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REGULATION OF THE ISG15 ACTIVITY BY SUMO

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REGULATION OF THE ISG15 ACTIVITY BY SUMO

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1. ABREVIATIONS

2APro	Poliovirus-encoded protease 2A
ADC	Adenocarcinoma
ANOVA	Analysis of variance
APL	Acute promyelocytic leukemia
ARF	Alternate reading frame protein product of the CDKN2A locus
ARIH1	Ariadne RBR (RING-between-RING) E3 ubiquitin protein ligase 1
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
c-Myc	Cellular myelocytomatosis oncogene
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CRL	Cullin-RING E3 ubiquitin ligase
CVB3	Coxsackievirus B3
DAPI	4',6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOXY	Doxycycline
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
DUBs	Deubiquitinating enzymes
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epithelial growth factor receptor
EGRF	Epidermal growth factor receptor
eIF4G1	Eukaryotic Translation Initiation Factor 4γ1
EMT	Epithelial-mesenchymal transition
ERK1	Extracellular-signal regulated kinase 1
ESRP1	Epithelial splicing regulatory protein 1
FAT10	Human leukocyte antigen F locus adjacent transcript 10
FGFR1	Fibroblast growth factor receptor 1
FGFR2	Fibroblast growth factor receptor 2
FITC	Fluorescein isothiocyanate
GAP	Gtpase-activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GST	Glutathione S-transferase
H&L	2 heavy chains (H) and 2 light chains (L) linked by disulfide bonds
HA	Hemagglutinin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma

HCMV	Human cytomegalovirus
Herc5	HECT (Homologous to the E6-AP Carboxyl Terminus) and RLD (RCC1(Regulator of Chromosome Condensation 1)-like domains) domain containing E3 ubiquitin protein ligase 5
HIS	Histidine
HIV-1	Human immunodeficiency virus type 1
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IAV	Influenza A virus
IBV	Infectious bronchitis virus
ICP0	Infected cell polypeptide 0
IFN	Interferon
IFNAR1	Interferon α and β receptor subunit 1
IFNAR2	Interferon α and β receptor subunit 2
IgG	Immunoglobulin G
IL-10	Interleukin-10
IL-12	Interleukin-12
IPT	Total protein extract
IPTG	Isopropyl-ß-D-Thiogalactopyranoside
IQGAP1	Ras gtpase-activating-like protein
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
IRF9	Interferon regulatory factor 9
ISG	Interferon-stimulated gene
ISG15	Interferon-stimulated gene 15
ISGF3	Interferon-stimulated gene factor-3
ISRE	Interferon-sensitive response element
JAK1	Janus kinase 1
kDa	Kilodalton
KO	Knockout
KRas	Kirsten rat sarcoma viral oncogen homolog
LB	Luria bertani
Lbpro	Leader protease
LFA1	Lymphocyte function-associated antigen
LMP1	Latent membrane protein 1
LPS	Lipopolysaccharide
LRRC25	Leucine-rich repeat-containing protein 25
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MHC	Major histocompatibility complex
miRNA	Micro-ribonucleic acid

MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEDD4	Neural precursor cell-expressed developmentally down-regulated 4
NEDD8	Neural precursor cell-expressed developmentally down-regulated 8
NF-κB	Nuclear factor-κb
NK	Natural killer
NMIIA	Non-muscle myosin II A
NP	Nucleoprotein
NP40	Nonidet P-40
NS1/A	Non-structural protein 1 of Influenza A virus
NS1B	Non-structural protein 1 of Influenza B virus
NS5A	Nonstructural protein 5A
OD	Optical density
OTUs	Ovarian tumor domain proteases
PaCSCs	Pancreatic cancer stem cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDNA	Plasmid cloning DNA
PEI	Polyethylenimine
PFU	Plaque-forming unit
PI	Propidium iodide
PIAS1	Protein inhibitor of activated STAT 1
PKB	Protein kinase B
PKR	Protein kinase R
PLCγ	Phospholipase cγ
PLPs	Papain-like proteases
PML	Promyelocytic leukemia protein
PTM	Post-translational modification
RARα	Retinoic acid receptor alpha
RIG-1	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RNF11	RING finger protein 11
RNF4	RING finger protein 4
ROS	Reactive oxygen species
SAE	SUMO activating enzyme
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SENP	SUMO-specific peptidase 1
SIM	SUMO interaction motif
SKP2	S-phase kinase-associated protein-2

STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STING	Stimulator of interferon genes
STUbLs	SUMO-targeted ubiquitin ligases
SUBEs	Small Ubiquitin-like Modifier- SUMO- binding entities
SUMO	Small Ubiquitin-like Modifier
TBK1	TANK (TRAF-associated NF-κb activator) binding kinase 1
TIR	Toll-interleukin-1 receptor
TNBC	Triple-negative breast cancer
TNFα	Tumor necrosis factor-α
TOM20	Translocase of the outer mitochondrial membrane complex subunit 20
TPCK	Tosyl phenylalanyl chloromethyl ketone
TRIF	TIR domain-containing adaptor-inducing interferon b
TRIM25	Tripartite motif-containing 25
TSG101	Tumor susceptibility gene 101
TTBS	Tween20 Tris-buffered saline
TYK2	Non-receptor tyrosine-protein kinase 2
U-ISGF3	Unphosphorylated Interferon-stimulated gene factor 3
Ub	Ubiquitin
Ubc9	Ubiquitin-conjugating enzyme 9
UbcH8	Ubiquitin-conjugating enzyme H8
UBE1L	Ubiquitin-activating enzyme E1-like protein
Ubl	Ubiquitin-like protein
UCRP	Ubiquitin cross-reactive protein
UFM1	Ubiquitin fold modifier 1
USP18	Ubiquitin-like carboxy-terminal hydrolase 18
UV	Ultraviolet
V5	V proteins of the paramyxovirus of simian virus 5
vRNP	Viral ribonucleoprotein
VSV	Vesicular stomatitis virus
WB	Western blot
WT	Wild type
XIAP	X-linked inhibitor of apoptosis protein
YAP	Yes-associated protein

