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ISGylation: a key to lock the cell gates for preventing the spread of threats

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Abstract

Interferon stimulated gene 15 (ISG15) is an ubiquitin-like protein, whose expression and conjugation to targets (ISGylation) is induced by infection, interferon (IFN)- α/β , ischemia, DNA damage, and aging. Attention has historically focused on the antiviral effects of ISGylation, which blocks the entry, replication or release of different intracellular pathogens. However, recently, new functions of ISGylation have emerged that implicate it in multiple cellular processes, such as DNA-repair, autophagy, protein translation or exosome secretion. In this Review, we discuss the induction and conjugation of ISG15, as well as the functions of ISGylation in the prevention of infections and in cancer progression. We also offer a novel perspective with regard to the latest findings on this pathway, with special attention to the role of ISGylation in the inhibition of exosome secretion, which is mediated by fusion of multivesicular bodies with lysosomes. Finally, we propose that under conditions of stress or infection, ISGylation acts as a defense mechanism to inhibit normal protein translation by modifying protein kinase R (PKR), while any newly synthesized proteins are being tagged and thus marked as potentially dangerous. Then, the endosomal system is re-directed towards protein degradation at the lysosome, to effectively 'lock' the cell gates and thus prevent the spread of pathogens, prions, and deleterious aggregates through exosomes.

1 **Introduction**

2 Interferon stimulated gene 15 (ISG15) is a 15 kDa protein that belongs to the family of
3 ubiquitin-like modifiers (UBLs) (Haas et al., 1987). The structure of ISG15 resembles a
4 tandem orientation of two ubiquitin folds (Daczkowski et al., 2017) and it can modify
5 cellular proteins at the posttranslational level by conjugating its C-terminal glycine to
6 lysine side-chain amino groups of targets (Loeb and Haas, 1992). However, ISG15 can
7 also exert some of its roles as a free intracellular molecule (Dos Santos and Mansur,
8 2017), and it can even be secreted and thus function as a cytokine (Bogunovic et al.,
9 2012), although its cellular receptors still remain unknown.

10 ISG15 conjugates to proteins through the sequential action of three enzymes: E1
11 (ubiquitin-activating enzyme E1-like protein, Ube1L), E2 (ubiquitin-carrier protein H8,
12 UbcH8), and E3 (HECT domain and RCC1-like domain-containing protein 5, HERC5,
13 and estrogen-responsive finger protein, EFP, in humans; HERC6 in mice). Its
14 deconjugation is mediated by an ISG15-specific protease called USP18 or UBP43
15 (reviewed in (Skaug and Chen, 2010)) (Fig. 1). ISG15 is constitutively expressed at low
16 levels, but transcription of both ISG15 and its conjugating and deconjugating enzymes is
17 strongly induced by interferon (IFN)- α/β through the binding of IFN regulatory factors
18 (IRFs) to interferon-stimulated response element (ISRE)-containing promoters (Sadler
19 and Williams, 2008). Remarkably, HERC5 binds to and ISGylates IRF3; this prevents its
20 association with peptidyl-prolyl isomerase NIMA-interacting 1 (Pin1), a protein that
21 promotes IRF3 ubiquitination and degradation; therefore, ISGylation of IRF3 blocks its
22 degradation and enhances IFN-sustained activation (Shi et al., 2010). However, USP18
23 can bind to the IFN receptor IFNAR2 through STAT2 (Arimoto et al., 2017) and inhibit
24 sustained JAK/STAT signaling, but this activity is independent of its ISG15-
25 deconjugating activity (Malakhova et al., 2006). ISG15 expression is also induced by
26 activation of the NF κ B pathway (Li et al., 2001) (Fig.1).

27 ISG15 expression can also be induced independently of IFN by viral infection and double
28 stranded (ds)RNA (Sen and Sarkar, 2007). Strikingly, p53 stimulates the expression of
29 ISG15 and its conjugation enzymes (Park et al., 2016), and is also required for optimal
30 ISG15 induction by dsRNA, but not by interferon or viral infection (Hummer et al., 2001).
31 In addition, ISG15 expression is induced by several other compounds, such as Poly I:C,
32 lipopolysaccharide (LPS), tumor necrosis factor (TNF)- α , vascular endothelial growth

factor (VEGF) and IFN- γ (Chairatvit et al., 2012; Doyle et al., 2002; Liu et al., 2009; Liu et al., 2016; Taylor et al., 1996), as well as by various other stimuli, including DNA damage, irradiation, ischemia and telomere shortening (Lou et al., 2009; Nakka et al., 2011; Park et al., 2016). Interestingly, many ISG15-inducing stimuli also induce inducible nitric oxide synthase (iNOS), which enhances the production of nitric oxide (NO), and nitrosylation of ISG15 cysteine residues by NO increases the total amount of ISGylation by impairing ISG15 dimerization (Okumura et al., 2008b).

