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Review Article

Regulation of Cellular Antiviral Signaling by Modifications of Ubiquitin and Ubiquitin-like Molecules

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
AIM, absent in melanoma; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; CRL, cullin-RING E3 ligase; CYLD, cylindromatosis; dsDNA, double-stranded DNA; DUB, deubiquitinating enzyme; E1, activating enzyme; E2, conjugating enzyme; E3, ligase; IFI, interferon-induced protein; IFN, interferon; IKK, IκB kinase; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; MAVS, mitochondrial antiviral-signaling protein; MDA, melanoma differentiation-associated gene; MITA,

ABSTRACT

The initiation of cellular antiviral signaling depends on host pattern-recognition receptors (PRRs)-mediated recognition of viral nucleic acids that are known as classical pathogen-associated molecular patterns (PAMPs). PRRs recruit adaptor proteins and kinases to activate transcription factors and epigenetic modifiers to regulate transcription of hundreds of genes, the products of which collaborate to elicit antiviral responses. In addition, PRRs-triggered signaling induces activation of various inflammasomes which leads to the release of IL-1β and inflammation. Recent studies have demonstrated that PRRs-triggered signaling is critically regulated by ubiquitin and ubiquitin-like molecules. In this review, we first summarize an updated understanding of cellular antiviral signaling and virus-induced activation of inflammasome and then focus on the regulation of key components by ubiquitin and ubiquitin-like molecules.

Keywords: Innate immune responses; Signal transduction; Regulation; Ubiquitin; SUMO; NEDD8; ISG15

INTRODUCTION

About 3 decades ago, the late master Charles Janeway Jr. proposed the concept that recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) activated effector functions of primitive immune systems prior to the development of adaptive immune responses and played a key role in host defense in both invertebrates and vertebrates (1). Since then, tremendous efforts have been taken to characterize the PAMPs, PRRs and molecules involved in primitive immunity, from which we know that viral nucleic acids are classical PAMPs recognized by at least 2 types of PRRs, membrane-located toll-like receptors (TLRs) and cytosol-located receptors (2). After binding to viral nucleic acids, these PRRs either recruit adaptor proteins or produce immuno-stimulating factors to trigger signaling to activate transcription of hundreds of downstream genes (3). For example, TLR3, TLR7/8, and TLR9 recruit adaptor proteins toll/interleukin-1 receptor-domain containing adaptor-inducing interferon (IFN)-β (TRIF) or myeloid differentiation protein 88 (MyD88) for signal transduction in immune cells such as dendritic cells and macrophages, and cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) recruit adaptor proteins virus-induced signaling adaptor (VISA) (also

mediator of interferon regulatory factor 3 activation; MyD88, myeloid differentiation protein 88; NEDD8, neural precursor cell expressed, developmentally downregulated 8; NEDP1, NEDD8-specific protease 1; NIK, NF-κB-inducing kinase; NLR, nucleotide oligomerization domain-like receptor; NLRP3, nucleotide oligomerization domain-like receptor family pyrin domain containing 3; OTUB1, OTU domain-containing ubiquitin aldehyde-binding protein 1; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; RIG-I, retinoic acid-inducible gene I; RLR, retinoic acid-inducible gene I-like receptor; RNF, ring finger protein; ROS, reactive oxygen species; SENP, small ubiquitin-related modifier-specific protease; TBK, Tank-binding kinase; TLR, toll-like receptor; TRIF, Toll/interleukin-1 receptor-domain containing adaptor-inducing interferon-β; TRIM, tripartite motif containing; Ubc, ubiquitin-conjugating enzyme; UBL, ubiquitin-like molecules; USP, ubiquitin-specific peptidase; VISA, virus-induced signaling adaptor; WWP, WW domain-containing protein

Author Contributions

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known as mitochondrial antiviral-signaling protein [MAVS], IFN-β promoter stimulator 1 [IPS-1], and caspase recruitment domain [CARD] adapter inducing IFN-β [Cardif]) and function in almost all types of cells (4-7), whereas the double-stranded DNA (dsDNA) sensors cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS) and RNA polymerase III generate cyclic GMP-AMP (cGAMP) and 5′ppp RNA which activate MITA (also known as stimulator of IFN genes [STING]), and RIG-I-VISA pathways, respectively (8-13). The adaptor proteins further recruit kinases including the canonical and noncanonical inhibitor of κB kinases to activate transcription factors IFN regulatory factor (IRF) 3, IRF7, and NF-κB, which collaborate to induce transcription of type I IFNs and pro-inflammatory cytokines. In addition to TLRs, RLRs, and dsDNA sensors, cytosolic nucleotide oligomerization domain-like receptors (NLRs) are also involved in recognition of viral nucleic acids. It has been reported that NLR family pyrin domain containing 3 (NLRP3) and absent in melanoma 2 (AIM2) detect RNA and DNA viruses and activate apoptosis-associated speck-like protein containing a CARD (ASC)-dependent signaling (14,15). In contrast to induction of gene transcription, ASC-mediated signaling activates caspase 1 which cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 to trigger robust inflammatory responses.

