ROLE OF TRIM41 IN ANTIVIRAL DEFENSE AGAINST INFLUENZA AND VESICULAR STOMATITIS VIRUS

By

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Abstract:
TRIM41 is a member of TRIM family of host proteins. TRIM proteins are E3 ubiquitin ligase proteins and play important roles in various cellular functions like tumorigenesis, autophagy, innate, and intrinsic antiviral immunity. Structurally 3 domains of proteins are preserved throughout the family and the C-terminal domains are variable. TRIM41 is a protein that is ubiquitous and constitutively expressed in variety of cell types. N-terminal RING, B-BOX and Coiled Coil domains are conserved domains where as TRIM41 possesses a C- terminal SPRY domain. Our proteomics studies of host-viral protein interaction network revealed interaction between TRIM41 and nucleoprotein of Influenza A virus (IAV).We found that TRIM41 interacts with nucleoprotein of these Influenza A through its SPRY domain. Overexpression of TRIM41 in cells reduces the IAV infection whereas depletion of TRIM41 increases susceptibility of host cells to the infection. TRIM41 like all other TRIM proteins is a E3 ubiquitin ligase and it polyubiquitinates IAV nucleoprotein in-vitro and in cells. TRIM41 lacking E3 ligase activity fail to restrict viral infection indicating the importance of E3 ligase activity for TRIM41 in its antiviral function. During the study, TRIM41 was also identified as an inhibitor of vesicular stomatitis virus (VSV) infection. TRIM41 through its SPRY domain interacts with nucleoprotein of VSV. RING domain plays a crucial role in antiviral activity of TRIM41 against VSV. The interaction with TRIM41 leads to polyubiquitination and proteasomal degradation of VSV nucleoprotein. Taken together, TRIM41 is a host intrinsic immune protein that functions against IAV and VSV by targeting their nucleoprotein for polyubiquitination and subsequent proteasomal degradation.

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CHAPTER I

REVIEW OF LITERATURE

Earlier version of this chapter has already been published as Girish Patil, Shitao Li. Tripartite motif proteins: an emerging antiviral protein family. Future virology, 2019; 14(2):107-122

Virus must engage with host cells to replicate and spread, which warrants profound changes in host cellular signaling pathways, especially the instigation of host immune responses. Host defenses are complex systemic immune responses at different levels, including intrinsic, innate and adaptive immunity. Recent studies show that the tripartite motif (TRIM) containing proteins play a versatile role in all layers of host immune defenses. TRIM proteins are a family of ubiquitin E3 ligases which share a characteristic N-terminal tripartite motif (also known as RBCC motif) consisting of a highly conserved order of three domains: the really interesting new gene (RING) domain, one or two B-box domains and a coiled coil region1. The RING domain confers TRIM proteins the ubiquitin E3 ligase activity 2. Ubiquitination is a translational modification which alters target protein stability, trafficking, subcellular localization, enzymatic activation and protein recruitment etc. TRIM proteins have been found to promote the host immune response by ubiquitination of critical signaling molecules in immunity and to interfere with viral infection by directly targeting viral proteins for degradation.

TRIM proteins are also known to participate in other biological processes, such as development and carcinogenesis [3], however, TRIM proteins are gaining in importance in the field of viral immunity in recent years and the studies of TRIM proteins in host defense is one of the fastest growing subjects in the field of immunity. Thus, a systematic and comprehensive understanding of the role of TRIM proteins in multiple layers of host defense will not only help elucidate the antiviral mechanisms of TRIM proteins but also provide potential for developing new antiviral therapeutics. In this review, we focus on the role and the antiviral mechanisms of TRIM proteins in each layer of host immune defense, elaborate viral evasion strategy by targeting TRIM proteins, and discuss the future direction.

TRIM PROTEIN: STRUCTURE, CLASSIFICATION & EXPRESSION

TRIM protein family consists of 76 members, which all share the RBCC motif. RBCC domain consists of three zinc binding motifs which are RING, B-box type 1 & 2 and coiled coil domain. The RING zinc finger motif is 40-60 amino acid long, which is essential for the ubiquitin E3 ligase activity and the binding with E2 conjugating enzyme [4]. RING domain plays a crucial role in TRIM proteins-mediated ubiquitination [5]. B-box domain presents in either single or paired form and plays a role in self-assembly of TRIM proteins and interaction with their substrates [6]. Coiled coil is a common hyper-helical structure which involves protein-protein interaction, including protein dimerization[1,7]. Similarity, the coiled coil of TRIM mediates dimerization and self-association of TRIM proteins [8,9]. Additionally, the coiled coil sequesters TRIM proteins in the cytoplasm or the nuclear bodies [1]. The RBCC motif is followed by one or more domains located on the C-terminus of TRIM proteins. The TRIM superfamily is classified into 11 groups according to the C-terminal domains (Figure 1). Group 1 is featured with multiple domains, including the C-terminal subgroup one signature (COS), fibrinolectin type 3 (FN3) and SPRY domain. COS box domain is responsible for microtubule binding [10]. FN3 binds heparin and DNA [11]. The SPRY domain (also termed as B30.2) is involved in protein-protein interaction and RNA binding [12].

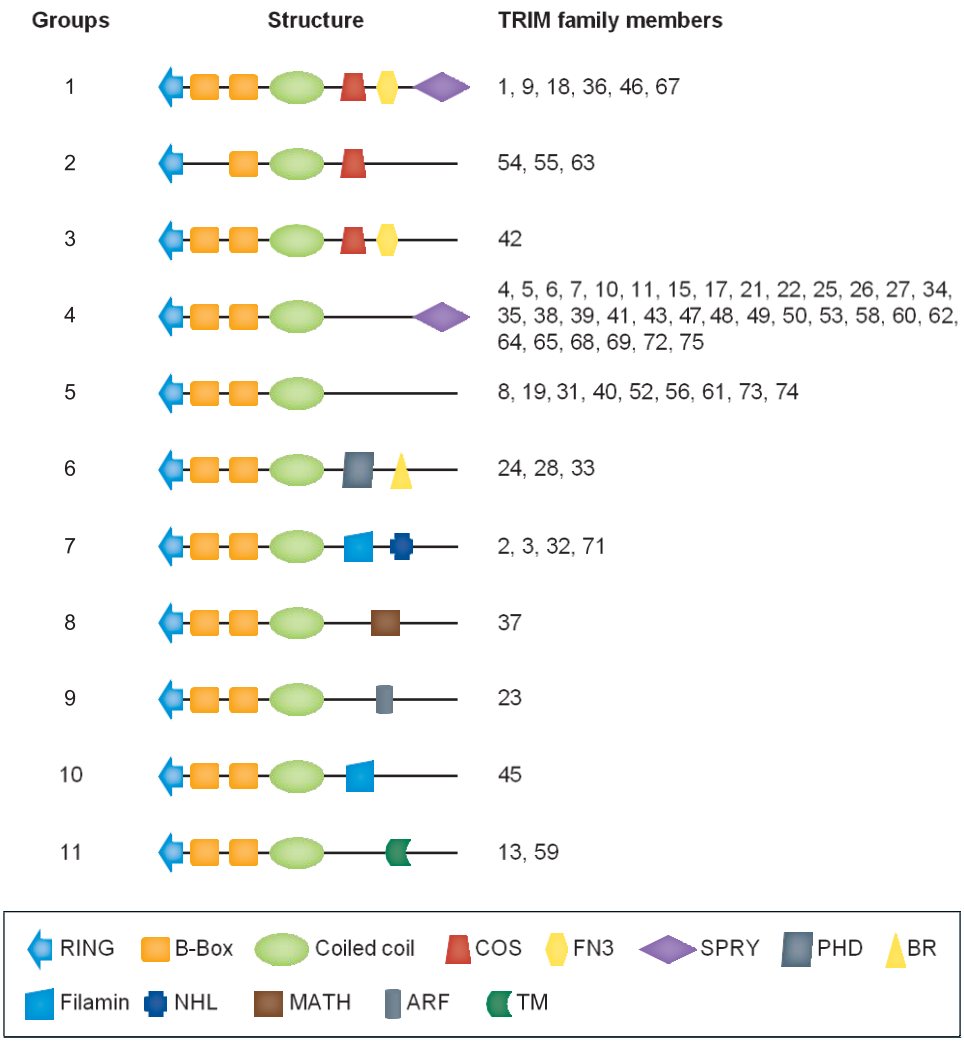

Figure 1. The schematics of the subgroups of TRIM proteins Each number in the column of TRIM family members is corresponding to the designated TRIM protein number. For example, 1 stands for TRIM1. TRIM: Tripartite motif.

Group 2 has a single COS domain whereas Group 3 has both COS and FN3 domains. Group 4 is the largest subfamily of TRIM proteins characterized by a SPRY domain at the C terminus. Group 5 has no defined motif in the C-terminal region. Group 6 has the PHD-BROMO domains while group 7 possesses the NHL repeats. Bromodomains bind acetylated lysine residue on the N-terminal of histones and repress transcription [13]. NHL repeats mediate protein-protein interaction. Groups 8-10

are featured with MATH, ARF and filamin domain, respectively. Filament type immunoglobulin domains are responsible for dimerization and actin crosslinking whereas ADP ribosylation factor like (ARF) domains stimulates vesicular trafficking [14,15]. MATH domain is important for receptor-TRAF protein interaction [9]. Group 11 comprises a transmembrane domain in their C-terminal region. Taken together, the variable C-terminal domains of TRIM proteins contribute to the versatile binding ability to their substrate.

TRIM proteins are found in many types of cells, such as immune cells. Interestingly, TRIM proteins are expressed differentially in B-cells, T-cells, dendritic cells, mast cells, NK-cells and macrophages [8], suggesting a regulatory mechanism for TRIM. In addition, TRIM proteins show different subcellular localizations. Many of them form cytoplasmic bodies when over-expressed [16,17]. Mutations in the RING, coiled coil domain or B-box have been reported to cause aberrant localization [11]. More importantly, viral infection results in some cytosolic TRIM proteins trafficking into the nucleus [18].

TRIM PROTEINS AND UBIQUITINATION

Ubiquitin is a highly conserved and ubiquitously expressed 76-amino acid protein. The ubiquitination process is a reversible covalent conjugation of ubiquitin to substrate by a three-stepwise enzymatic system (Fig. 2). In the first step, ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent reaction, in which the C-terminal carboxyl group of ubiquitin links to the sulfhydryl group of an E1 via a thioester bond. In the second step, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2), forming an E2-Ub thioester. The E2 then acts with the ubiquitin ligase (E3) to conjugate ubiquitin to the substrate protein via an isopeptide bond between the C-terminal glycine of ubiquitin and the lysine of substrate protein. To date, only two E1s (UBA1 and UBA6) and about 35 E2s have been identified in humans [19,20]. Typically, a single E2 can interact with multiple E3s. Regarding to TRIM proteins, the Cys_3-His-Cys_4 zinc finger in the RING domain binds

several E2s, including UBE2D and UBE2E [21]. Additionally, some TRIM proteins lack a RING domain, however, their B-box motifs comprise a zinc finger similar to the RING and confer the ubiquitin E3 ligase activity [22].

The ubiquitination process may be terminated after the attachment of a single ubiquitin moiety (mono-ubiquitination) or repeated either with additional ubiquitin molecules linked to the ubiquitin molecule previously attached to the substrate, forming a polyubiquitin chain. The C-terminus of ubiquitin can be conjugated to one of the seven lysines (K6, K11, K27, K29, K33, K48, K63) on another ubiquitin, forming polyubiquitin chain of different linkages. The linkage of polyubiquitin can influence the fate of the substrate, adding another layer of complexity to this modification. Majority of TRIM proteins mediate either K48- or K63-linked polyubiquitination of target proteins [23,24]. The K48-linked ubiquitination is usually involved in directing proteins for proteasome-dependent degradation while the K63-linked ubiquitination involves non-proteolytic processes, such as subcellular localization, signalosome stability and activation, and trafficking etc. For example, TRIM32 targets influenza PB1 protein for K48-linked polyubiquitination, which results in PB1 protein degradation and viral restriction[25]. In contrast, TRIM25 mediates K63-linked ubiquitination of RIG-I, which facilitates RIG-I multimerization and activation[26].

Although most TRIM proteins are ubiquitin E3 ligases, several TRIM proteins also can act as a E3 ligase for ubiquitin-like molecules, such as SUMO and ISG15. For instance, TRIM28 mediates sumoylation of IRF7, which leads to inhibition of IRF7 transactivation and IFN expression [27]. It is also reported that TRIM25 is the ISG15 E3 ligase for 14-3-3 sigma protein, however, the biological consequence is not clear [28].

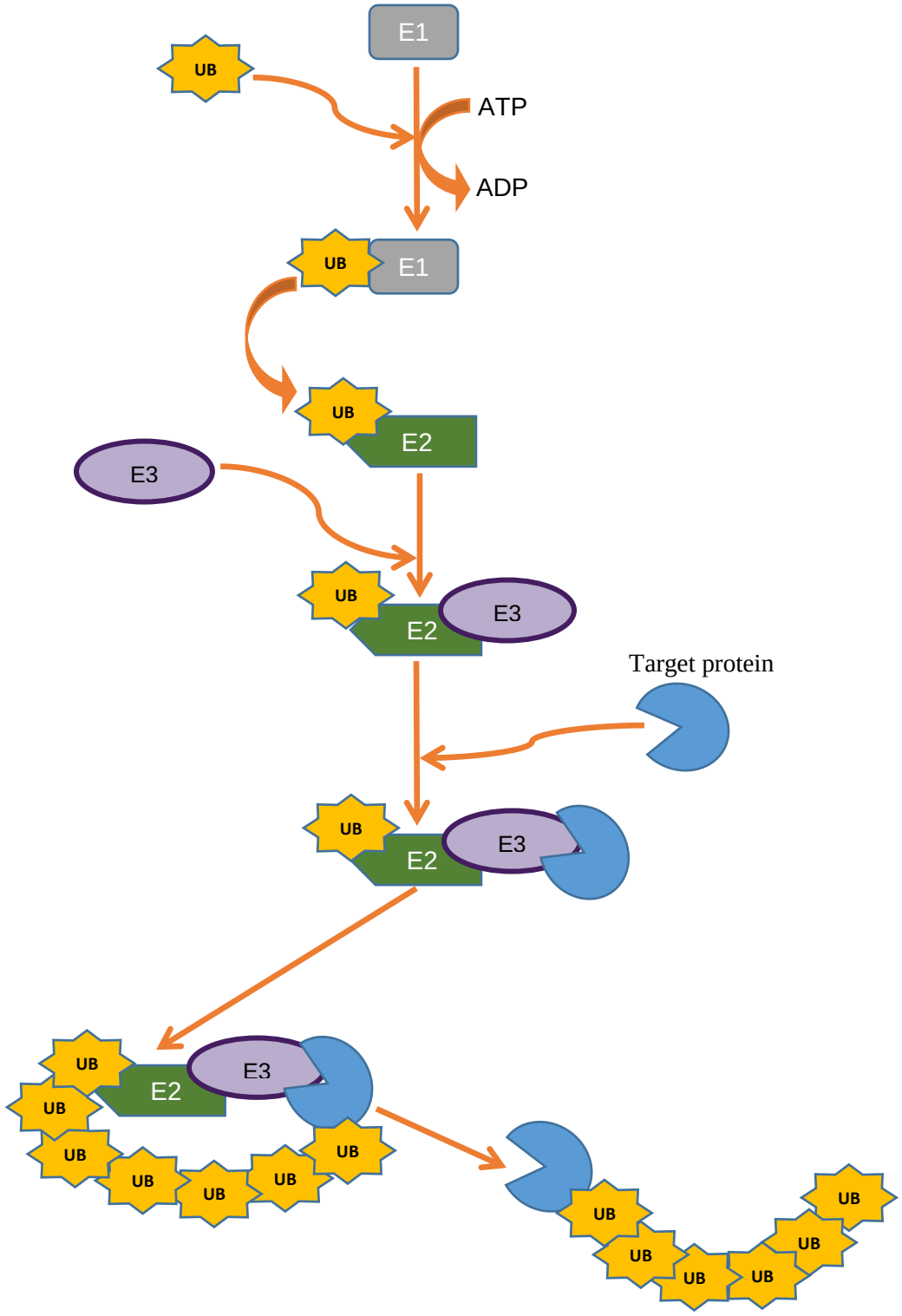

Figure 2. The process of ubiquitination. Ubiquitin (Ub) is first activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2). Finally, E3 ligase transfers the Ub from E2 to target protein.

TRIM PROTEINS & INTRINSIC IMMUNITY

Intrinsic immunity is the instant antiviral response by a group of constitutively expressed host factors through diverse mechanisms. The constitutive expression of these host factors guarantees the rapid and direct inhibition of viruses at almost every step of viral life cycle. A group of TRIM proteins belongs to these intrinsic factors which have been reported to curb viral infection through direct interaction with viral proteins. These TRIM proteins employ distinct mechanisms to inhibit viral entry, replication or dissemination. The intrinsic antiviral mechanism of each of these TRIM proteins will be discussed below (Fig. 3).

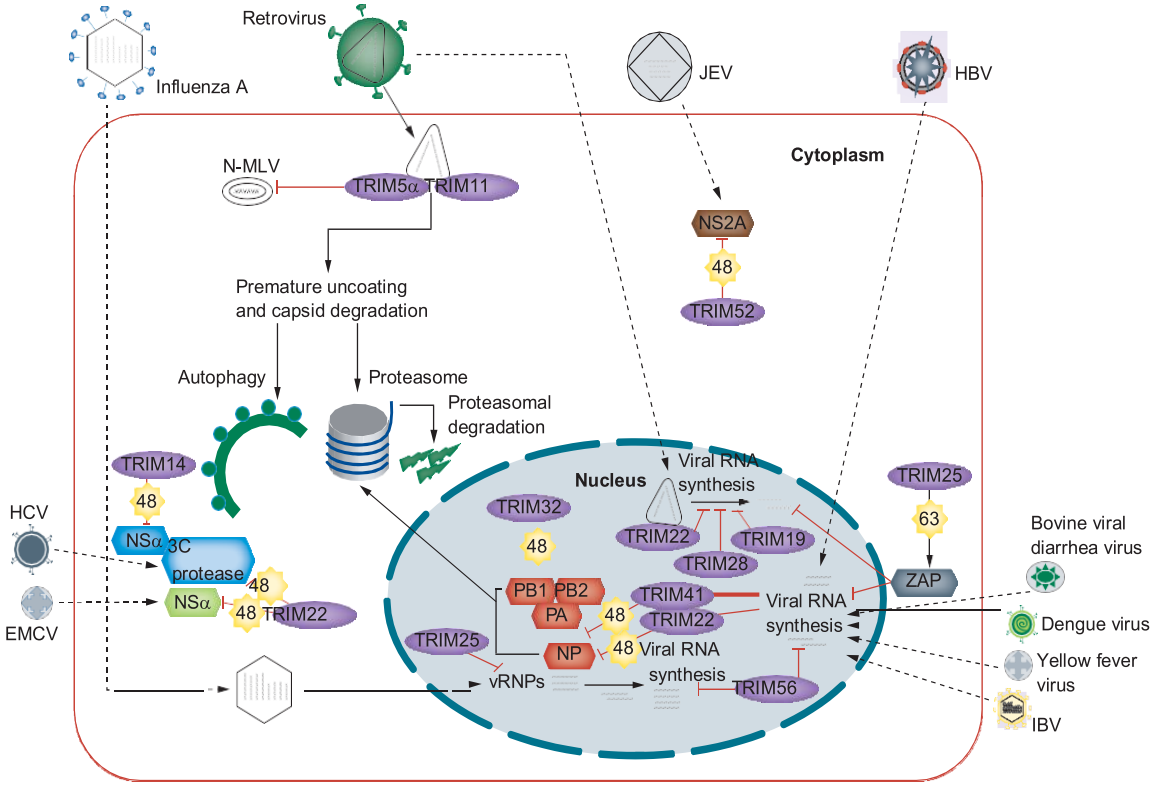

Figure 3. The role of TRIM proteins in intrinsic immunity This figure illustrates that TRIM proteins employ distinct mechanisms to inhibit viral entry, replication or dissemination by interacting with viral proteins. TRIM: Tripartite motif.

Table 1. Summary of the role of tripartite motif proteins in intrinsic immunity.

TRIM protein	Mode of action
TRIM5α	SPRY domain of TRIM5a interacts with the capsid of HIV and restricts the viral uncoating and replication.
TRIM11	TRIM11 restricts HIV-1 reverse transcription by accelerating premature viral uncoating
TRIM14	RINGIess TRIM14 interacts with HCV NS5A protein through SPRY domain and mediates its k48 polyubiquitination and degradation.
TRIM19	TRIM19/PML blocks HIV viral replication at two distinct steps. First is at early postentry stage and it also causes silencing of transcription
TRIM22	TRIM22 blocks the intracellular trafficking of the HIV viral structural protein Gag to the surface of the cell through its E3 ligase activity
TRIM22	TRIM22 interacts with class II interactor and sp1, thus inhibits HIV viral gene transcription initiation and elongation
TRIM22	TRIM22 targets influenza nucleoprotein and mediates its k48 polyubiquitination and degradation
TRIM22	TRIM22 inhibits the activity of HBV core promoter by interacting through C-terminal SPRY domain and acting as an E3 ligase.
TRIM22	TRIM22 interacts with HCV NS5A protein through SPRY domain and mediates its k48 polyubiquitination and degradation
TRIM22	TRIM22 interacts with the viral 3C protease (3C[PRO]) of encephalomyocarditis virus and mediates its ubiquitination and degradation
TRIM25	TRIM25 inhibits viral RNA synthesis results from its binding to viral ribonucleoproteins (vRNPs) of influenza.
TRIM28	TRIM28 binds to acetylated integrase and indeed deacetylation of it which restricts

	integration of HIV viral cDNA into the host genome
TRIM32	TRIM32 interacts with the PB1 of the influenza RNA polymerase complex and mediates its ubiquitination and proteasomal degradation
TRIM52	TRIM52 act against Japanese encephalitis virus infection by targeting and degrading viral NS2A through ubiquitination
TRIM56	TRIM56 inhibits BVDV replication by targeting an intracellular viral RNA replication
TRIM56	TRIM56 restricts YFV and DENV2 through E3 ligase activity and C-terminal domain interaction
TRIM56	TRIM56 specifically impede intracellular influenza virus RNA synthesis without acting as an E3 ligase.

BVDV: bovine viral diarrhea virus; DENV: dengue virus; PML: Promyelocytic leukemia protein; TRIM: Tripartite motif; YFV: yellow fever virus.

TRIM proteins-mediated intrinsic immunity to HIV-1

TRIM5α is the prototype of TRIM proteins in intrinsic immunity. TRIM5α is a well-known cytosolic host restriction factor to the cross-species transmission of retroviruses, which protects the Old World monkeys including rhesus macaques from infection of HIV-1[29-32] . The restriction specificity of TRIM5α is dependent on the its C-terminal PRY/SPRY domain which mediates the interaction with HIV-1 capsid [33,34]. On the HIV-1 capsid surface, TRIM5 proteins assembles into hexagonal net with the SPRY domains centered on the edges and the B-box and RING domains at the vertices [35]. The conformational flexibility of the hexagonal net allows TRIM5α to accommodate the variable curvature of retroviral capsids. TRIM5α-capsid interaction results in interference with the capsid uncoating process, thereby preventing reverse transcription and transport to the nucleus of the viral genome[34,36]. The ubiquitin E3 ligase activity of TRIM5α is required for restriction, however,

TRIM5α-mediated ubiquitination of HIV-1 capsid has not been detected[37]. TRIM5α also binds and restricts N tropic mouse leukemia virus (N-MLV)[38], but the precise mechanism is yet to be determined. In addition, it also reported that TRIM5α inhibits retroviral infection by activating innate immune signaling. We will discuss it in the below section of TRIM proteins and Innate Immunity.

Several other TRIM proteins also exhibit intrinsic anti-retroviral activity, including TRIM11[39], TRIM28[40], TRIM22[41,42]and TRIM19[43]. Like TRIM5α, TRIM11 also interacts with HIV-1 capsid. But in contrast to TRIM5α, TRIM11 restricts HIV-1 reverse transcription by promotes premature viral uncoating[39]. TRIM28 (also known as KAP1) limits HIV-1 by binding the acetylated integrase. The acetylation of integrase is essential for the integration of HIV-1 cDNA into the host genome. TRIM28 mediates integrase deacetylation through the formation of a protein complex which includes the deacetylase HDAC1[40]. TRIM22 restricts HIV infection by two distinct mechanisms. First, TRIM22 controls the trafficking of the gag protein to the plasma membrane and interferes with assembly of new viral particles[41]. Secondly, TRIM22 co-opts with class II transactivator (CIITA) and Sp1 to inhibit viral gene transcription initiation and elongation [44]. TRIM19, also known as promyelocytic leukemia (PML), restricts the HIV virus by reducing the reverse transcription products in the host and interfering with the HIV-1 transcription[43].

TRIM proteins-mediated intrinsic immunity to influenza A virus

Similar to HIV-1, influenza A virus (IAV) is targeted by multiple TRIM proteins. Our laboratory reported that TRIM32 is a host intrinsic immune factor to IAV. TRIM32 senses and targets IAV PB1 polymerase protein for ubiquitination and protein degradation [25]. TRIM22, an interferon-stimulated gene, also ubiquitinates and degrades IAV nucleoprotein[45]. TRIM56 inhibits the replication of influenza viruses by specifically targeting viral RNA synthesis through its C-terminal tail [46]. Furthermore, TRIM25, a critical TRIM in innate immunity (discussed in below section), inhibits IAV through a direct mechanism that is independent of its ubiquitin ligase activity and the interferon

pathway. TRIM25 binds IAV ribonucleoproteins and inhibits viral RNA synthesis [47](Figure 3). These recent studies show that TRIM proteins either target viral proteins for protein degradation or inhibit viral RNA synthesis. It is clear that TRIM proteins mediate viral protein degradation through K48-linked polyubiquitination; however, the mechanism for the inhibition of viral RNA synthesis is elusive. As the ubiquitin E3 ligase activity is dispensable, these TRIM proteins might disrupt IAV polymerase complex by directly binding NP or other subunit of viral RNP.

TRIM proteins-mediated intrinsic immunity to other viruses

In addition to IAV, TRIM22 restricts many other viruses (Figure 3). First, Hepatitis B virus is inhibited by TRIM22 and TRIM41. TRIM41 inhibits HBV enhancer II activity, which is dependent on its E3 ubiquitin ligase activity and the integrity of C-terminal domain [48]. TRIM22 inhibits HBV by suppressing the core promoter responsible for viral pre-genomic RNA synthesis [49,50]. TRIM22 also restricts hepatitis C virus (HCV) by targeting NS5α for ubiquitination and degradation[51]. Similarly, TRIM14 inhibits the HCV infection by targeting the NS5α protein for degradation[52]. Japanese encephalitis virus (JEV), another virus from the family Flaviviridae, is inhibited by TRIM52. TRIM52 interacts with the nonstructural protein 2A of JEV and mediates NS2A ubiquitination and protein degradation [53].

In addition to the viruses mentioned, TRIM22 also restricts encephalomyocarditis virus (EMCV) by targeting viral 3C protease for ubiquitination and degradation[50]. Like TRIM22, several TRIM proteins are reported to have a broad-spectrum antiviral activity. TRIM19 has been shown to inhibit herpes virus and adeno-associated virus[54,55]. TRIM21 restricts adenoviruses and rhinoviruses by proteasomal degradation of viral particles[56,57]. TRIM56 restricts IBV, HIV, yellow fever virus and dengue virus by reducing the vRNA levels through its C-terminal domain[58-60]. Additionally, TRIM25 mediates K63-linked ubiquitination of ZAP (zinc finger antiviral protein) to promotes ZAP antiviral activity to retroviruses, alphaviruses, filoviruses and HBV[61-63].

TRIM PROTEINS & INNATE IMMUNITY

Innate immune responses are triggered by exogenous microbial products which are common to many pathogens but are absent in the host. These microbial products, also known as pathogen-associated molecular patterns (PAMPs), engage with the pattern-recognition receptors (PRRs) which either present on the cell surface or inside the cells [64,65]. The engagement activates a series of signal cascades to stimulate the expression of a cohort of chemokines, cytokines and genes critical for host defense [66]. PRRs consist of several subgroups, including Toll-like receptor (TLR), RIG-I-like receptor (RLR), the cyclic GMP-AMP synthase (cGAS) and NOD-like receptor (NLR). TLR, RLR and cGAS signaling pathways converge to the hub, TANK-binding kinase 1 (TBK1) and IKK epsilon (IKKε). TBK1 and IKKε phosphorylate the interferon regulatory factors (IRFs) [67-70]. For example, TBK1 phosphorylates serines in the C-terminal domain of IRF3, which triggers the dimerization and nuclear translocation of IRF3. In the nucleus IRFs form active transcriptional complexes that bind to interferon stimulation response elements (ISRE) and activate type I IFN genes expression [67-71]. Type I IFN is the master cytokine in innate immunity and induces mRNA expression of > 300 IFN-stimulated genes (ISGs), such as protein kinase R, Mx1 and IFITM. Interestingly, some TRIM proteins are ISGs, including TRIM22, TRIM25 and TRIM5[72]. In this section, we discuss the role of TRIM proteins in the following innate immune signaling pathways (Fig. 4).

TRIM proteins & TLR signaling

TLRs are membrane proteins residing on cell surface or endosomes. The TLRs on endosomes recognize viral nucleic acids. For example, TLR3 detects viral double-strand RNA while TLR7 and TLR8 sense viral single-strand RNA [73]. TLR9 recognizes unmethylated CpG motifs within viral DNA [73]. Although all TLRs activates TBK1/IKKε and downstream IRF signaling, they recruit different adaptors. TLR3 recruits TRIF whereas TLR7, 8 and 9 relay signals through MyD88[73].

Several TRIM proteins regulate TLR signaling pathways. TRIM8 act as an essential regulator of IRF7 in an E3 ubiquitin ligase independent manner.[74] TRIM21 (also known as Ro52) has been reported to negatively regulates IFN production by targeting IRFs, including IRF3, IRF7 and IRF5 for ubiquitination and degradation [75-77]. However, another study presented an opposite result by showing that TRIM21 interferes with the interaction between Pin1 (peptidyl-prolyl cis/trans isomerase, NIMA-interacting 1) and IRF3, thus preventing IRF3 ubiquitination and degradation[78] . TRIM protein also regulates TLR adaptors. For example, TRIM56 promotes TLR3 activation by interacting with TRIF, but its E3 ligase activity is not required [79]. By contrast, TRIM38 impairs TLR3 signaling by targeting TRIF for proteasomal degradation [80]. In addition, TRIM38 also catalyzes K48-linked polyubiquitination of TRAF6 (TNF receptor associated factor 6) [81], NAP1[82] and lysosomal dependent degradation of TAB2[83] to negatively regulate NF-κB signaling pathway. The discovery of TRIM protein as a negative regulator of host immune response is important because it represents a new self-control mechanism to prevent over-activation of immune response.

TRIM proteins & RLR signaling pathway

The RLRs, including RIG-I, MDA5 and LGP2, recognize the double-stranded RNA (dsRNA) or 5' triphosphate RNA generated by viral replication of RNA viruses in the cytoplasm [84-89]. Engagement of viral RNA activates RIG-I and MDA5, which bind the mitochondrial antiviral signaling protein (MAVS) and initiate the TBK1-IRF3 signaling cascade to induce type I IFN expression [67-71,90-95].

TRIM proteins & the RIG-I-like receptors

TRIM proteins play both positive and negative roles in modulating the RLR signaling pathway. TRIM25 mediates K63-linked ubiquitination of RIG-I and the polyubiquitin chain stabilizes the oligomerization of RIG-I [24], which promotes MAVS aggregation and subsequent activation [96]. Similarly, TRIM4 conjugates K63-linked polyubiquitin chains the CARD domain of RIG-I [97]. Interestingly, TRIM25 also synthesizes unanchored K63-linked polyubiquitin chain which helps RIG-

I oligomerization and stability to supports the interaction with MAVS[24,98]. MDA5, another RLR, also undergoes K63-linked ubiquitination, however, TRIM65 is the cognate ubiquitin E3 ligase. Similar to RIG-I ubiquitination, the K63 ubiquitination of MDA5 is critical for MDA5 oligomerization and activation [99]. By contrast, TRIM13 negatively regulate the MDA5-dependent IRF activation although it not clear whether the ubiquitin E3 ligase activity is required[100]. In addition, TRIM38 positively regulates both RIG-I and MDA5 by SUMOlytion, which prevents K48-linked polyubiquitination and degradation[101]. It is clear that TRIM proteins regulate the activity of the cytosolic RNA sensor, RIG-I and MDA5, however, how these TRIM proteins are regulated upon external signal stimulation, such as RNA virus infection, is elusive. Furthermore, whether there is functional redundancy of these TRIM proteins and how these TRIM proteins coordinate need further investigation.

TRIM proteins & MAVS

Multiple TRIM proteins have been found to regulate MAVS on the mitochondria with distinct outcomes. TRIM31 mediates K63-linked polyubiquitination of MAVS and promotes the formation of prion-like aggregates, the active form of MAVS [102]. TRIM14 bridges NEMO to MAVS by the polyubiquitin of TRIM14 to activate IRF3 and NF-κB pathways [103]. Unexpectedly, one study reported that TRIM25 mediated K48-linked polyubiquitination of MAVS, which permits IRF3 signaling but inhibits NF-kB, another branch of MAVS signaling[104]. Additionally, TRIM44 has been to show to prevent K48-linked polyubiquitination of MAVS[105].