Despite its structural similarity with ubiquitin, ISG15 conjugation has not been reported to induce proteasomal degradation of its substrates. Actually, some of the effects of ISGylation are exerted by interference with the ubiquitin system. This interference can be mediated through the conjugation of ISG15 to different E2 and E3 ubiquitin-conjugating enzymes (Okumura et al., 2008a; Takeuchi and Yokosawa, 2005), or even through the formation of ubiquitin-ISG15 mixed chains (Fan et al., 2015a). As a result, ISGylation can decrease the levels of polyubiquitinated proteins and downregulate protein turnover by the proteasome system (Desai et al., 2006; Fan et al., 2015a). Also unlike ubiquitin, no poly-ISG15 chains or specific ISG15-interacting motifs have been identified so far.

Despite its wide expression and induction by a variety of stimuli, which are mostly related to stress situations, ISG15 has mainly been studied in relation to its functions as an antiviral molecule owing to its function in hampering the infection of different intracellular pathogens (Lenschow et al., 2007; Radoshevich et al., 2015). Here we review the classical aspects of ISG15 function as an antiviral molecule and also discuss the most novel findings for the pathway, paying special attention to its role in the inhibition of exosome secretion by the induction of multivesicular body (MVB) fusion with the lysosome.

The role of ISG15 in viral and bacterial infections

ISG15 has been shown to restrict infection of multiple viruses both *in vitro* (Pincetic et al., 2010) and *in vivo* (Lenschow et al., 2007), and, consequently, ISG15-knockout (KO) mice are more susceptible to infection with various pathogens, including the clinically relevant etiologic agents Influenza A and B virus (IAV and IBV), Herpes simplex virus (HSV), norovirus and Chikungunya virus (CHIKV) (Lenschow et al., 2007; Rodriguez et al., 2014; Werneke et al., 2011) (Fig. 2). The antiviral effects of ISG15 can be mediated

1 by either ISGylation or by unconjugated ISG15. Indeed, ISG15-KO but not Ube1L-KO
2 mice have elevated susceptibility to CHIKV (Werneke et al., 2011), pointing a
3 mechanism that is dependent on ISG15, but does not require ISGylation, whereas
4 susceptibility to IAV infection has been shown to be mediated by ISGylation (Morales et
5 al., 2015). Remarkably, knock-in mice expressing enzymatically inactive USP18 have
6 increased viral resistance against vaccinia virus (VACV) and IBV; however, these mice
7 do not show the IFN hypersensitivity, brain injury, and increased mortality that is
8 observed in USP18-KO mice (Goldmann et al., 2015; Ketscher et al., 2015), suggesting
9 that these phenotypes are ISG15-independent and depend on USP18-mediated, negative
10 regulation of IFN signaling.

11 ISG15 can act at different levels of viral infection. For example, ISG15 restricts norovirus
12 infection at an early step, probably at the entry of the virus or during its uncoating
13 (Rodriguez et al., 2014). During IAV infection, ISGylation of viral NS1 protein inhibits
14 its association with importin1, thus affects its nuclear import and, consequently, virus
15 replication (Zhao et al., 2010). ISGylated IBV nucleoprotein (NP) acts as a dominant-
16 negative inhibitor of IBV NP oligomerization and inhibits the formation of the viral
17 ribonucleoproteins that catalyze RNA synthesis, therefore decreasing viral protein
18 synthesis and virus replication (Zhao et al., 2016). ISG15 can also block the formation
19 and release of viral particles. For example, ISGylation of Nedd4 decreases the
20 ubiquitination of viral matrix proteins and the release of Ebola VP40 virus-like particles
21 (Okumura et al., 2008a). In addition, ISGylation can affect the ESCRT complex
22 component CHMP5, as ISGylation induces its aggregation and the sequestration of the
23 Vps4 cofactor LIP5. As a consequence, the membrane recruitment of Vps4 is abolished
24 and its interaction with the Gag budding complex of avian sarcoma leukosis virus (ASLV)
25 and human immunodeficiency virus 1 (HIV-1) disrupted, resulting in the inhibition of
26 virus budding (Pincetic et al., 2010). In addition, the ESCRT complex component
27 TSG101 is also ISGylated during IAV infection, which results in an inhibition of the
28 trafficking of viral hemagglutinin A (HA) protein to the cell surface, thereby impairing
29 release of virus (Sanyal et al., 2013). ISGylation is also involved in the establishment and
30 maintenance of latency in infection by Kaposi sarcoma virus (KSHV), through its role in
31 regulating viral microRNAs involved in lytic reactivation (Dai et al., 2016), and by HSV,
32 through the formation of autophagic clusters (Katzenell and Leib, 2016). Finally, ISG15
33 also regulates immune cell responses to viral infection, as ISG15 KO macrophages

display reduced activation, phagocytic activity, and cell death in response to VACV (Yanguéz et al., 2013).

