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Early innate immune response triggered by the human respiratory syncytial virus and its regulation by ubiquitination/deubiquitination processes

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Abstract

The human respiratory syncytial virus (HRSV) causes severe lower respiratory tract infections in infants and the elderly. An exuberant inadequate immune response is behind most of the pathology caused by the HRSV. The main targets of HRSV infection are the epithelial cells of the respiratory tract, where the immune response against the virus begins. This early innate immune response consists of the expression of hundreds of pro-inflammatory and anti-viral genes that stimulates subsequent innate and adaptive immunity. The early innate response in infected cells is mediated by intracellular signaling pathways composed of pattern recognition receptors (PRRs), adapters, kinases, and transcription factors. These pathways are tightly regulated by complex networks of post-translational modifications, including ubiquitination. Numerous ubiquitinases and deubiquitinases make these modifications reversible and highly dynamic. The intricate nature of the signaling pathways and their regulation offers the opportunity for fine-tuning the innate immune response against HRSV to control virus replication and immunopathology.

Keywords: Ubiquitination, Innate immunity, Immune Response Regulation, Respiratory Syncytial Virus

Introduction

Human Respiratory Syncytial Virus (HRSV) is the leading cause of severe lower respiratory tract infections such as bronchiolitis and pneumonia in infants [17]. It also produces severe infections in the elderly and immunocompromised adults [36]. Worldwide, HRSV causes more than 33 million infections in children under five per year, of which approximately 3 million require hospital admission, and about 60.000 die [119, 150]. Bronchiolitis caused by HRSV infection is characterized by

inflammation of the bronchial tubes and bronchioles of infected patients. Together with mucus, this inflammation obstructs the airway lumen reducing airflow through the airways. In the infant population, the airways are narrower, so they become more easily blocked, increasing the disease severity [135].

HRSV belongs to the *Orthopneumovirus* genus within the *Pneumoviridae* family. It is an enveloped virus with a non-segmented, single-stranded, negative RNA genome [2]. The HRSV genome contains ten genes that codify for 11 proteins. These include the attachment glycoprotein (G), the fusion protein (F), and the small hydrophobic (SH) protein, which are located on the virus surface. The nucleoprotein (N), phosphoprotein (P), large polymerase protein (L), M2 protein, and the matrix (M) protein are all placed inside the virion. Finally, the virus has two non-structural (NS1 and NS2) proteins [25, 53].

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The early immune response against HRSV

The immune response against HRSV begins in the epithelial cells from respiratory airways, the main targets of virus infection. These cells produce multiple cytokines and chemokines (including CCL2, CCL3, CCL5, CCL7, CXCL10, CXCL11, IL-8, and IL-15) that trigger a pro-inflammatory/anti-viral response essential for virus control [111, 168, 192]. Conversely, the pro-inflammatory response also plays a prominent role in the pathogenesis of the disease [9, 11, 28, 56, 118, 136].

The HRSV infection is detected in epithelial cells by Pattern Recognition Receptors (PRR) that recognize Pathogen-Associated Molecular Patterns (PAMPs), which, in HRSV infection, are mainly viral RNAs with 5'-triphosphate end and the double-strand RNA (dsRNA) produced during viral replication [52, 74, 96]. Those cell receptors trigger intracellular signaling involving different adaptors, kinases, and transcription factors (Fig. 1). The main PRRs that detect HRSV infection in epithelial cells are RIG-I Like Receptors