2. SUMMARY

2.1 RESUMO

O xene estimulado por interferón (IFN)-15 (ISG15) é unha proteína de 15 kDa semellante á ubiquitina (Ub), inicialmente descrita como unha proteína con reacción cruzada coa ubiquitina (UCRP) debido á reacción cruzada dos anticorpos contra a ubiquitina con ISG15 libre. En condicións normais pódense detectar niveis baixos de ISG15 en células e tecidos, pero a súa expresión, de acordo co seu nome, é fortemente inducida polo IFN tipo I. Ademais, moitos outros estímulos, como infeccións virais, algúns axentes xenotóxicos, lipopolisacáridos (LPS) ou ácido retinoico, tamén poden inducir a expresión de ISG15, en concordancia co seu papel en diferentes condicións de estrés. ISG15 exprésase nunha forma inmadura de 17 kDa, e a escisión do motivo carboxi-terminal da proteína pola proteasa específica de ISG15, a ubiquitina carboxi-terminal hialuronidasa 18 (USP18), expón o motivo de diglicina necesario para a súa conxugación a substratos. Esta proteína de 15 kDa corresponde á ISG15 madura. A proteína ISG15 madura pódese conxugar con outras proteínas a través dun proceso enzimático de tres pasos chamado ISGilación.

O primeiro paso deste proceso consiste na activación de ISG15. Require unha enzima activadora E1 específica para a ISGilación chamada UBE1L e conduce á formación dun enlace tioéster dependente de ATP con ISG15. Despois da activación, ISG15 transfírese ao residuo cisteína activo da enzima conjugadora E2, a UBCH8. Finalmente, a enzima E2 transfire ISG15 a unha enzima ligasa E3 (TRIM25, ARIH1 ou HERC5) que une covalentemente ISG15 a residuos lisina específicos dos substratos. O terceiro e último paso require unha enzima ligasa E3 que favorece a conxugación de ISG15 ao seu substrato como un monómero. Cómpre sinalar que o dominio carboxi-terminal de ISG15 establece interaccións coas enzimas de ISGilación E1 e E2, mentres que ao mesmo tempo, o dominio amino-terminal está implicado na discriminación e selección da enzima ligasa E3. Como un proceso dinámico, a ISGilación é reversible grazas á acción da enzima desconxugante USP18, que amosa unha notable especificidade para ISG15 sen actividade detectable contra ubiquitina ou outras proteínas semellantes á ubiquitina, como NEDD8 ou SUMO. USP18 utiliza o seu residuo Cys61 para executar un ataque electrofílico mediado polo tiois, conducindo á hidrólise da unión amida e á liberación de ISG15 do seu substrato proteico. HERC5 considérase a principal enzima ligasa E3 para ISG15 en humanos. Propúxose que a ISGilación ocorre principalmente nos ribosomas debido á localización de HERC5.

A conxugación de ISG15 a un substrato pode modular a súa localización subcelular, función, oligomerización ou estabilidade. A conxugación de ISG15 non dirixe os substratos á degradación. De feito, e moi común que a ISGilación dos substratos inhiba a ubiquitinación da proteína mediante a competencia coa ubiquitina para conxugarse co substrato. Ademais, propúxose que a formación de cadeas mixtas ubiquitina-ISG15 tamén pode inhibir a degradación de proteínas ubiquitinadas. Ademais da súa conxugación cos seus substratos, ISG15 existe como unha proteína non conxugada. A interacción non covalente de ISG15 con proteínas dentro da célula modula as súas funcións.
A ISGilación xoga un dobre papel ao mellorar e regular negativamente as vías de simalización antiviral. A conxugación de ISG15 cos seus substratos aumenta a produción de IFN tipo I e xenes estimulados por IFN (ISGs) ao estender a activación de proteínas de sialización como IRF3 e STAT1, reforzando así as respostas antivirais. Non obstante, para previr reaccións inmunolóxicas excesivas, ISG15 exerce simultaneamente unha retroalimentación negativa na sinalización de IFN tipo I a través de varios mecanismos. Así, nas células humanas, a

interacción non covalente de ISG15 con USP18 estabiliza USP18 ao previr a súa degradación mediada pola ubiquitinación dependente de SKP2 regulando negativamente a sinalización de IFN tipo I. Por conseguinte, a depleción de USP18 e/ou ISG15 nas células humanas resulta nunha sinalización prolongada de IFN tipo I e maiores niveis de ISGs. Importante, aínda que o USP18 de rato tamén reduce a sinalización de IFN de maneira semellante, non é estabilizado por ISG15 de rato ou humano, indicando que as funcións de ISG15 poden variar entre organismos. Outro mecanismo polo cal ISG15 regula a sinalización de IFN implica a súa interacción con a proteína rica en repeticións ricas en leucina 25 (LRRC25), para facilitar a degradación autofágica da proteína RIG-I inducida polo ácido retinoico (RIG-I). Ademais, ISG15 inhibe directamente a sinalización do receptor de IFN tipo I, uníndose á subunidade 2 do receptor (IFNAR2) a través de STAT-2. Isto impide a dimerización da subunidade IFNAR2 e evita o reclutamento de JAK1, crucial para a fosforilación e activación de STAT1.

ISG15 detéctase no soro dos ratos infectados con virus e pacientes tratados con IFN e propúxose que ISG15 pode actuar como unha citocina, uníndose ao antíxeno asociado á función do linfocito 1 (LFA1), e modulando varias funcións inmunomoduladoras, como: (i) desencadear a maduración e proliferación de células dendríticas e células asasinas naturais (NK), respectivamente; (ii) ser un factor quimiotáctico para neutrófilos e (iii) activar a produción de IFNγ e a secreción de interleucina-10 (IL-10) a partir de células primadas con IL-12.

O mecanismo preciso detrás da liberación de ISG15 aínda é descoñecido. ISG15 carece do péptido sinal para a secreción, pero algúns informes demostran que ISG15 pode ser liberado a través de exosomas, durante a apoptose (Dos Santos and Mansur 2017), ou despois da liberación de gránulos de neutrófilos. Recentemente, Huibregtse e o seu equipo identificaron aminoácidos específicos en ISG15 (os residuos L72A, S83A e L85F) como necesarios para a súa secreción pola célula. Demostrouse que a proteína non estrutural 1 do virus da gripe B (NS1B) únese a ISG15 e desactiva o residuo L72, inhibindo a liberación de ISG15 da célula, o que revela a relevancia destes aminoácidos para a secreción celular de ISG15.

