Induced TRIM21 ISGylation by IFN-β enhances p62 ubiquitination to prevent its autophagosome targeting

Jie Jin, Xianbin Meng, Yi Huo and Haiteng Deng

The tripartite motif-containing protein 21 (TRIM21) plays important roles in autophagy and innate immunity. Here, we found that HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5), as an interferon-stimulated gene 15 (ISG15) E3 ligase, catalyzes the ISGylation of TRIM21 at the Lys260 and Lys279 residues. Moreover, IFN-β also induces TRIM21 ISGylation at multiple lysine residues, thereby enhancing its E3 ligase activity for K63-linkage-specific ubiquitination and resulting in increased levels of TRIM21 and p62 K63-linked ubiquitination. The K63-linked ubiquitination of p62 at Lys7 prevents its self-oligomerization and targeting to the autophagosome. Taken together, our study suggests that the ISGylation of TRIM21 plays a vital role in regulating self-oligomerization and localization of p62 in the autophagy induced by IFN-β.

INTRODUCTION

Type I interferons (IFNs) are ubiquitously expressed in host cells upon stimulation by pathogen-associated molecular patterns, such as nucleic acids and lipopolysaccharide, derived from viruses and bacteria. They are known to play versatile roles in the innate and acquired immune responses partially by inducing the expression of a series of downstream genes, known as interferon-stimulated genes (ISGs) to eliminate exogenous pathogens from the host body [1]. Interferon-stimulated gene 15 (ISG15), a highly inducible ISG, was first identified by Haas et al. [2] as a ubiquitin-like protein expressing a 17-kDa precursor protein comprising 165 amino acid residues. The phenomenon of ISGylation involves the proteolytic processing of the C-terminus of ISG15 to expose the LRLRGG hexapeptide motif prior to the conjugation of the same to its target proteins at specific lysine residues [3]. Similar to ubiquitination, ISGylation is a reversible process catalyzed by its specific E1 activating enzyme (ubiquitin-activating enzyme E1-like; UBE1L [4]), E2 conjugating enzyme (ubiquitin-conjugating enzyme H8; UBC8 [5]), and E3 ligases (HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5) [6, 7], estrogen-responsive finger protein (EFP) [8], or human homolog of Ariadne (HHARI) [9]) in a stepwise manner, while the delISGylation from its targets is mediated by ubiquitin-specific peptidase 18 (USP18) [10] reversibly. A direct relationship between ISGs and autophagy has been confirmed in a recently published study, wherein ISGylation of BECN1 induced by type I IFNs was demonstrated to be critical in regulating autophagy by competing for its K63-linked ubiquitination [11]. Inspired by this vital mechanistic insight, we aimed to identify other ISGylated proteins involved in the regulation of autophagy. Although hundreds of cellular proteins were documented as being ISGylated in two proteomic profiling studies [6, 12], the results from these studies still remain to be validated, and little is known regarding the functional regulation of these ISGylated proteins.

In the present study, we verified tripartite motif-containing protein 21 (TRIM21) as a substrate of ISGylation, which was also detected in a recent publication [6]. TRIM21 is a dual function protein that functions not only as a cytosolic Fc receptor [13, 14] sensing intracellular incoming antibody-bound viruses to initiate immune responses [15, 16], but also as an E3 ubiquitin ligase catalyzing self-ubiquitination [17] and the ubiquitination of substrates with different linkages. For instance, TRIM21 catalyzes the mono-ubiquitination of IKKβ [18], K27-linked ubiquitination of MAVS [19], and K63-linked ubiquitination of Nmi [20], p62 functions as a selective autophagy receptor responsible for selecting ubiquitin-tagged cytoplasmic components destined for lysosomal degradation. It undergoes self-oligomerization and co-aggregation with ubiquitinated substrates for autophagic cargo segregation, which is precisely regulated by post-translational modifications [21]. Coincidentally, the self-oligomerization of p62, a prerequisite for its localization to the autophagosome [22], is blocked by the TRIM21-mediated K63-linked ubiquitination of its Lys7 site [23]. Autophagy, the highly conserved intracellular pathway for recycling damaged proteins and organelles, also participates in innate and acquired immune responses, including the elimination of intracellular pathogens, inflammation control, and MHC-restricted antigen presentation during viral and bacterial infection [24, 25]. Consistently, autophagy is assumed to be activated by the nutrient-sensing pathways because of the consumption of intracellular nutrients by invading pathogens, and it is integrated with pattern recognition receptor-mediated immune-sensing pathways [24]. Furthermore, it has been recently reported that autophagy can be directly induced by type I IFNs in

