

Supporting Information for

USP16 is an ISG15 cross-reactive deubiquitinase that targets pro-ISG15 and ISGylated proteins involved in metabolism

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SI Methods.

Cells and Cell Culture

Human HEK293T (Cat# ATCC® CRL-3216™) and HeLa (Cat# ATCC® CCL-2™) cells were cultured under standard conditions in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 8% FCS (Biowest) and 1% penicillin/streptomycin at 37 °C and 5% CO₂. Chronic myelogenous leukemia (CML)-derived HAP1 WT (Horizon #C631), HAP1 USP18KO (Horizon #HZGHC000492C011) (1) and HAP1 USP16KO cells (self-made based HAP1 WT) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 8% FCS (Biowest), 1% penicillin/streptomycin at 37 °C and 5% CO₂. All cell lines were tested for mycoplasma contamination using MycoAlert™ Mycoplasma Detection Kit (Lonza, Catalog #: LT07-318) on a monthly basis.

Gene knockdown by RNA interference

For siRNA transfections, non-targeting siRNA control pools (#D-001206-13-05) and USP16 siRNA oligos (#MQ-006067-01-0002) were purchased from Dharmacon, including siUSP16#1 (Cat# D-006067-01), siUSP16#2 (Cat# D-006067-02), siUSP16#3 (Cat# D-006067-03). Silencing was performed in HAP1 WT cells as follows: for the 6-well plate format, 200 µL siRNA (500 nM stock) were incubated with 4 µL Dharmafect reagent 1 (Dharmacon) diluted in 200 µL medium without supplements (total volume of 200 µL transfection mix) with gentle shaking for 20 min at room temperature (RT). A total of 80,000 cells resuspended in 1.6 mL of growth medium were added to transfection mixes to a final volume of 2 mL per well and cultured for 3 days prior to further analysis.

DNA Transfection

HEK293T cells were seeded to achieve 50–60% confluence and transfected using PEI (polyethylenimine, Polysciences Inc., Cat# 23966) as follows: 200 µL DMEM medium without supplements was mixed with DNA and PEI (1 mg/mL) at a ratio of 1:3 (eg: 1 µg DNA : 3 µL PEI), incubated at RT for 20 min, added drop-wise to the cells and incubated for 24 h prior to further analysis. Overexpression constructs are listed in Table S2.

Stimulation of cells

Cells were treated with 1000 U/mL of recombinant human IFN-β (PeproTech #300-02BC) or recombinant human IFN-α2 (PBL Assay Science #11105-1) for the indicated times.

Generation of HAP1 USP16KO cells

HAP1 USP16 KO cell lines were generated using CRISPR/Cas9. The guide RNA sequences targeting exon 4 (USP16 gRNA #B: 5'-CACCGTATTGTCTAGTCTTACAGTCT-3' and 5'-AAACAGACTGTAAAGACTGACAATAC) or exon 6 (USP16 gRNA #A: 5'-CACCGAATCAACCACTTGACCCAAAC-3' and 5'-AAACGTTGGGTCAAGTGGTTGATTC-3') were used as before (2). Annealed gRNAs were ligated into the lentiCRISPR_v2 vector (3). HAP1 WT cells were transfected with gRNA-annealed lentiCRISPR_v2 vectors using PEI reagent and selected with puromycin at 1 µg/mL for 3 days. Individual clones were expanded and screened for mutations in the USP16 gene by PCR and immunoblotting. PCR products were sequenced and analysed for indel mutations using the ICE CRISPR Analysis Tool from Synthego (<https://ice.synthego.com/#/>) (4) and by manual inspection of the sequencing profiles with Snapgene.

Real-time Quantitative RT-PCR

The mRNA level of endogenous USP18 and ISG15 was assessed by RT-qPCR (5). In brief, total RNAs were extracted using the NucleoSpin RNA II kit (MACHEREY-NAGEL) following the manufacturer's instructions. 1 µg of total RNA were reverse transcribed using RevertAid First Stand cDNA synthesis Kits (Thermo Fisher). Real-time quantitative PCR experiments were performed using SYBR Green (Promega) in a CFX connect Real-Time PCR detection system (Bio-Rad). All values for target gene expression were normalized to GAPDH. All experiments were repeated independently at least three times. Primers used for RT-qPCR were as follows: USP18 Forward primer CCTGAGGCAAATCTGTCAGTC, USP18 Reverse primer

CGAACACCTGAATCAAGGAGTTA, ISG15 Forward primer CTCATCTTTGCCAGTACAGGAG, ISG15 Reverse primer CCAGCATCTTCACCGTCAG, GAPDH Forward primer TGCACCACCAACTGCTTAGC and GAPDH Reverse primer CTCATGACCACAGTCCATGCC.

ISG15-PA, Ub-PA and M20-PA probe labeling assays

The Biotin / Rhodamine-tagged human C terminal domain hISG15_{CTD}-PA, Rhodamine-tagged mouse C terminal domain mISG15_{CTD}-PA, Biotin-tagged mouse full-length ISG15-PA, Rhodamine-tagged mouse full-length ISG15-PA, Ub-PA, and Rhodamine-tagged M20-PA probe came from previously prepared stocks (1, 6-9).

For labelling of cell lysates, cell pellets were resuspended in HR buffer (50 mM Tris-HCl, 5 mM MgCl₂, 250 mM sucrose, 2 mM TCEP and protease inhibitor t (Roche), pH 7.4), and lysed by sonication (Bioruptor, Diagenode, high intensity for 10 minutes with an ON/OFF cycle of 30 seconds) at 4°C. In SDS-PAGE or immunoblot assays, 25-40 µg of clarified cell lysate in 20 µL was labelled with the indicated probe (final concentration 1 µM) at 37 °C for 30 min. Reactions were stopped by the addition of LDS (lithium dodecyl sulfate) sample buffer (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 2.5% β-mercaptoethanol, followed by boiling for 7 minutes.

