs such as cGAS and STING, which sense DNA viruses [91]. Specifically, TRIM56 can monoubiquitinate cGAS at

K335, allowing cGAS to better recognize foreign DNA and produce the secondary messenger cGAMP [92]. TRIM56 interactions with STING promote downstream signaling and a complex with UBXN3B and TRIM56 may promote STING K63-linked polyubiquitination [93, 94]. Aside from the prototypical K63 and K48 ubiquitin linkages, other chain types can also be critical in certain situations. For example, in addition to its role in autophagy, K27-linked polyubiquitin mediates the recruitment of MAVS to TBK1, leading to IRF3 activation and IFN-I production in response to viral infection [95, 96]. Upon infection with several viruses including HCV, Newcastle disease virus (NDV), SeV, VSV and Coxsackie virus B3 (CVB3), TRIM21 is expressed and interacts with MAVS through its PRY-SPRY domain. This association allows for the conjugation of K27-linked polyubiquitin onto the K325 residue of MAVS for downstream signalling [95, 96].

TRIM regulation extends to additional immune pathways beyond IFN-I. TRIM52 promotes NF-κB signalling and pro-inflammatory cytokine production (TNFα and IL-6 cytokines) but does not affect levels of either IκBα of p-p65, suggesting a non-canonical activation of the NF-κB program [97]. Curiously, even in the presence of irreversible IKKα inhibitors that block the degradation of IκBα, overexpression of TRIM52 was still able to induce a NF-κB luciferase reporter [97]. Due to its expression in both the nucleus and the cytoplasm, it is plausible that TRIM52 may become activated by pro-inflammatory cytokine signalling, leading to a non-canonical activation of NF-κB signalling [97]. Regulation of immune signalling pathways is critical for eliciting the appropriate responses for pathogen clearance while mitigating host damage. TRIM28 is a regulator of IFN-I and pro-inflammatory signalling by balancing their expression as a transcriptional co-repressor during infection with human strains of IAV [98]. The delicate balance of these TRIM-regulated mechanisms can become easily distorted as highly pathogenic avian influenza (HPAIV) strains alter the effect TRIM28 has on the immune response. During HPAIV infection, TRIM28 becomes phosphorylated at S473, resulting in a forfeiture of its repressor functions and a subsequent IFN-I and pro-inflammatory cytokine storm [98]. This effect was found not to originate from TRIM28's known roles in the DNA damage response nor from ROS production during IAV infection, but rather from the ability of HPAIV strains to trigger the PKR/p38/MSK1 phosphorylation cascade, ultimately leading to phosphorylation of TRIM28 and an environment more permissive to harmful inflammation [98]. Successful clearance of foreign microbes requires their rapid identification by host PRRs. Incoming virions, therefore, have an early advantage as the viral RNA and DNA genomes that possess the PAMPs can be concealed by viral capsid structures. However, TRIM21 counteracts this challenge to afford the host an early opportunity to commence innate immune signalling. TRIM21 can recognize antibody-coated virions that enter the cell, activating K63-linked polyubiquitin formation that stimulates components of innate immune signalling [80, 99, 100].

Innate antiviral signalling functions of TRIM25: RIG-I-dependent or RIG-I-independent? Riplet or TRIM25?

To date, most studies have implicated TRIM25 as a critical mediator of innate immune signalling through ubiquitination of RIG-I [2, 11, 101–118], while only a handful of studies have proposed RIG-I-independent mechanisms [46, 119]. Recent years have brought both exciting discoveries and rousing controversies regarding how ubiquitin and E3 ligases precisely function to activate RIG-I for antiviral signalling, with some groups suggesting a single factor operates as the critical component while others have put forth a cooperative model based on trends gleaned from numerous studies. For viruses that are sensed by the RIG-I PRR, initial recognition of vRNA and binding of the viral 5′ tri- and diphosphate PAMPs occurs via the RIG-I helicase and C-terminal domains (CTDs) [108, 120, 121]. Identification of vRNA triggers a conformational change in RIG-I, freeing up the N-terminal 2CARD domain which would otherwise be suppressed by the CTD and a linker region between the helicase and CTD. Tetramerization of free 2CARDs allows for MAVS association and further activation of the signalling pathway [108]. Regulation of RIG-I signalling was initially found to rely on K63-linked polyubiquitination on residue K172 of its N-terminal 2CARD domain by TRIM25 (Fig. 5) [101]. Later studies showed that regulation of the CTD release step relied on another E3 ligase called Riplet, which shares high homology with the TRIM family but lacks a B-box domain [104, 122–124]. Riplet was found to not only promote RIG-I activation and signalling, but also to directly ubiquitinate RIG-I (Fig. 5) [122]. However, the importance of TRIM25 in the regulation of RIG-I activity cannot be understated as evidence collected from numerous groups over the past 12 years demonstrates a clear involvement of TRIM25 in RIG-I ubiquitination and activation. Targeting of TRIM25 alone for silencing using siRNA or shRNA in several human and mouse cell types, or TRIM25 gene deletion in MEFs and in various human cells using CRISPR-Cas9, showed strongly impaired innate immune responses following stimulation with physiological RIG-I ligands (e.g. HCV-PAMP), or upon live virus infection (e.g. SeV, HCV, IAV, HPV) [101, 102, 105, 113, 114, 125]. Additional evidence showing regulation of RIG-I by TRIM25 comes from studies that have uncovered several host proteins that regulate the E3 ligase activity of TRIM25 (e.g. NDR2, NLRP12, Caspase-12, Lnczc3h7a), thereby affecting downstream activation of RIG-I [103, 114, 116–118]. Furthermore, direct interactions between endogenous TRIM25 and RIG-I have been demonstrated during viral infection [105, 110].