© The Author(s) 2021

Cell Death and Disease (2021) 12:697; https://doi.org/10.1038/s41419-021-03989-x
performed LC-MS/MS analysis. We hypothesize that there were at least two ISGylation sites in components of the ISG15-conjugating system were co-overexpressed in HEK293 cells and TRIM21-Flag proteins were constituting the ISG15-conjugating system, including ISG15, UBCH8, HERCS, EFP, HHARI, and a subset of potential target genes reported previously [6, 12]. These potential target genes were then respectively co-transfected with ISG15, UBE1L, UBCH8, HERCS, EFP, HHARI, and a subset of potential target genes reported previously [6, 12]. The ISGylated bands were undetectable when the deISGylation enzyme USP18 was introduced into the system (Fig. 1B). To confirm whether TRIM21 is also ISGylated under physiological conditions, we treated human non-small-cell lung carcinoma A549 cells with IFN-β or IFNA2 and found that the expression of all the studied components of the ISG15-conjugating system were highly elevated, and the two ISGylated bands of TRIM21 were evident (Figs. 1C, D and S2). Taken together, our results demonstrated that TRIM21 is a substrate of ISGylation. The lower ISGylated TRIM21 band was also observed in untreated A549 cells (Figs. 1C, D and S2), suggesting that TRIM21 ISGylation might play other unknown biological roles in the resting state without exogenous IFN-β stimulation.

Results

TRIM21 is ISGylated by HERC5

To identify new substrates of ISGylation, we cloned all genes encoding two ISGylation sites in TRIM21 and that mutation of these residues indicated that Lys260 and Lys279 residues serve as the major ISGylation sites in TRIM21 and that mutation of these residues results in ISGylation on other Lys residues. Considering that the main inducible ISGylation band of TRIM21 by IFN-β was the top band (Fig. 1C, D), we chose the TRIM21(5KR) mutant for subsequent functional experiments.

ISGylation upregulates the K63-linkage-specific E3 ligase activity of TRIM21

TRIM21 belongs to the RING finger domain containing E3 ubiquitin ligase family and cooperates with numerous E2 ubiquitin-conjugating enzymes, such as UbcH5B, Ube2W, and Ube2N/Ube2V2 heterodimer, to modify itself with mono-ubiquitination and K48- or K63-linked ubiquitination sequentially [17, 31]. To validate these results, we co-transfected Flag-tagged TRIM21 with HA-tagged ubiquitin (WT) or ubiquitin mutants, denoted as K48R in which Lys48 was mutated to Arg, K63R in which Lys63 was mutated to Arg, AKR in which all lysine residues were mutated to arginine, K48O (K48 Only) in which all lysine residues except Lys48 were mutated to arginine, and K63O (K63 Only) in which all lysine residues except Lys63 were mutated to arginine. The results showed that self-ubiquitination of TRIM21 was weaker in the K48R and K63R groups than that in the WT group, and stronger in the K48O and K63O groups than that in the AKR group (Fig. 4A). The findings strongly suggest that TRIM21 catalyzes K48- and K63-linked self-ubiquitination.

Previous reports suggested that ISGylation affects the biological function of targets by competing for K63-linked ubiquitination (Becn1 [11]), or enhancing their E3 ubiquitin ligase enzyme activity (CHIP [32] and parkin [33]). To investigate whether ISGylation increases or reduces the enzymatic activity of TRIM21, we constructed an artificial ISG15-fused TRIM21 by adding an ISG15 mutant with a Gly (G) deletion in the LRLRGG hexapeptide motif to the N-terminus of TRIM21 (Fig. 4B) as previously reported [11, 32]. Interestingly, this ISG15-fused TRIM21, simulating the ISGylation of TRIM21, significantly increased K63-linked, but not K48-linked, self-ubiquitination of TRIM21 (Fig. 4C) in the co-transfection and ubiquitination assays. Additionally, co-expression of the ISG15-conjugating system with TRIM21, which led to the ISGylation of TRIM21, also increased K63-linked ubiquitination of TRIM21 (Fig. 4D). These data collaboratively demonstrate that ISGylation of TRIM21 increases the K63-linkage-specific E3 ligase activity of TRIM21.