Highlighting its crucial role in antiviral defense, some viruses such as IBV, VACV and cytomegalovirus (HCMV) have developed strategies to counteract ISG15 activity. With regard to IBV, its NS1 (NS1B) protein is able to bind ISG15 and interfere with its covalent linkage to target proteins, thus blocking ISGylation of proteins *in vitro* and *in vivo* (Yuan and Krug, 2001). Moreover, NS1B binds to dysfunctional ISGylated viral proteins, blocking the dominant-negative effect of ISGylated NP in inhibiting viral RNA synthesis (Zhao et al., 2016). In the case of VACV, the viral protein E3 is able to block ISGylation when overexpressed *in vitro*, although ISGylation levels were found to remain unaltered in infected cells (Guerra et al., 2008). Regarding HCMV, its viral protein IE1 blocks ISG15 expression by a STAT-1 dependent mechanism (Kim et al., 2016), whereas its viral protein UL26 suppresses ISG15 conjugation (Kim et al., 2016). ISGylation is also mitigated by nairovirus and arterivirus ovarian tumor (OTU)-domain-containing proteases, a family of cysteine proteases capable of de-ISGylating proteins (Frias-Staheli et al., 2007). In addition, papain-like proteases (PLpro) from Coronavirus, which are cysteine proteases with an essential role in the virus replication, are also able to cleave ISG15 from its substrates. Interestingly, PLpro inhibitor protects mice from lethal infection with Coronavirus *in vivo* (Deng et al., 2014). Finally, Hepatitis C virus is even able to exploit the ISG15 pathway to its own benefit by converting it into a proviral factor that negatively regulates the retinoic acid-inducible gene I (RIG-I) pathway that senses the presence of foreign RNA in the cellular cytoplasm, thereby promoting virus production. Here, ISGylation of RIG-I interferes with its ubiquitination, which is required for a correct induction of IFN during the infection; this therefore, results in a more permissive environment for HCV to replicate and disseminate (Arnaud et al., 2011).

Unexpectedly, human patients with ISG15 deficiency did not show increased susceptibility to virus infection, but are more susceptible to mycobacterial disease (Bogunovic et al., 2012). This appears to be due to the reduced secretion of IFN γ by lymphocytes, which is caused by the absence of free extracellular ISG15 (Bogunovic et al., 2012). Moreover, cells derived from ISG15-deficient patients do not only show a lack of increase in susceptibility to viruses, but even exhibit an enhanced antiviral protection (Speer et al., 2016). This species-specific gain-of-function in antiviral immunity appears to be based on the requirement of ISG15 to sustain USP18 levels in humans, but not in

1 mice. In these patients, the absence of intracellular ISG15 leads to an increased
2 degradation of USP18, which is a negative regulator of IFN signaling (Meuwissen et al.,
3 2016), thereby resulting in an amplification of IFN responses. Consequently, ISG15-
4 deficient patients also display cellular, immunological and clinical signs of enhanced
5 IFN- α/β immunity including higher levels of IFN-stimulated mRNAs, which is
6 reminiscent of the autoinflammatory interferonopathies Aicardi-Goutieres syndrome and
7 spondyloenchondroplasia (Zhang et al., 2015). In contrast, increased IFN response has
8 not been reported in ISG15-deficient mice (Osiak et al., 2005). Furthermore, the IFN
9 hypersensitivity observed in USP18 KO mice cannot be rescued with the additional
10 deletion of ISG15, and is therefore independent of it (Knobeloch et al., 2005), providing
11 further evidence for species specificity in ISG15 functions. Nevertheless, as in humans,
12 ISG15 also plays an important role in the defense against intracellular bacteria in mice.
13 For example, ISG15 expression is induced upon *Listeria* infection and restricts infection
14 *in vitro* and *in vivo*. Surprisingly, this induction is IFN-independent and depends on the
15 cytosolic DNA sensing pathway, which acts through STING, TBK1, IRF3, and IRF7
16 (Radoshevich et al., 2015).

17 Therefore, ISGylation is an important cellular defense mechanism to restrict infections
18 by affecting the entry, replication, trafficking and/or release of pathogens. As a
19 consequence, several viruses have evolved diverse strategies to counteract the effects of
20 ISGylation. In addition, ISG15 also has an important role in host defense against
21 pathogens through ISGylation-independent mechanisms, which are mostly related to the
22 regulation of the IFN pathway. Importantly, significant differences have been observed
23 in this context between human and mice systems, pointing to a species-specificity in
24 several functions carried out by ISG15.