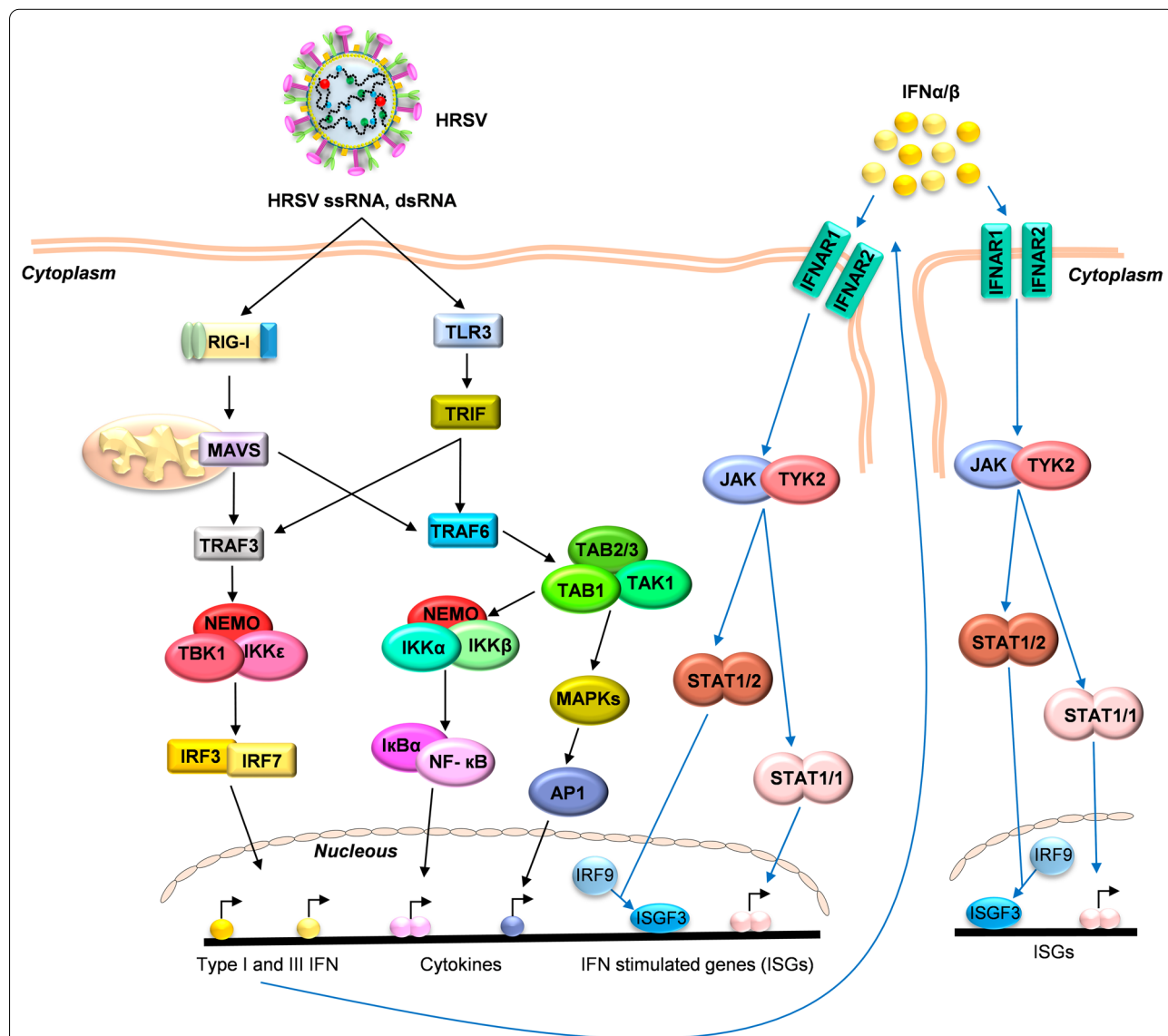


Fig. 1 Main pathways activated in early innate anti-viral immunity after HRSV infection. The signaling pathways begin with the recognition of HRSV RNA by RIG-I and TLR3 receptors. The signal is transduced through adaptor proteins (MAVS, TRIF) to TRAF proteins (TRAF3/6), which activate the kinase complexes (NEMO, TBK1, IKKα/β/ε, TAB1, and TAK1), triggering the activation of transcription factors IRF3/7, NF-κB and AP1 to express type I/III IFN, cytokines, and anti-viral genes. The released IFNs binds to their receptors (IFNAR1/IFNAR2) in an autocrine and paracrine manner to induce JAK/STAT-mediated expression of multiple ISGs

(RLRs) and Toll-Like Receptors (TLRs) [34, 63, 78, 96, 107, 146]. These PRRs activate the transcription factors nuclear factor- κ B (NF- κ B) and interferon regulatory factors 3 and 7 (IRF3/7) (Fig. 1). The activation and translocation of these transcription factors to the cell nucleus triggers the expression of type I interferon (IFN-I), cytokines, chemokines, and anti-viral molecules [59, 73, 96, 112, 148].

RIG-I mediated signaling

RIG-I (Retinoic acid Inducible Gene I), a member of the RLR family, is the principal PRR involved in HRSV recognition in respiratory epithelial cells [96, 110, 183]. RIG-I silencing by siRNA inhibited the activation of both NF- κ B and IRF3 transcription factors and the expression of IFN- β , CXCL10, CCL5, ISG15, TNF- α , and IL-6 at early times after HRSV infection [96, 110]. RIG-I comprises two N-terminal Caspases Activation and Recruitment Domains (CARDs), a central DExD/H box RNA helicase domain, and a regulatory C-Terminal Domain (CTD). The receptor is localized in the cell cytoplasm and, after viral infection, recognizes the 5'-triphosphate ends from single or double-strand viral RNAs [27, 143, 161, 184]. The viral RNA recognition induces a conformational change in RIG-I, leading to interaction with Mitochondrial Antiviral-Signaling proteins (MAVS, also known as Cardif, IPS1, or VISA). MAVS mediates the expression of most of the HRSV-induced genes in the lungs of infected mice, including IFN-I, IL-6, IL-1 β , TNF- α , CCL2, CXCL1, and CXCL2 [12, 82, 129]. MAVS proteins contain TRAF-interacting motifs to interact with TRAF family proteins such as TRAF3/6 (TNF Receptor Associated Factor 3 and 6). This interaction leads to kinase-mediated activation of the transcription factors IRF3/7 and NF- κ B and the subsequent expression of type I/III IFN, cytokines, chemokines, and anti-viral proteins [7, 10, 12–15, 23, 38, 40, 47, 52, 54, 58, 97, 98, 101, 113–115, 139, 140, 155, 159, 164–168, 183] (Fig. 1).

TLR3 mediated signaling

TLRs are among the best-characterized families that detect PAMPs from extracellular media, intracellular endosomes, and lysosomes [162]. In HRSV infection, the RNA recognition by TLR3 triggers the TRAF3/6-mediated signaling pathway and the transcription of several inflammatory and anti-viral immune response genes [55, 95, 96, 121, 141, 162] (Fig. 1). Following HRSV infection of airway epithelial cells, TLR3 is induced by RIG-I-dependent IFN- β secretion, indicating that RIG-I and TLR3 mediate the HRSV-induced innate immune response at different times postinfection [96].

IFN-mediated signaling

HRSV NS1 and NS2 proteins suppress type I IFN production [159]. Therefore, a robust IFN response is not observed in nasal secretions of HRSV infected infants [117]. However, type I and III interferons are produced in epithelial cells following HRSV-mediated RIG-I activation [96, 138, 185]. The transcription factors mediating IFN production are IRF3/7, activated by the kinase complex TBK1/IKK ϵ /NEMO [39, 57, 99] (Fig. 1). The secreted IFN can act in a paracrine or autocrine manner by binding to its receptor (IFNAR), activating intracellular signaling pathways and leading to the expression of IFN-stimulated genes (ISGs). The IFNAR receptor is a cell surface transmembrane receptor composed of two subunits; IFNAR1 (IFN- α receptor 1) and IFNAR2. Both are associated with cytoplasmic tyrosine kinase 2 (Tyk2) and Janus-activated kinase 1 (Jak1). After IFN interaction, the tyrosine residues in the IFNAR cytoplasmic domains are phosphorylated to recruit and phosphorylate the “Signal Transducers and Activators of Transcription 1 and 2” (STAT1 and STAT2), leading to the formation of STAT1/STAT2 heterodimers and STAT1/STAT1 homodimers [49, 60, 83, 158] (Fig. 1). The STAT1 homodimers translocate to the nucleus, binding to IFN-gamma-activated sequences (GAS) sites in gene promoters and activating its transcription. On the other hand, STAT1/STAT2 heterodimers interact with IRF9 to form the ISGF3 (Interferon Stimulated Gene Factor 3) complex that binds to the interferon-stimulated response elements (ISRE) site, leading to the transcription of many ISGs, and establishing an anti-viral state [41, 89, 156].