Ubiquitination is mediated through sequential catalytic reactions by 3 enzymes referred to as activating enzyme (E1), conjugating enzyme (E2), and ligase (E3), leading to the conjugation of mono- or poly-ubiquitin chains on the ε-amino (ε-NH2) group of lysine residues in target proteins. It is well recognized that different linkages of polyubiquitin chains exert distinct functions (16). For example, K63-linked ubiquitination of RIG-I catalyzed by tripartite motif containing (TRIM) 25, Riplet (also known as ring finger protein [RNF] 135 and REUL), TRIM4 and MEX3C is believed to promote the activation of RIG-I, whereas K48-linked ubiquitination of RIG-I by RNF125, c-Cbl, and IFN-induced protein (IFI) 35 destabilizes RIG-I.

In addition to ubiquitin, host genome encodes several ubiquitin-like molecules, including small ubiquitin-related modifier (SUMO), neural precursor cell expressed, developmentally downregulated 8 (NEDD8), and IFN-stimulated gene 15 (ISG15), which are powerful regulators of cellular antiviral signaling (17-20). The ubiquitin-like modifications are also mediated by E1, E2, and E3. Specifically, ubiquitin-conjugating enzyme (Ubc) 9, Ubc12, and UbcH8 are well characterized E2s for SUMOylation, NEDDylation, and ISGylation, respectively. It has been reported that these ubiquitin-like modifiers regulate the function of targeted proteins through at least three mechanisms. First, the ubiquitin-like modification interferes with ubiquitination on the same lysine residues of targeted protein, and thereby modulates its activity or stability. Second, the ubiquitin-like modification provides steric obstacles or docking sites, and thereby affecting the assembly of signaling complexes. Third, the ubiquitin-like modification remodels the structures and physical or chemical properties, thereby affecting the function or localization of targeted proteins. For example, SUMOylation of poly(C)-binding protein 2 (PCBP2) promotes its translocation from nucleus to cytosol, where it associates with and degrades VISA, whereas SUMOylation of IRF3 on Lys70/87 prevents its ubiquitination and degradation (21,22).

Considering the important roles of ubiquitin and ubiquitin-like modifications in cellular antiviral responses, it is conceivable that the reverse deconjugation process of ubiquitin and ubiquitin-like modifiers would be as equally important. The reversal reactions are exerted by deubiquitinating enzymes (DUBs) which remove ubiquitin modifications and ubiquitin-like proteases including sentrin/SUMO-specific protease (SENP), NEDD8-specific protease 1 (NEDP1, also known as SENP8 or DEN1) and COP9 signalosome complex subunit 5 (CSN5), and UBP43 (also known as ubiquitin-specific peptidase [USP] 18) which catalyze removal

of SUMO, NEDD8, and ISG15 from the target proteins, respectively (17-20). Their roles in cellular innate antiviral signaling have been emerging (19,23).

In this review, we will summarize the most recent advancement in our understanding of innate cellular antiviral signaling and then focus on the regulation of key components involved in these pathways by ubiquitin and ubiquitin-like molecules.

VIRUS-TRIGGERED PRRS-MEDIATED SIGNALING

Signaling leading to type I IFN induction

The induction of type I IFNs is one of the hallmarks after viral infection and is dependent on the transcription factors IRF3/7 and NF-κB, which are activated by the PRR-adaptor-kinase signaling cascades (**Fig. 1**). Binding of the PRRs to viral nucleic acids induces dimerization or oligomerization of adaptor proteins, which further recruit kinases in a ubiquitin- and phosphorylation-dependent manner. It has been reported that VISA is ubiquitinated by TRIM31 and MITA is conjugated with TRIM56- or TRIM32-mediated K63-linked ubiquitination and AMFR/gp98-mediated K27-linked ubiquitination, all of which are essential for the recruitment of Tank-binding kinase (TBK) 1 and IκB kinase (IKK) ε (2–27). In addition, the TRAF family