For labelling of purified recombinant enzymes, the enzymes were diluted in assay buffer containing 50 mM Tris-HCl, 100 mM NaCl, 0.5 mg/mL CHAPS and 5 mM TCEP, pH 7.6, to a final concentration of 5 µM. Pure ISG15-PA, and Ub-PA probes were added at a 1:1 molar ratio and incubated for up to 60 min at room temperature. Reactions were stopped by boiling with LDS sample buffer as described above. The recombinant enzymes are listed in Table S3.

DeISGylation assays in cell lysates

HAP1 USP18KO cells or HAP1 USP16KO#B cells were stimulated with 1000 U/mL IFN-β for 48 hours to induce ISGylation. Cell pellets were lysed in EMBO lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5 % NP40, 8 mM TCEP). Cell lysates (40 or 80 µg in 10 µl) were incubated with recombinant USP16 CD^{WT} or USP16CD^{C205S} at a final concentration of 5 µM at 37 °C for 2 hours. The reaction was stopped by boiling with LDS sample buffer as described above.

ProISG15 cleavage assays

Recombinant human pro-ISG15 protein (R&D Systems, #UL-615-500) was diluted in EMBO lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5 % NP40, 8 mM TCEP) to a final concentration of 5 µM, and incubated with 0.5 µM of the indicated enzymes at 37 °C for the indicated times. The reaction was stopped by boiling with LDS sample buffer as described above. The recombinant enzymes are listed in Table S3.

ISG15-FP and Ub-FP substrate cleavage assays

Fluorescence polarization (FP) assays were performed as reported previously (10, 11). In brief, two-fold serial dilutions (200–25 nM) of the indicated enzyme solutions (the recombinant enzymes are listed in Table S3) were prepared in assay buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 100 mM NaCl, 1 mg/mL CHAPS and 0.5 mg/mL bovine gamma globulin (BGG). Ten microliters of each of the dilution steps was added to the empty wells of a microtiter plate (Corning 3820, black, low volume 384 well microplate, LBS, round wells, flat bottom). The reaction was started by addition of 10 µL of the full-length ISG15-FP substrate (10) or the Ub-FP substrate (11) (200 nM final concentration). Fluorescence intensities in the S (parallel) and P (perpendicular) directions were recorded at intervals of 60 or 90 seconds on a BMG Labtech PHERAstar plate reader (excitation, 540 nm; emission, 590 nm). From these S and P values, the FP values (in mP) were calculated by adjustment of the FP value (L) of the tracer molecule TAMRA-KG to 50 mP:

$$\text{Polarization (mP)} = \frac{S - (G \times P)}{S + (G \times P)} \times 1000 \quad \text{where} \quad G = \frac{\text{average } S}{\text{average } P} \times \frac{1 - L/1000}{1 + L/1000}$$

Analysis of ISGylation in HEK293T cells

To analyse ISGylation on Myc-tagged substrates, ChromoTek Myc trap pulldown assays were performed (12). HEK293T cells, transfected as indicated, were lysed in 300 µL lysis buffer 1 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100,

10 mM *N*-methyl maleimide as a general DUB inhibitor diluted in DMSO, freshly added, and protease inhibitors (Roche Diagnostics, EDTA-free, freshly added). Then, 100 μ L lysis buffer 2 (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 2% SDS) were added to the crude lysates; samples were sonicated (Fisher Scientific FB120 Sonic Dismembrator, 3 pulses, amplitude 40%). SDS was then diluted by the bringing the sample volume to 1 mL with lysis buffer 1. After centrifugation (20 min, 4 °C, 20,817 g), lysates were incubated with 5 μ L Myc Trap Agarose (Chromotek) overnight at 4 °C. Beads were washed 5 times with lysis buffer. Bound proteins were released and denatured with sample buffer by heating at 95 °C for 7 min.

Electrophoresis and immunoblots

Samples were resolved on precast Bis-Tris NuPAGE Gels (Invitrogen, including 4-12%, 10% and 12% for different samples) using MOPS buffer (Invitrogen Life Technologies, Carlsbad, CA, USA). For fluorescence detection, labelled enzymes were visualized by in-gel fluorescence using a Typhoon FLA 9500 imaging system (GE Healthcare Life Sciences) (Rhodamine channel for activity-based probes, Cy5 channel for protein marker). For immunoblots, the gel was transferred to Nitrocellulose membranes. The signal was visualized using a LICOR Odyssey system. The primary antibodies used for immunoblots are listed in Table S1.

Activity-based protein profiling (ABPP) with the biotin-ISG15-PA probe

ABPP was performed as previously described (13). HAP1 cells were lysed using glass beads in lysis buffer (GBL: 50 mM Tris, pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA, and 250 mM Sucrose). Protein concentrations were determined using the Pierce BCA Assay. One mg of protein was labelled with Biotin-ISG15-PA at the pre-determined optimum ratio and incubated for 30 min (Viva Biotech Ltd) at 37 °C. The reaction was quenched by addition of 5% (w/v) SDS and 10% NP-40 to each sample. Samples were then diluted by adding NP-40 lysis buffer and NeutrAvidin Agarose beads (Thermo Fisher) were added prior incubation for 16 hours at 4 °C (with rotation). The beads were washed with NP-40 Lysis four times in total. Probe-labelled proteins were eluted from the beads by addition of LSLB with 3 mM biotin and boiled (10 min, 95 °C). Eluted materials were subjected to immunoblotting and LC/MS/MS analysis (13).