26 **The role of ISG15 in cancer**

27 The expression of ISG15 and its conjugating enzymes is deregulated in many types of
28 cancers (Desai et al., 2012; Kiessling et al., 2009; Li et al., 2014; Wood et al., 2012).
29 However, there is not consensus on whether this pathway has a pro-tumoral or a tumor
30 suppressor effect. For instance, in breast cancer, ISG15 overexpression correlates with an
31 unfavorable prognosis and poor response to chemotherapy and radiotherapy, and
32 vaccination with ISG15 peptides has been shown to reduce primary and metastatic tumor

burden in mice implanted with a metastatic mammary tumor cell line (Wood et al., 2012). In contrast, ISG15 downregulation has also been shown to decrease sensitivity of breast cancer cells to chemotherapy with camptothecins, possibly by blocking ISG15-mediated inhibition of proteasome-mediated repair of topoisomerase I-DNA covalent complexes (Desai et al., 2008). ISG15 has also been shown to disrupt F-actin architecture and the formation on focal adhesions in breast cancer cells; this promotes cancer cell migration by decreasing the proteasome-mediated turnover of proteins that are implicated in cell motility, invasion, and metastasis (Desai et al., 2012). Oncogenic Ki-Ras induces ISG15 and ISGylation, which in turn stabilize Ki-Ras by inhibiting its degradation in breast cancer cells. Importantly, silencing of ISG15 or UbcH8 in these cells reverses the Ki-Ras mutation-associated phenotypes, such as cell proliferation, anchorage-independent growth, cell migration and EMT (Burks et al., 2014). ISG15 and its conjugation enzymes are also upregulated in prostate cancer (Kiessling et al., 2009) and hepatocellular carcinoma (Li et al., 2014), where they promote proliferation and migration by stabilizing the apoptosis inhibitor Survivin through the sequestration of X-linked inhibitor of apoptosis protein (XIAP), the E3 ubiquitin ligase responsible for its degradation; in this model, ISG15 knockdown inhibited xenografted tumor growth and prolonged lifespan of tumor-bearing mice (Li et al., 2014). ISG15 is also overexpressed in nasopharyngeal carcinoma, where it promotes a cancer stem cell (CSC) phenotype and cisplatin resistance (Chen et al., 2016). Similarly, tumor-associated macrophages secrete ISG15, which enhances CSC phenotypes in pancreatic ductal adenocarcinoma *in vitro* and *in vivo*, thereby promoting CSC self-renewal, invasive capacity and tumorigenic potential (Sainz et al., 2014).

In contrast, the ISG15-conjugating enzyme Ube1L is downregulated in lung cancer, and its overexpression suppresses lung cancer growth by inhibiting cyclin D1, which is required for progression through the G1 phase of the cell cycle (Feng et al., 2008). Ube1L and ISG15 are also involved in the suppression of acute promyelocytic leukemia (APL) cancers that express the promyelocytic leukemia (PML) fused to retinoic acid (RA) receptor alpha (RARalpha); these cancers have shown clinical remissions through leukemic cell differentiation after retinoic acid (RA) treatment, as Ube1L is a RA-regulated gene that triggers PLM-RARalpha degradation and apoptosis during RA-induced differentiation of APL (Pitha-Rowe et al., 2004). Extracellular free ISG15 has also been shown to suppress tumor growth by stimulating the functions of natural killer

(NK) cells and of CD8⁺ lymphocytes (Burks et al., 2015; Villarreal et al., 2015). ISG15 also has a potential tumor suppressor function under conditions that elicit DNA damage owing to its effects on the p53 pathway: DNA-damaging agents induce ISGylation of p53, which enhances its binding to target gene promoters (e.g. BAX, CDKN1 and p53), resulting in suppression of cell growth and of tumorigenesis (Park et al., 2016). Furthermore, ISGylation of the dominant-negative splice variant Δ Np63 α inhibits its ability to promote anchorage-dependent growth and tumor formation *in vivo* (Jeon et al., 2012).

In summary, the ISG15 pathway is deregulated in different types of tumors, but whether it has a pro-tumor or a tumor suppressor effect remains controversial and appears to depend on the cancer and cell type. This could be partially explained by the fact that ISGylation can interfere with ubiquitination-induced proteasome degradation, which controls the stability of many different proteins involved in a wide variety of processes. Therefore, depending on the particular pathway that is altered or governs the behavior of a specific cancer or cell type, the impact of ISGylation is different; for example, it can affect migration if motility proteins are altered, or result in cell cycle arrest or apoptosis if cell cycle regulators or caspases are deregulated. Also, the presence of specific circumstances, such as treatment with retinoid acid (Pitha-Rowe et al., 2004) or DNA-damage agents that activate the p53 pathway (Park et al., 2016), will determine the overall effect of ISGylation in a specific cancer. Finally it has also been proposed that ISGylation has a tumor-promoting effect, whereas free ISG15 acts as a tumor suppressor (Desai, 2015).