O resultado funcional da conxugación de ISG15 ao seu substratos aínda non se coñece en detalle por moitas razóns. Ata agora, identificáronse centos de proteínas por espectrometría de masas como posibles candidatas a seren ISGiladas, pero só se probaron e validaron unhas poucas. Ademais, só unha porción limitada do conxunto completo de proteínas se ve afectada pola modificación de ISG15, o que supón un desafío para comprender a influencia máis ampla da ISGilación na funcionalidade global dunha proteína.
Apoiando as diferentes funcions que pode exercer o ISG15 segundo a especie, observouse que mentres os ratos que non expresan ISG15 mostran unha maior susceptibilidade a infección con varios virus, os pacientes humanos deficientes en ISG15 teñen unha condición autoinflamatoria de IFN tipo I e mostran unha resistencia reforzada á infección viral. Esta diverxencia entre humanos e ratos destaca as diferenzas complexas e específicas de cada especie na vía de ISG15. Ademais, durante o estudo de ISG15 describíronse moitos resultados contradictorios. Parte desta contradición atribúese ao feito de que a actividade de ISG15 pode depender do tipo celular, do tipo de virus, do contexto celular e da forma de ISG15 (conxugada ou non conxugada).
A expresión de ISG15 está fortemente regulada á alza en varios tipos de cancros e demostrouse que ISG15 é un marcador pronóstico en pacientes con diferentes neoplasias. Sen embargo, ISG15 ten funcións tanto protumorais como inmunomoduladoras antitumorais, e as

consecuencias da expresión de ISG15 na supervivencia dos pacientes con cancro dependen do tipo de cancro.
Desde a súa descuberta en 1975, identificáronse varios proteínas semellantes á ubiquitina (Ubls). Estas Ubls, que inclúen o xene estimulado por interferón 15 (ISG15), varios parálogos do modificador pequeno semellante á ubiquitina (SUMO), o locus F da antíxeno leucocitario humano 10 (FAT10), e o modificador de plegado de ubiquitina 1 (UFM1), entre outros, exhiben semellanzas estructurais e evolutivas coa ubiquitina e funcionan como moduladores críticos de moitos procesos celulares, como o control do ciclo celular, a reparación do ADN, a autofaxia, a transducción de sinal, a transcripción, etc.

O modificador pequeno semellante á ubiquitina (SUMO) xoga un papel crítico en numerosos procesos celulares, incluíndo o transporte nuclear, a transcripción, a remodelación da cromatina, a reparación do ADN e a bioxénesis ribosomal. A conxugación de SUMO aos seus substratos ou SUMOilación regula frecuentemente interaccións intermoleculares e cambios conformacionais, afectando os seus substratos a nivel molecular. Os efectos da conxugación de SUMO adoitan depender de interaccións non covalentes proteína-proteína entre SUMO ou proteínas SUMOiladas e proteínas que conteñen dominios de interacción con SUMO (SIM). Frecuentemente, os substratos de SUMO conteñen dominios SIM. Curiosamente, un estudo recente suxire que case o 90% das proteínas de unión a SUMO que conteñen dominios SIM tamén son substratos de SUMOilación, o que indica unha forte asociación funcional entre a interacción covalente e non covalente de SUMO nos substratos proteicos. Do mesmo xeito que a ISGilación, o IFN tamén induce a SUMOilación global, pero neste caso a través dun mecanismo mediado polo eixo miRNA Lin28/Let7. Ademais, a SUMOilación emerxiu como un regulador importante da vía de sinalización do IFN tipo I e da defensa antiviral. SUMO conxúgase con STAT1 e inhibe a fosforilación de STAT1 inducida polo virus. Ademais, a SUMOilación dos factores de transcripción IRF3 e IRF7 inhibe a síntese de IFN inducida polo virus. Pola contra, a actividade antiviral de diferentes factores de restrición, como PML, PKR e p53, require a súa SUMOilación. Numerosas investigacións informaron dun aumento da expresión das enzimas que participan na SUMOilación, SAE1/SAE2, Ubc9 e E3 ligasas de SUMO en diferentes tipos de cancros. A implicación substancial da SUMOilación na tumorigénese humana tornouse cada vez máis evidente. Os cambios na expresión ou función de varios compoñentes da vía de sinalización de SUMO teñen un impacto profundo na fisioloxía celular. A SUMOilación ten a capacidade de modular a proliferación celular, conferir resistencia á apoptose e potenciar o potencial metastático ao regular proteínas implicadas na carcinoxénese.

Descoñécese se ISG15 é un dos substratos regulados por SUMO. Neste estudo, noso obxetivo foi avaliar se a SUMOilación modula as propiedades e/ou funcións de ISG15.

Aquí, demostramos a SUMOilación de ISG15 por SUMO1 e SUMO2 *in vitro*, en células transfectadas e a niveis endóxenos. Esta modificación non estaba limitada a ISG15 humano, xa que tamén se SUMOilou a ISG15 do ratos. Nos ensaios de SUMOilación *in vitro* usando ISG15 e na presenza de SUMO1, observáronse diferentes bandas, o que suxire que SUMO pode conxugarse a diferentes residuos de lisina en ISG15. O análise *in silico* tamén identificou varios residuos de lisina en ISG15 como potenciais aceptores para a conxugación de SUMO. Finalmente, o análise de mutantes de ISG15 en sitios potenciais de SUMOilación confirmou que os residuos de lisina K8, K29, K35, K77, K90, K108, K129 e K143 en ISG15 son aceptores de SUMO. É ben sabido que moitos substratos de SUMOilación tamén poden interactuar de

maneira non covalente con SUMO ou outras proteínas SUMOiladas a través dun motivo de interacción con SUMO (SIM). Neste estudo, demostramos que ISG15 é un destes substratos de SUMOilación. Demostramos que ISG15 pode interactuar con SUMO de maneira non covalente a través dos dominios SIM 72-LLVVD-76 e 82-LSILV-86. A mutación dos dominios SIM en ISG15 non só interrompe a súa interacción non covalente con SUMO, senón que tamén inhibe a súa SUMOilación.
Unha das funcións propostas de SUMO, principalmente SUMO2/3, é participar na resposta celular a condicións de estrés. En acordo con esta función, a SUMOilación de moitas proteínas é desencadeada por estreses específicos. Aquí mostramos que a SUMOilación de ISG15 é inducida polo estrés causado polo choque térmico, irradiación UV ou infección viral, o que indica unha función fisiolóxica.

