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IDENTIFICATION AND VALIDATION OF ISG15 TARGET PROTEINS

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Abstract

ISG15 is an interferon-induced ubiquitin-like protein (Ubl) that has antiviral properties. The core El, E2 and E3 enzymes for conjugation of human ISG15 are Ube1L, UbcH8 and Herc5, all of which are induced at the transcriptional level by Type 1 interferon signaling. Several proteomics studies have, together, identified over 300 cellular proteins as ISG15 targets. These targets include a broad range of constitutively expressed proteins and approximately 15 interferon-induced proteins. This chapter provides an overview of the target identification process and the validation of these targets. We also discuss the limited number of examples where the biochemical effect of ISG15 conjugation on target proteins has been characterized.

INTRODUCTION

The innate immune response of mammalian cells is a first-line defense against viral and microbial infections.[1] The system is nonspecific with respect to antigen recognition and is instead geared to recognize classes of molecules that are commonly produced by microbes or viruses, such as lipopolysaccharides, flagellin and double-stranded RNA. These molecules are referred to as PAMPs, for Pattern Associated Molecular Patterns. Pathogen Recognition Receptors (PRRs), such as Toll-like receptors, RIG-I and double-stranded RNA-activated protein kinase (PKR), directly recognize PAMPs and trigger signaling pathways that lead to the production of Type I interferons (IFNs).[2] The two major forms of Type I IFNs are IFNα and β. IFNα is produced primarily by leukocytes, while IFNβ is produced by a wide range of cell types, including fibroblasts and epithelial cells. Type 1 IFNs are secreted by infected cells and bind to the interferon receptor (IFNAR) of surrounding cells, as well as the initially infected cell, resulting in the transcription of hundreds of interferon stimulated genes (ISGs). The expression of ISGs leads to the generation of a multi-faceted defense system that serves to limit viral or microbial infection.

ISG15 is an ISG denoted by the approximate molecular mass of the protein, although the actual molecular mass of the mature form of the protein is 17.1 kD. It was first identified based on its rapid induction in Ehrlich ascites tumor cells after IFN stimulation.[3] About a decade later. Art Haas and coworkers recognized ISG15 as what would turn out to be the first of many ubiquitin-like protein (Ubl) modifiers.[4,5] As discussed further below, it would be nearly another two decades before ISG15 was shown to actually have antiviral activity

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against a range of viruses, including Influenza, Sindbis, Herpes, HIV and Ebola virus.[6–9] There have so far been no reports demonstrating an antimicrobial function for ISG15. A timeline of discovery for ISG15, spanning 30 years from 1979 to 2009, is shown in Figure 1.

Like ubiquitin (Ub) and other Ubls, ISG15 is synthesized as a precursor protein that must be processed to reveal a C-terminal glycine residue. The identity of the processing enzyme is unknown, but Ubp43, an ISG15 deconjugating enzyme, is clearly not essential for processing as the Ubp43 knockout mouse still generates processed and conjugation-competent ISG15.[10] There are also inconsistencies in the literature concerning the role of murine Ubp43 as a specific ISG15 deconjugating enzyme. It has been reported to have activity against both ubiquitin and ISG15[11,12] and neurologic phenotypes of the Ubp43 knockout mouse were also seen in the Ubp43/ISG15 double knockout mouse,[10,13] indicating that accumulation of ISG15 conjugates cannot account for the Ubp43$^{-/-}$ phenoytpes. The difficulty is identifying a specific deISGylating enzyme, if one indeed exists, is likely complicated by the fact that the last six residues of ISG15 are identical to those of ubiquitin (LRLRGG) and these residues must be recognized by the active site of deconjugating enzymes. Consistent with this, other presumed deubiquitinating enzymes have also been shown to have activity against ISG15 conjugates.[14]