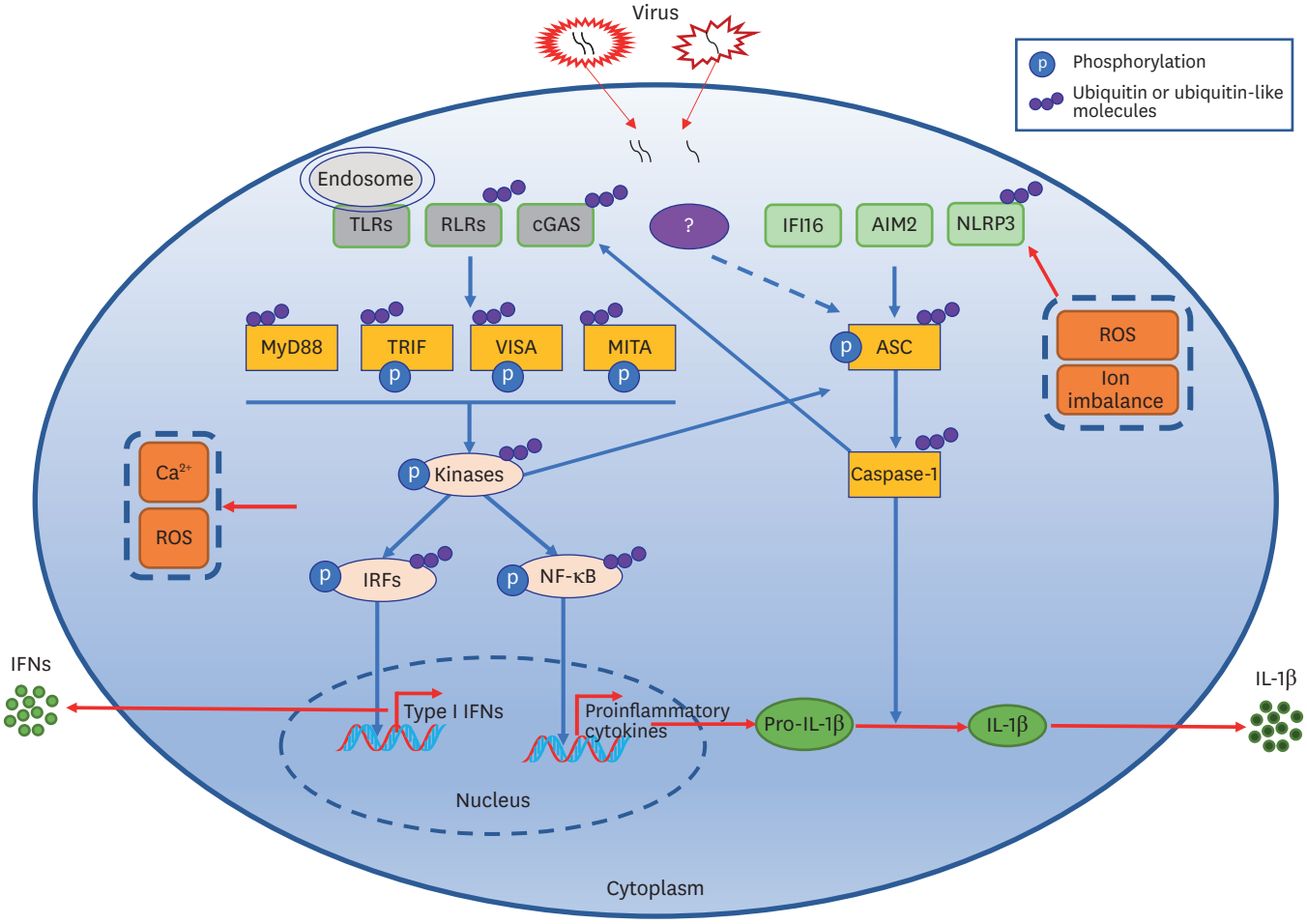

Figure 1. Virus-triggered PRRs-mediated signaling pathways leading to type I IFN and IL-1β induction.

proteins, TRAF2, TRAF3, TRAF5, and TRAF6 and death domain-containing (DD) family proteins, tumor necrosis factor receptor-associated death domain protein (TRADD), Fas-associated death domain protein (FADD), and receptor-interacting protein (RIP) act as scaffolding proteins to promote the recruitment of TBK1 and IKKε and their ubiquitination is required for this process (28,29). In the adaptor-scaffold-kinase complex, TBK1 or IKKε phosphorylates conserved serine residues on TRIF, VISA, or MITA, thereby providing a negative charged platform to recruit the transcription factor IRF3 through the charged surfaces on the C terminus of IRF3 (30). Although it is believed that IRF7 is activated in a manner similar to IRF3, the direct experimental evidence is still lacking. In contrast, IRF7 undergoes ubiquitination by TRAF6 and is phosphorylated by IKKα in plasmacytoid dendritic cells (pDCs) downstream of TLR7 or TLR9 and MyD88, which induces robust expression of IFN-α (31-33).

The ubiquitination and oligomerization of adaptor proteins also recruits TAK1 and IKKα/β/γ complex in a manner decedent on the TRAF and DD family proteins. The IKKα/β/γ complex phosphorylates IκBα that results in its ubiquitination and degradation, releasing the p65/p50 dimer from the cytoplasm into nucleus. IKKα/β also phosphorylates p65 to further enhance its transcriptional activity, as inhibition of IKKα/β-p65 association by INKIT impairs p65 activation and induction of type I IFNs (34). On the other hand, infection by RNA viruses activates NF-kB-inducing kinase (NIK)-IKKα which phosphorylates p100 and induces the partial degradation of p100 into p52 and p52 enters into nucleus and binds to the promoter of *IFNB* gene to inhibit its transcription (35). How viral infection triggers NIK-IKKα activation is less clear but at least requires the viral RNA receptor RIG-I. In addition, whether and how the NIK-IKKα-p100 axis is activated upon DNA virus infection are of great interest and await for further investigations.

Signaling leading to IL-1β production

Accumulating evidence suggests that the induction of IL-1β is another hallmark after viral infection, which is essential for host defense against viruses and involves a 2-step transcription and cleavage process (**Fig. 1**) (36). The transcription of pro-IL-1β depends on viral infection-induced activation of NF-κB, whereas the maturation of IL-1β requires activation of inflammasomes which are multi-component complex consisting of PRRs, adaptor protein ASC and caspase 1 (36,37). The PRRs initiating the inflammasome assembly after viral infection include NACHT, LRR, and PYD domains-containing protein 3 (NLPR3), AIM2, RIG-I, and IFI16. AIM2 and IFI16 are dsDNA sensors that directly recruit ASC and caspase 1, leading to cleavage of pro-IL-1β into mature IL-1β (38,39). It is thus conceivable that infection of various DNA viruses and retroviruses (whose reverse transcription generates ssDNA that adopts stem-loop structures) activate the AIM2 or IFI16 inflammasome to induce IL-1β production (40). However, it is less clear whether and how the AIM2 and IFI16 inflammasomes function cooperatively or in a virus-specific manner. For example, AIM2 inflammasome-induced IL-1β induction is essential for defense against vaccinia virus and mouse cytomegalovirus, whereas IFI16 inflammasome detects HSV-1 infection to trigger IL-1β induction (38,40). One possible explanation for this is that diverse inflammasomes increase the chance to counteract the evading strategies evolved by different viruses.