For analysis by LC/MS/MS, the eluted materials were diluted with ultra-pure water and reduced DTT (200 mM in 0.1 M Tris, pH 7.8) for 30 min at 37 °C. Samples were alkylated with iodoacetamide (100 mM in 0.1 M Tris, pH 7.8) for 15 min at room temperature in the dark. Protein samples were precipitated with methanol, chloroform, and water. Samples were centrifuged at 17,000 g for 3 min, and the resultant upper aqueous phase was discarded. Proteins were further precipitated by addition of methanol and, following a second extraction to remove remaining SDS, precipitated proteins were resuspended in 50 μ L of 6 M urea. Urea was then diluted to <1 M urea with 20 mM HEPES (pH 8.0) buffer. Trypsin digestion was carried out by adding enzyme (from a 1 mg/mL stock in 1 mM HCl) at a 1:100 ratio at room temperature overnight. Following digestion, samples were acidified with 1% trifluoroacetic acid and desalted on C18 solid-phase extraction cartridges (SEP-PAK plus, Waters), dried, and re-suspended in buffer A.

immunoprecipitation to recover the ISG15 interactome

Immunoprecipitations were performed as described (1) with the following modifications. HAP1 cells were lysed with lysis buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 0.2% NP-40, 10 % glycerol, 5 mM NEM, phosphatase and protease inhibitor cocktails) and subjected to immunoprecipitation using 5 μ g of anti-ISG15 antibody (Boston Biochem #A-380) and 25 μ L of protein G Sepharose slurry (Invitrogen; #15920-10) for 16 h at 4 °C. Beads were washed 4 times with lysis buffer. Immune complexes were eluted with 2X Laemmli sample buffer. One-tenth of the eluates was used for immunoblotting with the indicated antibodies. The remaining eluate was prepared for analysis by MS as previously described (14) using suspension traps (S-Traps). Proteins were reduced with 200 mM DTT in 0.1 M Tris pH 7.8, followed by alkylation with 200 mM iodoacetamide in 0.1 M Tris pH 7.8 in the dark. Samples were acidified by addition of 12% phosphoric acid and captured on S-TrapTM midi columns (C02-midi, ProtiFi). Columns were washed with 90% methanol in 100 mM triethylammonium bicarbonate (TAEB) by centrifugation at 4,000 g. Captured proteins were digested with trypsin (1:100 w/w) overnight at room temperature. Peptides were dried and dissolved in Buffer A (98 % MilliQ-H₂O, 2 % CH₃CN and 0.1 % TFA).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed using a Dionex Ultimate 3000 nano-ultra high-pressure reverse-phase chromatography instrument coupled on-line to a Fusion Lumos (ISG15 interactome) or a Q Exactive (ISG15 ABPP) mass spectrometer (Thermo Scientific) as described previously (13). In brief, samples were separated on an EASY-Spray PepMap RSLC C18 column (500 mm × 75 µm, 2 µm particle size, Thermo Scientific) over a 60 min (120 min in the case of the matching proteome) gradient of 2–35% acetonitrile in 5% dimethyl sulfoxide (DMSO), 0.1% formic acid at 250 nL/min. MS1 scans were acquired at a resolution of 60,000 at 200 m/z and the top 12 most abundant precursor ions were selected for fragmentation by high collision dissociation (HCD).

Data analysis

The raw MS files were used for searches against the UniProtKB human sequence data base (92,954 entries). Label-free quantitation was performed using MaxQuant Software (v1.5.5.1). Search parameters include carbamidomethyl (C) as a fixed modification, oxidation (M) and deamidation (NQ as variable modifications, maximum 2 missed cleavages, matching between runs, and LFQ quantitation was performed using unique peptides. Label-free interaction data analysis was performed using Perseus (v1.6.0.2), and volcano and scatter plots were generated using a t-test with permutation FDR = 0.01 for multiple-test correction and $s_0 = 0.1$ as cut-off parameters.

Other graphs were generated using GraphPad PRISM 8 and Excel. For statistical analysis, we applied two-way ANOVA tests, including multiple comparison testing via the Dunnett method, available through the GraphPad Prism software.

Immunoblot protein bands were quantified using Image Studio Lite Version 5.2.5. quantification Statistical evaluations applied Student's t test (two-tailed distribution) with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, NS: not significant). All error bars correspond to the mean ± S.E.M.

Gene ontology and STRING network analysis

GO analysis was performed using the GO consortium web tool (<http://geneontology.org/>). The PANTHER overrepresentation test (released 20230510) was used for the evaluation of enriched GO terms of the identified USP16-dependent ISG15 interactome proteins. Proteins were analysed for overrepresentation of PANTHER GO-Slim biological process, PANTHER GO-Slim cellular component, and PANTHER GO-Slim molecular function terms using the Fischer exact test.

Network analysis of USP16-dependent ISG15 interactome proteins (Dataset S3) was performed using the online STRING database v11.5 (<https://string-db.org/>). The following setting were applied: Output settings: medium confidence interaction score (0.4), edges show protein connections based on textmining, experiments, databases, co-expression, neighborhood, co-occurrence, and gene fusion. The network was subsequently exported as a TVS (tab separated values) file and imported into Cytoscape version 3.9.1 for further visualization and network analysis. The cytoscape plug-in MCODE (molecular complex detection) version 2.0.2 was used to identify highly connected subclusters of proteins using a degree cutoff of two, cluster finding: haircut, a node score cutoff of 0.2, a K-core of two and a max depth of 100.