Conjugation of human ISG15 to target proteins occurs via an enzymatic cascade consisting of a single E1 enzyme (Ube1L), E2 enzyme (UbcH8) and a single major E3 enzyme (Herc5). The genes encoding these proteins are all transcriptionally induced by IFNα/β[15] although their induction is delayed relative to that of ISG15. Therefore, while free (unconjugated) ISG15 is rapidly induced by interferon stimulation,[3] conjugation of ISG15 does not occur to an appreciable degree until approximately 18–24 hours after stimulation.[5] It is not understood why this delay is built into the conjugation system, although it cannot be ruled out that that free ISG15 has a function independent of conjugation. Ube1L is the ISG15 E1 enzyme and this is a monomeric E1-like enzyme,[16] unlike the heterodimeric E1 enzymes for Nedd8 and Sumo. The E2 enzyme for ISG15 is UbcH8/Ube2L6.[17–19] While UbcH8 has been reported to be an E2 for ubiquitin,[20–26] determination of kinetic constants of Ube1L and Ube1 (E1^{Ub}) for UbcH8 and UbcH7 indicated that UbcH8 is unlikely to function as a ubiquitin E2 in vivo.[17] The major E3 for ISG15 in human cells is Herc5, a HECT domain ligase with N-terminal RCC1 repeats.[15,27] Herc5 depletion leads to a dramatic decrease in overall ISG15 conjugation activity, affecting conjugation to the vast majority of cellular target proteins.[15] While additional proteins may be required for ISG15 conjugation, Ube1L, UbcH8 and Herc5 form the core IFN-induced components of the system, as their co-expression with ISG15 reconstitutes robust ISG15 conjugation in non-IFN-stimulated cells.[15,27,28] ISGylation of target proteins has so far not been reconstituted in vitro. The difficulty appears to lay at the level of the E3 enzyme, as ISG15, Ube1L and UbcH8 are all biochemically active in vitro.[17]

Interestingly, human Herc5 does not have a direct equivalent in mice or rats.[29] However, the human Herc5 and Herc6 genes are adjacent to each other on chromosome 4 and are clearly related to each other through a gene duplication event.[15] Both genes are transcriptionally regulated by interferon signaling and the human Herc5 and Herc6 proteins are 50% identical with very similar domain organizations.[29] Mouse Herc6 is interferon-induced and located at

the corresponding genomic position as human Herc6. While it was proposed that Herc5 was the result of a gene duplication of Herc6 in the primate lineage,[29] this is likely incorrect since other mammals (dogs, cow, sheep) have both the Herc5 and Herc6 genes in a similar arrangement as in the human genome, suggesting that Herc5 was lost in the evolution of the rodent lineage. Although there is no evidence that human Herc6 plays a significant role in ISG15 conjugation, it is conceivable that murine Herc6 plays the equivalent role to human Herc5 in conjugation of murine ISG15. To our knowledge, this possibility has not yet been experimentally tested.

Support for an antiviral function for ISG15 was first reported in 2001 when it was found that influenza B virus blocked the conjugation of ISG15 to cellular proteins.[16,30] Since then, ISG15 has been reported to have antiviral effects against Influenza, Sindbis, Vaccinia (VACV), Herpes Simplex I, murine γ-Herpesvirus, HIV-1 and Ebola virus.[6–9,31] Recent studies with mice lacking Ube1L highlight the importance of ISG15 conjugation, as Ube1L-null mice infected with either Sindbis or influenza B virus produced free ISG15, but no conjugates and were shown to have increased susceptibility to both viruses.[32,33] In addition to Influenza B, other viruses have also evolved mechanisms for interfering with ISG15 conjugation: SARS coronavirus encodes a protease that can deconjugate ISG15 from target proteins,[34] while vaccinia virus encodes a viral early protein, E3, which binds ISG15 and prevents ISGylation.[35]

Importantly, the precise biochemical function of ISG15 and the biochemical basis of its antiviral activities remain unknown. In order to understand the mechanism by which ISG15 conjugation contributes to antiviral responses, it is important to identify the proteins targeted by the ISG15 pathway. As described below, proteomic studies have identified over 300 cellular ISG15 targets. The functional consequences of ISGylation will also be discussed, although this has only been determined for a small number of target proteins.