The notion of different E3 ligases playing critical roles in the activation of RIG-I has recently been challenged with some groups identifying Riplet, and not TRIM25, as the required E3 ligase [126–128]. The challenge was raised when Riplet$^{-/-}$, but not TRIM25$^{-/-}$, cell lines failed to induce innate immune signalling upon stimulation [126], although these experiments did not address different time points or other stimulation conditions that may trigger RIG-I activation, or the possibility of functional redundancy. Furthermore, it

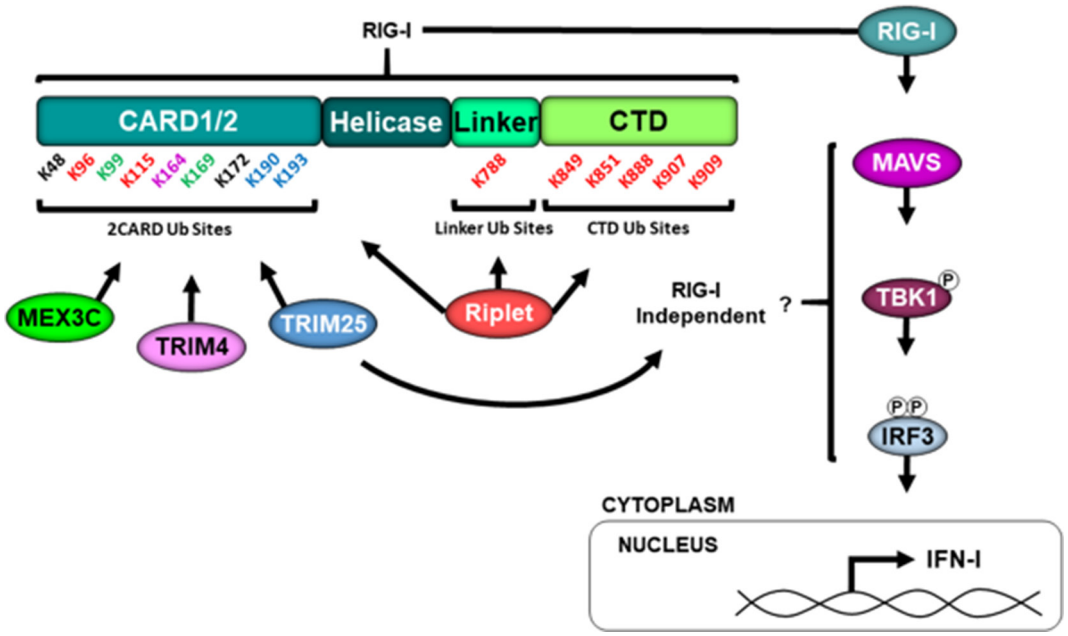

Fig. 5. Ubiquitination of RIG-I. TRIM25, TRIM4, MEX3C and Riplet activate RIG-I innate immune signalling through ubiquitination at various RIG-I Lys residues. Residues coloured in blue (K190, K193), pink (K164), green (K99, K169), red (K96, K115, K788, K849, K851, K888, K907, K909) and black (K48, K172) are ubiquitinated by TRIM25, TRIM4, MEX3C, Riplet and several of the aforementioned E3 ligases, respectively.

is unclear why different reports using the same TRIM25$^{-/-}$ MEF cell lines have yielded different results. Additionally, the authors examined a single TRIM25$^{-/-}$ HEK293T clone which would not eliminate the possibility of off-target effects as a result of the guide RNA used, especially because two TRIM25$^{-/-}$ HEK293 clones from another study showed a reduced IFN-I response during SeV infection [114]. Riplet$^{-/-}$ mice and cell lines are known to have significant losses in both IFN-I and pro-inflammatory responses to infection [123, 126, 128]. Riplet is also known to bind and ubiquitinate several Lys residues in the CTD and linker region of RIG-I while TRIM25 only targets Lys residues in the 2CARD domain (Fig. 5) [101, 108, 122–124]. Whereas complementation of RIG-I with Riplet enhanced IFN-I promoter activity, mutation of the RIG-I linker region Lys (K788R) prevented ubiquitination at that residue by Riplet and abolished the observed IFN response [124]. Riplet-mediated ubiquitination of the CTD Lys residues could precede RIG-I unfolding, thereby allowing TRIM25 access to the exposed 2CARD. Knockout cell lines would not address this confounding possibility and cannot eliminate TRIM25 as a requirement for RIG-I signalling, especially if Riplet somehow influences TRIM25 activation or function. An additional challenge from the aforementioned study was that the authors claimed only Riplet, and not TRIM25, could promote RIG-I ubiquitination in a dsRNA-dependent manner using an *in vitro* ubiquitination assay. However, they also showed that TRIM25 is able to ubiquitinate the 2CARD domain of RIG-I, albeit at higher concentrations of TRIM25 [126]. This study only utilized the E2 enzyme Ubc13 alone or with Uev1A and ignored other E2 enzymes (Ubc5c) found to promote TRIM25-mediated polyubiquitin chain synthesis [21]. Recently, another group has identified TRIM25 as being capable of promoting the K63-linked polyubiquitination of full-length RIG-I and that this effect can be further enhanced by other host factors such as NDR2 [116]. Whether other stimulation conditions during infection may regulate activation of one E3-ligase versus the other also cannot be excluded. In addition, the fact that some TRIMs may be able to coordinate substrate ubiquitination using multiple E2-conjugating enzymes [129] is also difficult to rule out. Another recent study also proposed that Riplet, but not TRIM25, is responsible for RIG-I-dependent type-I and type-III IFN induction against IAV or SeV in CRISPR knockout cell lines [128]. However, TRIM25 knockout mice in this same study did have increased IAV titres in the lungs that correlated with reduced IFNβ protein (although the authors curiously disregard this finding), demonstrating the importance of TRIM25 for IFN induction *in vivo*. This raises the question of whether TRIM25 may have cell-type- or species-specific roles, or whether the effects observed with TRIM25 depend on the experimental conditions. Giving strength to this argument is a recent study using the murine macrophage RAW264.7 cell line that unveiled signalling for IFN-I production required an interaction between TRIM25 and RIG-I that depended on the long non-coding RNA (lncRNA) Lnczc3h7a [118]. One additional challenge that has been ignored in these CRISPR knockout studies is the possibility that TRIM25 has different isoforms with potential regulatory functions, which could also have cell-type-specific expression patterns and functions.