As consecuencias da SUMOilación dependen do substrato: SUMO pode modular a actividade de factores de transcripción, regular a localización de proteínas, controlar as funcións de enzimas ou a estabilidade dos substratos, entre outras. Aínda que a proteína ISG15 se detecta principalmente no citoplasma celular, tamén se atopou en todos os compartimentos celulares. Nos nosos ensaios, observamos que a ISG15-WT se distribuí de maneira difusa principalmente no citoplasma celular, independentemente da súa conxugación a substratos, pero tamén detectamos que a ISG15 co-localiza co PML-NB no núcleo. A mutación nos sitios de SUMOilación ou nos dominios SIM levou a unha localización predominantemente nuclear de ISG15 de maneira independente do tipo celular. Estes resultados suxiren que a interacción de SUMO con ISG15 facilita a súa localización citoplasmática. Como se mencionou antes, a proteína ISG15 tamén pode encontrarse no compartimento extracelular como resultado da súa secreción desde o citoplasma. Observamos que a mutación nos motivos de SUMOilación ou dos dominios SIM en ISG15 estaba asociada á ausencia de proteína ISG15 no sobrenadante celular, independentemente da súa conxugación a substratos. Isto concorda con descubrimentos recentes que identificaron os aminoácidos L72, S83 e L85 na proteína ISG15, contidos no dominio SIM, como necesarios para a súa secreción. A diminución dos niveis de ISG15 no sobrenadante pode indicar que SUMO, ao interactuar con ISG15, facilita a súa secreción, ou pode ser unha consecuencia da súa localización predominante no núcleo celular. Dado que a SUMOilación de ISG15 pode inducirse en resposta a diferentes estreses, sería interesante avaliar se os niveis de ISG15 no sobrenadante celular aumentan en células sometidas a estes estreses.
O estres non é a única condición que estimula a SUMOilación de ISG15. A proteína supresora de tumores p14ARF desempeña un papel crucial na indución do arresto do ciclo celular, a senescencia ou a apoptose en resposta ao estres oncoxénico. Ademais, propúxose que p14ARF tamén contribúe á estimulación inmunitaria. Aínda que algunhas das actividades de p14ARF ocorren a través dunha vía dependente de p53, p14ARF é capaz de exercer algunhas das súas actividades de maneira independente de p53. Así, p14ARF induce a SUMOilación de moitos interactores, incluíndo p53 e MDM2, de maneira independente de p53. Aquí observamos que a sobreexpresión de p14ARF induciu a translocación de ISG15 ao núcleo e un aumento marcado na SUMOilación de ISG15. Este fenómeno coincide con descubrimentos previos que suxiren que p14ARF, cando está sobreexpresado, pode sequestrar aos seus socios de interacción no nucleo, inducindo así a súa SUMOilación.
Aquí mostramos que a proteína ISG15 protexe as células A549 da apoptose inducida pola resposta ao IFN e que a interacción de SUMO con ISG15 contribúe á súa actividade anti-apoptótica. Con todo, é importante notar que as células A549 son células p53 WT e carecen de p14ARF. Será importante avaliar o papel de ISG15 e da interacción ISG15-SUMO nunha liña

celular de cancros que non exprese p53 pero que exprese p14ARF. A indución de SUMOilación en resposta á sobreexpresión de p14ARF e a contribución de SUMO á actividade anti-apoptótica de ISG15 lévanos a hipotetizar que a SUMOilación de ISG15 podería ser un dos mecanismos polos cales p14ARF exerce a súa actividade anti-tumoral.
Ademais, os nosos datos revelaron a ISG15 como un novo regulador de p14ARF. Sábese que p14ARF pode ser poliubiquitinado no seu extremo N-terminal, aínda que non ten ningún residuo de lisina. O impacto da conxugación de ISG15 en ARF é descoñecido, pero hipotetizamos que pode servir como un mecanismo de estabilización para p14ARF xa que ISG15 competitía con ubiquitina para conxugarse a p14ARF.
Outro supresor de tumores capaz de inducir a SUMOilación dos seus interactores é PML. O PML-NB serve como un punto central para as proteínas SUMO e recoñécese como un lugar crucial para a SUMOilación de proteínas. Curiosamente, tamén se propuxo un papel de p14ARF na SUMOilación inducida polo PML-NB. O noso estudo indicou que unha pequena fracción de ISG15 detéctase nos PML-NB, en acordo coa súa capacidade de interactuar con SUMO. Con todo, tamén observamos que a mutación no sitio de SUMOilación ou nos dominios SIM en ISG15 aumentaba a fracción de ISG15 detectada nos PML-NB. O mecanismo molecular que leva a unha fracción maior dos mutantes SUMO nos PML-NB é descoñecido. Unha explicación posible é que a localización principalmente nuclear dos mutantes de interacción con SUMO poida facilitar a súa interacción cos PML-NBs. Ademais, tamén se informou que PML se asocia con proteínas mal plegadas e componentes do proteosoma. Polo tanto, é posible que a maior colocalización entre PML e os mutantes de SUMOilación reflicta a acumulación dos mutantes de ISG15 xunto co PML no proteosoma. Ademais, dado que se informou que a ISG15 modifica o PML, tamén é posible que abolir a interacción de SUMO con ISG15 poida promover a acumulación e agregación de PML. Son necesarias máis investigacións para validar estas hipóteses.
A ISG15 foi identificada como un xogador relevante na resposta antiviral do hospedeiro. Con todo, o papel da ISG15 é específico da especie e depende do virus. Os experimentos en células A549 WT ou nas que non expresan ISG15 revelaron que a ISG15 favorece a replicación doVSV nestas células. Polo contrario, a SUMOilación inhibe a replicación do VSV, como se revela polo aumento da replicación viral en resposta ao tratamento co inhibidor de SUMOilación, ML-792. Con todo, este efecto foi independente da expresión ou non de ISG15, indicando mecanismos de acción adicionais. O análise de células A549 que non expresan ISG15 reconstituídas con ISG15 WT ou cos diferentes mutantes de ISG15 revelou que a interacción de ISG15 con SUMO é necesaria para facilitar a replicación de VSV en células A549. O mecanismo polo que a interacción de SUMO con ISG15 facilita a replicación de VSV aínda está en estudo. A nosa hipótese actual é que a interacción de SUMO con ISG15 é esencial para estabilizar USP18 e, polo tanto, inhibir a sinalización de interferón tipo I.
As mitocondrias son orgánulos vitais responsables de procesos como a produción de enerxía, a señalización de calcio, a apoptose e a regulación do sistema inmunitario. Estudos recentes revelaron unha conexión entre a ISG15 e as mitocondrias, enfatizando o seu papel crñítico na fisioloxía celular, na resposta inmunitaria e no metabolismo celular. A proteína SUMO tamén foi implicada na bioxénese mitocondrial. Estes datos leváronos a estudar o papel da interacción de SUMO con ISG15 na biogénesis da mitocondria. O noso estudo revelou un aumento na fragmentación mitocondrial nas células A549 carentes de ISG15 ou nas tratadas có inhibidor da SUMOilación. Ademais, os nosos datos suxiren que a interacción de ISG15 con SUMO é esencial para manter mitocondrias funcionais. Con todo, son necesarios estudos adicionais para confirmar esta hipótese.