In addition to inducing transcription of the genes encoding ASC, pro-caspase 1 and pro-IL-1β, RIG-I also engages inflammasome activation by directly interacting with ASC (41). However, whether similar mechanisms apply to RIG-I mediated recruitment of VISA and ASC requires further investigations. TLR7 and TLR8 are required for inflammasome activation in human monocytes and macrophages after HIV or HCV infection, and it is unknown whether they function to induce pro-IL-1β and/or ASC transcription or directly activate ASC-dependent inflammasomes (42). It is likely that during TLRs or RLRs signaling and viral replication, a number of danger

signals such as reactive oxygen species (ROS) and ion influx or efflux are generated as byproducts which are detected by other sensors such as NLRP3 to activate inflammasomes (discussed below). Whether exist cytoplasmic RNA sensors that simultaneously recognize RNA and associates with ASC-pro-caspase 1 to activate inflammasome is of great interest.

NLRP3 belongs to NLR family and directly interacts with ASC and pro-caspase 1 to form an inflammasome, in which pro-caspase 1 is cleaved into active caspase 1 to cleave pro-IL-1β into IL-1β (43). Unlike TLRs, RLRs or cytoplasmic DNA sensors that directly bind to viral nucleic acids, NLRP3 lacks a nucleic acid binding motif and senses a variety of danger signals such as ROS, ion imbalance, cathepsin B and extracellular ATP for activation (43). Viral infection activates the danger signals either by triggering nucleic acid sensors-mediated signaling or by the virus-encoding viroporins. For example, influenza virus infection induces ROS during viral internalization or by activating RIG-I or TLR7 signaling and the ion channel protein M2 encoded by the influenza genome disturbs the ionic milieu in the cytoplasm, whereas influenza virus infection-induced cell death leads to release of ATP to the local infected matrix (44,45). DNA viruses such as adenovirus and myxoma virus trigger the induction of ROS and cathepsin B to activate the NLRP3 inflammasome (46). Thus, the NLRP3 inflammasome provides a house-keeping guard in detection of danger signals generated during infection and replication of DNA and RNA viruses, although the precise mechanism of NLRP3 inflammasome activation in response to the danger signals requires additional investigations.

The signaling pathways involved in the induction IFNs and IL-1β cross-talk with each other extensively. The kinase IKKε phosphorylating IRF3 also phosphorylates ASC at Ser58 to promote the translocation of ASC into cytoplasm, and the kinase IKKα phosphorylating IκBα phosphorylates ASC at Ser19/193 to inhibit its oligomerization (47). Recently, it has been demonstrated that inflammasome-activated caspase 1 cleaves cGAS and thereby impair DNA virus-induced expression of type I IFNs (48). It seems that the mutual regulation facilitates appropriate cellular immune responses against invading viruses.

REGULATION OF VIRUS-TRIGGERED PRRS-MEDIATED SIGNALING

Virus-triggered PRRs-mediated signaling must be tightly controlled to mount protective immune responses and avoid harmful immune pathology. Cumulative evidence has suggested that posttranslational modifications by ubiquitin and ubiquitin-like molecules are critically involved in this process by conjugating to the ε-NH2 group of lysine residues through the conserved C terminal-GG motif (**Fig. 2**) (49). Here we will discuss the most

		E2s	Deconjugating enzymes
Ubiquitin:	69-VLRLRGG-76	Ubc13, etc	DUBs
NEDD8:	70-VLALRGG-76	UBE2M/UBE2F	SENP8/CSN5
ISG15:	151-NLRLRGG-157	UbcH8	USP18
Sumo1:	89-EVYQEQTGG-97	Ubc9	SENP1/2/3/7
Sumo2:	85-DVFQQQTGG-93		
Sumo3:	122-DVFQQQTGG-130		
Sumo4:	85-DVFQQPTGG-93		

Figure 2. The C terminal conserved amino acids of ubiquitin and ubiquitin-like molecules.

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recent progresses on the regulation of virus-triggered PRRs-mediated signaling by ubiquitination, ISGylation, SUMOylation, and NEDDylation (**Table 1**).

Table 1. Ubiquitin and ubiquitin-like modifications in virus-triggered PRRs-mediated signaling pathways

