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Review

ISG15 and immune diseases

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ABSTRACT

ISG15, the product of interferon (IFN)-stimulated gene 15, is the first identified ubiquitin-like protein, consisting of two ubiquitin-like domains. ISG15 is synthesized as a precursor in certain mammals and, therefore, needs to be processed to expose the C-terminal glycine residue before conjugation to target proteins. A set of three-step cascade enzymes, an E1 enzyme (UBE1L), an E2 enzyme (UbcH8), and one of several E3 ligases (e.g., EFP and HERC5), catalyzes ISG15 conjugation (ISGylation) of a specific protein. These enzymes are unique among the cascade enzymes for ubiquitin and other ubiquitin-like proteins in that all of them are induced by type I IFNs or other stimuli, such as exposure to viruses and lipopolysaccharide. Mass spectrometric analysis has led to the identification of several hundreds of candidate proteins that can be conjugated by ISG15. Some of them are type I IFN-induced proteins, such as PKR and RIG-I, and some are the key regulators that are involved in IFN signaling, such as JAK1 and STAT1, implicating the role of ISG15 and its conjugates in type I IFN-mediated innate immune responses. However, relatively little is known about the functional significance of ISG15 induction due to the lack of information on the consequences of its conjugation to target proteins. Here, we describe the recent progress made in exploring the biological function of ISG15 and its reversible modification of target proteins and thus in their implication in immune diseases.

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1. Introduction

Since the discovery of type I interferons (IFN α and IFN β) in 1957, they have widely been used as clinical drugs [1,2]. For example, IFN α has been used for the treatment of chronic hepatitis B and hepatitis C viruses and of several cancers, such as leukemia, and IFN β is effective for treating multiple sclerosis. Type I IFNs exert their effects through the activation of Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway [3]. Upon binding of the IFNs to their cell surface receptors (IFNAR), the receptor-associated kinases JAK1 and tyrosine kinase 2 (TYK2) become activated and phosphorylate tyrosine residues in the cytoplasmic tails of the receptor subunit, IFNAR1. The phosphorylated subunit provides specific docking sites for the activation of STATs by JAK1/TYK2-mediated phosphorylation [4,5]. Activated STATs dissociate from the receptor and translocate into the nucleus, where they act as transcription factors that bind to the promoter regions of IFN-stimulated genes (ISGs) [6]. STAT1/STAT2 heterodimers associate with IFN regulatory factor 9 (IRF-9) to form the transcription complex IFN-stimulated gene factor 3 (ISGF3), which binds to IFN stimulatory response elements (ISREs) within the promoters of ISGs [7]. On the other hand, homodimers and heterodimers of STAT1 and STAT3 bind to gamma-activated sequence (GAS) response elements [8]. The ISG proteins

generated by these pathways play key roles in the induction of innate and adaptive immune responses [9,10].

Protein modifications by ubiquitin and ubiquitin-like proteins (Ubls), including SUMO and Nedd8, have emerged as critical regulatory processes, such as in the control of cell cycle, stress response, signaling transduction, and immune response [11–15]. Moreover, deregulation of the modification systems gives rise to numerous human diseases, such as cancers, neurodegenerative diseases, and immune diseases. Conjugation of ubiquitin and Ubls to target proteins involves three-step cascade enzymes: ubiquitin- and Ubl-activating E1 enzymes, ubiquitin- and Ubl-conjugating E2 enzymes, and ubiquitin- and Ubl E3 ligases. Protein modifications by ubiquitin and Ubls are reversible processes that are catalyzed by isopeptidases, called deubiquitinating enzymes (DUBs) and Ubl-specific proteases (ULPs), respectively.

ISG15, a member of the Ubl family, shows a significant sequence homology to ubiquitin. Like ubiquitin, ISG15 is conjugated to numerous cellular proteins via isopeptide bonds. This ISG15 conjugation (ISGylation) utilizes UBE1L as E1 enzyme, UbcH8 as E2 enzyme, and some ubiquitin E3 ligases, such as EFP and HERC5, as E3 enzymes (Fig. 1). On the other hand, UBP43 (also called USP18) acts as a major ISG15-specific deconjugating enzyme. ISG15 is not present in yeast, nematode (*Caenorhabditis*), or insect (*Drosophila*), indicating that the functions of ISG15 and its modification are restricted to higher animals with evolved IFN signaling.

ISG15 is strongly induced by type I IFNs [16,17]. Viral infection also strongly induces ISG15 [18,19] because one of its major host responses is the production of type I IFNs. A number of proteins

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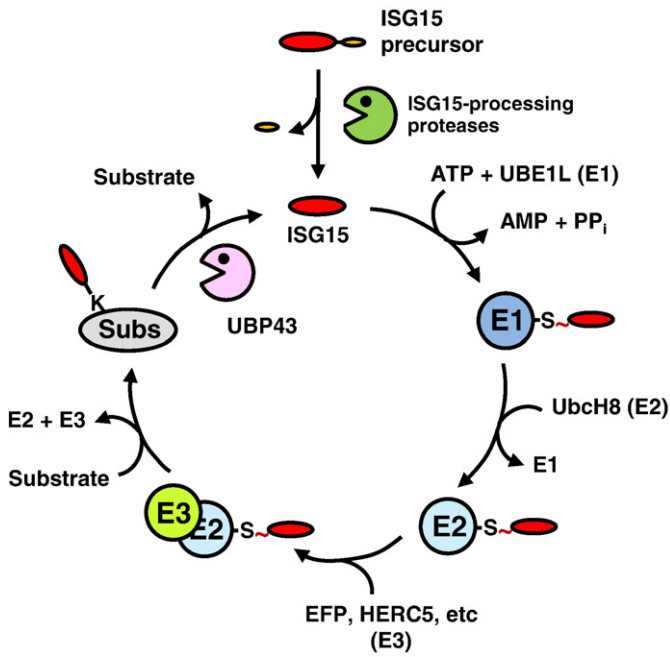


Fig. 1. Protein modification by ISG15. ISG15-specific proteases cleave off the C-terminal extensions from ISG15 precursors to generate matured ISG15 molecules. ISG15 is activated by UBE1L (E1) at the expense of ATP and is subsequently linked to the activating enzyme via thioester bond. ISG15 linked to UBE1L is transferred to UbcH8 (E2) and then to a target protein with the aid of an ISG15 E3 ligase, such as EFP and HERC5. UBP43 functions in the reversal of the ISGylation process by cleaving off ISG15 molecules that are conjugated to the substrate proteins via isopeptide bonds.

that are involved in antiviral signaling pathways, including RIG-I, MDA-5, Mx1, PKR, STAT1, and JAK1, have been identified as target proteins for ISGylation. Moreover, recent studies have explored the biological functions of ISG15 and its conjugation to target proteins. ISG15 and its conjugation impair viral replication in vivo [20–26]. Conversely, certain viruses induce viral specific proteins that can deconjugate ISG15 from its target proteins or prevent the genera-

tion of ISGylated proteins, thus abrogating the antiviral response [18,27,28]. However, functional consequences of reversible ISG15 modification of most target proteins, which are induced by viral infection, are still largely unknown.

One of the key components of the innate immune response in regulating cancer development is the activation of type I IFN signaling pathways [29,30]. Type I IFNs suppress cell proliferation and promote apoptosis and, therefore, have been used in the treatment of several cancers, such as leukemia [31]. Notably, ISG15 appears to function as an oncogenic protein as well as a tumor suppressor protein [32]. The findings that cancer chemotherapeutics cause an increase in the level of ISG15 conjugates suggest the role of ISG15 as a tumor suppressor [33–37]. Conversely, the observations that deregulated overexpression of ISG15 and enhanced ISGylation are positively correlated with carcinogenesis implicate the oncogenic potential of ISG15 protein [38–40]. However, since IFNs are induced on the development of many cancers and ISG15 is an interferon-inducible protein, the enhanced expression of ISG15 and its conjugation could be a side product of IFN response to cancer and may not play a general role in carcinogenesis. Thus, further studies are required for clarification of the functional relationship between ISG15 and carcinogenesis.

