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REVIEW

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

Carolina Villarroya-Beltri[1,2,3], Susana Guerra[4] and Francisco Sánchez-Madrid[1,2,3,*]

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 and -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 and 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, also known as EIF2AK2), 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.

KEY WORDS: Exosomes, ISG15, Pathogen, Post-translational modification, Ubiquitin-like

Introduction

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

ISG15 forms conjugates to proteins through the sequential action of three enzymes: an E1 (ubiquitin-activating enzyme E1-like protein, Ube1L; also known as UBA7), an E2 (ubiquitin-carrier protein H8, UbcH8; also known as UBE2L6), and an E3 [HECT domain and RCC1-like domain-containing protein 5 (HERC5), and either estrogen-responsive finger protein (EFP, also known as TRIM25) in humans or HERC6 in mice]. Its deconjugation is mediated by an ISG15-specific protease called USP18 or UBP43 (reviewed in Skaug and Chen, 2010) (Fig. 1). ISG15 is constitutively expressed at low levels, but transcription of both ISG15 and its conjugating and deconjugating enzymes is strongly induced by interferon (IFN)-α and -β through the binding of IFN regulatory factors (IRFs) to interferon-stimulated response element (ISRE)-containing promoters (Sadler and Williams, 2008). Remarkably, HERC5 binds to and ISGylates IRF3; this prevents its association with peptidyl-prolyl isomerase NIMA-interacting 1 (Pin1), a protein that promotes IRF3 ubiquitylation and degradation; therefore, ISGylation of IRF3 blocks its degradation and enhances IFN-sustained activation (Shi et al., 2010). However, USP18 can bind to the IFN receptor IFNAR2 through STAT2 (Arimoto et al., 2017) and inhibit sustained JAK/STAT signaling, but this activity is independent of its ISG15-deconjugating activity (Malakhova et al., 2006). ISG15 expression is also induced by activation of the NF-κB pathway (Li et al., 2001) (Fig. 1).

ISG15 expression can also be induced independently of IFN by viral infection and double-stranded (ds)RNA (Sen and Sarkar, 2007). Strikingly, p53 (TP53) stimulates the expression of ISG15 and its conjugation enzymes (Park et al., 2016), and is also required for optimal ISG15 induction by dsRNA, but not by interferon or viral infection (Hummer et al., 2001). In addition, ISG15 expression is induced by several other compounds, such as poly I:C, lipopolysaccharyde (LPS), tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF) and IFN-γ (Chairatvit et al., 2012; Doyle et al., 2002; Liu et al., 2009, 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; also known as NOS2), which enhances the production of nitric oxide (NO); nitrosylation of cysteine residues of ISG15 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 through 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 mixed ubiquitin–ISG15 chains (Fan et al., 2015a). As a result, ISGylation can decrease the levels of polyubiquitylated proteins and downregulate protein turnover by the proteasome system (Desai et al., 2006; Fan et al., 2015a). Also, unlike with ubiquitin, no poly-ISG15 chains or specific ISG15-interacting motifs have been identified so far.

[1]Vascular Pathophysiology Area, Centro Nacional de Investigaciones Cardiovasculares (CNIC), 28029 Madrid, Spain. [2]Immunology Service, Hospital de la Princesa, Universidad Autónoma de Madrid, 28006 Madrid, Spain. [3]CIBER de Enfermedades Cardiovasculares, Instituto de Salud Carlos III, 28029 Madrid, Spain. [4]Preventive Medicine Department, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain.

*Author for correspondence (fsmadrid@salud.madrid.org)

C.V., 0000-0001-5303-0762

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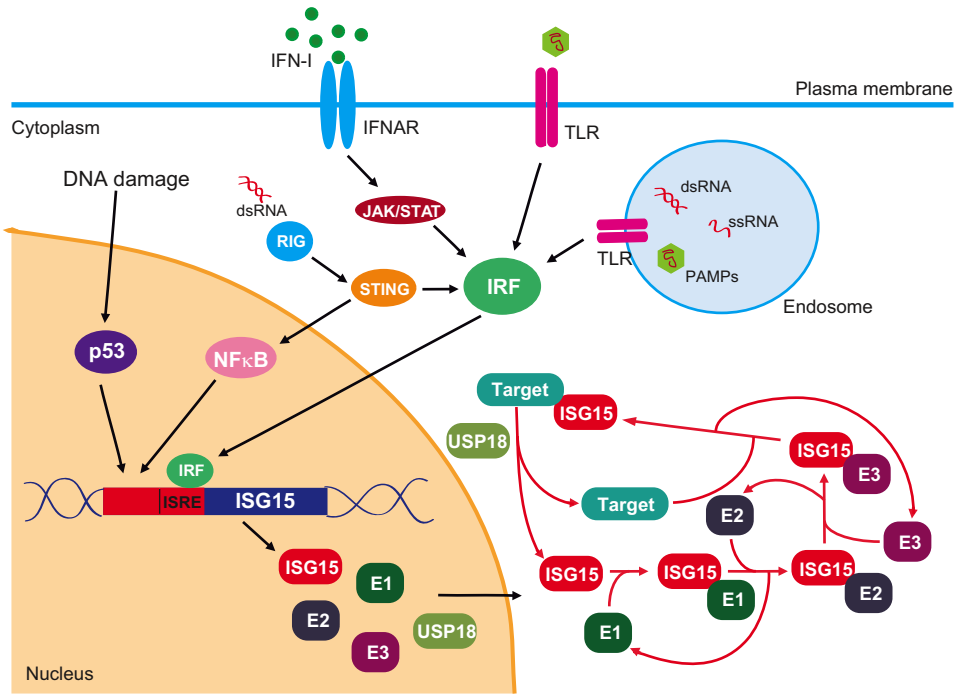

Fig. 1. ISG15 induction and conjugation pathways. ISG15 expression is induced upon binding of interferon response factors (IRF) to the interferon-stimulated response element (ISRE) located in the ISG15 promoter. This binding is induced by type I interferon (IFN-I) through activation of IFN receptor (IFNAR) and JAK/STAT signaling, 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: an E1 (Ube1L), an E2 (UbcH8), and an E3 (HERC5, and EFP in humans or HERC6 in mice), and it can be deconjugated by the protease USP18.