Newly described ISG15 functions

In addition to the above mentioned roles of ISG15 in restricting intracellular pathogen infection and in cancer progression, several recent studies have pointed to broader cell biology functions for ISG15 (Fig. 2). For example, ISGylation has been shown to regulate termination of error-prone translesion DNA synthesis (TLS) (Park et al., 2014). In response to DNA damage, PCNA is mono-ubiquitinated and triggers TLS by recruiting polymerase- η . The ISG15 E3 ligase EFP then binds to mono-ubiquitinated PCNA and promotes its ISGylation. ISGylated PCNA tethers USP10 for deubiquitination and the subsequent release of polymerase- η from PCNA. Eventually, PCNA is deISGylated by USP18 and can be reloaded onto replicative DNA polymerases with resumption of normal DNA replication. Accordingly, ISGylation-defective mutations in PCNA, or knockdown

1 of either ISG15 or EFP, lead to persistent recruitment of mono-ubiquitinated PCNA and
2 polymerase- η to nuclear foci, thereby causing an increase in mutation frequency (Park et
3 al., 2014).

4 ISG15 also has an important role in autophagy. In fact, ISG15 interacts with p62 and
5 histone-deacetylase 6 (HDAC6) in insoluble fractions, where protein aggregates
6 accumulate (Nakashima et al., 2015). Both proteins have been shown to control
7 autophagic clearance of protein aggregates: p62 interacts with Lys63-polyubiquitinated
8 aggregation-prone proteins and facilitates their degradation by directly recruiting LC3-
9 phagophores (Pankiv et al., 2007), whereas HDAC6 recruits misfolded proteins to dynein
10 motors for their transport to the aggresome (Kawaguchi et al., 2003) and promotes
11 autophagosome-lysosome fusion (Lee et al., 2010). p62 also interacts with HDAC6 and
12 regulates its deacetylase activity (Yan et al., 2013). Moreover, ISG15 conjugation of
13 proteins, such as GFP or TSG101, has been shown to promote their aggregation and
14 degradation by the lysosome (Nakashima et al., 2015; Villarroya-Beltri et al., 2016).
15 Together these observations suggest that ISGylation of proteins may promote their
16 aggregation and degradation by selective autophagy through the interaction of ISG15
17 with p62 and HDAC6. In addition, ISG15 is upregulated in ataxia telangiectasia cells and
18 enhances their autophagic flux, probably to compensate the impaired proteasomal
19 function that is caused by their constitutive activation of ISG15 (Desai et al., 2013).
20 Furthermore, HSV infection triggers the formation of ISG15- and p62-decorated
21 autophagosome clusters, which are involved in the establishment of latency (Katzenell
22 and Leib, 2016). These autophagosomes are not induced by rapamycin or starvation (the
23 typical inducers of autophagy) and therefore are likely to be related to selective
24 autophagy, rather than global autophagy. Accordingly, ISGylation has been shown to
25 induce fusions of MVBs with the autophagosome/lysosome compartment, without
26 inducing general autophagy (Villarroya-Beltri et al., 2016). It should be noted that, in
27 contrast to the above studies and a previous report that IFN induces autophagy
28 (Schmeisser et al., 2013), a different study suggested that IFN-I-induced ISGylation of
29 Beclin 1 (BECN1) negatively regulates autophagy (Xu et al., 2015). BECN1 positively
30 regulates autophagy by stimulating the lipid kinase activity of phosphatidylinositol 3-
31 kinase catalytic subunit type 3 (PI3KC3), which generates phosphatidylinositol 3-
32 phosphate and enables the recruitment of proteins involved in the nucleation of the
33 autophagosome. BECN1 activity is induced by its ubiquitination, which disrupts the

1 association between BECN1 and its inhibitor partner BCL-2 (Abrahamsen et al., 2012).
2 According to the above-mentioned study, ISGylation of BECN1 impairs its ubiquitination
3 and consequently inhibits BECN1-mediated activation of PI3KC3 and thus autophagy
4 (Xu et al., 2015). The authors suggest that a plausible explanation for the discrepancy
5 between their work and previous work showing that IFN-I positively regulates autophagy
6 (Schmeisser et al., 2013) is that IFN-I-induced autophagy is transient and that long-term
7 IFN-I treatment inhibits autophagy through BECN1 ISGylation. It is conceivable that
8 ISGylation may have a negative effect on rapamycin-induced general autophagy, but
9 nevertheless positively regulates selective autophagy. However, further investigation will
10 be needed to uncover the exact role of ISGylation during autophagy.

11 ISGylation is also involved in the regulation of protein synthesis. ISG15 conjugation to
12 protein kinase R (PKR) induces its activation in the absence of viral infection, resulting
13 in phosphorylation of eukaryotic initiation factor 2 alpha (eIF2 α) and the subsequent
14 downregulation of protein translation (Okumura et al., 2013). In addition, ISGylation of
15 the translation suppressor 4EHP increases its cap-binding activity and its ability to
16 compete with the translation initiation factor for mRNA binding, resulting in translation
17 initiation inhibition (Okumura et al., 2007).