Table 2. Summary of the role of tripartite motif proteins in innate immunity	
TRIM protein	**Mode of action**
TRIM4	TRIM4 induces the Lys 63-linked ubiquitination of CARD domain RIG-I, which is crucial for the cytosoiic RIG-I signaling pathway
TRIM5	TRIM5 couples innate viral sensing and CD8+ T-cell activation to increase species barriers against retrovirus infection.
TRIM6	TRIM6 synthesize unanchored K48-Iinked polyubiquitin chains, which activate IKKt for subsequent STAT1 phosphorylation.
TRIM11	TRIM11 inhibits RIG-l-mediated IFN-p production by targeting the TBK1 signaling complex. Interaction is dependent on CC domain of TRIM11
TRIM13	TRIM13 enhances RIG-I activation and positively regulate signaling pathway
TRIM13	TRIM13 regulates the type I interferon response through inhibition of MDA5 activity
TRIM14	TRIM14 interacts with MAVS through its SPRY domain and undergoes k63 linked autoubiquitination and recruits NEMO to the MAVS complex.
TRIM21	The IRF association domain of IRF5 interacts with TRIM21 via its PRY/SPRY domain and mediates its K48 and k63 ubiquitination I regulation.
TRIM21	TRIM21 interacts with IRF3 through its SPRY domain and inhibits IRF3 ubiquitination and positively regulates it.
TRIM21	TRIM21 acts as an E3 ligase and mediates monoubiquitination of IKKþ downregulates NF-nB Signalling
TRIM21	TRIM21 interacts with Fc fragment of swine immunoglobulin G (sFc) fused VP1 of FMDV and thereby causing its K4Mnked degradation.
TRIM23	TRIM23 through itsARF domain Interacts with NEMO resulting in its K27-

	linked ubiquitination and positively regulate downstream pathway
TRIM24	T-Cell-lntrinsic TRIM24 is required for IL-ip-mediated activation of TH2 cells
TRIM25	TRIM25 induces the Lys 63-linked ubiquitination of CARD domain RIG-I, which is crucial for the cytosoiic RIG-I signaling pathway
TRIM25	Short, unanchored. K63-linked ubiquitin chains mediated by TRIM25 activate RIG-I
TRIM25	TRIM25 mediates k48 linked polyubiquitination of MAVS and positively regulates IRF3 pathway through regulating oligomerization
TRIM26	Autoubiquitination of TRIM26 helps TBK1-NEMO interaction and recruits TBK1 to the MAVS signalsome.
TRIM27	TRIM27 negatively regulates signaling involved in the antiviral response and inflammation by targeting the IKKs
TRIM27	TRIM27 mediates K-48 polyubiquitination of PI3KC2P, leading to a decrease in PI3K activity and negatively regulate CD4 T cells
TRIM27	TRIM27 through its SPRY domain interacts with NDB domain of NOD2 and targets it for K48-linked ubiquitination proteasomal degradation
TRIM28	TRIM28 acts as a SUMO E3 ligase and negatively regulates IRF7 by SUMOylation
TRIM29	TRIM29 induces NEMO ubiquitination through K48 linkage which results in proteasomal degradation and supression of downstream pathway
TRIM29	TRIM29 targets STING for K48 ubiquitination and degradation and negatively regulates STING dependent pathway
TRlM30a	TRIM30a interacts with TAK1-TAB2-TAB3 and promotes the k48 linked ubiquitination of degradation of TAB2 and TAB3 thus supressing signaling

TRIM30a	TRIM30a prevents TRAF6 autoubiquitination, diminishes IkBo phosphorylation and negatively regulates the NF-kB pathway
TRIM30a	TRIM30a promotes the degradation of STING via K48-linked ubiquitination and negatively regulates STING dependent pathway
TRIM30a	TRIM30 negatively regulates NLRP3 intlammasome activation by modulating and inhibiting ROS production
TRIM31	TRIM31 interacted with MAVS and catalyzed the Lys63 (K63)-linked polyubiquitination and regulates downstream pathway positively
TRIM31	TRIM31 directly binds to NLRP3, promotes K48-linked polyubiquitination and proteasomal degradation of NLRP3.
TRIM32	TRIM32 targets STING for K63-linked ubiquitination through its E3 ligase activity, which promoted the interaction of STING with TBK1.
TRIM33	TRIM33 targets DHX33 for K63 linked ubiquitination which activates the formation of the DHX33-NLRP3 inflammasome complex.
TRIM38	TRIM38 interacts with and mediates k48 linked ubiquitination and degradation of TRIF and negatively regulates TLR3/4 dependent pathway
TRIM38	TRIM38 activates MDA5- and RIG-I through their SUMOylation which enhances dephosphorylation and activation and inhibits its degradation
TRIM38	TRIM38 targets NAP1 for ubiquitination and subsequent proteasome-mediated degradation and supresses IFN-þ response.
TRIM38	TRIM38 Interacts with TAB2/3 through Its PRY-SPRY domain and mediates k48-linked ubiqutination, degradation and translocation
TRIM38	TRIM38 sumoylates STING during the early phase of viral infection, promoting both STING activation and protein stability

TRIM38	TRIM38 binds to TRAF6 and promotes its K48-linked polyubiquitination and proteasomal degradation thus supresses downstream pathway
TRIM44	TRIM44 acts as a deubiquitinase enzyme and prevents k48 linked degradation of MAVS positively regulate MAVS dependent pathway
TRIM56	TRIM56 interacts with TRIF through its C-terminal domain and enhances TLR3 pathway in an E3 ligase independent manner.
TRIM56	TRIM56 interacts with STING and targets it for K63-linked ubiquitination which initiates dimerization and facilitates STING–TBK1 interaction
TRIM56	TRIM56 induces the Lys335 monoubiquitination of cGAS which increases of its dimerization, DNA-binding activity and cGAMP production.
TRIM65	TRIM65 interacts and promotes K63-linked ubiquitination of MDA5 at lysine 743, which is critical for MDA5 oligomerization and activation.
TRIM9s	TRIM9s undergoes Lys-63-linked autopolyubiquitination and serves as a platform to bridge GSK3þ to TBK1, activation of IRF3 signaling.
FMDV: Foot and mouth disease virus; IKKþ: inhibitor of nuclear factor kappa-B kinase subunit beta; MAVS: Mitochondrial antiviral signaling protein; STAT: Signal transducer and activator of transcription; TRAF6: TNF receptor-associated factor 6; TRIM: Tripartite motif.	

TRIM proteins & the TBK1 protein complex

Several TRIM proteins target the TBK1 kinase complex for IFN regulation. First, TRIM26 undergoes autoubiquitination upon viral infection and the polyubiquitin of TRIM26 bridges NEMO to TBK1, thus facilitating TBK1 activation [106]. Secondly, the short form of TRIM9 (TRIM9s) also undergoes K63-linked auto-polyubiquitination upon viral infection. The polyubiquitin of TRIM9s serves as a

platform to bridge GSK3β to TBK1, leading to the activation of IRF3 signaling [107]. Thirdly, TRIM11 inhibits TBK1 activity by blocking TBK1 interaction with its binding partners, NAP1 and TANK[108]. Lastly, TRIM38 facilitates K48 linked polyubiquitination and degradation of NAP1, resulting in a reduced activity of IRF3[82]. As the TBK1 kinase complex is the hub of various innate immune signaling pathways, whether these TRIM proteins are specific to the RLR signal pathway or also work on other innate signaling pathways need to be clarified in the future.

TRIM proteins & cGAS signaling

Cytosolic viral DNA is recognized by the recently identified DNA sensor, cyclic GMP-AMP synthase (cGAS, also known as MB21D1) [109]. After binding to DNA, cGAS produces cyclic GMP-AMP (cGAMP). cGAMP is a second messenger and binds to the endoplasmic reticulum membrane protein, stimulator of interferon genes (STING, also known as TMEM173, MPYS, MITA and ERIS), which leads the dimerization of STING [110-112]. Subsequently, STING recruits TANK-binding kinase 1(TBK1) to endoplasmic reticulum and activates TBK1. Activated TBK1 phosphorylates interferon regulatory factors (IRFs) [67-70].

TRIM56 and TRIM32 have been reported to catalyze K63-linked polyubiquitination of STING, which is essential for activation of IFNβ expression and restriction of DNA viruses [113,114]. In contrast, TRIM30α and TRIM29 promote the degradation of STING via K48-linked ubiquitination through a proteasome-dependent pathway[115,116]. Interestingly, TRIM38-meidated sumoylation of STING counteracts the K48-linked ubiquitination of STING and prevents STING from protein degradation [117]. Similarly, TRIM38 also mediates sumoylation of cGAS, thus preventing cGAS polyubiquitination and degradation[117] . Recently, the ubiquitin E3 ligase for STING, TRIM56, is found to catalyze monoubiquitination of cGAS, which promotes cGAS dimerization, DNA-binding activity, and cGAMP production [118]. In addition, TRIM21 facilitates the exposure of the viral genome in the cytosol, thus sensitizing cGAS and RIG-I signaling [119](Figure 4). Notably, TRIM38 and TRIM56 regulate both cGAS and STING. However, STING is a membrane protein on the ER while cGAS is a

cytosolic and nuclear protein. The subcellular localization of these TRIM proteins and the intracellular trafficking of these proteins are the questions that need to be addressed.

TRIM proteins & NLR signaling

NLRs comprise a family of intracellular sensors which recognize microbial products and mount proinflammatory and antimicrobial immune response[120,121]. Upon stimulation, NLR forms inflammosomes, a large and highly ordered cytosolic complex, which leads to caspase-mediated proteolytic activation and IL-1β and IL-18 [122]. TRIM33 binds and ubiquitinates DHX33, a cytosolic receptor upstream of NLRP3, which facilitates inflammosome activation [123]. Coiled Coil domain of TRIM31 interacts with NACHT domain of NLRP3 and mediates K48-linked ubiquitination that leads to the degradation of NLRP3 [124]. TRIM30α negatively regulates NLRP3 inflammasome activation by modulating reactive oxygen species production [125]. In addition, TRIM27 degrades NOD2 and inhibits the NOD2 receptors during viral infection [126-128].

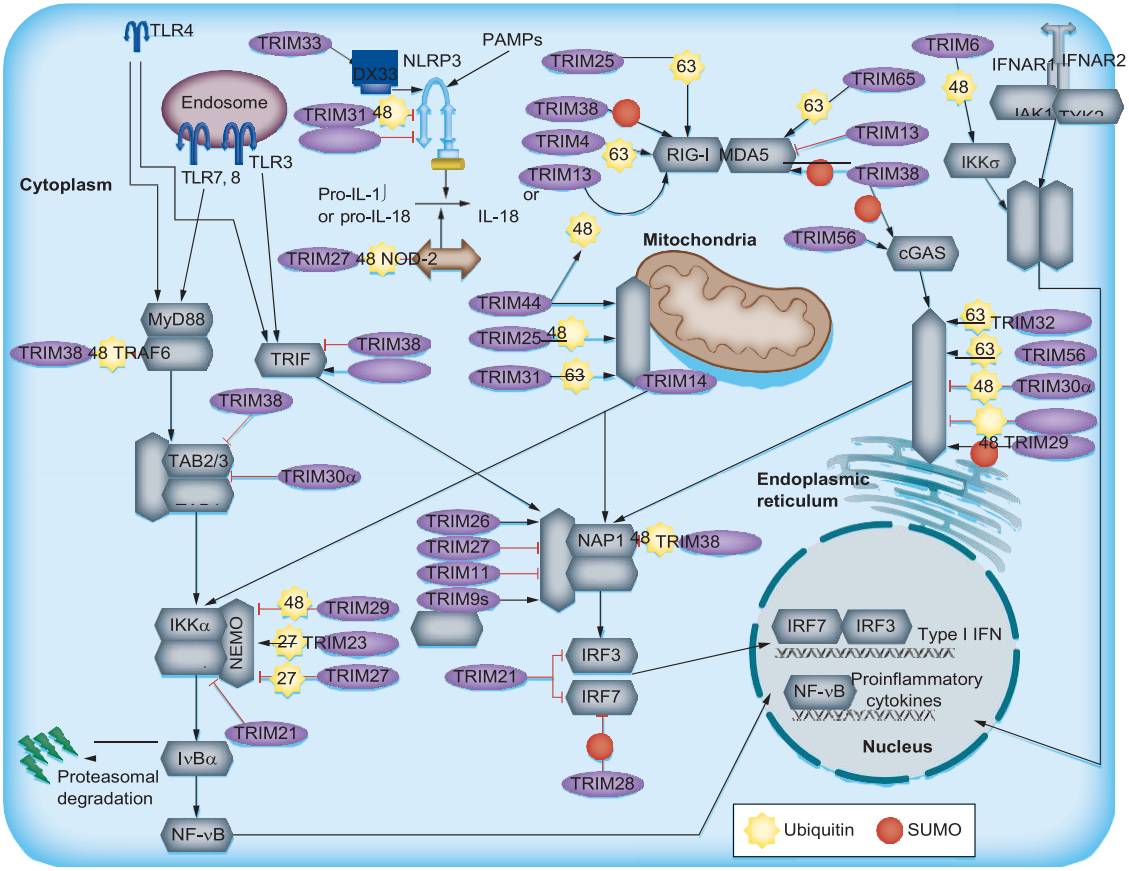

Figure 4. The role of TRIM proteins in innate immunity This figure illustrates that TRIM proteins play both positive and negative roles in TLR, RLR and cGAS signaling pathways.

cGAS: Cyclic GMP–AMP synthase; RLR: RIG-I-like receptor; TLR: Toll-like receptor; TRIM: Tripartite motif.

TRIM proteins & other innate immune signaling pathways

The NF-κB transcription factors play a critical role in various antiviral immune signaling pathways[129]. A wide range of soluble and membrane-bound extracellular ligands activate NF-κB, such as TNF and IL-1 etc. Take TNFα signaling for example, the TNF receptor recruits a series of intermediary adaptors after TNF stimulation. These adaptors further recruit and activate the IKK kinase complex consisting of IKKα, IKKβ and NEMO. The activated IKK complex phosphorylates IκBα, which leads to IκBα protein degradation and subsequent release and activation of NF-κB. The IKK complex is

regulated by several TRIM proteins. TRIM23 binds NEMO through the C terminal ARF domain and targets NEMO for K27-linked ubiquitination, which leads to increased activation of NF-κB signaling [130]. By contrast, TRIM30α mediates protein degradation of TAB2/3, the adaptors of TAK1, thus inhibiting TAK1 activity and downstream IKK kinase activation [131]. TRIM21 inhibits the NF-κB pathway through mono-ubiquitination of IKKβ [132]. Several TRIM proteins regulate both NF-κB and IRF signaling pathways. For example, TRIM27 inhibits NF-κB and IRF3 via interaction with IKK, but the mechanism is not clear [133]. TRIM29 also suppresses both pathways by degradation of NEMO[134].

IFN binds IFN receptors to activate JAK (Janus –kinase)-STAT (signal transducer and activator of transcription) pathway, which stimulates hundreds of ISGs to execute the antiviral actions. TRIM6 is found to catalyze unanchored K48-linked polyubiquitin chains, which activates IKKε to phosphorylate STAT1. The phosphorylation results in the induction of a subset of ISGs essential for the antiviral response [135].

TRIM PROTEINS & ADAPTIVE IMMUNITY

Most TRIM proteins are found to regulate intrinsic and innate immunity, however, there are also several TRIM proteins regulate adaptive immunity. TRIM5 couples innate viral sensing and CD8(+) T-cell activation to increase species barriers against retrovirus infection [136]. TRIM8 is a Th17-specific HIV dependency factor and an important anti-HIV factor [137]. TRIM24 regulates the expression of Th2-type cytokines [138]. TRIM27 inhibits the activation of $CD4^+$ cells through K48-linked polyubiquitination of PI3K-C2β [139]. Additionally, TRIM21 plays an important role in detection of antibody opsonized viruses, such as the foot and mouth disease virus (FMDV). TRIM21 binds to the highly conserved Fc region of immunoglobulins and targets the virus for degradation by proteasome [140]. Overall, there are only a few TRIM proteins are discovered to regulate adaptive immunity because

of the limits of research tools. Nonetheless, the list will grow as more animal models for TRIM proteins are generated.

TARGETING TRIM PROTEINS: VIRAL EVASION STRATEGY

Targeting TRIM proteins by RNA virus

The arms race against host defense has forced viruses to evolve new strategies for evasion. Thus, it is not a surprise to find some viruses target the TRIM proteins to dampen host de defense. Firstly, several RNA viruses target TRIM25, the regulator of RIG-I. The non-structural protein 1 (NS1) of Influenza A virus directly interacts with the coiled coil domain of TRIM25, thereby preventing its multimerization which is essential for the E3 ligase activity. Defective TRIM25 fails to catalyze K63-linked ubiquitination of RIG-I, thus blocking type I IFN expression[141,142]. Similarly, the nucleoprotein of SARS-associated coronavirus (SARS-CoV), MERS-CoV and the severe fever with thrombocytopenia virus (SFTSV) also interact with TRIM25 and prevent TRIM25-RIG-I interaction, thereby inhibiting RIG-I signaling[143]. The Non-coding RNAs of PR-2b strain of Dengue virus bind to the host TRIM25 and prevents USP15-mediated deubiquitination, which is important for RIG-I activation[144,145]. Secondly, TRIM6 is the target of two RNA viruses. The matrix protein of Nipah virus induces TRIM6 degradation to suppress TRIM6 –mediated IFN response [146] whereas Ebola virus utilizes TRIM6 to ubiquitinate its VP35 protein, thereby promoteing VP35 activity and virus replication[147]. Lastly, several RNA viruses utilize TRIM proteins to suppress host innate immune responses. The NS5 protein of yellow fever virus interacts with TRIM23, which results in K6-linked poly-ubiquitination of NS5. The polyubiquitination facilitates NS5 interaction with STAT2, thereby inhibiting type I IFN response[148]. JEV upregulates the expression of TRIM21, which suppresses the IFN-I response[149], however, the mechanism is not clear. The vpr protein of HIV-1 negatively regulates TRIM11 via unknown mechanism[150].

Targeting TRIM proteins by DNA virus

Like RNA viruses, DNA viruses also target TRIM protein to subvert immune response by several distinct mechanisms. The HBV X protein suppresses TRIM22 mRNA expression by CpG methylation of the 5`-UTR of TRIM22 gene[151]. Epstein - Barr virus (EBV) targets TRIM28 for phosphorylation at serine residue 824, which impairs antiviral function of TRIM28[152]. In addition, several DNA viruses target TRIM proteins for ubiquitination. The ICP0 protein of HSV-1 ubiquitinates and degrades TRIM27, resulting in the inhibition of immune response[153]. Similarly, Gamma herpesvirus MHV-68 degrades TRIM19[154].

CONCLUSION

The co-evolution of host and virus has kept shaping the viral survival strategy and host defense system. Although these mechanisms have existed for a very long time, many of them await for discovery, including the antiviral mechanism of the TRIM protein family. Since TRIM5a was discovered as a species barrier to restrict viral infection, the number of antiviral TRIM proteins had kept increasing. Several recent systematic screenings suggest that more TRIM proteins are involved in host immunity and viral restriction. Despite of the increasing knowledge of TRIM proteins, there are several questions needed to be addressed or directions to be explored in the future. First, what are the underlying mechanisms for the broad-spectrum antiviral activity and specificity of TRIM proteins? As discussed above, some TRIM proteins, such as TRIM22, TRIM19 and TRIM56, restrict multiple viruses. To determine the broad-spectrum antiviral activity, it is important to identify the common mechanism. For example, TRIM22 ubiquitinates viral proteins to restrict viral infection. It will be interesting to know whether the interacting viral proteins share a common motif for TRIM interaction. Conversely, multiple TRIM proteins may attack the same virus. For example, HIV-1 has been shown to be limited by several TRIM proteins. However, detailed biochemical and genetic analyses are further needed to clarify the functional redundancy among TRIM proteins. Secondly,

how TRIM proteins are activated and regulated upon viral infection? Most studies focus on the mechanisms by which TRIM proteins modulate host immunity and viral infection. Little is known about the regulatory mechanisms for TRIM proteins. Elucidation of the regulatory mechanism for TRIM proteins will help develop a novel strategy to boost host immunity to viral infection. Thirdly, the *in vivo* role of most TRIM proteins is unknown. Recently, several TRIM protein transgenic and knockout mice have been generated. These mice are precious tools to study the *in vivo* role of TRIM proteins. However, most of these mouse models have not been examined for viral infection. Lastly, is there any inherent relevance of the genetic mutations of TRIM proteins to human immune diseases? Genetic mutations in the NHL repeats of TRIM32 cause recessive hereditary muscle disorders, including limb girdle muscular dystrophy 2H (LGMD2H) [155-158]. Recent genome-wide association (GWA) studies implied a link of a group of TRIM proteins to the susceptibility of neuropsychiatric disorders, developmental diseases, cardiovascular and metabolic diseases[159]. However, whether genetic mutations of TRIM proteins dampen human host immune defense is not clear. Taken all together, studies in the above directions will open a new spectrum in anti-viral research and will aid development of strategies for prevention and control of viral infections.

REFERENCES

1 Reymond, A. *et al.* The tripartite motif family identifies cell compartments. *The EMBO journal* **20**, 2140-2151, doi:10.1093/emboj/20.9.2140 (2001).

2 Meroni, G. & Diez-Roux, G. TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases. *BioEssays : news and reviews in molecular, cellular and developmental biology* **27**, 1147-1157, doi:10.1002/bies.20304 (2005).

3 Hatakeyama, S. TRIM Family Proteins: Roles in Autophagy, Immunity, and Carcinogenesis. *Trends in biochemical sciences* **42**, 297-311, doi:10.1016/j.tibs.2017.01.002 (2017).

4 Saurin, A. J., Borden, K. L., Boddy, M. N. & Freemont, P. S. Does this have a familiar RING? *Trends Biochem Sci* **21**, 208-214 (1996).

5 McEwan, W. A. *et al.* Intracellular antibody-bound pathogens stimulate immune signaling via the Fc receptor TRIM21. *Nature immunology* **14**, 327-336, doi:10.1038/ni.2548 (2013).

6 Massiah, M. A., Simmons, B. N., Short, K. M. & Cox, T. C. Solution structure of the RBCC/TRIM B-box1 domain of human MID1: B-box with a RING. *J Mol Biol* **358**, 532-545, doi:10.1016/j.jmb.2006.02.009 (2006).

7 Esposito, D., Koliopoulos, M. G. & Rittinger, K. Structural determinants of TRIM protein function. *Biochemical Society transactions* **45**, 183-191, doi:10.1042/bst20160325 (2017).

8 Rajsbaum, R., García-Sastre, A. & Versteeg, G. A. TRIMmunity: The Roles of the TRIM E3-Ubiquitin Ligase Family in Innate Antiviral Immunity. *Journal of Molecular Biology* **426**, 1265-1284, doi:http://doi.org/10.1016/j.jmb.2013.12.005 (2014).

9 Ozato, K., Shin, D.-M., Chang, T.-H. & Morse, H. C. TRIM family proteins and their emerging roles in innate immunity. *Nature reviews. Immunology* **8**, 849-860, doi:10.1038/nri2413 (2008).

10 Bernardi, R. & Pandolfi, P. P. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nature reviews. Molecular cell biology* **8**, 1006-1016, doi:10.1038/nrm2277 (2007).

11 Ozato, K., Shin, D. M., Chang, T. H. & Morse, H. C., 3rd. TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* **8**, 849-860, doi:10.1038/nri2413 (2008).

12 Nisole, S., Stoye, J. P. & Saib, A. TRIM family proteins: retroviral restriction and antiviral defence. *Nature reviews. Microbiology* **3**, 799-808, doi:10.1038/nrmicro1248 (2005).

13 Le Douarin, B., Nielsen, A. L., You, J., Chambon, P. & Losson, R. TIF1 alpha: a chromatin-specific mediator for the ligand-dependent activation function AF-2 of nuclear receptors? *Biochemical Society transactions* **25**, 605-612 (1997).

14 Nie, Z., Hirsch, D. S. & Randazzo, P. A. Arf and its many interactors. *Current opinion in cell biology* **15**, 396-404 (2003).

15 Holthuis, J. C. & Burger, K. N. Sensing membrane curvature. *Developmental cell* **5**, 821-822 (2003).

16 Ichimura, T. *et al.* 14-3-3 proteins sequester a pool of soluble TRIM32 ubiquitin ligase to repress autoubiquitylation and cytoplasmic body formation. *Journal of cell science* **126**, 2014-2026, doi:10.1242/jcs.122069 (2013).

17 Kawaguchi, Y. *et al.* TRIM32-Cytoplasmic-Body Formation Is an ATP-Consuming Process Stimulated by HSP70 in Cells. *PLoS One* **12**, e0169436, doi:10.1371/journal.pone.0169436 (2017).

18 Versteeg, Gijs A. *et al.* The E3-Ligase TRIM Family of Proteins Regulates Signaling Pathways Triggered by Innate Immune Pattern-Recognition Receptors. *Immunity* **38**, 384-398, doi:https://doi.org/10.1016/j.immuni.2012.11.013 (2013).

19 van Wijk, S. J. & Timmers, H. T. The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *FASEB journal : official publication of the*

Federation of American Societies for Experimental Biology **24**, 981-993, doi:10.1096/fj.09-136259 (2010).

20 Groettrup, M., Pelzer, C., Schmidtke, G. & Hofmann, K. Activating the ubiquitin family: UBA6 challenges the field. *Trends in biochemical sciences* **33**, 230-237, doi:10.1016/j.tibs.2008.01.005 (2008).

21 Napolitano, L. M., Jaffray, E. G., Hay, R. T. & Meroni, G. Functional interactions between ubiquitin E2 enzymes and TRIM proteins. *The Biochemical journal* **434**, 309-319, doi:10.1042/bj20101487 (2011).

22 Bell, J. L. *et al.* TRIM16 Acts as an E3 Ubiquitin Ligase and Can Heterodimerize with Other TRIM Family Members. *PLOS ONE* **7**, e37470, doi:10.1371/journal.pone.0037470 (2012).

23 Pertel, T. *et al.* TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* **472**, 361-365, doi:10.1038/nature09976 (2011).

24 Zeng, W. *et al.* Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. *Cell* **141**, 315-330, doi:10.1016/j.cell.2010.03.029 (2010).

25 Fu, B. *et al.* TRIM32 Senses and Restricts Influenza A Virus by Ubiquitination of PB1 Polymerase. *PLoS pathogens* **11**, e1004960, doi:10.1371/journal.ppat.1004960 (2015).

26 Gack, M. U. *et al.* TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* **446**, 916-920, doi:10.1038/nature05732 (2007).

27 Liang, Q. *et al.* Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. *Journal of immunology (Baltimore, Md. : 1950)* **187**, 4754-4763, doi:10.4049/jimmunol.1101704 (2011).

28 Zou, W. & Zhang, D. E. The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase. *The Journal of Biological Chemistry* **281**, 3989-3994, doi:10.1074/jbc.M510787200 (2006).

29 Nisole, S., Lynch, C., Stoye, J. P. & Yap, M. W. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 13324-13328, doi:10.1073/pnas.0404640101 (2004).

30 Sayah, D. M., Sokolskaja, E., Berthoux, L. & Luban, J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* **430**, 569-573, doi:10.1038/nature02777 (2004).

31 Stremlau, M. *et al.* The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* **427**, 848-853, doi:10.1038/nature02343 (2004).

32 Yap, M. W., Nisole, S., Lynch, C. & Stoye, J. P. Trim5alpha protein restricts both HIV-1 and murine leukemia virus. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 10786-10791, doi:10.1073/pnas.0402876101 (2004).

33 Sebastian, S. & Luban, J. TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* **2**, 40, doi:10.1186/1742-4690-2-40 (2005).

34 Anderson, J. L. *et al.* Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. *Journal of virology* **80**, 9754-9760, doi:10.1128/jvi.01052-06 (2006).

35 Li, Y. L. *et al.* Primate TRIM5 proteins form hexagonal nets on HIV-1 capsids. *eLife* **5**, doi:10.7554/eLife.16269 (2016).

36 Wu, X., Anderson, J. L., Campbell, E. M., Joseph, A. M. & Hope, T. J. Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 7465-7470, doi:10.1073/pnas.0510483103 (2006).

37 Kutluay, S. B., Perez-Caballero, D. & Bieniasz, P. D. Fates of retroviral core components during unrestricted and TRIM5-restricted infection. *PLoS pathogens* **9**, e1003214, doi:10.1371/journal.ppat.1003214 (2013).

38 Yap, M. W., Nisole, S. & Stoye, J. P. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Current biology : CB* **15**, 73-78, doi:10.1016/j.cub.2004.12.042 (2005).

39 Yuan, T., Yao, W., Tokunaga, K., Yang, R. & Sun, B. An HIV-1 capsid binding protein TRIM11 accelerates viral uncoating. *Retrovirology* **13**, 72, doi:10.1186/s12977-016-0306-5 (2016).

40 Allouch, A. *et al.* The TRIM family protein KAP1 inhibits HIV-1 integration. *Cell host & microbe* **9**, 484-495, doi:10.1016/j.chom.2011.05.004 (2011).

41 Barr, S. D., Smiley, J. R. & Bushman, F. D. The interferon response inhibits HIV particle production by induction of TRIM22. *PLoS Pathog* **4**, e1000007, doi:10.1371/journal.ppat.1000007 (2008).

42 Forlani, G. *et al.* The MHC-II transactivator CIITA inhibits Tat function and HIV-1 replication in human myeloid cells. *Journal of translational medicine* **14**, 94, doi:10.1186/s12967-016-0853-5 (2016).

43 Masroori, N., Merindol, N. & Berthoux, L. The interferon-induced antiviral protein PML (TRIM19) promotes the restriction and transcriptional silencing of lentiviruses in a context-specific, isoform-specific fashion. *Retrovirology* **13**, 19, doi:10.1186/s12977-016-0253-1 (2016).

44 Forlani, G. & Accolla, R. S. Tripartite Motif 22 and Class II Transactivator Restriction Factors: Unveiling Their Concerted Action against Retroviruses. *Frontiers in immunology* **8**, 1362, doi:10.3389/fimmu.2017.01362 (2017).

45 Di Pietro, A. *et al.* TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. *Journal of virology* **87**, 4523-4533, doi:10.1128/jvi.02548-12 (2013).

46 Liu, B. *et al.* The C-Terminal Tail of TRIM56 Dictates Antiviral Restriction of Influenza A and B Viruses by Impeding Viral RNA Synthesis. *J Virol* **90**, 4369-4382, doi:10.1128/JVI.03172-15 (2016).

47 Meyerson, N. R. *et al.* Nuclear TRIM25 Specifically Targets Influenza Virus Ribonucleoproteins to Block the Onset of RNA Chain Elongation. *Cell host & microbe* **22**, 627-638.e627, doi:10.1016/j.chom.2017.10.003 (2017).

48 Zhang, S., Guo, J. T., Wu, J. Z. & Yang, G. Identification and characterization of multiple TRIM proteins that inhibit hepatitis B virus transcription. *PLoS One* **8**, e70001, doi:10.1371/journal.pone.0070001 (2013).

49 Gao, B., Duan, Z., Xu, W. & Xiong, S. Tripartite motif-containing 22 inhibits the activity of hepatitis B virus core promoter, which is dependent on nuclear-located RING domain. *Hepatology (Baltimore, Md.)* **50**, 424-433, doi:10.1002/hep.23011 (2009).

50 Eldin, P. *et al.* TRIM22 E3 ubiquitin ligase activity is required to mediate antiviral activity against encephalomyocarditis virus. *The Journal of general virology* **90**, 536-545, doi:10.1099/vir.0.006288-0 (2009).

51 Yang, C. *et al.* Interferon alpha (IFNalpha)-induced TRIM22 interrupts HCV replication by ubiquitinating NS5A. *Cellular & molecular immunology* **13**, 94-102, doi:10.1038/cmi.2014.131 (2016).

52 Wang, S. *et al.* TRIM14 inhibits hepatitis C virus infection by SPRY domain-dependent targeted degradation of the viral NS5A protein. *Scientific reports* **6**, 32336, doi:10.1038/srep32336 (2016).

53 Fan, W. *et al.* TRIM52 inhibits Japanese Encephalitis Virus replication by degrading the viral NS2A. *Sci Rep* **6**, 33698, doi:10.1038/srep33698 (2016).

54 Chelbi-Alix, M. K., Quignon, F., Pelicano, L., Koken, M. H. & de The, H. Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. *Journal of virology* **72**, 1043-1051 (1998).

55 Geoffroy, M. C. & Chelbi-Alix, M. K. Role of promyelocytic leukemia protein in host antiviral defense. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **31**, 145-158, doi:10.1089/jir.2010.0111 (2011).

56 Hauler, F., Mallery, D. L., McEwan, W. A., Bidgood, S. R. & James, L. C. AAA ATPase p97/VCP is essential for TRIM21-mediated virus neutralization. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 19733-19738, doi:10.1073/pnas.1210659109 (2012).

57 Mallery, D. L. *et al.* Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). *Proc. Natl. Acad. Sci. U. S. A.* **107**, 19985-19990, doi:10.1073/pnas.1014074107 (2010).

58 Wang, J. *et al.* TRIM56 is a virus- and interferon-inducible E3 ubiquitin ligase that restricts pestivirus infection. *Journal of virology* **85**, 3733-3745, doi:10.1128/jvi.02546-10 (2011).

59 Kane, M. *et al.* Identification of Interferon-Stimulated Genes with Antiretroviral Activity. *Cell host & microbe* **20**, 392-405, doi:10.1016/j.chom.2016.08.005 (2016).

60 Liu, B. *et al.* Overlapping and distinct molecular determinants dictating the antiviral activities of TRIM56 against flaviviruses and coronavirus. *Journal of virology* **88**, 13821-13835, doi:10.1128/jvi.02505-14 (2014).

61 Li, M. M. *et al.* TRIM25 Enhances the Antiviral Action of Zinc-Finger Antiviral Protein (ZAP). *PLoS pathogens* **13**, e1006145, doi:10.1371/journal.ppat.1006145 (2017).

62 Zheng, X. *et al.* TRIM25 Is Required for the Antiviral Activity of Zinc Finger Antiviral Protein. *Journal of virology* **91**, doi:10.1128/jvi.00088-17 (2017).

63 Li, M. M., MacDonald, M. R. & Rice, C. M. To translate, or not to translate: viral and host mRNA regulation by interferon-stimulated genes. *Trends in cell biology* **25**, 320-329, doi:10.1016/j.tcb.2015.02.001 (2015).

64 Kawai, T. & Akira, S. TLR signaling. *Seminars in immunology* **19**, 24-32, doi:10.1016/j.smim.2006.12.004 (2007).

65 Kawai, T. & Akira, S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *International immunology* **21**, 317-337, doi:10.1093/intimm/dxp017 (2009).