2. ISG15, the product of interferon-stimulated gene 15

2.1. Properties of ISG15

ISG15 was originally identified by Farrell et al. [16]. Because some antibodies directed against ubiquitin also react with ISG15, it was initially named as an ubiquitin cross-reactive protein (UCRP) [17]. This cross-reactivity is explained by the fact that the 15-kDa ISG15 protein consists of two domains, each of which bears high sequence homology to ubiquitin. The primary sequences of the two ubiquitin-like domains that correspond to the N- and C-terminal regions of ISG15 share 29% and 31% identities with ubiquitin, respectively (Fig. 2). Furthermore, both the N- and C-terminal domains of ISG15 show a striking similarity in their tertiary structures to ubiquitin (Fig. 3) [41]. Like ISG15, FAT10, which is also a member of the Ubl family,

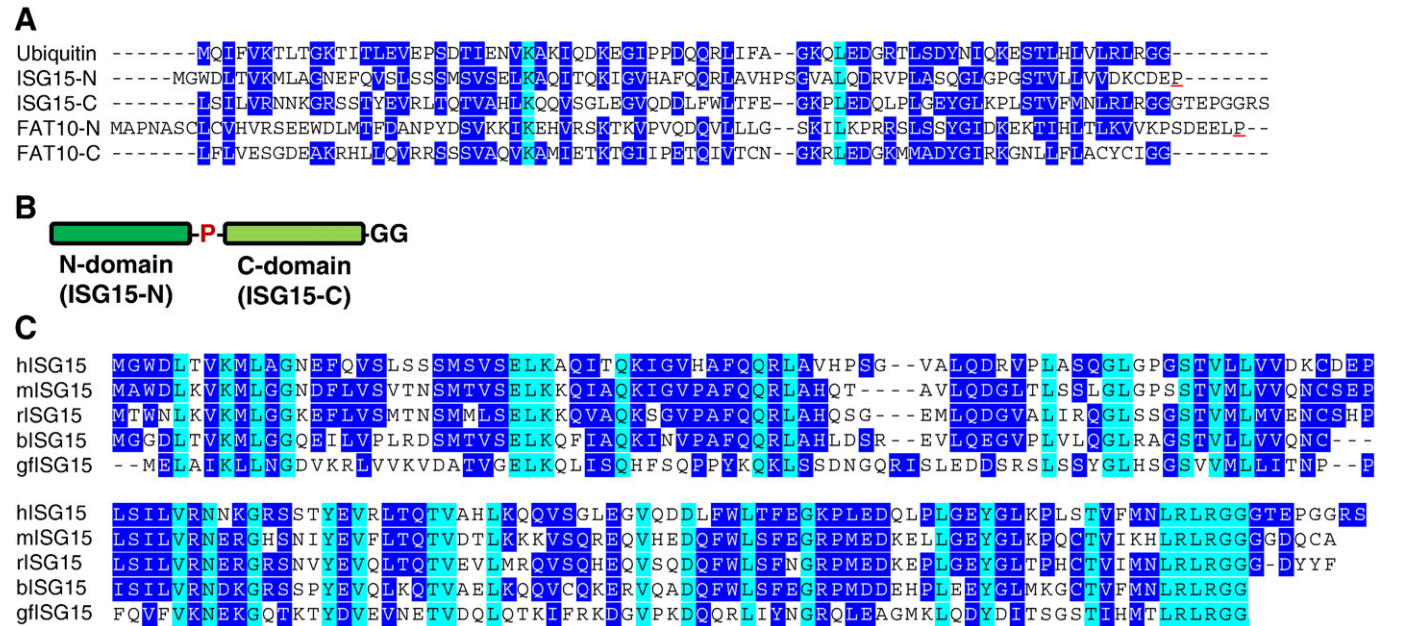


Fig. 2. Comparison of the amino acid sequence of ISG15 with that of ubiquitin and FAT10. (A) The amino acid sequences of ubiquitin, ISG15, and FAT10 are aligned. Similar amino acids are indicated by dark blue and the identical amino acids are by light blue. (B) Schematic diagram shows the N- and C-terminal ubiquitin-like domains that are flanked by a flexible linker region containing a Pro residue. (C) The amino acid sequences of ISG15 from various species are aligned. Note that ISG15 molecules from human, mouse, and rat have the C-terminal extensions of 5–8 amino acids, whereas ISG15 molecules from bovine and fish do not.

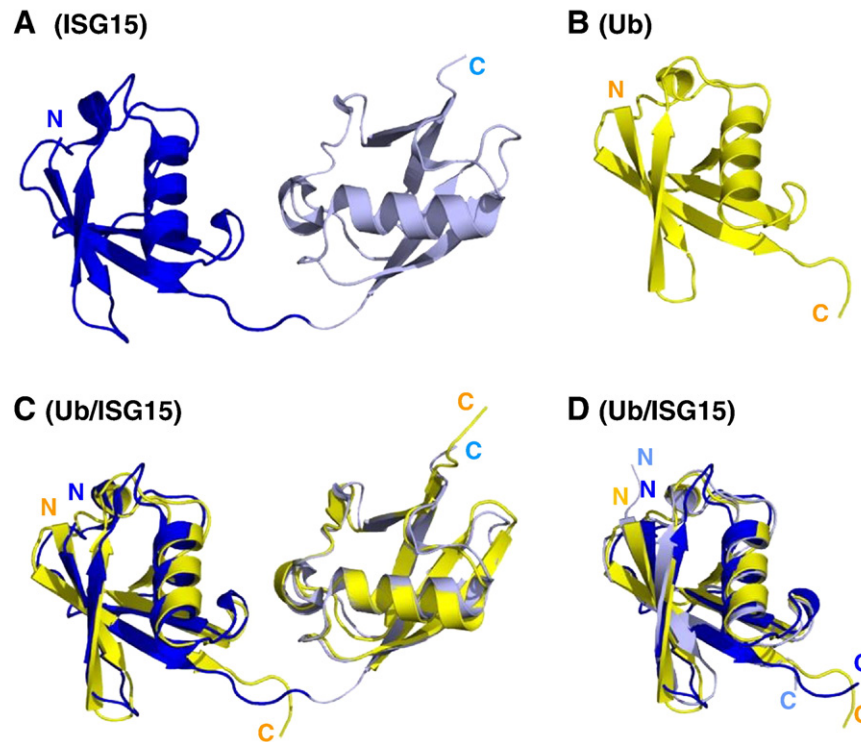


Fig. 3. Comparison of the tertiary structure of ISG15 with that of ubiquitin. (A) ISG15 alone. (B) Ubiquitin alone. (C) Ubiquitin is overlapped to the N- and C-terminal domains of ISG15. (D) All of ubiquitin and the N- and C-terminal domains of ISG15 are overlapped. Information for structures was from the protein data bank (PDB): ubiquitin from UCH-L3-Ub complex (PDB code: 1XD3) and ISG15 (PDB code: 1Z2M). The photographic images were made by using the PyMol program.

comprises two ubiquitin-like domains [42]. FAT10 is conjugated to a limited number of cellular proteins [43,44].

Like ubiquitin, ISG15 proteins in some species are synthesized as precursors that need to be processed before conjugation to target proteins (Fig. 2) [45]. While ISG15 molecules in human, mouse, and rat are translated as precursors containing the extensions of 5–8 amino acids in their C-termini, ISG15 proteins in most other species, including fish and bovine, are synthesized as matured forms [46]. The C-terminal LRLGG sequence of ISG15, as that of ubiquitin, is essential not only for its conjugation to substrates but also for its recognition by the relevant processing proteases. However, this hexapeptide sequence is absent in the analogous position of the N-terminal domain. Instead, a Pro residue is present at the position 81 of ISG15, thus preventing the incorrect processing that might generate the two cleaved ubiquitin-like domains of the 15-kDa polypeptide [47].