IFN-β-induced TRIM21 ISGylation promotes K63-linked ubiquitination of p62

Given that ISGylation stimulates K63-linkage-specific E3 ligase activity of TRIM21, we next investigated whether ISGylation affects

Identification of Lys260 and Lys279 in TRIM21 as the major ISGylation sites

Unlike ubiquitin, only one ISG15 molecule is conjugated to the lysine residue in the target proteins [30]. We identified two ISGylated bands of TRIM21 from HEK293 cells in which the ISG15-conjugating system was overexpressed (Fig. 1A, B) or from IFN-β-treated A549 cells (Fig. 1C, D). These findings allowed us to hypothesize that there were at least two ISGylation sites in TRIM21. To determine the ISGylation sites in TRIM21, we performed LC-MS/MS analysis. Briefly, the TRIM21-Flag and components of the ISG15-conjugating system were co-precipitated in HEK293 cells and TRIM21-Flag proteins were immunoprecipitated with anti-Flag affinity gel and then separated by SDS-PAGE, followed by in-gel digestion with trypsin and LC-MS/MS analysis. Our analysis revealed that TRIM21 was ISGylated at the Lys260 site (Fig. 3A) in the bottom band and at both Lys260 and Lys279 in the top band (Fig. 3B, C). To confirm that Lys260 and Lys279 truly serve as functional ISGylation sites, we mutated these two Lys (K) residues to Arg (R) residues and found that those two ISGylated bands were still visible (Fig. 3D). Moreover, as expected, the top band was evidently lighter in the TRIM21(2KR) mutant than in the wild-type TRIM21 (Fig. 3D). Considering that neighboring Lys residues can be alternatively ISGylated [11, 30], we generated a quintuple mutant TRIM21(5KR) by mutating the additional three lysine residues Lys214, Lys217, and Lys280 in addition to Lys260 and Lys279. The ISGylation assay indicated that the top band, but not the bottom band, disappeared in the TRIM21(5KR) mutant (Fig. 3D). To identify other potential ISGylation sites, the bottom band of the ISGylated TRIM21(5KR) mutant was subjected to in-gel digestion and LC-MS/MS analysis. Subsequent data interpretation identified nine additional ISGylation sites (Fig. S3A–I). However, mutation of all nine Lys residues in TRIM21(5KR) prohibited the protein expression (data not shown). Taken together, our data indicate that Lys260 and Lys279 residues serve as the major ISGylation sites in TRIM21 and that mutation of these residues results in ISGylation on other Lys residues. Considering that the main inducible ISGylation band of TRIM21 by IFN-β was the top band (Fig. 1C, D), we chose the TRIM21(5KR) mutant for subsequent functional experiments.
Fig. 1 TRIM21 can be ISGylated in HEK293 and A549 cells. 

A) TRIM21-Flag, UBE1L-Myc (E1), and UBCH8-Myc (E2) were overexpressed together with HA-ISG15(GG) or HA-ISG15(AA) in HEK293 cells. Cells were collected and lysed 24 h after transfection, and cell lysates were incubated with mouse Flag antibodies and protein A/G agarose at 4 °C for 4 h or overnight, followed by western blot with HA and TRIM21 antibodies. Cell lysates were also detected with Flag, Myc, and HA antibodies.

B) TRIM21-Flag, UBE1L-Myc (E1), UBCH8-Myc (E2), and HA-ISG15 were co-expressed with or without USP18-Myc in HEK293 cells. Cell lysates were subjected to immunoprecipitation with mouse Flag antibodies. The immunoprecipitates and the lysates were analyzed with TRIM21, Flag, Myc, and HA antibodies.

C) Flag-ISG15 overexpressing A549 cells were treated with 2000 unit/ml IFN-β for 48 h. ISGylated proteins were pulled down by mouse Flag antibodies and detected by TRIM21 antibodies. Cell lysates were subjected to western blot with Flag antibodies.

D) A549 cells treated with 2000 unit/ml IFN-β for 48 h were lysed and immunoprecipitated with ISG15 antibodies, followed by immunoblotting with TRIM21 antibodies. Cell lysates were analyzed with ISG15 antibodies. Data shown are representative of three independent experiments.
the K63-linked ubiquitination levels of other substrates of TRIM21. Three proteins, Nmi [20], Keratin17 [34], and p62 [23], have been documented to be modified by TRIM21-mediated K63-linked ubiquitination under different physiological conditions. Among them, p62 is the only protein that participates in autophagy, a physiological process that was recently reported to be induced by type I IFNs [26–28]. Inspired by the mechanistic studies of ISGylation of BECN1 [11] and CHIP [32], we hypothesized that ISGylation of TRIM21 regulates the autophagy pathway by promoting the K63-linked ubiquitination of p62.