66 Kawai, T. & Akira, S. Regulation of innate immune signalling pathways by the tripartite motif (TRIM) family proteins. *EMBO Mol Med* **3**, 513-527, doi:10.1002/emmm.201100160 (2011).

67 Sharma, S. *et al.* Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**, 1148-1151, doi:10.1126/science.1081315 (2003).

68 Fitzgerald, K. A. *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* **4**, 491-496, doi:10.1038/ni921ni921 [pii] (2003).

69 Hemmi, H. *et al.* The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J Exp Med* **199**, 1641-1650, doi:10.1084/jem.20040520 (2004).

70 McWhirter, S. M. *et al.* IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proc Natl Acad Sci U S A* **101**, 233-238, doi:10.1073/pnas.2237236100 (2004).

71 Takeuchi, O. & Akira, S. Innate immunity to virus infection. *Immunol Rev* **227**, 75-86, doi:10.1111/j.1600-065X.2008.00737.x (2009).

72 Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-stimulated genes: a complex web of host defenses. *Annual review of immunology* **32**, 513-545, doi:10.1146/annurev-immunol-032713-120231 (2014).

73 Jimenez-Dalmaroni, M. J., Gerswhin, M. E. & Adamopoulos, I. E. The critical role of toll-like receptors--From microbial recognition to autoimmunity: A comprehensive review. *Autoimmunity reviews* **15**, 1-8, doi:10.1016/j.autrev.2015.08.009 (2016).

74 Maarifi, G. *et al.* TRIM8 is required for virus-induced IFN response in human plasmacytoid dendritic cells. *Science advances* **5**, eaax3511, doi:10.1126/sciadv.aax3511 (2019).

75 Higgs, R. *et al.* The E3 ubiquitin ligase Ro52 negatively regulates IFN-beta production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3. *Journal of immunology (Baltimore, Md. : 1950)* **181**, 1780-1786 (2008).

76 Higgs, R. *et al.* Self protection from anti-viral responses--Ro52 promotes degradation of the transcription factor IRF7 downstream of the viral Toll-Like receptors. *PLoS One* **5**, e11776, doi:10.1371/journal.pone.0011776 (2010).

77 Lazzari, E. *et al.* TRIpartite motif 21 (TRIM21) differentially regulates the stability of interferon regulatory factor 5 (IRF5) isoforms. *PLoS One* **9**, e103609, doi:10.1371/journal.pone.0103609 (2014).

78 Yang, K. *et al.* TRIM21 is essential to sustain IFN regulatory factor 3 activation during antiviral response. *Journal of immunology (Baltimore, Md. : 1950)* **182**, 3782-3792, doi:10.4049/jimmunol.0803126 (2009).

79 Shen, Y. *et al.* TRIM56 is an essential component of the TLR3 antiviral signaling pathway. *The Journal of biological chemistry* **287**, 36404-36413, doi:10.1074/jbc.M112.397075 (2012).

80 Hu, M. M. *et al.* TRIM38 Negatively Regulates TLR3/4-Mediated Innate Immune and Inflammatory Responses by Two Sequential and Distinct Mechanisms. *Journal of immunology (Baltimore, Md. : 1950)* **195**, 4415-4425, doi:10.4049/jimmunol.1500859 (2015).

81 Zhao, W., Wang, L., Zhang, M., Yuan, C. & Gao, C. E3 ubiquitin ligase tripartite motif 38 negatively regulates TLR-mediated immune responses by proteasomal degradation of TNF receptor-associated factor 6 in macrophages. *Journal of immunology (Baltimore, Md. : 1950)* **188**, 2567-2574, doi:10.4049/jimmunol.1103255 (2012).

82 Zhao, W. *et al.* Tripartite motif-containing protein 38 negatively regulates TLR3/4- and RIG-I-mediated IFN-beta production and antiviral response by targeting NAP1. *Journal of*

immunology (Baltimore, Md. : 1950) **188**, 5311-5318, doi:10.4049/jimmunol.1103506 (2012).

83 Hu, M. M. *et al.* TRIM38 inhibits TNFalpha- and IL-1beta-triggered NF-kappaB activation by mediating lysosome-dependent degradation of TAB2/3. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 1509-1514, doi:10.1073/pnas.1318227111 (2014).

84 Pichlmair, A. *et al.* RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997-1001, doi:10.1126/science.1132998 (2006).

85 Hornung, V. *et al.* 5'-Triphosphate RNA is the ligand for RIG-I. *Science* **314**, 994-997, doi:10.1126/science.1132505 (2006).

86 Yoneyama, M. *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* **5**, 730-737, doi:10.1038/ni1087 (2004).

87 Kang, D. C. *et al.* mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc Natl Acad Sci U S A* **99**, 637-642, doi:10.1073/pnas.022637199 (2002).

88 Kato, H. *et al.* Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101-105, doi:10.1038/nature04734 (2006).

89 Cui, S. *et al.* The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. *Mol Cell* **29**, 169-179, doi:10.1016/j.molcel.2007.10.032 (2008).

90 Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805-820, doi:10.1016/j.cell.2010.01.022 (2010).

91 Wilkins, C. & Gale, M., Jr. Recognition of viruses by cytoplasmic sensors. *Curr Opin Immunol* **22**, 41-47, doi:10.1016/j.coi.2009.12.003 (2010).

92 Kawai, T. *et al.* IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* **6**, 981-988, doi:10.1038/ni1243 (2005).

93 Meylan, E. *et al.* Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**, 1167-1172, doi:10.1038/nature04193 (2005).

94 Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**, 669-682, doi:10.1016/j.cell.2005.08.012 (2005).

95 Xu, L. G. *et al.* VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* **19**, 727-740, doi:10.1016/j.molcel.2005.08.014 (2005).

96 Okamoto, M., Kouwaki, T., Fukushima, Y. & Oshiumi, H. Regulation of RIG-I Activation by K63-Linked Polyubiquitination. *Frontiers in immunology* **8**, 1942, doi:10.3389/fimmu.2017.01942 (2017).

97 Yan, J., Li, Q., Mao, A. P., Hu, M. M. & Shu, H. B. TRIM4 modulates type I interferon induction and cellular antiviral response by targeting RIG-I for K63-linked ubiquitination. *Journal of molecular cell biology* **6**, 154-163, doi:10.1093/jmcb/mju005 (2014).

98 Oshiumi, H., Matsumoto, M. & Seya, T. Ubiquitin-mediated modulation of the cytoplasmic viral RNA sensor RIG-I. *Journal of biochemistry* **151**, 5-11, doi:10.1093/jb/mvr111 (2012).

99 Lang, X. *et al.* TRIM65-catalized ubiquitination is essential for MDA5-mediated antiviral innate immunity. *The Journal of experimental medicine* **214**, 459-473, doi:10.1084/jem.20160592 (2017).

100 Narayan, K. *et al.* TRIM13 is a negative regulator of MDA5-mediated type I interferon production. *Journal of virology* **88**, 10748-10757, doi:10.1128/jvi.02593-13 (2014).

101 Hu, M. M., Liao, C. Y., Yang, Q., Xie, X. Q. & Shu, H. B. Innate immunity to RNA virus is regulated by temporal and reversible sumoylation of RIG-I and MDA5. *The Journal of experimental medicine* **214**, 973-989, doi:10.1084/jem.20161015 (2017).

102 Liu, B. *et al.* The ubiquitin E3 ligase TRIM31 promotes aggregation and activation of the signaling adaptor MAVS through Lys63-linked polyubiquitination. *Nature immunology* **18**, 214-224, doi:10.1038/ni.3641 (2017).

103 Zhou, Z. *et al.* TRIM14 is a mitochondrial adaptor that facilitates retinoic acid-inducible gene-I-like receptor-mediated innate immune response. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E245-254, doi:10.1073/pnas.1316941111 (2014).

104 Castanier, C. *et al.* MAVS ubiquitination by the E3 ligase TRIM25 and degradation by the proteasome is involved in type I interferon production after activation of the antiviral RIG-I-like receptors. *BMC biology* **10**, 44, doi:10.1186/1741-7007-10-44 (2012).

105 Yang, B. *et al.* Novel function of Trim44 promotes an antiviral response by stabilizing VISA. *Journal of immunology (Baltimore, Md. : 1950)* **190**, 3613-3619, doi:10.4049/jimmunol.1202507 (2013).

106 Ran, Y. *et al.* Autoubiquitination of TRIM26 links TBK1 to NEMO in RLR-mediated innate antiviral immune response. *Journal of molecular cell biology* **8**, 31-43, doi:10.1093/jmcb/mjv068 (2016).

107 Qin, Y. *et al.* TRIM9 short isoform preferentially promotes DNA and RNA virus-induced production of type I interferon by recruiting GSK3beta to TBK1. *Cell research* **26**, 613-628, doi:10.1038/cr.2016.27 (2016).

108 Lee, Y., Song, B., Park, C. & Kwon, K. S. TRIM11 negatively regulates IFNbeta production and antiviral activity by targeting TBK1. *PLoS One* **8**, e63255, doi:10.1371/journal.pone.0063255 (2013).

109 Sun, L., Wu, J., Du, F., Chen, X. & Chen, Z. J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **339**, 786-791, doi:10.1126/science.1232458 (2013).

110 Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674-678, doi:10.1038/nature07317 (2008).

111 Zhong, B. *et al.* The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* **29**, 538-550, doi:10.1016/j.immuni.2008.09.003 (2008).

112 Sun, W. *et al.* ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proc Natl Acad Sci U S A* **106**, 8653-8658, doi:10.1073/pnas.0900850106 (2009).

113 Tsuchida, T. *et al.* The ubiquitin ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA. *Immunity* **33**, 765-776, doi:10.1016/j.immuni.2010.10.013 (2010).

114 Zhang, J., Hu, M. M., Wang, Y. Y. & Shu, H. B. TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination. *J Biol Chem* **287**, 28646-28655, doi:10.1074/jbc.M112.362608 (2012).

115 Wang, Y. *et al.* TRIM30alpha Is a Negative-Feedback Regulator of the Intracellular DNA and DNA Virus-Triggered Response by Targeting STING. *PLoS pathogens* **11**, e1005012, doi:10.1371/journal.ppat.1005012 (2015).

116 Li, Q. *et al.* TRIM29 negatively controls antiviral immune response through targeting STING for degradation. *Cell discovery* **4**, 13, doi:10.1038/s41421-018-0010-9 (2018).

117 Hu, M. M. *et al.* Sumoylation Promotes the Stability of the DNA Sensor cGAS and the Adaptor STING to Regulate the Kinetics of Response to DNA Virus. *Immunity* **45**, 555-569, doi:10.1016/j.immuni.2016.08.014 (2016).

118 Seo, G. J. *et al.* TRIM56-mediated monoubiquitination of cGAS for cytosolic DNA sensing. *Nature communications* **9**, 613, doi:10.1038/s41467-018-02936-3 (2018).

119 Watkinson, R. E., McEwan, W. A., Tam, J. C., Vaysburd, M. & James, L. C. TRIM21 Promotes cGAS and RIG-I Sensing of Viral Genomes during Infection by Antibody-Opsonized Virus. *PLoS pathogens* **11**, e1005253, doi:10.1371/journal.ppat.1005253 (2015).

120 Lamkanfi, M. & Dixit, V. M. Mechanisms and functions of inflammasomes. *Cell* **157**, 1013-1022, doi:10.1016/j.cell.2014.04.007 (2014).

121 Latz, E., Xiao, T. S. & Stutz, A. Activation and regulation of the inflammasomes. *Nature reviews. Immunology* **13**, 397-411, doi:10.1038/nri3452 (2013).

122 Bauernfeind, F. *et al.* Inflammasomes: current understanding and open questions. *Cellular and molecular life sciences : CMLS* **68**, 765-783, doi:10.1007/s00018-010-0567-4 (2011).

123 Weng, L. *et al.* The E3 ubiquitin ligase tripartite motif 33 is essential for cytosolic RNA-induced NLRP3 inflammasome activation. *Journal of immunology (Baltimore, Md. : 1950)* **193**, 3676-3682, doi:10.4049/jimmunol.1401448 (2014).

124 Song, H. *et al.* The E3 ubiquitin ligase TRIM31 attenuates NLRP3 inflammasome activation by promoting proteasomal degradation of NLRP3. *Nature communications* **7**, 13727, doi:10.1038/ncomms13727 (2016).

125 Hu, Y. *et al.* Tripartite-motif protein 30 negatively regulates NLRP3 inflammasome activation by modulating reactive oxygen species production. *Journal of immunology (Baltimore, Md. : 1950)* **185**, 7699-7705, doi:10.4049/jimmunol.1001099 (2010).

126 Kapoor, A., Forman, M. & Arav-Boger, R. Activation of nucleotide oligomerization domain 2 (NOD2) by human cytomegalovirus initiates innate immune responses and restricts virus replication. *PLoS One* **9**, e92704, doi:10.1371/journal.pone.0092704 (2014).

127 Zurek, B. *et al.* TRIM27 negatively regulates NOD2 by ubiquitination and proteasomal degradation. *PLoS One* **7**, e41255, doi:10.1371/journal.pone.0041255 (2012).

128 Moreira, L. O. & Zamboni, D. S. NOD1 and NOD2 Signaling in Infection and Inflammation. *Frontiers in immunology* **3**, 328, doi:10.3389/fimmu.2012.00328 (2012).

129 van Tol, S., Hage, A., Giraldo, M. I., Bharaj, P. & Rajsbaum, R. The TRIMendous Role of TRIMs in Virus-Host Interactions. *Vaccines* **5**, doi:10.3390/vaccines5030023 (2017).

130 Arimoto, K. *et al.* Polyubiquitin conjugation to NEMO by triparite motif protein 23 (TRIM23) is critical in antiviral defense. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 15856-15861, doi:10.1073/pnas.1004621107 (2010).

131 Shi, M. *et al.* TRIM30 alpha negatively regulates TLR-mediated NF-kappa B activation by targeting TAB2 and TAB3 for degradation. *Nature immunology* **9**, 369-377, doi:10.1038/ni1577 (2008).

132 Wada, K., Niida, M., Tanaka, M. & Kamitani, T. Ro52-mediated monoubiquitination of IKK{beta} down-regulates NF-{kappa}B signalling. *Journal of biochemistry* **146**, 821-832, doi:10.1093/jb/mvp127 (2009).

133 Zha, J. *et al.* The Ret finger protein inhibits signaling mediated by the noncanonical and canonical IkappaB kinase family members. *Journal of immunology (Baltimore, Md. : 1950)* **176**, 1072-1080 (2006).

134 Xing, J. *et al.* Identification of a role for TRIM29 in the control of innate immunity in the respiratory tract. *Nature immunology* **17**, 1373-1380, doi:10.1038/ni.3580 (2016).

135 Rajsbaum, R. *et al.* Unanchored K48-linked polyubiquitin synthesized by the E3-ubiquitin ligase TRIM6 stimulates the interferon-IKKepsilon kinase-mediated antiviral response. *Immunity* **40**, 880-895, doi:10.1016/j.immuni.2014.04.018 (2014).

136 Jimenez-Moyano, E. *et al.* Nonhuman TRIM5 Variants Enhance Recognition of HIV-1-Infected Cells by CD8+ T Cells. *Journal of virology* **90**, 8552-8562, doi:10.1128/jvi.00819-16 (2016).

137 Cleret-Buhot, A. *et al.* Identification of novel HIV-1 dependency factors in primary CCR4(+)CCR6(+)Th17 cells via a genome-wide transcriptional approach. *Retrovirology* **12**, 102, doi:10.1186/s12977-015-0226-9 (2015).

138 Perez-Lloret, J. *et al.* T-cell-intrinsic Tif1alpha/Trim24 regulates IL-1R expression on TH2 cells and TH2 cell-mediated airway allergy. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E568-576, doi:10.1073/pnas.1522287113 (2016).

139 Cai, X. *et al.* Tripartite motif containing protein 27 negatively regulates CD4 T cells by ubiquitinating and inhibiting the class II PI3K-C2beta. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 20072-20077, doi:10.1073/pnas.1111233109 (2011).

140 Fan, W. *et al.* Swine TRIM21 restricts FMDV infection via an intracellular neutralization mechanism. *Antiviral research* **127**, 32-40, doi:10.1016/j.antiviral.2016.01.004 (2016).

141 Sanchez-Aparicio, M. T., Ayllon, J., Leo-Macias, A., Wolff, T. & Garcia-Sastre, A. Subcellular Localizations of RIG-I, TRIM25, and MAVS Complexes. *J Virol* **91**, doi:10.1128/JVI.01155-16 (2017).

142 Gack, M. U. *et al.* Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* **5**, 439-449, doi:10.1016/j.chom.2009.04.006 (2009).

143 Hu, Y. *et al.* The Severe Acute Respiratory Syndrome Coronavirus Nucleocapsid Inhibits Type I Interferon Production by Interfering with TRIM25-Mediated RIG-I Ubiquitination. *J Virol* **91**, doi:10.1128/jvi.02143-16 (2017).

144 Manokaran, G. *et al.* Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness. *Science (New York, N.Y.)* **350**, 217-221, doi:10.1126/science.aab3369 (2015).

145 Pauli, E. K. *et al.* The ubiquitin-specific protease USP15 promotes RIG-I-mediated antiviral signaling by deubiquitylating TRIM25. *Sci Signal* **7**, ra3, doi:10.1126/scisignal.2004577 (2014).

146 Bharaj, P. *et al.* The Matrix Protein of Nipah Virus Targets the E3-Ubiquitin Ligase TRIM6 to Inhibit the IKKepsilon Kinase-Mediated Type-I IFN Antiviral Response. *PLoS Pathog* **12**, e1005880, doi:10.1371/journal.ppat.1005880 (2016).

147 Bharaj, P. *et al.* The Host E3-Ubiquitin Ligase TRIM6 Ubiquitinates the Ebola Virus VP35 Protein and Promotes Virus Replication. *Journal of virology* **91**, doi:10.1128/jvi.00833-17 (2017).

148 Laurent-Rolle, M. *et al.* The interferon signaling antagonist function of yellow fever virus NS5 protein is activated by type I interferon. *Cell host & microbe* **16**, 314-327, doi:10.1016/j.chom.2014.07.015 (2014).

149 Manocha, G. D. *et al.* Regulatory role of TRIM21 in the type-I interferon pathway in Japanese encephalitis virus-infected human microglial cells. *Journal of neuroinflammation* **11**, 24, doi:10.1186/1742-2094-11-24 (2014).

150 Yuan, T., Yao, W., Huang, F., Sun, B. & Yang, R. The human antiviral factor TRIM11 is under the regulation of HIV-1 Vpr. *PLoS One* **9**, e104269, doi:10.1371/journal.pone.0104269 (2014).

151 Lim, K. H. *et al.* Suppression of interferon-mediated anti-HBV response by single CpG methylation in the 5'-UTR of TRIM22. *Gut* **67**, 166-178, doi:10.1136/gutjnl-2016-312742 (2018).

152 Li, X., Burton, E. M. & Bhaduri-McIntosh, S. Chloroquine triggers Epstein-Barr virus replication through phosphorylation of KAP1/TRIM28 in Burkitt lymphoma cells. *PLoS pathogens* **13**, e1006249, doi:10.1371/journal.ppat.1006249 (2017).

153 Conwell, S. E., White, A. E., Harper, J. W. & Knipe, D. M. Identification of TRIM27 as a novel degradation target of herpes simplex virus 1 ICP0. *J Virol* **89**, 220-229, doi:10.1128/jvi.02635-14 (2015).

154 Ling, P. D., Tan, J., Sewatanon, J. & Peng, R. Murine gammaherpesvirus 68 open reading frame 75c tegument protein induces the degradation of PML and is essential for production of infectious virus. *Journal of virology* **82**, 8000-8012, doi:10.1128/jvi.02752-07 (2008).

155 Neri, M. *et al.* A patient with limb girdle muscular dystrophy carries a TRIM32 deletion, detected by a novel CGH array, in compound heterozygosis with a nonsense mutation. *Neuromuscul Disord* **23**, 478-482, doi:S0960-8966(13)00059-X [pii]10.1016/j.nmd.2013.02.003 (2013).

156 Guglieri, M., Straub, V., Bushby, K. & Lochmuller, H. Limb-girdle muscular dystrophies. *Curr Opin Neurol* **21**, 576-584, doi:10.1097/WCO.0b013e32830efdc2 (2008).

157 Saccone, V. *et al.* Mutations that impair interaction properties of TRIM32 associated with limb-girdle muscular dystrophy 2H. *Hum Mutat* **29**, 240-247, doi:10.1002/humu.20633 (2008).

158 Schoser, B. G. *et al.* Commonality of TRIM32 mutation in causing sarcotubular myopathy and LGMD2H. *Ann Neurol* **57**, 591-595, doi:10.1002/ana.20441 (2005).

159 Watanabe, M. & Hatakeyama, S. TRIM proteins and diseases. *Journal of biochemistry* **161**, 135-144, doi:10.1093/jb/mvw087 (2017).

CHAPTER II

TRIM41-Mediated Ubiquitination of Nucleoprotein Limits Influenza A Virus Infection

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ABSTRACT

Influenza A virus (IAV) is a highly transmissible respiratory pathogen and a major cause of morbidity and mortality around the world. Nucleoprotein (NP) is an abundant IAV protein essential for multiple steps of the viral life cycle. Our recent proteomic study of the IAV-host interaction network found that TRIM41 (tripartite motif-containing 41), a ubiquitin E3 ligase, interacted with NP. However, the role of TRIM41 in IAV infection is unknown. Here, we report that TRIM41 interacts with NP through its SPRY domain. Furthermore, TRIM41 is constitutively expressed in lung epithelial cells, and overexpression of TRIM41 inhibits IAV infection. Conversely, RNA interference (RNAi) and knockout of TRIM41 increase host susceptibility to IAV infection. As a ubiquitin E3 ligase, TRIM41 ubiquitinates NP in vitro and in cells. The TRIM41 mutant lacking E3 ligase activity fails to inhibit IAV infection, suggesting that the E3 ligase activity is indispensable for TRIM41 antiviral function. Mechanistic analysis further revealed that the polyubiquitination leads to NP protein degradation and viral inhibition. Taking these observations together, TRIM41 is a constitutively expressed intrinsic IAV restriction factor that targets NP for ubiquitination and protein degradation.

INTRODUCTION

Influenza A virus is a member of the Orthomyxoviridae family and a human respiratory pathogen that causes seasonal epidemics and occasional global pandemics with considerable economic and social impact [1,2]. Virus engages with the host cellular protein interaction network during infection. The engagement either facilitates virus hijack of host molecular machinery to fulfill viral life cycle or triggers host immune defense to eliminate the virus. In recent years, host intrinsic restriction factors are gaining increasing importance in IAV inhibition [3]. Host intrinsic restriction factors usually limit viral infection by direct interaction with viral proteins. For example, plakophilin 2 (PKP2) competes with PB2 for PB1 binding, thus disrupting IAV polymerase complex and inhibiting viral replication [4]. The therapeutics targeting at intrinsic immunity factors are more promising because cellular proteins are less likely to mutate under drug-mediated selective pressure.

The tripartite motif (TRIM) family members have been increasingly recognized as intrinsic immunity factors that inhibit viral infection. For example, TRIM5α is well known for the species-specific retroviral restriction by binding to the viral capsid and inducing premature uncoating [5]. TRIM79α restricts tick-borne encephalitis virus [6]. TRIM28, also known as KAP1, restricts murine leukemia virus as well as facilitates the establishment of viral latency [7,8]. Recently, TRIM52 has been found to interact with the NS2A protein of Japanese encephalitis virus and target NS2A for proteasome-mediated destruction [9].

Several TRIM proteins have been found to inhibit IAV infection. For example, TRIM32 ubiquitinates PB1 and subsequently degrades PB1, thereby limiting viral infection [10]. TRIM19 (also known as PML), TRIM22 and TRIM56 display a broad intrinsic antiviral activity and inhibit multiple viruses, including IAV [11-13]. By contrast, IAV evolves to subvert host immunity

by targeting TRIM proteins [14]. TRIM25 ubiquitinates and activates RIG-I-mediated innate immunity [15]. The NS1 of IAV impairs interferon (IFN)-dependent innate immune response by impeding TRIM25 multimerization and activation of RIG-I [16,17].

Our recent study on the IAV-host protein interaction network found that TRIM41 interacted with the NP [4]. TRIM41, also known as the RING finger-interacting protein with C kinase (RINCK), regulates PKC kinase signaling [18]. TRIM41 is also found to interact with the nucleotide binding oligomerization domain containing 2 (NOD2), but how TRIM41 regulates NOD2 signaling is not clear [19]. Recently, a screening of TRIM proteins found that TRIM41 along with other seven TRIM proteins inhibited hepatitis B virus (HBV) transcription [20]. However, the role of TRIM41 in IAV infection is unknown. Here, we characterized the physical interaction between TRIM41 and NP. Furthermore, overexpression of TRIM41 inhibits IAV infection while depletion of TRIM41 increases host susceptibility to viral infection. As a ubiquitin E3 ligase, TRIM41 ubiquitinates NP and the ubiquitination leads to NP protein degradation. Thus, our study establishes the role of TRIM41 as a new host restriction factor in IAV infection.

MATERIALS AND METHODS

Cells and viruses. HEK293 cells (ATCC, # CRL-1573) and MDCK cells (ATCC, # CCL-34) were maintained in Dulbecco's Modified Eagle Medium (Life Technologies) containing antibiotics (Life Technologies) and 10% fetal bovine serum (Life Technologies). A549 cells (ATCC, # CCL-185) were cultured in RPMI Medium 1640 (Life Technologies) plus 10% fetal bovine serum and 1X MEM Non-Essential Amino Acids Solution (Life Technologies). Primary human tracheal epithelial cells and supporting medium were purchased from Lifeline Technology (Frederick, MD) (# FC-0035 and # LL-0023).

Influenza A/Puerto Rico/8/34 (H1N1) (Charles River Laboratories, # 10100374), A/WSN/33 (H1N1) (a kind gift of Dr. Peter Palese, Mount Sinai School of Medicine, NY), and A/New York/18/2009 (H1N1) pdm09 (BEI Resources). Influenza PR8-GLuc virus was a generous gift from Dr. Peter Palese and features a *Gaussia* luciferase (Gluc) gene inserted downstream of PB2 [23]. IAV propagation in specific pathogen-free fertilized eggs and $TCID_{50}$ assay was performed as described by Szretter et al. [37]. Sendai virus with a luciferase gene (SeV-Luc) was a kind gift from Dr. Charles Russell (St. Jude's Hospital, Memphis, TN). VACV with a firefly luciferase gene (VACV-Luc) was a gift from D.L. Bartlett (University of Pittsburg, Pittsburg, PA).

Plasmids and antibodies. Human TRIM41-HA was a generous gift from Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine, NY) [30]. TRIM41 was cloned into pCMV-3Tag-8 (Strategene) to generate TRIM41-FLAG [38]. Deletion mutant of TRIM41-FLAG was constructed using a Q5® Site-Directed Mutagenesis Kit (New England Biolabs).

Anti-β-actin (Abcam, # ab8227), anti-FLAG (Sigma, # F3165), anti-IFITM3 (GeneTex, GTX63349), anti-ubiquitin (Santa Cruz Biotechnology, # sc-8017), anti-TRIM41 (Aviva Systems

Biology, # ARP34763_P050), anti-HA epitope (Cell Signaling Technology, # 3724), anti-NP (BEI resources, # NR-4282), anti-NP (EMD Millipore, # MAB8800), anti-NP (Genscript, # A01506-40). Goat anti-Mouse IgG-HRP (Santa Cruz Biotechnology, # sc-2055), Goat anti-Rabbit IgG-HRP (Santa Cruz Biotechnology, # sc-2030), Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) (Life Technologies, # A11005), Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) (Life Technologies, # A11034).

Sample preparation, western blotting and immunoprecipitation. Approximately 1 X 10^6 cells were lysed in 500 µl of tandem affinity purification (TAP) lysis buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.5% Nonidet P40, 10% glycerol, Complete EDTA-free protease inhibitor cocktail tablets (Roche)] for 30 min at 4^0C. The lysates were then centrifuged for 30 min at 15,000 x *g*. Supernatants were collected and mixed with 1X Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific). Western blotting and immunoprecipitation were performed as described in a previous study [4].

Immunofluorescence assay. Cells were cultured in the Lab-Tek II CC2 Chamber Slide System 4-well (Thermo Fisher Scientific). After the indicated treatment, the cells were fixed and permeabilized in cold methanol for 10 min at -20 ^{0}C. Then, the slides were washed with 1 X PBS for 10 min and blocked with Odyssey Blocking Buffer (LI-COR Biosciences) for 1 h. The slides were incubated in Odyssey Blocking Buffer with appropriately diluted primary antibodies at 4 ^{0}C for 12 h. Images were captured and analyzed using an iRiS™ Digital Cell Imaging System (Logos Biosystems).

Real-time PCR. Total RNA was prepared using RNeasy columns (Qiagen). 1 µg quantity of RNA was transcribed into cDNA using a QuantiTect reverse transcription kit (Qiagen). For one

real-time reaction, 10 µl of SYBR Green PCR reaction mix (Eurogentec) including a 1/10 volume of the synthesized cDNA plus an appropriate oligonucleotide primer pair were analyzed on a 7500 Fast Real-time PCR System (Applied Biosystems). The comparative C*t* method was used to determine the relative mRNA expression of genes normalized by the housekeeping gene GAPDH. The primer sequences: IP10, forward primer 5`- TTCAAGGAGTACCTCTCTCTAG-3`, reverse primer 5`- CTGGATTCAGACATCTCTTCTC-3`; IFN□, forward primer 5`-CAGCAATTTTCAGTGTCAGAAGC-3`, reverse primer 5`- TCATCCTGTCCTTGAGGCAGT-3`; TRIM41, forward primer 5`- AGAATCCAGGAGCCACAAAC-3`, reverse primer 5`-TCTTCAGCCAGAAACCGTG-3`; GAPDH, forward primer 5`- AGGTGAAGGTCGGAGTCA-3`, reverse primer 5`-GGTCATTGATGGCAACAA-3`.

Plasmid transfection. Plasmid transfection. HEK293 cells were transfected using Lipofectamine 2000 or Lipofectamine 3000 transfection reagent (Life Technologies) according to the manufacturer's protocol. For coimmunoprecipitation experiments, a total of 2.5 µg of plasmids was transfected into approximately 1.2×10^6 cells. For other experiments, a total of 0.5 µg of plasmids was transfected into approximately 0.2×10^6 cells.

RNAi depletion. RNAi target sequences (sense strand): TRIM41 siRNA #1: GAGGCGAGTGACAGAACTGAA; TRIM41 siRNA #2: AAGGCGTGCTGTGGAAATAAA; TRIM41 siRNA #3: TTCAATAGGTGTGAAGAGGTA. siGENOME Non-Targeting Control siRNA (Dharmacon, # D-001210-02-05) was used as the control siRNA. siRNA duplexes were transfected into A549 cells (5 pmol) using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer’s protocol.

Cell viability. Cell viability was assessed by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions.

CRISPR/Cas9. The single guide RNA (sgRNA) sequence targeting human TRIM41 is: GTAGTCTTCATCCCGCATGG. The sgRNA was cloned into lentiCRISPR v2 [24] (Addgene). The lentiviral construct was transfected into HEK293 cells using Lipofectamine 2000. Cells were selected with 10 μg/ml puromycin for 14 days. Single clones were expanded for knockout confirmation by western blotting and DNA sequencing.

***In vitro* ubiquitination.** *In vitro* ubiquitination assay was performed according to the manufacturer's manual (Boston Biochem). Ubiquitin (3 μg), E1 (200 ng), UBCH5A (300 ng) (Boston Biochem), NP-FLAG (0.5 μg) bound to the anti-FLAG resin (Sigma) and TRIM41-HA or ΔRING-HA (0.5 μg) were incubated at 30°C in the ubiquitin assay buffer (20 mM Tris-HCl pH 7.5 plus 1 x ERS buffer) for 2 h. Two hours later, the anti-FLAG resin was washed with 1 M urea for 15 min to exclude potential binding of unanchored polyubiquitin. Then the resin was incubated with 40 μL of 0.2 μg/ml FLAG peptide to elute NP protein. The eluates were subsequently analyzed by SDS-PAGE followed by western blotting.

Statistical analysis. The sample size was sufficient for data analysis using paired two-tailed Student's *t*-test. For all statistical analysis, differences were considered to be statistically significant at values of $P < 0.05$.

RESULTS

TRIM41 interacts and co-localizes with NP. Our previous proteomics study showed that TRIM41 associated with NP, but the interaction is not well defined (4). Thus, we first validated the protein interaction between TRIM41 and NP. FLAG-tagged NP from influenza A/Puerto Rico/8/1934 (PR8) or A/New York/1682/2009 (NY) was co-transfected with HA-tagged TRIM41 into HEK293 cells. Co-immunoprecipitation confirmed the interaction between TRIM41 and the two NP proteins (Fig. 5).

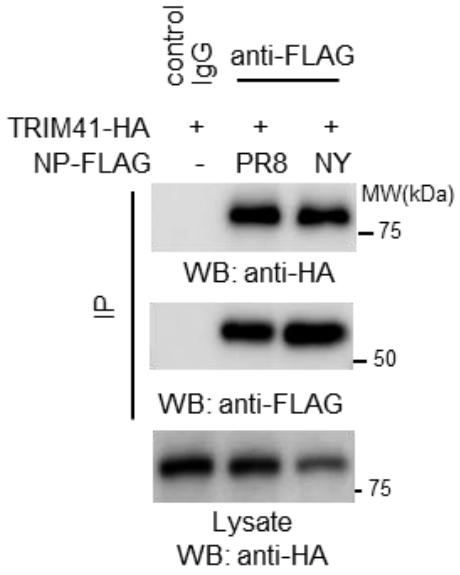

Figure.5: **TRIM41 – NP interaction.** TRIM41-HA (1.25 μg) was cotransfected with 1.25 μg of FLAG-tagged NP from the IAV PR8 or NY strain into HEK293 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody or control IgG and then blotted as indicated. Molecular weights are indicated

Next, we examined NP-TRIM41 protein interaction during viral infection. A549 cells were infected with 1 MOI of PR8 IAV for 12 hr, and then cell lysates were immunoprecipitated with anti-NP antibody or control IgG. As shown in Figure 2, viral NP interacted with endogenous TRIM41. Furthermore, RNase treatment had little effects on this interaction (Fig. 6), suggesting NP-TRIM41 interaction is independent of RNA.