DETECTION OF ISG15 CONJUGATION

ISGylation of target proteins can be detected by two cell-based methods: either by treatment of interferon-responsive cells (e.g., A549, HeLa) with purified IFN-β, or by cotransfection of non-interferon-stimulated cells with four plasmids expressing the core ISG15 conjugation components (ISG15, Ube1L, UbcH8 and Herc5). In HeLa cells, conjugation of ISG15 can be detected approximately 18 hours after treatment with IFNβ, with maximal accumulation of conjugates by 48 hours posttreatment.[5,16] For cotransfection experiments, HEK293T cells can be transfected with plasmids expressing Ube1L, UbcH8, Herc5 and tagged (3X-FLAG) or untagged ISG15. In both cases, extracts are typically prepared 24–48 hours after IFNβ treatment or transfection and total cellular proteins are separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane is then probed with anti-ISG15 antibody (Santa Cruz Antibodies) or with anti-FLAG antibody (Sigma) to detect conjugation of FLAG-ISG15. It is often useful to use a 12% acrylamide gel in order to observe the free (unconjugated) ISG15. Conjugates typically range in size from approximately 45 kD to very high molecular weight adducts that migrate near the top of the gel. Figure 2 shows a time course of ISG15 conjugation of FLAG-ISG15 following cotransfection of plasmids expressing 3XFLAG-ISG15, Ube1L, UbcH8 and Herc5. It should be noted that, at least in

our hands, HEK293T respond poorly to IFNβ stimulation and little if any endogenous ISG15 conjugation can be detected in these cells.

IDENTIFICATION OF ISG15 TARGETS

For ubiquitin, large-scale mass spectrometry-based identification of target in yeast proteins was first reported in 2003.[36] This approach involved expression of an N-terminally tagged (6xHis) ubiquitin molecule, affinity purification of conjugates under denaturing conditions and multidimensional liquid chromatography coupled with tandem mass spectrometry to identify conjugated proteins. The basis of mass spectrometry identification of ubiquitin and Ubl modifiers and the criteria for considering a protein as target have been discussed elsewhere.[37,38] Based on this general principle, three proteomics studies were performed to identify cellular proteins conjugated to ISG15.[27,39,40] Zhao, et al, expressed a double-tagged ISG15 protein (6xHis and FLAG N-terminal tags), along with Ube1L and UbcH8, in HeLa cells and then treated the transfected cells with IFN-β. Extracts were prepared 24 hours after IFN treatment and conjugates were purified under denaturing conditions on Ni-NTA resin, followed by purification on anti-FLAG beads. Purification under denaturing conditions assured that conjugated proteins would be identified, as opposed to proteins associated with conjugated proteins. Isolated proteins were separated by SDS-PAGE and regions of the gel were subjected to trypsin digestion, peptides were separated and identified by liquid chromatography and mass spectrometry, as described.[40] This study identified approximately 158 high-confidence targets. In a second study, Giannakopolous, et al, identified ISG15 conjugates in interferon-treated human cells (U937 cells) and in mouse Ubp43-null MEFs.[39] Conjugates were immunopurified using antibody recognizing endogenously expressed ISG15. Mass spectrometry identified a total of 76 mouse and human proteins in the immunoprecipitates. Finally, Wong, et al, stably expressed FLAG-ISG15 in A549 cells and immunopurified conjugates after 48 hours of IFNβ treatment.[27] 174 ISG15 target proteins were identified in this study and together, these three studies identified slightly more than 300 unique proteins as potential ISG15 target proteins.

The identification of ISG15 target proteins was highly anticipated, as it was hoped that this would provide valuable insight into the biologic function of ISG15. This, for example, was the case for identification of targets of Sumo, where target identification revealed a very high percentage of targets to be nuclear proteins involved in regulation of DNA- and RNA-related processes.[41,42] Unfortunately, the identification of ISG15 target proteins was not particularly revealing. The targets were largely abundant constitutively expressed proteins, with the exception of approximately 15 targets that were themselves interferon-induced proteins. The interferon-induced targets included some of the better-characterized antiviral ISGs, including PKR, p56, MxA and RIG-I.[43,44] PKR and RIG-I, as mentioned above, are pathogen recognition receptors. p56 is a translational inhibitor and MxA is an endoplasmic reticulum-associated GTPase. The constitutively expressed proteins included both cytoplasmic and nuclear proteins involved in a diverse array of cellular functions, including cytoskeletal organization, stress responses, translation, transcription, RNA splicing and general metabolism. Therefore, a clear prediction of the biologic function for ISG15 could not be inferred from this diverse set of target proteins. Finally, it should be noted that a common primary sequence motif has not been identified within this set of target proteins

that might serve as a recognition signal for ISGylation and the basis for substrate recognition by the ISGylation machinery remains unknown.