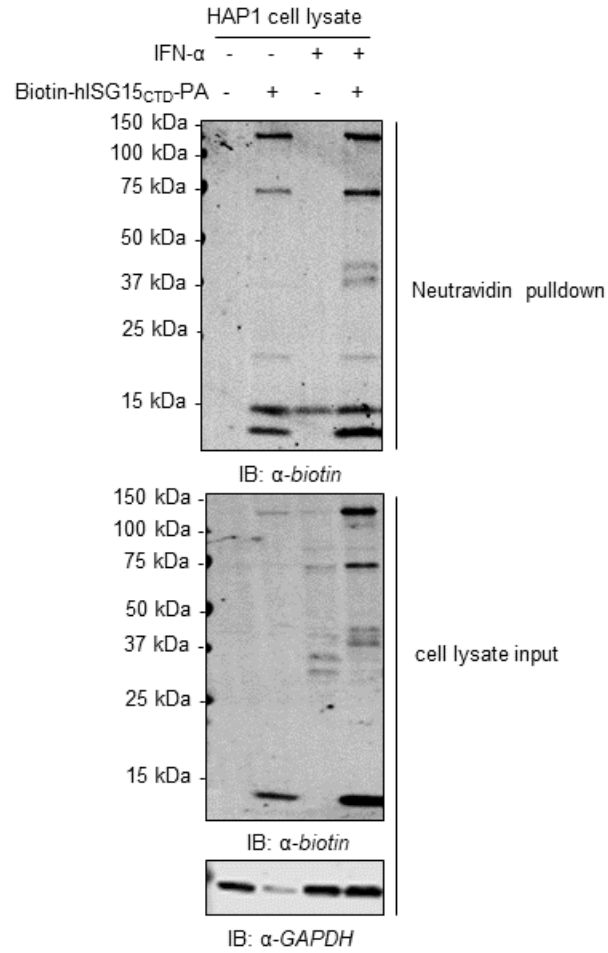


Fig. S1. Activity-based probe labelling of ISG15-reactive proteases in human HAP1 WT cells. Anti-biotin immunoblot shows ISG15-reactive proteases enriched by the Biotin-hISG15_{CTD}-PA probe applied to human HAP1 WT cells. Recovery of biotinylated proteins was done on Streptavidin beads and following denaturing wash steps, corresponding to Figure 1A.

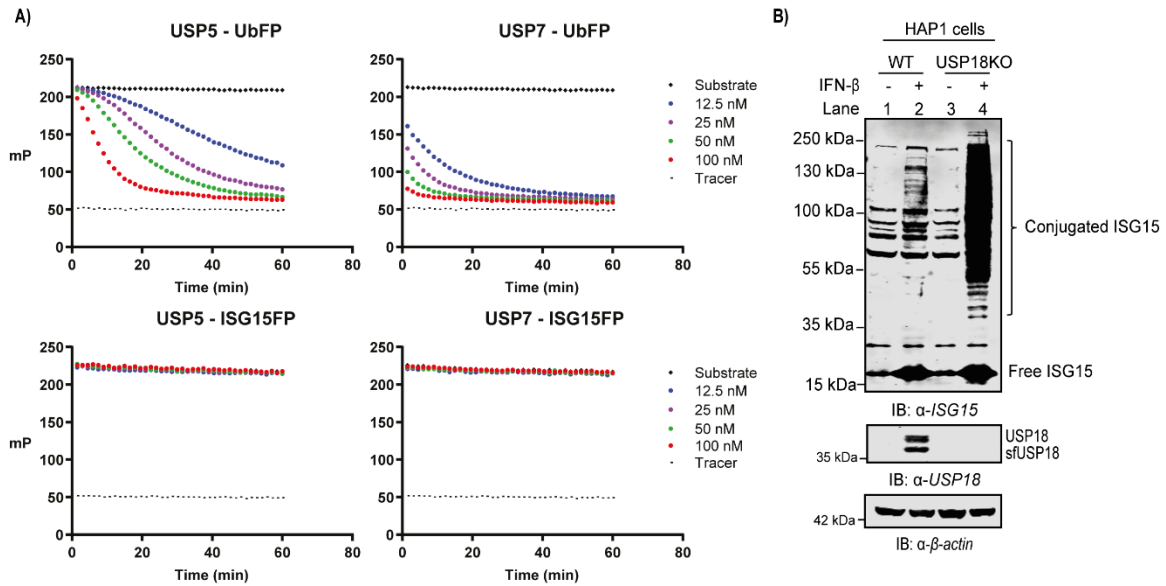


Fig. S2. USP5 and USP7 showed no activity on the ISG15FP substrate *in vitro*. USP18 KO cells show an increase in cellular ISGylation upon stimulation with interferon. (A) Catalytic activity of recombinant human USP5 and USP7 towards the isopeptide-linked Ub-FP and ISG15-FP substrates. The indicated amounts of USP16 FL/CD enzymes were incubated with 200 nM Ub-FP or ISG15-FP. Substrate cleavage was monitored on the basis of the change in fluorescence polarization (in millipolarization units (mP)). Representative data of two (n=2) independent experiments. (B) HAP1 WT and USP18KO cells were stimulated with 1000 U/ml of IFN- β for 24 hours to induce ISGylation and expression of USP18. The loss of USP18 in KO cells strongly increases cellular ISGylation. Corresponding to Figure 2.