Despite its wide expression and induction by a variety of stimuli, mostly related to stress situations, ISG15 has mainly been studied in relation to its function as an antiviral molecule because it can hamper 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 newly discovered roles for ISGylation in other pathways, 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 Chikunguya virus (CHIKV) (Lenschow et al., 2007; Rodriguez et al., 2014; Werneke et al., 2011) (Fig. 2). The antiviral effects of ISG15 can be mediated by either ISGylation or by unconjugated ISG15. Indeed, ISG15-KO but not Ube1L-KO mice have elevated susceptibility to CHIKV (Werneke et al., 2011), pointing to a mechanism that is dependent on ISG15, but does not require ISGylation, whereas susceptibility to IAV infection has been shown to be mediated by ISGylation (Morales et al., 2015). Remarkably, knock-in mice expressing enzymatically inactive USP18 have increased viral resistance against vaccinia virus (VACV) and IBV; however, these mice do not show the IFN hypersensitivity, brain injury and increased mortality that is observed in USP18-KO mice (Goldmann et al., 2015; Ketscher et al., 2015), suggesting that these phenotypes are ISG15-independent and depend on USP18-mediated negative regulation of IFN signaling.

ISG15 can act at different levels of viral infection. For example, ISG15 restricts norovirus infection at an early step, probably at the entry of the virus or during its uncoating (Rodriguez et al., 2014). During IAV infection, ISGylation of viral NS1 protein inhibits its association with importin 1, thus affecting its nuclear import and, consequently, virus replication (Zhao et al., 2010). ISGylated IBV nucleoprotein (NP) acts as a dominant-negative inhibitor of IBV NP oligomerization and inhibits the formation of the viral ribonucleoproteins that catalyze RNA synthesis, therefore decreasing viral protein synthesis and virus replication (Zhao et al., 2016). ISG15 can also block the formation and release of viral particles. For example, ISGylation of Nedd4 decreases the ubiquitylation of viral matrix proteins and the release of Ebola VP40 virus-like particles (Okumura et al., 2008a). In addition, ISGylation can affect the ESCRT complex component CHMP5, as ISGylation induces its aggregation and the sequestration of the Vps4 cofactor LIP5 (also known as VTA1). As a consequence, the membrane recruitment of Vps4 is abolished and its interaction with the Gag budding complex of avian sarcoma leukosis virus (ASLV) and human immunodeficiency virus 1 (HIV-1) is disrupted, resulting in the inhibition of virus budding (Pincetic et al., 2010). In addition, the ESCRT complex component TSG101 is also ISGylated during IAV infection, which results in an inhibition of the trafficking of viral hemagglutinin A (HA) protein to the cell surface, thereby impairing release of virus (Sanyal et al., 2013). ISGylation is also involved in the establishment and maintenance of latency in infection by Kaposi sarcoma virus (KSHV) through its role in regulating viral microRNAs involved in lytic reactivation (Dai et al., 2016), and in HSV infection, through the formation of autophagic clusters (Katzenell and Leib, 2016). Finally, ISG15 also regulates immune cell responses to viral infection, as ISG15-KO macrophages display reduced activation, phagocytic activity and cell death in response to exposure to VACV (Yanguez 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

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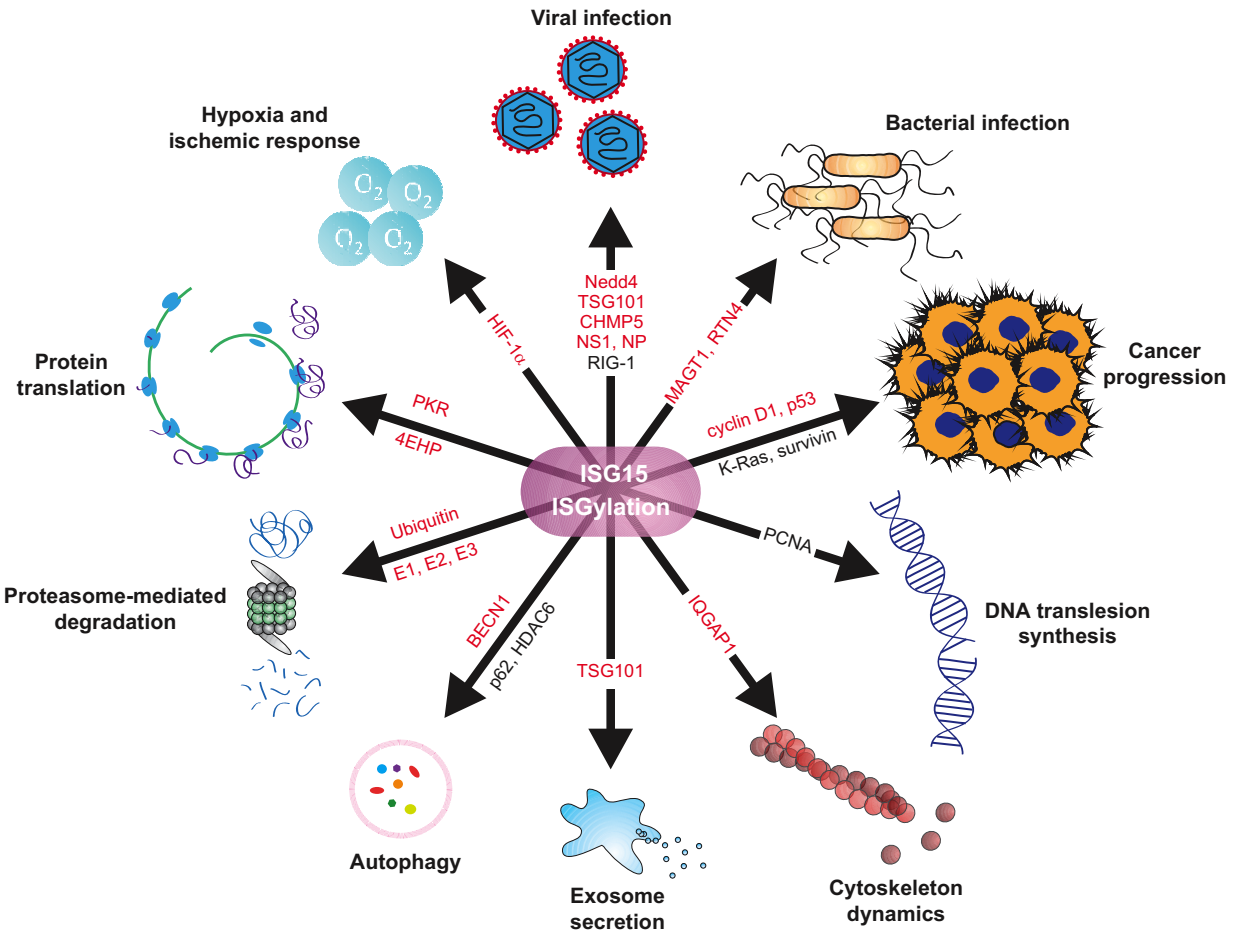

Fig. 2. Overview of ISG15 functions. ISG15 restricts viral infections by conjugating to cellular proteins, such as Nedd4, TSG101 and CHMP5, or to viral proteins, such as influenza NS1 and NP proteins. ISGylation can also facilitate the infection of some viruses such as hepatitis C by modifying RIG-I. 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 GTPase-activating protein IQGAP1. 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-1α. Proteins whose ISGylation inhibits the process are shown in red and those where ISGylation activates the process are shown in black.