To investigate the proposed hypothesis, we generated a TRIM21−/− A549 cell line using CRISPR/Cas9 technology with a

Fig. 2 HERC5 catalyzes the ISGylation of TRIM21. A TRIM21-Flag was co-expressed with HHARI-Myc, EFP-Myc, or HERC5-Myc in HEK293 cells respectively. Cell lysates were immunoprecipitated with mouse control IgG or mouse Flag antibodies, and the immunoprecipitates were subjected to western blot with rabbit Flag antibodies and mouse Myc antibodies. Cell lysates were also subject to western blot to detect target proteins. B TRIM21-Flag, as well as UBE1L-Myc (E1), UBCH8-Myc (E2), and HA-ISG15 were co-transfected with HHARI-Myc, EFP-Myc, or HERC5-Myc into HEK293 cells. Cell lysates were subjected to immunoprecipitation with mouse Flag antibodies, followed by immunoblotting with HA antibodies to detect the ISGylated TRIM21 bands. Cell lysates were analyzed with Myc antibodies. C, D A549 cells stably transfecting with shControl, shHHARI, shEFP, and shHERC5 were treated with or without IFN-β for 24 h. Cells were collected and lysed with RIPA buffer and subjected to immunoblotting with indicated antibodies (C), or immunoprecipitation with ISG15 antibodies, followed by immunoblotting with TRIM21 antibodies (D). Data shown are representative of three independent experiments.
linked ubiquitination of p62 was significantly increased in TRIM21/+/−, but not TRIM21−/− A549 cells 24 h after IFN-β stimulation (Fig. 5D). Furthermore, it cannot be ruled out that the enhancement of K63-linked ubiquitination of p62 was caused by increased TRIM21 expression. To further assess the function of TRIM21 ISGylation in catalyzing K63-linked ubiquitination of p62, an empty vector and vector carrying wild-type TRIM21 or TRIM21 (5KR) mutant were reintroduced into TRIM21−/− A549 cells. These A549 cells were then stimulated with IFN-β for varying timepoints and harvested for endogenous denaturing immunoprecipitation assay. The results demonstrated that the K63-linked ubiquitination of p62 was obviously upregulated upon IFN-β stimulation only in TRIM21−/− A549 cells stably expressing wild-type TRIM21, but not in the empty vector or TRIM21 (5KR) mutant (Fig. 5E). Additionally, in TRIM21−/− A549 cells stably expressing TRIM21 (K279R) mutant, which blocked most multi-ISGylation (top band) on TRIM21 (Fig. 3D), the upregulation of p62 K63-linked ubiquitination was evidently abolished as compared to TRIM21−/− A549 cells stably expressing wild-type TRIM21 (Fig. 5F). Taken together, our data suggest that ISGylated TRIM21 indeed promotes the K63-linked ubiquitination level of p62 due to its enhanced K63-linkage-specific E3 ligase activity.

Fig. 3 Identification of ISGylation sites in TRIM21. A–C TRIM21-Flag, as well as the ISG15-conjugating system were overexpressed in HEK293 cells. Cell lysates were pulled down with anti-Flag affinity c-gel, and then subjected to SDS-PAGE separation and in-gel digestion. Iodoacetamide was used in in-gel digestion to avoid false positive result. LC-MS/MS analysis identified Lys260 as original ISGylation sites in the bottom ISGylated TRIM21 band (A), and Lys260, Lys279 as original ISGylation sites in the top ISGylated TRIM21 band (B, C). D HEK293 cells were transfected for 24 h with ISG15-conjugating system and TRIM21 or indicated TRIM21 mutants, K260R, K279R, 2KR (K260/279R), 3KR (K260/279/280R), and 5KR (K214/217/260/279/280R). Cell lysates were subjected to anti-Flag pull-down, followed by immunoblotting with anti-HA and anti-Flag antibody. Data shown are representative of three independent experiments.