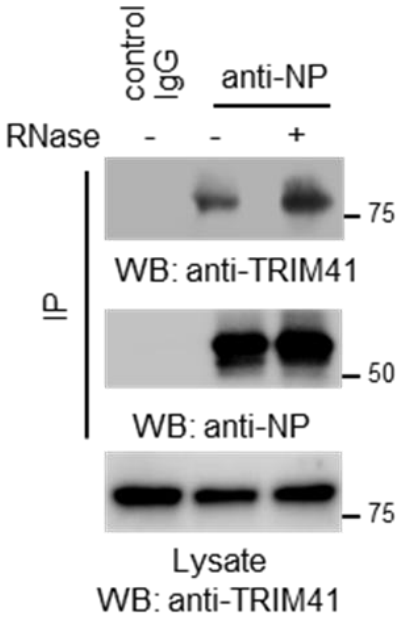

Figure 6: **RNA independent TRIM41- NP interaction.** A549 cells were infected at an MOI of 1 with PR8 IAV for 12 h. Cell lysates were treated with or without RNase A and then immunoprecipitated with anti-NP antibody or control IgG.

We also examined the co-localization between TRIM41 and NP in A549 lung epithelial cells. Consistent with a previous report (21), endogenous TRIM41 was expressed in the cytoplasm and nucleus (Fig. 7). Following IAV infection, NP was expressed and co-localized with TRIM41 in the cytoplasm and nucleus (Fig. 7).

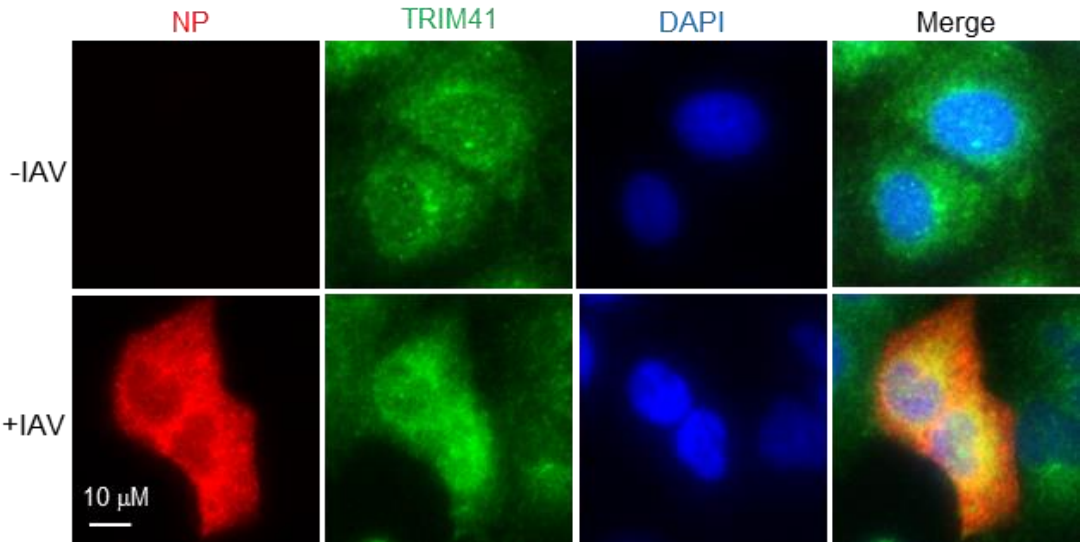

Figure 7: **TRIM41- NP co-localization.** A549 cells were mock infected or infected with PR8 IAV. After 12 h, cells were fixed with cold methanol and incubated with anti-NP and anti-TRIM41 antibodies. DAPI, 4′,6′-diamidino-2-phenylindole.

Finally, to determine which domain of TRIM41 is required for NP interaction, we generated a series of TRIM41 truncates by mutagenesis (Fig.8).

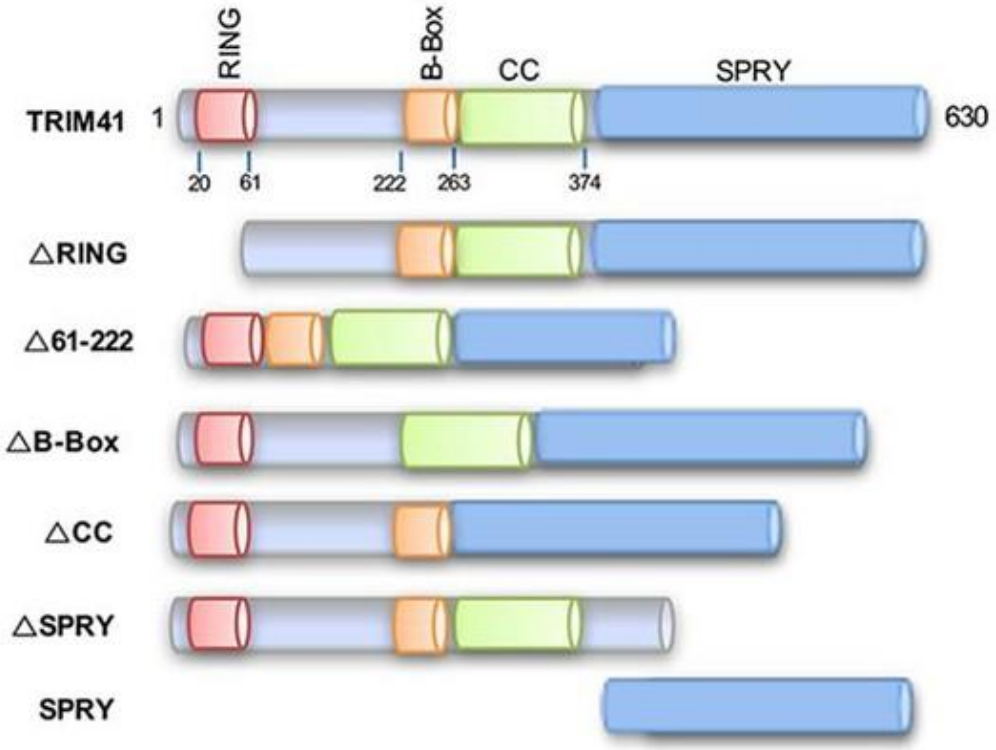

Figure 8: **The schematics of TRIM41 mutants**. RING, really interesting new gene; B-Box, B-box type zinc finger; CC, coiled coil; SPRY, SPla and the RYanodine receptor.

As shown in Figure 9, deletion of RING, the region of amino acid 61-222, B-box, or coiled-coil (CC) had little effects on TRIM41-NP interaction while deletion of SPRY abolished the interaction suggesting that the C-terminal SPRY domain is required. Indeed, the SPRY domain alone is sufficient for NP interaction. Thus, TRIM41 interacts with NP through the SPRY domain on the C-terminus.

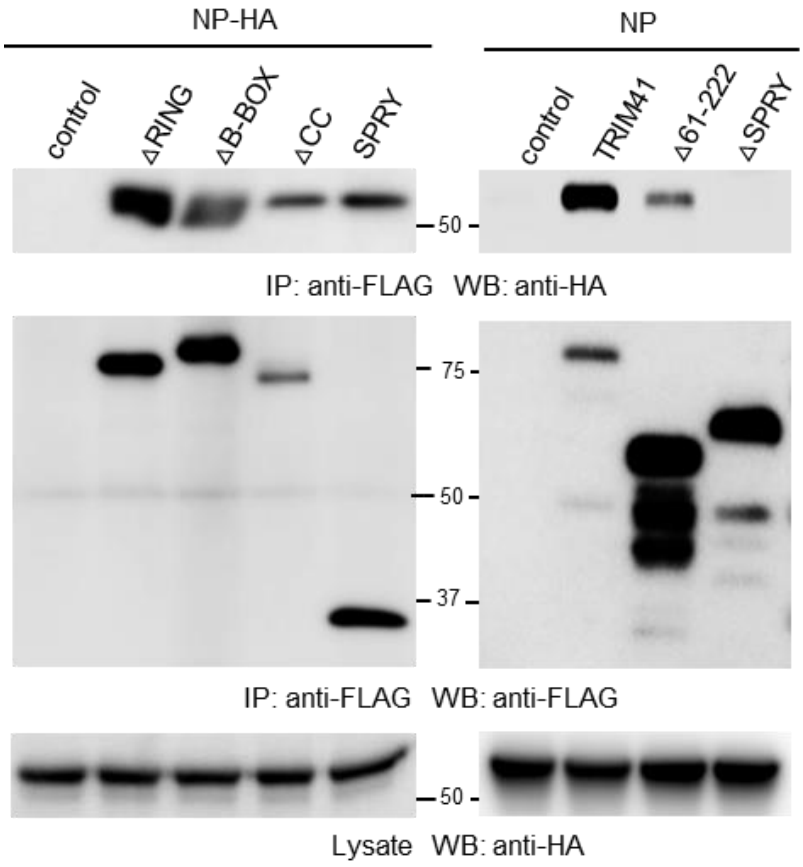

Figure 9: **TRIM41 interacts with NP through the SPRY domain.** HA-tagged NP (NP-HA; 1.25 μg) was cotransfected with 1.25 μg of the indicated FLAG-tagged TRIM41 mutants into HEK293 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with the indicated antibodies. WB, Western blotting.

TRIM41 expression is not induced by type I interferon (IFN)

As IAV is intrinsically sensitive to the antiviral action of IFN, we speculated that type I IFN might regulate TRIM41 expression. Therefore, we treated the cells with IFN or infected A549 lung epithelial cells with the PR8 IAV mutant with NS1 gene deletion (PR8 ΔNS1) (22). TRIM41 mRNA and protein expression were then examined. However, IFN stimulation or IAV infection marginally modulates TRIM41 protein and mRNA levels (Figs. 10-13), suggesting that TRIM41 is constitutively expressed in A549 human lung epithelial cells.

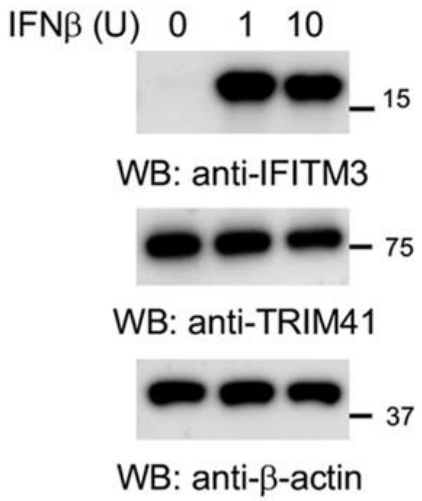

Figure 10: **Effect of Interferon treatment on TRIM41 expression.** A549 cells were treated with the designated units (U) of IFN-β for 12 h. Cell lysates were blotted with the indicated antibodies. The interferon-induced transmembrane protein 3 (IFITM3) was included as a positive control.

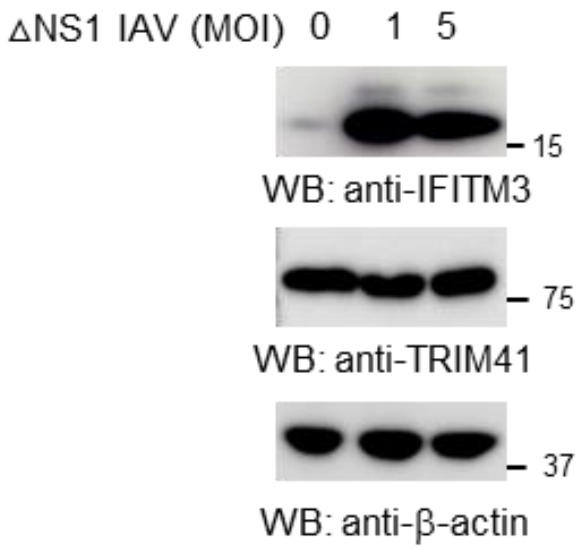

Figure 11: **Effect of IAV infection on TRIM41 expression.** A549 cells were infected at the indicated MOI with PR8 ΔNS1 IAV for 12 h. Cell lysates were blotted with the indicated antibodies. IFITM3 was included as a positive control.

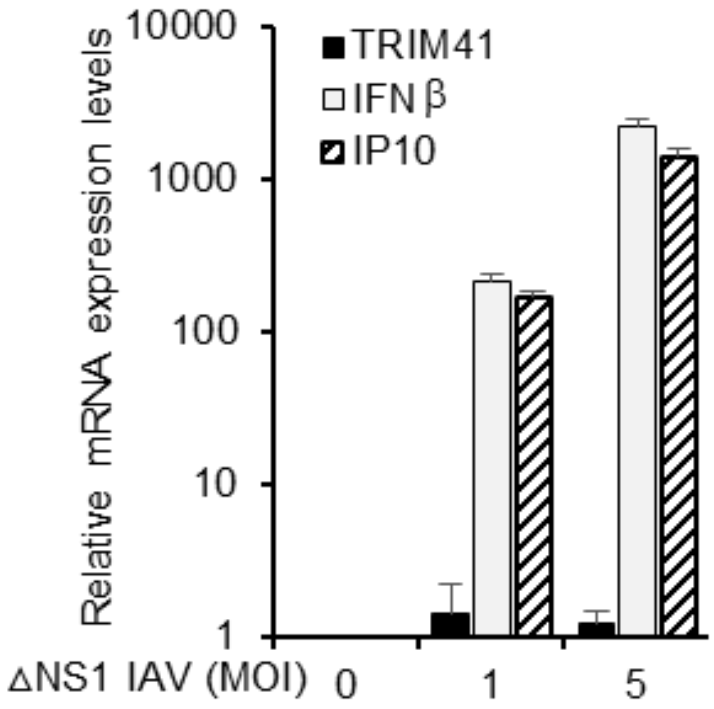

Figure 12: **Effect of IAV infection on TRIM41 mRNA levels.** A549 cells were infected at the indicated MOI with the ΔNS1 mutant virus for 4 h. mRNA was extracted, and real-time PCR was performed to determine the relative levels of the indicated genes. IFN-β and IFN-γ-inducible protein 10 (IP10) are the positive-control genes that are known to be induced by viral infection.

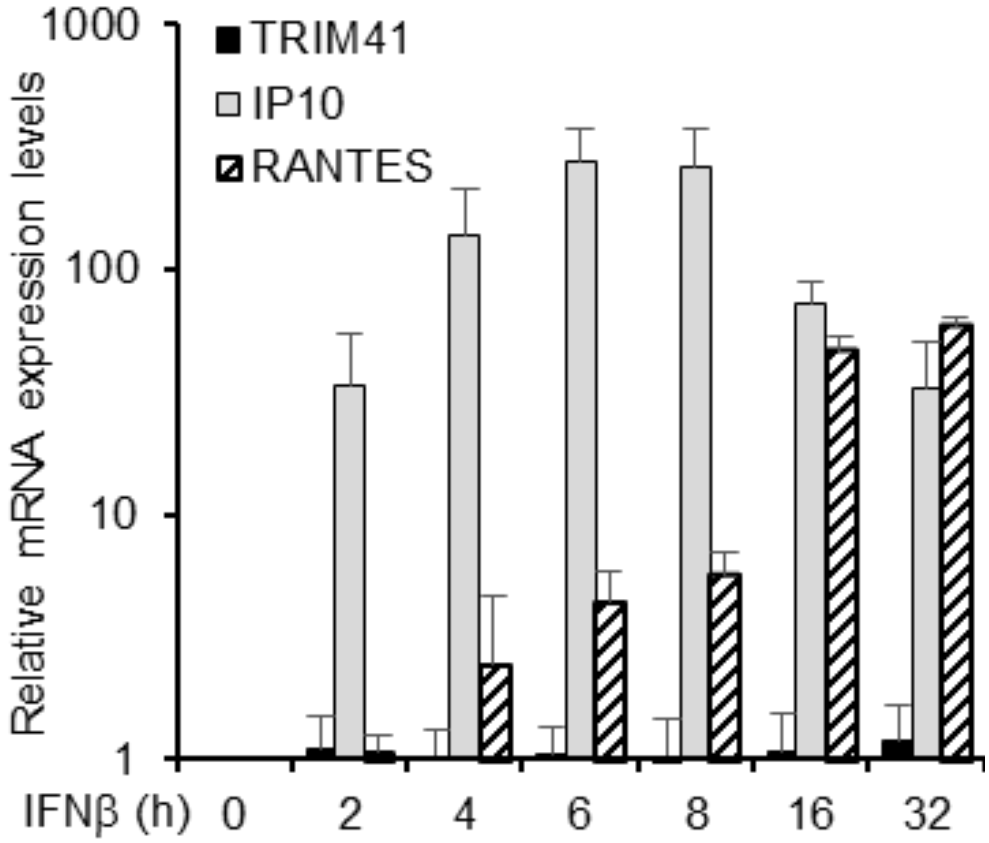

Figure 13: **Effect of interferons on TRIM41 mRNA levels**. A549 cells were treated with 1 unit of IFN-β for the designated times. mRNA was extracted, and real-time PCR was performed to determine the relative levels of the indicated genes. All experiments were biologically repeated three times. RANTES (regulated on activation, normal T cell expressed and secreted) and IP10 were the positive controls.

TRIM41 limits IAV infection.

These initial observations led to our working hypothesis that TRIM41 modulates IAV infection. To test this hypothesis, four assays (reporter assay, western blotting, immunofluorescence and TCID50 assay) were adopted to evaluate the biological effects of TRIM41 overexpression on IAV infection. First, FLAG-tagged TRIM41 or vector was transfected into HEK293 cells, followed by infection with the PR8-Gaussia luciferase reporter virus (PR8-Gluc) (23). Reporter

assay showed that TRIM41 overexpression inhibited IAV replication activity (Fig. 10). We also examined whether TRIM41 restricted other viruses. HEK293 cells expressing TRIM41-FLAG were also infected with two other reporter viruses, sendai virus with a firefly luciferase gene (SeV-Luc) or vaccinia virus with a firefly luciferase gene (VACV-Luc). TRIM41 selectively restricted IAV replication (Fig. 14).

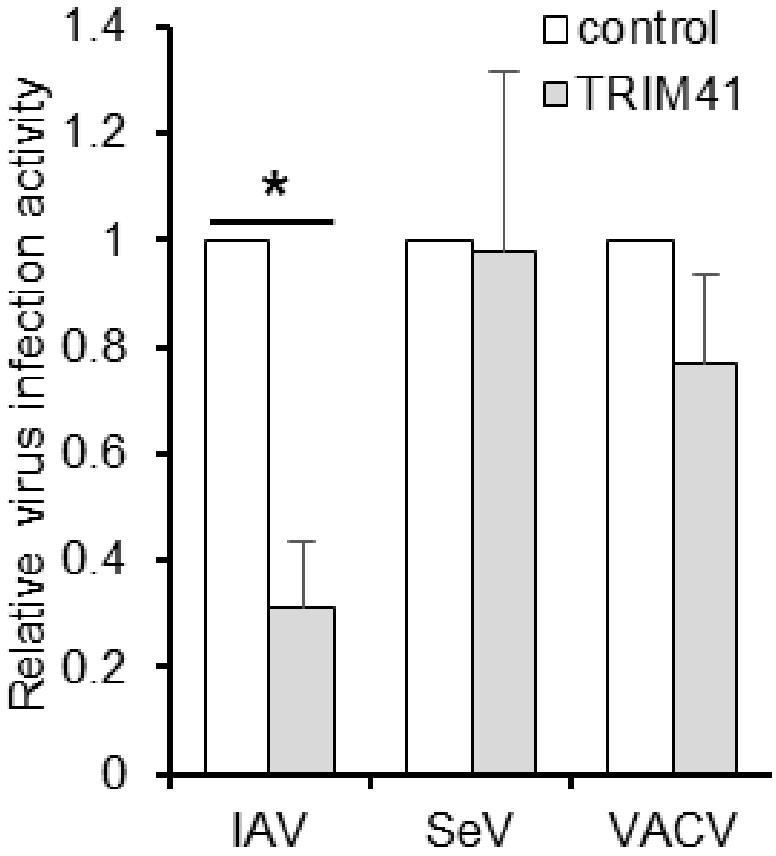

Figure 14: **TRIM41 selectively restricted IAV replication.** HEK293 cells transfected with 0.5 μg of TRIM41-FLAG or pCMV-3Tag-8 vector were infected at an MOI of 0.1 with IAV-Gluc, SeV-Luc, and VACV-Luc for 12 h. Relative luciferase activities were examined. Data represent means ± standard deviations of three independent experiments. The P value was calculated (two-tailed Student's t test) by comparison of results with those with the vector control (*, $P < 0.05$).

We then determined the effect of TRIM41 overexpression on viral infection by examining NP expression of PR8 IAV and A/WSN/1933 (WSN). TRIM41 overexpression impaired the replication of both IAV strains, as indicated by decreased levels of NP protein (Figs. 15-16).

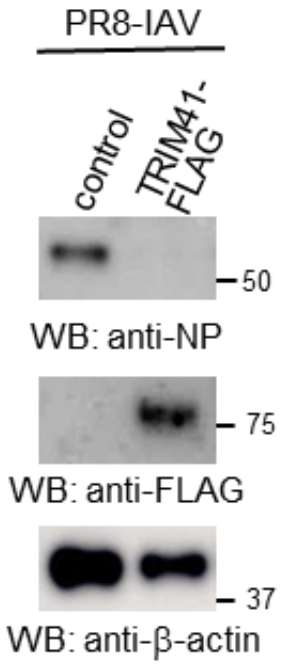

Figure 15: **Effect of TRIM41 overexpression on PR8 IAV nucleoprotein.** HEK293 cells were transfected with 0.5 μg of pCMV-3Tag-8 vector or TRIM41-FLAG for 24 h and then infected at an MOI of 1 with PR8 IAV for 12 h. Cell lysates were blotted using the indicated antibodies.

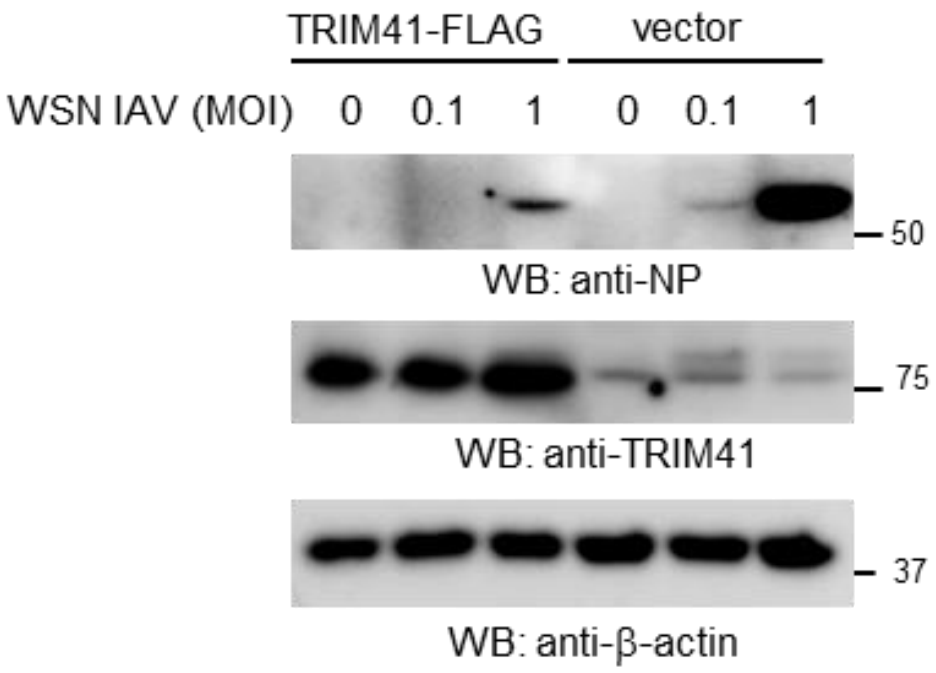

Figure 16: **Effect of TRIM41 overexpression on WSN IAV nucleoprotein.** HEK293 cells were transfected with 0.5 μg of pCMV-3Tag-8 vector or TRIM41-FLAG for 24 h and then infected at the indicated MOI with WSN IAV for 12 h. Cell lysates were blotted using the indicated antibodies.

Immunofluorescence assay (IFA) was also performed to visualize viral restriction. Ectopic expression of TRIM41 restricted NY IAV infection in HEK293 cells, indicated by the number of infected cells (Fig. 17).

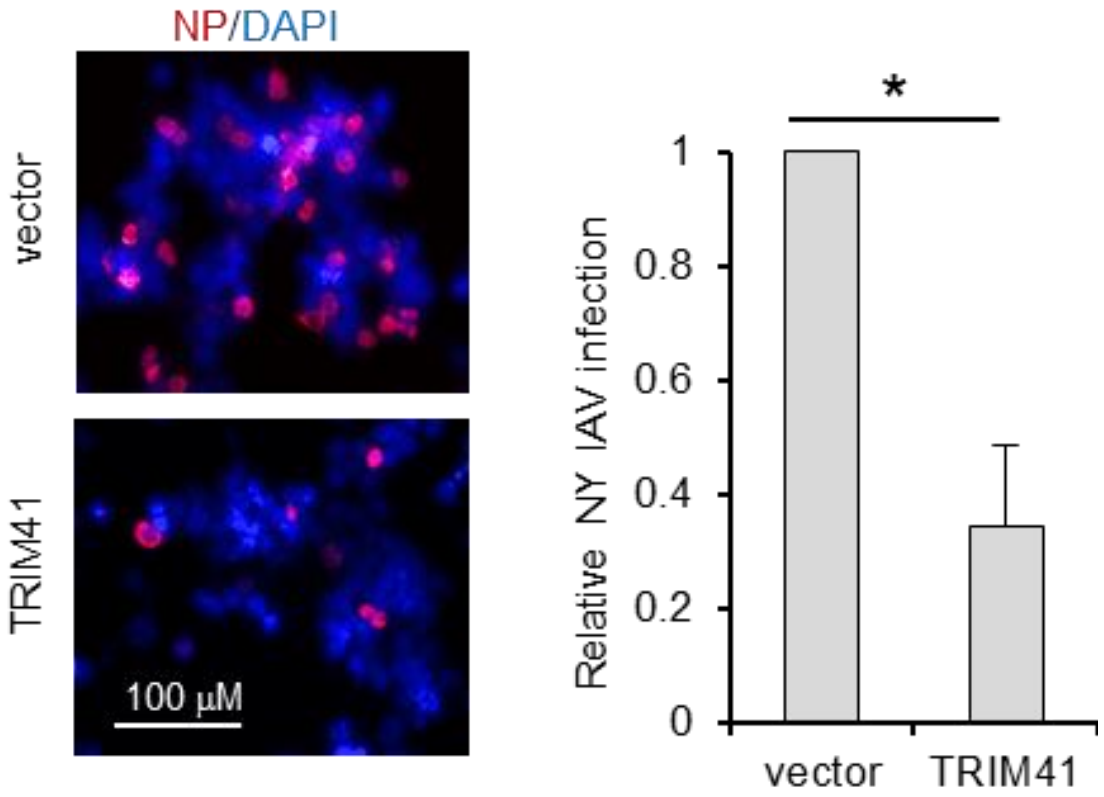

Figure 17: **Effect of TRIM41 overexpression on NY IAV.** HEK293 cells were transfected with 0.5 μg of pCMV-3Tag-8 vector or TRIM41-FLAG for 24 h and then infected at an MOI of 1 with NY IAV for 12 h. Fixed cells were stained with anti-NP antibody. The percentage of stained cells is summarized in the graph. *, $P < 0.05$.

TCID50 assay was used to determine the effect of TRIM41 on the production of infectious IAV particles. Overexpression of TRIM41 consistently reduced viral titers of WSN IAV (Fig. 18). Taken together, these findings indicate that TRIM41 is an IAV restriction factor.

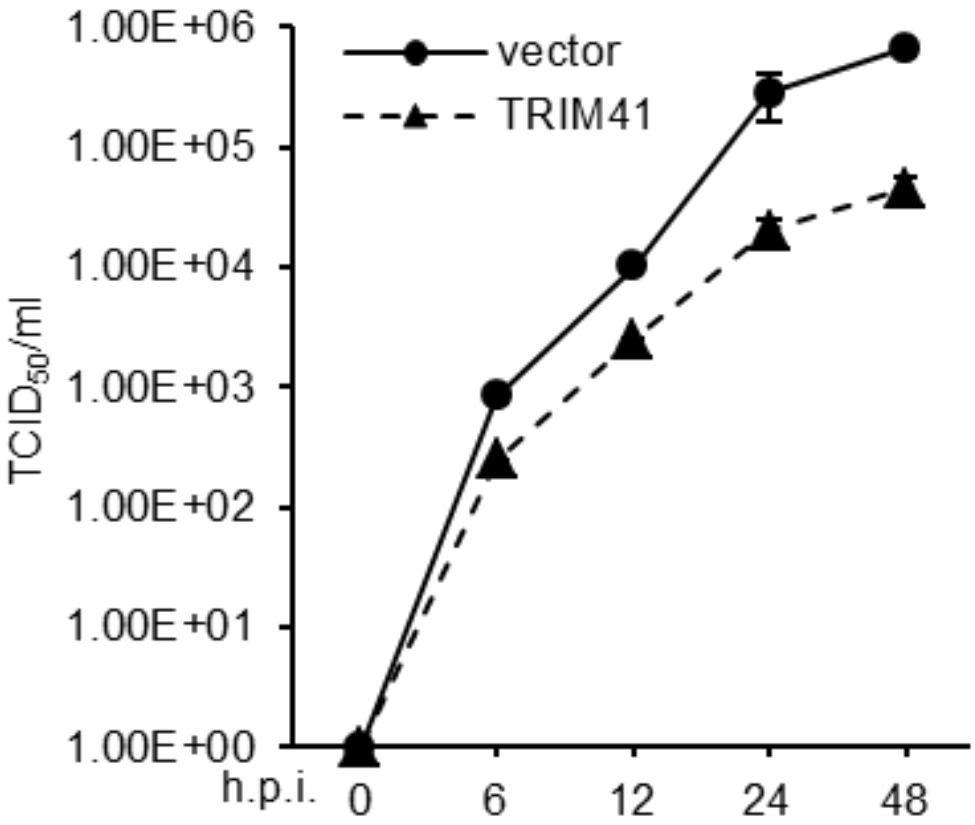

Figure 18: **Effect of TRIM41 overexpression on IAV titers.** HEK293 cells were transfected with 0.5 μg of pCMV-3Tag-8 vector or TRIM41-FLAG. After 24 h, cells were infected at an MOI of 0.001 with WSN/33 IAV. After the designated hour post infection (h.p.i.), virus titers were determined by TCID50 assay in MDCK cells. All experiments were biologically repeated three times.

TRIM41 deficiency increases host susceptibility to IAV.

To complement the above gain-of-function, we first depleted TRIM41 using the small interfering RNA (siRNA).

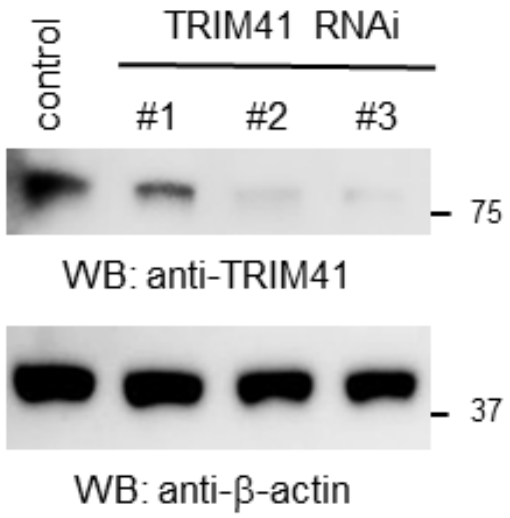

Figure 19: **Knockdown of TRIM41 using RNA interference.** A549 cells were transfected with 5 pmol of a scrambled control siRNA or three different siRNA duplexes against TRIM41. After 48 h, cell lysates were blotted with anti-TRIM41 or anti-β-actin antibody.

Three pairs of siRNA duplex against TRIM41 were individually transfected into A549 cells. After 48 h, cells were infected with PR8-Gluc IAV for 12 h. Reduced TRIM41 protein expression was correlated with increased IAV reporter activity in A549 lung cells (Figs.20).

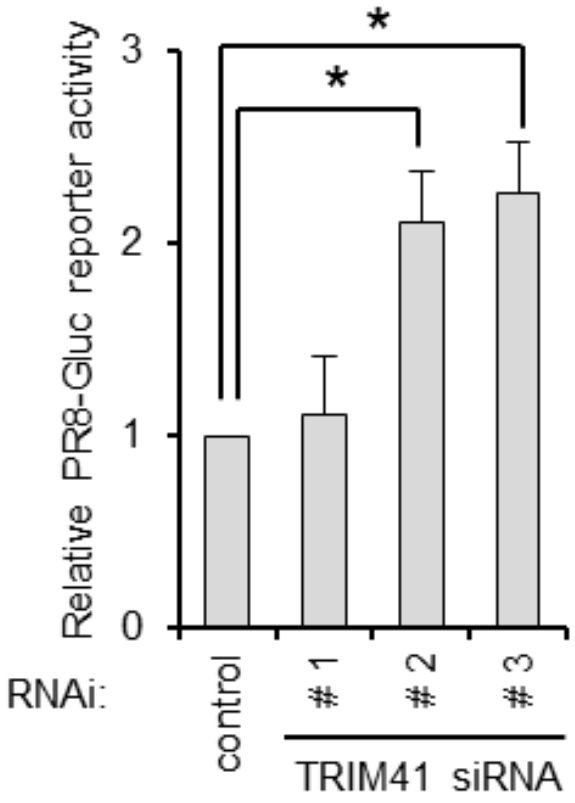

Figure 20: **Effect of TRIM41 knockdown on PR8 IAV infection.** A549 cells were transfected with 5 pmol of the control siRNA or the indicated siRNA duplex against TRIM41. After 48 h, the cells were infected at an MOI of 0.1 with IAV PR8-Gluc for 12 h. Relative luciferase activities were examined. All experiments were biologically repeated three times. Data represent means ± standard deviations of three independent experiments. The P value was calculated (two-tailed Student's t test) by comparison of results with those of the siRNA control in each cell group (*, $P < 0.05$).