Target molecules	E3 ligases-mediated ubiquitination	Modification sites	References
PRRs			
RIG-I	RNF122 (K48 linkage)	Lys115, Lys 146	Wang et al. (56)
	CHIP (K48 linkage)	ND	Zhao et al. (136)
	CypA-TRIM25 (K63 linkage)	Lys172	Liu et al. (50)
			Gack et al. (51)
	p97-RNF125 (K48 linkage)	Lys181	Hao et al. (55)
	TRIM40 (K27 and K48 linkage)	ND	Zhao et al. (59)
	Ube2D3, Ube2N (K63 linkage)	Lys48, Lys96, Lys172	Shi et al. (52)
	MUL1 (SUMOylation)	ND	Doiron et al. (105)
	USP15 (K63 linkage)	-	Zhang et al. (60)
	UbcH8 (ISGylation)	ND	Arimoto et al. (118)
	TRIM38 (SUMOylation)	Lys96, Lys888	Hu et al. (106)
	SENP2	-	-
MDA5	TRIM65 (K63 linkage)	Lys743	Lang et al. (67)
	TRIM40 (K27 and K48 linkage)	Lys23, Lys43, Lys68	Zhao et al. (59)
	PIAS2β (SUMOylation)	ND	Fu et al. (104)
	TRIM38 (SUMOylation)	Lys43, Lys865	Hu et al. (106)
cGAS	RNF185 (K27 linkage)	ND	Wang et al. (68)
	TRIM38 (SUMOylation)	Lys83, Lys231, Lys479	Hu et al. (107)
	SENP2	-	-
	SENP7	Lys335, Lys372, Lys382	Cui et al. (108)
	USP14 (K48 linkage)	Lys414	Chen et al. (69)
NLRP3	FBXL12 (K48 linkage)	Lys689	Han et al. (71)
	MARCH7 (K48 linkage)	ND	Yan et al. (72)
AIM2, IFI16	ND	-	-
Adaptor proteins			
TRIF	TRIM38 (K48 linkage)	Lys228	Xue et al. (73)
			Hu et al. (74)
	WWP2 (K48 linkage)	ND	Yang et al. (75)
	TRIM32 (ubiquitin-independent)	-	Yang et al. (76)
MyD88	CYLD (K63 linkage)	Lys231	Lee et al. (77)
	Smurf1/2 (K48 linkage)	Lys231, Lys262	Naiki et al. (78)
			Strickson et al. (79)
	Pellino (K48 linkage)	ND	Ji et al. (80)
	ND (NEDDylation)	ND	Yan et al. (131)
VISA	Tetherin-MARCH8 (K48 linkage)	Lys7	Jin et al. (82)
	TRIM31 (K63 linkage)	Lys10, Lys311, Lys461	Liu et al. (24)
	IRTKS-PCBP2 (SUMOylation)	-	Xia et al. (21)
MITA	TRIM29 (K48 linkage)	ND	Xing et al. (83)
	MUL1 (K63 linkage)	Lys224	Ni et al. (84)
	TRIM30α (K48 linkage)	Lys275	Wang et al. (85)
	AMFR (K27 linkage)	-	Wang et al. (25)
	USP21 (K27/63 linkage)	-	Chen et al. (88)
	USP18-USP20 (K48 linkage)	-	Zhang et al. (86)
	USP13 (K27 linkage)	-	Sun et al. (87)
	TRIM38 (SUMOylation)	Lys338	Hu et al. (107)
ASC	TRAF3 (K63 linkage)	Lys174	Guan et al. (95)
TRAF3, TRAF6	ERα (K48 linkage)	ND	Wang et al. (90)
	cIAP1/2 (K63 and K48 linkage)	-	Mao et al. (89)
	USP25 (K48 linkage)	-	Lin et al. (93)
	MYSM1 (K63 linkage)	-	Panda et al. (94)
	HSCARG-OTUB1 (K48 linkage)	-	Peng et al. (91)

(continued to the next page)

Table 1. (Continued) Ubiquitin and ubiquitin-like modifications in virus-triggered PRRs-mediated signaling pathways

Target molecules	E3 ligases-mediated ubiquitination	Modification sites	References
Kinases and transcription factors			
TBK1	MIB2 (K63 linkage)	ND	Ye et al. (137)
	RNF128 (K63 linkage)	Lys30, Lys401	Song et al. (98)
	USP1–UAF1 (K48 linkage)	ND	Yu et al. (138)
	DYRK2-DTX4 (K48 linkage)	Lys670	Cui et al. (139)
			An et al. (96)
	ND (SUMOylation)	Lys694	Saul et al. (140)
IRF3	c-Cbl (K48 linkage)	ND	Zhao et al. (99)
	LUBAC (linear)	Lys193, Lys313	Chattopadhyay et al. (100)
	ND (SUMOylation)	Lys152	Kubota et al. (110)
	PIAS1 (SUMOylation)	ND	Li et al. (112)
	SENP2	Lys70, Lys87	Yang et al. (22)
	HERC5 (ISGylation)	Lys193, Lys360, Lys366	Lu et al. (119)
			Shi et al. (120)
IRF7	TRIM28 (SUMOylation)	Lys444, Lys446	Liang et al. (111)
	ND (SUMOylation)	Lys406	Kubota et al. (110)
	TRAF6 (K63 linkage)	Lys44, Lys446, Lys452	Ning et al. (101)
Pro-IL-1β	A20 (K63 linkage, unanchored)	Lys133	Duong et al. (141)

ND, not determined.

UBIQUITINATION AND DEUBIQUITINATION

Regulation of PRRs

It has been shown that TRIM25 catalyzes K63-linked ubiquitination of RIG-I at Lys172, and this process is promoted by the peptidyl-prolyl cis/trans isomerase cyclophilin A (CypA) (50,51), leading to potentiated recruitment of VISA and induction of type I IFNs. In addition to TRIM25, multiple E3s such as TRIM4, Riplet, and MEX3C have been suggested to mediate K63-linked ubiquitination and activation of RIG-I. Specifically, Ube2D3 promotes Riplet-mediated conjugation of polyubiquitin chains to RIG-I and Ube2N facilitates Riplet to sythsize unanchored polyubiquitin chains, which are prerequisites for full activation of RIG-I (52). These E3s may function in a synergistic way, as supported by recent studies that use a systemic biology approach to demonstrate that TRIM4 and TRIM25 exhibit dose-dependent synergism for RIG-I activation and catalyze ubiquitination of RIG-I on multiple lysine residues in a hierarchical way, which provides an efficient and optimal synergistic regulatory module in antiviral immune responses (53,54). Recently, it has been reported that the E3 ubiquitin ligase RNF122 delivers K48-linked ubiquitination to the Lys115 and Lys146 residues of RIG-I and p97 recruits RNF125 to ubiquitinate RIG-I at Lys 181 and thereby promote RIG-I degradation, leading to the inhibition of RIG-I-mediated signaling (55,56). RNF125 and c-Cbl have been shown to catalyze K48-linked ubiquitination and degradation of RIG-I (57,58). A more recent study has shown that TRIM40 catalyzes K27- and K48-linked ubiquitination of RIG-I, leading to its proteasome-dependent degradation and suppression of antiviral responses (59). However, it is unknown whether the negative ubiquitin signals on RIG-I also conform to the synergistic regulatory model.

