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Review

Review

Regulation of cellular innate antiviral signaling by ubiquitin modification

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Abstract

Host pattern-recognition receptors (PRRs) recognize pathogen-associated molecular patterns generated by invading viruses and initiate a series of signaling cascades that lead to the activation of interferon-regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB) and subsequent induction of type I interferons (IFNs). Posttranslational modification of proteins by ubiquitin plays an essential role in mediating or regulating the virus-triggered PRRs-mediated signaling. Deubiquitination is the reversible process of ubiquitination and its role in regulating PRRs-mediated signaling has recently been explored. In this review, we first summarize the ubiquitination events in PRRs-mediated signaling that is triggered by viral nucleic acid and then focus on host and viral deubiquitinating enzymes-mediated regulation of virus-triggered signaling that modulates the activation of IRF3 and NF-κB and subsequent induction of type I IFNs.

Key words: ubiquitin modification, pattern-recognition receptor, cellular innate antiviral signaling

Introduction

Innate immune responses are initiated by the recognition of pathogen-associated molecular patterns (PAMPs) via host pattern-recognition receptors (PRRs). The viral PAMPs include structurally conserved RNA or DNA molecules generated during the infection and replication life cycle of viruses. After binding to the viral PAMPs, PRRs activate downstream adaptors and kinases to trigger a series of signaling cascades that lead to the activation of transcription factors nuclear factor-κB (NF-κB) and interferon-regulatory factor (IRF) 3/7 and subsequent induction of type I interferons (IFNs) and other cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) [1]. Type I IFNs then activate the JAK-STAT pathway and induce the expression of a large array of antiviral genes, the products of which collaborate to inhibit viral replication, induce apoptosis of infected cells and elicit cellular innate antiviral responses [2]. To date, two groups of PRRs have been identified to recognize viral nucleic acid and activate the induction of type I IFNs. One group is the intracellular membrane-located Toll-like receptors (TLRs: TLR3, TLR7/8, and TLR9) which signal through adaptor proteins TIR domain-containing adaptor inducing interferon-β (TRIF) or MyD88 and mainly function in immune cells such as dendritic cells, macrophages, and B cells. The other group is localized in the cytoplasm, including retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and cyclic GMP-AMP synthase (cGAS) which recruit adaptor proteins VISA (also known as MAVS, IPS-1, and Cardif) or MITA (also known as STING) and function in almost all types of cells [3–17]. In addition, a number of cytosol DNA sensors have been identified, including RNA polymerase III, DNA-dependent activator of IRFs (DAI), IFN-inducible protein-16 (IFI16), LSm14A, and DDX41 which might recognize cytosolic DNA in a cell-type and/or ligand-specific manner [18–23].

It has been well established that virus-triggered PRRs-mediated activation of NF-κB and IRF3 is critically regulated by various posttranslational modifications. For example, the activation of IRF3 requires phosphorylation by the TANK-binding kinase 1 (TBK1) [24,25], whereas the phosphatase protein phosphatase 2A deactivates IRF3 by catalyzing the dephosphorylation of IRF3 [26]. The phosphorylation status of IRF3 determines its dimerization and nuclear translocation. Like phosphorylation, protein ubiquitination is a reversible process and plays an essential role in PRRs-triggered signaling. In resting cells, the NF-κB dimer p65/p50 is sequestered in the cytoplasm by inhibitor of κB (IκB). Upon viral infection, IκBα is phosphorylated by

IκB kinase (IKK) complex. The E3 ligase β-TrCP binds to and ubiquitinates phosphorylated IκBα, leading to the degradation of IκBα and thereby releasing p65/p50 into the nucleus. NF-κB and dimerized IRF3 bind to the conserved DNA sequences on the promoters of type I IFN genes to initiate their transcriptions.