The C-terminal ubiquitin-like domain is sufficient for its activation by UBE1L and subsequent thioesterification of UBE1L and UbcH8 [48]. When overexpressed, the C-terminal domain of ISG15 can be conjugated to cellular proteins. However, the level of cellular proteins conjugated by the C-terminal domain alone is much lower than that by full-length ISG15, suggesting that both domains of ISG15 are required for efficient conjugation to cellular proteins.

Interestingly, the replacement of Arg153 in human ISG15 (Arg151 in mouse) by Ala ablates the binding of ISG15 to UBE1L and subsequent transesterification of UbcH8 [49]. Accordingly, the ability of the mutant ISG15 to form protein conjugates in 293T cells is markedly diminished. Furthermore, while expression of wild-type ISG15 protects *IFNAR*^{-/-} mice from lethality after Sindbis virus (SNV) infection, expression of the Arg-to-Ala mutant form of ISG15 confers no survival benefit. This mutation also attenuates the ability of ISG15 to decrease SNV replication in *IFNAR*^{-/-} mice or prolong the survival of *ISG15*^{-/-} mice. Thus, Arg153 appears to serve as a binding site for UBE1L.

2.2. Control of ISG15 induction

Type I IFNs are capable of inducing ISG15 [47]. Notably, the induction of ISG15 shows biphasic kinetics. After IFN β treatment, the increase in the level of free ISG15 molecules is first observable within 2 h, continues for the next 10 h, and becomes maximal by about 18 h. On the other hand, ISG15 conjugates are observable at least 12 h after the exposure to IFN β , and their level dramatically increases between 12 and 18 h and continuously increases thereafter although at a slower rate. This delayed induction of ISG15 conjugates indicates the requirement of ISG15-conjugating machinery that is expressed in the later periods after the treatment with type I IFNs.

ISG15 is strongly induced by viral infection [18,19]. Like other IFN-stimulated genes, the *ISG15* gene has a 5'-cis-regulatory sequence called ISRE (interferon-stimulated response element) [50]. A number of IRFs, including IRF-3 and IRF-9, bind to the ISRE for ISG15 induction (Fig. 4) [50–52]. IRF-3 forms a complex with CBP/p300 coactivators for ISG15 induction [53,54]. On the other hand, IRF-9 interacts with STAT1 and STAT2, leading to the formation of ISGF3 complex that also induces ISG15. Upon dsRNA treatment, however, IRF-3 induces ISG15 independently of IFNs [55].

ISG15 is also strongly induced by lipopolysaccharide (LPS) [56–58]. When macrophages are stimulated by LPS, ISG15 can be detected as early as 1 h and its level becomes maximal at about 4 h [59]. Infection of mice with bacille Calmette–Guérin (BCG) markedly induces ISG15 in macrophages. Moreover, this bacterial infection leads to ISGylation of serpin2a (serine protease inhibitor 2a), which was the first identified target protein, although its biological significance remains unknown. ISGylation of serpin2a may protect macrophages from lysosomal enzymes because a variety of lysosomal proteases are upregulated by IFN γ for the destruction of ingested bacteria and other pathogens. In addition, it has been shown that Trif/IRF-3 signaling pathway is responsible for LPS-mediated induction of ISG15 and its conjugation to target proteins [60].

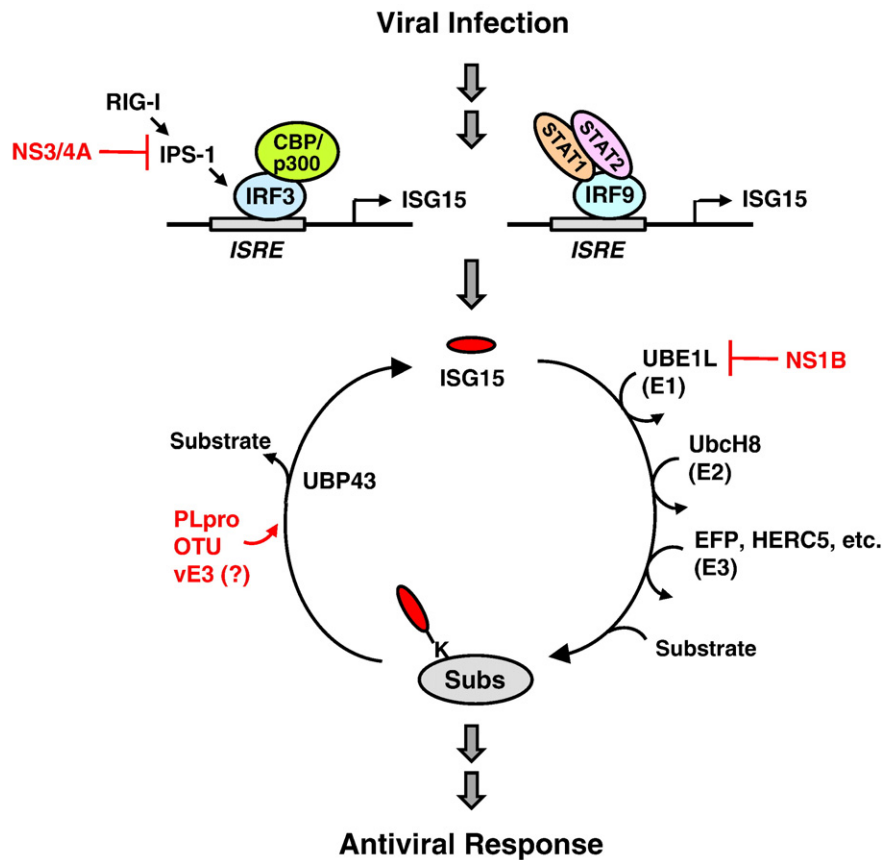


Fig. 4. Viral immune-evasion proteins. NS3/4A is involved in the cleavage of IPS-1, thus blocking the RIG-I signaling for IRF-3-mediated induction of type I IFN as well as of ISG15. NS1B inhibits the thioesterification of UBE1L and thereby the generation of ISG15 conjugates that are required for antiviral response. PLpro, OTU protease, and viral E3 protein mediate the removal of ISG15 from its conjugates.

ISG15 is a target gene of NF- κ B. LPS induces ISG15 in 70Z/3 cells that recapitulate aspects of the pre-B to immature B-cell transition in response to NF- κ B activation, but not in 1.3E, which is a NF- κ B signaling defective mutant cell line, indicating that NF- κ B activation is required for ISG15 induction [56]. Ataxia telangiectasia (AT) is a multifaceted autosomal recessive genetic disorder, characterized by the loss of coordination, progressive neuronal degeneration, immunodeficiency, and cancer proneness [61]. NF- κ B is constitutively activated in human fibroblasts derived from AT patients [62]. Moreover, the level of ISG15 is constitutively elevated in the AT cells [63]. Because activated NF- κ B is capable of inducing the expression of type I IFNs, it appears that the elevation of ISG15 level in the AT cells is due to abnormal production of type I IFNs.

ISG15 also is a target gene of p53 [64–66]. Activation of temperature-sensitive p53 leads to ISG15 induction in the absence of exogenous stimuli. Since cycloheximide treatment does not influence the increase in the level of ISG15 mRNA, ISG15 induction appears to be a primary response to p53. While type I IFNs induce the accumulation of ISG15 mRNA in both wild-type or p53-deficient cells, dsRNA induces it only in wild-type cells, indicating that p53 is required for ISG15 induction by dsRNA but not for that by type I IFNs. In addition, sequence analysis of the human ISG15 gene has led to the identification of a putative p53-responsive element that is located adjacent to the ISG15-specific ISRE [50]. Thus, in virus-infected cells, p53 seems to exert its antiviral function by the induction of ISG15 in addition to the induction of apoptotic signaling [67].