En resumo, este estudo revela unha nova regulación de ISG15 a través da súa interacción covalente e non covalente con SUMO, o impacto destas interaccións na proteína ISG15 e as súas posibles implicacións funcionais. Este traballo contribúe a avanzar na nosa comprensión das complexas redes de regulación que gobernan as respostas celulares e abre as portas para investigacións adicionais neste campo.

2.2 SUMMARY

The Interferon (IFN)-Stimulated-Gene 15 (ISG15) is a ubiquitin-like (Ubl) protein which expression is induced by type I IFN, DNA damage and other stresses. ISG15 can conjugate to its substrates through an enzymatic and reversible process called ISGylation. ISG15 can modulate the subcellular localization, oligomerization, activity or stability of its substrates. ISG15 frequently increases the stability of its substrates by competing with ubiquitin to bind to its substrates or through the formation of mixed ISG15-ubiquitin chains. ISG15 also exist as an unconjugated protein inside the cell or it can be secreted from the cell by unclear mechanisms. Free extracellular ISG15 has been reported to function as a cytokine with immunomodulatory activities.

Although ISG15 is minimally expressed under physiological conditions, its levels are abnormally elevated in different pathological conditions such as different types of cancer and neurodegenerative disorders. Contradictory results have been reported on the role of ISG15 protein on the pathogenesis of different diseases and it has been proposed that ISG15 can perform different functions depending on the cell type, the specie, physiological state, virus type or the ISG15 form. How ISG15 functions are modulated is still unknown. Understanding the mechanisms of regulation of ISG15 activities could help to develop new strategies to intervene in the progression of many diseases.

Increasing evidences indicate that the crosstalk between different Ubl proteins may a mechanism to fine-tune responses to different stresses. Another important Ubl is the small ubiquitin modifier (SUMO). The attachment of SUMO to lysine residues in the target proteins or SUMOylation is considered as a key regulatory mechanism modulating the functional properties of a large number of proteins. Conjugation of SUMO to substrates regulates the interaction of the substrate with other proteins or nucleic acid affecting their stability, localization or activity, and influencing many different processes such as the cell cycle, DNA repair, tumor progression, and virus infection. The consequences of SUMO conjugation vary greatly depending on the substrate or the virus type. Thus, SUMO activity can promote or inhibit cellular proliferation, migration, oncogenic transformation or virus replication. The relevance of SUMO not only stand for their conjugation to target proteins. One characteristic of many SUMOylated proteins is to contain SIMs that mediate their non-covalent interaction with SUMO or with other SUMOylated proteins allowing the formation of big complexes with a function in many processes such as the tress response, virus-cell interplay or immune response.

Recent data support the existence of an interplay between SUMOylation and ISGylation. In response to IFN, SUMO3 has been reported to upregulate ISG15 as well as the ISG15 E2 conjugating enzyme UBCH8 and the E3 ISG15 ligases Trim28 and HERC5 by still unknown mechanisms. We hypothesized that SUMO could also interact with ISG15 and that this interplay may impact ISG15 properties and/or activities.

We demonstrated the SUMOylation of human ISG15 by SUMO1 and SUMO2 *in vitro*, in transfected cells, and at totally endogenous levels. We show that mouse ISG15 was also SUMOylated and we identified several SUMOylation sites in ISG15. In addition, we demonstrated that ISG15 can interact with SUMO in a non-covalent manner trough two SIM domains. Importantly, the SIM domains in ISG15 are required for its SUMOylation. In agreement with a role of SUMO2/3 in the cellular response to stress, and with a physiological function for ISG15 SUMOylation, we observed that the SUMOylation of ISG15 is induced upon heat shock stress, UV irradiation, or viral infections. Our results revealed that the tumor suppressor p14ARF protein also triggers ISG15 SUMOylation. Moreover, we identified ISG15 as a new regulatory layer of p14ARF. Another tumor suppressor capable of inducing SUMOylation of its interactors is PML. Interestingly, our study indicated that ISG15 can co-localize with PML-NBs and that mutation of the SUMOylation sites or of SIM domains in ISG15 enhanced the fraction of ISG15 co-localizing with the PML-NBs.

Our data suggest that the interaction of SUMO with ISG15 facilitates its cytoplasmic localization. In addition, we demonstrated that the SUMO binding domains in ISG15 are required for its secretion.

Analysis of A549 cells depleted from ISG15 revealed that ISG15 protect A549 cells from apoptosis induced in response to IFN treatment and that the interaction of SUMO with ISG15 contributes to its anti-apoptotic activity. In addition, we demonstrated that ISG15 exerts a proviral activity on VSV in A549 cells and that the interaction of ISG15 with SUMO is required for this proviral activity on VSV.