Previous studies, including our own, have shown that TRIM25 may play an antiviral IFN-I-mediated role via RIG-I-independent pathways. For example, knockdown of TRIM25 in human monocyte-derived dendritic cells reduced IFNβ and ISG induction upon stimulation with TLR2 and TLR4 ligands [39]. Triggering of the antiviral state by TRIM25 can also be observed with the ISG zinc-finger antiviral protein (ZAP) [119]. Binding and viral replication assays showed that TRIM25 interacts with ZAP through its SPRY domain and that ZAP's antiviral activity relied on a functional TRIM25 [119]. The ZAP–TRIM25 interaction was revealed to impede Sindbis virus (SINV) translation, consistent with its known roles in targeting vRNA [119]. The K63-linked ubiquitination of RIG-I by TRIM25 is well established, but additional E3 ubiquitin ligases such as Riplet, MEX3C and TRIM4 have been identified as playing critical roles in fully activating the RIG-I pathway (Fig. 5) [101, 108]. Therefore, the fact that TRIM25 may be involved in different aspects of antiviral signalling does not exclude the possibility that TRIM25 and Riplet have redundant functions in the activation of RIG-I, and that one factor may be dominant over the other. A perfect example of this type of redundancy has been described for the kinases TBK1 and IKKε, which are both known to phosphorylate IRF3 and IRF7 for IFN-I induction [130–132]. TBK1 is constitutively expressed in many cell types and knockout cells almost completely lose IFN induction, whereas in some conditions, or cell types, knockout or knockdown of IKKε has only limited effects [133, 134].

There is abundant experimental evidence that TRIM25 plays a role in activation of RIG-I. For example, a full *in vitro* reconstitution of the RIG-I pathway utilizing TRIM25 as the E3 ligase is known to potently activate RIG-I signalling through unanchored K63-linked polyubiquitin chains [21]. In addition, viral products have been found to directly antagonize TRIM25, consequently reducing RIG-I ubiquitination and downstream signalling, including paramyxoviruses V protein, the papillomavirus E6 oncoprotein, the severe acute respiratory syndrome-related coronavirus (SARS-CoV) N protein, the NSs protein of severe fever with thrombocytopenia syndrome virus (SFTSV), the nucleocapsid protein (N) of porcine reproductive and respiratory syndrome virus (PRRSV) and potentially DENV subgenomic RNA [106, 107, 111, 113, 115, 135]. Specifically, the IAV NS1 protein inhibits both TRIM25 and Riplet, and this occurs in a species-specific manner [104]. Importantly, this study also showed that the roles of TRIM25 and Riplet in ubiquitinating mouse or human RIG-I may be different, especially because the residue K172 in the 2CARD domain of human RIG-I is not conserved in mouse RIG-I [104]. This study also suggested that, at least in mice, Riplet is unlikely to ubiquitinate the 2CARD domain of RIG-I because IAV NS1 did not inhibit ubiquitination of the murine 2CARD [104]. Therefore, any data from new studies suggesting TRIM25 does not play a role in RIG-I activation should be carefully interpreted given the differences observed with cell types, host species, stimulatory conditions and pathway redundancies.

A final challenge to the current dogma of TRIM25 involvement in RIG-I signalling dawned from the remaining presence of an innate immune response in TRIM25$^{-/-}$ cells upon stimulation [126]. However, in 2014 two additional E3 ligases (MEX3C and TRIM4) were identified as important components in mediating the K63-linked polyubiquitination of RIG-I 2CARD and facilitating downstream signalling (Fig. 5) [136, 137]. Compensatory mechanisms in antiviral host defence are commonplace as viruses are continually evolving to circumvent these barriers. TRIMs exemplify this as multiple TRIMs can cooperatively regulate a common target. K172 in the RIG-I 2CARD domain is the critical residue for TRIM25-mediated RIG-I activation and yet TRIM4 and Riplet also target K172, indicating a redundant role (Fig. 5) [127, 137]. Indeed, cooperative inhibition of viral antagonism by TRIM25, Riplet and TRIM4 was described during IAV and SeV infection in two independent studies [104, 137]. Of note was the additional findings from the challenging study that Riplet can interact with dsRNA-bound RIG-I, leading to cross-bridging of RIG-I–dsRNA oligomers. This formation of higher order structures was independent of Riplet's RING domain and induced both MAVS aggregation and IFN-I production [126]. This ubiquitin-independent function could implicate the E3 ligase structure as a novel aspect of RIG-I activation that may be applicable to other E3s and innate immune pathways. In conclusion, there is overwhelming evidence that TRIM25 is indeed involved in RIG-I-mediated IFN induction, and that it also retains non-redundant functions in the IFN response that are independent of RIG-I. Although it is clear that Riplet plays dominant roles in RIG-I activation, redundancy of these and other ubiquitin ligases cannot be excluded.

TRIM5α and ubiquitin in immune signalling

In the mid-2000s, TRIM5α was identified as a restriction factor that specifically recognizes incoming retroviral capsids [63, 138, 139]. In addition to its strong affinity, TRIM5α can become polyubiquitinated in response to the presence of these capsids. The importance of TRIM5α ubiquitination was demonstrated by Campbell and colleagues utilizing a modified version of TRIM5α which was fused to the small catalytic domain of the herpes simplex virus (HSV) UL36 DUB [65]. The placement of this DUB on the N-terminal RING domain provided a means to deconjugate ubiquitin chains associated with TRIM5α and allowed for a proteasome inhibitor-independent way of assessing the antiviral function of TRIM5α ubiquitination. This DUB-fused TRIM5α allowed for the presence of more intact retroviral capsids and an increase in HIV reverse transcription products compared to controls [65]. Notably, this lack of ubiquitination correlated with a failure to induce the NF-κB pathway [65]. A thorough examination of the mechanism behind TRIM5α ubiquitination was recently provided by Fletcher and colleagues [140]. TRIM5α itself can be monoubiquitinated at its N terminus, leading to its proteasomal degradation before formation of higher-order assemblies seen during infection [64, 140]. This monoubiquitination occurs as a result of an interaction with

the E2 conjugase Ube2W that cooperates with an additional E2 (Ube2N/Ubc13) to conjugate additional ubiquitin moieties onto TRIM5α [64, 71]. Upon retroviral capsid recognition and higher-order assembly, the trimer of RING domains that are now in proximity can operate with Ube2N in a 'two-plus-one' manner where a RING dimer and Ube2N conjugate several ubiquitin molecules onto the N-terminal monoubiquitin of the third RING domain [140]. This process continues to extend the K63-linked polyubiquitin chain needed for NF-κB signalling. Fascinatingly, TRIM5α receives its N-terminal monoubiquitin early, creating a 'standby' protein ready for degradation in the absence of infection and higher-order assemblies. This stepwise progression of capsid recognition, higher-order assembly, K63-linked polyubiquitination and NF-κB signalling function as checkpoints, thereby allowing TRIM5α to act as its own antiviral signalling platform with an established criterion system for what is sufficient for activation and signal induction (Fig. 4) [140, 141]. TRIM5 has therefore been proposed to act as a PRR by recognizing the retroviral capsid and promoting activation of the TAK1 kinase via synthesis of unanchored K63-linked polyubiquitin chains [63]. Further discussion of TRIM5α can be found in recent reviews [4, 32].