JNK induces the expression of ISGs, such as ISG15, ISG12, and IGTP [68]. Swiss 3T3 cells expressing constitutively active MKK7–JNK1 β fusion protein show increased resistance to apoptosis induced by vesicular stomatitis virus (VSV) infection, suggesting the involvement

of JNK signaling pathway in antiviral response. Recently, PI3K has been shown to control both IFN α - and IFN γ -induced expression of ISGs, including ISG15, at both transcriptional and translational levels [69]. IFN-mediated antiviral response is defective in cells lacking p85 α /p85 β , the regulatory subunits of PI3K, suggesting the involvement of PI3K signaling pathway in innate immune response.

ISG15 is induced by certain genotoxic stresses. For example, treatment with camptothecin, an inhibitor of topoisomerase I, leads to an increase in the level of ISG15 mRNA, and this increase requires protein synthesis and a functional p53 protein [35]. Interestingly, IFNs and JAK/STAT are dispensable for camptothecin-mediated induction of ISG15. ISG15 conjugates generated by camptothecin are distinct from those produced by type I IFNs, suggesting that different protein substrates are targeted for ISGylation. However, camptothecin markedly increases IFN-induced ISGylation of cellular proteins. Furthermore, treatment with both IFNs and camptothecin causes synergistic killing of colorectal cancer xenografts in nude mice, suggesting that the combination of the drugs can be an effective therapeutic strategy [36].

Retinoic acid upregulates the levels of ISG15, its conjugates, and UBE1L in acute promyelocytic cells [33,34]. The retinoic acid-mediated accumulation of ISG15 and its conjugates occurs in retinoic acid-sensitive leukemic cells but not in retinoic acid-resistant cells and the pattern of accumulated ISG15 conjugates is similar to that observed by the exposure to type I IFNs. In addition, several of the type I IFN-induced proteins, such as IRF-1 and (2'-5') oligoadenylate synthetase, are induced by retinoic acid [70–72]. Treatment with retinoic acid also leads to an increased secretion of type I IFNs into culture media. Blockade of IFNAR with a neutralizing antibody prevents retinoic acid-mediated accumulation of ISG15 and its conjugates. Thus, retinoic

acid seems to elevate the levels of ISG15 and its conjugates by stimulating cells to secrete IFNs.

3. Enzymes for ISG15 modification

3.1. ISG15-activating E1 enzyme

UBE1L is a 112-kDa protein that shows a 45% identity in amino acid sequence to the human ubiquitin-activating E1 enzyme (UBE1) [73]. UBE1L expressed in baculovirus forms a thioester bond with ISG15 but not with ubiquitin [18], indicating that it is a specific ISG15-activating E1 enzyme. In addition, UBE1L has a C-terminal ubiquitin-fold domain that is required for the transfer of ISG15 from UBE1L to UbcH8 as well as for the binding of UBE1L to UbcH8 [74]. As expected, UBE1L-deficient mice are not capable of producing ISG15 conjugates upon stimuli [75]. In *UBE1L*^{-/-} macrophages treated with LPS and in *UBE1L*^{-/-} mouse embryonic fibroblasts (MEFs) treated with IFNs, ubiquitination of cellular proteins occurs normally, but ISGylation of proteins is completely abolished, confirming the strict requirement of UBE1L for ISGylation.

The *UBE1L* gene is located in the D3F15S2 locus of chromosome 3q21 [76]. UBE1L can be detected in the jejunum, colon, lung, liver, tonsils, testis, and skin but not in the spleen and pancreas. Significantly, UBE1L is not detectable in all tested human lung cancer cell lines [73,77], implicating a tumor-suppressive role of UBE1L. This repressed expression in lung cancer cell lines is intriguing because a region of chromosome 3q, in which the *UBE1L* gene resides, is often deregulated in preneoplastic or neoplastic epithelial tissues [78]. However, upon generation of *UBE1L*^{-/-}/*K-ras*^{LA2} mice, it has recently been shown that the loss of ISGylation does not affect tumor spectrum, tumor pathology, or survival of *K-ras*^{LA2} mice [79]. Nonetheless, it is possible that UBE1L deficiency and *K-ras* mutation are two separate pathways in lung cancer development. Additional works with other lung cancer mouse models are necessary to clarify the potential tumor suppressor function of UBE1L in *K-ras* mutation-independent human lung cancers.

3.2. ISG15-conjugating E2 enzymes

UbcH8, an ISG15-conjugating E2 enzyme, is induced by type I IFNs and viral infection [80–82]. UbcH6 is also induced by type I IFNs and forms a thioester intermediate with ISG15, suggesting that UbcH6 has the potential to function as an ISG15-conjugating E2 enzyme [83]. However, the amount of the thioester intermediate formed by UbcH6 is much lower than that by UbcH8, indicating that UbcH8 is a major E2 enzyme for ISG15.

UbcH8 was originally identified as an ubiquitin-conjugating E2 enzyme by a yeast two-hybrid screening for its interaction with E6AP [84]. UbcH8 was later shown to also interact with other ubiquitin E3 ligases, such as Parkin, Dorfin, Staring, EFP, and RLIM and function as an ubiquitin-conjugating E2 enzyme [85–90]. Thus, UbcH8 serves as an E2 enzyme for both ubiquitin and ISG15. However, although UbcH7 is the most closely related E2 to UbcH8, it does not function in ISG15 conjugation [74]. Moreover, UbcH8 shows a higher affinity to UBE1L than UbcH7, while UbcH7 binds more strongly to UBE1 than UbcH8. In addition, it has been shown that two structural elements within the E2 N-terminal region are responsible for the differential interaction of UbcH8 and UbcH7 with UBE1L. Thus, UbcH8 may preferentially, but not exclusively, function as an E2 enzyme for ISG15.

Interestingly, UbcH8 and ISG15 can act as functional regulators of RNF125, an ubiquitin E3 ligase of RIG-I [91]. In the absence of ISG15, UbcH8 interacts with RNF125 and interferes with RIG-I ubiquitination. Upon overexpression of ISG15, however, the interaction of UbcH8 with ISG15 leads to the dissociation of UbcH8 from RNF125, thus allowing the association of RNF125 with UbcH5 for RIG-I ubiquitination.

3.3. ISG15 E3 ligases

The overlapping function of UbcH8 as an E2 enzyme for both ubiquitin and ISG15 raises a possibility that some UbcH8-interacting ubiquitin E3 ligases can also function as ISG15 E3 ligases. Indeed, the UbcH8-interacting protein EFP (estrogen-responsive finger protein) that has a RING finger domain serves as an ISG15 E3 ligase [92]. In the absence of ISG15, UbcH8 and EFP may function as E2 and E3 enzymes for ubiquitination, respectively. Upon ISG15 induction, however, ISG15 may compete with ubiquitin for binding to UbcH8, allowing UbcH8 and EFP to serve as the enzymes for ISGylation of 14-3-3 σ . Replacement of the active site Cys residue in the RING domain disrupts EFP-mediated ISGylation of 14-3-3 σ . Like UbcH8, EFP is a type I IFN-inducible protein [93]. Interestingly, EFP can ISGylate itself on the Lys117 residue and this auto-ISGylation negatively regulates the ISG15 E3 ligase activity of EFP toward 14-3-3 σ , suggesting that a feedback loop is operating for the control of the protein ISGylation [94,95]. An additional RING-containing ubiquitin E3 ligase, called HHARI (human homolog of *Drosophila ariadne*), also serves as an ISG15 E3 ligase for 4EHP [96].

HERC5 (HECT domain and RCC1-like domain containing protein 5) is an ISG15 E3 ligase that contains the HECT domain [97–99]. Like EFP, HERC5 is a type I IFN-inducible protein. Knockdown of HERC5 by using siRNA prevents IFN-mediated increase in the total level of ISGylated cellular proteins, unlike that of EFP, which blocks the ISGylation of 14-3-3 σ with little or no effect on the total level of ISGylated proteins. Thus, it appears that HERC5 serves as a general IFN-induced ISG15 E3 ligase. Like EFP, HERC5 itself is a target for ISGylation, but its functional significance is unknown.