In HRSV infection, IFN signaling plays an essential role in inducing pro-inflammatory cytokines and anti-viral genes [75, 83]. Goritzka et al. described the critical role of IFNAR in the innate resistance to HRSV infection in mice [50]. IFNAR1 deficient mice showed increased viral load and reduced type I/II/III IFN, pro-inflammatory cytokines, and chemokines in the lung in response to HRSV infection, indicating that signaling through IFNAR is necessary for coordinating the inflammatory response against HRSV [50]. Makris et al. reported that cytokine production is abolished in alveolar macrophages (AM) from IFNAR deficient mice infected with HRSV [106]. These data showed that the IFN pathway is critical for the innate immune response following HRSV infection. Therefore, inhibition of IFN signaling may help reduce inflammation in HRSV infections. However, the IFN pathway is also necessary to inhibit virus replication, so a fine-tune control of the innate immune response should be done to avoid immunopathological damage while restricting viral replication.

The ubiquitination process

The ubiquitin is a small protein of 76 amino acids conserved among different eukaryotic organisms. Ubiquitin can be conjugated to other proteins by covalent attachment between its C-terminal di-glycine motif and lysine (K) residues in the target protein. This covalent attachment, known as ubiquitination, modifies the activity or functionality of the target protein [6, 29, 134]. Ubiquitination is a three-step enzymatic process; firstly, the ubiquitin-activating enzyme (E1) activates the ubiquitin molecule in an ATP-dependent process. Secondly, the E1 protein transfers the activated ubiquitin to the ubiquitin-conjugating enzyme (E2). Lastly, the ubiquitin ligase (E3) interacts with E2 to attach the activated ubiquitin to the target protein through an isopeptide bond [24, 64, 81, 182] (Fig. 2A). The E3 enzyme determines the specificity of the target protein [29, 105, 197].

One (monoubiquitination), two (diubiquitination), or several (polyubiquitination) ubiquitin molecules can be attached to the target protein. The links between different ubiquitin molecules are also formed through covalent attachment of the C-Terminal di-glycine motif of one ubiquitin and the epsilon amino lysine residue of a second ubiquitin. The ubiquitin molecule has seven internal lysines (K6, K11, K27, K29, K33, K48, and K63), and therefore, several different types of ubiquitin chains with distinct functions can be formed (Fig. 2B) [29, 70, 81, 116]. The best-characterized are: (i)

Ubiquitin linkage through lysine at position 48 (K48), which labels the target protein for proteasome recognition and degradation. (ii) Ubiquitin linkage through K63 activates intracellular signaling pathways by stabilizing substrates or acting as a scaffold that facilitates the formation of an active signaling complex [29, 200]. (iii) The linear ubiquitin chains are formed by covalent bonding between the C-terminal carboxyl group of one ubiquitin and the N-terminal methionine of another ubiquitin molecule [29].

Ubiquitination is a reversible process. Ubiquitin molecules can be removed from the target proteins by deubiquitinases (DUBs), which have protease and metalloprotease activity. The ubiquitin molecules are released to the cytosol for recycling or degraded in the proteasome [29] (Fig. 2A).

Innate immune response regulation by ubiquitination/deubiquitination

In viral infections, the intracellular pathways activated by PRRs are tightly regulated by ubiquitination/deubiquitination and phosphorylation processes. These post-translational modifications modulate the activity, stability, or location of proteins involved in signaling pathways to ensure a proper immune/anti-viral response [57, 79, 108, 131, 142, 179] (Table 1).