18 ISGylation has also been shown to regulate cytoskeletal dynamics. Indeed, ISG15 and
19 UbcH8 disrupt F-actin architecture and the formation of focal adhesions in breast cancer
20 cells (Desai et al., 2012). In addition, prolonged loss of the Rac1/CDC42 guanine-
21 exchange factor DOCK6 reduces the expression of ISG15. Consequently, decreased
22 ISGylation of the Ras GTPase-activating-like protein IQGAP1 increases the levels of
23 active CDC42 and Rac1 to restore F-actin function and compensate for DOCK6
24 disruption (Cerikan et al., 2016).

25 ISG15 is also induced in other physiological and pathological contexts, which reveal it
26 might potentially function beyond restriction infection. Indeed, ISG15 is elevated upon
27 traumatic brain injury (Rossi et al., 2015) and after cerebral focal ischemia (Nakka et al.,
28 2011), where it has a neuroprotective effect as shown by increased mortality, exacerbated
29 infarction, and worsened neurologic recovery of ISG15-KO mice when they are subjected
30 to transient middle cerebral artery occlusion (Nakka et al., 2011). Interestingly,
31 ISGylation of hypoxia induced factor 1-alpha (HIF-1 α) disrupts its dimerization and
32 attenuates HIF-1 α mediated gene expression, resulting in decreased tumor growth (Yeh

et al., 2013). Furthermore, ISG15 conjugation is activated during erythroid differentiation, with a significant decrease in the proportion of fully differentiated erythrocytes and a concomitant increase in the proportion of erythroid precursors (Maragno et al., 2011). Finally, ISGylation also exacerbates intestinal inflammation and colitis-associated colon cancer in mice by negatively regulating the ubiquitin-proteasome system in macrophages, which results in an increase in reactive oxygen species (ROS); this then enhances the activation of p38 MAP kinase, which induces the expression of inflammation-related cytokines (Fan et al., 2015b). Thus, ISGylation is induced upon a variety of stimuli, apart from IFN and infection and has been shown to participate in multiple cellular processes, such as translesion DNA synthesis, autophagy, cytoskeletal dynamics and protein translation.

Effects of ISG15 on secretion: locking the cell gates

Exosomes are vesicles of 50 to 100nm in size that are secreted to the extracellular environment by most cells. They form in specific endosomal compartments called multivesicular bodies (MVBs). Upon the fusion of MVBs with the plasma membrane, the intraluminal vesicles (ILVs) that they contain are released as exosomes, which can be taken up by recipient cells, thereby mediating intercellular communication (Raposo and Stoorvogel, 2013). ISG15 has been recently shown to inhibit exosome secretion both *in vitro* and *in vivo* (Villarroya-Beltri et al., 2016). Here, inhibition of exosome secretion was not observed when ISG15 is mutated so that its conjugation to proteins is impaired, whereas exosome secretion is increased in mouse cells that express a catalytically inactive form of the de-ISGylase USP18 and thereby show enhanced ISGylation, indicating that inhibition of exosome secretion is mediated by ISGylation and not by free ISG15. ISGylation decreases the number of MVBs without significantly affecting their biogenesis, suggesting that ISGylation is involved in inducing their degradation. Indeed, ISGylation induces the co-localization between the endosome marker hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and lysosome-associated membrane protein 1 (LAMP-1). Exosome secretion can be recovered when the fusion of MVBs with lysosomes or autophagosomes is blocked, suggesting that ISGylation inhibits exosome secretion by inducing the fusion of MVBs with lysosomes and autophagosomes (Villarroya-Beltri et al., 2016). ISG15 also conjugates to the endosomal protein TSG101 (Sanyal et al., 2013; Villarroya-Beltri et al., 2016), which was shown to control the secretion of exosomes (Colombo et al., 2013). Indeed, ISGylation of TSG101 is sufficient

1 to inhibit exosome secretion (Villarroya-Beltri et al., 2016); however, because other
2 endosomal proteins are modified by ISG15 (Kuang et al., 2011), the inhibition of
3 exosome secretion could also be mediated by the ISGylation of additional proteins.

4 Exosomes can be hijacked by viruses as ‘Trojan horses’ to evade the immune system
5 during their dissemination (Mittelbrunn and Sanchez-Madrid, 2012). Therefore, the
6 inhibition of exosome secretion by ISGylation could be a cellular defense strategy to
7 impair the spreading of pathogens through this route and to promote viral protein
8 degradation by the lysosome. In addition, pathogens use the endolysosomal system for
9 their entrance into cells and their replication; therefore positioning the ISGylation
10 machinery towards the endosomal compartment will facilitate its encounter with viral
11 components and thus their degradation at sites of virus entry, replication, and egress.
12 Protein aggregates and prions are also enriched in and spread through exosomes (Guo et
13 al., 2016), which has been suggested to contribute to the progression of prion diseases
14 and aggregopathies (Baker et al., 2016; Eitan et al., 2016; Quek and Hill, 2017). In
15 addition, ISGylated proteins interact with the aggresome marker p62 (Nakashima et al.,
16 2015), which in turn interacts with LC3-II in the membrane of autophagosomes, thereby
17 facilitating the clearance of protein aggregates. Furthermore, ISGylated proteins also
18 interact with HDAC6 as noted above (Nakashima et al., 2015), which promotes the
19 autophagic degradation of protein aggregates (Kawaguchi et al., 2003; Lee et al., 2010).
20 It is therefore conceivable that ISGylation also facilitates the degradation of prions and
21 toxic protein aggregates that often accumulate in a cell with age and prevents their
22 dissemination through exosomes.