3.4. ISG15-specific proteases

3.4.1. UBP43 and other ISG15-specific proteases

UBP43 cleaves off ISG15 from its protein conjugates that are linked via isopeptide bonds [100]. UBP43 can also cleave the peptide bonds immediately after the LRLGG sequence in ISG15 fusions [100]. Thus, UBP43 appears to also function in the processing of ISG15 precursors to generate matured ISG15 molecules. However, apart from UBP43, additional ISG15-specific proteases must exist because ISG15 precursor is processed to its matured form in UBP43-deficient mice [101]. The overlapping function of some E2 and E3 enzymes in the conjugation of both ISG15 and ubiquitin also implies the existence of promiscuous DUBs that can serve as ISG15-specific proteases. Indeed, several DUBs, including USP2, USP5, USP13, and USP14, have been identified as the candidates that can function as ISG15-processing and/or deconjugating enzymes [102]. In addition, it has been reported that recombinant ISG15 precursors can be properly processed by a 100-kDa enzyme in the extracts of human lung fibroblasts cell line A549, whose activity is unaffected by type I IFN stimulation [103]. This 100-kDa enzyme is a cysteine protease and shows a partial similarity in its amino acid sequence with that of the human ortholog of yeast Ubp1 or Ubp1-related protein. However, deletion of the *UBP43* gene in mouse leads to a massive increase of ISG15 conjugates in tissues without affecting the level of ubiquitin conjugates, indicating that UBP43 is the major ISG15-specific protease that deconjugates ISG15 from its target proteins. Thus, it appears likely that the above-mentioned DUBs as well as the 100-kDa cysteine protease function primarily in the processing of ISG15 precursors to generate matured ISG15 molecules.

UBP43 is induced by type I IFNs, and this induction requires a functional JAK/STAT signaling pathway [104]. IFN β induces UBP43 more strongly than IFN α and dsRNA, but IFN γ barely induces it [105]. UBP43 is also induced by LPS [58]. IRF-3 is responsible for LPS-mediated induction of UBP43, while IRF-2 is involved in its induction to a basal level. However, both IRF-2 and IRF-3 are required for optimal responsiveness to LPS. Interestingly, UBP43 induction is upregulated by

the acute myelogenous leukemia (AML1)–ETO fusion protein that is created by t(8;21), suggesting a possible involvement of UBP43 in hematopoiesis [106]. However, it is unknown how AML1-ETO affects the up-regulation of UBP43 induction. In addition, UBP43 has been identified as a substrate for Skp2 [107]. Skp2 promotes the ubiquitination of UBP43 and subsequent degradation by the proteasomes, suggesting that the SCF^{Skp2} may be involved in the regulation of type I IFN signaling by controlling the stability of UBP43.

The human *UBP43* gene maps to the chromosome 22q11.2 [108]. This region, known as DiGeorge syndrome critical region, is consistently deleted in DiGeorge syndrome and related disorders, suggesting that UBP43 may be involved in the development of thymus or differentiation of T cells.

3.4.2. *UBP43 as a negative regulator of innate immune responses*

UBP43-deficient mice are viable and resistant to the fatal lymphocytic choriomeningitis and myeloencephalitis that develop in wild-type mice upon intracerebral inoculation of lymphocytic choriomeningitis virus (LCMV) and VSV, respectively [101,109]. Furthermore, survival of *UBP43*^{-/-} mice after LCMV infection correlates with severe inhibition of LCMV replication as well as with an increase in the level of ISG15 conjugates in the brain. None of the *UBP43*^{-/-} mice infected with LCMV died or developed clinical symptoms by day 11 after infection, whereas all LCMV-infected wild-type mice died by day 7 infection. These findings strongly suggest that UBP43 deficiency causes an unfavorable environment for LCMV replication. In addition, *UBP43*^{-/-} MEF cells exhibit enhanced type I IFN-mediated resistance to cytopathic effect caused by VSV and SNV. These findings strongly suggest that UBP43 serves as a negative regulator of innate immune response against viral infection. However, the enhanced resistance to viral infection in *UBP43*^{-/-} mice cannot be rescued in *UBP43*^{-/-}/*ISG15*^{-/-} or *UBP43*^{-/-}/*UBE1L*^{-/-} double knockouts, indicating that the phenotypic alterations are not associated with the protein modification by ISG15 [75,110]. Thus, UBP43, independently of its isopeptidase activity, may have another biological function.

UBP43^{-/-} cells are hypersensitive to type I IFNs, resulting in a dramatic increase in the level of ISGylated proteins, which is associated with the enhanced and prolonged JAK/STAT signaling [111]. However, UBP43, independently of its catalytic activity, also functions as a negative regulator of type I IFN signaling [112]. This UBP43 action is achieved through a direct interaction between UBP43 and IFNAR2, a subunit of type I IFN receptor. Binding of IFNAR2 to UBP43 interferes with the interaction between JAK and the receptor, leading to the inhibition of downstream phosphorylation cascade and other signaling events. In addition, complementation of *UBP43*^{-/-} cells with a catalytically inactive mutant of UBP43 leads to the inhibition of STAT1 phosphorylation to a level seen by that with wild-type UBP43. Moreover, down-regulation of the total level of ISG15 conjugates by siRNA-mediated knockdown of UBE1L in *UBP43*^{-/-} cells shows little or no effect on STAT1 phosphorylation in comparison with that seen in *UBP43*^{+/+} cells, indicating that the deISGylating activity of UBP43 is not required for its inhibitory effect on type I IFN signaling. This conclusion is further corroborated with the findings that phenotypic alterations in *UBP43*^{-/-} mice are not influenced by the lack of ISG15 in *UBP43*^{-/-}/*ISG15*^{-/-} double knockout mice [75,110].

The isopeptidase-independent action of UBP43 is also involved in the replication of HBV (hepatitis B virus) [113]. *UBP43*^{-/-} cells show an increased induction of ISGs in response to type I IFNs, indicating that the lack of UBP43 results in a strengthened immune response. Consistently, the steady-state level of HBV DNA is substantially reduced in *UBP43*^{-/-} mice in comparison with that in *UBP43*^{+/+} mice. Thus, approaches that modulate UBP43 level could be used as therapeutic potentials in treating viral infection, especially for viruses sensitive to type I IFN signaling. In addition to the protection against HBV, UBP43 deficiency increases the resistance to oncogenic transformation by BCR–ABL (breakpoint cluster region Abelson leukemia

protein), a fusion protein consisting of the N-terminal portion of BCR joined to most of the ABL tyrosine kinase [114]. This resistance to leukemia development is heavily dependent on the activation of type I IFN signaling pathway. Loss of type I IFN signaling through the loss of IFNAR results in a reversal of the original resistance to the leukemia development observed in mice that received a transplant of BCR–ABL-expressing UBP43-deficient donor cells. Thus, it appears that inhibition of the negative effect of UBP43 on type I IFN signaling could enhance innate immune response against cancer development.

4. Biological functions of ISG15 and its conjugation

By using a combination of affinity purification and mass spectrometry, hundreds of target proteins for ISGylation have been identified. Some of them are type I IFN-induced proteins, including PKR, MxA, Hup56, and RIG-I [115]. Some are key regulators that are involved in type I IFN signaling, such as PLCγ1, JAK1, ERK1, and STAT1 [116]. Most other targets are constitutively expressed and function in diverse cellular pathways, including translation, glycolysis, cell motility, protein modification, intracellular protein trafficking, RNA splicing, chromatin remodeling, cytoskeletal organization, and stress responses [97,115,117]. These findings implicate the role of protein ISGylation in type I IFN-induced immune responses as well as in the control of numerous fundamental cellular pathways.