(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 through a STAT1-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 ISG15 into a proviral factor that negatively regulates the retinoic acid-inducible gene I (RIG-I; also known as DDX58) pathway that senses the presence of foreign RNA in the cellular cytoplasm, thereby promoting virus production. Here, ISGylation of RIG-I interferes with its ubiquitylation, which is required for a correct induction of IFN during the infection; this, therefore, results is a more permissive environment for HCV to replicate and disseminate (Arnaud et al., 2011).

Unexpectedly, it was found that human patients with ISG15 deficiency did not show increased susceptibility to virus infection, but were more susceptible to mycobacterial disease (Bogunovic et al., 2012). This appears to be due to the reduced secretion of IFNγ 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 no increase in their 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 mice. In these patients, the absence of intracellular ISG15 leads to an increased degradation of USP18, which is a negative regulator of IFN signaling (Meuwissen et al., 2016), thereby resulting in an

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amplification of IFN responses. Consequently, ISG15-deficient patients also display cellular, immunological and clinical signs of enhanced IFN-α and -β immunity, including higher levels of IFN-stimulated mRNAs, which is reminiscent of the autoinflammatory interferonopathies Aicardi–Goutieres syndrome and spondyloenchondroplasia (Zhang et al., 2015). In contrast, increased IFN response has not been reported in ISG15-deficient mice (Osiak et al., 2005). Furthermore, the IFN hypersensitivity observed in USP18-KO mice cannot be rescued with the additional deletion of ISG15, and is therefore independent of it (Knobeloch et al., 2005), providing further evidence for species specificity in ISG15 functions. Nevertheless, as in humans, ISG15 also plays an important role in the defense against intracellular bacteria in mice. For example, ISG15 expression is induced upon Listeria infection and restricts infection *in vitro* and *in vivo*. Surprisingly, this induction is IFN-independent and depends on the cytosolic DNA-sensing pathway, which acts through STING (TMEM173), TBK1, IRF3 and IRF7 (Radoshevich et al., 2015).

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

The role of ISG15 in cancer

The expression of ISG15 and its conjugating enzymes is deregulated in many types of cancers (Desai et al., 2012; Kiessling et al., 2009; Li et al., 2014; Wood et al., 2012). However, there is not consensus on whether this pathway has a pro-tumoral or a tumor suppressor effect. For instance, in breast cancer, ISG15 overexpression correlates with an unfavorable prognosis and poor response to chemotherapy and radiotherapy, and 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 covalent topoisomerase-I–DNA 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 K-Ras induces ISG15 and ISGylation, which in turn stabilize K-Ras by inhibiting its degradation in breast cancer cells. Importantly, silencing of ISG15 or UbcH8 in these cells reverses the K-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 (also known as BIRC5) 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 the 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 protein (PML) fused to retinoic acid (RA) receptor α (RARα); these cancers have shown clinical remission because of leukemic cell differentiation after retinoic acid (RA) treatment, as Ube1L is a RA-regulated gene that triggers PLM-RARα 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. those encoding BAX (also known as BCL2L4), CDKN1A and p53], resulting in suppression of cell growth and of tumorigenesis (Park et al., 2016). Furthermore, ISGylation of the dominant-negative splice variant ΔNp63α 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 ubiquitylation-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. In addition, the specific circumstances, such as treatment with RA (Pitha-Rowe et al., 2004) or DNA-damage-inducing 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-ubiquitylated and triggers TLS by recruiting polymerase-η, which has a lower fidelity than other DNA polymerases. The ISG15 E3 ligase EFP then binds to mono-ubiquitylated PCNA and promotes its ISGylation. ISGylated PCNA tethers USP10 for deubiquitylation 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 of either ISG15 or EFP, lead to persistent recruitment of mono-ubiquitylated PCNA and polymerase-η to nuclear foci, thereby causing an increase in mutation frequency (Park et al., 2014).

ISG15 also has an important role in autophagy. In fact, ISG15 interacts with p62 (also known as SQSTM1) and histone deacetylase 6 (HDAC6) in insoluble cell fractions, where protein aggregates accumulate (Nakashima et al., 2015). Both proteins have been shown to control autophagic clearance of protein aggregates: p62 interacts with Lys63-polyubiquitylated aggregation-prone proteins and facilitates their degradation by directly recruiting phagophores containing LC3A and LC3B (also known as MAP1LC3A and MAP1LC3B) (Pankiv et al., 2007), whereas HDAC6 recruits misfolded proteins to dynein motors for their transport to the aggresome (Kawaguchi et al., 2003) and promotes autophagosome–lysosome fusion (Lee et al., 2010). p62 also interacts with HDAC6 and regulates its deacetylase activity (Yan et al., 2013). Moreover, conjugation of ISG15 to proteins such as GFP or TSG101 has been shown to promote their aggregation and degradation by the lysosome (Nakashima et al., 2015; Villarroya-Beltri et al., 2016). Taken together, these observations suggest that ISGylation of proteins may promote their aggregation and degradation by selective autophagy through the interaction of ISG15 with p62 and HDAC6. In addition, ISG15 is upregulated in ataxia telangiectasia cells and enhances their autophagic flux, probably to compensate for the impaired proteasomal function that is caused by their constitutive activation of ISG15 (Desai et al., 2013). Furthermore, HSV infection triggers the formation of ISG15- and p62-decorated autophagosome clusters, which are involved in the establishment of latency (Katzenell and Leib, 2016). These autophagosomes are not induced by rapamycin or starvation (the typical inducers of autophagy) and therefore are likely to be related to selective autophagy, rather than global autophagy. Accordingly, ISGylation has been shown to induce fusion of MVBs with the autophagosome or lysosome compartment, without inducing general autophagy (Villarroya-Beltri et al., 2016). It should be noted that, in contrast to the above studies and a previous report showing that IFN induces autophagy (Schmeisser et al., 2013), a different study has suggested that IFN-I-induced ISGylation of Beclin 1 (BECN1) negatively regulates autophagy (Xu et al., 2015). BECN1 positively regulates autophagy by stimulating the lipid kinase activity of phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3), which generates phosphatidylinositol 3-phosphate and enables the recruitment of proteins involved in the nucleation of the autophagosome. BECN1 activity is induced by its ubiquitylation, which disrupts the association between BECN1 and its inhibitor partner BCL-2 (Abrahamsen et al., 2012). According to the above-mentioned study, ISGylation of BECN1 impairs its ubiquitylation and consequently inhibits BECN1-mediated activation of PI3KC3 and thus autophagy (Xu et al., 2015). The authors suggest that a plausible explanation for the discrepancy between their work and previous work showing that IFN-I positively regulates autophagy (Schmeisser et al., 2013) is that IFN-I-induced autophagy is transient and that long-term IFN-I treatment inhibits autophagy through BECN1 ISGylation. It is conceivable that ISGylation may have a negative effect on rapamycin-induced general autophagy, but nevertheless positively regulates selective autophagy. However, further investigation will be needed to uncover the exact role of ISGylation during autophagy.