Silencing TRIM41 also enhanced IAV propagation in A549 cells, as detected by IFA and western blotting (Figs. 21-22).

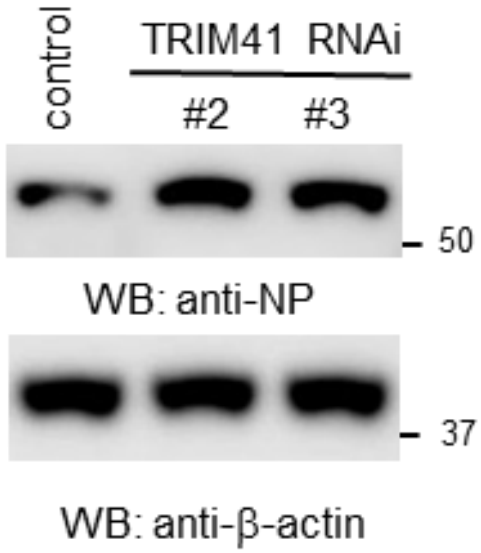

Figure 21: **Effect of TRIM41 knockdown on WSN IAV nucleoprotein.** A549 cells were transfected with 5 pmol of the control siRNA or the TRIM41 siRNA 2 or 3 duplex for 48 h and then infected at an MOI of 1 with WSN IAV for 12 h. Cell lysates were blotted using the indicated antibodies.

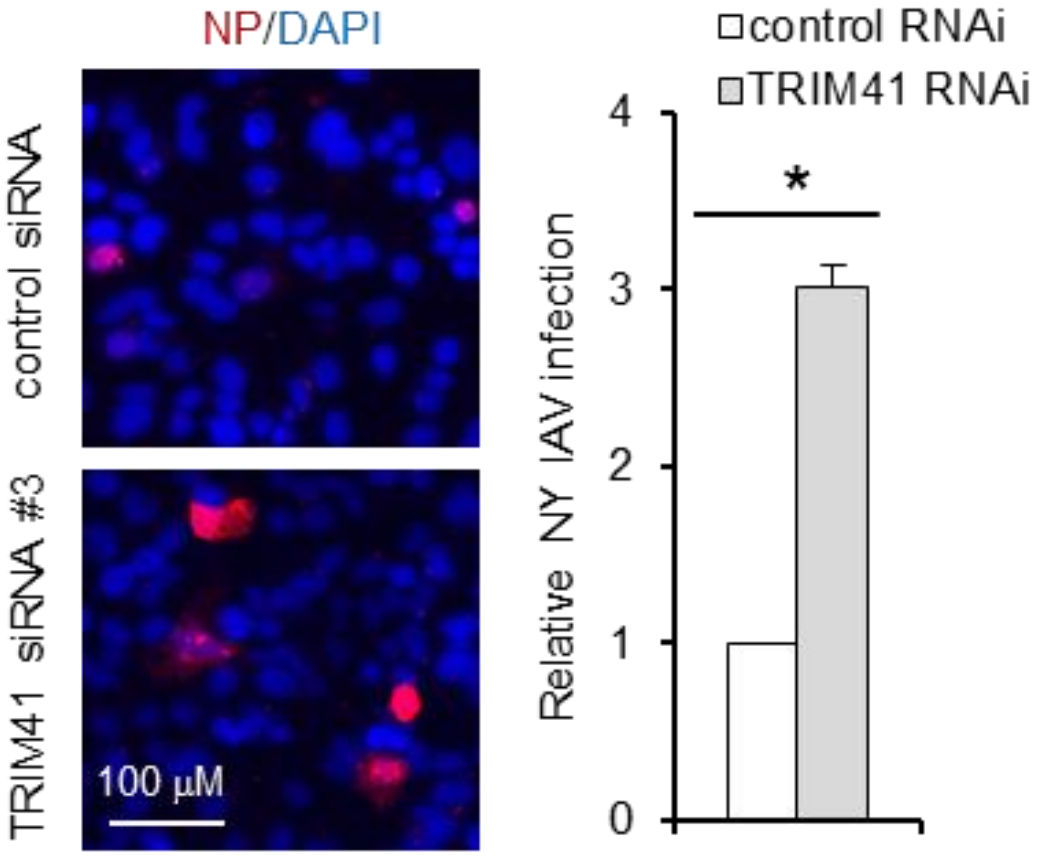

Figure 22: **Effect of TRIM41 knockdown on NY IAV infection.** A549 cells were transfected with 5 pmol of the control siRNA or the TRIM41 siRNA 3 duplex for 48 h and then infected at an MOI of 0.1 with NY IAV for 12 h. Fixed cells were stained with anti-NP antibody. The percentage of stained cells is summarized in the graph. *, $P < 0.05$. DAPI, 4′,6′-diamidino-2-phenylindole.

Furthermore, we knocked down TRIM41 in human primary tracheal cells and then infected these cells with PR8-Gluc IAV. Luciferase assays showed that RNAi depletion of TRIM41 increased IAV infection in primary tracheal cells (Fig. 23).

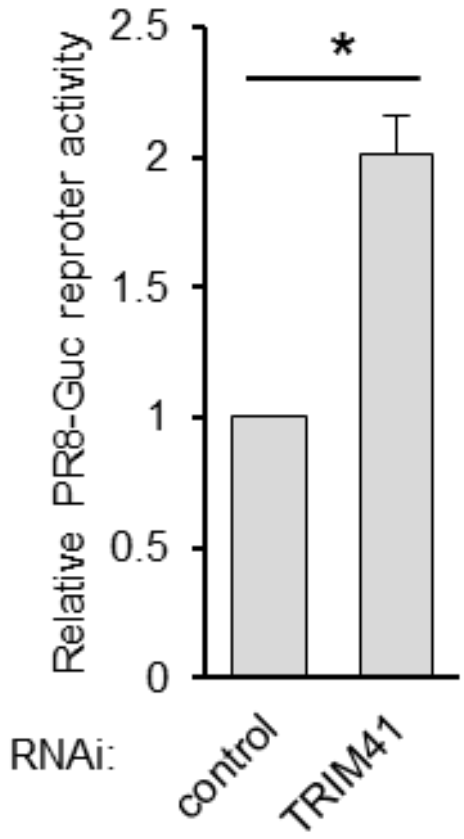

Figure 23: **Effect of TRIM41 knockdown on PR8 IAV infection in primary cells.** Primary human tracheal epithelial cells were transfected with 5 pmol of the control siRNA or the TRIM41 siRNA 3 duplex. After 48 h, the cells were infected at an MOI of 0.1 with PR8-Gluc for 16 h. The relative luciferase activity was examined. Data represent means ± standard deviations of three independent experiments. *, $P < 0.05$.

Knockout of TRIM41 increases host susceptibility to IAV infection

We next examined the effects of TRIM41 knockout on IAV infection. In this regard, a single guide RNA (sgRNA) was cloned into the lentiCRISPR v2 containing Cas9 (24) and transfected into HEK293 cells. After 48 h, cells were selected using puromycin. Single clones were picked and expanded for knockout confirmation by western blotting. TRIM41 expression is abolished in the CRIPSR knockout clone #4 as shown by western blotting (Fig. 24). DNA sequencing found a 1-base pair insertion in the DNA genome of the knockout clone #4.

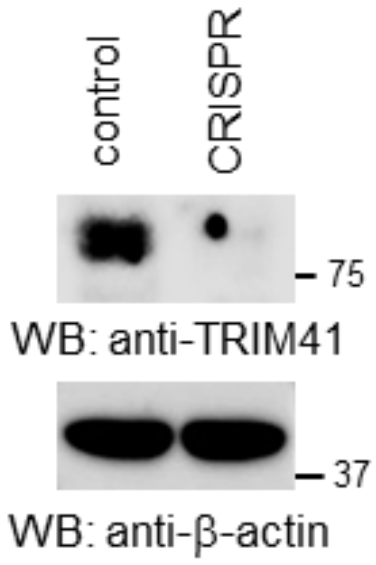

Figure 24: **TRIM41 knockout using CRISPR – CAS9 technique.** CRISPR knockout of TRIM41 in HEK293 cells. Cell lysates were blotted as indicated.

The increase in PR8-GLuc reporter activity was noted in TRIM41 knockout cells compared to wild type controls (Fig. 25).

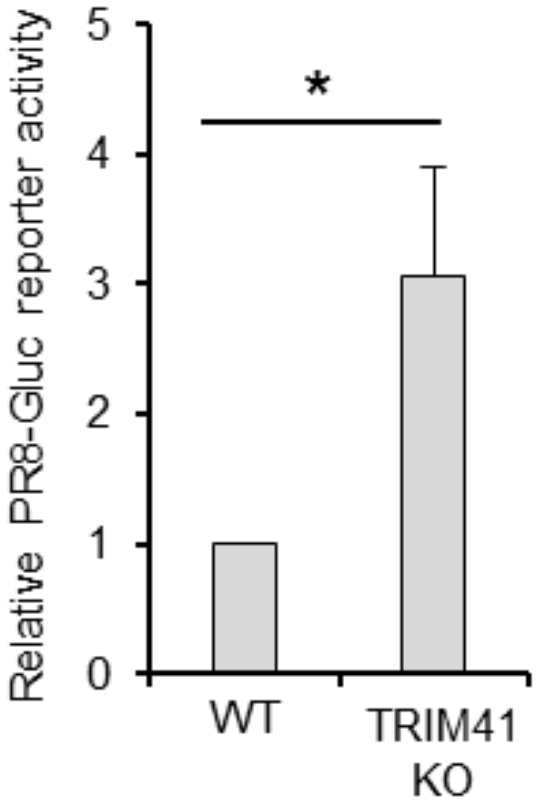

Figure 25: **Effect of TRIM41 knockout on PR8 IAV infection.** Wild-type (WT) HEK293 and TRIM41 knockout (KO) cells were infected at an MOI of 0.1 with IAV PR8-Gluc for 12 h. Relative luciferase activities were examined. All experiments were biologically repeated three times. *, $P < 0.05$.

In line with these results, significantly more TRIM41 knockout cells were stained with anti-NP antibody (Fig. 26).

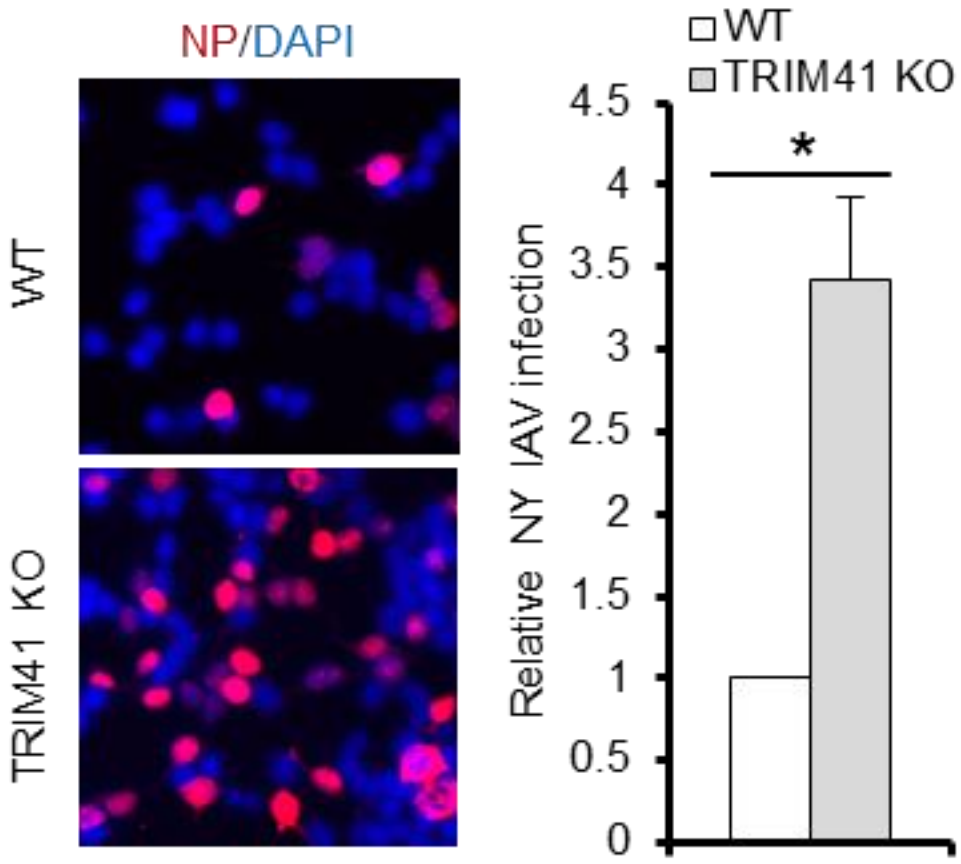

Figure 26: **Effect of TRIM41 knockout on NY IAV infection.** Wild-type and TRIM41 knockout cells were infected at an MOI of 1 with NY IAV for 12 h. Cells were fixed and stained with anti-NP antibody. The percentage of stained cells is summarized in the graph. *, $P < 0.05$.

We also evaluated the impact of TRIM41 on viral propagation using TCID50 assay. As predicted, TRIM41 knockout cells produce more infectious viral particles than control HEK293 cells (Fig. 27).

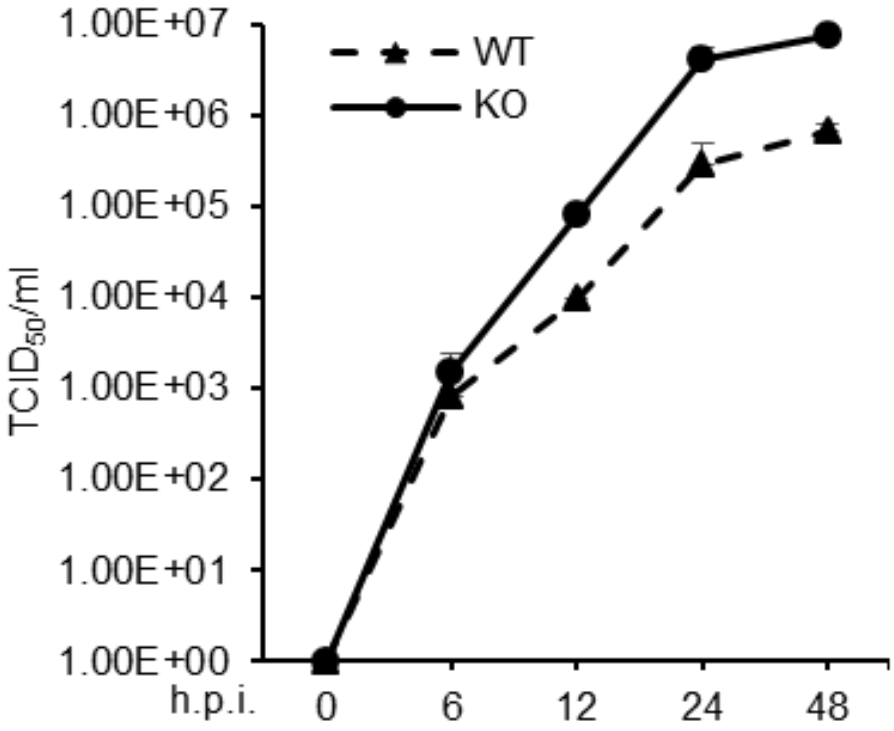

Figure 27: **Effect of TRIM41 knockout on WSN IAV titers.** Wild-type and TRIM41 knockout cells were infected at an MOI of 0.001 with WSN/33 IAV. After the designated hour postinfection, virus titers were determined by TCID50 assay in MDCK cells. All experiments were biologically repeated three times.

Taken together, these data suggest that endogenous TRIM41 is essential for host restriction to IAV.

Ubiquitin E3 ligase activity is required for TRIM41 antiviral function

RING domain is critical for the E3 ligase function of TRIM proteins. Thus, we evaluated the role of the RING domain in TRIM41-mediated viral restriction. TRIM41 or the RING deletion (ΔRING) mutant was transfected into HEK293 cells followed by IAV infection. Ectopic expression of wild type TRIM41 attenuates infection with the IAV reporter virus while the ΔRING mutant failed to defend against IAV infection (Fig. 28).

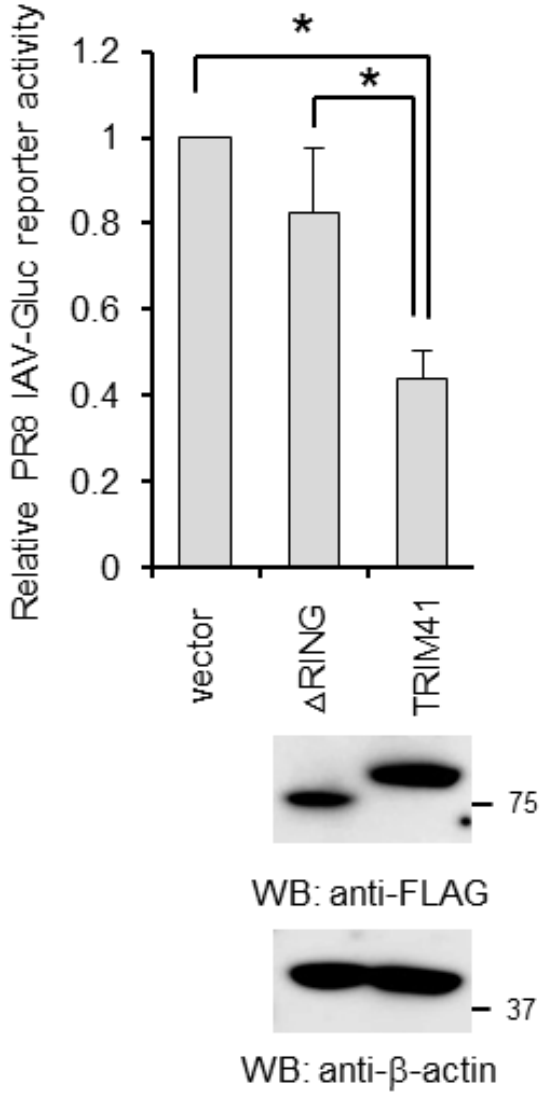

Figure 28: **Effect of deletion of RING domain on anti-IAV action of TRIM41.** HEK293 cells were transfected with 0.5 μg of pCMV-3Tag-8 vector, FLAG-tagged TRIM41, or the ΔRING mutant. After 24 h, cells were infected at an MOI of 0.1 with PR8-Gluc for 12 h. Relative luciferase activities were examined. Data represent means ± standard deviations of three independent experiments. The P value was calculated by two-tailed Student's t test (*, $P < 0.05$).

Furthermore, wild type TRIM41, but not the mutant, restored antiviral activity in the TRIM41 knockout cells (Fig. 29).

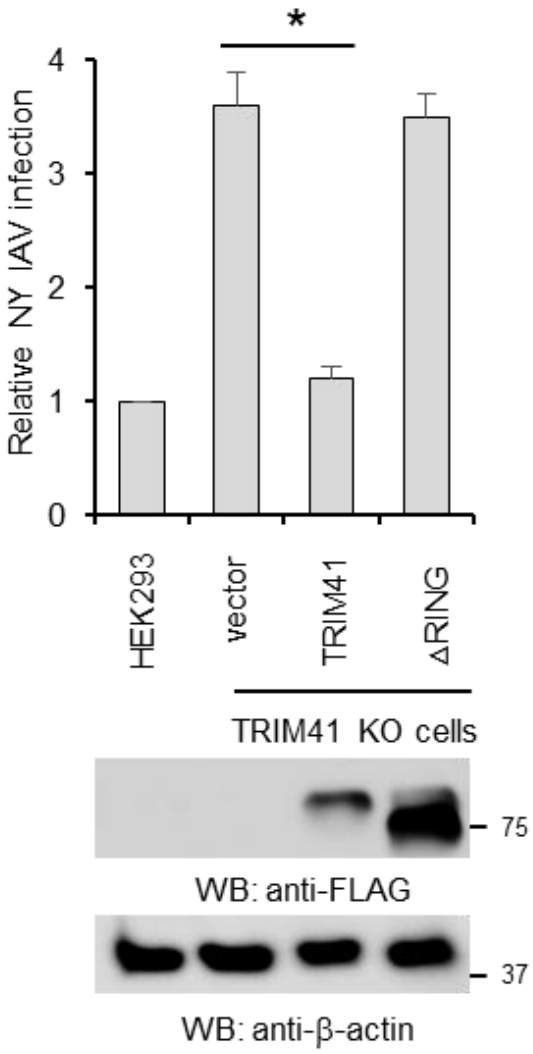

Figure 29: **Effect of RING domain deletion on TRIM41 anti-IAV action in knockout cells.** TRIM41 knockout cells were transfected with 0.5 μg of pCMV-3Tag-8 vector, TRIM41-FLAG, or the ΔRING mutant for 24 h and then infected at an MOI of 1 with NY IAV for 12 h. HEK293 cells were included as the control. IFA was performed, and the percentage of stained cells was used to determine the infection activity. *, $P < 0.05$.

Similarly, the mutant failed to inhibit IAV infection in the TRIM41 knockout cells as determined by TCID50 assay (Fig. 30).

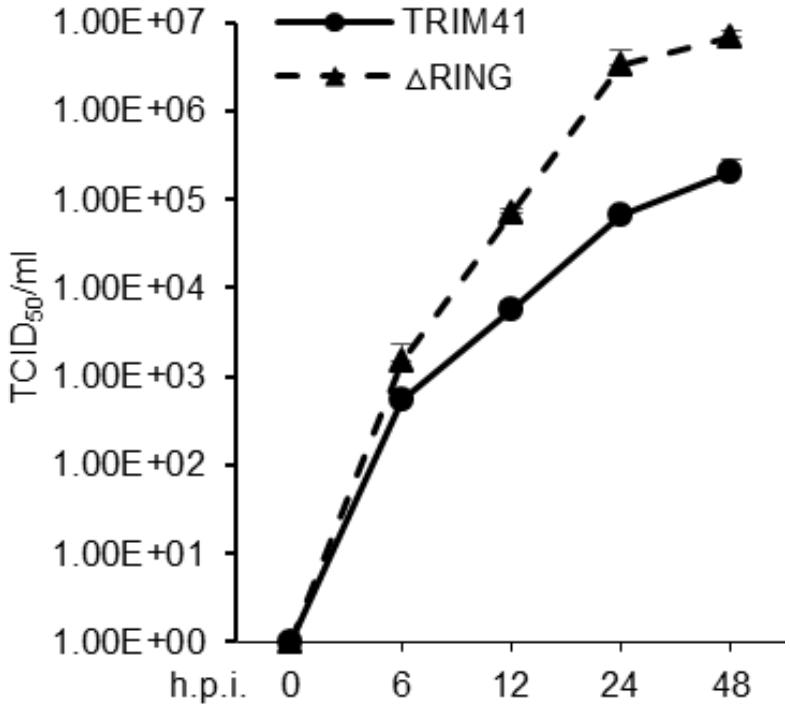

Figure 30: **Effect of TRIM41 RING domain deletion on WSN IAV titers.** TRIM41 knockout cells were transfected with 0.5 μg of pCMV-3Tag-8 vector, TRIM41-FLAG, or the ΔRING mutant. After 24 h, cells were infected at an MOI of 0.001 with WSN/33 IAV. After the designated hour post infection, virus titers were determined by TCID50 assay in MDCK cells. All experiments were biologically repeated three times.

Thus, we concluded that E3 ligase activity is critical for the antiviral activity of TRIM41.

TRIM41 ubiquitinates and degrades NP

The requirement of E3 ligase activity implicates that TRIM41 may ubiquitinate NP. To determine whether TRIM41 can couple ubiquitin onto NP, we first performed an in vitro ubiquitination assay. TRIM41 is able to heavily conjugate ubiquitin onto NP in vitro, but the ΔRING mutant failed to conjugate ubiquitin onto NP (Fig. 31).

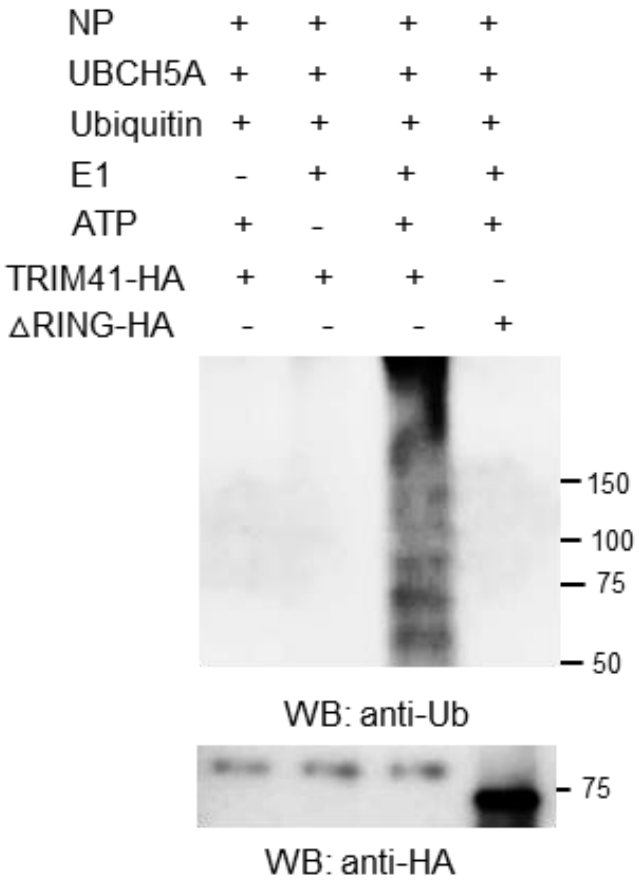

Figure 31: **TRIM41 mediated in vitro ubiquitination of NP.** In vitro ubiquitination of NP by TRIM41. FLAG-tagged NP, HA-tagged TRIM41, and the RING deletion mutant plus E1, E2 (UBCH5A), ATP, and ubiquitin were added as indicated and incubated at 30°C for 2 h. Samples were blotted with the indicated antibodies. Ub, ubiquitin.

We next examined NP ubiquitination in cells. NP was transfected with vector, TRIM41 or the ΔRING mutant into HEK293 cells. As shown in Figure 7B, TRIM41 increased the levels of NP ubiquitination. However, ectopic expression of the ΔRING mutant showed little or no effect on NP ubiquitination (Fig. 32).

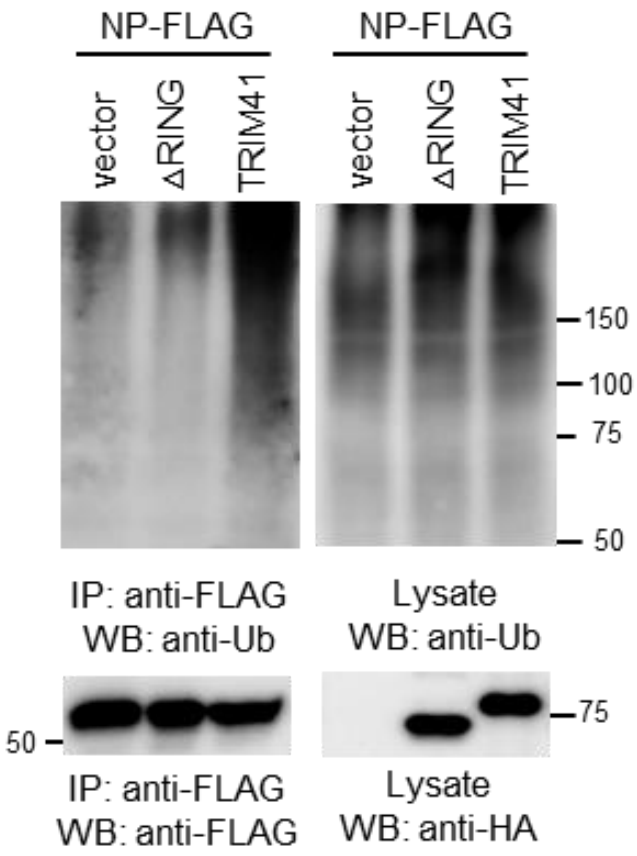

Figure 32: **TRIM41 mediated polyubiquitination of NP.** NP-FLAG (0.5 μg) was cotransfected with 2 μg of vector, TRIM41-HA, or the HA-tagged ΔRING mutant into HEK293 cells. Cells were treated with 1 μM MG132 for 12 h to prevent NP protein degradation. Cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with the indicated antibodies.

We also examined whether overexpression of TRIM41 increases NP ubiquitination during IAV infection. HEK239 cells were transfected with vector or TRIM41, and then infected with 1 MOI of PR8 IAV for 12 h. As shown in Figure. 33, TRIM41 increased the levels of NP ubiquitination during IAV infection.

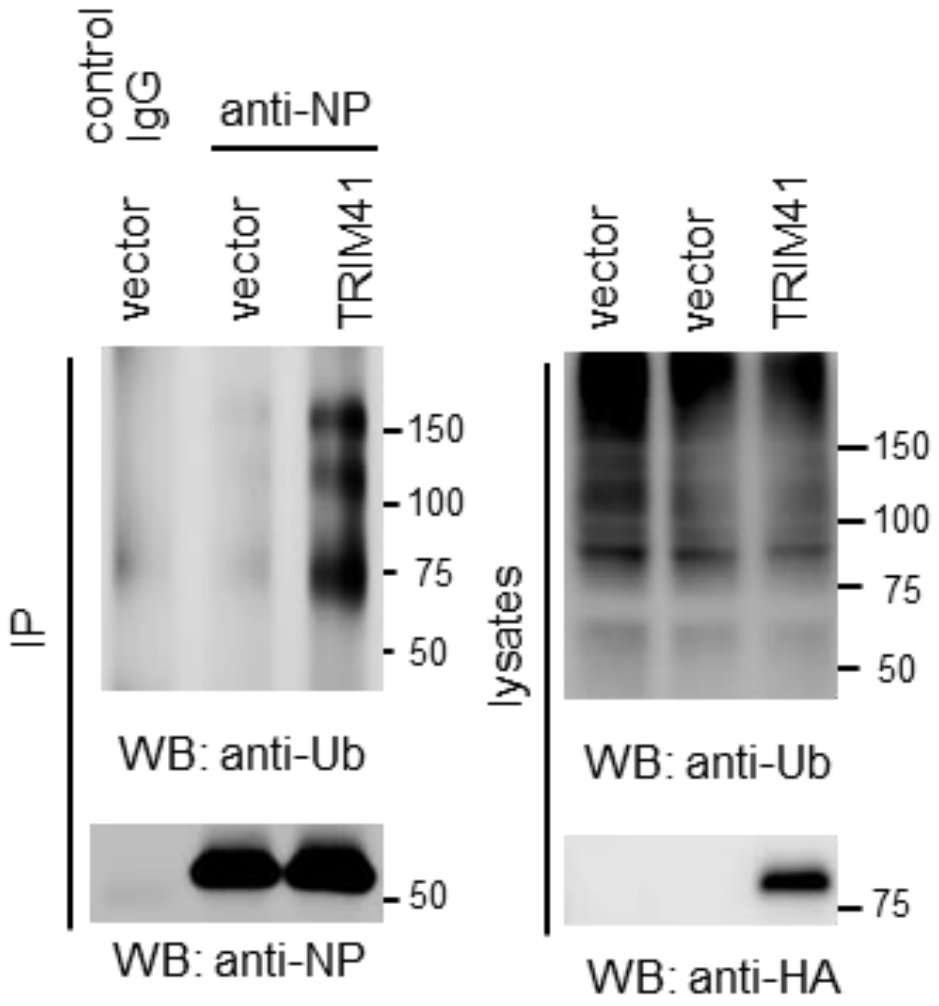

Figure 33: **TRIM41 mediated polyubiquitination of NP in cells.** HEK293 cells were transfected with 2.5 μg of vector or TRIM41. After 24 h, cells were infected at an MOI of 1 with PR8 IAV and treated with 1 μM MG132 for 12 h. Cell lysates were immunoprecipitated with anti-NP antibody and blotted with the indicated antibodies.

Consistently, ubiquitinated NP was barely detected in TRIM41 knockout cells (Fig. 34).

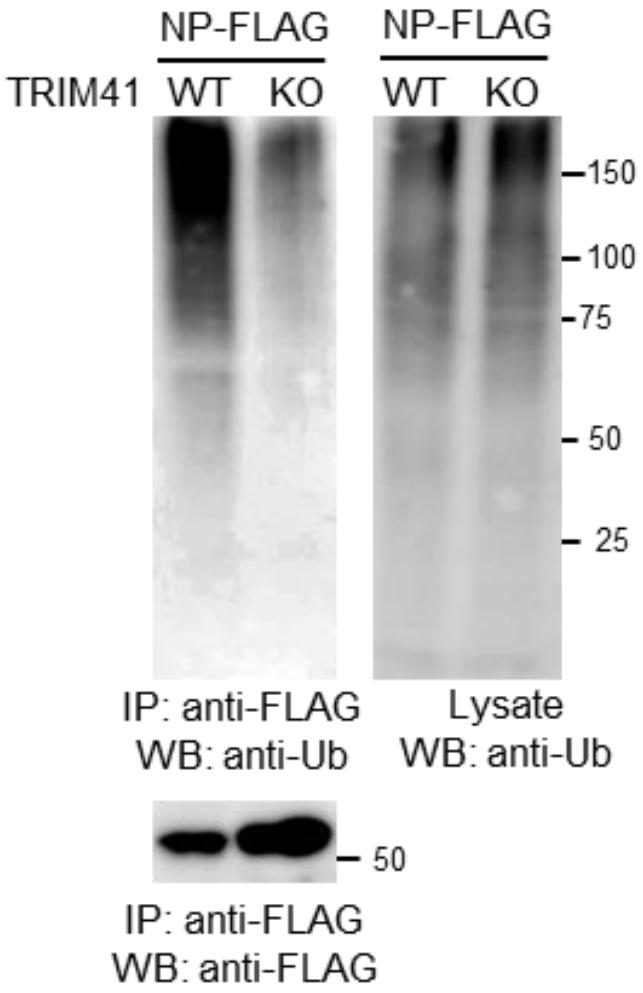

Figure 34: Effect of TRIM41 knockout on NP ubiquitination. Wild-type and TRIM41 knockout cells were transfected with 0.5 μg of NP-FLAG. Cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with the indicated antibodies.