Deubiquitination is a reversible process of ubiquitination and is mediated by deubiquitinating enzymes (DUBs). About 90 DUBs are encoded by human genome and belong to 5 different families, the ubiquitin-specific protease (USP) (56 members), the ubiquitin C-terminal hydrolases (4 members), the ovarian tumor domain protease (OTU) (14 members), the Machado-Joseph disease protein domain protease (4 members), and the Jab1/Pab1/MPN metalloenzyme motif protease (JAMM) (12 members) [27]. The major functions of DUBs fall into three categories. (i) DUBs hydrolyze ubiquitin precursors which are translated as liner multiple ubiquitin chains or liner ubiquitin fused with ribosomal proteins into free ubiquitin molecules. (ii) DUBs recycle ubiquitin molecules that are conjugated to target proteins for degradation through proteasome, lysosome, or autophagosome. (iii) DUBs modulate the ubiquitin conjugation of target proteins and thereby regulate their activities and signaling, which are involved in various biological processes including immune and inflammatory responses, chromatin remodeling and development [28]. Recently, it has been demonstrated that DUBs also play important roles in cellular innate antiviral signaling. In addition to DUBs encoded by the host, viral genomes also encode DUBs (vDUBs) which may interfere with antiviral signaling and facilitate viral replication.

In this review, we briefly discuss the ubiquitination events involved in virus-triggered PRRs-mediated induction of type I IFNs and then focus on host and viral DUBs-mediated regulation of virus-triggered signaling that modulates the activation of IRF3 and NF-κB and subsequent induction of type I IFNs.

Virus-triggered PRRs-mediated Cellular Innate Antiviral Signaling

TLR-mediated signaling

Among all the TLRs identified, TLR3, TLR7/8, and TLR9 have been demonstrated to sense viral nucleic acid, which induces the expression of type I IFNs. TLR3 recognizes double-strand RNA (dsRNA) and signals through the adaptor protein TRIF, whereas TLR7/8 and TLR9 detect viral single-strand RNA (ssRNA) and unmethylated CpG DNA, respectively, and initiate signaling transduction through the adaptor protein MyD88. TRIF then recruits TNF receptor-associated factor 3 (TRAF3) and TBK1 for the activation of IRF3 and recruits TRAF6 or receptor-interacting protein 1 (RIP1) for the activation of NF-κB, while MyD88 recruits interleukin-1 receptor-associated kinase (IRAK) 1/4 and TRAF6 to form a complex which further interacts with TAK1 and TBK1 to activate NF-κB and IRF7, respectively [29,30]. TRIF- and MyD88-mediated signaling involves a series of ubiquitination events, which could serve as both ‘positive signals’ and ‘negative signals’. Triad3A ubiquitinates and promotes degradation of TLR3 and TLR9, but not TLR2 to inhibit TLR3- and TLR9-triggered signaling [31]. Following TLR3 activation, TRAF3 and TRAF6 catalyze self-ubiquitination and/or undergo ubiquitination by the E3 ligases cIAP1 and cIAP2 and thereby promote activation of IRF3 and NF-κB [32]. Pellino1 promotes K63-linked polyubiquitination of RIP1 and thus selectively facilitates TLR3-mediated activation of NF-κB and induction of pro-inflammatory cytokines [33]. In contrast, the E3 ligases WWP2, TRIM38, and Nrdp1 catalyze K48-linked ubiquitination and induce the proteasome-dependent degradation of TRIF and MyD88, respectively, turning down TLR-mediated signaling [34–36].

RLR-mediated signaling

RLRs include three family members, RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). It is clear that RIG-I binds to 5′-triphosphorylated ssRNA, short fragmented dsRNA, and panhandle-like RNA, and MDA5 recognizes long fragmented dsRNA [37–42]. LGP2 facilitates the binding and recognition of the viral RNA ligands by RIG-I or MDA5 [43]. After binding to the RNA ligands, RIG-I is ubiquitinated and activated by TRIM25, Riplet (also known as RNF135 and REUL), TRIM4, and MEX3C. The binding of Ubc5-dependent free K63-linked ubiquitin chains to RIG-I further promotes its tetramer formation and activation [44–49]. In contrast, RNF125, C-cbl, and interferon-inducible protein IFI35 induce K48-linked ubiquitination and degradation of RIG-I [50–52]. Although viral infection induces ubiquitination of MDA5, it is currently unknown which E3(s) mediate(s) this process.