4.1. *ISG15 as a cytokine modulating immune responses*

ISG15 is synthesized in many cell types and secreted from human monocytes and lymphocytes [118]. Both native and recombinant ISG15 induce the synthesis and secretion of IFNγ from B-cell-depleted lymphocytes, implicating the role of ISG15 as a cytokine that modulates immune response [119]. Treatment with human ISG15, but not its precursor form, leads to an increase in DNA synthesis in cultured primary blood lymphocytes in a dose-dependent fashion, suggesting that ISG15 can act as a mitogen and that the processing of ISG15 precursor is required for the generation of biologically active ISG15 [120]. Furthermore, ISG15 has been identified as a member of the cytokine cascades. Upon viral infection, type I IFNs produced in infected cells induce the synthesis of ISG15. These ISG15 molecules can be secreted or released by lysis of the infected cells. They may then induce the production of IFNγ from T cells, augment NK cell proliferation, activate non-major histocompatibility complex-restricted cytolytic lymphocytes, and activate monocytes and macrophages via the induced IFNγ.

4.2. *Regulation of signal transduction pathways*

Filamins are nonmuscle actin-binding proteins that comprise a family of three members: filamin A, B, and C [121,122]. These filamin isoforms are large cytoplasmic proteins that play important parts in cross-linking cortical actin filaments into a dynamic three-dimensional structure. Recently, it has been shown that filamin B functions as a scaffold that links between activated Rac1 and a JNK cascade module for mediating type I IFN signaling [123,124]. Filamin B interacts with Rac1, MEKK1, MKK4, and JNK and enhances their sequential activation in response to type I IFNs, thereby promoting JNK activation and apoptosis. This acceleration of JNK-mediated apoptosis provides a biological basis for antitumor and antiviral functions of type I IFNs. Furthermore, it has been revealed that type I IFNs induce ISGylation of filamin B, which leads to dissociation of Rac1, MEKK1, and MKK4 from the scaffold protein and thus to the prevention of prolonged activation of type I-induced JNK signaling pathway [124,125]. In contrast, blockade of filamin B ISGylation by substitution of the ISG15 acceptor site Lys2467 with Arg or by siRNA-mediated knockdown of

UBE1L prevents the release of the signaling molecules from filamin B, resulting in persistent promotion of JNK activation and JNK-mediated apoptosis. These findings indicate that ISGylation of filamin B serves as a negative feedback regulatory gate for desensitization of type I IFN-induced JNK signaling, thus providing an important mechanism for the survival of uninfected bystander cells.

PP2C β dephosphorylates TAK1 and suppresses TAK1/TAB1-mediated I κ B α degradation, thus controlling NF- κ B signaling pathway, which plays a critical role in innate and adaptive immunity and cancer [126–129]. PP2C β is a target for ISGylation, and this modification blocks the suppressive function of the protein phosphatase against TAK1/TAB1-mediated NF- κ B activation [130]. This conclusion is based on the observation that overexpression of UBE1L, UbcH8, and ISG15 blocks the suppressive effect of PP2C β on NF- κ B activation, but not that of its mutant, in which the ISG15 acceptor sites Lys12 and Lys142 are replaced by arginine. Thus, ISGylation of PP2C β seems to play a role in the control of NF- κ B pathway.

4.3. Regulation of ubiquitination

Ubc13, an ubiquitin-conjugating E2 enzyme, is a target for ISGylation [131,132]. ISG15 is conjugated to Lys92 of Ubc13, which is very closely located to its active site, thus preventing the formation of a thioester bond with ubiquitin. Since Mms2, which forms a heteromeric complex with Ubc13, interacts with both unmodified and ISGylated Ubc13, the inhibitory effect of Ubc13 ISGylation on the thioesterification of ubiquitin seems to be achieved by the inability of ISGylated Ubc13 to accept ubiquitin to its active site or by blocking the recognition of ubiquitin E1 enzyme and subsequent transfer of ubiquitin from the E1 enzyme to Ubc13. Ubc13 is known to mediate the generation of Lys63-linked poly-ubiquitin chains that are conjugated to a variety of signaling molecules [133,134]. Thus, it is possible that ISGylation of Ubc13 may play an important role in the control of signal transduction pathways, such as NF- κ B pathway, which are associated with Lys63-linked poly-ubiquitination [132]. UbcH8 and UbcH6 also are targets for ISGylation [83]. ISGylation of UbcH6 occurs in response to type I IFNs and blocks the thioesterification of ubiquitin, like that of Ubc13, suggesting that ISGylation of UbcH6 may also lead to the suppression of its ubiquitin-conjugating activity.

4.4. Regulation of antiviral responses

Initial efforts that intend to determine the role of ISG15 in antiviral responses appeared unsuccessful. *UBE1L*^{-/-} mice are fertile and exhibit normal antiviral responses against VSV and LCMV infection, indicating that UBE1L and protein ISGylation may not be essential for IFN signaling [75]. Furthermore, *ISG15*^{-/-} mice are also fertile and display no obvious abnormalities [135]. Lack of ISG15 does not affect the development and composition of the main cellular immune system. The IFN-induced antiviral and immune responses directed against VSV and LCMV are not significantly altered in the absence of ISG15. In addition, IFN- or endotoxin-induced STAT1 phosphorylation as well as the expression of typical STAT1 target genes remain unaffected by the lack of ISG15, indicating that ISG15 is dispensable for STAT1-mediated IFN signaling. However, the function of ISG15 as an antiviral effector has come to the front.

Unlike the infection by VSV and LCMV, *UBE1L*^{-/-} mice display markedly increased susceptibility to influenza B virus infection, with only 25% survival of *UBE1L*^{-/-} mice for 21 days, compared to 86% survival of *UBE1L*^{+/+} mice [20]. Furthermore, both of the kinetics of lethality and the overall survival of *UBE1L*^{-/-} mice are identical to those of *ISG15*^{-/-} mice, indicating that the antiviral activity of ISG15 against influenza B virus is mediated by its conjugation to target proteins. The predominant site of ISG15 action during influenza virus infection resides within a stromal cell population. A likely candidate

is the respiratory epithelium, since it is the site of influenza virus replication. This is the first phenotype described for *UBE1L*^{-/-} mice, which were previously found to have no defect in response to VSV or LCMV infection [75]. In addition, it has recently been shown that both the synthesis of influenza A virus protein and the early rate of the virus replication are inhibited by ISG15 conjugation of cellular proteins [21].

ISG15^{-/-} mice also exhibit increased susceptibility to infection by a number of other viruses, such as influenza A/WSN/33 and B/Lee/40 viruses, herpes simplex virus type 1 and murine gamma herpes virus 68, and SNV [22]. In addition, ISG15 is induced after SNV infection, and this induction is markedly attenuated in *IFNAR*^{-/-} mice, indicating that induction of ISG15 by SNV infection is dependent on type I IFNs [136]. ISG15 induction protects against SNV-induced lethality and decreases the virus replication in multiple organs. The increased susceptibility of *IFNAR*^{-/-} mice to SNV infection can be rescued by the expression of wild-type ISG15 having the C-terminal diglycine residues but not by that of an ISG15 mutant, of which the C-terminal diglycine is replaced by dialanine, again indicating that the antiviral action of ISG15 requires its conjugation to target proteins.

Type I IFN-mediated inhibition of human immunodeficiency virus (HIV) assembly and release has been shown to correlate with the induction of ISG15 [23]. Furthermore, overexpression of ISG15 mimics the IFN effect and inhibits the release of HIV-1 virions without having any effect on the synthesis of the viral proteins in infected cells [24]. In cells infected with HIV-1 provirus, overexpression of ISG15 and UBE1L causes a complete inhibition of HIV-1 replication. On the other hand, coexpression of UBP43 can partially rescue the release of HIV-1 in ISG15-expressing cells. Intriguingly, ISG15 expression specifically inhibits the ubiquitination of Gag and Tsg101 and disrupts their interaction, thereby preventing assembly and release of virions from infected cells. Expression of ISG15 alone (i.e., without UBE1L) does not block the viral release, indicating that ISGylation of target proteins, but not ISG15 itself, is required for the inhibition of Gag ubiquitination, although ISGylation of either Gag or Tsg101 could not be detected. Thus, ISGylation of certain unknown viral and/or host proteins appears to play a critical role in the IFN-mediated inhibition of HIV-1 assembly and release.