VALIDATION OF ISG15 TARGETS

Validation of ISG15 targets can be performed in several different ways depending on the availability of an antibody to the target, the expression level of the endogenous target protein and the degree to which the target protein is modified with ISG15. In the simplest case, IFNβ can be used to induce the ISG15 conjugation system for 24–48 hours, followed by immunoblotting for the endogenous target protein. The presence of ISG15 conjugates is then seen as the appearance of modified forms of the protein corresponding to the 15 kD Ubl. Multiple conjugates at 15 kD intervals are seen among on some targets. There is no evidence that these represent poly-ISG15 chains and mass spectrometry based mapping of modification sites on individual target proteins strongly suggests that these represent single ISG15 moieties at multiple lysine residues of the target protein (unpublished results). The pattern of modified bands may appear more complicated due to the fact that mono-ISGylated proteins modified at different sites of a target protein may result in slightly different migration on SDS-PAGE gels. In any case, these modified forms can be confirmed to be ISG15 conjugates by siRNA knockdown of one or more of the ISG15 conjugation enzymes. Importantly, only a small number of target proteins have been validated by direct western blotting of IFN-treated cells and these have primarily been those that are IFN-induced and highly expressed, such as p56 and MxA.[38] This may primarily be due to the fact that ISGylation is an inefficient process. Typically, the most robust conjugation observed will be on the order of 10–20% of the total pool of the target and in most cases it is much lower. Alternatively, immunoprecipitation of a target protein, followed by immunoblotting for ISG15 (IP-westerns), may serve to enrich for the target protein and result in more sensitive detection of conjugates. ISG15 conjugates can also be concentrated by immunoprecipitating total conjugates and then immunoblotting for an individual target protein.

The single most successful way to validate ISGylation of targets has been to transiently express the target protein along with the conjugation components (ISG15, Ube1L, UbcH8, with or without Herc5) in non-IFN-treated cells. Prior to the identification of Herc5 as the major ISG15 E3, modification of targets was in some cases validated by co-expression of the target with ISG15, Ube1L and UbcH8.[39,40] In retrospect, this combination of components was likely sufficient for modification of some proteins because of a basal level of expression of Herc5.[15] That is, while conjugation can be detected in HEK293T or HeLa cells by co-expression of ISG15, Ube1L and UbcH8, an siRNA against Herc5 dramatically decreases the level of conjugates, while co-expression of Herc5 boosts the level of conjugation.[15] Figure 3 shows the modification of four epitope-tagged target proteins (V5-Ran, FLAG-p56, V5-moesin and TAP-Hsc70) in the presence of a triple (ISG15, Ube1L, UbcH8) or quadruple (ISG15, Ube1L, UbcH8, Herc5) combination of the conjugation components. This demonstrates that modification can in some cases be detected with only basal levels of Herc5 expression, but that it in all cases it is strongly enhanced by co-expression of Herc5.

THE EFFECTS OF ISGYLATION ON TARGET PROTEIN FUNCTION

As described above, there are a number of cell-based approaches can be used to generate ISGylated proteins and validate modification of individual proteins. However, it is important to note that an in vitro ISGylation system has not yet been established. Without such a system, it is not possible to easily generate purified ISGylated target proteins for biochemical analyses. This is a key reason why only modest progress has been made on understanding the biochemical function of ISG15 conjugation. Nevertheless, a small number of studies have examined modification of different target cellular target proteins and described biochemical consequences of modification. The four targets discussed here are Ubc13, filamin B, PP2Cβ, and 4EHP.

Ubc13 is a ubiquitin E2 enzyme that functions in the generation of K63-linked polyubiquitin chains. Ubc13 was identified as a cellular target of ISGylation in one of the three proteomic studies discussed above.[40] Two subsequent reports showed that Ubc13 is ISGylated at lysine residue K92, which lies near the active-site cysteine residue (C87) of the protein. Both studies showed that the ISGylated form of Ubc13 was defective for ubiquitin thioester formation.[45,46] Given the proximity of C87 to K92 in the structure, the inhibition of thioester formation is likely to be simply a result of steric occlusion of C87 by the conjugated ISG15 molecule. The downstream effects of this inhibition have been suggested to result in prevention of NFkB activation,[47] as K63-linked polyubiquitination catalyzed by the Ubc13/Mms2 complex is a critical component of the signaling pathway that leads to NFkB activation. NFkB activation, in turn, is critical for transcriptional activation of genes involved in the innate immune response, including IFNβ. An unresolved problem with this proposed mechanism is that only a small fraction of Ubc13 is ISGylated and it is not clear how this would lead to a significant inhibition of overall Ubc13 activity.