Cooperative antiviral mechanisms of TRIM22 and TRIM19

The inhibition of pathogens can sometimes involve multiple host restriction factors working in concert. HIV-1 transcription through the host Specificity protein 1 (Sp1) is known to be inhibited by TRIM22 [142]. This is achieved via blockade of the interaction between Sp1 and the HIV-1 LTR promotor region, although this does not involve a direct interaction between TRIM22 and Sp1, suggesting TRIM22 involvement in an inhibition complex [142, 143]. Interestingly, TRIM22 was found to interact with HDAC, a repressor of HIV-1 viral transcription, and this interaction was necessary for continual proviral suppression [144]. Both human and murine TRIM19 are capable of preventing HIV-1 replication by downregulating expression of the viral LTR [145, 146]. This effect holds true for several isoforms of TRIM19, including I, II, IV and VI, which can be enhanced further by IFN-I stimulation [145]. Recently, the HIV-1 restriction factor class II transactivator (CIITA) was identified as a TRIM22 binding partner that resulted in re-localization of both components into TRIM22 nuclear bodies (NBs) [147]. Furthermore, TRIM19 was also recruited to TRIM22 NBs and IFN stimulation alone was sufficient for NB formation and integration of both CIITA and TRIM19 [147]. Due to CIITA's role as a countermeasure to Tat-driven HIV-1 LTR transactivation and TRIM19's aforementioned abilities, it is possible that TRIM22 NBs can function as a central hub for prevention of retroviral transcription and establishment of latency [144, 147].

Innate antiviral functions of fish TRIMs

The importance of TRIMs extends beyond the mammalian immune system. Involvement of TRIMs in the immune response to aquatic pathogens has garnered increased interest [148]. Three TRIMs from orange spotted grouper (EcTRIM39, EcTRIM25 and EcTRIM32) were shown to counteract pathogens that can be detrimental to aquaculture farms, including Singapore grouper iridovirus (SGIV) and red spotted grouper nervous necrosis virus (RGNNV) [149–151]. Although the exact means of antiviral activity of these fish TRIMs is unknown, their presence positively enhanced IFN and pro-inflammatory pathway markers [149–151]. Interestingly, this antiviral function appears to rely on cellular re-localization as RING domain knockout mutants not only failed to activate innate immune responses, but also appeared misplaced in the cell with EcTRIM39-ΔRING trapped in the nucleus and EcTRIM25-ΔRING forming aggregates and filaments [149, 150]. The presence of multigenic TRIM subsets in fish led to the identification of the fish novel TRIMs (finTRIMs) present in teleosts with functions in innate immunity [31, 148]. Some recent examples of the antiviral functions of finTRIMs can be noted with finTRIM36- and finTRIM83-mediated activation of the IFN-I pathway and subsequent restriction of RNA and DNA viruses [152, 153]. It remains to be determined whether finTRIM restriction relies on the activation of immune pathways or inhibition of viral factors. However, their antiviral function was shown to rely on functional RING and PRY-SPRY domains [152]. This requirement suggests a similar regulation of immune pathways between mammalian and fish TRIMs [148, 154].

Indirect restriction of viral replication

Alteration of host cell processes and structures presents another way for TRIMs to indirectly inhibit viral invasion. TRIM2 reduces infection of New World arenaviruses such as the Junín virus (JUNV) vaccine strain Candid 1 but not Old World arenaviruses such as Lassa or LCMV [155]. Interestingly, restriction by TRIM2 halted infection of target cells without preventing interaction [155]. Furthermore, the restrictive capability of TRIM2 was independent of the RING domain yet required the FIL domain, suggesting TRIM2 operates at a post-receptor binding step in the viral life cycle that is independent of E3 ligase activity [155]. Interactome profiling of TRIM2 identified signal regulatory protein α (SIRPA) as the necessary interacting molecule for halting JUNV entry. SIRPA's role in preventing phagocytosis is harnessed by TRIM2, resulting in the blockade of JUNV internalization [155]. In a TRIM RNAi screening, TRIM43 was found to be important in limiting the reactivation of latent herpesviruses including Kaposi's sarcoma-associated herpesvirus (KSHV) [156]. TRIM43 expression was also strongly induced for a number of herpesvirus members, including EBV, human cytomegalovirus (HCMV), and HSV-1 but not for DENV or VSV, indicating TRIM43 may attenuate an inherent trait found in herpesviruses. MS analysis identified the centrosomal protein pericentrin (PCNT) as a binding partner, consistent with the centrosomal localization of TRIM43 [156]. The importance of this interaction was evident during HSV-1 and KSHV infection where TRIM43-mediated ubiquitination and proteasomal degradation of PCNT was key in the control of herpesvirus infection primarily through

alterations in nuclear lamina integrity as a consequence to PCNT destruction [156]. Without defined nuclear lamina, associations between herpesvirus chromatin and host chromatin are inhibited, thereby lowering viral replication [156].

Negative regulation of host antiviral signalling

Some TRIMs can enhance viral infection as a consequence of their negative regulatory functions in the host [4]. TRIM27 expression becomes induced during infection or IFN-I stimulation, which coincides with an increase in HCV replication [157]. In this case, the presence of TRIM27 attenuates the antiviral response of both the IFN-I and pro-inflammatory signalling pathways [157]. Although the precise mechanism is unknown, it is likely to operate in a similar manner to what was observed by other groups using different RNA and DNA viruses. Stimulation with either VSV, HSV or SeV also led to a reduction in IFN-I production as a result of TRIM27 [158]. Specifically, infection with these viruses promoted Siglec1 gene expression, leading to the formation of a negative feedback signalling complex consisting of DAP12, SHP2 and TRIM27 [158]. Recruitment of TRIM27 leads to the targeting of TBK1 for K48-linked polyubiquitination and proteasomal degradation, thereby quenching the IFN-I signal and increasing viral replication [158]. Additional characterization of the TRIM27-TBK1 signalling axis revealed an important role for the DUB USP7 in facilitating TRIM27's activity. TRIM27 has a short half-life and can be ubiquitinated, suggesting the protein is under constant turnover, possibly mediated by the proteasome [159]. USP7 was found to bind and remove ubiquitin from TRIM27 allowing for the ubiquitination and degradation of TBK1 (Fig. 6) [159].