ISGylation is also involved in the regulation of protein synthesis. Conjugation of ISG15 to protein kinase R (PKR) induces its activation in the absence of viral infection, resulting in phosphorylation of eukaryotic initiation factor 2α (eIF2α) and the subsequent downregulation of protein translation (Okumura et al., 2013). In addition, ISGylation of the translation suppressor 4EHP (also known as EIF4E2) increases its cap-binding activity and its ability to compete with the translation initiation factor for mRNA binding, resulting in inhibition of translation initiation (Okumura et al., 2007).

ISGylation has also been shown to regulate cytoskeletal dynamics. Indeed, ISG15 and UbcH8 disrupt F-actin architecture and the formation of focal adhesions in breast cancer cells (Desai et al., 2012). In addition, prolonged loss of the Rac1 and Cdc42 guanine-exchange factor DOCK6 reduces the expression of ISG15. Consequently, decreased ISGylation of the Ras GTPase-activating-like protein IQGAP1 increases the levels of active Cdc42 and Rac1 to restore F-actin function and compensate for DOCK6 disruption (Cerikan et al., 2016).

ISG15 is also induced in other physiological and pathological contexts, which reveal it might potentially function beyond restriction infection. Indeed, ISG15 is elevated upon traumatic brain injury (Rossi et al., 2015) and after cerebral focal ischemia (Nakka et al., 2011), where it has a neuroprotective effect as shown by increased mortality, exacerbated infarction and worsened neurologic recovery of ISG15-KO mice when they are subjected to transient middle cerebral artery occlusion (Nakka et al., 2011). Interestingly, ISGylation of hypoxia-inducible factor 1α (HIF-1α) disrupts its dimerization and attenuates HIF-1α-mediated gene expression, resulting in decreased tumor growth (Yeh et al., 2013). Furthermore, ISGylation 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 in ISG15-KO mice (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 MAPK family proteins, 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 100 nm in size that are secreted to the extracellular environment by most cells. They form in specific endosomal compartments called 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). In those systems, inhibition of exosome secretion was not observed when ISG15 was mutated so that its conjugation to proteins was impaired, whereas exosome secretion was increased in mouse cells that expressed a catalytically inactive form of the de-ISGylase USP18 (and thereby showed enhanced

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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 colocalization between the endosome marker hepatocyte growth factor-regulated tyrosine kinase substrate (HRS; also known as HGS) and lysosome-associated membrane protein 1 (LAMP-1). Exosome secretion in the above systems can be rescued 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 forms conjugates with the endosomal protein TSG101 (Sanyal et al., 2013; Villarroya-Beltri et al., 2016), which has been shown to control the secretion of exosomes (Colombo et al., 2013). Indeed, ISGylation of TSG101 is sufficient to inhibit exosome secretion (Villarroya-Beltri et al., 2016); however, because other endosomal proteins are modified by ISG15 (Kuang et al., 2011), the inhibition of exosome secretion could also be mediated by the ISGylation of additional proteins.

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

With regard to the effect of ISG15 on the secretion of molecules through the classical endoplasmic reticululum (ER)-to-Golgi pathway, ISG15 appears to not inhibit but rather enhance the secretion of cytokines via this route (Fan et al., 2015b; Radoshevich et al., 2015), which might allow the secretion of signaling molecules that are required to counteract infection. This appears to be mediated by ISGylation of ER proteins such as reticulon 4 (RTN4) (Radoshevich et al., 2015). In addition, ISGylation has been shown to increase the cell surface expression of major histocompatibility complex I (MHC-I) (Burks et al., 2015), which is responsible for presenting non-self and other intracellularly synthesized peptides that reflect a danger situation (e.g. oncogenic proteins) to cytotoxic cells, thereby triggering the killing of the presenting cells (Fig. 3). Furthermore, the major ISG15 E3 ligase HERC5 interacts with the polysome and promotes the conjugation of ISG15 to nascent proteins during their translation; it has been suggested that this labels any new proteins as suspicious as they might be of intracellular pathogen origin (Durfee et al., 2010). In addition, PKR and 4EHP ISGylation results in downregulation of global protein translation (Okumura et al., 2013, 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 towards 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 to proteins 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

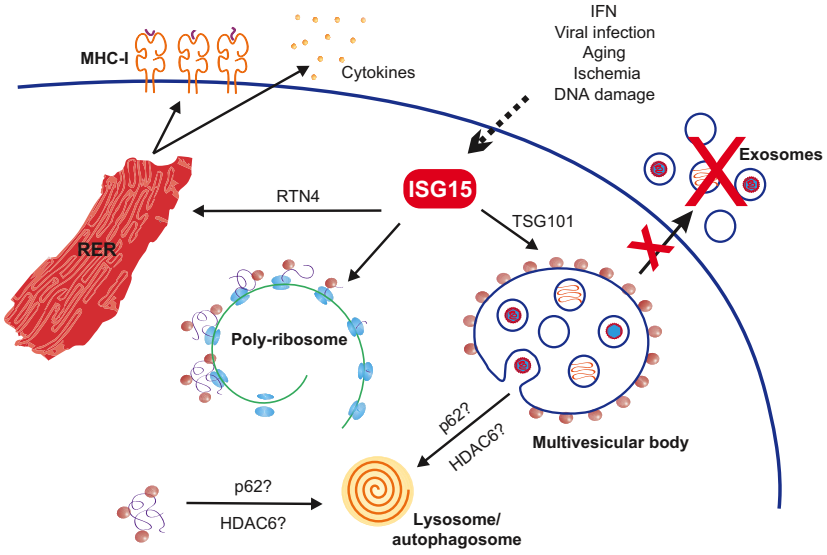

Fig. 3. Model for the role of ISG15 in preventing the spreading of a threat. ISG15 is induced during a variety of stress situations. ISG15 conjugates endosomal proteins such as TSG101, promoting the fusion of multivesicular bodies with lysosomes or autophagosomes, likely through the interaction of ISG15 with p62 and HDAC6. This inhibits exosome secretion, thus preventing the spread of virus, prions and protein aggregates. ISG15 is also conjugated to proteins at the polyribosome, tagging newly synthesized proteins as suspicious (potentially pathogen origin). 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.