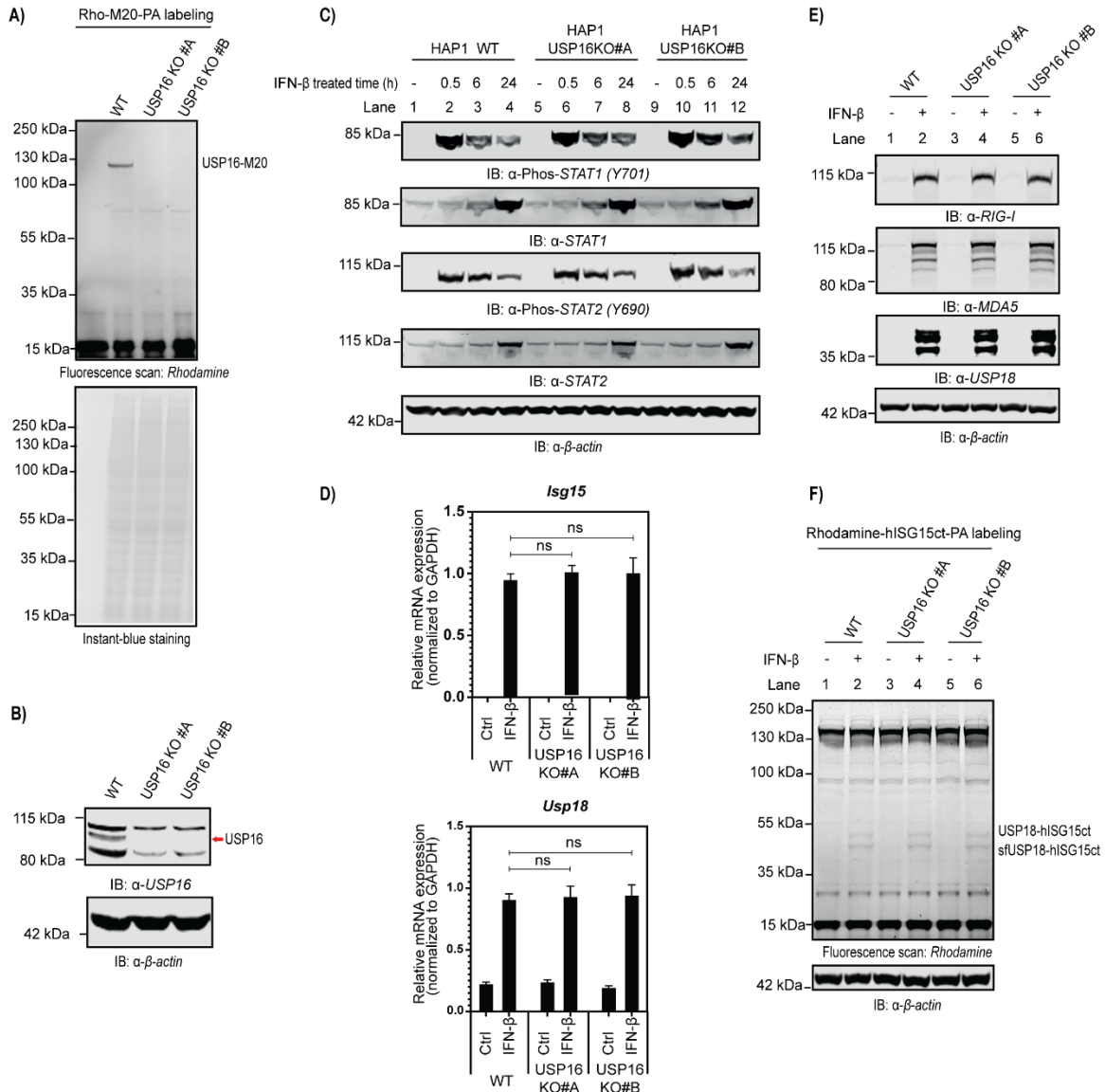


Fig. S3. Loss of USP16 increases cellular ISGylation without affecting type I IFN signaling or changing interferon-induced transcript levels of ISG15 and USP18. (A-B) Characterization of USP16 knockout (KO) HAP1 cells generated by CRISPR/Cas9 using guide RNAs that target exon 6 (KO #A) or exon 4 (KO #B). (A) protein Analysis of endogenous USP16 by labeling with the USP16-specific Rhodamine-M20-PA probe. Representative data of two (n=2) independent experiments. (B) Endogenous USP16 protein Analysis of endogenous USP16 by immunoblotting in USP16 knockout (KO) HAP1 cells. Representative data of two (n=2) independent experiments. (C) Immunoblot analysis of type I IFN signaling. HAP1 WT and USP16KO cells were stimulated with 1000 U/ml of IFN-β as indicated. Cell lysates were then analysed by immunoblotting using the indicated antibodies. (D) Quantitative RT-PCR for *Isg15* and *Usp18* transcripts in HAP1 WT and USP16KO cells stimulated with 1000 U/ml of IFN-β for 24 hours. Bars represent means ± S.E.M (n=3). Data are representative of three independently performed experiments. (E) Immunoblot analysis of selected type I IFN-inducible proteins (RIG-1, MDA5, and USP18). HAP1 WT and USP16KO cells were stimulated with 1000 U/ml of IFN-β for 24 hours. Cell lysates were then analysed by immunoblotting using the indicated antibodies. (F) ISG15-PA probe labeling of lysates of HAP1 wt and USP16KO cells. Cells were treated with IFN-β (1000 U/ml for 24 hours) where indicated; lysates were incubated with Rhodamine-hISG15ct-PA probe, resolved by SDS-PAGE,

and the gels scanned for Rhodamine fluorescence. The position of migration of endogenous USP18 is indicated. Immunoblots for β -actin are shown to confirm equal loading. Corresponding to Figure 3