Finally, our data revealed that proper mitochondria biogenesis requires ISG15 and SUMOylation. In addition, our data suggested that the interaction of ISG15 with SUMO is essential for maintaining functional mitochondria.

In summary, this study reveals a novel regulation of ISG15 through its covalent and non-covalent interaction with SUMO, the impact of these interactions on the ISG15 protein and their potential functional implications. This work contributes to advancing our understanding of the complex regulatory networks governing cellular responses and lays the groundwork for further investigations in the field.

3. INTRODUCTION

3.1 ISG15

3.1.1 ISG15 protein structure

The Interferon (IFN)-Stimulated-Gene 15 (ISG15) is a 15 kDa ubiquitin (Ub)-like (Ubl) protein described as a ubiquitin cross-reactive protein (UCRP) due to the cross-reactivity of anti-ubiquitin antibodies with free ISG15 (Farrell et al., 1979). Upon transcription, the ISG15 mRNA undergoes translation to produce a 17 kDa polypeptide consisting of 165 amino acids as a precursor form of ISG15 (Pro-ISG15). Subsequent processing events involve the removal of the N-terminal methionine, and a proteolytic cleavage that eliminates eight amino residues from the C-terminus. This process exposes the C-terminal LRLRGG sequence, which is also present at the C terminus of mature ubiquitin (Potter et al. 1999). This motif is required for the covalent conjugation of ISG15 to substrates via lysine residues following an enzymatic process called ISGylation. The mature ISG15 polypeptide is a soluble 156 amino acid protein with a molecular weight of 15 kDa. ISG15 is composed of two Ubl domains that correspond to the N- and C- terminal regions of the protein, separated by a flexible linker. The ISG15 structure comprises two beta-grasp folds that is characteristic of ubiquitin. The beta-grasp fold is composed of a five-stranded beta-sheet partially wrapped around a central alpha-helix (Narasimhan et al. 2005). The N- and C-terminal regions of ISG15 share 29% and 31% sequence identity with ubiquitin, respectively (Blomstrom et al. 1986; Dao 2005; Haas et al. 1987; Narasimhan et al. 2005). Unlike ubiquitin, which has almost 100% cross-species conservation, ISG15 is found only in vertebrates and the ISG15 primary sequence is poorly conserved across mammalian species, with human and murine ISG15 sharing approximately 63% sequence identity at the protein level (Kang, Kim, and Jeon 2022). Importantly, it has been proposed that the diversity of ISG15 among species may influence tertiary structure characteristics.

3.1.2 ISG15 gene expression

ISG15 can be detected at low level in cells and tissues in normal conditions but its expression, according to its name, is highly induced by type I IFN. The binding of type I IFN to the IFNα/β receptor 1 (IFNAR1) and IFNAR2, leads to the activation of the receptor-associated Janus kinase 1 (JAK1) and the non-receptor tyrosine-protein kinase 2 (TYK2) ((Stark et al. 1998; Levy 2001; Negishi, Taniguchi, and Yanai 2018). This leads to the recruitment and the phosphorylation of STAT1 and STAT2 which interact with the interferon regulatory factor 9 (IRF9) and form the IFN-stimulated gene factor 3 (ISGF3) complex that binds to the IFN-sensitive response elements (ISRE) present in the ISG15 promoter (Briscoe J et al. 1996; Darnell 1998; Darnell, Kerr, and Stark 1994) and induces its expression (Fu et al. 1992; Reich et al. 1987). Importantly, expression of members of the ISG15 conjugation machinery is also strongly induced by type I IFN. In the case of chronic viral infection, both interferon beta and type III interferons stimulate the formation of an unphosphorylated ISGF3 complex (U-ISGF3) (Cheon et al. 2013; Cheon and Stark 2009), which maintains the expression of a subset of interferon-stimulated genes (ISGs), including *ISG15* (Sung et al. 2015).