Similar studies have uncovered a variety of TRIMs that can also potentiate the replication of pathogens as a result of their function as suppressors of excessive inflammation. Activation of IFN-I through the vRNA sensors RIG-I and MDA5 were found to be under the pressure of both TRIM13 and TRIM40 [160, 161]. In both cases, suppression of MDA5-mediated IFN-I production was achieved through binding with either TRIM, leading to enhancement of viral replication (Fig. 6) [160, 161]. For TRIM40, this dampening of the antiviral immune response involved an interaction between its CC domain and the 2CARD domain of MDA5, leading to its K27- and K48-linked polyubiquitination and removal via the proteasome [161].

Impairment of both vRNA and vDNA sensing pathways by a single TRIM highlight how host homeostasis mechanisms are exploited by various pathogens. Suppression of IFN-I pathway activation in response to both RNA and DNA viruses has been shown with TRIM29. Stimulation with IAV, Reovirus and EBV induces the expression of TRIM29 in bone marrow-derived dendritic cells (BMDCs), macrophages (BMDMs), and alveolar epithelial cells (AECs) [162–165]. RNA virus infection led TRIM29 to target both NEMO and MAVS for proteasomal destruction through K48-linked and K11-linked polyubiquitination, respectively [162, 164]. In the case of EBV infection, TRIM29 directly interacted with the cytosolic DNA sensor STING for ubiquitin-mediated degradation showing

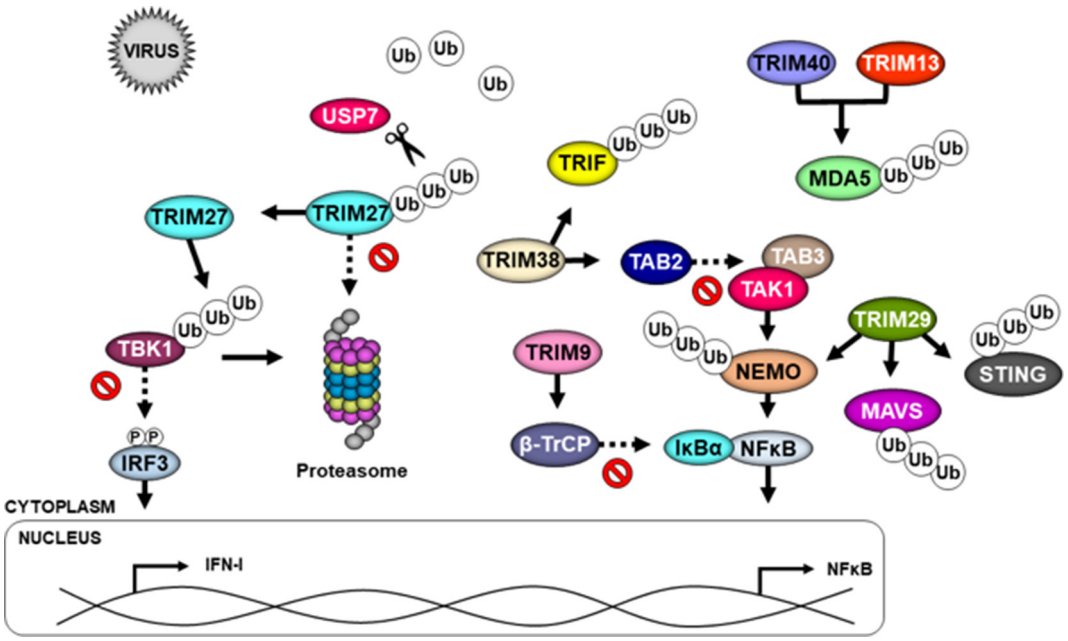

Fig. 6. TRIM regulation of antiviral signalling. TRIM27 targets TBK1 for ubiquitin-mediated proteasomal degradation, a process made possible by the deubiquitination of TRIM27 by USP7. TRIM38 prevents NF-κB signalling through the proteasomal degradation of TRIF and the sequestration of TAB2 from forming the TAK1 complex. TRIM40 and TRIM13 both target MDA5 for ubiquitination and subsequent proteasomal degradation. TRIM9 prevents β-TrCP from targeting IκBα for the release of NF-κB. TRIM29 can inhibit multiple innate immune pathways by independently targeting NEMO, MAVS and STING for ubiquitination and destruction.

TRIM29's indirect involvement in viral replication by eliminating critical PAMP sensors (Fig. 6) [163].

Negative regulation of innate immunity is also achieved by suppressing pro-inflammatory cytokine production. In an overexpression screening of TRIMs that alter NF-κB levels, TRIM9 was shown to lower NF-κB promoter activity 10–20-fold [166]. TRIM9 is present in high concentrations in neuronal tissues during all life stages and thwarts the induction of NF-κB through sequestration of the pathway component *β*-TrCP (Fig. 6) [166]. As an E3 ubiquitin ligase, *β*-TrCP targets the regulatory component of NF-κB, IκBα, for ubiquitination and proteasomal destruction allowing for NF-κB nuclear translocation. This barrier to pro-inflammatory cytokine production may explain the abundance of TRIM9 expression in immunoprivileged sites such as the brain where it can limit the prospect of neuroinflammation. Interestingly, TRIM9's function appears conserved across different species, as overexpression of the recombinant oyster homologue, ChTRIM9, in HEK293T cells resulted in a reduction to NF-κB promoter activity [167]. Furthermore, the shrimp homologues of TRIM9 and *β*-TrCP (LvTRIM9 and Lv*β*-TrCP, respectively) can also interact and depletion of LvTRIM9 during white spot syndrome virus (WSSV) infection invoked higher levels of antimicrobial peptides [168]. However, TRIM9 expression in immune cells appears to be cell-type-specific, with high levels of expression in CD4 regulatory T cells that produce high levels of IL-10 cytokine [28]. Whether TRIM9 is involved in regulating IL-10 expression for the development of specific T-cell subsets remains to be studied.