USP15 is a DUB that deubiquitinates K63-linked ubiquitination of RIG-I, thereby turning down type I IFNs induction (60). Interestingly, however, results from another study suggest that USP15 deconjugates K48-linked ubiquitin chains from TRIM25 and promotes its stability and thus facilitates K63-linked ubiquitination of RIG-I and virus-triggered expression of type I IFNs (61). The reasons for discrepancies between these 2 studies are unknown. In addition, a number of DUBs including cylindromatosis (CYLD), USP3, USP21, USP4, and USP17 are also involved in the regulation of K63- or K48-linked ubiquitination of RIG-I (62-66). It is

so far unclear whether these DUBs function redundantly or hierarchically in cellular innate antiviral responses.

The observation that TRIM25 targets RIG-I but not melanoma differentiation-associated gene (MDA) 5 for ubiquitination prompts investigators to characterize E3s that are responsible for MDA5 ubiquitination and activation. The identification of TRIM65 as the E3 catalyzing K63-linked ubiquitination of MDA5 has filled in gap in our understanding of the mechanisms by which MDA5 triggers signaling. Unlike the activation of RIG-I requires ubiquitination on the N terminal CARD domain, TRIM65 promotes K63-linked ubiquitination of MDA5 at the C terminal Lys743, which is critical for MDA5 oligomerization and activation. Trim65 deficiency abolishes MDA5 agonist- or encephalomyocarditis virus (EMCV)-induced type I IFN production (67). Whether there exist E3s specifically regulating the stability of MDA5 and whether there are DUBs counteracting this process require further investigations.

The DNA sensor cGAS undergoes K27-linked ubiquitination by RNF185 after HSV-1 infection, which promotes its enzymatic activity (68). Interestingly, cGAS is modified by K48-linked ubiquitination at Lys414, which leads to p62-dependent selective autophagic degradation. TRIM14 recruits USP14 to deconjugate such a modification, thereby promote cGAS signaling (69). Upon challenge, AIM2 and NLRP3 undergo ubiquitination and recruit the adaptor p62 for autophagic degradation (70), and blocking autophagy potentiates AIM2 and NLRP3 inflammasome activity. While FBXL2 and MARCH7 target NALP3 for ubiquitination and degradation (71,72), the E3s responsible for the degradative ubiquitin modification of cGAS and AIM2 remain to be characterized.

Regulation of adaptor proteins

The TLR3-TRIF and TLR7/8-MyD88 pathways mediate immune responses against certain viruses such as West Nile virus and influenza virus. It has been shown that WW domain-containing protein (WWP) 2 and TRIM38 promote K48-linked ubiquitination of TRIF to induce its proteasome-dependent degradation, whereas TRIM32 induces the autophagic degradation of TRIF in a manner independent of ubiquitination (73-76). These E3s may function redundantly or cooperatively in a cell-type or signal specific manner. For example, WWP2 functions in bone marrow-derived macrophages (BMDMs) but not bone marrow-derived dendritic cells (BMDCs) and specifically regulates TLR3- but not TLR4-mediated innate immune and inflammatory responses, whereas TRIM38 and TRIM32 function in various types of cells and regulate both TLR3 and TLR4 signaling. The activation of MyD88 requires K63-linked ubiquitination at Lys231 after *Haemophilus* influenzae infection which is deconjugated by CYLD (77) and the E3s for this modification remain to be identified. The TLR-MyD88 signaling is turned down by TGF-β treatment which induces Smurf1/2-mediated K48-linked ubiquitination and degradation of MyD88 (78,79). In addition, Ndrp1 and Drosophila Pellino catalyze K48-linked ubiquitination and degradation of MyD88 (80,81). Although these E3s function as negative or positive regulators of TRIF or MyD88, their role in regulating cellular antiviral responses remains uninvestigated.

A number of E3s have been identified to catalyze K48-linked ubiquitination and proteasome-dependent degradation of VISA. Recently, tetherin has been found to recruit the E3 ubiquitin ligase membrane-associated RING-CH-8 (MARCH8) to catalyze K27-linked ubiquitin chains on MAVS at lysine 7 for autophagic degradation (82), and TRIM31 catalyzes K63-linked ubiquitination of VISA at Lys10, Lys311, and Lys461 to promote aggregation of VISA and the recruitment of TBK1 (24), thereby inhibiting or promoting cellular antiviral responses

and type I IFN induction. It has been so far unclear whether and how DUBs antagonize this process. About one dozen of E3s have been reported to catalyze K11-linked (RNF26), K27-linked (AMFR), K48-linked (RNF5, TRIM30α, and TRIM29) or K63-linked (TRIM32, TRIM56, and MUL1) ubiquitination of MITA at various lysine residues (49,83-85). Recently, we demonstrated that USP18 recruits USP20 to deubiquitinate and stabilize MITA to promote type I IFN induction and USP13 directly removes K27-linked ubiquitin chains from MITA to turn down antiviral signaling (86,87). In addition, another study has shown that USP21 deconjugates K27- and K63-linked ubiquitin chains on MITA and restricts cellular antiviral signaling and autoimmunity (88). These studies indicate a highly dynamic regulation of MITA which facilitates efficient and appropriate immune responses against viruses.