23 With regard to the effect of ISG15 on the secretion of molecules through the classical
24 endoplasmic reticulum (ER)-to-Golgi pathway, ISG15 appears to not inhibit but rather
25 enhance the secretion of cytokines via this route (Fan et al., 2015b; Radoshevich et al.,
26 2015), which might allow the secretion of signaling molecules that are required to
27 counteract the infection. This appears to be mediated by ISGylation of ER proteins such
28 as reticulon 4 (RTN4) (Radoshevich et al., 2015). In addition, ISGylation has been shown
29 to increase the cell-surface expression of major histocompatibility complex I (MHC-I)
30 (Burks et al., 2015), which is responsible of presenting non-self and other intracellularly
31 synthesized peptides that reflect a danger situation (e.g. oncogenic proteins) to cytotoxic
32 cells, thereby triggering to kill the presenting cells (Fig. 3). Furthermore, the major ISG15
33 E3 ligase HERC5 interacts with the polysome and promotes the conjugation of ISG15 to

nascent proteins during their translation, which has been suggested to serve to label any new proteins as suspicious as they might be of intracellular pathogen origin (Durfee et al., 2010). Also, PKR and 4EHP ISGylation results in downregulation of global protein translation (Okumura et al., 2013; Okumura et al., 2007).

Hence, the induction of ISGylation results in the tagging—and probably also the degradation and presentation—of newly synthesized ‘suspicious’ proteins, the downregulation of protein translation and the re-direction of the endosomal system toward degradation at the lysosome to ‘lock’ the cell gates and prevent the spread of pathogens, prions, and protein aggregates (Fig. 3).

Concluding remarks and future perspectives

Despite being the first UBL identified, ISG15 has for many years been studied almost exclusively with regard to its antiviral activity. However, IFN and viral infection are not the only activators of ISG15, and several stimuli, mostly related to stress conditions, have been shown to induce its expression and conjugation in a variety of cell types. In addition, novel and unexpected ISG15 functions have emerged in recent years. For instance, ISGylation inhibits the release of exosomes (Villarroya-Beltri et al., 2016), probably to prevent the spread of potential pathogens or protein aggregates without decreasing the secretion of cytokines that are needed to signal and neutralize the threat (Radoshevich et al., 2015). ISGylation also downregulates protein translation (Okumura et al., 2013) and tags newly synthesized proteins (Durfee et al., 2010), distinguishing them from proteins present before the stress challenge and possibly increasing their presentation to cytotoxic cells. In addition, ISGylation enhances p53 functions (Park et al., 2016). We therefore hypothesize that ISGylation, which is activated in stress situations, orchestrates a cellular response that arrest the cell functions by inhibiting translation and enhancing p53, triggers the degradation of endosomal and newly synthesized proteins by the autophagosome/lysosome and signals a state of alert to induce a response by the immune system. Given that ISG15 conjugation is transient and can be reversed by specific proteases, this modification may allow the recovery of the homeostatic state once the stress has ceased.

Many unanswered questions remain regarding the role of ISG15. For example, the receptors for free ISG15 have not been identified. Furthermore, the reasons underlying