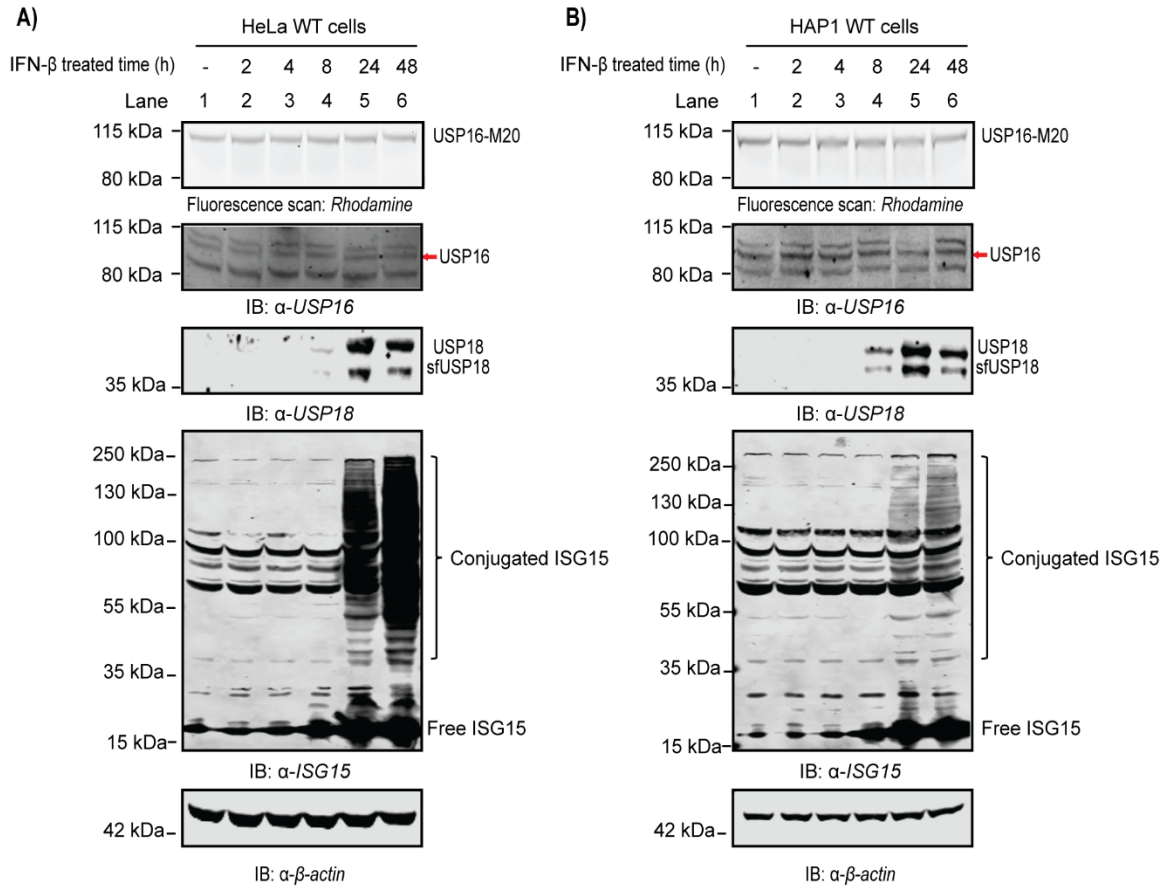


Fig. S4. Activity and protein levels of USP16 are not affected by IFN- β . Assessment of USP16 enzymatic activity and protein expression in HeLa cells (A) and HAP1 cells (B) stimulated with 1000 U/ml of IFN- β as indicated. Cell lysates were labeled with the USP16-specific Rhodamine-M20-PA probe, and analyzed by immunoblotting using the indicated antibodies. Representative data of two (n=2) independent experiments. Corresponding to Figure 3