As mentioned above, autophagy, as well as ISGylation, was found to be induced by type I IFNs recently [3, 26–28]. However, the relationship between them remains to be fully investigated. p62, a well-known autophagy selective receptor, was demonstrated to be modified by ISGylated TRIM21-catalyzed K63-linked ubiquitination (Fig. 5D, E), which has been shown to block its self-oligomerization.
Since self-oligomerization is required for p62 targeting to the autophagosome formation site [22], p62 should be sequestered from the autophagosome by ISGylated TRIM21 upon stimulation by IFN-β. To verify our results, we constructed mCherry-LC3 overexpressing A549 cell lines with lentiviruses and performed immunofluorescence staining and imaging experiments with p62 antibody to examine the co-localization of p62 puncta and mCherry-LC3-labeled autophagosomes. Compared to TRIM21+/− A549 cells, IFN-β treatment led to significant increases in the numbers of co-localized puncta of p62 and autophagosomes (Figs. 6A, C and S7C). Consistent with these findings, reconstitution of wild-type TRIM21, but not the TRIM21(5KR) mutant, led to diminished co-location of the puncta of p62 and autophagosomes (Figs. 6B, D and S7D). Together, these results demonstrate that ISGylation of TRIM21 induced by IFN-β plays a vital role in p62 oligomerization and autophagosome targeting by catalyzing its K63-linked ubiquitination.

DISCUSSION
In this study, we demonstrated that the E3 ubiquitin ligase TRIM21 is a substrate of ISGylation, which is catalyzed by E3 ISG15 ligase HERCS and deISGylated by USP18. Surprisingly, the ISGylated TRIM21 bands were detectable, irrespective of the treatment of A549 cells with IFN-β. The difference is that only the top band, but not the bottom band, was inducible by IFN-β stimulation. This result indicates that mono-ISGylation of TRIM21 plays more universal and as yet unknown roles in unidentified physiological processes. In the HEK293 overexpression system, we identified two major ISGylation sites, Lys260 and Lys279, and a subset of alternative sites that were ISGylated when Lys260 and Lys279 were mutated to arginine, using immunoprecipitation coupled with LC-MS/MS analysis. Consistent with the results of recent studies [11, 30], mutations in the major ISGylation sites, Lys260 and Lys279, as well as neighboring lysine residues, Lys214, Lys217, and Lys280, blocked the formation of the top ISGylation band of TRIM21. Furthermore, mutation of these five lysine residues to arginine prevents the formation of the multi-ISGylated TRIM21(5KR) in the top band, resulting in the marked increase of mono-ISGylated TRIM21(5KR) in the low band.

Similar to the cases of CHIP [32] and parkin [33], we found that the E3 ubiquitin ligase activity of TRIM21 was also enhanced by ISGylation. However, unlike ISGylation of CHIP [32] and parkin [33], TRIM21 ISGylation enhanced its activity for K63-linked ubiquitination, but not for K48-linked ubiquitination. Furthermore, ISGylation of TRIM21 induced by IFN-β also led to enhanced K63-linkage-specific E3 ligase activity in A549 cells, which increased the K63-linked ubiquitination levels of TRIM21 and p62. It was recently reported that TRIM21 maintains its E3 ligase activity at a low level
through B-Box domain-mediated auto-inhibition at resting state, and can be activated by phosphorylation at Ser80 upon stimulation [37]. Thus it is rational to hypothesize that ISGylation of TRIM21 induced by IFN-β stimulation has similar effects as that of Ser80 phosphorylation of TRIM21.

TRIM21-mediated K63-linked ubiquitination of p62 at Lys7 has been reported to inhibit its self-oligomerization [23], which is essential for its localization to autophagosome [22]. Our findings suggest that ISGylated TRIM21 blocks p62 self-oligomerization and subsequent packaging by autophagosomes in IFN-β-induced autophagy. Consistent with our findings, more and larger p62 puncta were formed and localized to autophagosomes 24 h after IFN-β treatment in TRIM21−/− A549 cells than in the TRIM21+/+ A549 cells (Figs. 6A, C and S7C). Additionally, reconstitution of wild-type TRIM21, but not the TRIM21(5KR) mutant, into TRIM21−/− A549 cells reduced the number and size of co-localized p62 puncta in autophagosomes 24 h after IFN-β treatment (Figs. 6B, D and S7D). Nonetheless, there seemed to be no significant differences in the numbers of total LC3B puncta between IFN-β-treated TRIM21+/+ and TRIM21−/− A549 cells (Fig. S7A) or TRIM21−/− A549 cells stably expressing empty vector, wild-type TRIM21, and TRIM21(5KR) mutant (Fig. S7B).