Filamin B is one of three related actin binding proteins that are critical for crosslinking of cortical actin filaments[48] and it was identified in one of the three large-scale ISG15 proteomics projects discussed above.[40] At early time points after interferon stimulation, filamin B tethers RAC1 and a MAP kinase module, which promotes activation of JNK and JNK-mediated apopotosis. The ISGylation of filamin B was shown to lead to release of RAC1, MEKK1 and MKK4 from the scaffold, preventing JNK activation.[49] A model was proposed whereby ISGylation of filamin B, at relatively late time points after interferon stimulation, leads to the inactivation of JNK.

PP2Cβ is a protein phosphatase identified in two ISG15 proteomics studies.[40,50] PP2Cβ dephosphorylates the TAK1 and IKK kinases, leading to inhibition of NFkB signaling and it was shown that ISGylation of PP2Cβ inhibited its activity. Finally, in a case where ISGylation activates, rather than inactivates, a target protein, the 4EHP mRNA cap binding protein was reported to have increased cap binding activity following ISGylation.[51] The biochemical basis of this enhanced binding is unknown.

CONCLUSION

A clearer picture has been to emerge of the enzymes required for ISGylation, the identity of the cellular targets of ISGylation and the spectrum of viruses sensitive to the antiviral effects of ISGylation. Some of the major gaps in our understanding concern (1) the basis for enzyme-substrate recognition in the conjugation process (that is, how a single major E3 enzyme target over 300 cellular proteins?), (2) how the ISGylation of the known targets are directly related to antiviral activities and (3) the precise biochemical effect of ISG15 on target proteins. The establishment of an in vitro ISGylation system will be important for addressing all of these problems, as will a better understanding of the precise steps that ISG15 inhibits in the course of the life cycles of relevant viruses.

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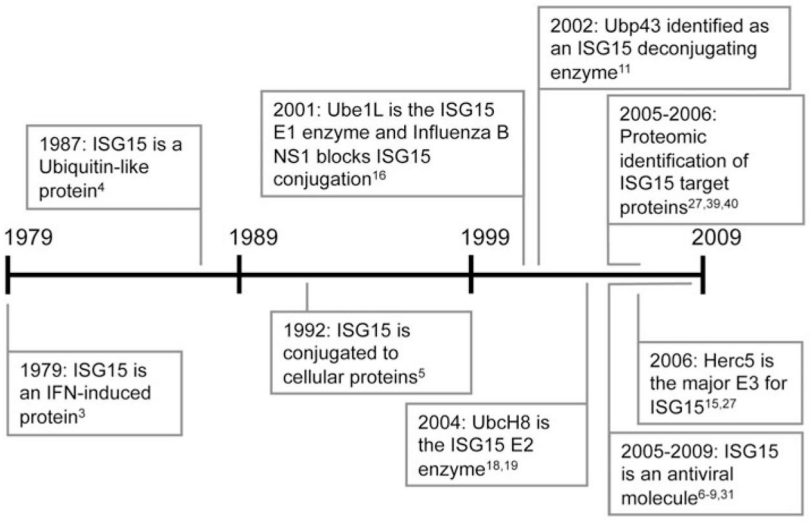

Figure 1.

Important discoveries in the ISG15 field. The timeline highlights the progress made since the discovery of ISG15 in 1979.