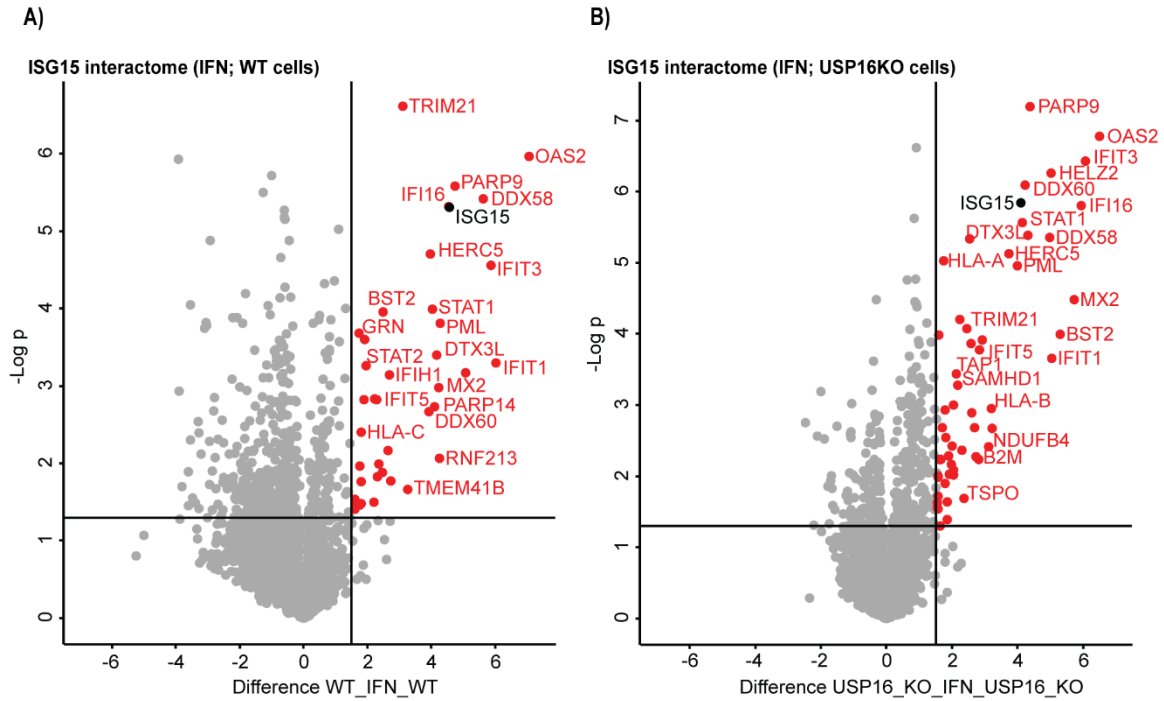
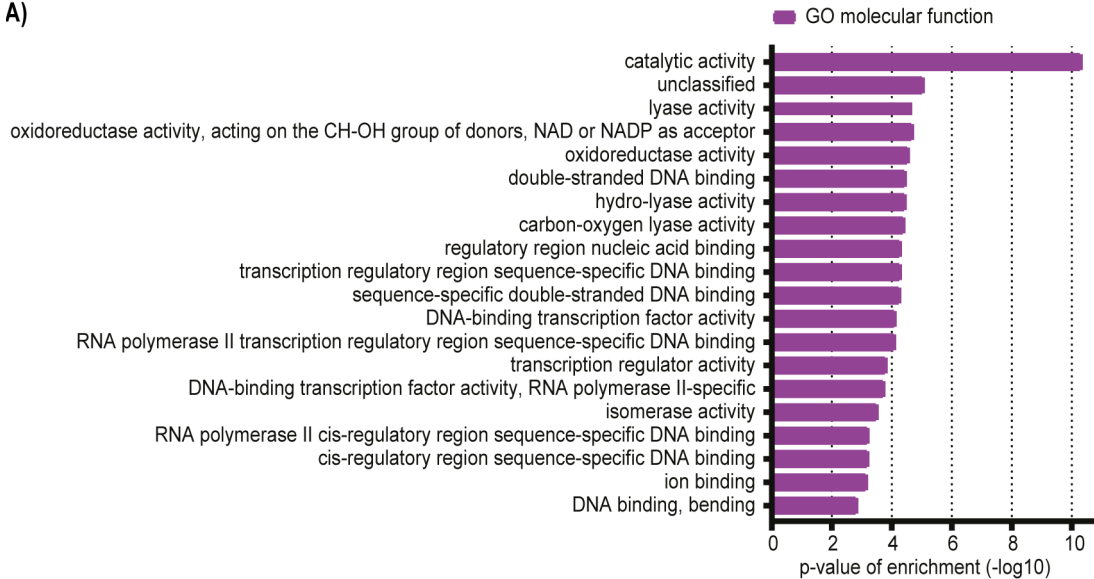


Fig. S5. ISG15 interactome in HAP1 WT and USP16KO cells. (A) Volcano plot showing all identified proteins within the IFN- β stimulated WT samples compared to unstimulated WT. Lines indicate a cut-off difference of the Log2-transformed intensities bigger than 1.5 and a p-value of 0.05 ($-\log_{10} = 1.3$), $n = 2$ independent experiments. Proteins upregulated in the IFN- β stimulated WT cells, referred to as “ISG15 interactome in HAP1 WT cells” are shown in red. (B) Volcano plot showing all identified proteins within the IFN- β stimulated USP16KO samples compared with unstimulated USP16KO. Lines indicate a cut-off difference of the Log2-transformed intensities bigger than 1.5 and a p-value of 0.05 ($-\log_{10} = 1.3$), $n = 2$ independent experiments. Proteins upregulated in the IFN- β stimulated USP16KO cells, referred to as “ISG15 interactome in HAP1 USP16KO cells” are shown in red. Corresponding to Figure 4

A)



B)