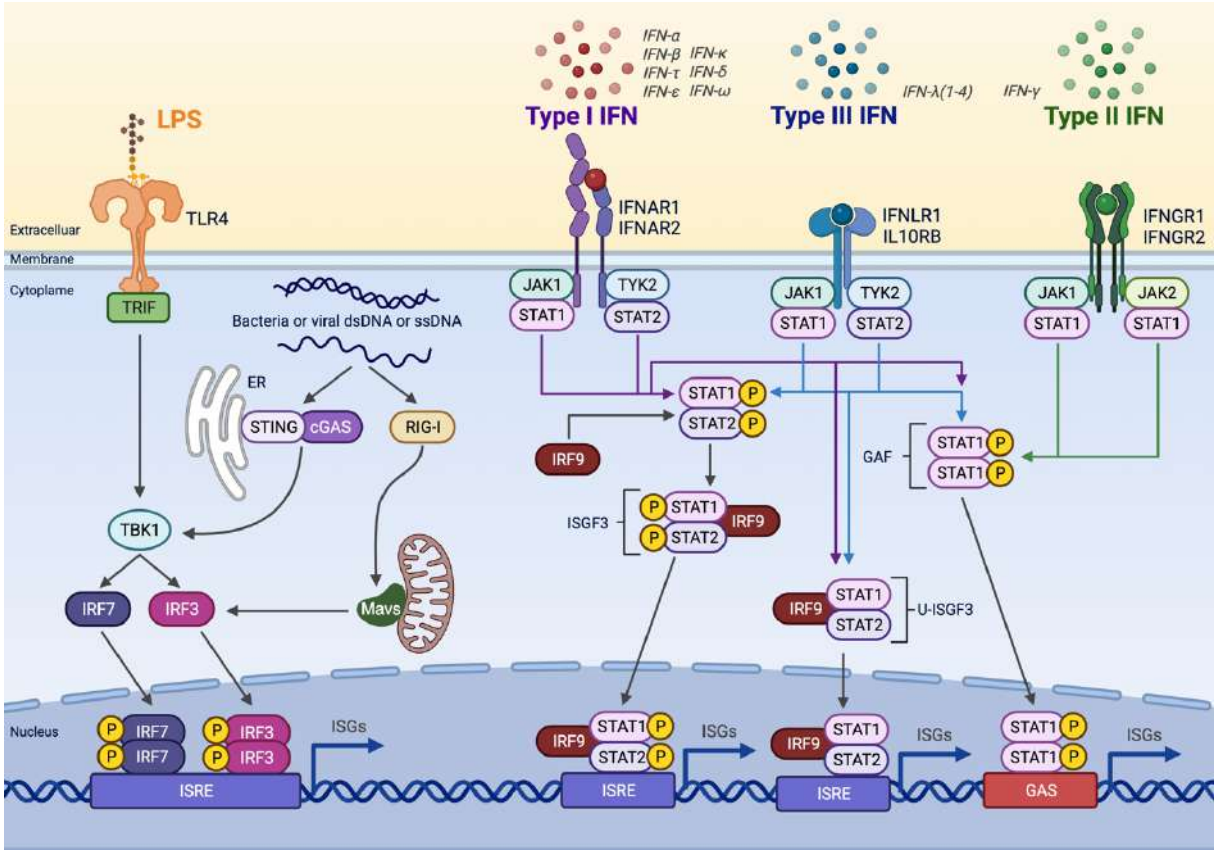


Figure 1. ISG15 induction.

ISG15 expression is strongly induced by type I interferon (IFN). The binding of type I IFN to IFNAR1 and IFNAR2 heterodimers, leads to the activation of the receptor-associated JAK1 and TYK2. This leads to the recruitment and the phosphorylation of STAT1 and STAT2 which interact with IRF9 and form the ISGF3 complex that binds to ISRE elements in gene promoters to induce the transcription of ISGs. In the case of chronic viral infection, both interferon beta and type III interferons stimulate the formation of a U-ISGF3 complex which maintains the expression of a subset of ISGs, including ISG15. LPS induces ISG15 expression through a TRIF-IRF3 signalling pathway. Binding of type II IFN to dimers of heterodimers of IFNGR1 and IFNGR2 leads to phosphorylation of JAK1 and JAK2, the phosphorylation of STAT1, and its homodimerization to form the IFN-gamma activator factor (GAF) which translocate to the nucleus to induce genes regulated by gamma-activated sequence (GAS) promoter elements, including ISG15. Other stimuli such as dsRNA or foreign DNA can also induce ISG15 expression in an IFN-independent manner. dsRNA can induce ISG15 through IRF3, and bacterial DNA via the STING-TBK1-IRF3/7 pathway. (Figure designed with Biorender)

Expression of ISG15 in response to type II IFN has also been reported. Binding of type II IFN to dimers of heterodimers of IFNGR1 and IFNGR2 leads to phosphorylation of JAK1 and JAK2, the phosphorylation of STAT1, and its homodimerization to form the IFN-gamma activator factor (GAF) which translocate to the nucleus to induce genes regulated by gamma-activated sequence (GAS) promoter elements, including ISG15 (Tecalco-Cruz and Mejía-Barreto, 2017 J Cell Commun Signal). ISG15 is induced by other stimuli, such as foreign RNA or DNA or lipopolysaccharide (LPS). In the case of LPS, it induces ISG15 expression through the TIR domain-containing adapter protein inducing IFNβ (TRIF)-IRF3 signaling pathway (Malakhova et al. 2002). Retinoic acid induces type I IFN and consequently triggers ISG15 expression (Pitha-Rowe, Hassel, and Dmitrovsky 2004). However, ISG15 expression is

induced by double-stranded RNA (dsRNA) through IRF3 in an interferon-independent manner (Mémet et al. 1991). Similarly, bacterial DNA can induce ISG15 in a IFN-independent manner via the pathway composed by the stimulator of interferon genes protein (STING), the serine/threonine-protein kinase TBK1, IRF3, and IRF7 (Radoshevich et al. 2015) (Figure 1). Importantly, the promoters of ISG15 and ISGylation enzymes contain p53-responsive elements explaining why stimuli inducing p53, such as DNA damage agents, transactivate the ISG15 pathway (Liu et al. 2004; Park et al. 2016).

3.1.3 Conjugation of ISG15 or ISGylation

As mentioned above, ISG15 is expressed as an immature protein with a mass of 17 kDa which is cleaved at its carboxy-terminus through the action of the ubiquitin specific peptidase 18 (USP18), generating a mature 15 kDa protein (Yuan 2001;Potter et al. 1999). Mature ISG15 protein can conjugate to other proteins through a three steps enzymatic process called ISGylation (Figure 2).

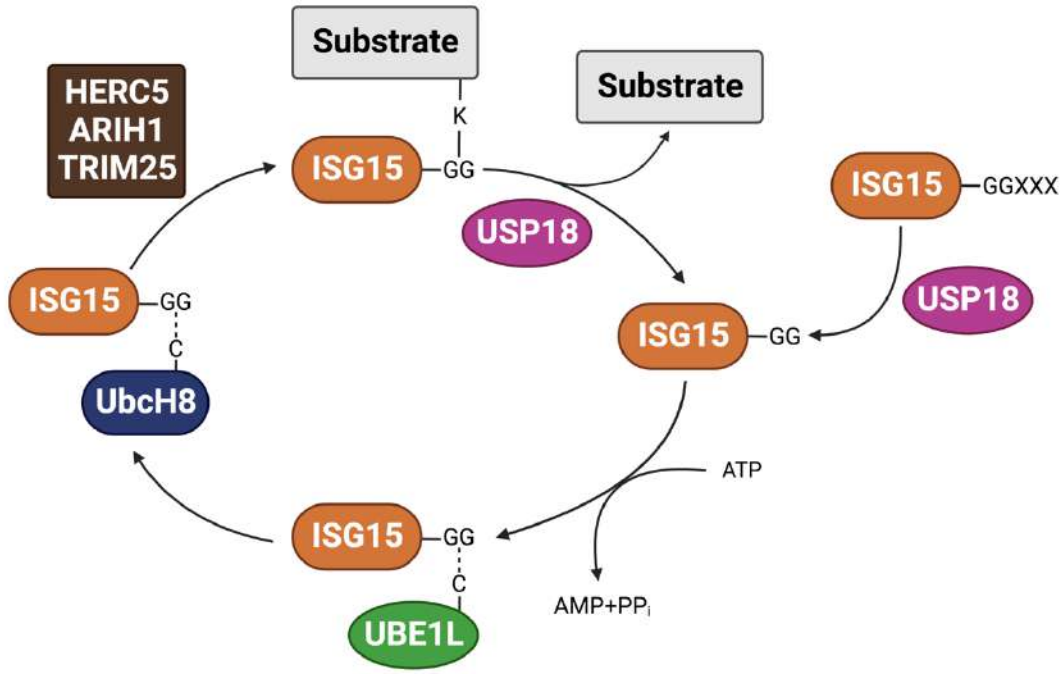


Figure 2. ISG15 and the ISGylation machinery.