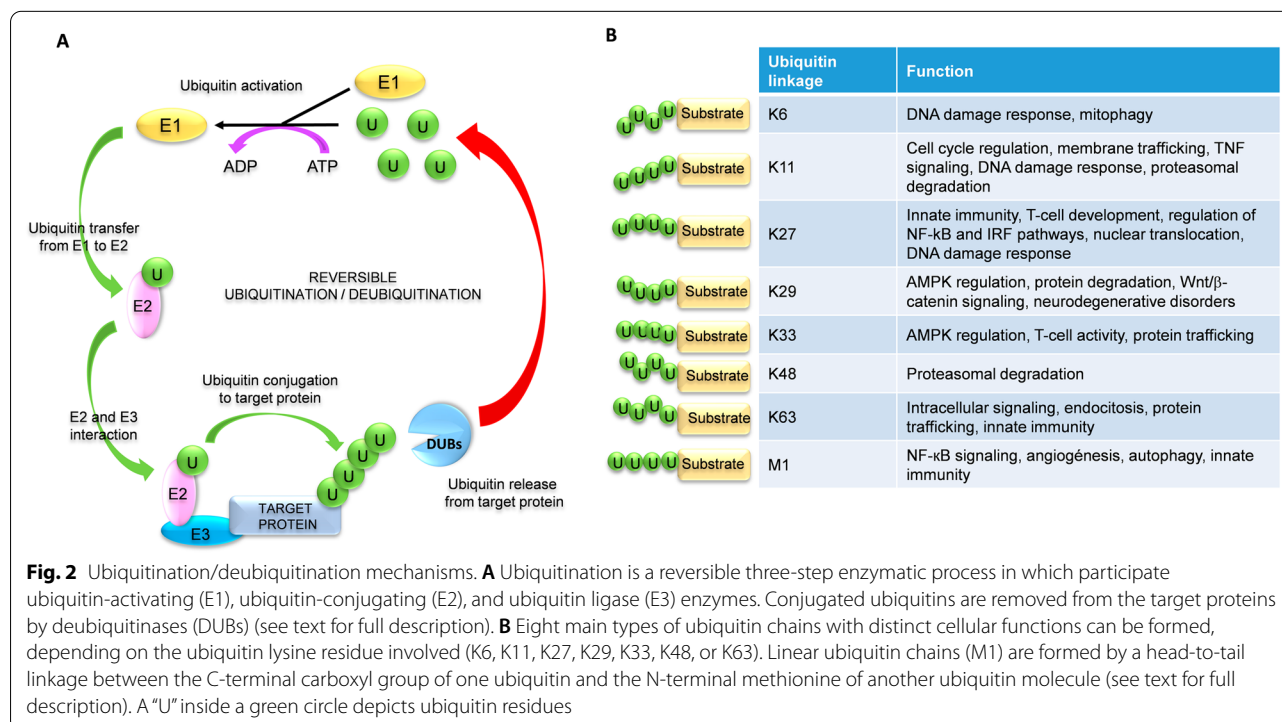


Table 1 Ubiquitinases (E3 ligases) and deubiquitinases (proteases) that regulate intracellular signaling

Target protein	Enzyme (symbol)	Name	Activity	Ubiquitin linkage	Refs.	
RIG-I	TRIM25	Tripartite Motif-containing protein 25	E3 ligase	K63	[44]	
	RNF135	RING Finger Protein 135	E3 ligase	K63	[122]	
	TRIM4	Tripartite Motif-containing protein 4	E3 ligase	K63	[181]	
	MEX3C	Mex-3 RNA binding family member C	E3 ligase	K63	[84]	
	USP4	Ubiquitin Specific Protease 4	Protease	K48	[172]	
	RNF122	RING Finger Protein 122	E3 ligase	K48	[174]	
	RNF125	RING Finger Protein 125	E3 ligase	K48	[5]	
	CBL (c-Cbl)	Casitas B lineage lymphoma	E3 ligase	K48	[19]	
	TRIM40	Tripartite Motif-containing protein 40	E3 ligase	K27 and K48	[193]	
	CYLD	Cylindromatosis	Protease	K63	[42]	
	USP21	Ubiquitin Specific Protease 21	Protease	K63	[37]	
MAVS	TRIM31	Tripartite Motif-containing protein 31	E3 ligase	K63	[93]	
	TRIM21	Tripartite Motif-containing protein 21	E3 ligase	K27	[180]	
	RNF125	RING Finger Protein 125	E3 ligase	K48	[5]	
	SMURF1/2	SMAD Specific E3 ubiquitin-protein ligase 1/2	E3 ligase	K48	[126, 176]	
	ITCH	Itchy E3 ubiquitin-protein ligase	E3 ligase	K48	[22, 187]	
	RNF5	RING Finger Protein 5	E3 ligase	K48	[199]	
	MARCH5	Membrane-associated RING-CH 5	E3 ligase	K48	[186]	
	TRIM25	Tripartite Motif-containing protein 25	E3 ligase	K48	[16]	
	MARCH8	Membrane-associated RING-CH 8	E3 ligase	K27	[77]	
	TRIM29	Tripartite Motif-containing protein 29	E3 ligase	K11	[178]	
YOD1 (OTUD2)	YOD1 (OTUD2)	Ovarian Tumor Deubiquitinase2	Protease	K63	[94]	
	TRAF3	RNF166	RING Finger Protein 166	E3 ligase	K63	[18]
		HECTD3	HECT Domain E3 Ubiquitin Protein Ligase 3	E3 ligase	K63	[86]
		RNF216 (Triad3A)	RING Finger Protein 216	E3 ligase	K48	[120]
		OTUB1/2	OTU Deubiquitinase, Ubiquitin Aldehyde Binding 1 and 2	Protease	K63	[88]
ZC3H12A (MCP1P1)		Monocyte Chemotactic Protein-Induced Protein-1	Protease	K63	[20, 91]	
TRAF6	TRAF6	TNF Receptor Associated Factor 6	E3 ligase	K63	[85]	
	RNF166	RING Finger Protein 166	E3 ligase	K63	[18]	
	ZC3H12A (MCP1P1)	Monocyte Chemotactic Protein-Induced Protein-1	Protease	K63	[91]	
	OTUB1/2	OTU Deubiquitinase, Ubiquitin Aldehyde Binding 1 and 2	Protease	K63	[88]	
	TNFAIP3 (A20)	TNFα Induced Protein 3	Protease	K63	[3, 130, 149]	
TRIM38	TRIM38	Tripartite Motif-containing protein 38	E3 ligase	K48	[195]	
	TBK1	MIB1/2	MIB E3 ubiquitin-protein ligase	E3 ligase	K63	[87]
		RNF41 (Nrdp1)	RING Finger Protein 41	E3 ligase	K63	[170]
		RNF128	RING Finger Protein 128	E3 ligase	K63	[152]
		DTX4	Deltex E3 Ubiquitin Ligase 4	E3 ligase	K48	[26]
TRAIIP (TRIP)		TRAF Interacting Protein	E3 ligase	K48	[191]	
TRIM27	TRIM27	Tripartite Motif-containing protein 27	E3 ligase	K48	[198]	
	NEMO	TRAF6	TNF Receptor Associated Factor 6	E3 ligase	K63	[31]
		TRIM23	Tripartite Motif-containing protein 23	E3 ligase	K27	[4]
		LUBAC	Linear Ubiquitin chain Assembly Complex	E3 ligase	M1	[169]
		TRAF7	TNF Receptor Associated Factor 7	E3 ligase	K29	[201]
TRIM29		Tripartite Motif-containing protein 29	E3 ligase	K48	[177]	
TAK1	TRAF6	TNF Receptor Associated Factor 6	E3 ligase	K63	[171]	
	CYLD	Cylindromatosis	Protease	K63	[1]	
	ITCH	Itchy E3 ubiquitin-protein ligase	Protease	K63	[1]	
TBK1-IKK complex	TNFAIP3 (A20)	TNFα Induced Protein 3	Protease	K63	[46, 127, 144]	