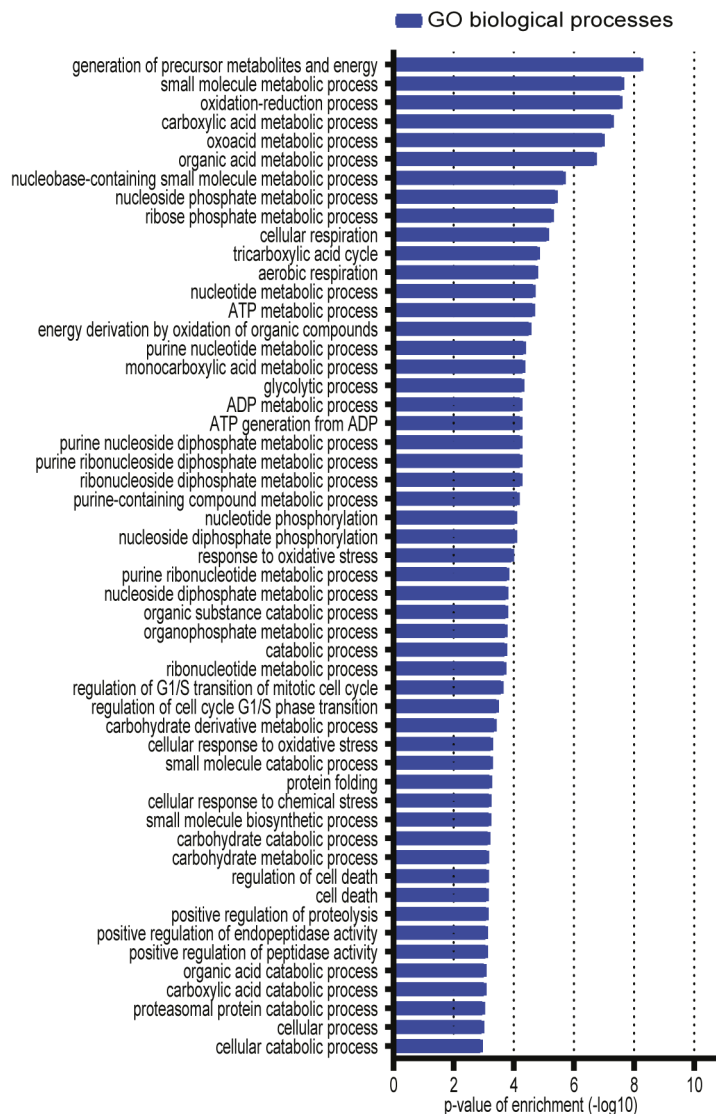


Fig. S6. Gene Ontology (GO) enrichment analysis of the USP16-dependent ISG15 interactome with full GO terms. (A) The bar graph shows the full GO terms for molecular functions (MF) of the USP16-dependent ISG15 interactome against the annotated human proteome. (B) The bar graph shows the full GO terms for biological processes (BP) of the USP16-dependent ISG15 interactome against the annotated human proteome. Corresponding to Figure 4 and Figure 5.

Table S1. Details of the antibodies used in this study

Antibody	Application	Supplier	Cat. No.
USP18 (D4E7)	Immunoblot	Cell Signaling Technology	4813
ISG15	Immunoblot	rabbit antiserum raised against full-length hISG15, provided by Klaus-Peter Knobeloch (15)	
ISG15	Immunoprecipitation	R&D Systems	A-830
USP16	Immunoblot	Bethyl Laboratories	A301-614A
USP5	Immunoblot	Bethyl Laboratories	A301-542A
USP7	Immunoblot	Enzo Life Sciences	BML-PW0540-0100
USP14	Immunoblot	Bethyl Laboratories	A300-920A
GAPDH (GA1R)	Immunoblot	Invitrogen	MA5-15738
β actin (AC-15)	Immunoblot	Sigma-Aldrich	A5441
STAT1 (D4Y6Z)	Immunoblot	Cell Signaling Technology	14995
phospho STAT1 (Y701) (58D6)	Immunoblot	Cell Signaling Technology	9167
STAT2 (D9J7L)	Immunoblot	Cell Signaling Technology	72604
phospho STAT2 (Y690) (D3P2P)	Immunoblot	Cell Signaling Technology	88410
MDA5 (D74E4)	Immunoblot	Cell Signaling Technology	5321
RIG-I (D14G6)	Immunoblot	Cell Signaling Technology	3743
HA (16B12)	Immunoblot	Enzo Life Sciences	ENZ-ABS120-0200
GFP	Immunoblot	rabbit antiserum raised against full-length GFP in house (16)	
Flag (M2)	Immunoblot	Sigma-Aldrich	F3165
Flag	Immunoblot	Sigma-Aldrich	F7425
S tag	Immunoblot	Millipore	71549
Myc tag (9E10)	Immunoblot	Sigma-Aldrich	13-2500

Table S2. Details of the plasmids used in this study

Gene name	Vector	cloning method or resource
USP16 WT	pDEST-CMV-N-EGFP (addgene #122842) (17)	gateway cloning
USP16 C205S	pDEST-CMV-N-EGFP (addgene #122842) (17)	site-directed mutation from USP16 WT (Forward primer: 5'-GAAACACAagTTTCTTCAATGCAGTTATGCAGAACTTGTCAC-3'; reverse primer: 5'-CATTGAAGAAActTGTGTTTCCCAAATTACTGAGTCCTTTCAC-3')
UBE1L	pCAGGS-HA-hUBE1L (addgene #12438)	(18)
UbcH8	pFlagCMV2-UbcH8 ((addgene #12442)	(18)
HERC5	pTriEx2	(15)
ISG15	pEGFP-C1 vector (EGFP was replaced by 4*Flag tag)	Restriction enzyme pairs (Bgl II-Hind III)
USP18 WT	pTriEx2	(6)
USP18 C64A	pTriEx2	(6)
MDH1	pEGFP-C1 vector (EGFP was replaced by 2*Myc tag)	Restriction enzyme pairs (Bgl II-EcoR I)
SOD1	pEGFP-C1 vector (EGFP was replaced by 2*Myc tag)	Restriction enzyme pairs (Bgl II-EcoR I)
ALDOA	pEGFP-C1 vector (EGFP was replaced by 2*Myc tag)	Restriction enzyme pairs (Bgl II-EcoR I)
MDH2	pEGFP-C1 vector (EGFP was replaced by 2*Myc tag)	Restriction enzyme pairs (Sal I-Apa I)
GOT1	pEGFP-C1 vector (EGFP was replaced by 2*Myc tag)	Restriction enzyme pairs (Sal I-Apa I)

Table S3. Details of the recombinant enzymes used in this study.

DUB	Species	Tag	Domain	Resource
USP16	Homo Sapiens	His-3C	FL (isoform3, 22-823 aa)	in-house stock from previous study (19)
			CD ^{WT} (196-823 aa)	
			CD ^{C205S} (196-823 aa)	
USP5	Homo Sapiens	6His	FL	Ubiquigent. #64-0002-050
USP7	Homo Sapiens	-	FL	in-house stock from previous study(19)
USP18	Homo Sapiens	-	FL (16-372 aa)	in-house stock from previous study (6)
USP18	Mus musculus	-	FL (46-368 aa)	in-house stock from previous study (10)

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