It has recently been amply documented that TRIM21 functions as a cytosolic Fc receptor sensing incoming antibody-coated viruses and then inducing the expression of type I IFNs through immune signaling as the first wave immune response [16]. With the help of AAA ATPase p97/VCP, TRIM21 mediates the proteasomal degradation of viral capsid and antibody by...
Fig. 6  IFN-β-induced TRIM21 ISGylation prevents p62 oligomerization and autophagosome targeting. A TRIM21+/+ and TRIM21−/− A549 cells were untreated or treated with IFN-β for 24 h. Cells were fixed, permeabilized, and then subjected to staining with anti-p62 antibody (green). Co-localization of p62 puncta and mCherry-LC3 labeled autophagosomes (red) were observed under Andor Dragonfly confocal microscope. B TRIM21−/− A549 cells stably expressing VECTOR, TRIM21, or TRIM21(5KR) mutant were untreated or treated with IFN-β for 24 h. Cells were subjected to staining with anti-p62 antibody and observed under confocal microscope as A. C, D p62 puncta in TRIM21+/+ and TRIM21−/− A549 cells (C) or TRIM21−/− A549 cells stably expressing VECTOR, TRIM21, or TRIM21(5KR) mutant (D) untreated or treated with IFN-β for 24 h were quantified with Imaris. Data shown were the statistical result of 25 cells for each cell line in 28 independent experiments plotted with GraphPad Prism 5. Data were analyzed with Student’s t test and represented as mean ± standard error of the mean (n.s. not significant. ***P < 0.001). E A working model for regulation of p62 self-oligomerization and autophagosome targeting by TRIM21 ISGylation. Induction of TRIM21 ISGylation by IFN-β activates TRIM21 to catalyze K63-linked ubiquitination of itself and p62, which results in blocking of p62 self-oligomerization and subsequent localizing to autophagosome formation site.
catalyzing autoubiquitination [38, 39]. In addition, TRIM21 catalyzes not only the K27-linked ubiquitination of MAVS [19] and K63-linked ubiquitination of Nmi [20] to regulate RIG-I signaling, but also the mono-ubiquitination of IKKβ [18, 40] and K48-linked ubiquitination of IRF3 [41] to regulate the activation of transcription factors NF-κB and IRF3. In facilitating the proteasomal degradation of viral capsid and antibody, TRIM21 itself is also degraded by proteasome. We propose that the upregulation of TRIM21 induced by type I IFNs may function as a complementary mechanism to maintain the antiviral responses.

In summary, the current study provides a novel regulatory mechanism of p62 oligomerization and autophagosome targeting in autophagy induced by IFN-β via TRIM21 ISGylation in human non-small-cell lung carcinoma A549 cells.

MATERIALS AND METHODS

Cell culture and transfection

HEK293 and A549 cells were cultured in DMEM (WISENT INC., 319-005-CL) and RPMI 1640 (WISENT INC., 350-600-CL) respectively containing 10% FBS (PAN-Biotec, P30-3302) and 1% streptomycin-penicillin (WISENT INC., 450-201-s) at 37 °C in 5% CO2. HEK293 and A549 cells used were purchased from the global bioresource center ATCC and kept in our laboratory. DNA constructs were transiently transfected into HEK293 cells with polyethylenimine (PEI) (Sigma-Aldrich, 9002-98-6) and A549 cells with Lipofectamine 3000 (Invitrogen, L3000-015).

Co-immunoprecipitation and western blot assays

Cells were harvested from 10 cm dishes 24 h after transfection and lysed with 500 μl Western & IP lysis buffer (Beyotime, P0013) containing 1× PMSF (Solarbio, P0100), protease inhibitor cocktail (bimake, B14002) and phosphatase inhibitor cocktail (bimake, B15001). Supernant cell lysates (400 μl) was taken to mix with 1 μg of control IgG or specific antibody and protein A/G agarose (Thermo Scientific, 20422) in a new 1.5 ml tube for immunoprecipitation. The mixture was rotated for 4 h or overnight at 4 °C, followed by centrifugation at 4000 rpm for 2 min and washed for five times with Western & IP lysis buffer. The immunoprecipitates and the rest cell lysates (100 μl) were processed for western blot analysis by boiling at 98 °C for 10 min in equal volume 2× sample loading buffer. The samples were subjected to SDS-PAGE and then transferred to 0.45 μm nitrocellulose (NC) membrane (Merck millipore, HATF00010). NC membranes were blocked in 5% non-fat milk prepared with Tris-buffered saline with Tween 20 (TBST) for 1 h, and incubated with corresponding primary antibody, second HRP-linked IgG in 5% non-fat milk for 2 h each successively. After each incubation, NC membranes were washed five times with TBST for 5 min each. Target proteins were detected with ECL reagent (Bio-rad, 1705061) and photographed by ChemiDoc™ system (Bio-Rad, 1708265).