the differences found between ISG15 function in mice and human remain unclear. The structure of ISG15 may provide some clues in this regard, as structural information for USP18 has recently revealed that the origin of its reactivity towards ISG15 and not ubiquitin lies on a critical hydrophobic patch in USP18 that interacts with a hydrophobic region unique to ISG15 (Basters et al., 2017). In addition, it is unknown how ISG15 is able to target specific substrates. Thus, unlike sumoylation, there is no consensus sequence for ISGylation, and in contrast to ubiquitination, only two ISG15 E3 conjugating enzymes are known in humans and only one in mice. There are also no poly-ISG15 chains or any associated enzymes described thus far. However, recent reports have identified mixed ubiquitin-ISG15 chains, which interfere with the ubiquitin-proteasome system (Fan et al., 2015a). It will be interesting to investigate whether ISG15 can form mixed chains and interfere with the function of other ubiquitin-like proteins such as SUMO. It also remains unknown how the outcome of ISGylation is decided, and thus how it exerts the versatile effects observed. The subcellular localization of a target protein may be important for determining the fate of the ISGylated product. For example, for an endosome-located protein, ISGylation may result in degradation by the lysosome if the interaction between endosomal and lysosomal components is promoted. In this regard, it is also not clear currently how ISG15 targets endosomal proteins. This could be driven by E3 localization at the endosome, or by interaction of ISG15 with phospho-inositol-enriched domains, as has been described for SUMO conjugation (Kunadt et al., 2015). It is also possible that ISG15 interacts with the ubiquitin-binding ESCRT proteins, as has been shown for the ubiquitin-binding proteins p62 (Nakashima et al., 2015) or Nedd4 (Okumura et al., 2008a), although there is not experimental evidence of a direct interaction of ISG15 with ubiquitin-binding domains so far. Another possibility is that the fate of the modified protein depends on the ISGylation level, which in turn could depend on several factors, such as the type or the strength of the induction stimulus, the affinity of the substrate for E3 ligases, the rate of its translation, or the number and distribution of lysines present. High levels of ISGylation of a substrate may result in its aggregation and degradation by selective autophagy, whereas low levels may not be sufficient to induce aggregation and degradation, but could still exert an effect by affecting the ability of the substrate to interact with other proteins or molecules. The overall effect of ISGylation could vary considerably, from changes in subcellular localization or stability, to inactivation owing to the inability to interact with activating partners, or to activation through the suppression of any inter- or intramolecular inhibitory

1 interactions. ISGylation-mediated protein inactivation could be effective even when only
2 a small fraction of the protein is modified, if the modified protein acts as dominant-
3 negative mutant that sequesters the remaining functional pool. Further insights on the
4 ISGylation pathway will provide valuable information on how cells respond to pathogens
5 and other stress challenges, and might prove useful in developing novel strategies to treat
6 infections, as well as other diseases, such as cancer or autoimmune disorders.

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FIGURE LEGENDS

Fig.1. ISG15 induction and conjugation pathways. ISG15 expression is induced upon binding of interferon response factors (IRF) to the interferon-stimulated response element (ISRE) located in the ISG15 promoter. This binding is induced by type I interferon (IFN-I) through activation of the IFN receptor (IFNAR) and JAK/STAT, as well as by single strand (ss)RNA, double-strand (ds)RNA, or other viral compounds (pathogen-associated molecular patterns, PAMPs); this is mediated through toll-like receptors (TLR) or cytosolic receptors such as retinoic acid-inducible gene 1-like receptors (RIG). The same stimuli also induce the enzymes responsible for ISG15 conjugation or deconjugation. Expression of ISG15 and the ISGylation machinery is also stimulated by activation of the p53 and NFκB pathways. ISG15 is conjugated to target proteins through the consecutive action of three enzymes: E1 (Ube1L), E2 (UbcH8), and E3 (HERC5/EFP in humans and HERC6 in mice), and it can be deconjugated by the protease USP18. PM: plasma membrane; ENDO: endosome; CYT: cytosol; NUC: nucleus.

Fig. 2. Overview of ISG15 functions. ISG15 restricts viral infections by conjugating to cellular proteins, such as Nedd4, TSG101 and CHMP5, or to viral proteins, such as Influenza NS1 and NP proteins. ISGylation can also facilitate the infection of some viruses such as Hepatitis C by modifying RIG-I. ISGylation restricts bacterial infection by modifying proteins such as MAGT1 and RTN4. ISG15 can also promote the progression of some cancers through ISGylation of K-Ras or survivin, whereas, in other cancers, ISG15 has an inhibitory role owing to its modification of cyclin D1 or p53. ISGylation is involved in translesion DNA synthesis termination through the modification of PCNA. Furthermore, ISGylation controls actin cytoskeleton dynamics by modifying the GTP-ase activating protein IQGAP. ISGylation inhibits exosome secretion by modifying TSG101. Moreover, the degradation of ISGylated proteins through selective autophagy is promoted by the interaction of ISG15 with p62 and HDAC6, whereas ISG15 inhibits general autophagy by modifying BECN1. ISG15 also inhibits proteasome-mediated degradation by affecting either ubiquitin, or the E1, E2 and E3 ubiquitin conjugating enzymes. In addition, ISGylation of PKR and 4EHP inhibits protein translation. Finally, ISG15 is involved in induction of hypoxia by modifying HIF-2α.

Fig. 3. Model for the role of ISG15 in the prevention of a threat spreading. ISG15 is induced during a variety of stress situations. ISG15 conjugates endosomal proteins such as TSG101, promoting the fusion of multivesicular bodies with lysosomes/autophagosomes, likely through the interaction of ISG15 with p62 and HDAC6. This inhibits exosome secretion, thus preventing the spread of virus, prions, and protein aggregates. ISG15 also conjugates proteins at the polyribosome, tagging newly-synthesized proteins as suspects. ISG15 also targets proteins at the endoplasmic reticulum (RER) such as reticulon 4 (RTN4), and enhances the secretion of cytokines and the surface expression of MHC-I, which are necessary to signal and counteract the threat.