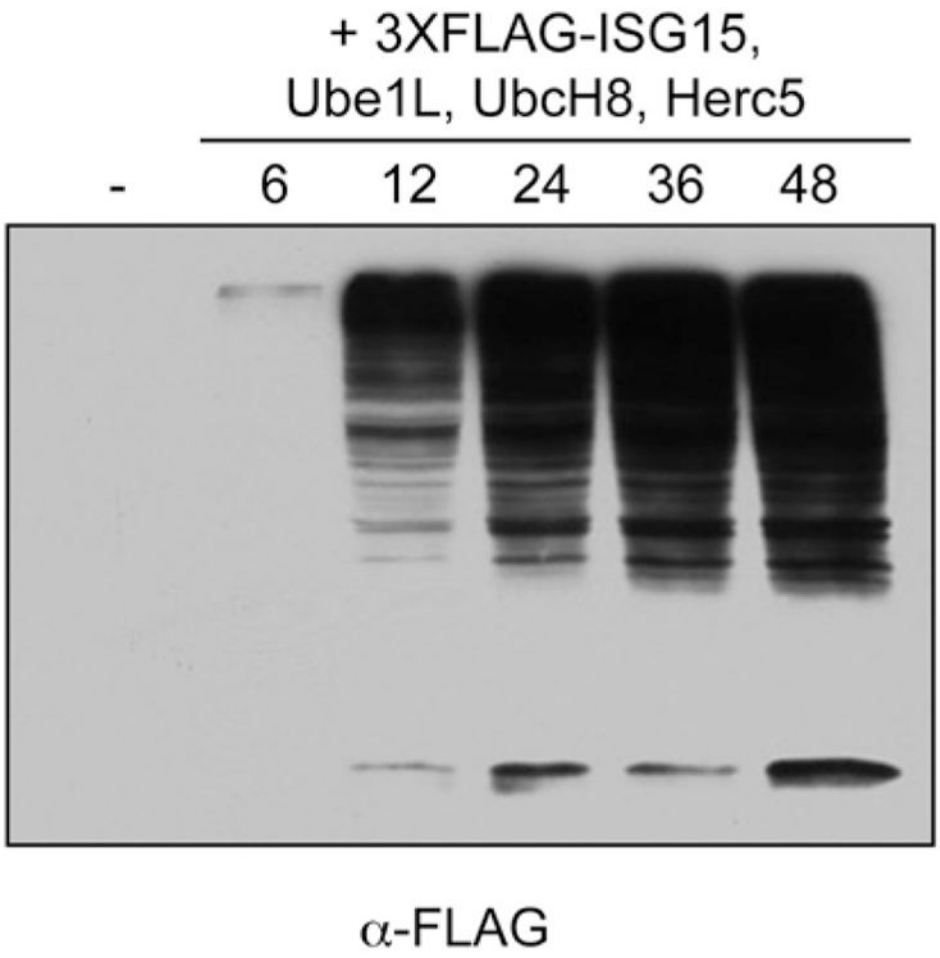

Figure 2.

Timecourse of total ISG15 conjugation in non-interferon-treated cells. HEK293T cells were transfected with plasmids expressing Ube1L, UbcH8, Herc5 and 3X-FLAG-ISG15. Cell extracts were collected at the indicated time points and analyzed by immunoblotting with anti-FLAG antibody.

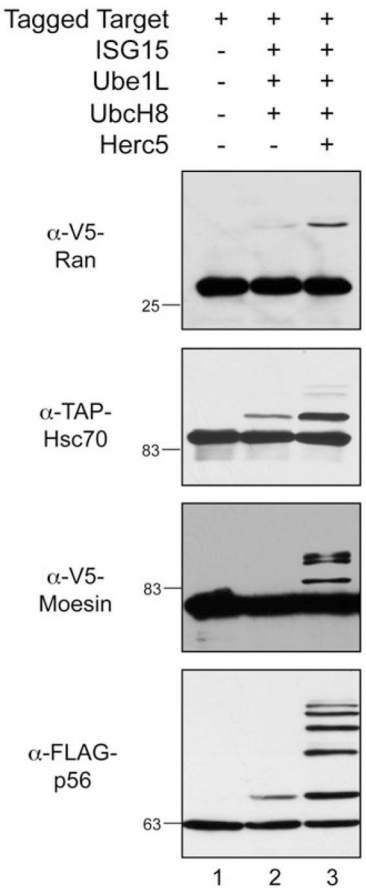

Figure 3.

ISG15 conjugation to exogenously expressed targets is enhanced by the co-expression of Herc5. HEK293T cells were transfected with a plasmid expressing an epitope-tagged target protein either alone (lane 1), or combined with Ube1L, UbcH8 and ISG15 (lane 2), or combined with Ube1L, UbcH8, ISG15 and Herc5 (lane 3). Cell extracts were prepared 48 hours posttransfection and analyzed by immunoblotting using the indicated antibodies.