VISA is the sole adaptor protein downstream of RLRs. The activated RLRs induce the oligomerization of VISA and promote VISA to form prion-like structures for full activation [53]. Although it is not known whether and how the activation of VISA requires ubiquitination modification, it has been shown that K63-linked ubiquitination of VISA mediates the recruitment of inhibitor of IKKε to the mitochondria [54,55]. Several studies have demonstrated that E3s deactivate VISA by promoting K48-linked ubiquitination and degradation of VISA, including AIP4-Itch, RNF5, PSMA7, TRIM25, and Smurf1/2 [56–60]. It is possible that by targeting VISA, these E3s function redundantly or cooperatively to finely tune the innate antiviral responses in different types of cells and/or at different stages after viral infection.

VISA then recruits multiple E3 ligases to activate antiviral signaling cascades, including TRAF3, TRAF2, TRAF5, and TRAF6 [61]. These TRAFs promote ubiquitination that is critical for the recruitment of NF-κB essential modulator (NEMO), leading to the activation of the kinases TBK1 and IKK complex after viral infection. Triad3A and UbL-UBA domain-containing protein RAD23A induce K48-linked ubiquitination of TRAF3 and TRAF2, respectively, and inhibit RLRs-mediated signaling [62,63]. The E3 ligases Nrdp1 and MIB2 (mindbomb E3 ubiquitin protein ligase 2) promote K63-inked ubiquitination of TBK1 and thereby activate IRF3, whereas TRIP and DTX4 inhibit IRF3 activation by mediating K48-linked ubiquitination and degradation of TBK1 [36,64–66]. The linear ubiquitin assembly complex (LUBAC, consisting of E3s HOIP and HOIL and the accessory protein SHARPIN) inhibits virus-triggered activation of IRF3 through two different mechanisms. (i) LUBAC catalyzes linear ubiquitination of NEMO which leads to the recruitment of TRAF3 and disruption of VISA–TRAF3 interaction [67]. (ii) LUBAC promotes ubiquitination and degradation of TRIM25 which impairs RIG-I activation [68]. FOXO1 and two E3s RBCK1 and RAUL have been reported to catalyze K48-linked ubiquitination and degradation of IRF3, thereby inhibiting IRF3-dependent type I IFN induction [69–71]. Interestingly, Zheng *et al*. [72] have reported that ubiquitination of IRF3 is required for its nuclear translocation, although the E3s that mediate the process remain to be characterized.

Cytoplasmic DNA sensor-mediated signaling

So far, about half dozen of cytoplasmic dsDNA sensors have been identified, including RNA polymerase III, DAI, IFI16, DDX41, LSm14A, and cGAS. However, it is generally accepted that cGAS is a 'universal' sensor that functions in most types of cells and recognizes different types of dsDNA. Upon binding to dsDNA, cGAS catalyzes GTP and ATP into cyclic GMP-AMP (cGAMP) [73]. cGAMP binds to the adaptor protein, mediator of IFN regulatory transcription factor 3 activation (MITA, also known as STING and ERIS) and induces oligomerization/dimerization and activation of MITA [74]. MITA then recruits TRAF3, TBK1, and TRAF6 to activate IRF3 and NF-κB [16]. TRIM56 and TRIM32 catalyze K63-linked ubiquitination of MITA, whereas RNF5 catalyzes K48-linked ubiquitination and degradation of MITA [75–77]. Recently, we have identified an E3 ring finger protein 26 (RNF26) that promotes K11-linked ubiquitination of MITA which competitively inhibits RNF5-mediated K48-linked ubiquitination of MITA, thereby promoting the protein stability of MITA and efficient type I IFN induction after viral infection [78]. Whether and how other types of ubiquitin linkage are targeted to MITA, and the functions of ubiquitination are of great interest (**Table 1**).