TRIMs may be involved in regulation of multiple signalling pathways, which is exemplified by TRIM38 [169]. TRIM38 negatively regulates initial IFN-I and pro-inflammatory cytokine production by targeting the TRIF adaptor protein for K48-linked polyubiquitination and degradation after PAMP recognition by TLR3/4 [170]. Further inhibition is observed at the level of pro-inflammatory cytokine signalling where TRIM38 prevents TNFα and IL-1*β* receptor pathways by degrading the TAB2 component of the TAB2/3-TAK1 signalling platform [171, 172]. Remarkably, this destruction of TAB2 is independent of TRIM38's E3 ligase activity and instead relies on an interaction between TAB2 and TRIM38's PRY-SPRY domain that ferries TAB2 to degradative lysosomes [171]. TRIM38's ability to regulate multiple steps of a pathway through different mechanisms highlights the important role TRIMs have in moderating the innate immune response (Fig. 6).

VIRAL ANTAGONISM OF THE IMMUNE RESPONSE INVOLVING TRIMs

As mentioned above, since its identification as a critical component for induction of RIG-I-mediated innate immunity, TRIM25 has received much attention not only for its importance in antiviral defence, but also for how a diverse range of pathogens have zeroed in and neutralized its function [101, 102, 104, 106, 107, 109, 111–113, 115]. Besides TRIM25, other TRIMs have been found to be targeted by viruses. A viral structural protein that moonlights as an antagonist of host innate immunity is the matrix protein from Nipah virus (NiV-M) [173]. In what was the first description of a henipavirus structural protein targeting a host component of innate immunity, NiV-M was found to prevent IFN-I production by inhibiting the IKKε kinase. Specifically, NiV-M bound and eliminated TRIM6, the E3 ubiquitin ligase responsible for synthesizing the K48-linked unanchored polyubiquitin required for IKKε oligomerization and cross-phosphorylation [22, 173]. Curiously, the degradation of TRIM6 by NiV-M was independent of the proteasome and lysosome, but instead was dependent on the preservation of NiV-M compartmental trafficking [173]. The K258A mutant of NiV-M with improper localization still retained TRIM6 binding, but was incapable of degrading it, suggesting that proper localization of NiV-M affords the recruitment of additional factors involved in TRIM6 destruction [173].

In an effort to combat selective pressures, viruses have evolved in a manner that allows their limited proteome to retain numerous functions, thereby augmenting their capacity to counteract the various methods with which host cells restrict viral replication. The HSV-1 US11 protein antagonizes host immune responses by limiting the efficacy of TRIM23-mediated autophagy [55, 174]. Autoubiquitination of TRIM23 during infection with K27-linked polyubiquitin activates its GTPase programing that is required for TBK1-mediated activation of the autophagy pathway component p62 [55]. US11 binds TRIM23 and prevents the necessary incorporation of TBK1 into the budding autophagy initiation complex consisting of TRIM23, TBK1 and Hsp90 [174]. Additional herpesvirus members including HCMV rely on similar mechanisms to abrogate the formation of host antiviral complexes. HCMV IE1 can directly target and accumulate near forming TRIM19 NBs to prevent assembly of these structures and ultimately inhibit innate immune responses [175].

Several TRIM genes are IFN-inducible, allowing for induction of their potent antiviral effect only when deemed necessary. Some viruses, such as HBV, have developed methods for preventing such rapid activation of immune programmes through epigenetic manipulation of these host genes. LC-MS/MS screening of IFN-inducible genes whose expression is affected during HBV infection was performed using stable cell lines expressing a major epigenetic modifier of host genes, HBx [176]. TRIM22 was found to be downregulated during IFN stimulation in the presence of HBx, implicating it as a viral target. The promoter regions of several IFN-inducible TRIMs, including TRIM14 and TRIM22, are under control of the IRF1 transcription factor [177]. A single CpG methylation site located in the 5′-UTR of the TRIM22 gene was identified as the target for HBx-mediated restriction. Coincidentally, this site is also part of the TRIM22-IRF1 binding region revealing a mechanism by which HBV regulates IFN-induced host gene expression [176].

NOVEL PRO-VIRAL ROLES OF TRIMs AND OTHER E3 UBIQUITIN LIGASES

In spite of the numerous instances where TRIMs function as critical components in antiviral innate immunity, a previously unrecognized role of TRIMs is starting to emerge on the opposite side of the spectrum by promoting the activities of an invading pathogen. This can include indirectly assisting viruses in establishing a productive infection by preventing the full breadth of the innate immune response as a consequence of their programming to avoid uncontrolled inflammation. Incidentally, TRIMs can also become beneficial to viruses if they are absent from their posts, which can be brought about through forced dismissal by viral components. Finally, in line with the Red Queen hypothesis, a theory proposing that any evolutionary advancement in a species is countered by an equal advancement in a coexisting species [178, 179], TRIMs intended for host defence can become tools for viruses through appropriation and repurposing to directly promote viral survival.

Thus far, a majority of TRIM-related studies have focused on the roles TRIMs play as antiviral factors. The original works identifying TRIMs as restriction factors sparked a flurry of investigations thereafter focused on these antiviral and innate immune roles, and with this the field missed an important potential characteristic of TRIM function. Only recently have new TRIM studies begun to uncover a novel aspect of TRIM–pathogen interactions where the same viruses that TRIMs target for inhibition can also co-opt these TRIMs and take advantage of their ubiquitin ligase activities to directly promote viral replication. This presents itself as a striking mechanism utilized by viruses because hijacking a known antiviral factor and repurposing its functions to enhance replication would give double the advantage. Indeed, we have previously shown that TRIM6 knockout cell lines, which have a reduced IFN-I-mediated antiviral response, do not provide an optimal replication environment for Ebola virus (EBOV) [22, 180]. Interestingly, further evidence exists in recent studies that utilized genome-wide siRNA knockdown or CRISPR/Cas9 knockout screens on the potential pro-viral roles of TRIMs (e.g. TRIM23) [181]. This study does not mark the first instance of TRIM23 being identified in a screen as a pro-viral factor as the NS5 component of yellow fever virus (YFV) was found to require K63-linked polyubiquitin from TRIM23 in order to interact with STAT2 and inhibit IFN-I signalling [182]. Before these studies, TRIM23 was known as an antiviral signalling component [183]. Other screens have identified additional TRIMs as potential pro-viral factors for flaviviruses, for example TRIM7 [184]. Unfortunately, results from some of these screens have not been pursued, probably due to the low statistically significant changes observed in viral replication when TRIMs are targeted. TRIM7 has also been reported to have antiviral restriction activity against Noroviruses (NoVs) [185], and plays a role in different immune signalling pathways including activation of c-Jun/AP-1 transcription factors [186], and potentially in induction of inflammatory cytokines upon TLR4 stimulation in macrophages [187]. If more TRIMs behave similarly to TRIM6, TRIM7 and TRIM23 during infection, the inconclusive results generated by screens could be explained by simultaneous antiviral and pro-viral functions, or by specific effects from TRIM isoforms and cell types.