Table 1 (continued)

Target protein	Enzyme (symbol)	Name	Activity	Ubiquitin linkage	Refs.
IRF3	TRIM26	Tripartite Motif-containing protein 26	E3 ligase	K48	[173]
	TRIM21	Tripartite Motif-containing protein 21	E3 ligase	K48	[66]
	RBCK1 (RNF54)	RING Finger Protein 54	E3 ligase	K48	[190]
	CBL (c-Cbl)	Casitas B lineage lymphoma	E3 ligase	K48	[196]
	UBE3C (RAUL)	Ubiquitin Protein Ligase E3C	E3 ligase	K48	[188]
IRF7	UBE3C (RAUL)	Ubiquitin Protein Ligase E3C	E3 ligase	K48	[188]
	TRIM21	Tripartite Motif-containing protein 21	E3 ligase	K48	[65]
NF-κB	MKRN2	Makorin Ring Finger Protein 2	E3 ligase	K48	[151]
	PDLIM1	PDZ And LIM Domain 1	E3 ligase	K48	[163]
	COMMD1/Cul2	Copper Metabolism Domain Containing 1/Cullin 2	E3 ligase	K48	[48]
	TRAF7	TNF Receptor Associated Factor 7	E3 ligase	K29	[201]
STAT1	RNF31	RING Finger Protein 31	E3 ligase	M1	[202]
	OTULIN	OTU deubiquitinase with Linear linkage specificity	Protease	M1	[202]

Ubiquitination/deubiquitination processes regulate many proteins involved in RIG-I and TLR3 signaling pathways. Different E3 ligases (add ubiquitin chains to the target protein) or proteases (remove the ubiquitin chains from the target protein) modify the activity, localization, or stability of the target proteins. E3 ligases add ubiquitin residues to the target proteins; proteases (deubiquitinases) remove ubiquitin residues from the target proteins

RIG-I

RIG-I receptor is tightly modulated by complex ubiquitination and deubiquitination processes [44, 122, 125, 133, 189]. Its activity is positively regulated by TRIM25 and RNF135 (also known as RIPLET or REUL). Both TRIM25 and RNF135 contain an N-terminal RING (Really Interesting New Gene) domain with E3 ligase activity and a C-terminal PRY-SPRY domain [44, 45, 62, 109, 122, 123, 125]. RIG-I recognizes the 5'-triphosphate ends from small uncapped viral RNAs by its CTD. This interaction induces a conformational change in RIG-I that exposes its CARDs domains to interact with TRIM25. Subsequently, TRIM25 activates RIG-I via K63 polyubiquitination, while RNF135 activates RIG-I by K63-linked ubiquitin chains on its CTD domain [21, 44, 67, 122, 124, 145, 189]. RIG-I polyubiquitination promotes its interaction with MAVS and triggers downstream intracellular inflammatory and anti-viral responses [43, 44, 80, 137, 179]. Two other ubiquitin ligases have been reported to polyubiquitinate CARDs on RIG-I, namely TRIM4 and MEX3C [84, 181]. TRIM4 belongs to the TRIM family and adds ubiquitin residues through K63 to one of the CARDs of RIG-I, contributing to the RIG-I activation [181]. K63-linked ubiquitin residues added by MEX3C on different CARDs lysines of RIG-I increase the type-I IFN induction [84]. Additionally, the deubiquitinase USP4 removes K48-linked polyubiquitin chains on CARDs of RIG-I, allowing signal transduction [172].

There are also negative regulators of RIG-I activity: (i) RNF125 and RNF122 belong to the RING domain and E3-ligase family proteins, which ubiquitinate the

CTD and CARDs in RIG-I through K48, respectively [5, 105, 123, 174]. (ii) c-Cbl (CBL) also ubiquitinates RIG-I through K48 on its CTD domain [19]. (iii) TRIM40 ubiquitinates RIG-I through K27 and K48 at the first CARD domain. In all these cases, these types of ubiquitination induce RIG-I proteasomal degradation [193]. (iv) CYLD is a deubiquitinase of the USP family that removes the K63 ubiquitin residues from RIG-I, inhibiting the interaction between RIG-I and MAVS [42]. (v) USP21 deubiquitinates the K63-linked ubiquitin chains on CARDs of RIG-I anchored by TRIM25 and RNF135 [37]. In all instances, the result is the inhibition of the RIG-I-mediated intracellular signaling pathway.

In HRSV infection, the K63 ubiquitination of RIG-I by TRIM25 induces the innate signaling pathways [7, 44, 102, 110]. The carboxy-terminal SPRY domain of TRIM25 interacts with the N-terminal CARDs or RIG-I to ubiquitinate Lys 172 of RIG-I [44]. Moreover, the E3 ubiquitin ligase FBXW7 ubiquitinates and degrades the SHP2 protein disrupting the SHP2/c-Cbl complex that mediates RIG-I degradation [153]. In both cases, these modifications promote RIG-I-mediated signaling.

MAVS

In HRSV infection, levels of MAVS are increased and mediate signaling pathways triggering the innate and adaptive immune response [12, 30, 129]. Upon RIG-I activation, CARD-CARD interaction between RIG-I and mitochondria-anchored MAVS induces a conformational change in MAVS to form a prion-like structure that activates downstream signaling [68]. The prion-like

structure of MAVS recruits different TRAFs proteins like TRAF3/6, which promotes the activation of: (i) TBK1 complex (TBK1, IKK ϵ , and NEMO (IKK γ)) that induces the IRF3/7 phosphorylation and their translocation into the nucleus to induce the transcription of type I IFN genes [100, 142]. (ii) IKK complex (IKK α/β and NEMO) that activates the NF- κ B transcription factor and production of pro-inflammatory cytokines [100, 104, 162] (Fig. 1).