Regulation of Cellular Innate Antiviral Signaling by Host and Viral DUBs

Ubiquitination is a reversible process and protein deubiquitination is mediated by a group of DUBs. Because ubiquitination is critically involved in virus-triggered type I IFN induction, it is conceivable that deubiquitination is essential to regulate virus-triggered type I IFN induction by modulating the ubiquitination status of the molecules involved in cellular innate antiviral responses. In addition, the ubiquitination status of the signaling molecules could be targeted by DUBs encoded by viral genomes, which interferes the antiviral signaling and thereby benefits viruses.

Deubiquitination of PRRs

Although it has been reported that TLRs and some cytoplasmic dsDNA sensors (for example, DDX41) are ubiquitinated for proteasome-mediated degradation, little is known about the

Table 1. Ubiquitination and deubiquitination events in virus-triggered PRRs-mediated signaling pathways

Target molecules	E3 ligases-mediated ubiquitination	Host DUBs-mediated deubiquitination	Viral DUBs-mediated deubiquitination
PRRs			
TLR3/9	Triad3A (K48 linkage)	ND	ND
RIG-I	TRIM25 (K63 linkage)	USP15 (K63 linkage)	NSP2 of Arterivirus (K63 linkage)
	Riplet (K63 linkage)	USP17 (ND)	L protein of Nairovirus (K63 linkage)
	TRIM4 (K63 linkage)	CYLD (K63 linkage)	ORF64 of KSHV (K63 linkage)
	Ubc5 (K63 linkage free ubiquitin chains)	USP3 (K63 linkage)	L^{pro} of FMDV (K63 linkage)
	MEX3C (K63 linkage)	USP21 (K63 linkage)	ORF1 of HEV (K63 linkage)
	c-Cbl (K48 linkage)	USP4 (K48 linkage)	VP2 of bocavirus (K48 linkage)
	RNF125 (K48 linkage)		
MDA5	ND	USP17 (ND)	ND
DDX41	TRIM21 (K48 linkage)	ND	ND
IFI16, DAI, LSm14A, RNA Pol III	ND	ND	ND
cGAS	ND	ND	ND
Adaptor proteins			
TRIF	WWP2 (K48 linkage)	ND	ND
	TRIM38 (K48 linkage)		
MyD88	Nrdp1 (K48 linkage)	ND	ND
VISA	Itch (K48 linkage)	ND	ND
	RNF5 (K48 linkage)		
	PSMA7 (K48 linkage)		
	Smurf1/2 (K48 linkage)		
	TRIM25 (K48 linkage)		
	Unknown (K63 linkage)		
MITA	RNF5 (K48 linkage)	ND	ND
	TRIM56 (K63 linkage)		
	TRIM32 (K63 linkage)		
	RNF26 (K11 linkage)		
TRAF3	cIAP1/2 (K63 and K48 linkage)	DUBA (k63 linkage)	ND
	Triad3A (K48 linkage)	OTUB1/2 (K63 linkage)	
TRAF6	cIAP1/2 (K63 and K48 linkage)	OTUB1/2 (K63 linkage)	BPLF1 of EBV (K63 linkage)
		A20 (K63 linkage)	
Kinases and transcription factors			
NEMO	TRAF6 (K63 linkage)	CYLD (K63 linkage)	ND
TBK1, IKKε	TRAF3 (K63 linkage)	A20 (K63 linkage)	NSP3 of MHV-A59 (K63 linkage)
	Others		NSP3 of SARS-CoV (K63 linkage)
IRF3	RBCK1 (K48 linkage)	ND	NSP3 of MHV-A59 (K63 linkage)
	RAUL (K48 linkage)		NSP3 of SARS-CoV (K63 linkage)
	FOXO1 (K48 linkage)		
	Others (K63 linkage?)		