Proteins modified with polyubiquitin are classic targets for proteasomal degradation. To determine the role of TRIM41 in NP protein turnover, we transfected HEK293 cells with NP together with wild type TRIM41 or the ΔRING mutant. Wild type TRIM41, but not the mutant, reduced NP protein expression (Fig. 35), indicating that the presence of TRIM41 has a destabilizing effect on NP expression.

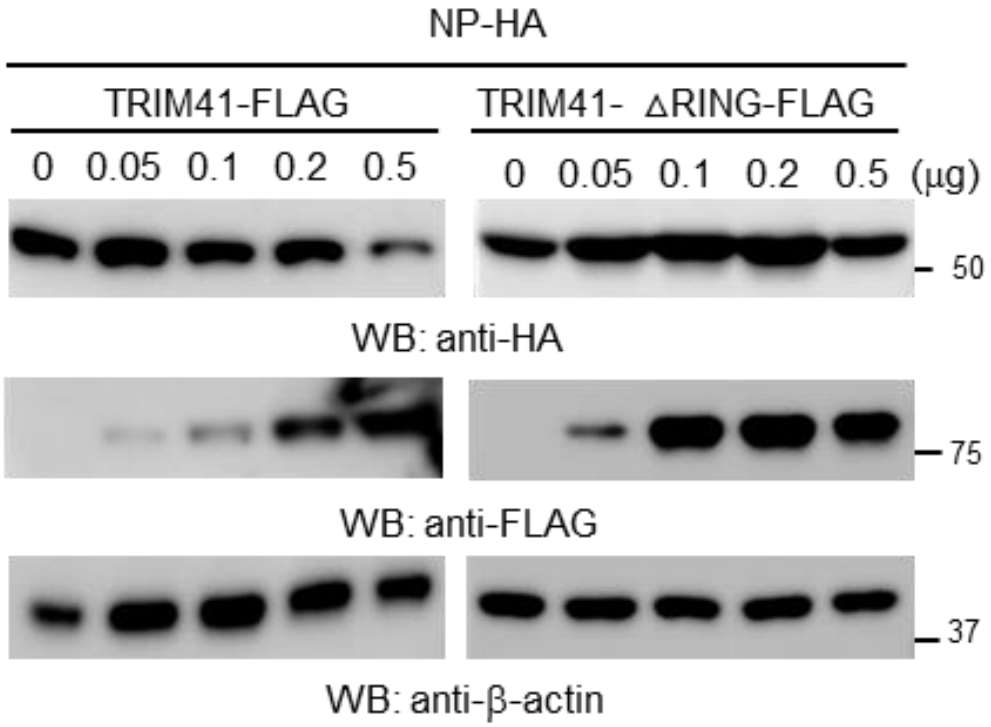

Figure 35: **Effect of TRIM41 RING domain deletion on NP degradation.** NP-HA (0.25 μg) was cotransfected with the designated amounts of FLAG-tagged TRIM41 or the ΔRING mutant into HEK293 cells. Cell lysates were blotted as indicated.

To corroborate the role of TRIM41 in NP degradation, HEK293 cells were transfected with NP along with TRIM41 or vector. After 24 h, cells were treated with the protease inhibitor MG132 for 12 h. Western blotting found that MG132 prevented TRIM41-mediated NP degradation (Fig. 36).

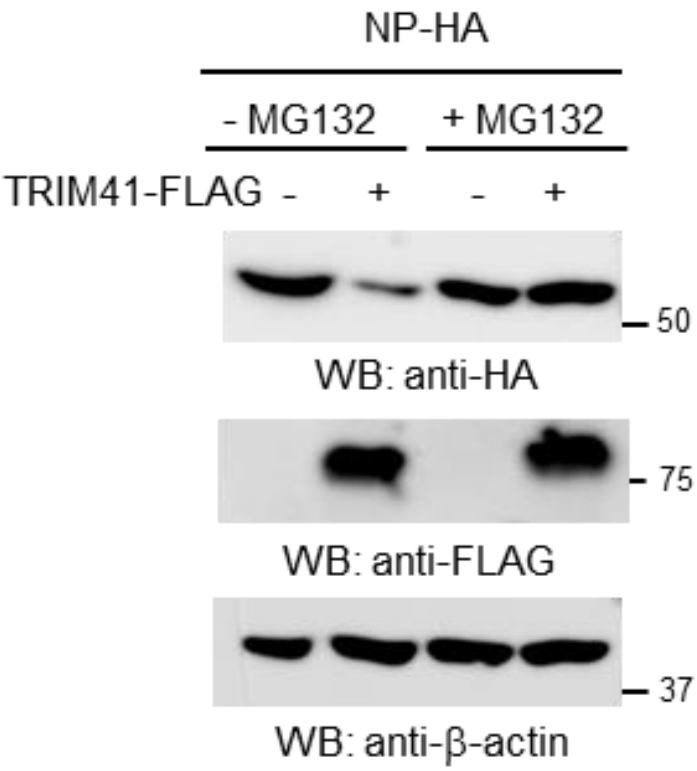

Figure 36: **TRIM41 mediated degradation of NP.** NP-HA (0.25 μg) was cotransfected with 0.25 μg of vector or TRIM41-FLAG into HEK293 cells. After 24 h, cells were treated with dimethyl sulfoxide or 1 μM MG132 for 12 h. Cell lysates were then blotted with the indicated antibodies.

Taken together, the combined data indicate that TRIM41 mediates ubiquitination of NP, which leads to NP degradation and viral inhibition.

DISCUSSION

The innate immune system uses the pattern recognition receptors (PRRs) in different cellular compartments to discriminate microbial components that mark invading viruses. Many of these PRRs have been characterized, including the Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) [25,26]. TLRs and RLRs sense viral nucleic acids and indirectly inhibit viral infection by eliciting signaling cascades that activate the expression of type I IFN. The type I IFN acts as a master cytokine that activates hundreds of interferon-stimulated genes (ISGs), which in turn inhibits viral infection. For example, the interferon-induced transmembrane proteins (IFITMs) recruits and coordinates with the zinc metalloprotease ste24-like protein (ZMPSTE24) to block influenza virus entry on endosomes [27,28]. By contrast, intrinsic immunity impairs viral infection by direct interaction between host factors and virus [3]. Several TRIM proteins have been reported as intrinsic restriction factors, such as TRIM5α [5], TRIM32 [10] and TRIM22 [12]. Similarly, our study demonstrates that TRIM41 directly restricts viral infection through ubiquitination of NP. Thus, TRIM41 is a new intrinsic immune factor that inhibits IAV infection.

TRIM proteins are ubiquitin E3 ligases that share a conserved N-terminal tripartite motif (comprising RING, B-box and coiled-coil) and a versatile C-terminal domain [29-32]. TRIM41 belongs to the subgroup of TRIM family that possesses a SPRY domain on the C-terminus. Our data showed that the SPRY of TRIM41 is responsible for the interaction with NP. More importantly, the RING domain of TRIM proteins confers the ubiquitin E3 ligase activity. Our study found the requirement of E3 ligase activity for TRIM41 antiviral activity. Interestingly, a recent study reported that the E3 ligase activity of TRIM41 is also required for HBV inhibition [20],

but the antiviral mechanism is unknown. However, our study further revealed that TRIM41 mediates the ubiquitination of NP, which leads to NP protein degradation and viral inhibition.

NP has an essential role in the IAV life cycle, including nuclear transport of vRNP, transcription and replication of the viral genome [3]. Several host factors have been shown to inhibit IAV infection by interacting with NP. For example, the myxovirus resistance protein 1 (Mx1) interacts with NP and suppresses the influenza virus transcription [33]. RuvB-like protein 2 (RUVBL2) disrupts vRNP assembly by interfering with NP oligomerization [34]. Interferon stimulated gene 20 (ISG20) interacts with NP and inhibits viral protein expression [35]. Similarly, our study showed that TRIM41 modulates IAV infection by interaction with NP, which grants NP ubiquitination and subsequent protein degradation.

TRIM22 was also reported to ubiquitinate NP and target it for protein degradation [12]. Unlike TRIM41, TRIM22 is an IFN-stimulated gene and the previous showed that the protein expression of TRIM22 is very low or undetectable in cells without IFN stimulation. Thus, TRIM22 might not be present in the cells during the early stage of viral infection before host IFN response is elicited. By contrast, TRIM41 is constitutively expressed and its expression is not modulated by IFN and IAV. Therefore, it is possible that TRIM41 and TRIM22 cooperate to limit NP expression at different stages of IAV life cycle. Future experiments will investigate the distinct roles of TRIM41 and TRIM22 in IAV restriction. Furthermore, CNOT4 is also reported to ubiquitinate NP [36]. However, CNOT4-mediated ubiquitination promotes viral RNA replication by monoubiquitination, but not polyubiquitination of NP [36].

CONCLUSION

We demonstrate that TRIM41 inhibits IAV by targeting NP for proteasomal degradation and provides intrinsic cellular restriction against IAV infection. Up-regulation of TRIM41 E3 ligase activity may boost host immunity to limit viral infection. Thus, future investigation of the regulation of TRIM41 activity might provide the insights and opportunities needed for therapeutic development.

REFERENCES

1. Tscherne DM, Garcia-Sastre A. 2011. Virulence determinants of pandemic influenza viruses. J Clin Invest 121:6-13.
2. Fukuyama S, Kawaoka Y. 2011. The pathogenesis of influenza virus infections: the contributions of virus and host factors. Curr Opin Immunol 23:481-486.
3. Zhao M, Wang L, Li S. 2017. Influenza A Virus-Host Protein Interactions Control Viral Pathogenesis. Int J Mol Sci 18.
4. Wang L, Fu B, Li W, Patil G, Liu L, Dorf ME, Li S. 2017. Comparative influenza protein interactomes identify the role of plakophilin 2 in virus restriction. Nat Commun 8:13876.
5. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427:848-853.
6. Taylor RT, Lubick KJ, Robertson SJ, Broughton JP, Bloom ME, Bresnahan WA, Best SM. 2011. TRIM79alpha, an interferon-stimulated gene product, restricts tick-borne encephalitis virus replication by degrading the viral RNA polymerase. Cell Host Microbe 10:185-196.
7. Wolf D, Goff SP. 2007. TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell 131:46-57.
8. Rauwel B, Jang SM, Cassano M, Kapopoulou A, Barde I, Trono D. 2015. Release of human cytomegalovirus from latency by a KAP1/TRIM28 phosphorylation switch. Elife 4.
9. Fan W, Wu M, Qian S, Zhou Y, Chen H, Li X, Qian P. 2016. TRIM52 inhibits Japanese Encephalitis Virus replication by degrading the viral NS2A. Sci Rep 6:33698.
10. Fu B, Wang L, Ding H, Schwamborn JC, Li S, Dorf ME. 2015. TRIM32 Senses and Restricts Influenza A Virus by Ubiquitination of PB1 Polymerase. PLoS Pathog 11:e1004960.

11. Liu B, Li NL, Shen Y, Bao X, Fabrizio T, Elbahesh H, Webby RJ, Li K. 2016. The C-Terminal Tail of TRIM56 Dictates Antiviral Restriction of Influenza A and B Viruses by Impeding Viral RNA Synthesis. J Virol 90:4369-4382.
12. Di Pietro A, Kajaste-Rudnitski A, Oteiza A, Nicora L, Towers GJ, Mechti N, Vicenzi E. 2013. TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. J Virol 87:4523-4533.
13. Chelbi-Alix MK, Quignon F, Pelicano L, Koken MH, de The H. 1998. Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. J Virol 72:1043-1051.
14. Van Tol S, Hage A, Giraldo MI, Bharaj P, Rajsbaum R. 2017. The TRIMendous Role of TRIMs in Virus-Host Interactions. Vaccines (Basel) 5.
15. Gack MU, Shin YC, Joo CH, Urano T, Liang C, Sun L, Takeuchi O, Akira S, Chen Z, Inoue S, Jung JU. 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature 446:916-920.
16. Rajsbaum R, Albrecht RA, Wang MK, Maharaj NP, Versteeg GA, Nistal-Villan E, Garcia-Sastre A, Gack MU. 2012. Species-specific inhibition of RIG-I ubiquitination and IFN induction by the influenza A virus NS1 protein. PLoS Pathog 8:e1003059.
17. Sanchez-Aparicio MT, Ayllon J, Leo-Macias A, Wolff T, Garcia-Sastre A. 2017. Subcellular Localizations of RIG-I, TRIM25, and MAVS Complexes. J Virol 91.
18. Chen D, Gould C, Garza R, Gao T, Hampton RY, Newton AC. 2007. Amplitude control of protein kinase C by RINCK, a novel E3 ubiquitin ligase. J Biol Chem 282:33776-33787.
19. Thiebaut R, Esmiol S, Lecine P, Mahfouz B, Hermant A, Nicoletti C, Parnis S, Perroy J, Borg JP, Pascoe L, Hugot JP, Ollendorff V. 2016. Characterization and Genetic Analyses of New Genes Coding for NOD2 Interacting Proteins. PLoS One 11:e0165420.
20. Zhang S, Guo JT, Wu JZ, Yang G. 2013. Identification and characterization of multiple TRIM proteins that inhibit hepatitis B virus transcription. PLoS One 8:e70001.

21. Tanaka M, Fukuda Y, Mashima K, Hanai R. 2005. Intracellular localization and domain organization of human TRIM41 proteins. Mol Biol Rep 32:87-93.
22. Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 252:324-330.
23. Heaton NS, Leyva-Grado VH, Tan GS, Eggink D, Hai R, Palese P. 2013. In Vivo Bioluminescent Imaging of Influenza A Virus Infection and Characterization of Novel Cross-Protective Monoclonal Antibodies. Journal of Virology 87:8272-8281.
24. Sanjana NE, Shalem O, Zhang F. 2014. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods 11:783-784.
25. Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. Cell 140:805-820.
26. Wilkins C, Gale M, Jr. 2010. Recognition of viruses by cytoplasmic sensors. Curr Opin Immunol 22:41-47.
27. Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. Cell 139:1243-1254.
28. Fu B, Wang L, Li S, Dorf ME. 2017. ZMPSTE24 defends against influenza and other pathogenic viruses. J Exp Med 214:919-929.
29. Hatakeyama S. 2017. TRIM Family Proteins: Roles in Autophagy, Immunity, and Carcinogenesis. Trends Biochem Sci 42:297-311.
30. Versteeg GA, Rajsbaum R, Sanchez-Aparicio MT, Maestre AM, Valdiviezo J, Shi M, Inn KS, Fernandez-Sesma A, Jung J, Garcia-Sastre A. 2013. The E3-ligase TRIM family of proteins regulates signaling pathways triggered by innate immune pattern-recognition receptors. Immunity 38:384-398.

31. Ozato K, Shin DM, Chang TH, Morse HC, 3rd. 2008. TRIM family proteins and their emerging roles in innate immunity. Nat Rev Immunol 8:849-860.
32. Kawai T, Akira S. 2011. Regulation of innate immune signalling pathways by the tripartite motif (TRIM) family proteins. EMBO Mol Med 3:513-527.
33. Turan K, Mibayashi M, Sugiyama K, Saito S, Numajiri A, Nagata K. 2004. Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome. Nucleic Acids Research 32:643-652.
34. Kakugawa S, Shimojima M, Neumann G, Goto H, Kawaoka Y. 2009. RuvB-like protein 2 is a suppressor of influenza A virus polymerases. J Virol 83:6429-6434.
35. Qu H, Li J, Yang L, Sun L, Liu W, He H. 2016. Influenza A Virus-induced expression of ISG20 inhibits viral replication by interacting with nucleoprotein. Virus Genes 52:759-767.
36. Lin YC, Jeng KS, Lai MMC. 2017. CNOT4-Mediated Ubiquitination of Influenza A Virus Nucleoprotein Promotes Viral RNA Replication. MBio 8.
37. Szretter KJ, Balish AL, Katz JM. 2006. Influenza: propagation, quantification, and storage. Curr Protoc Microbiol Chapter 15:Unit 15G 11.
38. Chen X, Bonne S, Hatzfeld M, van Roy F, Green KJ. 2002. Protein binding and functional characterization of plakophilin 2. Evidence for its diverse roles in desmosomes and beta -catenin signaling. J Biol Chem 277:10512-10522.

CHAPTER III

TRIM41-Mediated Ubiquitination of Nucleoprotein Limits Vesicular Stomatitis Virus Infection

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Earlier version of this chapter has already been published as Girish Patil, Lingling Xu, Yakun Wu, Kun Song, Wenzhuo Hao, Fang Hua, Lingyan Wang, Shitao Li. TRIM41-Mediated Ubiquitination of Nucleoprotein Limits Vesicular Stomatitis Virus Infection. Viruses.2020; 12(2):131.

ABSTRACT

Vesicular stomatitis virus (VSV) is a zoonotic, negative-strand RNA virus of the family Rhabdoviridae. The nucleoprotein (N) of VSV protects the viral genomic RNA and plays an essential role in viral transcription and replication, which makes the nucleoprotein an ideal target of host defense. However, whether and how host innate/intrinsic immunity limits VSV infection by targeting the N protein are unknown. In this study, we found that the N protein of VSV (VSV-N) interacted with a ubiquitin E3 ligase, tripartite motif protein 41 (TRIM41). Overexpression of TRIM41 inhibited VSV infection. Conversely, depletion of TRIM41 increased host susceptibility to VSV. Furthermore, the E3 ligase defect mutant of TRIM41 failed to limit VSV infection, suggesting the requirement of the E3 ligase activity of TRIM41 in viral restriction. Indeed, TRIM41 ubiquitinated VSV-N in cells and in vitro. TRIM41-medaited ubiquitination leads to the degradation of VSV-N through proteasome, thereby limiting VSV infection. Taken together, our study identifies TRIM41 as a new intrinsic immune factor against VSV by targeting the viral nucleoprotein for ubiquitination and subsequent protein degradation.

INTRODUCTION

Vesicular stomatitis virus (VSV) belongs to the *Rhabdoviridae* that encapsidates a single-stranded RNA genome encoding five major proteins [1]. VSV is a zoonotic virus with epidemic potential in horses, cattle, and swine, which causes significant economic burden to the livestock industry [2]. To infect host cells, VSV binds to the cell receptors through its glycoprotein (G), which triggers endocytosis followed by uncoating of the helical nucleocapsid (NC) in the cytoplasm. The nucleoprotein (N) encapsidates the viral RNA to form the NC, which is the template for full-length NC replication and for transcription of subgenomic mRNAs [3,4]. The NC is tightly shielded by N protein and only opens transiently for RNA synthesis by the L polymerase and phosphoprotein (P) [5,6]. Since the N protein is critical for VSV capsid and viral replication, it

is an ideal target for host immune system. However, whether and how the N protein is targeted by host innate or intrinsic immunity is unknown. Filling this knowledge gap will not only deepen our understanding of host defense to VSV but also pave an avenue for the development of therapeutics for VSV infectious disease.

The tripartite motif (TRIM) proteins are a group of ubiquitin E3 ligases which play critical roles in innate immune signaling as well as intrinsic immunity. The ubiquitin E3 ligase activity is conferred by the conserved N-terminal RBCC motif comprising three domains, RING, B box, and coiled-coil. TRIM proteins have variable C-terminal domains that determine the interaction between the target proteins and TRIM proteins. It is well known that TRIM proteins mediate host innate immune activation and promote the induction of antiviral cytokines and chemokines, such as type I interferon (IFN). For instance, TRIM25 ubiquitinates the cytosolic RNA sensor, RIG-I, which is critical for subsequent RIG-I oligomerization and activation [7,8]. TRIM5 interacts with HIV capsid and activates NF-κB signaling [9]. Recent studies found that TRIM proteins are also intrinsic immune factors, which limit virus infection by directly targeting viral proteins [10]. For example, TRIM32 ubiquitinates the polymerase basic 1 (PB1) of influenza A virus, which leads to PB1 proteasomal degradation, thereby inhibiting viral infection [11].

Several TRIM proteins, including TRIM41, participate in both innate immunity and intrinsic immunity. First, TRIM41 regulates NOD2 and cGAS innate immune signaling pathways. TRIM41 interacts with NOD2, but the underlying mechanism by which TRIM41 regulates NOD2 signaling is not clear [12]. TRIM41 also mediates monoubiquitination of the cytosolic DNA sensor cGAS, thereby promoting type I IFN production [13]. Secondly, TRIM41 is an intrinsic immunity factor that inhibits viral infection. Our recent study discovered that TRIM41 restricts influenza by polyubiquitination and degradation of viral nucleoprotein [14]. Recently, a screening of TRIM

proteins found that TRIM41 and other seven TRIM proteins inhibited hepatitis B virus (HBV) transcription [15]. However, whether TRIM41 inhibits other viruses, such as VSV, is unknown.

In this study, we demonstrate that TRIM41 inhibited VSV infection by both gain- and loss-of-function studies. Co-Immunoprecipitation found that TRIM41 interacted with the N protein of VSV (VSV-N). Furthermore, TRIM41 ubiquitinated VSV-N in cells and *in vitro*, which led to proteasomal degradation of VSV-N, thus limiting VSV replication. Overall, our study establishes TRIM41 as a new intrinsic immune host factor against VSV.

MATERIALS AND METHODS

Cells and viruses. HEK293 cells (ATCC, # CRL-1573) and Vero cells (ATCC, # CCL-81) were maintained in Dulbecco's Modified Eagle Medium (Life Technologies) containing antibiotics (Life Technologies) and 10% fetal bovine serum (Life Technologies). A549 cells (ATCC, # CCL-185) were cultured in RPMI Medium 1640 (Life Technologies) plus 10% fetal bovine serum and 1X MEM Non-Essential Amino Acids Solution (Life Technologies).

VSV Indiana strain was purchased from ATCC (VR-1238). The VSV carrying a luciferase gene (VSV-Luc) and the VSV expressing a GFP gene (VSV-GFP) were a kind gift from Dr. Sean Whelan (Harvard Medical School, MA). Viral titration was performed as described in a previous study [16]. Briefly, Vero cells were infected with a serial diluted VSV. After 45 min, media was removed and replaced by the DMEM plus 10% FBS and 1% agarose. After 48 h, cells were fixed using the methanol–acetic acid (3:1) fixative and stained using a Coomassie blue solution.

Constructs and Reagents. TRIM41-HA was a generous gift from Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine, NY) [17]. TRIM41 was cloned into pCMV-3Tag-8 (Stratagene)

to generate TRIM41-FLAG. Deletion mutants of TRIM41-FLAG were constructed using a Q5® Site-Directed Mutagenesis Kit (New England Biolabs). VSV-N was cloned into pCMV-3Tag-8 (Stratagene) and pCMV-3Tag-8-HA to generate FLAG-tagged and HA-tagged VSV-N, respectively.

Anti-β-actin (Abcam, # ab8227), anti-FLAG (Sigma, # F3165), anti-ubiquitin (Santa Cruz Biotechnology, # sc-8017), anti-TRIM41 (Aviva Systems Biology, # ARP34763_P050), anti-HA epitope (Cell Signaling Technology, # 3724), anti-VSV (Imanis Life Sciences, # REA005), anti-VSV-N (KeraFAST, # EB0009). Goat anti-Mouse IgG-HRP (Santa Cruz Biotechnology, # sc-2055), Goat anti-Rabbit IgG-HRP (Santa Cruz Biotechnology, # sc-2030), Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) (Life Technologies, # A11005), Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) (Life Technologies, # A11034).

Sample preparation, Western blotting and immunoprecipitation. Approximately 1 X 10^6 cells were lysed in 500 µl of tandem affinity purification (TAP) lysis buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.5% Nonidet P40, 10% glycerol, Complete EDTA-free protease inhibitor cocktail tablets (Roche)] for 30 min at 4^oC. The lysates were then centrifuged for 30 min at 15,000 x *g*. Supernatants were collected and mixed with 1X Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific). Western blotting and immunoprecipitation were performed as described in a previous study [18].

Immunofluorescence assay. Cells were cultured in the Lab-Tek II CC2 Chamber Slide System 4-well (Thermo Fisher Scientific). After the indicated treatment, the cells were fixed and permeabilized in cold methanol for 10 min at -20 ^0C. Then, the slides were washed with 1 X DPBS for 10 min and blocked with Odyssey Blocking Buffer (LI-COR Biosciences) for 1 h. The slides were incubated in Odyssey Blocking Buffer with 1:100 diluted primary antibodies at 4 ^0C

for 12 h. Images were captured and analyzed using an iRiS™ Digital Cell Imaging System (Logos Biosystems).

Plasmid transfection. Transfections using Lipofectamine 2000 or Lipofectamine 3000 Transfection Reagent (Life Technologies) were performed according to the manufacturer's protocol. For co-IP experiments, a total of 2.5 µg of plasmids was transfected into approximately 1.2 x 10^6 cells. For other experiments, a total of 0.5 µg of plasmids was transfected into approximately 0.2 x 10^6 cells.

RNA interference. RNAi target sequences (sense strand): TRIM41 siRNA #2: AAGGCGTGCTGTGGAAATAAA; TRIM41 siRNA #3: TTCAATAGGTGTGAAGAGGTA. siGENOME Non-Targeting Control siRNA (Dharmacon, # D-001210-02-05) was used as the control siRNA. 5 pmol of siRNA duplexes were transfected into A549 cells using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer's protocol.

CRISPR/Cas9. The single guide RNA (sgRNA) sequence targeting human TRIM41 is: GTAGTCTTCATCCCGCATGG. The sgRNA was cloned into lentiCRISPR v2 [19] (Addgene). 0.5 µg of lentiviral construct was transfected into HEK293 cells using Lipofectamine 2000. Cells were selected with 10 µg/ml puromycin for 14 days. Single clones were expanded for knockout confirmation by Western blotting and DNA sequencing.

***In vitro* ubiquitination.** *In vitro* ubiquitination assay was performed according to the manufacturer's manual (Boston Biochem). Ubiquitin, E1, UBCH5A (Boston Biochem), VSV-N-FLAG, and TRIM41-HA or Del-RING-HA bound to the anti-HA resin (Sigma) were incubated at 30°C in the ubiquitin assay reaction buffer (Boston Biochem) for 2 h. The anti-HA resin was

washed with 1 M urea for 15 min to exclude potential binding of unanchored polyubiquitin. Then the resin was incubated with 45 μL of 0.5 mg/ml HA peptide to elute VSV-N protein. The elutes were subsequently analyzed by SDS-PAGE followed by Western blotting.

Statistical analysis. The sample size was sufficient for data analysis using paired two-tailed Student's *t*-test. For all statistical analysis, differences were considered to be statistically significant at values of $P < 0.05$.

RESULTS

TRIM41 restricts VSV infection.

To examine the effect of TRIM41 on VSV infection, we first transfected FLAG-tagged TRIM41 into HEK293 cells. After 48 h, cells were infected with a VSV reporter virus carrying a luciferase gene in the viral genome (VSV-Luc). As shown in Figure 1A, the ectopic expression of TRIM41 inhibited VSV replication activity.

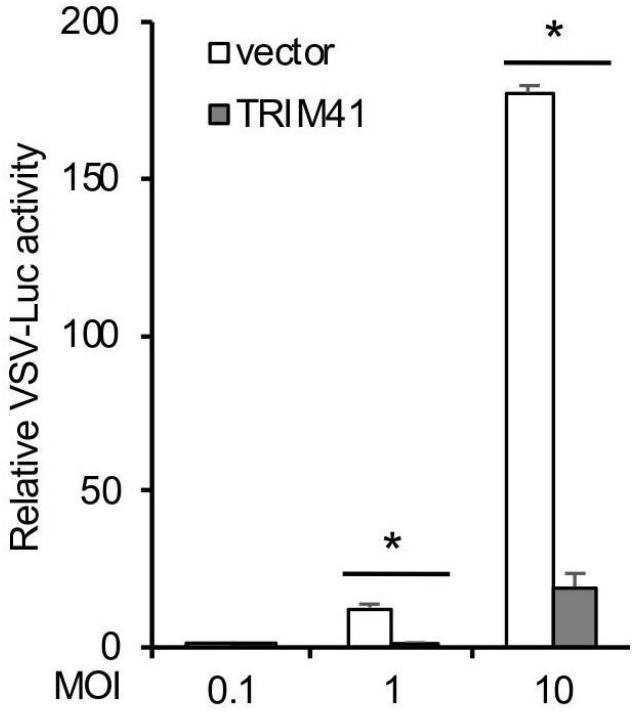

Figure 37: **Effect of TRIM41 overexpression on VSV infection- Reporter Assay.** HEK293 cells transfected with TRIM41-FLAG or pCMV-3Tag-8 vector were infected with 0.1 MOI of VSV-Luc for 12 h. Relative luciferase activities were examined. Data represent means ± s.d. of three independent experiments. The P value was calculated (two-tailed Student's t-test) by comparison with the vector control. An asterisk indicates $P < 0.05$.

To corroborate this finding, we further examined the effect of TRIM41 on the infection of VSV-GFP virus that expresses a GFP protein. TRIM41 or empty vector was transfected in HEK293 cells followed by the infection of VSV-GFP for 12 h. TRIM41 also limited VSV-GFP infection in HEK293 cells, indicated by the decreased GFP positive cells.

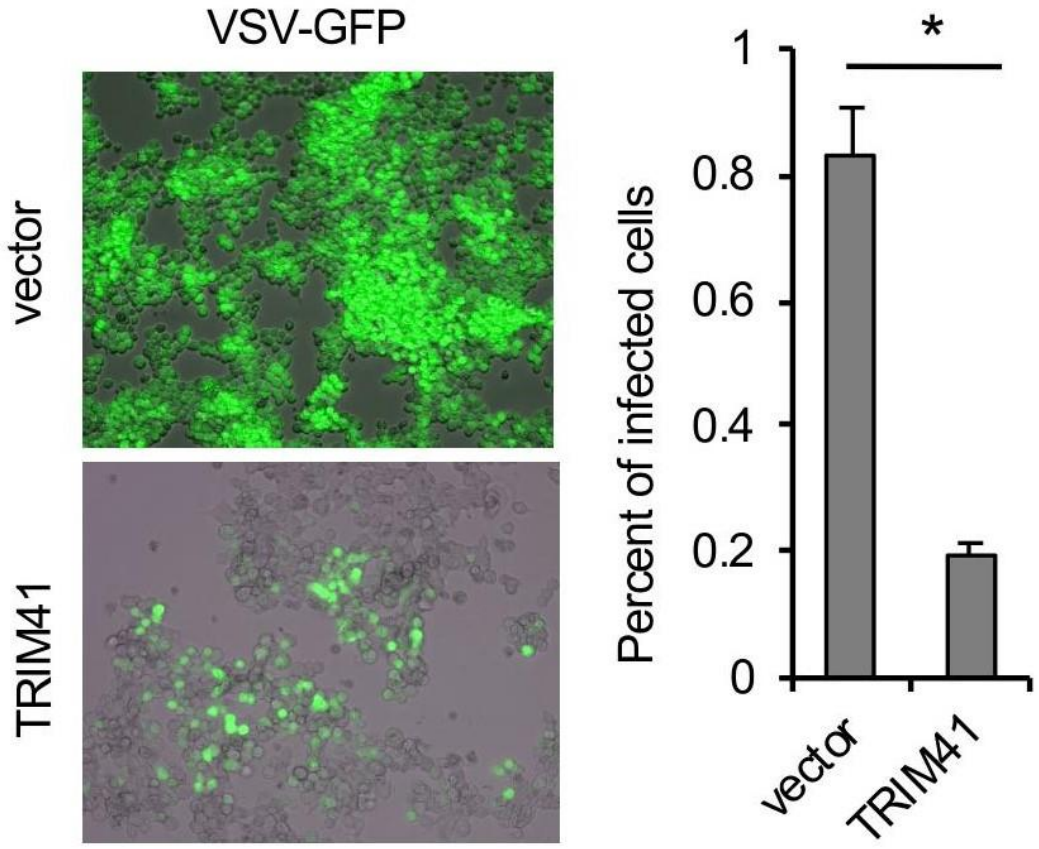

Figure 38: **Effect of TRIM41 overexpression on VSV infection- Fluorescence Assay.** HEK293 cells were transfected with pCMV-3Tag-8 vector or TRIM41-FLAG for 24 h, then infected with 1 MOI of VSV-GFP for 12 h. The percentage of GFP-positive cells is summarized in the right graph. An asterisk indicates $P < 0.05$.

Next, we examined the effect of TRIM41 overexpression on viral protein expression. HEK293 cells were transfected with vector or TRIM41, then were infected with different multiplicity of infections (MOIs) of VSV. Consistently, Western blot analysis showed that TRIM41 reduced VSV protein expression.