Post-translational modifications tightly regulate the MAVS adaptor to ensure a proper immune response. TRIM31 and TRIM21 are TRIM family members with E3 ubiquitin ligase activity that have been proposed as positive regulators of MAVS. TRIM31 catalyzes the formation of the prion-like aggregates through K63 ubiquitination of MAVS [93]. TRIM21 ubiquitinates MAVS through K27, enhancing its interaction with TBK1. In both cases, IRF3 and NF- κ B signaling pathways are activated [180].

Numerous E3 ubiquitin ligases mediate the K48-linked ubiquitination and subsequent proteasome degradation of MAVS: RNF125 [5], Smurf1/2 [126, 176], ITCH [22, 187], RNF5 [199], MARCH5 [186], and TRIM25 [16]. Moreover, other E3 ligases, TRIM29 and MARCH8, ubiquitinate MAVS through K11 and K27, promoting MAVS degradation by the proteasome and autophagy, respectively [77, 178]. Recently, it has been described that the deubiquitinase YOD1 (OTUD2) removes K63-linked ubiquitin chains on MAVS, thereby abolishing the formation of MAVS prion-like aggregates and attenuating downstream signaling [94].

TRAF

TRAF3 and TRAF6 are involved in inducing the early innate immune response against HRSV. Thus, TRAF3 seems to be relevant in the signaling pathways induced by HRSV, as indicated by the NS1 and NS2-induced decrease of TRAF3 levels in HRSV infections [160]. TRAF6 is required for RIG-I-mediated p65 phosphorylation and subsequent activation of NF- κ B transcription factor [183].

TRAF3/6 belongs to the TRAF family of adapter proteins, characterized by one conserved TRAF domain at the C-terminal end necessary to interact with other proteins and one RING domain at its N-terminal end with E3 ubiquitin ligase activity [72, 131]. MAVS recruits TRAF3/6 proteins to activate intracellular signaling [100] (Fig. 1). TRAF3/6 interaction with MAVS induces TRAF3/6 K63-linked autoubiquitination and activation [72].

Following activation, TRAF3 recruits TBK1 and IKK γ/ϵ to phosphorylate IRF3 and IRF7. The phosphorylated transcription factors translocate to the nucleus to induce

type I IFNs and ISGs expression [121, 142] (Fig. 1). TRAF3 activity is modified by ubiquitination and deubiquitination processes. The RNF166 and HECTD3 are E3 ubiquitin ligases that ubiquitinate and activate TRAF3 via K63, inducing the signaling pathway [18, 86]. In contrast, another E3 ubiquitin ligase, RNF216 (also known as Triad3A), adds K48-linked ubiquitin residues to TRAF3, inducing its proteasomal degradation and inhibiting signal transduction [120]. Finally, TRAF3 can be negatively regulated by MCPIP1 (also known as ZC3H12A), OTUB1, and OTUB2. The MCPIP1 protein, as well as OTUB1 and OTUB2, removes K63-linked ubiquitin moieties from TRAF3, inhibiting cell signaling [20, 88, 91].

TRAF6 E3 ligase activity mediates K63-linked polyubiquitination of its substrates, including itself and NEMO [31, 85, 171]. Ubiquitinated TRAF6 serves as a scaffold for the recruitment and activation of the TAK1/TAB1/TAB2/3 complex and subsequent NF- κ B activation (Fig. 1) [31, 157, 194]. Like TRAF3, TRAF6 is positively regulated by RNF166-mediated K63 ubiquitination [18]. Conversely, TRAF6 is negatively regulated by some proteins, including MCPIP1 [91], OTUB1, OTUB2 [88], TNFAIP3, and TRIM38. TNFAIP3 (also known as A20) removes K63-linked ubiquitin residues on TRAF6, inhibiting its activity and subsequent signaling [3, 130, 149]. The E3 ubiquitin ligase TRIM38 negatively regulates TRAF6 through K48 ubiquitination and subsequent degradation by the proteasome [195].

IKK and IKK-related kinases

The I κ B kinase (IKKs), IKK α and IKK β , and the IKK-related kinases TBK1 (TANK Binding Kinase 1) and IKK ϵ are the last proteins to transduce the RLR-signaling pathway upstream of the transcription factors (Fig. 1). Both types of kinases interact with NEMO (IKK γ), a scaffold protein essential in the RIG-I-MAVS-mediated response against HRSV in infected cells [98]. NEMO recruits TBK1 (TANK Binding Kinase 1) and IKK ϵ to form a complex that phosphorylates IRF3/7. Phosphorylated IRF3/7 translocate to the nucleus to induce the expression of type I/III IFNs, pro-inflammatory cytokines, and chemokines [148, 194] (Fig. 1).

In the case of NF- κ B, its p65 (RelA) and p50 subunits are retained in the cytosol by the I κ B α protein (Inhibitor of NF- κ B proteins). The complex formed by NEMO, IKK α , and IKK β phosphorylates I κ B α , triggering its ubiquitination and subsequent proteasome-dependent degradation. Degradation of I κ B α leads to the release of NF- κ B and its translocation to the nucleus to express pro-inflammatory cytokines [61].

In HRSV infections, IKK β is required for p65 phosphorylation and subsequent NF- κ B translocation to the nucleus [33, 183]. Moreover, Haerberle et al. have reported

that IKK β and NEMO association is critical for NF- κ B-mediated chemokine expression and lung inflammation [58]. Interestingly, after HRSV infection, IKK ϵ appears to mediate both IRF3 and NF- κ B-dependent gene expression [8, 71, 160]. Additionally, a decrease of TBK1 has been observed after treating cells with a potential drug against HRSV, indicating that TBK1 may participate in the HRSV-mediated immune signaling [69]. Finally, inhibition of TAK1 expression reduces HRSV-induced NF- κ B-dependent gene expression [33].

The activity of these kinases is regulated by ubiquitination and deubiquitination processes. TBK1 is activated by the E3 ubiquitin ligases Mib1/2 [87], Nrdp1 (also known as RNF41) [170], and RNF128 [152], all of them add K63-linked ubiquitin residues to TBK1, promoting the downstream signaling pathway. On the other hand, DTX4 [26], TRIP (also known as TRAIIP) [191], and TRIM27 [198], ubiquitinate TBK1 via K48 and label the protein for proteasomal degradation, inhibiting the RLR-mediated signaling cascade.