In addition to autoubiquitination, TRAF3 and TRAF6 are also ubiquitinated by cIAP1/2, which is required for virus-triggered signaling (89). Interestingly, a recent study shows that estrogen receptor (ER) α inhibits vesicular stomatitis virus (VSV)-induced IRF3 activation via promoting K48-linked ubiquitination of TRAF3, and whether cIAP1/2 or other E3s are involved in this process is unknown (90). The K48-linked ubiquitination of TRAF3 can be reversed by OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1), in which NmrA like redox sensor 1 (NMRAL1 also known as HSCARG) is required (91). However, our previous study has demonstrated that OTUB1/2 remove K63-linked polyubiquitin chains from TRAF3 and TRAF6, thereby inhibiting virus-triggered induction of type I IFNs (92). We and others have shown that USP25 and MYSM1 deconjugate K48- and K63-linked ubiquitin chains from TRAF3 and promote or inhibit cellular antiviral signaling, respectively (93,94). It should be noted that TRAF3 also catalyzes K63-linked ubiquitination of ASC to promote inflammasome activation, indicating an essential crosstalk between virus-triggered type I IFN induction and IL-1β production pathways (95).

Regulation of kinases and transcription factors

It has been previously shown that TBK1 undergoes deltex (DTX) 4-mediated K48-linked ubiquitination, and later we found that the kinase DYRK2-mekdiated phosphorylation on Ser527 of TBK1 is a prerequisite for its ubiquitination and degradation (95,96). The DLAIS motif of VISA recruits MIB2 to promote K63-linked ubiquitination of TBK1 (97) and Song et al have characterized another E3 ligase RNF128 as an E3 mediating K63-linked ubiquitination of TBK1 at Lys30 and Lys401 to promote activation of TBK1 and type I IFN induction after viral infection (98). The activation and regulation of IKKε by ubiquitination is less clear.

We and others have shown that forkhead box protein O1 (FOXO1) and multiple E3s such as RBCK1 and RAUL catalyze K48-linked ubiquitination and degradation of IRF3 (49). Recently, another E3 c-Cbl promotes K48-linked polyubiquitination and proteasomal degradation of IRF3 (99). In addition, linear ubiquitin chain assembly complex (LUBAC) catalyzes linear ubiquitination of IRF-3 at Lys193 and Lys313 to induce RLR-triggered apoptosis and blocks viral pathogenesis (100). IRF7 is ubiquitinated by TRAF6 and involved in TLR7/9-meidated type I IFN induction (33,101). The DUBs responsible for the reverse modifications of the kinases or transcription factors remain to be characterized.

SUMOYLATION AND DE-SUMOYLATION

SUMOylation has been shown to essentially engage PRRs-mediated signaling and type I IFN induction (102). It has previously been observed that RIG-I and MDA5 undergo SUMOylation

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after viral infection, and MUL1 and PIAS2b are E3s for RIG-I and MDA5 SUMOylation, respectively (103-105). In a recent study, Hu et al. (106) have elegantly shown that TRIM38 mediates SUMOylation of RIG-I and MDA5 at Lys96/888 or Lys43/865, respectively. This SUMOylation prevents the K48-linked ubiquitination and degradation. At late time points after viral infection, the SUMOylation of RIG-I and MDA5 is removed by SENP2, which allows for K48-linked ubiquitination and degradation (106). Moreover, they further demonstrate that cGAS and MITA are also Sumoylated by TRIM38 and de-Sumoylated by SENP2 in a similar way (107). Results from another study shows that cGAS is Sumoylated at Lys335/372/382, and SENP7 removes Sumo molecules from cGAS, rendering the ability to sense DNA by cGAS (108). These studies together reveal dynamic SUMOylation and de-SUMOylation and the crosstalk with ubiquitination in modulating innate antiviral signaling.

The kinase TBK1 is also sumoylated at Lys694 which confers it antiviral activity, although the E3s for this process are unknown (109). Viral infection induces SUMOylation of IRF3 and IRF7 at Lys152 and Lys406, respectively, which negatively regulates their transcription activity (110). The E3s TRIM28 and PIAS1 mediate SUMOylation of IRF7 and IRF3 to repress their transcription activity (111,112). However, in another study, viral infection induces SUMOylation of IRF3 at Lys70 and Lys87 to stabilize IRF3, which are K48-linked ubiquitination sites. SENP2 de-Sumoylates IRF3 and renders IRF3 sensitive to ubiquitination and proteasome degradation (22). These data together suggest SUMOylation on different lysine residues of IRF3 or IRF7 may facilitate positive and negative roles of innate antiviral signaling. A systemic analysis of SUMOylation of host proteins has been conducted recently and it is hoped that more sumoylated proteins and their roles in viral infection will be uncovered in a near future (113,114).