RNA extraction and qRT-PCR

Total RNA of A549 cells was extracted with TRNzol Universal Reagent (TIANGEN, DP424), reverse transcribed by using 5× EasyQuick RT MasterMix (CWBio, CW2020M), and amplified and detected by LightCycler® (Roche) with 2× UltraSYBR Mixture (CWBio, CW0957M) in a final reaction volume of 10 μl. qRT-PCR primers were designed using Primer3 website, and the primer sequences for p62 were 5′-CACCTGTCAGGGCTTCCT-3′ and 5′-CACACTCCTCCAAAACTG-3′. Data were normalized by actin mRNA levels using primers, 5′-GGACTCTGCGAAGAGATG-3′ and 5′-AGCAC TGGTGGGCGGTAC-3′.

Statistical analysis

Statistical analysis acquired in qRT-PCR and cell imaging are analyzed with Two-way ANOVA and Student’s t test by using GraphPad Prism 5, and are presented as mean ± standard error of the mean (S.E.M). P < 0.05 were considered statistically significant.

Ubiquitination assays

Ubiquitination assays were performed in the denaturing RIPA lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium pyrophosphate, 0.1% SDS) (Beyotime, P0013K) and protease inhibitor cocktail (bimake, B14002), with appropriate antibody to avoid disturbance by the ubiquitination of non-covalently interacting proteins. Unlike Co-Immunoprecipitation, the immunoprecipitates were washed for three times with RIPA lysis buffer. The saved cell lysates and the immunoprecipitates were subjected to western blot analysis to detect the target protein and its ubiquitination.

Generation of TRIM21 KO A549 cells

gRNA targeting TRIM21 (sgTRIM21 F: 5′-caccgCATGTGTGCTGTCG ATGAT-3′, sgTRIM21 R: 5′-aaccATCGACGACGTCAGAACATG-3′) were synthesized, annealed and cloned into the px458 vector, which containing CRISPR/Cas9 and GFP encoding sequences. The px458-sgTRIM21 vector was transiently transfected into A549 cells with Lipofectamine 3000 and single clones were sorted into 96 wells plates by flow cytometry 24 h later when GFP was already expressed in the cells. Once the sorted cells were expanded into 24-wells plates, half of the cells were subjected to genome DNA extraction with TIANamp Genomic DNA Kit (TIANGEN, DP304-02), and amplification by PCR with identification primers (iTRIM21 F: 5′-CAGCGAAAACCTCCTAAAGGT-3′, iTRIM21 R: 5′-AGAGGTTGCTCTT TCCATT-3′). The PCR products were identified by DNA sequencing to choose genome edited clones.

Construction of stable cell lines with lentivirus

pLVX-IRES-ZsGreen1 empty vector or vectors encoding genes of interest, together with packaging system VSV-G, pRRE and RSV-Rev were co-transfected into HEK293 cells with PEI. The medium was replaced by fresh medium with 10% FBS and 1% streptomycin-penicillin six hours later. The supernatants were harvested and concentrated with PEG6000 48 h later to infect A549 cells in the presence of 10 μg/ml polybrene.

LC-MS/MS analysis

Flag-TRIM21 along with ISG15-conjugating system overexpressed in HEK293 cells was immunoprecipitated with anti-Flag affinity gel (bimake, B23102) 24 h after transfection and subjected to SDS-PAGE separation and Coomassie Blue staining. Target ISGylated bands were taken for in-gel digestion with trypsin according to a standard protocol, followed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis on Thermo Scientific™ Orbitrap Fusion™ coupled with EASY-nLC™ 1200. The MS/MS spectra of modified peptides were acquired by searching the raw data with Proteome Discoverer Program (Version 1.4).