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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 arrests the cell functions by inhibiting translation and enhancing p53, triggers the degradation of endosomal and newly synthesized proteins by the autophagosome and 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 humans 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 ubiquitylation, only two ISG15 E3 conjugating enzymes are known in humans and only one in mice. There have also been no poly-ISG15 chains or enzymes involved in the formation of poly-ISG15 chains 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 phosphatidylinositol-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) and 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 lysine residues 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 intra-molecular inhibitory interactions. ISGylation-mediated protein inactivation could be effective even when only a small fraction of the protein is modified if the modified protein acts as a dominant-negative mutant that sequesters the remaining functional pool. Further insights on the ISGylation pathway will provide valuable information on how cells respond to pathogens and other stress challenges, and might prove useful in developing novel strategies to treat infections, as well as other diseases, such as cancer or autoimmune disorders.

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Competing interests
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References
Abrahamsen, H., Stenmark, H. and Platta, H. W. (2012). Ubiquitination and phosphorylation of Beclin 1 and its binding partners: tuning class III phosphatidylinositol 3-kinase activity and tumor suppression. *FEBS Lett.* **586**, 1584-1591.

Arimoto, K.-I., Lochte, S., Stoner, S. A., Burkart, C., Zhang, Y., Miyauchi, S., Wilmes, S., Fan, J.-B., Heinisch, J. J., Li, Z. et al. (2017). STAT2 is an essential adaptor in USP18-mediated suppression of type I interferon signaling. *Nat. Struct. Mol. Biol.* **24**, 279-289.

Arnaud, N., Dabo, S., Akazawa, D., Fukasawa, M., Shinkai-Ouchi, F., Hugon, J., Wakita, T. and Meurs, E. F. (2011). Hepatitis C virus reveals a novel early control in acute immune response. *PLoS Pathog.* **7**, e1002289.

Baker, S., Polanco, J. C. and Götz, J. (2016). Extracellular vesicles containing P301L mutant tau accelerate pathological tau phosphorylation and oligomer formation but do not seed mature neurofibrillary tangles in ALZ17 mice. *J. Alzheimers Dis.* **54**, 1207-1217.

Basters, A., Geurink, P. P., Röcker, A., Witting, K. F., Tadayon, R., Hess, S., Semrau, M. S., Storici, P., Ovaa, H., Knobeloch, K.-P. et al. (2017). Structural basis of the specificity of USP18 toward ISG15. *Nat. Struct. Mol. Biol.* **24**, 270-278.

Bogunovic, D., Byun, M., Durfee, L. A., Abhyankar, A., Sanal, O., Mansouri, D., Salem, S., Radovanovic, I., Grant, A. V., Adimi, P. et al. (2012). Mycobacterial disease and impaired IFN-gamma immunity in humans with inherited ISG15 deficiency. *Science* **337**, 1684-1688.

Burks, J., Reed, R. E. and Desai, S. D. (2014). ISGylation governs the oncogenic function of Ki-Ras in breast cancer. *Oncogene* **33**, 794-803.

Burks, J., Reed, R. E. and Desai, S. D. (2015). Free ISG15 triggers an antitumor immune response against breast cancer: a new perspective. *Oncotarget* **6**, 7221-7231.

Cerikan, B., Shaheen, R., Colo, G. P., Glasser, C., Hata, S., Knobeloch, K.-P., Alkuraya, F. S., Fässler, R. and Schiebel, E. (2016). Cell-intrinsic adaptation arising from chronic ablation of a key Rho GTPase regulator. *Dev. Cell* **39**, 28-43.

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L. F., Thery, C. and Raposo, G. (2013). Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J. Cell Sci.* **126**, 5553-5565.

Chairatvit, K., Wongnoppavich, A. and Choonate, S. (2012). Up-regulation of interferon-stimulated gene15 and its conjugates by tumor necrosis factor-alpha via type I interferon-dependent and -independent pathways. *Mol. Cell. Biochem.* **368**, 195-201.

Chen, R. H., Du, Y., Han, P., Wang, H. B., Liang, F. Y., Feng, G. K., Zhou, A. J., Cai, M. Y., Zhong, Q., Zeng, M. S. et al. (2016). ISG15 predicts poor prognosis and promotes cancer stem cell phenotype in nasopharyngeal carcinoma. *Oncotarget* **7**, 16910-16922.

Daczkowski, C. M., Dzimianski, J. V., Clasman, J. R., Goodwin, O., Mesecar, A. D. and Pegan, S. D. (2017). Structural insights into the interaction of coronavirus papain-like proteases and interferon-stimulated gene Product 15 from different species. *J. Mol. Biol.* **429**, 1661-1683.

Dai, L., Bai, L., Lin, Z., Qiao, J., Yang, L., Flemington, E. K., Zabaleta, J. and Qin, Z. (2016). Transcriptomic analysis of KSHV-infected primary oral fibroblasts: the role of interferon-induced genes in the latency of oncogenic virus. *Oncotarget* **7**, 47052-47060.

Deng, X., Agnihothram, S., Mielech, A. M., Nichols, D. B., Wilson, M. W., StJohn, S. E., Larsen, S. D., Mesecar, A. D., Lenschow, D. J., Baric, R. S. et al. (2014). A chimeric virus-mouse model system for evaluating the function and inhibition of papain-like proteases of emerging coronaviruses. *J. Virol.* **88**, 11825-11833.

Desai, S. D. (2015). ISG15: a double edged sword in cancer. *Oncoimmunology* **4**, e1052935.

Desai, S. D., Haas, A. L., Wood, L. M., Tsai, Y. C., Pestka, S., Rubin, E. H., Saleem, A., Nur, E. K. A. and Liu, L. F. (2006). Elevated expression of ISG15 in tumor cells interferes with the ubiquitin/26S proteasome pathway. *Cancer Res.* **66**, 921-928.

Desai, S. D., Wood, L. M., Tsai, Y.-C., Hsieh, T.-S., Marks, J. R., Scott, G. L., Giovanella, B. C. and Liu, L. F. (2008). ISG15 as a novel tumor biomarker for drug sensitivity. *Mol. Cancer Ther.* **7**, 1430-1439.

Desai, S. D., Reed, R. E., Burks, J., Wood, L. M., Pullikuth, A. K., Haas, A. L., Liu, L. F., Breslin, J. W., Meiners, S. and Sankar, S. (2012). ISG15 disrupts cytoskeletal architecture and promotes motility in human breast cancer cells. *Exp. Biol. Med. (Maywood)* **237**, 38-49.

Desai, S. D., Reed, R. E., Babu, S. and Lorio, E. A. (2013). ISG15 deregulates autophagy in genotoxin-treated ataxia telangiectasia cells. *J. Biol. Chem.* **288**, 2388-2402.