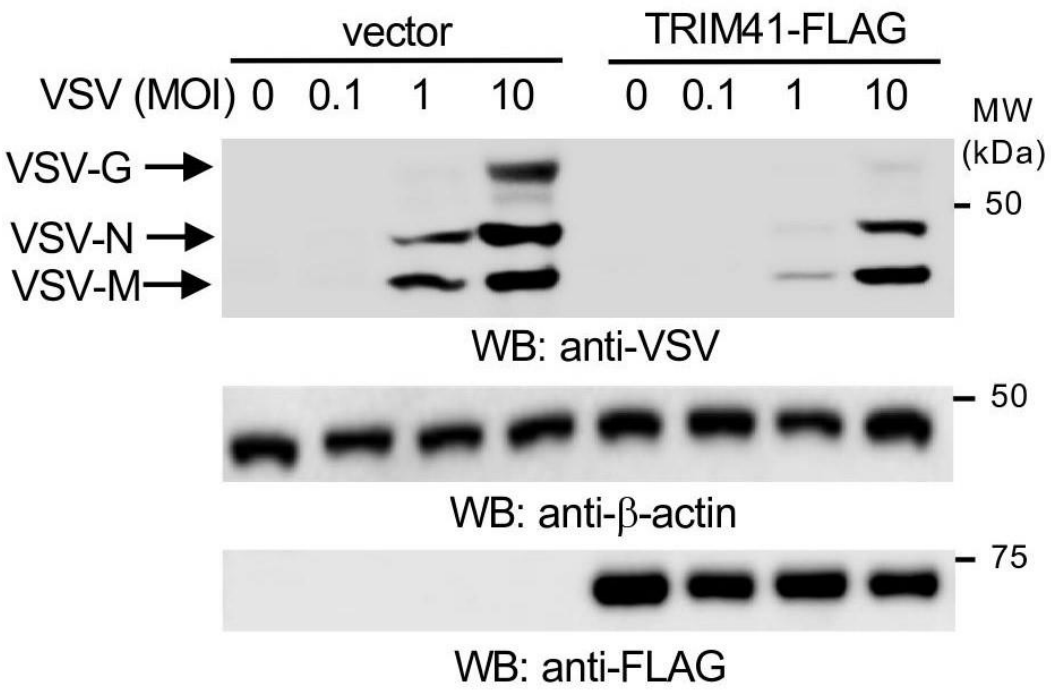

Figure 39: **Effect of TRIM41 overexpression on VSV protein expressions.** HEK293 cells were transfected with pCMV-3Tag-8 vector or TRIM41-FLAG for 24 h, then infected with different MOIs of VSV for 12 h. Cell lysates were blotted using the indicated antibodies.

Lastly, $TCID_{50}$ assay was performed to determine the effect of TRIM41 overexpression on the production of infectious VSV particles. Overexpression of TRIM41 reduced VSV viral titers significantly at the time course of 6 h to 48 h.

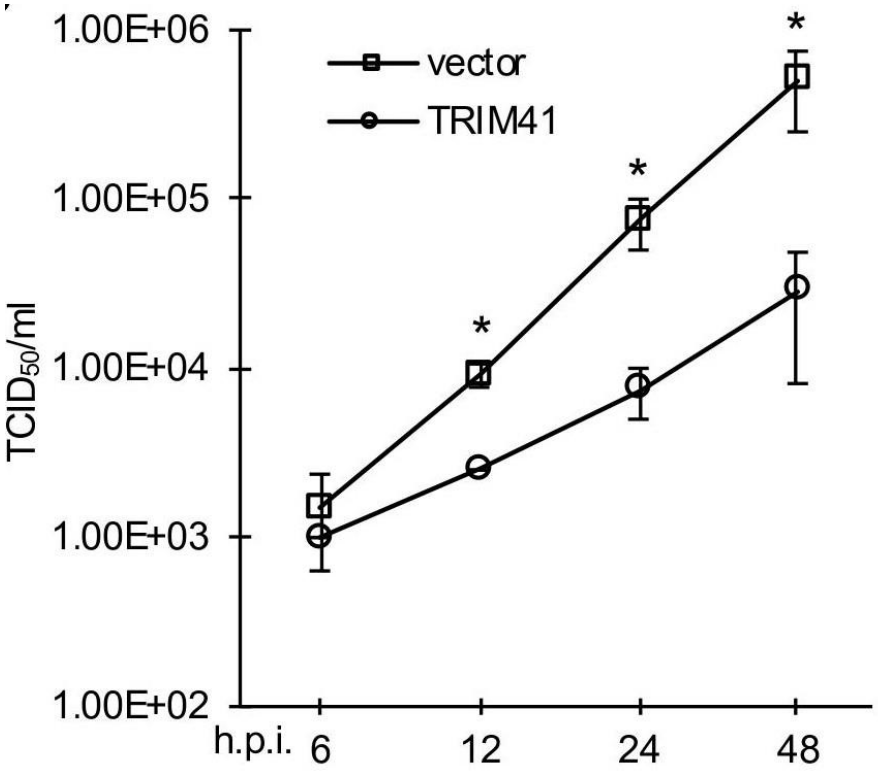

Figure 40: **Effect of TRIM41 overexpression on VSV titers.** HEK293 cells were transfected with pCMV-3Tag-8 vector or TRIM41-FLAG. After 24 h, cells were infected with 0.001 MOI of VSV. After the designated hour post infection (h.p.i.), virus titers were determined by $TCID_{50}$ in Vero cells. All experiments were biologically repeated three times. An asterisk indicates $P < 0.05$.

Taken together, these data suggest that TRIM41 is an anti-VSV host factor.

TRIM41 deficiency increases host susceptibility to VSV

To corroborate the gain-of-function of TRIM41, we further examined the effect of TRIM41 depletion on VSV infection. We first depleted TRIM41 using a small interfering RNA (siRNA). Two validated siRNA duplexes against TRIM41 (Patil et al., 2018) were individually transfected into A549 lung epithelial cells. After 48 h, cells were infected with VSV-Luc for 12 h. Knockdown of TRIM41 increased VSV infection activity in A549 cells.

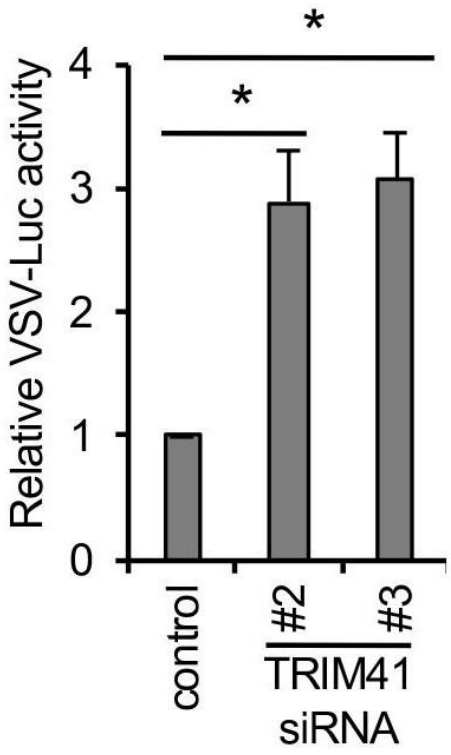

Figure 41: **Effect of TRIM41 knockdown on VSV infectious activity.** A549 cells were transfected with 5 pmol of the control siRNA or the indicated siRNA duplex against TRIM41. After 48 h, the cells were infected at an MOI of 0.1 with VSV-Luc for 12 h. Relative luciferase activities were examined. All experiments were biologically repeated three times. Data represent means ± standard deviations of three independent experiments. The *P* value was calculated (two-

tailed Student's t test) by comparison of results with those of the siRNA control in each cell group (* $P < 0.05$).

Secondly, wild type and the TRIM41 knockout HEK293 cells used in our previous study (Patil et al., 2018) were infected with different doses of VSV-Luc for 12 h. Reporter assays demonstrated the increased viral infection activity in TRIM41 knockout cells.

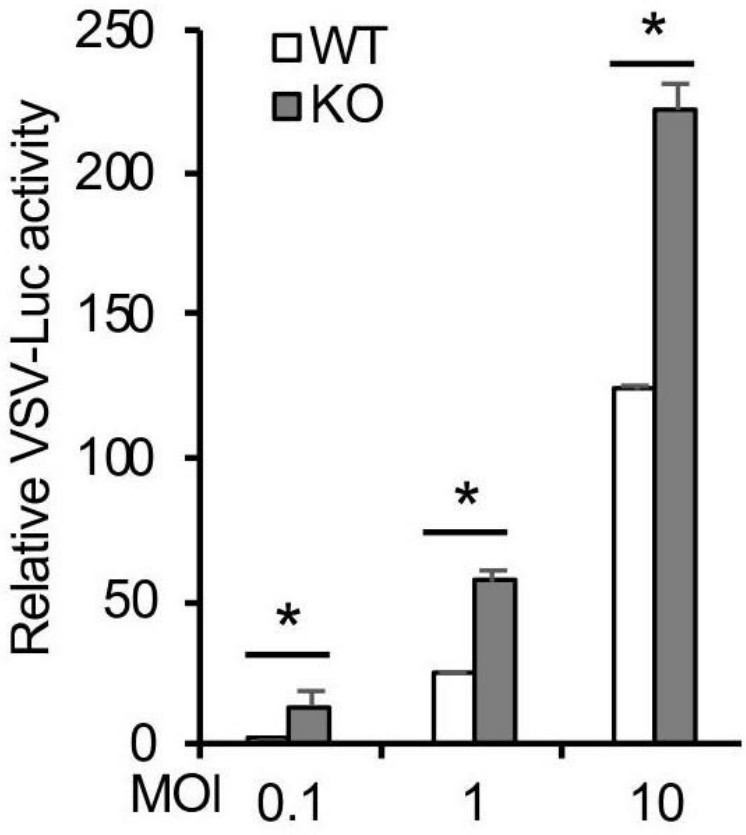

Figure 42: **Effect of TRIM41 knockout on VSV infectious activity.** Wild type (WT) HEK293 and TRIM41 knockout (KO) cells were infected with the indicated MOIs of VSV-Luc for 12 h. Relative luciferase activities were examined. All experiments were biologically repeated three times. An asterisk indicates $P < 0.05$.

Thirdly, we examined the infection of VSV-GFP in wild type and TRIM41 knockout HEK293 cells. Knockout of TRIM41 resulted in an increase of the number of GFP positive cells.

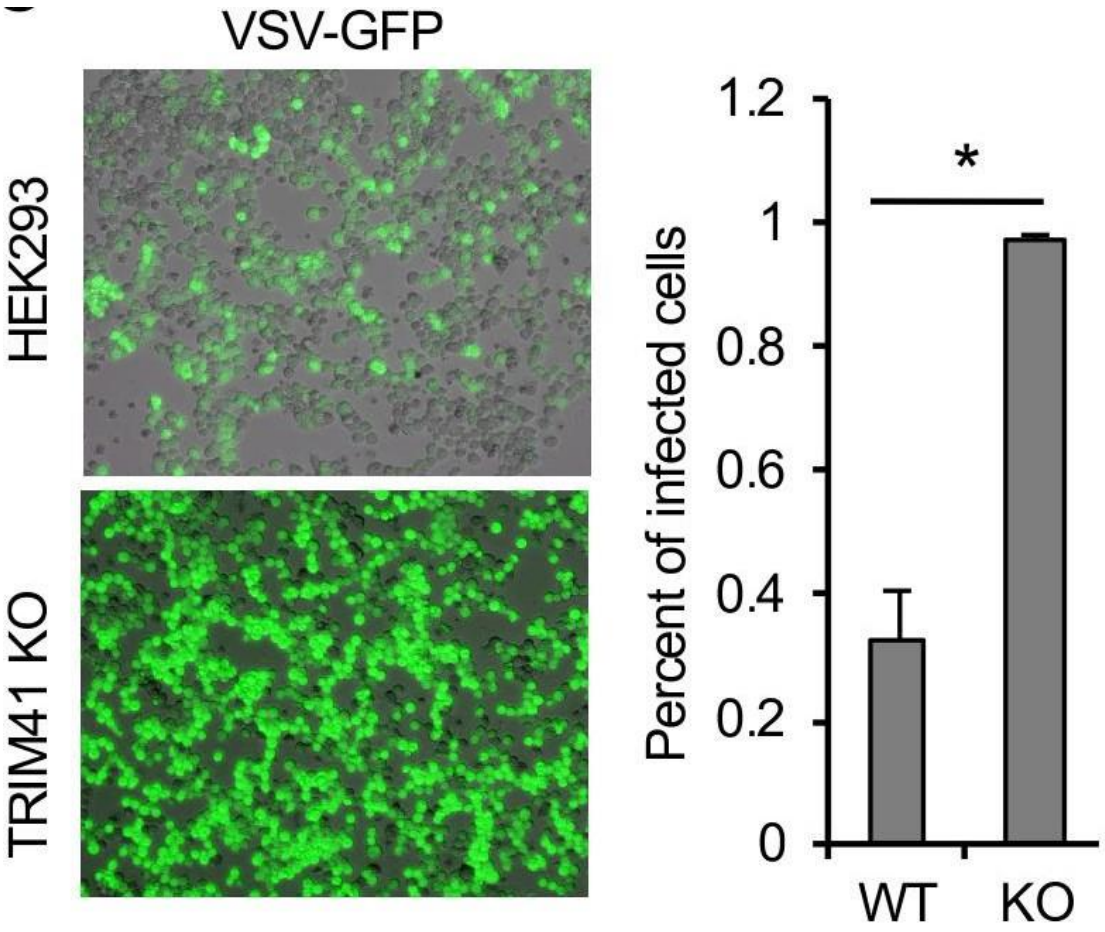

Figure 43: **Effect of TRIM41 knockout on VSV – Fluorescence assay.** Wild type and TRIM41 knockout cells were infected with 0.1 MOI of VSV-GFP for 12 h. The percentage of GFP-positive cells is summarized in the right graph. An asterisk indicates $P < 0.05$.

Fourthly, we examined the effect of TRIM41 deficiency on viral protein expression. TRIM41 wild type and knockout cells were infected with different doses of VSV for 12 h. As shown in Figure 44, viral protein expression dramatically increased in the knockout cells.

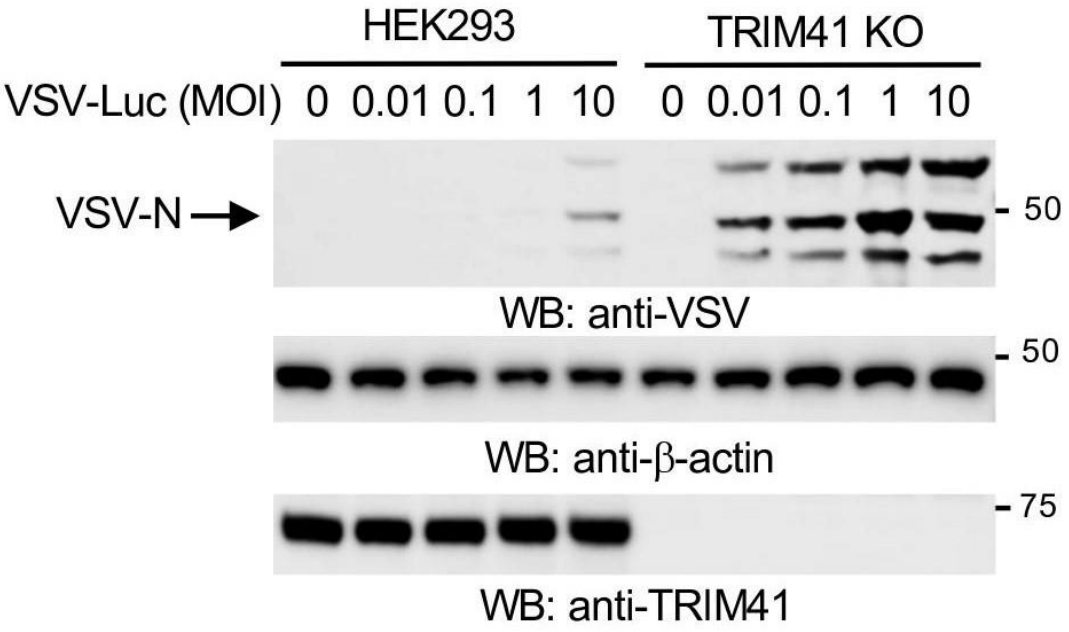

Figure 44: **Effect of TRIM41 knockout on VSV protein expression.** Wild type and TRIM41 knockout cells were infected with different MOIs of VSV for 12 h. Cell lysates were blotted using the indicated antibodies.

Lastly, viral titers were determined by $TCID_{50}$ assay in TRIM41 wild type vs. knockout cells. VSV viral titers increased about 10-fold in knockout cells compared to wild type cells (Figure 45), suggesting depletion of TRIM41 increases host susceptibility to VSV infection.

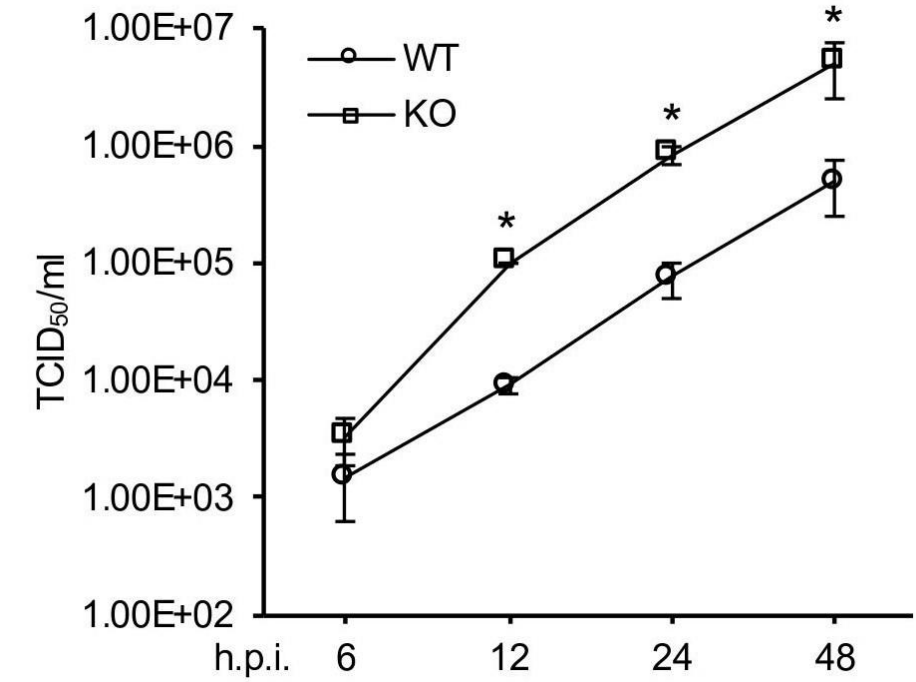

Figure 45: **Effect of TRIM41 knockout on VSV titers.** Wild type and TRIM41 knockout cells were infected with 0.001 MOI of VSV. After the designated hour post infection, virus titers were determined by $TCID_{50}$ assay in Vero cells. All experiments were biologically repeated three times. An asterisk indicates $P < 0.05$.

TRIM41 interacts with the nucleoprotein of VSV

We previously reported that TRIM41 interacted with influenza viral protein to limit viral infection (Patil et al., 2018). Therefore, it is plausible that TRIM41 also interacts with VSV protein(s) to inhibit viral infection. In this regard, FLAG-tagged G, P, or N of VSV was co-transfected with HA-tagged TRIM41 into HEK293 cells. Co-immunoprecipitation (co-IP) revealed the specific interaction between TRIM41 and the N protein of VSV (VSV-N).

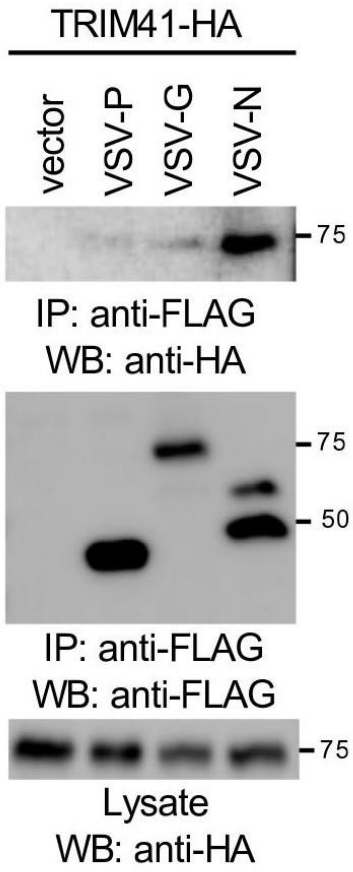

Figure 46: **Interaction of TRIM41 with VSV protein.** TRIM41-HA was co-transfected with FLAG-tagged phosphoprotein (P), glycoprotein (G) or nucleoprotein (N) of VSV into HEK293 cells. Cells were treated with 1 □M MG132 to prevent VSV protein from degradation. Cell lysates were immunoprecipitated with the anti-FLAG antibody, and then blotted as indicated.

Next, we examined the subcellular localization of TRIM41 and VSV-N by immunofluorescence assay. FLAG-tagged VSV-N and HA-tagged TRIM41 were transfected alone or together into A549 cells. The immunofluorescence assays showed that TRIM41 expressed in the cytoplasm and the nucleus while VSV-N only expressed in the cytoplasm.

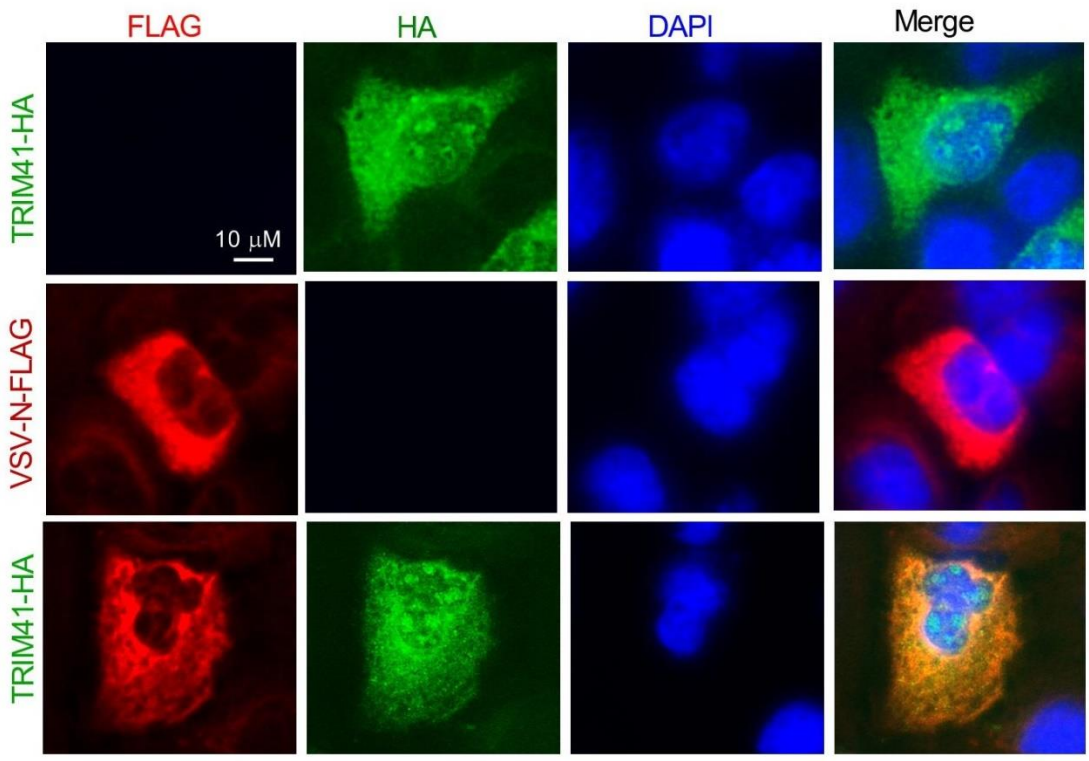

Figure 47: **TRIM41-VSV N co-localization.** A549 cells were transfected with HA-tagged TRIM41 (TRIM41-HA) and FLAG-tagged VSV-N (VSV-N-FLAG). Cells were treated with 1 μM MG132 to prevent VSV protein from degradation. Red: FLAG; Green: HA; Blue: DAPI, a nuclear stain.

We further examined the co-localization of endogenous TRIM41 and VSV-N during viral infection. A549 cells were cultured in a chamber slide and infected with 1 MOI of VSV for 24 h. Consistent with the ectopic expressing TRIM41, the endogenous TRIM41 was expressed in the cytoplasm and the nucleus. VSV-N only expressed in the cytoplasm and co-localized with TRIM41 in the cytoplasm.

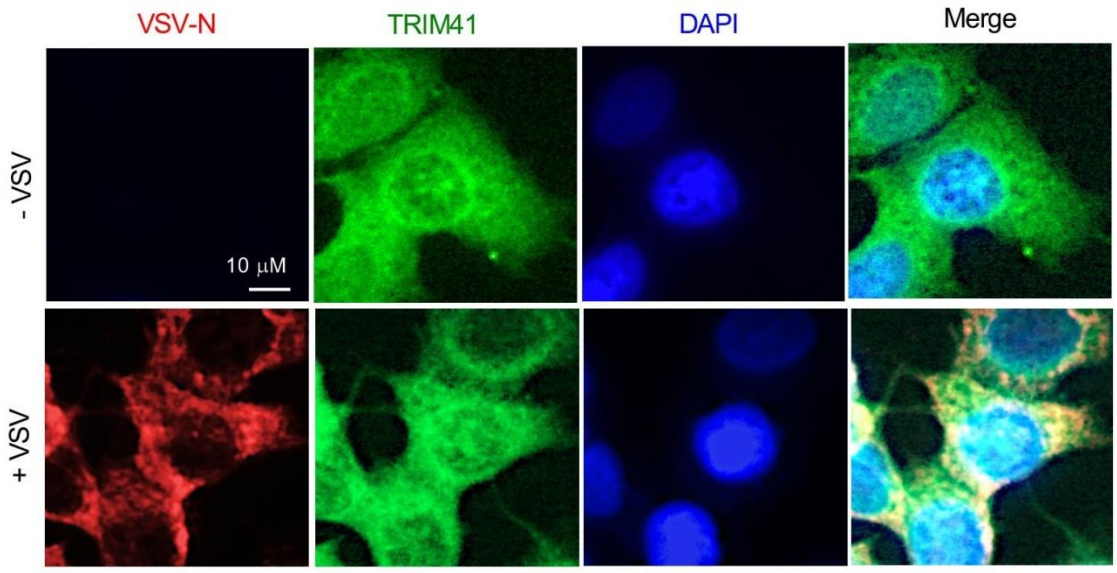

Figure 48: **VSV localization.** A549 cells were mock infected or infected with VSV. Cells were treated with 1 μM MG132 to prevent VSV-N from degradation. After 24 h, cells were fixed with cold methanol and incubated with anti-VSV-N and anti-TRIM41 antibodies. Red: VSV-N; Green: TRIM41; Blue: DAPI, a nuclear stain.

To identify the domain of TRIM41 essential for the interaction with VSV-N, full-length TRIM41 or domain deletion mutants from previous study were co-transfected with VSV-N into HEK293 cells. Co-IP showed that the B-box deletion and the coiled-coil deletion mutant interacted with VSV-N, indicating both domains are not essential for the interaction (Fig. 49). On the contrary, deletion of either the RING or the SPRY domain impaired the interaction, suggesting that both SPRY and RING domains are required for the interaction between TRIM41 and VSV-N.

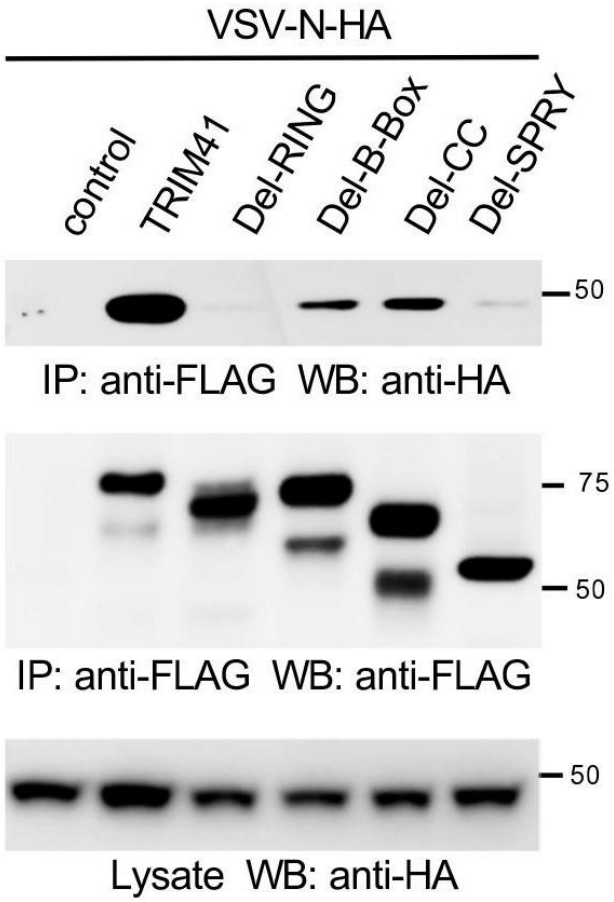

Figure 49: **TRIM41 mutant- VSV nucleoprotein interaction.** HA-tagged VSV-N (VSV-N-HA) was co-transfected with the indicated FLAG-tagged TRIM41 or indicated mutants into HEK293 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with the indicated antibodies.

SPRY and RING domains are required for TRIM41 antiviral function

Since the SPRY and RING domains interact with VSV-N, we examined the effect of SPRY deletion mutant (Del-SPRY) and RING deletion mutant (Del-RING) on VSV infection. TRIM41, Del-SPRY or the Del-RING was transfected into HEK293 cells, followed by infection with different MOIs of VSV-Luc for 12 h. Luciferase reporter assay showed that neither Del-SPRY nor Del-RING inhibited VSV infection (Figs. 50-51).

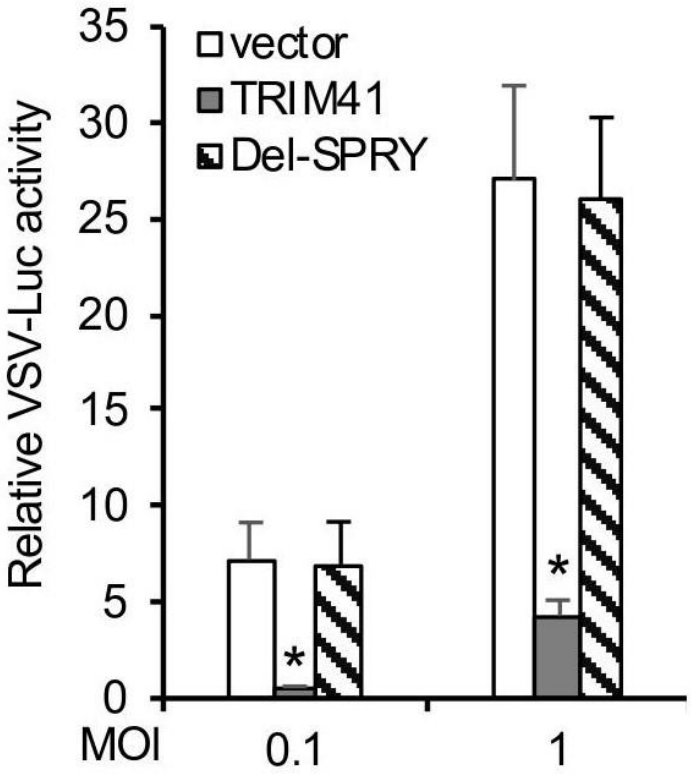

Figure 50: **Effect of SPRY deletion on anti-VSV action of TRIM41.** HEK293 cells were transfected with FLAG-tagged TRIM41 or the SPRY deletion mutant (Del-SPRY). After 24 h, cells were infected with designated MOIs of VSV for 12 h. Relative luciferase activities were examined. Data represent means ± s.d. of three independent experiments. The *P* value was calculated by two-tailed Student's *t*-test. An asterisk indicates $P < 0.05$.

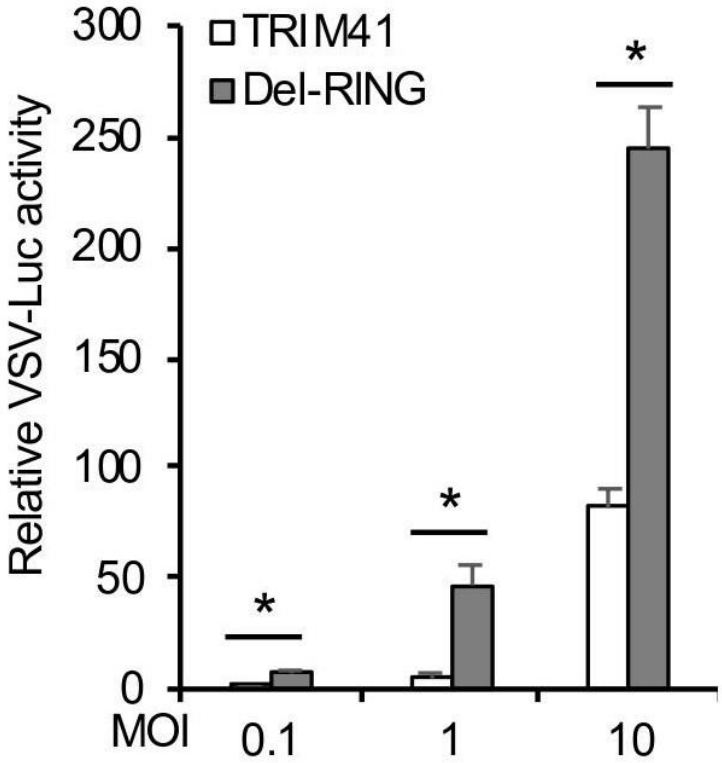

Figure 51: **Effect of RING deletion on anti-VSV action of TRIM41.** HEK293 cells were transfected with FLAG-tagged TRIM41 or the RING deletion mutant (Del-RING). After 24 h, cells were infected with designated MOIs of VSV for 12 h. Relative luciferase activities were examined. Data represent means ± s.d. of three independent experiments. The *P* value was calculated by two-tailed Student's *t*-test. An asterisk indicates $P < 0.05$.

We further reconstituted full-length TRIM41 or the Del-RING in TRIM41 knockout cells by transfection. After 48 h, cells were infected with VSV-GFP. As shown in Figure 4C, full-length TRIM41, but not the Del-RING, restored antiviral activity against VSV in the knockout cells.