The IKK complex is regulated at different steps. NEMO activity is positively regulated by TRAF6, which ubiquitinates NEMO through K63 linkages, promoting IKK complex activation and the subsequent phosphorylation of IRF3/7 and NF- κ B transcription factors [31]. Other E3 ubiquitin ligases, such as TRIM23 and LUBAC, promote the NEMO activity by adding K27 and M1-linked ubiquitin chains, respectively [4, 169]. In contrast, TRAF7 and TRIM29 induce NEMO degradation through K29 and K48-linked ubiquitination, respectively [177, 201]. TAK1 is also positively regulated by TRAF6 through the same mechanism as NEMO [171] but is negatively regulated by CYLD- and ITCH-mediated K63 deubiquitination [1].

Both TBK1 and IKK complexes are negatively regulated by the A20 deubiquitinase, which removes K63-ubiquitin chains on those proteins. In this role, A20 cooperates with TAX1BP1 (Tax1 Binding Protein 1) and ABIN1 (A20-binding inhibitor of NF- κ B activation, also known as TNIP1) to disrupt the TRAF3-TBK1-IKK ϵ complex and inhibit the IRF3 activation [46, 127, 144]. In line with this, our group found that downregulation of A20, TAX1BP1, or ABIN1 in HRSV infection increased the early innate immune response and reduced virus production in epithelial cells [108]. Accordingly, enhanced expression of inflammatory and anti-viral cytokines has been observed in TAX1BP1 knockout mice infected with HRSV [32].

Transcription factors: IRF3/7 and NF- κ B

The last step in the RLR-signaling pathway is the activation of the transcription factors IRF3/7 and NF- κ B [39, 57, 61, 99]. In HRSV infection, these transcription factors have been implicated in the induction of several

pro-inflammatory cytokines and chemokines [7, 10, 23, 33, 38, 40, 54, 59, 71, 92, 96, 97, 115, 128, 140, 155, 164, 166–168, 183].

IRF3 is negatively regulated by K48 ubiquitination that promotes its proteasomal degradation. The E3 ubiquitin ligases involved in this process are: TRIM26, TRIM21, RBCK1 (also known as RNF54), c-Cbl, and RAUL (also known as UBE3C) [66, 173, 188, 190, 196]. IRF7 is also modulated by the ubiquitin E3 ligases RAUL and TRIM21 through the same degradative mechanism [65, 188].

NF- κ B is also regulated by ubiquitination. The p65 (RelA) subunit is negatively regulated through K48 and K29 ubiquitination mediated by MKRN2, PDLIM1, COMMD1/Cul2, and TRAF7. Except for TRAF7, which ubiquitinates p65 through K29, all other ubiquitin E3 ligases add K48 ubiquitin chains to p65. However, both K29 and K48 ubiquitination result in p65 degradation and, consequently, the inactivation of NF- κ B transcription factor [48, 151, 163, 201].

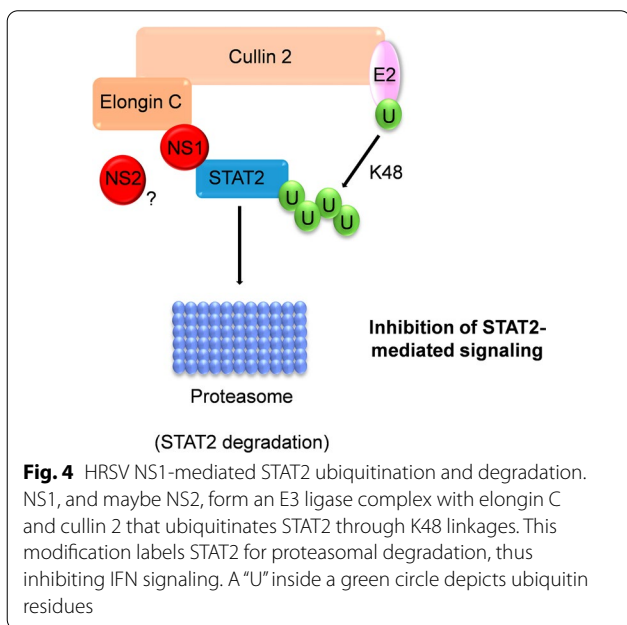
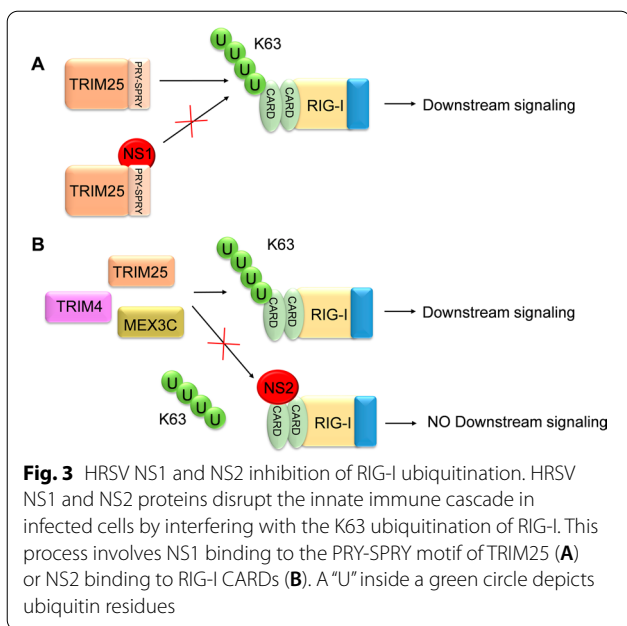
Components of the IFN signaling pathway

The HRSV innate immune response in epithelial cells begins with RIG-I activation, leading to type I/III IFN and pro-inflammatory cytokines expression. IFNs from infected cells trigger additional signaling pathways in the same and neighboring cells. These pathways are also tightly modulated by ubiquitination and deubiquitination processes. Thus, linear ubiquitination of STAT1 by RNF31 (also known as HOIP, a part of the LUBAC complex) prevents its interaction with IFNAR2. Consequently, STAT1 is not phosphorylated, and the anti-viral type I IFN signaling is inhibited [202]. As a positive regulator, the deubiquitinase OTULIN specifically removes linear ubiquitin chains from STAT1, allowing its phosphorylation and activation [202].