Dos Santos, P. F. and Mansur, D. S. (2017). Beyond ISGlylation: functions of free intracellular and extracellular ISG15. *J. Interferon Cytokine Res.* **37**, 246-253.

Doyle, S. E., Vaidya, S. A., O'Connell, R., Dadgostar, H., Dempsey, P. W., Wu, T.-T., Rao, G., Sun, R., Haberland, M. E., Modlin, R. L. et al. (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17**, 251-263.

Durfee, L. A., Lyon, N., Seo, K. and Huibregtse, J. M. (2010). The ISG15 conjugation system broadly targets newly synthesized proteins: implications for the antiviral function of ISG15. *Mol. Cell* **38**, 722-732.

Eitan, E., Hutchison, E. R., Marosi, K., Comotto, J., Mustapic, M., Nigam, S. M., Suire, C., Maharana, C., Jicha, G. A., Liu, D., et al. (2016). Extracellular vesicle-associated Aβ mediates trans-neuronal bioenergetic and Ca^{2+}-handling deficits in Alzheimer's disease models. *NPJ Aging Mech Dis.* **2**,16019.

Fan, J.-B., Arimoto, K.-I., Motamedchaboki, K., Yan, M., Wolf, D. A. and Zhang, D.-E. (2015a). Identification and characterization of a novel ISG15-ubiquitin mixed chain and its role in regulating protein homeostasis. *Sci. Rep.* **5**, 12704.

Fan, J.-B., Miyauchi-Ishida, S., Arimoto, K.-A., Liu, D., Yan, M., Liu, C.-W., Győrffy, B. and Zhang, D.-E. (2015b). Type I IFN induces protein ISGylation to enhance cytokine expression and augments colonic inflammation. *Proc. Natl. Acad. Sci. USA* **112**, 14313-14318.

Feng, Q., Sekula, D., Guo, Y., Liu, X., Black, C. C., Galimberti, F., Shah, S. J., Sempere, L. F., Memoli, V., Andersen, J. B. et al. (2008). UBE1L causes lung cancer growth suppression by targeting cyclin D1. *Mol. Cancer Ther.* **7**, 3780-3788.

Frias-Staheli, N., Giannakopoulos, N. V., Kikkert, M., Taylor, S. L., Bridgen, A., Paragas, J., Richt, J. A., Rowland, R. R., Schmaljohn, C. S., Lenschow, D. J. et al. (2007). Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. *Cell Host Microbe.* **2**, 404-416.

Goldmann, T., Zeller, N., Raasch, J., Kierdorf, K., Frenzel, K., Ketscher, L., Basters, A., Staszewski, O., Brendecke, S. M., Spiess, A. et al. (2015). USP18 lack in microglia causes destructive interferonopathy of the mouse brain. *EMBO J.* **34**, 1612-1629.

Guerra, S., Cáceres, A., Knobeloch, K.-P., Horak, I. and Esteban, M. (2008). Vaccinia virus E3 protein prevents the antiviral action of ISG15. *PLoS Pathog.* **4**, e1000096.

Guo, B. B., Bellingham, S. A. and Hill, A. F. (2016). Stimulating the release of exosomes increases the intercellular transfer of prions. *J. Biol. Chem.* **291**, 5128-5137.

Haas, A. L., Ahrens, P., Bright, P. M. and Ankel, H. (1987). Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. *J. Biol. Chem.* **262**, 11315-11323.

Hummer, B. T., Li, X.-L. and Hassel, B. A. (2001). Role for p53 in gene induction by double-stranded RNA. *J. Virol.* **75**, 7774-7777.

Jeon, Y. J., Jo, M. G., Yoo, H. M., Hong, S.-H., Park, J.-M., Ka, S. H., Oh, K. H., Seol, J. H., Jung, Y. K. and Chung, C. H. (2012). Chemosensitivity is controlled by p63 modification with ubiquitin-like protein ISG15. *J. Clin. Invest.* **122**, 2622-2636.

Katzenell, S. and Leib, D. A. (2016). Herpes simplex virus and interferon signaling induce novel autophagic clusters in sensory neurons. *J. Virol.* **90**, 4706-4719.

Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A. and Yao, T.-P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* **115**, 727-738.

Ketscher, L., Hannss, R., Morales, D. J., Basters, A., Guerra, S., Goldmann, T., Hausmann, A., Prinz, M., Naumann, R., Pekosz, A. et al. (2015). Selective inactivation of USP18 isopeptidase activity in vivo enhances ISG15 conjugation and viral resistance. *Proc. Natl. Acad. Sci. USA* **112**, 1577-1582.

Kiessling, A., Hogrefe, C., Erb, S., Bobach, C., Fuessel, S., Wessjohann, L. and Seliger, B. (2009). Expression, regulation and function of the ISGylation system in prostate cancer. *Oncogene* **28**, 2606-2620.

Kim, Y. J., Kim, E. T., Kim, Y.-E., Lee, M. K., Kwon, K. M., Kim, K. I., Stamminger, T. and Ahn, J.-H. (2016). Consecutive inhibition of ISG15 expression and ISGylation by cytomegalovirus regulators. *PLoS Pathog.* **12**, e1005850.

Knobeloch, K. P., Utermohlen, O., Kisser, A., Prinz, M. and Horak, I. (2005). Reexamination of the role of ubiquitin-like modifier ISG15 in the phenotype of UBP43-deficient mice. *Mol. Cell. Biol.* **25**, 11030-11034.

Kuang, Z., Seo, E. J. and Leis, J. (2011). Mechanism of inhibition of retrovirus release from cells by interferon-induced gene ISG15. *J. Virol.* **85**, 7153-7161.

Kunadt, M., Eckermann, K., Stuendl, A., Gong, J., Russo, B., Strauss, K., Rai, S., Kügler, S., Falomir Lockhart, L., Schwalbe, M. et al. (2015). Extracellular vesicle sorting of alpha-Synuclein is regulated by sumoylation. *Acta Neuropathol.* **129**, 695-713.

Lee, J.-Y., Koga, H., Kawaguchi, Y., Tang, W., Wong, E., Gao, Y.-S., Pandey, U. B., Kaushik, S., Tresse, E., Lu, J. et al. (2010). HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. *EMBO J.* **29**, 969-980.

Lenschow, D. J., Lai, C., Frias-Staheli, N., Giannakopoulos, N. V., Lutz, A., Wolff, T., Osiak, A., Levine, B., Schmidt, R. E., Garcia-Sastre, A. et al. (2007). IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc. Natl. Acad. Sci. USA*. **104**, 1371-1376.