Overexpression of ISG15 or UBE1L with UbcH8 has been shown to inhibit budding of Ebola virus VP40 virus-like particles (VLPs) [25,26]. Ebola virus is a member of the Filoviral family of negative-sense RNA viruses. The VP40 matrix protein is a key structural protein that is critical for the virion release. The efficient budding of VLPs requires the interaction of overlapping L-domains (late-budding domains) in the VP40 protein with Nedd4, a ubiquitin E3 ligase, as well as with other members of the ESCRT pathway (e.g., Tsg101) [137–140]. ISG15 overexpression decreases not only the level of VP40 detected in VLPs but also the levels of endogenous Nedd4 incorporated into budding VP40 VLPs. Nedd4 interacts with and is conjugated by ISG15. Moreover, ISG15 overexpression blocks the ability of Nedd4 to ubiquitinate VP40, leading to the prevention of L-domain-mediated budding of VP40 VLPs. In addition, it has been shown that free ISG15 specifically binds to Nedd4 and inhibits the interaction of the E3 ligase with ubiquitin E2 enzymes (e.g., UbcH6), thus preventing the transfer of ubiquitin from the E2 enzymes to Nedd4 [26]. These findings reveal a mechanism for the antiviral function of ISG15 that involves the ISGylation and inactivation of the host Nedd4 E3 ligase.

IRF-3 is a target for ISGylation, which can be induced by both type I IFNs and viral infection [141]. This ISGylation prevents the ubiquitination and degradation of IRF-3 in NDV (Newcastle disease virus)-infected human fibrosarcoma 2fTGH cells, and promotes the translocation of IRF-3 to the nucleus, where it binds to the *IFN* β promoter. The relative level of IRF-3 is significantly lower in NDV-infected *ISG15*^{-/-} cells than in *ISG15*^{+/+} cells, indicating that the subversion of antiviral response is mediated by proteolysis of IRF-3. Moreover, the degradation of IRF-3 can be counteracted by

the induction of ISG15. This finding provides a positive feedback mechanism for the induction of host antiviral response by ISGylation-dependent stabilization of IRF-3.

However, ISG15 is typically conjugated to only a small fraction of target proteins. Therefore, an important issue is how the small fractions of ISGylated proteins, including IRF-3, can exert their biological functions. It has been proposed that if the small fractions of proteins that are modified by UbIs (e.g., SUMO and ISG15) are localized to some functionally unique cellular site or the transient modification is sufficient to switch the protein into new state, their functions could efficiently operate [124,142,143]. Intriguingly, overexpression of ISG15 leads to the appearance of IRF-3 as punctates in the nucleus [141]. This nuclear retention could allow IRF-3 to exert its profound antiviral function, although only a small portion of IRF-3 is ISGylated upon the viral infection. An additional example is that upon overexpression of filamin B, UbcH8 is recruited to membrane ruffles where filamin B is also concentrated with actin [123,125]. This recruitment of UbcH8, which otherwise resides throughout the cytoplasm and the nucleus, should allow the E2 enzyme to efficiently function in filamin B ISGylation and thus in desensitization of type I IFN-induced JNK signaling (see above).

The inducible nitric oxide synthase (iNOS) is not expressed under normal conditions, but like ISG15, it is induced by various stimuli, such as exposure to cytokines, microbes, or microbial products, resulting in the sustained production of NO [144]. Upon stimuli, NO as well as the products generated by its interaction with ROS, such as peroxynitrite and peroxynitrous acid, are accumulated and utilized for the antibacterial or antiviral effects [144–146]. NO is also covalently attached to the thiol group of cysteine residues of proteins, causing their S-nitrosylation. This posttranslational modification serves as an important mechanism that mediates antibacterial and antiviral effects through the alteration in enzymatic activity, protein–protein interaction, and signal transduction [147]. Interestingly, the cysteine residue in ISG15 can also be modified by NO and this S-nitrosylation decreases the dimerization of ISG15, thereby increasing the availability as well as the solubility of monomeric ISG15 for its conjugation to cellular proteins [148]. Moreover, treatment with S-ethylisothiourea, an iNOS inhibitor, reduces the level of ISG15 conjugates, whereas overexpression of iNOS increases it. Thus, iNOS may play a role in the enhancement of innate immune responses by mediating ISG15 S-nitrosylation.

4EHP is an mRNA 5'cap structure-binding protein and acts as a translation suppressor by competing with eIF4E for binding to the cap structure [149]. 4EHP is a target protein for ISGylation and this modification substantially increases its cap structure-binding activity [96]. This is the first report that shows “gain of function” by IFN-induced ISGylation, thus providing a mechanism by which a small fraction of any ISGylated protein can generate profound biological effects in response to IFNs or pathogen infections. Interestingly, 4EHP fused with ISG15 at either of its N- or C-terminus dramatically enhances the cap structure-binding activity, indicating the ISG15 fusion protein can mimic the ISGylated 4EHP. Since IFNs inhibit the translation of viral mRNAs while allowing normal translation of the majority of cellular mRNAs [150], it is possible that ISGylated 4EHP acts as a viral mRNA-specific translation inhibitor in a cap binding-dependent manner.

RIG-I senses intracellular virus-specific nucleic acid structures and as an early antiviral response induces the production of type I IFNs, which in turn activates the expression of RIG-I, thus generating a positive feedback loop for further accumulation of RIG-I [151–155]. Intracellular dsRNA activates NF- κ B and IRF-3/IRF-7 through RIG-I and the mitochondrial adaptor protein IFN promoter stimulator 1 (IPS-1) [156–159]. Furthermore, RIG-I is a target protein for ISGylation [115,160]. However, ISGylation of RIG-I leads to a reduction in the levels of both basal and virus-induced IFN production. Consistently, the basal mRNA and protein levels of RIG-I are significantly higher in *UBE1L*^{-/-} cells than in *UBE1L*^{+/+} cells. Based on these observations, it has been proposed that a negative feedback loop is operating for fine-

tuning the strength of RIG-I-mediated signaling to maintain a balance between innate immune response and hypersensitivity during antiviral responses. In addition, ubiquitination of RIG-I by TRIM25 is required to mediate antiviral signaling responses, whereas that by RNF125 results in the proteasomal degradation of RIG-I [161]. Thus, multiple positive and negative mechanisms appear to contribute to the maintenance of optimal RIG-I level and thus to the control of RIG-I-mediated signaling.

4.5. Viral immune-evading mechanisms

Viruses escape from the antiviral activity of ISG15 by using different mechanisms. Influenza B virus strongly induces ISG15 during infection but specifically blocks ISGylation of host proteins [18]. This inhibition is mediated by the viral NS1B protein, which interacts with ISG15 and prevents the generation of ISG15 conjugates under in vitro conditions as well as in infected cells (Fig. 4). Purified NS1B inhibits the formation of ISG15-UBE1L-like thioester intermediate. ISG15 conjugates accumulate in IFN-treated cells, but not in cells infected by influenza B virus, although similar amounts of ISG15 are synthesized in both cells. Moreover, ISG15 conjugates are not detectable in cells infected with the virus expressing full-length NS1B but are markedly accumulated in cells infected with the virus expressing truncated NS1B, which cannot bind to ISG15, indicating that the interaction of NS1B with ISG15 is responsible for the inhibition of ISG15 conjugation and thus of the antiviral function of ISG15.

SARS-coronavirus (SARS-CoV) produces a papain-like protease, called PLpro, which can generate an authentic ISG15 molecule by cleaving off a protein that is fused to the C-terminus of ISG15 [162]. It is possible that the activity of PLpro may mimic the ISG15-deconjugating activity of UBP43, thus favoring viral replication by counteracting protein ISGylation.