ND, not determined.

deubiquitination process of these PRRs [79]. As discussed above, the activation of RIG-I is positively regulated through K63-linked ubiquitination by TRIM25, Riplet, and TRIM4 as well as K63-linked free polyubiquitin chains and negatively regulated through K48-linked ubiquitination by RNF125 and c-Cbl. The deubiquitination of RIG-I is also very complicated and at least six DUBs have been reported to deubiquitinate RIG-I. It has been reported that cylindromatosis (CYLD) deubiquitinates K63-linked polyubiquitin chains from RIG-I and inhibits virus-triggered activation of IRF3 and NF-κB [80]. We have demonstrated that knockdown of USP17 potentiated the ubiquitination of RIG-I and MDA5 and inhibited virus-triggered induction of type I IFNs, indicating that USP17 positively regulates type I IFN response by modulating ubiquitination status of RLRs [81]. However, the linkage of polyubiquitin chains targeting to RLRs remains to be determined. USP15 deubiquitinates LUBAC-mediated K48-linked ubiquitination of TRIM25 and promotes the protein stability of TRIM25, there by promoting K63-linked ubiquitination of RIG-I and potentiating virus-triggered expression of type I IFN genes [82]. USP4 deubiquitinates and stabilizes RIG-I to promote type I IFN induction after viral infection [83]. In addition, two DUBs USP3 and USP21 have been reported to remove the K63-linked polyubiquitin chains from RIG-I and inhibit virus-triggered signaling [84,85]. Future studies are required to determine whether these DUBs function redundantly in cellular innate antiviral responses.

Several studies have shown that deubiquitination of RLRs could be mediated by DUBs encoded by vDUBs. The nonstructural protein 2 (NSP2) of the Arterivirus family and the L protein (harboring the RNA-dependent RNA polymerase) of the Nairovirus genus catalyze deubiquitination of RIG-I and inhibit RIG-I-mediated activation of IFN-stimulated response element in reporter assays [86]. Kaposi's sarcoma-associated herpesvirus (KSHV) open reading frame 64 (ORF64) contains DUB activity and mediates deubiquitination of RIG-I to promote persistent infection [87]. The leader proteinase (L^{pro}) of foot-and-mouth disease virus is similar to human USP14 and removes K63-linked ubiquitination of RIG-I [88]. The ORF1 of hepatitis E virus contains the papain-like cysteine protease domain and deubiquitinates RIG-I to inhibit poly(I:C)-induced activation of IRF3 [89]. On the contrary, human bocavirus VP2 deubiquitinates RNF125-mediated K48-linked ubiquitination of RIG-I, promotes the stability of RIG-I and upregulates IFNβ induction [90]. However, these *in vitro* studies have been performed in an over-expression system or with purified proteins. Further investigations with recombinant viruses with inactive vDUBs are required to confirm the function of the vDUBs in regulating RLR-mediated signaling *in vivo*. Nonetheless, it is expected that the vDUBs are ideal targets for the treatment of viral infection-related diseases in the future.

In addition to DUBs, several reports also showed that host or viral proteins without DUB activity mediate deubiquitination of RIG-I. For example, a protein kinase, IFN-inducible double-stranded RNA-dependent inhibitor and repressor of p58 inhibits the ubiquitination and degradation of RIG-I and enhances the induction of type I IFNs [91]. The influenza A virus NS1 protein inhibits TRIM25-mediated ubiquitination of RIG-I and thus attenuates type I IFN induction after viral infection [92].

Deubiquitination of adaptor proteins

Four adaptor proteins are involved in PRRs-mediated antiviral signaling, including TRIF, MyD88, VISA, and MITA (herein designated as immediate adaptors), all of which have been reported to undergo ubiquitination. However, their deubiquitination is completely unknown. The TRAF proteins function as integrate adaptor proteins downstream of the immediate adaptors. DUBA is an OUT domain-containing DUB that deubiquitinates TRAF3 and inhibits TLR3- and RLRs-mediated activation of IRF3 [93]. We and others have demonstrated that OTUB1/2 and A20 catalyze the deubiquitination of TRAF3 and TRAF6, which shuts down virus-triggered induction of type I IFNs [94]. Epstein–Barr virus (EBV)-encoded BPLF1 interacts with and deubiquitinates TRAF6, leading to the inhibition of NF-κB activation and promotion of lytic infection [95].