Li, J., Peet, G. W., Balzarano, D., Li, X., Massa, P., Barton, R. W. and Marcu, K. B. (2001). Novel NEMO/IkappaB kinase and NF-kappa B target genes at the pre-B to immature B cell transition. *J. Biol. Chem.* **276**, 18579-18590.

Li, C., Wang, J., Zhang, H., Zhu, M., Chen, F., Hu, Y., Liu, H. and Zhu, H. (2014). Interferon-stimulated gene 15 (ISG15) is a trigger for tumorigenesis and metastasis of hepatocellular carcinoma. *Oncotarget* **5**, 8429-8441.

Liu, C., Chang, R., Yao, X., Qiao, W. T. and Geng, Y. Q. (2009). ISG15 expression in response to double-stranded RNA or LPS in cultured Fetal bovine lung (FBL) cells. *Vet. Res. Commun.* **33**, 723-733.

Liu, F., Gao, X., Wang, J., Gao, C., Li, X., Li, X., Gong, X. and Zeng, X. (2016). Transcriptome sequencing to identify transcription factor regulatory network and alternative splicing in endothelial cells under VEGF stimulation. *J. Mol. Neurosci.* **58**, 170-177.

Loeb, K. R. and Haas, A. L. (1992). The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. *J. Biol. Chem.* **267**, 7806-7813.

Lou, Z., Wei, J., Riethman, H., Baur, J. A., Voglauer, R., Shay, J. W. and Wright, W. E. (2009). Telomere length regulates ISG15 expression in human cells. *Aging (Albany NY)* **1**, 608-621.

Malakhova, O. A., Kim, K. I. I., Luo, J.-K., Zou, W., Kumar, K. G. S., Fuchs, S. Y., Shuai, K. and Zhang, D.-E. (2006). UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. *EMBO J.* **25**, 2358-2367.

Maragno, A. L., Pironin, M., Alcalde, H., Cong, X., Knobeloch, K.-P., Tangy, F., Zhang, D.-E., Ghysdael, J. and Quang, C. T. (2011). ISG15 modulates development of the erythroid lineage. *PLoS ONE* **6**, e26068.

Meuwissen, M. E. C., Schot, R., Buta, S., Oudesluijs, G., Tinschert, S., Speer, S. D., Li, Z., van Unen, L., Heijsman, D., Goldmann, T. et al. (2016). Human USP18 deficiency underlies type 1 interferonopathy leading to severe pseudo-TORCH syndrome. *J. Exp. Med.* **213**, 1163-1174.

Mittelbrunn, M. and Sanchez-Madrid, F. (2012). Intercellular communication: diverse structures for exchange of genetic information. *Nat. Rev. Mol. Cell Biol.* **13**, 328-335.

Morales, D. J., Monte, K., Sun, L., Struckhoff, J. J., Agapov, E., Holtzman, M. J., Stappenbeck, T. S. and Lenschow, D. J. (2015). Novel mode of ISG15-mediated protection against influenza A virus and Sendai virus in mice. *J. Virol.* **89**, 337-349.

Nakashima, H., Nguyen, T., Goins, W. F. and Chiocca, E. A. (2015). Interferon-stimulated Gene 15 (ISG15) and ISG15-linked proteins can associate with members of the selective autophagic process, histone deacetylase 6 (HDAC6) and SQSTM1/p62. *J. Biol. Chem.* **290**, 1485-1495.

Nakka, V. P., Lang, B. T., Lenschow, D. J., Zhang, D.-E., Dempsey, R. J. and Vemuganti, R. (2011). Increased cerebral protein ISGylation after focal ischemia is neuroprotective. *J. Cereb. Blood Flow Metab.* **31**, 2375-2384.

Okumura, F., Zou, W. and Zhang, D.-E. (2007). ISG15 modification of the eIF4E cognate 4EHP enhances cap structure-binding activity of 4EHP. *Genes Dev.* **21**, 255-260.

Okumura, A., Pitha, P. M. and Harty, R. N. (2008a). ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. *Proc. Natl. Acad. Sci. USA* **105**, 3974-3979.

Okumura, F., Lenschow, D. J. and Zhang, D.-E. (2008b). Nitrosylation of ISG15 prevents the disulfide bond-mediated dimerization of ISG15 and contributes to effective ISGylation. *J. Biol. Chem.* **283**, 24484-24488.

Okumura, F., Okumura, A. J., Uematsu, K., Hatakeyama, S., Zhang, D.-E. and Kamura, T. (2013). Activation of double-stranded RNA-activated protein kinase (PKR) by interferon-stimulated gene 15 (ISG15) modification down-regulates protein translation. *J. Biol. Chem.* **288**, 2839-2847.

Osiak, A., Utermohlen, O., Niendorf, S., Horak, I. and Knobeloch, K.-P. (2005). ISG15, an interferon-stimulated ubiquitin-like protein, is not essential for STAT1