To date, the involvement of TRIM family E3 ubiquitin ligases in host innate immunity and viral replication has centred on a general dynamic between TRIMs and viruses. TRIMs prevent viral invasion by governing innate immune defences or outright elimination of viral factors. Viruses establish infection by exploiting the inherent regulatory roles of TRIMs or through the calculated neutralization of TRIMs required for an antiviral state. However, these paradigms leave out the possibility of TRIMs directly participating in viral success as an unintentional cofactor. The novel concept of viruses hijacking TRIMs to directly participate in successful viral replication was recently reported by our group with TRIM6 [180]. TRIM6 is an important component in IFN production and signalling, although EBOV replication in TRIM6 knockout cells was not increased, suggesting that TRIM6 is required for optimal EBOV replication [22, 180]. The EBOV VP35 protein, a known IFN antagonist and cofactor of the viral polymerase, was found to interact with the SPRY domain of TRIM6, implicating VP35 as a target for ubiquitin [180, 188–190]. Indeed, MS identified K309 on VP35 as a target for ubiquitination and co-expression of TRIM6 enhanced ubiquitinated forms of VP35 [180]. Direct enhancement of EBOV replication by TRIM6 was observed in an EBOV minigenome assay where increasing amounts of TRIM6, but not the catalytically defective C15A RING mutant, enhanced EBOV polymerase activity [180]. Although a K309A mutation on VP35 reduced ubiquitination, the presence of mono-, di- and unanchored ubiquitinated forms of VP35 persisted, indicating VP35 is ubiquitinated at additional residues whose functional roles remain to be determined [180].

Viral co-opting of the host ubiquitin machineries for direct enhancement of replication has been described, with some studies identifying the involvement of different families of E3 ubiquitin ligases. Direct use of the host ubiquitin system for replication by IAV has been implicated at different stages of the viral life cycle including vRNA replication and progeny virion packaging. Ubiquitination of every subunit of the IAV vRNP (PB1, PB2, PA and NP) has been observed with a direct correlation between increasing ubiquitination and upregulation of viral polymerase function [191]. Monoubiquitination of the IAV NP by the host E3 ligase CNOT4 does not target NP for proteasomal degradation, but instead enhances viral replication and vRNP activity [192]. The avibirnavirus infectious bursal disease virus (IBDV) and the recently emerged duck tembusu virus (DTMUV) also show reliance on the host ubiquitin system for replication [193, 194]. The K63-linked polyubiquitination at K751 of VP1 promoted IBDV replication while mutant K751R viruses exhibited significant impairments [193]. Reduction of host ubiquitin lowered vRNA transcription of DTMUV and therefore impaired replication [194]. The need for a clearer understanding of how viruses utilize the host ubiquitin system extends beyond human

health as IBDV and DTMUV are both important animal pathogens responsible for significant losses to waterfowl industries [194].

Additional viral components outside of the replication complex have shown a requirement for ubiquitination that can directly impact viral propagation. Identification of K78 on the cytoplasmic domain of the IAV M2 protein as a target for ubiquitination uncovered a role for ubiquitin in the successful formation of progeny virions [195]. Mutant viruses harbouring a K78R switch in their M2 protein produced severely flawed viral particles that lacked vRNA and vRNPs and otherwise appeared empty under electron microscopy observation [195]. HCV assembly at the endoplasmic reticulum (ER) interface depends on the E3 ubiquitin ligase MARCH8 [196]. Screening of critical components of the UPS that interact with the HCV NS2 protein identified MARCH8 as a required host factor. MARCH8 interacts with NS2 at the ER and promotes its K63-linked polyubiquitination in order to facilitate HCV envelopment into the ER lumen and assembly of the infectious particle [196].

UNANCHORED UBIQUITIN IN IMMUNITY AND VIRUS REPLICATION

Free or unanchored polyubiquitin chains, which retain their free C-terminal Gly residue and thus are not covalently attached to any protein, have been proposed to play roles in both immune signalling and virus replication [20–22, 197, 198]. Although there has been debate as to whether these chains have biological functions or whether they might be detected in cells as an artefact after cleavage of covalent polyubiquitin by DUBs, there is abundant experimental evidence *in vitro* supporting a functional role [20–22]. Purified, unanchored K63-linked polyubiquitin chains added *in vitro* have been shown to promote the autophosphorylation and subsequent activation of TAK1 (Fig. 7) [20]. TRIM5α has also been proposed to be involved in the synthesis of unanchored K63-linked polyubiquitin chains that can activate TAK1 as a result of binding to the HIV-1 capsid [63]. Additional studies have also shown that unanchored K63-linked polyubiquitin chains synthesized by TRIM25 facilitate RIG-I oligomerization and stabilization, leading to downstream IRF3 phosphorylation *in vitro* (Fig. 7) [21]. Unanchored K63-linked polyubiquitin has also been shown to interact with MDA5, leading to oligomerization of its 2CARD domain [199]. Furthermore, recognition of the Fc receptor of pathogen-bound antibodies by TRIM21 can also lead to the production of unanchored K63-linked polyubiquitin that can activate the AP-1, IRF3 and NF-κB pathways [99, 200].

Evidence suggesting unanchored ubiquitin is indeed relevant comes from *in vivo* studies where an E3 ligase responsible for synthesis of unanchored ubiquitin, TRIM6, was knocked down in the lungs of IAV-infected mice using peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) [22]. *In vitro*, TRIM6 promotes the synthesis of unanchored K48-linked polyubiquitin chains which induce IKKε activation through oligomerization and autophosphorylation (Fig. 7). PPMO-mediated knockdown of TRIM6 reduced binding