Deubiquitination of kinases and transcription factors

The IKK complex and TBK1/IKKε are kinases responsible for the activation of NF-κB and IRF3, respectively. NEMO is the regulatory subunit of the IKK complex and CYLD is reported to deubiquitinate NEMO to inhibit the activation of NF-κB [96]. A20, an OUT domain-containing DUB, together with RNF11, removes K63-linked ubiquitination of TBK1, thereby impairing IRF3 activation [97]. L^{pro} of foot-and-mouth disease virus also catalyzes deubiquitination of TBK1 [88]. The papain-like domain 2 of the NSP3 of mouse hepatitis virus A59 (MHV-A59) and severe acute respiratory syndrome (SARS) coronavirus (CoV) deubiquitinates TBK1 and IRF3 and prevents the nuclear translocation of IRF3 [98].

Conclusions and Perspectives

The past decade has witnessed tremendous progress in the study of PRRs-mediated innate antiviral signaling which is heavily regulated by ubiquitination and deubiquitination. Although strategies based on intervention of the imbalance between ubiquitination and deubiquitination by small molecules may be hopefully developed to treat viral infection-caused diseases, more studies are needed to understand the systemic mechanisms by which the balance of ubiquitination and deubiquitination is maintained or disrupted by host or viral proteins.

Why does a ubiquitinated protein need to be deubiquitinated?

A target protein could be modified by active and inactive ubiquitin modifications and the balance of ubiquitination status determines the function and fate of the target. For example, RIG-I undergoes K63-linked ubiquitination for activation at the early stage of viral infection, while RNF125 is induced by viral infection and catalyzes K48-linked ubiquitination and degradation of RIG-I. USP3 and USP4 remove the active or inactive ubiquitin modification from RIG-I, respectively. It is conceivable that removing the active form of polyubiquitin chains from the target is to avoid excessive harmful immune response to the host. However, it remains enigmatic why ubiquitination-mediated inactivation (or throwaway) determined by the host could be rescued by host-encoded DUBs. A simplest explanation for this is that recycling a protein is more economical than re-synthesizing it. In addition, sufficient antiviral protein in the cell could equip the host to defense against a second-round infection more efficiently. Collectively, the DUBs work as a sentinel to 'check and balance' all the time and keep the cellular antiviral responses in check.

How do the DUBs exhibit substrate specificity?

Compared with ~600 E3s encoded by the human genome, only 90 DUBs have been identified in the human genome. The substrate specificity of DUBs might be determined by several factors: the linkage types of polyubiquitin chains, the substrate itself, and cofactors associated with the DUBs. For instance, USP15 interacts with and deubiquitinates TRIM25 when it is ubiquitinated by the E3 complex LUBAC. It is also possible that not all DUBs have been identified or that the DUBs display poor substrate specificity. In this context, it seems that vDUBs exhibit little specificity to the target.

How does ubiquitination correlate with other modifications?

In addition to ubiquitination, protein could undergo phosphorylation, methylation, acylation, sumoylation, ISGylation, and NEDDylation. There is much crosstalk between these modifications in virus-triggered type I IFN signaling. Phosphorylation-dependent degradation of IκBα and the inhibition of ubiquitination of IRF3 by sumoylation are two examples [99]. It has been demonstrated that RIG-I and MDA5 could be sumoylated and ISGylated and whether the sumoylation or ISGylation correlates with the ubiquitination is unclear [100–102]. Undoubtedly, further investigations are required to systemically characterize the role of DUBs in the innate antiviral responses.

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