ISGYLATION AND DE-ISGYLATION

ISG15 is an IFN-stimulated gene that can be conjugated to lysine residues of target proteins. The process is called ISGylation and required to mount efficient immune response against viruses. Firstly, it has been shown that genetically deletion of ISG15 in mice leads to hypersensitivity to viral infections (115). Secondly, deletion of USP18 or knock-in USP18^{C61A} increases ISGylation in cells and renders cells more resistant to viral infection after type I IFN treatment (116,117). Third, the USP18^{C61A} mice are resistant to viral infections (117). Later it has been shown that RIG-I and IRF3 are modified by ISG15, which impairs the K48-linked ubiquitination and degradation of RIG-I and IRF3 by RNF125 and Pin1 respectively and promotes type I IFN induction (118-120). Indeed, ISG15 is likely to target newly synthesized proteins including IFN-stimulated genes and viral proteins (120), which modulates the functions of host proteins and dominantly inhibits the functions of viral proteins. However, there is evidence suggesting that the free ISG15 also plays a role in antiviral responses, supported by the observation that deletion of E1 for ISGylation UBE1L protects mice from Chikungunya virus (CHIKV) infection (121). Together, the ISGylation and free ISG15 are essential for host defense against viral infections in mice.

The functions of ISG15 or ISGylation in humans may be different from those in mice. Human patients with ISG15 deficiency exhibit mycobacterial but not viral diseases, and human ISG15 deficient cells are not susceptible to viral infections. In contrast, the deficiency of ISG15 in human leads to reduced production of IFN-γ, indicating that human ISGylation is largely redundant for antiviral immunity and that ISG15 plays an essential role in IFN-γ production for

optimal antimycobacterial immunity (122). In another study, it is shown that ISG15-deficient patients display unanticipated cellular, immunological and clinical signs of autoinflammatory interferonopathies Aicardi-Goutières syndrome and spondyloenchondrodysplasia, which is due to the reduced expression of USP18, a negative regulator of type I IFN signaling (123). More recently, it has been demonstrated that USP18 deficiency in human increases ISGylation and hyper-activation of type I IFN signaling and results in the severe pseudo-toxoplasmosis, rubella, cytomegalovirus, and herpes simplex virus (TORCH) syndrome (124). These data collectively suggest that in humans ISGylation or ISG15 is essential to promote IFN-γ induction and restrict type I IFN signaling, thereby facilitating antibacterial immunity and avoiding interferonopathy. The targets of ISG15 modification and the mechanisms by which ISGylation exerts its function requires further investigations.

NEDDYLATION AND DE-NEDDYLATION

NEDD8 is a ubiquitin-like protein that activates the cullin-RING E3 ligases (CRLs) by conjugating to the conserved lysine residues on the C terminus of cullin proteins. The CRLs are widely involved in the regulation of ubiquitination in various processes including cellular antiviral responses. The most studied example is the F-box containing protein β-transducing repeat-containing protein (βTrCP), a subunit of the SKP1-cullin-F-box (SCF) complex which induces ubiquitination and degradation of phosphorylated IκBα, a prerequisite for NF-κB activation in response to various stimulations such as viral infection. Another CRL consisting of SOCS1 (suppressor of cytokine signaling)-cullin5-Rbx2 mediates ubiquitination and degradation of p65 (125). Although few CRLs have been identified to regulate antiviral signaling pathways, it is expected that dysfunction of NEDDylation regulates antiviral signaling by modulating ubiquitination of key proteins involved in the pathways. Indeed, recent studies have shown that inhibition of NEDDylation by the inhibitor MLN4924 disrupts viral latency and elicits cellular cytotoxicity in a dose-dependent manner (126) and that viruses can hijack the NEDDylation on CRLs to benefit replication (127,128). NEDDylation can occur on non-CRL proteins (18,129), but the E3s for this process remains largely unidentified. For example, TRIM40 promotes neddylation of IKKγ, which inhibits NF-κB activation (130) and MyD88 is neddylated which inhibits dimerization of MyD88 and NF-κB activity by antagonizing its ubiquitination (131).

The NEDDylation can be removed by CSN5 and NEDP1, which deconjugate NEDD8 from CRLs and non-CRL substrates or aberrant neddylated CRLs, respectively (132-134). It is so far unclear whether and how NEDP1-mediated de-NEDDylation of non-CRL substrates regulate virus-triggered PRRs-mediated signaling. A recent study has systemically analyzed neddylated proteins in the absence of NEDP1, which may provide information for future investigations (129).

CONCLUDING REMARKS

Although ubiquitination and deubiquitination have been extensively investigated in the regulation of cellular antiviral signaling, the exact dynamic regulation in specific cells in response to infection of distinct viruses still remains largely unknown and the roles of ubiquitin-like modifications in cellular antiviral signaling have just been emerging. The machinery involved in ubiquitin and ubiquitin-like modifications, the targets of such

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modifications, and the crosstalk among these modifications are of great interest and have attracted increasing attentions from researchers worldwide. In addition, the complicated machinery could be targeted or even hijacked by viruses to facilitate viral replication (49,135). Accumulating evidence has suggested that viral structural or nonstructural proteins could directly modulate ubiquitin or ubiquitin-like modifications on key components involved in antiviral signaling or indirectly utilize host machinery to interfere with the cellular antiviral signaling. It is expected that huge progresses will be made to understand the machinery and strategies targeting the machinery will be developed to treat viral infection-related diseases in the next decade.

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