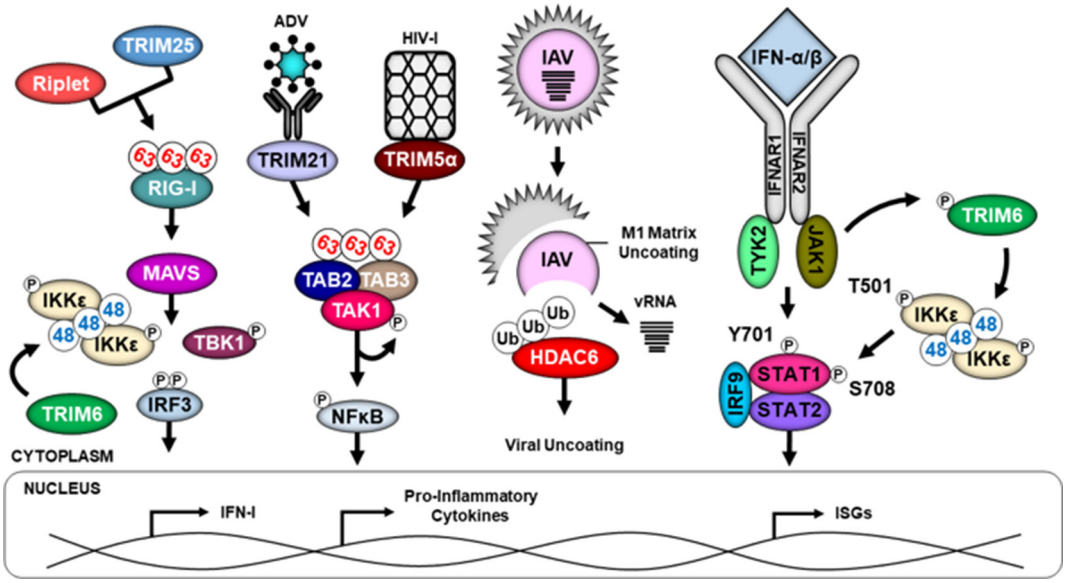

Fig. 7. Unanchored ubiquitin involvement in innate immunity. Unanchored K63-linked polyubiquitin synthesized by TRIM25, and potentially Riplet, can promote IFN-I production through the activation of RIG-I via an interaction with the 2CARD domain of RIG-I. TRIM21 and TRIM5α can recognize antibody-bound virions (Adenovirus) and retroviral capsids (HIV-1) respectively, leading to production of unanchored K63-linked polyubiquitin chains that activate TAK1. The influenza virion packages unanchored ubiquitin that aids in uncoating through the host aggresome component HDAC6. TRIM6 synthesizes unanchored K48-linked polyubiquitin that activates IKKε-mediated innate immune signalling. Regulation of TRIM6's activity may rely on phosphorylation by JAK1.

of IKKε to unanchored polyubiquitin and inhibited IKKε-mediated immune signalling *in vivo* [22]. Further *in vivo* evidence that TRIM6 and unanchored K48-linked polyubiquitin play an important physiological role comes from recent studies using knockdown and overexpression of TRIM6 in murine hearts, which showed that TRIM6 promotes myocardial infarction (cardiomyocyte apoptosis and heart attacks), via activation of IKKε and STAT1 phosphorylation [201].

In addition to the role of unanchored ubiquitin in immune signalling, it has also been proposed that IAV carries unanchored ubiquitin chains within the virion itself [197]. These chains can activate the host aggresomes pathway to help the virus uncoat during the entry stage of its life cycle (Fig. 7). Whether this viral strategy also inadvertently triggers host immune signalling through the recognition of these free ubiquitin chains after virus uncoating is currently unknown [198].

CONCLUSIONS AND FUTURE PERSPECTIVES

This review has focused on recent advancements in TRIM-mediated innate immunity and how viruses counteract these systems for their own benefit. We emphasize the well-characterized views of TRIMs as both critical components in regulating antiviral responses and as targets of viral antagonism while also bringing to attention the growing evidence of E3 ubiquitin ligases being hijacked to directly promote viral functions. The information extrapolated from these investigations identifies a thin line separating ubiquitin as a host evolutionary defence mechanism or as a useful component in viral evolution that warrants further attention.

The past decade of work on ubiquitin and TRIM proteins has led to countless discoveries in PTMs, immune signalling and regulation, and pathogen sensing and host–virus interactions. Although these advances have broadened our understanding of the complexity involved in cellular functions, there remain unresolved questions that the field is reluctant to address. Proper delineation of the roles between covalent and unanchored ubiquitin seems distant as it is currently impossible to generate ubiquitin knockout phenotypes that would result in either anchored or unanchored ubiquitin expression. Presently, the best available strategies involve removal of either the E2 or the E3 enzymes involved in their synthesis. New and more inventive approaches are needed to resolve the precise role of unanchored polyubiquitin.

The reality of cell-type- and isoform-specific effects also adds an element of difficulty in deciphering the different roles TRIMs have. Use of CRISPR-based gene knockouts in validating phenotypes observed in overexpression and knockdown studies has become an increasingly common tool for data robustness, and recent advancements suggest novel CRISPR-Cas systems (Cas13d) can be utilized for editing alternative splicing events, thereby modifying expression of protein isoform ratios [202, 203]. Implementing these systems could usher in a new era of isoform-specific CRISPR knockouts.

The high degree of redundancy between TRIMs has made isolating their individual contributions a challenge. Compensatory mechanisms and fail-safe systems, while ideal for counteracting an ever-evolving threat, have prompted controversies on the importance of one factor over another. An increase in the utilization of *in vitro* assays may aid in assessing the individual effect one TRIM may have on a particular pathway while simultaneously preventing unforeseen cross-talk from other TRIMs that have yet to be characterized.

Differentiation between antiviral and pro-viral roles for TRIMs is presently a murky subject. From a technical perspective, these mechanisms would be challenging to study as use of knockout cell lines for antiviral factors would alone be advantageous to viral replication, making it difficult to observe pro-viral phenotypes unless the antiviral factor in question is absolutely necessary for facilitating viral replication. One of the effects, either antiviral or pro-viral, would need to win in order to accurately assign TRIMs into either category. Subtle experimental details such as cell-type and tissue specificity, the particular virus being examined, and overly stringent *P*-values may all contribute to preventing the identification of TRIMs that are both antiviral and pro-viral. With currently available techniques, determining where a particular TRIM exists in the balance between aiding the host or the virus has proven to be a puzzling endeavor (Fig. 1).

The crossroads between ubiquitin, TRIMs and viruses shows beautifully how interconnected a host and pathogen are. Despite the wealth of knowledge garnered from examining how this intersection has come to be, it has proven to hold many secrets. Still, the difficulty in elucidating the unknown has spurred the very innovation needed to advance this field. Perhaps the next decade of research will find solutions to the current unanswered questions and lead to progress in the development of novel antiviral therapies.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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