Immunofluorescence staining and imaging

A549 cells seeded on glass bottom culture dishes (φ15mm, TC) (NEST, 802000) were fixed with 4% formaldehyde for 10 min. Next, the cells were permeabilized in 0.5% saponin for 10 min and blocked in 5% goat serum for 1 h. The glass bottoms were covered by anti-p62 primary rabbit antibody in 1% BSA and stained with Alexa Fluor 647 linked goat anti-rabbit secondary antibodies subsequently. The cells were washed for three times 5 min each with PBS between each steps. Imaging was performed on Andor Dragonfly turntable confocal microscope. p62 puncta in different cell lines untreated or treated with IFN-β were quantified with Imaris.

Antibody and reagents

Mouse control IgG (CST, 3900 S), HRP-linked horse anti-mouse IgG (CST, 7076 S) (1:2000), HRP-linked goat anti-rabbit IgG (CST,7074 S) (1:2000), Goat anti-rabbit IgG/Alexa Fluor 647 (Bioss, bs-0295G-AF647), rabbit anti-HA (CST, 3724 S) (1:1000), rabbit anti-Flag (CST, 2368 S) (1:1000), mouse anti-Flag (EASYBIO, BE2004-100), mouse anti-MyC (CST, 2276 S) (1:1000), rabbit anti-TRIM21 (proteintech, 12108-1-AP) (1:1000), rabbit anti-UBE1L (proteintech, 15818-1-AP) (1:1000), rabbit anti-LC3 (proteintech, 17278-1-AP) (1:1000), rabbit anti-HERCS (GeneTec, GTX130167) (1:1000), mouse anti-HHARI (Santa Cruz biotechnology, sc-390763) (1:1000), rabbit anti-ISG15 (Santa Cruz biotechnology, sc-50366) (1:1000), rabbit anti-actin (ABclonal, AC004) (1:1000), rabbit anti-p62 (CST, 5114 S) (1:1000), rabbit Anti-Ubiquitin, Lys63-Specific (Merk millipore, 05-1208) (1:1000), Bafilomycin A1 (Selleck, S1413), PNA2 (Sino Biological, 13833-HNAS), and IFN-β (Sino Biological, 10704-HNAS) were purchased from the corresponding manufactures.

Short hairpin RNAs

The short hairpin RNA (shRNA) lentiviral plasmids pLKO-shControl, pLKO-shHHARI, pLKO-shEFP, and pLKO-shHERC5 were purchased from Custom Glycerol-human shRNA library Facility, Tsinghua University. The appropriate lentiviral plasmid together with packaging vectors VSVG, pRRE, and RSV-Rev

Cell Death and Disease (2021) 12:697
were co-transfected into HEK293 cells to obtain lentiviruses. Packaged viruses were then used to infect A549 cells in the presence of polybrene (10 μg/ml) and then selected with puromycin (1 μg/ml) for 2 weeks. Control vector expressed shRNA targeting TurboGFP. The sequences of shRNAs were listed below. shHAR: 5'-CCGCGTACCTGGAGAAGATGATGCTGATTTGACATACGTTCTCAAGGTAATCTTGGT-3'; shEP: 5'-CCGCCGGAACGATCTGATGTTACTCTCATAATGTCATGCGGCAACCTTGTCATAACTTGGAGAAGATGATGCTGATTTGACATACGTTCTCAAGGTAATCTTGGT-3'. shHERCS: 5'-CCGGATGGCCACCTTGTCATAACTTGGAGAAGATGATGCTGATTTGACATACGTTCTCAAGGTAATCTTGGT-3'.

**REFERENCES**

1. McNab F, Mayer-Barber K, Sher A, Wack A, O’Garra A. Type I interferons in infectious disease. Nat Rev Immunol. 2015;15:87–103.

2. Haas AL, Ahrens P, Bright PM, Ankel H. Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. J Biol Chem. 1987;262:11315–23.

3. Han HG, Moon HW, Jeon YJ. ISG15 in cancer: beyond ubiquitin-like protein.

4. Yuan W, Krug RM. In...
FUNDING
This work was funded by the National Key Research and Development Program of China (Grant 2017YFA0505103), NSFC (21877068), and the Chinese Ministry of Science and Technology (2020YFC2002705).

COMPETING INTERESTS
The authors declare no competing interests.

ETHICAL APPROVAL
This study was approved by the ethical review board of School of Life Sciences, Tsinghua University. There were no animal models or human subjects involved in this study.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41419-021-03989-x.

Correspondence and requests for materials should be addressed to H.D.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021