Vaccinia virus (VACV) E3 protein is an early protein that interacts with ISG15 through its C-terminal domain [28]. Whereas siRNA-mediated knockdown of ISG15 enhances the viral replication, complementation of ISG15 to *ISG15*^{-/-} cells attenuates it. Notably, the level of ISG15 conjugates is much higher in ISG15-complemented *ISG15*^{-/-} cells infected with the E3-deficient virus than that with the wild-type virus, suggesting that VACV E3 protein is directly or indirectly involved in deconjugation of ISG15 from cellular proteins. Moreover, the virus lacking E3 protein that is unable to grow in *ISG15*^{+/+} cells can replicate in *ISG15*^{-/-} cells. These findings suggest a new strategy for poxviruses to evade the host antiviral response through the viral E3 protein-mediated control of protein ISGylation.

The ovarian tumor domain (OTU)-containing proteases from nairoviruses and arteriviruses, two unrelated groups of RNA viruses, are capable of deconjugating ubiquitin and ISG15, but not SUMO, from cellular target proteins [27]. Purified OTU protease cleaves off ubiquitin from both Lys48- and Lys63-linked poly-ubiquitin chains in vitro. Remarkably, the expression of OTU protease antagonizes the antiviral effects of ISG15 and enhances the susceptibility to SNV infection. It has been shown that approximately 70% of *IFNAR*^{-/-} mice survive after infection of the virus expressing both ISG15 and an OTU variant, of which the catalytic Cys residue is replaced by Ala. In contrast, only 20% of the mice survive when infected with the virus expressing both ISG15 and wild-type OTU protease, indicating that the antiviral response is mediated by ISG15, but not by other ISGs, and that the immune-evading effect of OTU protease requires its catalytic activity. These findings indicate that SNV uses OTU protease as a unique strategy to evade the host antiviral response.

Hepatitis C virus (HCV) is an enveloped virus that causes liver diseases, including cancer [163,164]. RIG-I signaling induces a host response that controls HCV RNA replication [165]. However, HCV can evade this response in part through its ability to antagonize the relay of RIG-I signaling to IRF-3 [166]. NS3/4A, a major serine protease expressed by HCV [164], cleaves IPS-1, causing the release of cleaved

IPS-1 from the mitochondrial membrane [167,168]. This cleavage results in the subcellular redistribution of IPS-1 and loss of its interaction with RIG-I, thereby preventing downstream activation of IRF-3 and IFN β induction. Intriguingly, in liver tissues chronically infected by HCV, the IPS-1 cleavage and its subcellular redistribution are associated with the lack of ISG15 and its conjugates. Among the HCV-infected tissues, ISG15 and its conjugates can be detected only in the liver from patients, which has predominantly uncleaved, full-length IPS-1 protein. These findings indicate that NS3/4A plays a critical role in evading the host antiviral response by attenuating the IFN amplification loop of RIG-I and thus the expression of ISGs, including ISG15, which are normally induced by RIG-I- and IRF-3-dependent pathways.

4.6. ISG15 in tumorigenesis

4.6.1. ISG15 as a tumor suppressor

UBE1L has been shown to play an important role in the suppression of lung cancer growth. UBE1L overexpression inhibits the growth of human bronchial epithelial cells and lung cancer cells. Furthermore, UBE1L promotes cyclin D1 ISGylation and this modification leads to the destabilization of cyclin D1, indicating that UBE1L confers growth suppression by targeting cyclin D1 [169]. These findings provide a mechanism for the tumor-suppressive role of UBE1L [37,73,77,170], although it is unknown how the stability of cyclin D1 is affected by its ISGylation.

Acute promyelocytic leukemia (APL) is characterized by the accumulation of oncogenic PML/RAR α fusion protein [171,172]. Retinoic acid induces UBE1L and subsequent ISGylation of PML/RAR α [33,34,37]. Moreover, UBE1L-mediated ISGylation of PML/RAR α leads to a decrease in the level of PML/RAR α , thus overcoming the oncogenic effects of the fusion protein [173,174]. On the other hand, treatment with *N*-acetyl-Leu-Leu-norleucinal, a proteasome inhibitor, prevents the degradation of ISGylated PML/RAR α , indicating that the proteasomes are involved in the control of PML/RAR α stability. These findings implicate an important role of UBE1L in the suppression of leukemia.

4.6.2. ISG15 as an oncogenic factor

In many tumors and tumor cell lines, ISG15 is highly elevated and extensively conjugated to cellular proteins [39]. The level of ISG15 mRNA is significantly higher in cancerous mammary epithelial cell lines, such as BT20, MDA-MB468, MDA-MB231, T47D, and MCF7, than in nonmalignant mammary cell lines, including HMEC and MCF10A. The level of ISG15 protein is also higher in breast tumors than in normal tissues [175]. In addition, a correlation between increased ISG15 level and unfavorable prognosis in the survival of patients has been reported, suggesting a potential role of ISG15 in breast cancer development, although the role of ISG15 in breast carcinogenesis is unknown.

Significantly, the increased level of ISG15 in tumor cells is associated with the decreased levels of ubiquitinated proteins, suggesting that ISG15 plays an antagonistic role against ubiquitin-mediated proteolysis and that deregulation of ubiquitin–proteasome pathway is related with tumorigenesis [39]. Since some of the E2 (e.g., UbcH8) and E3 enzymes (e.g., EFP) can utilize both ubiquitin and ISG15 for conjugation to target proteins, it seems possible that ISG15 may potentially interfere with the ubiquitination pathway at the level of E2 and E3 enzymes, thus decreasing the level of ubiquitinated proteins in tumor cells.

However, it has been shown that the level of ubiquitin conjugates in *UBP43*^{-/-} cells is nearly the same as that in *UBP43*^{+/+} cells [116], despite the fact that treatment with type I IFNs leads to a dramatic accumulation in the level of ISG15 conjugates in *UBP43*^{-/-} cells as compared with that in *UBP43*^{+/+} cells. Furthermore, treatment with lactacystin, an inhibitor of the proteasome, shows little or no effect on the level of ISG15 conjugates in either of *UBP43*^{+/+} or *UBP43*^{-/-} cells

whether or not exposed to IFN β , while as expected the level of ubiquitinated proteins markedly increases in both cells. Thus, it appears that ISG15 and its conjugates by themselves do not interfere with the ubiquitination of cellular proteins and their subsequent degradation.

The reason for the tumor-specific overexpression of ISG15 in different tumors is unclear. One possibility is that the elevated expression of ISG15 may be due to constitutively activated NF- κ B in many tumor cells. This possibility is supported by an observation that ISG15-overexpressing ZR-75-1 cells show a greater NF- κ B activity than ISG15-underexpressing BT474 breast cancer cells [176]. Alternatively, the deregulated expression of UBE1L and UBP43 in certain tumors could contribute to the variation in the levels of ISG15 conjugates in tumor biopsy samples.

Androgen receptor has been implicated in the initiation and progression of prostate cancer [177,178]. Significantly, UBE1L and UbcH8 are upregulated in prostate cancer lesions as compared to those in corresponding nonmalignant tissues [179]. Moreover, overexpression of UBE1L in LNCaP cells leads to a marked increase in the mRNA and protein levels of androgen receptor in addition to an increase in the rate of cell proliferation. On the other hand, siRNA-mediated knockdown of ISG15 and UBE1L in LNCaP cells results in a significant decrease in the transcript and protein levels of androgen receptor. These findings suggest that ISGylation machinery participates in a positive control of androgen receptor expression during the onset of prostate cancers, thereby promoting the tumor growth.

5. Concluding remarks

Since the discovery of IFNs, a vast knowledge on the role of the cytokines in innate immune responses has been accumulated. ISG15 is one of the most strongly induced ISGs upon exposure to type I IFNs, virus, LPS, and other stresses, including certain genotoxic stresses [16,17,35,56,64,180]. Considering that type I IFNs play critical roles in innate immune responses regulating the antiviral responses as well as the cancer development, there is no doubt that ISG15 and its conjugation to target proteins play critical roles in the type I IFN-induced immune responses. However, the biological significance of protein modification by ISG15 has been established only in several cases. Therefore, much studies are required for clarification of the role of ISG15 in innate immune responses against viral infection and cancer and thus for the control of immune diseases as well as for resolving the contradictory findings, such as the role of ISG15 as a tumor suppressor versus an oncogenic protein.

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