The HRSV infection activates and modulates STAT signaling pathways and subsequent ISGs expression [60, 75, 83, 138]. Wang et al. observed that the inhibition of HRSV replication by JAK-STAT1/2 activation is partially mediated by TRIM22 expression [175].

Regulation of ubiquitination processes by HRSV proteins

HRSV counteracts the host's innate immune response by different mechanisms. The viral proteins NS1 and NS2 play a crucial role in modulating RLR-mediated induction of type I and III IFN [76, 92, 103, 140, 147, 154]. The interaction between NS1 and TRIM25 is one of the best-known mechanisms to disrupting the RIG-I signaling pathway. The NS1 protein binds to the PRY-SPRY domain in TRIM25 to prevent K63 ubiquitination of RIG-I (Fig. 3A) and, consequently, its activation [7]. The HRSV NS2 protein also interferes with RIG-I activation by



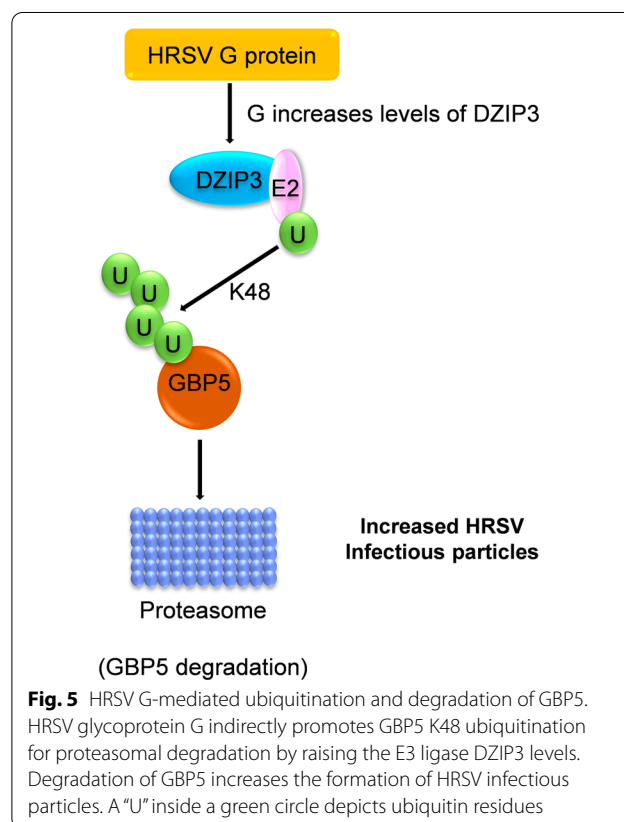
binding to the N-terminal CARD domain of RIG-I, preventing RNA recognition, ubiquitination of the domain, and its interaction with MAVS (Fig. 3B) [92, 132].

NS1 has a consensus sequence (VALLKITYTDK) for binding to the elongin C and cullin 2 E3 ligase. Thus, it has been suggested that the NS1 may interact with elongin C and cullin 2 to form an E3 ligase complex that may ubiquitinate STAT2 for proteasomal degradation (Fig. 4) [35]. Although the NS2 protein does not

appear to interact directly with the E3 ligase complex, it is necessary for effective STAT2 degradation, perhaps by bringing STAT2 closer to the NS1 E3 ligase complex or stabilizing or regulating the complex [35, 103]. However, Swedan et al. found a similar consensus sequence for potential binding to elongin C and cullin 2 in NS2 [160]. Therefore, the ability of NS2 to reduce STAT2 protein levels through a proteasomal mechanism may be mediated by this sequence (Fig. 4) [160].

Goswami et al. have described the so-called NS-degradosome (NSD), a large degradative complex containing the NS1 and NS2 proteins, as well as proteasomal and non-proteasomal proteases. Upon HRSV infection, NSD translocates to the mitochondria and interacts with MAVS. This association allows the degradation of several intermediates of the immune/anti-viral pathways, such as RIG-I, TRAF3, IKKε, or IRF3/7 [51]. However, it was not determined whether ubiquitination enzymes are structural components of the NSD or not.

Not only HRSV NS1 and NS2 proteins are involved in the regulation of the early innate immune response. GBP5 (Guanylate Binding Protein 5) is an IFNγ-inducible gene that belongs to the GTPase family and is involved in several cellular processes, such as inflammasome assembly, vesicle trafficking, and innate immunity. In HRSV



infection, GBP5 reduces cell-associated SH protein levels by promoting SH release in cell culture, resulting in defective HRSV particles. However, HRSV modulates GBP5 expression in infected cells through the HRSV G protein. The G protein upregulates DZIP3 (DAZ Interacting Zinc Finger Protein 3), an E3 ligase that ubiquitinates GBP5 inducing its proteasomal degradation, promoting the generation of viable HRSV particles (Fig. 5) [90].

Concluding remarks

Emerging data show that complex ubiquitination and deubiquitination processes are involved in the regulation of HRSV-induced early innate immunity. RIG-I, MAVS, TRAF3/6, and NEMO are the main proteins regulated by these processes. Ubiquitination/deubiquitination of K63 or K48-linked chains are the most frequent modifications.

Regulation of innate immune pathways in infected cells may impact HRSV dissemination and adaptative immunity. Therefore, proteins participating in those pathways are potential targets for controlling virus replication and immunopathology.

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Authors' contributions

Conceptualization: SR and IM. Data curation: MMV and IM. Funding acquisition: SR and IM. Investigation: MMV and IM. Supervision and visualization: SR and IM. Writing—original draft preparation: MMV and IM. Writing—Review and Editing: SR. All authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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