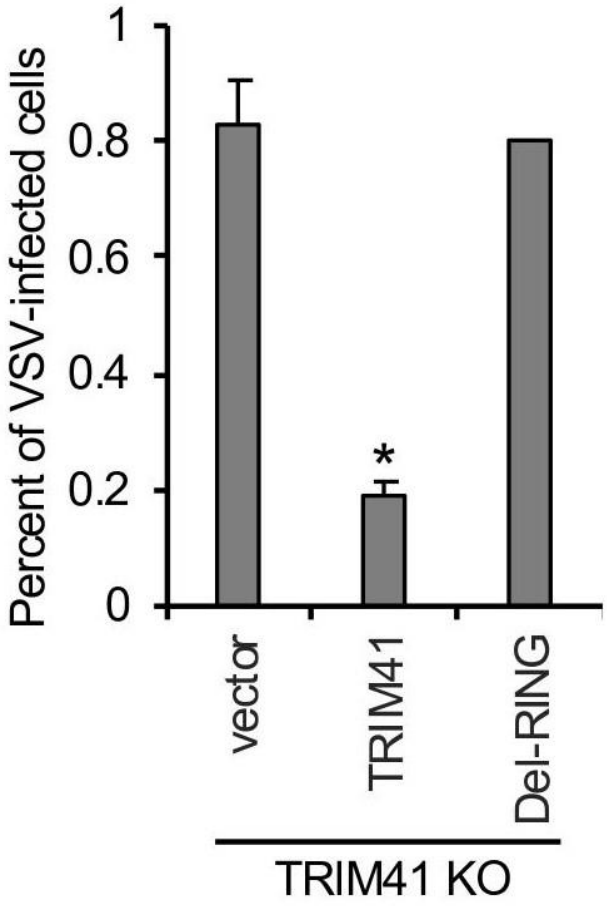

Figure 52: **Effect of RING deletion on anti-VSV action of TRIM41in knockout.** TRIM41 knockout HEK293 cells were transfected with pCMV-3Tag-8 vector, TRIM41-FLAG or the Del-RING for 24 h, then infected with 0.1 MOI of VSV-GFP for 12 h. The percentage of GFP-positive cells was adopted to determine the infection activity. An asterisk indicates $P < 0.05$.

Lastly, we examined viral titers in TRIM41 knockout cells reconstituted with full-length TRIM41 vs. the Del-RING. In line with the reporter assays, the Del-RING failed to suppress VSV infection evidenced by $TCID_{50}$ assay.

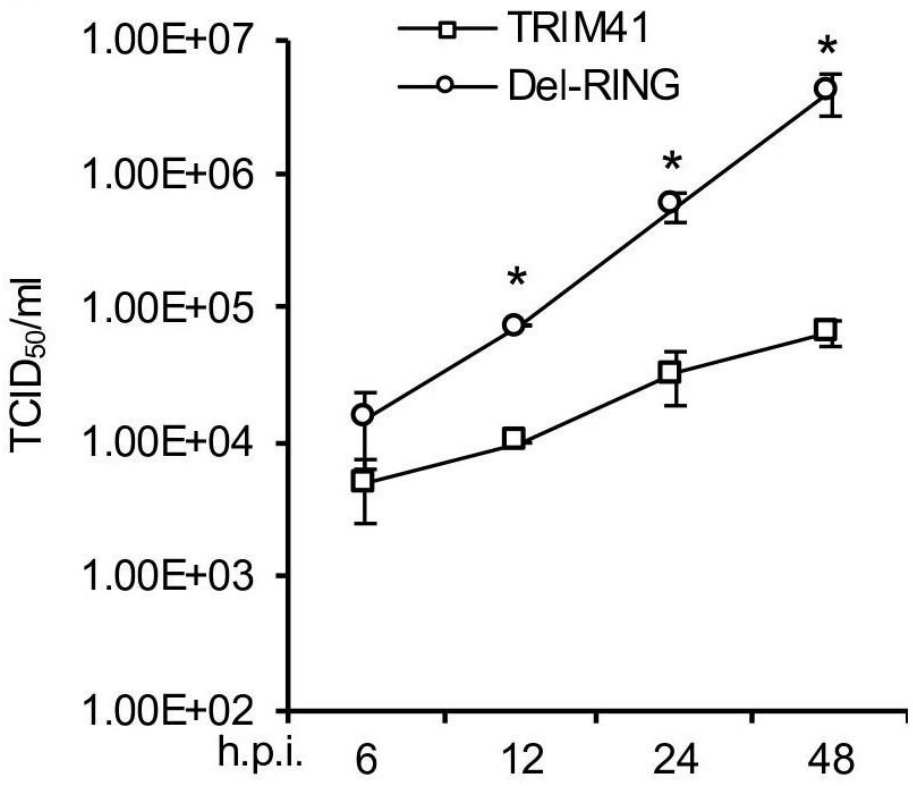

Figure 53: **Effect of RING deletion of TRIM41 on VSV titers.** HEK293 knockout cells were transfected with wild type TRIM41 or the Del-RING mutant. After 24 h, cells were infected with 0.001 MOI of VSV. After the designated hour post infection, virus titers were determined by $TCID_{50}$ assay in Vero cells. All experiments were biologically repeated three times.

Taken together, the interaction with VSV-N is indispensable for the antiviral function of TRIM41.

TRIM41 mediates ubiquitination and degradation of VSV-N

TRIM41 has been shown to promote cytosolic DNA-induced type I IFN production (Liu et al., 2018). Whether TRIM41 regulates type I IFN production induced by the cytosolic RNA, such as VSV RNA, is unknown. Thus, we transfected vector or TRIM41 together with NF-□B or the interferon-stimulated response element (ISRE) reporter into HEK293T cells. We used HEK293T cells because the HEK293T cells have an intact RNA sensing pathway, but the DNA sensing pathway is defective. As shown in Figure 54, the deficiency of TRIM41 has little effect on the reporter activity of either NF-κB or ISRE, suggesting TRIM41 is dispensable for cytosolic RNA-mediated innate immunity.

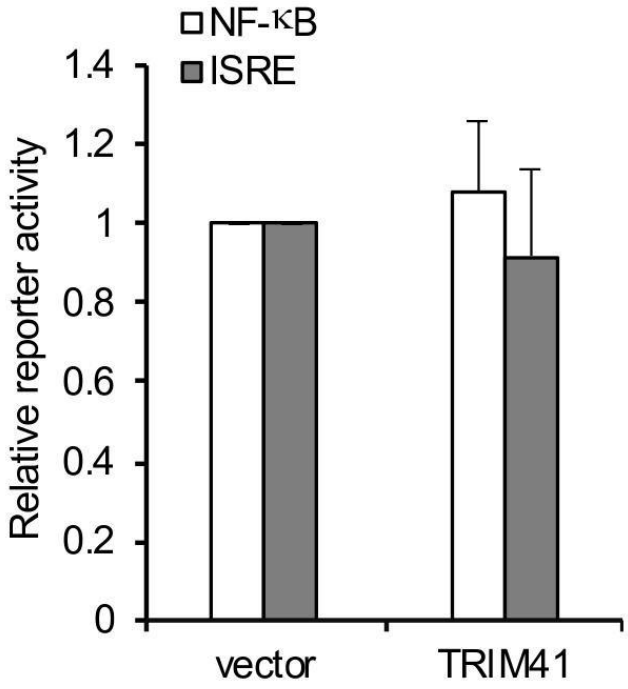

Figure 54: **Effect of TRIM41 on innate immunity.** One hundred ng of vector or FLAG-tagged FIP200 was transfected with 50 ng of pRL-SV40 (Renilla luciferase as an internal control), together with 100 ng of pISRE-Luc or NF-κB-Luc into HKE293 cells. After 48 h, cells were collected and the ratio of firefly luciferase to Renilla luciferase was calculated to determine the relative reporter activity. All experiments were repeated three times.

Although RING mediates the interaction with VSV-N, the RING domain is well known to confer the E3 ubiquitin ligase activity of TRIM proteins. Therefore, the requirement of RING also suggests that TRIM41 might inhibit VSV infection through ubiquitination of VSV-N. To examine whether TRIM41 can conjugate ubiquitin molecules onto VSV-N, we first performed an *in vitro* ubiquitination assay. As shown in Figure 55, TRIM41, but not the Del-RING, conjugated the ubiquitin molecules onto VSV-N.

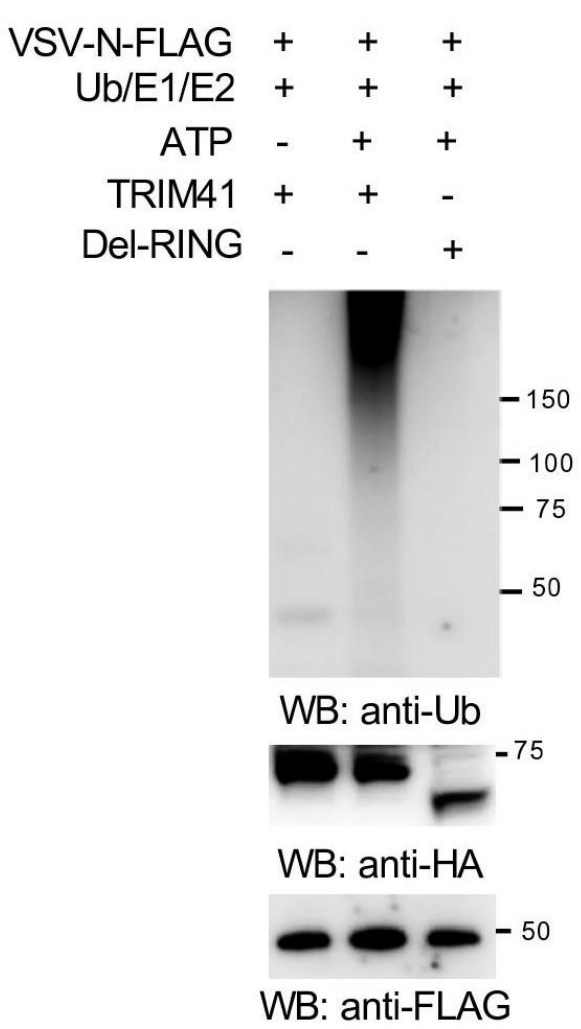

Figure 55: ***In vitro*** **ubiquitination of VSV-N by TRIM41.** FLAG-tagged VSV-N, HA-tagged TRIM41 and the Del-RING mutant, plus E1, E2 (UBCH5A), ATP and ubiquitin were added as indicated and incubated at 30°C for 2 h. Samples were blotted with the indicated antibodies.

Consistently, overexpression of wild type TRIM41, but not the Del-RING mutant, induced the ubiquitination of VSV-N in HEK293 cells.

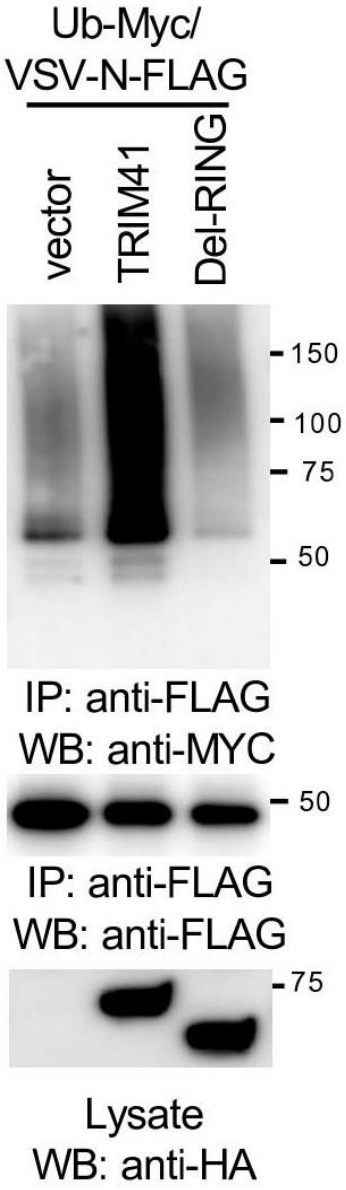

Figure 56: **TRIM41 mediated VSV N ubiquitination.** FLAG-tagged VSV-N was co-transfected with vector, TRIM41-HA or the HA-tagged Del-RING mutant together with Myc-tagged ubiquitin (Ub-Myc) into HEK293 cells. Cells were treated with 1 □M MG132 to prevent VSV-N

from degradation. Cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with the indicated antibodies.

Next, we examined VSV-N ubiquitination in TRIM41 knockout cells. VSV-N was transfected with HA-tagged ubiquitin into wild type and TRIM41 knockout HEK293 cells. Knockout of TRIM41 dramatically reduced the levels of polyubiquitin chain conjugated onto VSV-N.

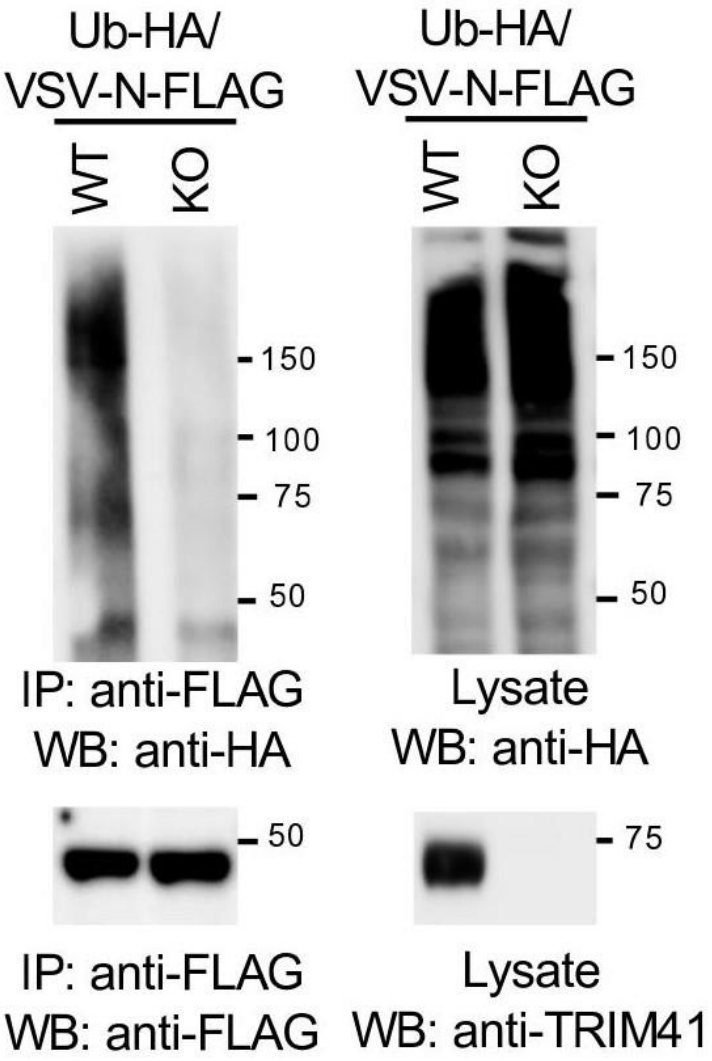

Figure 57: **Effect of TRIM41 knockout on VSV N ubiquitination.** Wild type and TRIM41 knockout HEK293 cells were transfected with FLAG-tagged VSV-N and HA-tagged ubiquitin

(Ub-HA). Cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with the indicated antibodies.

These experiments suggest that TRIM41 mediates polyubiquitination of VSV-N. Polyubiquitination often leads to proteasomal degradation of the targeted protein. Thus, we further examined whether TRIM41 mediates VSV-N protein degradation. VSV-N was co-transfected with TRIM41 or the Del-RING into HEK293 cells. Overexpression of full-length TRIM41 reduced the VSV-N expression; however, the Del-RING had a marginal effect on the expression of VSV-N protein.

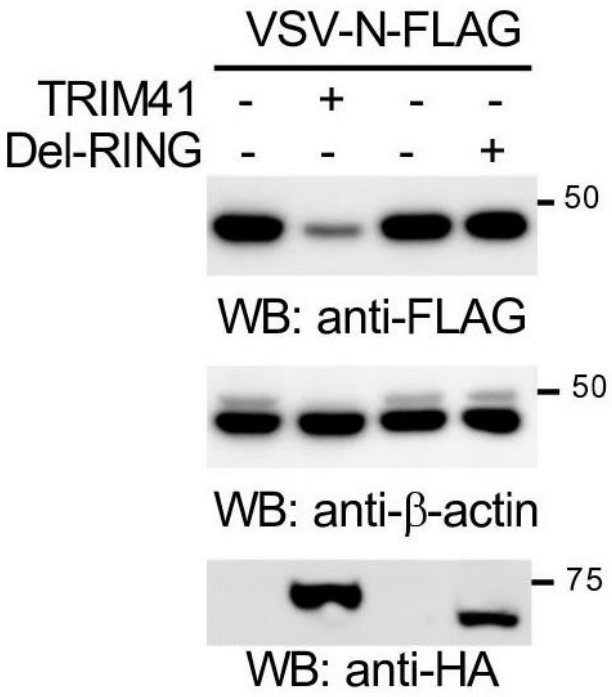

Figure 58: **RING dependent TRIM41 mediated VSV N degradation.** FLAG-tagged VSV-N (VSV-N-FLAG) was co-transfected with HA-tagged TRIM41 or the Del-RING mutant into HEK293 cells. Cell lysates were blotted as indicated.

Furthermore, we treated cells with the protease inhibitor MG132 to determine whether TRIM41-mediated protein degradation of VSV-N is proteasome-dependent. HEK293 cells were transfected with VSV-N along with TRIM41 or vector. After 48 h, cells were treated with the protease inhibitor MG132 for 6 h. Western blot analysis found that MG132 treatment blocked TRIM41-mediated VSV-N degradation.

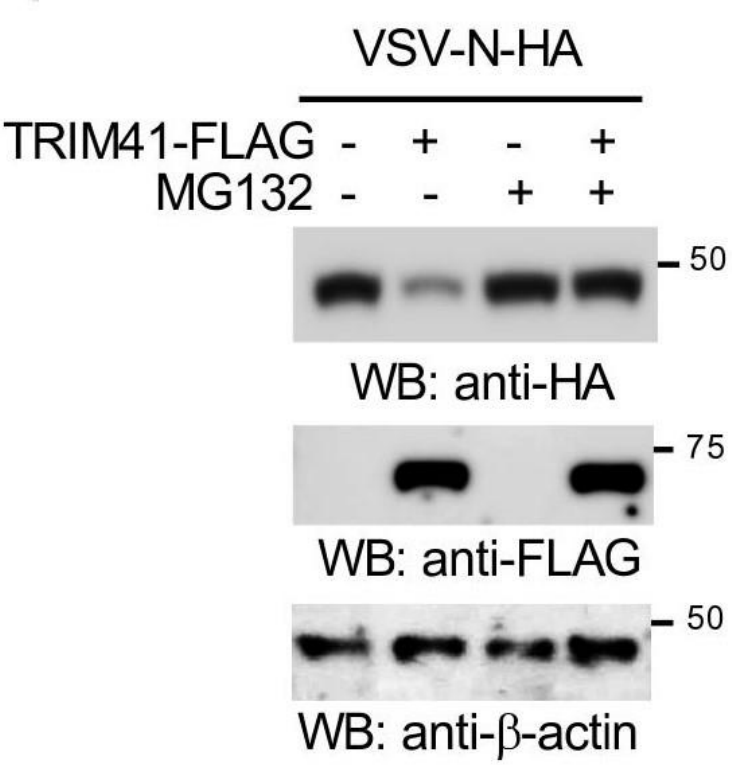

Figure 59: **TRIM41 mediated VSV N degradation.** HA-tagged VSV-N (VSV-N-HA) was co-transfected with vector or TRIM41-FLAG into HEK293 cells. After 24 h, cells were treated with DMSO or 1 □M MG132 for 12 h. Cell lysates were then blotted with the indicated antibodies.

In all, the combined data indicate that TRIM41 mediates ubiquitination of VSV-N, which leads to VSV-N proteasomal degradation, thereby inhibiting VSV infection.

DISCUSSION

Innate immunity and intrinsic immunity are the frontlines of host defense to the invading viruses. Innate immunity comprises of various signaling cascades leading to the expression of type I IFN and many other antiviral genes. These innate immune signaling pathways are triggered by the products and/or by-products of microbes, which are usually foreign to the host. The Toll-like receptor 7 (TLR7) and RIG-I are the sensors that recognize the 5`-ppp-RNA of VSV in the endosome and the cytoplasm, respectively [20,21]. Upon the engagement with viral RNA, TLR7 and RIG-I activate signaling cascades, which lead to the induction of type I IFN expression [22-26]. Unlike innate immunity factors, the intrinsic immunity factors are a group of constitutively expressed host factors. The "pre-existed" expression of these host factors guarantees a more rapid response and a direct inhibition of viral infection [10,27]. For example, the cytidine deaminase, apolipoprotein editing complex 3 G (APOBEC3G), introduces transversion mutations into the HIV genome, thereby impeding HIV infection [28]. It is well established for how innate immunity responds to VSV infection; however, the intrinsic immune response to VSV is still elusive. Here, we report that TRIM41 interacts with the N protein of VSV and subsequently targets it for ubiquitination and proteasomal degradation. Our previous study showed that TRIM41 is constitutively expressed [14]. Thus, TRIM41 is a new intrinsic immune factor to VSV.

TRIM41 belongs to the TRIM family that consists of more than 70 members. Recently, many TRIM proteins are identified as intrinsic immune factors that curb viral infection through directly targeting viral proteins. For instance, TRIM32 restricts influenza A virus by targeting PB1 protein for ubiquitination and degradation [11]. TRIM22 and TRIM14 restrict hepatitis C virus by targeting NS5α for ubiquitination and degradation [29,30]. TRIM52 interacts with the nonstructural protein 2A of Japanese encephalitis virus and mediates NS2A ubiquitination and protein degradation [31]. Our study showed TRIM41 inhibited VSV infection by mediating proteasomal degradation of the N

protein. It should be noted that another TRIM protein, PML (also known as TRIM19), has been implied a role in VSV infection. Overexpression of PML in CHO cells increased the resistance to infection of VSV [32]. Furthermore, PML deficiency increases mice susceptibility to VSV infection [33]. A recent mechanistic study showed that PML enhances IFN synthesis by regulating the cellular distribution of Pin1 (peptidyl-prolyl cis/trans isomerase). The interaction of PML with endogenous Pin1 prevents the degradation of activated IRF3, thereby potentiating IRF3-dependent production of IFN [34].

Our previous study demonstrates that TRIM41 directly restricts influenza A virus by ubiquitination and degradation of nucleoprotein [14]. TRIM41 also inhibits HBV activity, which is dependent on the ubiquitin E3 ligase activity and the C-terminal domain of TRIM41 [15]. Thus, TRIM41 has emerged as a broad-spectrum antiviral factor. Like TRIM41, several other TRIM proteins are reported to have a broad-spectrum antiviral activity. PML also inhibits herpesvirus and adeno-associated virus [35]. TRIM56 restricts influenza B virus, HIV, yellow fever virus, and dengue virus by reducing the viral RNA levels through its C-terminal tail [36,37]. TRIM22 restricts encephalomyocarditis virus, influenza A virus, hepatitis B virus, and hepatitis C virus [29,38-40]. However, the underlying mechanism for the broad-spectrum antiviral activity of TRIM proteins is unknown. To determine the broad-spectrum antiviral activity, it is important to identify the common mechanism. Future studies will determine whether the interacting viral proteins share a common motif for TRIM41 interaction.

Our previous study [14] and this study show the endogenous TRIM41 localizes in the cytoplasm and the nucleus. Furthermore, ectopic expression of TRIM41 also show the cytosolic and nuclear localization in this study. Consistently, previous studies by other groups also reported both cytosolic and nuclear localization of TRIM41 [41,42]. Viral infection may change the subcellular localization of TRIM proteins, such as influenza-induced TRIM32 nuclear localization [11].

However, we have not found that either VSV or influenza A virus alters cellular localization of TRIM41. The dual subcellular localization of TRIM41 makes it access to the substrate in the cytoplasm and the nucleus, thereby inhibiting cytosolic replication virus, such as VSV, and the nuclear replication virus, such as influenza A virus.

VSV life cycle comprise of four major steps: entry, replication and transcription, virion assembly, and progeny release. As TRIM41 is a cytosolic and nuclear protein, it unlikely TRIM41 interferes with viral entry and release because these steps involve host membrane proteins. For example, ZMPSTE24, an endosomal protein found in our previous study, blocks the entry of VSV and other viruses [43]. The N protein of VSV is critical for VSV RNA replication and transcription [3,4]. As TRIM41 mediates VSV-N for protein degradation, it is plausible that TRIM41 inhibits VSV replication and transcription. Indeed, the VSV reporter viruses, VSV-Luc and VSV-GFP, carry a reporter gene whose expression is controlled by VSV polymerase. The reporter activity of these two VSV viruses indicates the activity of VSV transcription activity. VSV-N is also a major viral protein for VSV nucleocapsid, therefore, TRIM41 might interfere with the assembly of the nucleocapsid. Future studies will investigate this hypothesis.

CONCLUSION

We demonstrate that TRIM41 targets the N protein to inhibit VSV infection. The N protein covers the entire VSV genomic RNA and plays an important role in viral transcription and replication, which makes the N protein an ideal drug target. Thus, our study provides a foundation for furure development of antiviral therapeutics.

REFERENCES

1. Albertini, A.A., Schoehn, G., Weissenhorn, W., and Ruigrok, R.W. (2008). Structural aspects of rabies virus replication. *Cell Mol Life Sci* 65, 282-294.
2. Basak, S., Mondal, A., Polley, S., Mukhopadhyay, S., and Chattopadhyay, D. (2007). Reviewing Chandipura: a vesiculovirus in human epidemics. *Biosci Rep* 27, 275-298.
3. Bonilla, W.V., Pinschewer, D.D., Klenerman, P., Rousson, V., Gaboli, M., Pandolfi, P.P., Zinkernagel, R.M., Salvato, M.S., and Hengartner, H. (2002). Effects of promyelocytic leukemia protein on virus-host balance. *J Virol* 76, 3810-3818.
4. Chelbi-Alix, M.K., Quignon, F., Pelicano, L., Koken, M.H., and De The, H. (1998). Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. *J Virol* 72, 1043-1051.
5. Chen, D., Gould, C., Garza, R., Gao, T., Hampton, R.Y., and Newton, A.C. (2007). Amplitude control of protein kinase C by RINCK, a novel E3 ubiquitin ligase. *J Biol Chem* 282, 33776-33787.
6. Di Pietro, A., Kajaste-Rudnitski, A., Oteiza, A., Nicora, L., Towers, G.J., Mechti, N., and Vicenzi, E. (2013). TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. *J Virol* 87, 4523-4533.
7. Diallo, J.S., Vaha-Koskela, M., Le Boeuf, F., and Bell, J. (2012). Propagation, purification, and in vivo testing of oncolytic vesicular stomatitis virus strains. *Methods Mol Biol* 797, 127-140.
8. El Asmi, F., Maroui, M.A., Dutrieux, J., Blondel, D., Nisole, S., and Chelbi-Alix, M.K. (2014). Implication of PMLIV in both intrinsic and innate immunity. *PLoS Pathog* 10, e1003975.

9. Eldin, P., Papon, L., Oteiza, A., Brocchi, E., Lawson, T.G., and Mechti, N. (2009). TRIM22 E3 ubiquitin ligase activity is required to mediate antiviral activity against encephalomyocarditis virus. *J Gen Virol* 90, 536-545.
10. Fan, W., Wu, M., Qian, S., Zhou, Y., Chen, H., Li, X., and Qian, P. (2016). TRIM52 inhibits Japanese Encephalitis Virus replication by degrading the viral NS2A. *Sci Rep* 6, 33698.
11. Fitzgerald, K.A., Mcwhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M., and Maniatis, T. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4, 491-496.
12. Fu, B., Wang, L., Ding, H., Schwamborn, J.C., Li, S., and Dorf, M.E. (2015). TRIM32 Senses and Restricts Influenza A Virus by Ubiquitination of PB1 Polymerase. *PLoS Pathog* 11, e1004960.
13. Fu, B., Wang, L., Li, S., and Dorf, M.E. (2017). ZMPSTE24 defends against influenza and other pathogenic viruses. *J Exp Med* 214, 919-929.
14. Fu, Z.F. (2005). Genetic comparison of the rhabdoviruses from animals and plants. *Curr Top Microbiol Immunol* 292, 1-24.
15. Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S., and Jung, J.U. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446, 916-920.
16. Gao, B., Duan, Z., Xu, W., and Xiong, S. (2009). Tripartite motif-containing 22 inhibits the activity of hepatitis B virus core promoter, which is dependent on nuclear-located RING domain. *Hepatology* 50, 424-433.
17. Geoffroy, M.C., and Chelbi-Alix, M.K. (2011). Role of promyelocytic leukemia protein in host antiviral defense. *J Interferon Cytokine Res* 31, 145-158.
18. Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004). The roles of two IkappaB kinase-related

kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J Exp Med* 199, 1641-1650.

19. Liu, B., Li, N.L., Wang, J., Shi, P.Y., Wang, T., Miller, M.A., and Li, K. (2014). Overlapping and distinct molecular determinants dictating the antiviral activities of TRIM56 against flaviviruses and coronavirus. *J Virol* 88, 13821-13835.
20. Liu, Z.S., Zhang, Z.Y., Cai, H., Zhao, M., Mao, J., Dai, J., Xia, T., Zhang, X.M., and Li, T. (2018). RINCK-mediated monoubiquitination of cGAS promotes antiviral innate immune responses. *Cell Biosci* 8, 35.
21. Luo, M., Green, T.J., Zhang, X., Tsao, J., and Qiu, S. (2007a). Conserved characteristics of the rhabdovirus nucleoprotein. *Virus Res* 129, 246-251.
22. Luo, M., Green, T.J., Zhang, X., Tsao, J., and Qiu, S. (2007b). Structural comparisons of the nucleoprotein from three negative strand RNA virus families. *Virol J* 4, 72.
23. Mcwhirter, S.M., Fitzgerald, K.A., Rosains, J., Rowe, D.C., Golenbock, D.T., and Maniatis, T. (2004). IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proc Natl Acad Sci U S A* 101, 233-238.
24. Okada, A., and Iwatani, Y. (2016). APOBEC3G-Mediated G-to-A Hypermutation of the HIV-1 Genome: The Missing Link in Antiviral Molecular Mechanisms. *Front Microbiol* 7, 2027.
25. Patil, G., and Li, S. (2019). Tripartite motif proteins: an emerging antiviral protein family. *Future Virology* 14.
26. Patil, G., Zhao, M., Song, K., Hao, W., Bouchereau, D., Wang, L., and Li, S. (2018). TRIM41-Mediated Ubiquitination of Nucleoprotein Limits Influenza A Virus Infection. *J Virol* 92.
27. Pertel, T., Hausmann, S., Morger, D., Zuger, S., Guerra, J., Lascano, J., Reinhard, C., Santoni, F.A., Uchil, P.D., Chatel, L., Bisiaux, A., Albert, M.L., Strambio-De-Castillia, C.,

Mothes, W., Pizzato, M., Grutter, M.G., and Luban, J. (2011). TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472, 361-365.

28. Rieder, M., and Conzelmann, K.K. (2009). Rhabdovirus evasion of the interferon system. *J Interferon Cytokine Res* 29, 499-509.
29. Sanchez, J.G., Chiang, J.J., Sparrer, K.M.J., Alam, S.L., Chi, M., Roganowicz, M.D., Sankaran, B., Gack, M.U., and Pornillos, O. (2016). Mechanism of TRIM25 Catalytic Activation in the Antiviral RIG-I Pathway. *Cell Rep* 16, 1315-1325.
30. Sanjana, N.E., Shalem, O., and Zhang, F. (2014). Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11, 783-784.
31. Sharma, S., Tenoever, B.R., Grandvaux, N., Zhou, G.P., Lin, R., and Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. *Science* 300, 1148-1151.
32. Takeuchi, O., and Akira, S. (2009). Innate immunity to virus infection. *Immunol Rev* 227, 75-86.
33. Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell* 140, 805-820.
34. Tanaka, M., Fukuda, Y., Mashima, K., and Hanai, R. (2005). Intracellular localization and domain organization of human TRIM41 proteins. *Mol Biol Rep* 32, 87-93.
35. Thiebaut, R., Esmiol, S., Lecine, P., Mahfouz, B., Hermant, A., Nicoletti, C., Parnis, S., Perroy, J., Borg, J.P., Pascoe, L., Hugot, J.P., and Ollendorff, V. (2016). Characterization and Genetic Analyses of New Genes Coding for NOD2 Interacting Proteins. *PLoS One* 11, e0165420.
36. Versteeg, G.A., Rajsbaum, R., Sanchez-Aparicio, M.T., Maestre, A.M., Valdiviezo, J., Shi, M., Inn, K.S., Fernandez-Sesma, A., Jung, J., and Garcia-Sastre, A. (2013). The E3-ligase TRIM family of proteins regulates signaling pathways triggered by innate immune pattern-recognition receptors. *Immunity* 38, 384-398.

37. Wang, J., Liu, B., Wang, N., Lee, Y.M., Liu, C., and Li, K. (2011). TRIM56 is a virus- and interferon-inducible E3 ubiquitin ligase that restricts pestivirus infection. *J Virol* 85, 3733-3745.

38. Wang, L., Fu, B., Li, W., Patil, G., Liu, L., Dorf, M.E., and Li, S. (2017). Comparative influenza protein interactomes identify the role of plakophilin 2 in virus restriction. *Nat Commun* 8, 13876.

39. Wang, S., Chen, Y., Li, C., Wu, Y., Guo, L., Peng, C., Huang, Y., Cheng, G., and Qin, F.X. (2016). TRIM14 inhibits hepatitis C virus infection by SPRY domain-dependent targeted degradation of the viral NS5A protein. *Sci Rep* 6, 32336.

40. Wilkins, C., and Gale, M., Jr. (2010). Recognition of viruses by cytoplasmic sensors. *Curr Opin Immunol* 22, 41-47.

41. Yang, C., Zhao, X., Sun, D., Yang, L., Chong, C., Pan, Y., Chi, X., Gao, Y., Wang, M., Shi, X., Sun, H., Lv, J., Gao, Y., Zhong, J., Niu, J., and Sun, B. (2016). Interferon alpha (IFNalpha)-induced TRIM22 interrupts HCV replication by ubiquitinating NS5A. *Cell Mol Immunol* 13, 94-102.

42. Zhang, S., Guo, J.T., Wu, J.Z., and Yang, G. (2013). Identification and characterization of multiple TRIM proteins that inhibit hepatitis B virus transcription. *PLoS One* 8, e70001.

43. Zhao, M., Wang, L., and Li, S. (2017). Influenza A Virus-Host Protein Interactions Control Viral Pathogenesis. *Int J Mol Sci* 18.

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