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signaling and responses against vesicular stomatitis and lymphocytic choriomeningitis virus. *Mol. Cell. Biol.* **25**, 6338-6345.
Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., Overvatn, A., Bjorkoy, G. and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* **282**, 24131-24145.
Park, J. M., Yang, S. W., Yu, K. R., Ka, S. H., Lee, S. W., Seol, J. H., Jeon, Y. J. and Chung, C. H. (2014). Modification of PCNA by ISG15 plays a crucial role in termination of error-prone translesion DNA synthesis. *Mol. Cell* **54**, 626-638.
Park, J. H., Yang, S. W., Park, J. M., Ka, S. H., Kim, J.-H., Kong, Y.-Y., Jeon, Y. J., Seol, J. H. and Chung, C. H. (2016). Positive feedback regulation of p53 transactivity by DNA damage-induced ISG15 modification. *Nat. Commun.* **7**, 12513.
Pincetic, A., Kuang, Z., Seo, E. J. and Leis, J. (2010). The interferon-induced gene ISG15 blocks retrovirus release from cells late in the budding process. *J. Virol.* **84**, 4725-4736.
Pitha-Rowe, I., Hassel, B. A. and Dmitrovsky, E. (2004). Involvement of UBE1L in ISG15 conjugation during retinoid-induced differentiation of acute promyelocytic leukemia. *J. Biol. Chem.* **279**, 18178-18187.
Quek, C. and Hill, A. F. (2017). The role of extracellular vesicles in neurodegenerative diseases. *Biochem. Biophys. Res. Commun.* **483**, 1178-1186.
Radoshevich, L., Impens, F., Ribet, D., Quereda, J. J., Nam Tham, T., Nahori, M. A., Bierne, H., Dussurget, O., Pizarro-Cerda, J., Knobeloch, K. P. et al. (2015). ISG15 counteracts Listeria monocytogenes infection. *Elife* **4**, e06848.
Raposo, G. and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* **200**, 373-383.
Rodriguez, M. R., Monte, K., Thackray, L. B. and Lenschow, D. J. (2014). ISG15 functions as an interferon-mediated antiviral effector early in the murine norovirus life cycle. *J. Virol.* **88**, 9277-9286.
Rossi, J. L., Todd, T., Daniels, Z., Bazan, N. G. and Belayev, L. (2015). Interferon-stimulated Gene 15 upregulation precedes the development of blood-brain barrier disruption and cerebral edema after traumatic brain injury in young mice. *J. Neurotrauma* **32**, 1101-1108.
Sadler, A. J. and Williams, B. R. G. (2008). Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* **8**, 559-568.
Sainz, B., Jr, Martin, B., Tatari, M., Heeschen, C. and Guerra, S. (2014). ISG15 is a critical microenvironmental factor for pancreatic cancer stem cells. *Cancer Res.* **74**, 7309-7320.
Sanyal, S., Ashour, J., Maruyama, T., Altenburg, A. F., Cragnolini, J. J., Bilate, A., Avalos, A. M., Kundrat, L., García-Sastre, A. and Ploegh, H. L. (2013). Type I interferon imposes a TSG101/ISG15 checkpoint at the Golgi for glycoprotein trafficking during influenza virus infection. *Cell Host Microbe* **14**, 510-521.
Schmeisser, H., Fey, S. B., Horowitz, J., Fischer, E. R., Balinsky, C. A., Miyake, K., Bekisz, J., Snow, A. L. and Zoon, K. C. (2013). Type I interferons induce autophagy in certain human cancer cell lines. *Autophagy* **9**, 683-696.
Sen, G. C. and Sarkar, S. N. (2007). The interferon-stimulated genes: targets of direct signaling by interferons, double-stranded RNA, and viruses. *Curr. Top. Microbiol. Immunol.* **316**, 233-250.
Shi, H. X., Yang, K., Liu, X., Liu, X. Y., Wei, B., Shan, Y. F., Zhu, L. H. and Wang, C. (2010). Positive regulation of interferon regulatory factor 3 activation by Herc5 via ISG15 modification. *Mol. Cell. Biol.* **30**, 2424-2436.
Skaug, B. and Chen, Z. J. (2010). Emerging role of ISG15 in antiviral immunity. *Cell* **143**, 187-190.
Speer, S. D., Li, Z., Buta, S., Payelle-Brogard, B., Qian, L., Vigant, F., Rubino, E., Gardner, T. J., Wedeking, T., Hermann, M. et al. (2016). ISG15 deficiency and increased viral resistance in humans but not mice. *Nat. Commun.* **7**, 11496.
Takeuchi, T. and Yokosawa, H. (2005). ISG15 modification of Ubc13 suppresses its ubiquitin-conjugating activity. *Biochem. Biophys. Res. Commun.* **336**, 9-13.
Taylor, J. L., D'Cunha, J., Tom, P., O'Brien, W. J. and Borden, E. C. (1996). Production of ISG-15, an interferon-inducible protein, in human corneal cells. *J. Interferon Cytokine Res.* **16**, 937-940.
Villarreal, D. O., Wise, M. C., Siefert, R. J., Yan, J., Wood, L. M. and Weiner, D. B. (2015). Ubiquitin-like molecule ISG15 acts as an immune adjuvant to enhance antigen-specific CD8 T-cell tumor immunity. *Mol. Ther.* **23**, 1653-1662.
Villarroya-Beltri, C., Baixauli, F., Mittelbrunn, M., Fernandez-Delgado, I., Torralba, D., Moreno-Gonzalo, O., Baldanta, S., Enrich, C., Guerra, S. and Sanchez-Madrid, F. (2016). ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins. *Nat. Commun.* **7**, 13588.
Werneke, S. W., Schilte, C., Rohatgi, A., Monte, K. J., Michault, A., Arenzana-Seisdedos, F., Vanlandingham, D. L., Higgs, S., Fontanet, A., Albert, M. L. et al. (2011). ISG15 is critical in the control of Chikungunya virus infection independent of UbE1L mediated conjugation. *PLoS Pathog.* **7**, e1002322.
Wood, L. M., Pan, Z. K., Seavey, M. M., Muthukumaran, G. and Paterson, Y. (2012). The ubiquitin-like protein, ISG15, is a novel tumor-associated antigen for cancer immunotherapy. *Cancer Immunol. Immunother.* **61**, 689-700.
Xu, D., Zhang, T., Xiao, J., Zhu, K., Wei, R., Wu, Z., Meng, H., Li, Y. and Yuan, J. (2015). Modification of BECN1 by ISG15 plays a crucial role in autophagy regulation by type I IFN/interferon. *Autophagy* **11**, 617-628.
Yan, J., Seibenhener, M. L., Calderilla-Barbosa, L., Diaz-Meco, M. T., Moscat, J., Jiang, J., Wooten, M. W. and Wooten, M. C. (2013). SQSTM1/p62 interacts with HDAC6 and regulates deacetylase activity. *PLoS ONE* **8**, e76016.
Yanguez, E., Garcia-Culebras, A., Frau, A., Llompart, C., Knobeloch, K. P., Gutierrez-Erlandsson, S., Garcia-Sastre, A., Esteban, M., Nieto, A. and Guerra, S. (2013). ISG15 regulates peritoneal macrophages functionality against viral infection. *PLoS Pathog.* **9**, e1003632.
Yeh, Y. H., Yang, Y. C., Hsieh, M. Y., Yeh, Y. C. and Li, T. K. (2013). A negative feedback of the HIF-1alpha pathway via interferon-stimulated gene 15 and ISGylation. *Clin. Cancer Res.* **19**, 5927-5939.
Yuan, W. and Krug, R. M. (2001). Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. *EMBO J.* **20**, 362-371.
Zhang, X., Bogunovic, D., Payelle-Brogard, B., Francois-Newton, V., Speer, S. D., Yuan, C., Volpi, S., Li, Z., Sanal, O., Mansouri, D. et al. (2015). Human intracellular ISG15 prevents interferon-alpha/beta over-amplification and auto-inflammation. *Nature* **517**, 89-93.
Zhao, C., Hsiang, T. Y., Kuo, R. L. and Krug, R. M. (2010). ISG15 conjugation system targets the viral NS1 protein in influenza A virus-infected cells. *Proc. Natl. Acad. Sci. USA* **107**, 2253-2258.
Zhao, C., Sridharan, H., Chen, R., Baker, D. P., Wang, S. and Krug, R. M. (2016). Influenza B virus non-structural protein 1 counteracts ISG15 antiviral activity by sequestering ISGylated viral proteins. *Nat. Commun.* **7**, 12754.