ISG15 is expressed as an immature polypeptide of 17 kDa which is cleaved at its carboxy-terminus by the action of USP18 to generate the 15 kDa mature protein. Mature ISG15 protein can conjugate to other proteins through a three steps enzymatic process called ISGylation. The E1 activation enzyme for ISG15, UBE1L, activates the C-terminal group of ISG15 leading to the formation of a thioester bond in an ATP-dependent manner. ISG15 is then transferred to the active-site cysteine of the E2 conjugating enzyme UBCH8. The E2 enzyme then transfers ISG15 to an E3 ligase (HERC5, TRIM25, ARIH1) which covalently attach ISG15 to specific lysine residues of substrates. ISGylation is a reversible process thanks to the action of the deconjugating enzyme USP18. (Figure designed with Biorender).

The first step of this process consists in the activation of ISG15. It requires an E1 activating enzyme for ISG15 called UBE1L and leads to the formation of a thioester bond in an ATP-dependent manner (Krug, Zhao, and Beaudenon 2005). After activation, ISG15 is transferred to the active-site cysteine of the E2 conjugating enzyme UBCH8 (Kim et al. 2004; Zhao et al. 2004). Finally, the E2 enzyme transfer ISG15 to an E3 ligase (TRIM25, ARIH1, HERC5) which covalently attach ISG15 to specific lysine residues of substrates (Okumura, Zou, and Zhang 2007; Zou and Zhang 2006). Both Ubl domains of ISG15 are required for its conjugation to substrates (Chang et al. 2008). The C-terminal domain of ISG15 establishes interactions with its E1 and E2 enzymes, while concurrently, the N-terminal domain is implicated in the discrimination and selection of the E3 ligase (Ashley et al. 2019). ISGylation is reversible thanks to the action of the deconjugating enzyme USP18 (Basters et al. 2017; Malakhov et al. 2002) which displays a remarkable specificity for ISG15 without detectable activity against ubiquitin or other Ubls such as NEDD8 or SUMO (Laval, Chaumont, and Demangel 2021; Monson et al. 2021). USP18 is able to reverse modification with ISG15 by hydrolyzing the covalent bond between its C-termini and substrate (Tanigawa et al. 2008). HERC5 is considered the major E3 ligase for ISG15 in humans (Dastur et al. 2006; Ketscher et al. 2012; Oudshoorn et al. 2012; Wong et al. 2006). ISGylation has been proposed to occur mainly at the ribosomes due to the localization of HERC5.

To date, hundreds of potential ISGylation substrates have been identified by mass spectrometry; however, only few substrates have been validated. The functional consequences of ISG15 conjugation is unclear. ISG15 conjugation does not target substrates for degradation (Liu, Li, and Hassel 2003). Studies of specific ISG15 targets have revealed that conjugation of ISG15 to a substrate can modulate its subcellular localization, function, oligomerization or stability (Jeon et al. 2009; Rahnefeld et al. 2014; Wardlaw and Petrini 2023). Sometimes, ISG15 compete with ubiquitin to conjugate to the substrates, inhibiting its ubiquitination (Okumura et al. 2006). In addition, it has been proposed that the formation of ISG15-ubiquitin mixed chains may also inhibit the degradation of ubiquitinated proteins (Fan, Arimoto, et al. 2015).

3.1.4 Free intracellular ISG15

In addition to its conjugation to target proteins, ISG15 exists as an unconjugated protein. The non-covalent interaction of ISG15 with proteins inside the cell modulates their functions (Loeb and Haas 1992a; Narasimhan, Potter, and Haas 1996). Thus, the binding of ISG15 to NEDD4 leads to the disruption of the NEDD4 interaction with Ubiquitin conjugating enzymes, preventing the transfer of ubiquitin to the active site of NEDD4, and consequently inhibiting ubiquitination of NEDD4 substrates (Malakhova and Zhang 2008; Okumura, Pitha, and Harty 2008). Free intracellular human ISG15 can also interact with and stabilize USP18 and by doing so, regulates type I IFN signaling (Zhang et al. 2015). Other mechanism by which ISG15 regulates IFN signaling is through its non-covalent interaction with leucine-rich repeat-containing protein 25 (LRRC25) and retinoic acid-inducible gene I (RIG-I) protein, to facilitate the autophagic degradation of RIG-I (Du et al. 2018; Nakashima et al. 2015).

3.1.5 Free extracellular ISG15

ISG15 has been detected in the serum of mice infected with virus and of patients treated with IFN (Loeb and Haas 1992b; Werneke et al. 2011). Consequently, it has been proposed that ISG15 can act as a cytokine, binding to the lymphocyte function-associated antigen 1 (LFA1), and exerting a variety of immunomodulatory functions such as: (i) triggering maturation and proliferation of dendritic and natural killer (NK) cells, respectively (Desai et al. 2006; Fan, Arimoto, et al. 2015) ; (ii) being a chemotactic factor for neutrophils (Loeb and Haas 1992) and

(iii) activating IFNγ production (Werneke et al. 2011) and interlekin-10 (IL-10) secretion from IL-12 primed cells (Swaim et al. 2017).

The precise mechanism behind the release of ISG15 is still unknown. ISG15 lacks the signal peptide for secretion and some reports demonstrate that ISG15 can be released via exosomes (Sun et al. 2016), during apoptosis (Dos Santos and Mansur 2017), or after the release of neutrophil granules (dos Santos and Mansur 2017). Recently, Huibregtse and his team identified specific amino acids in ISG15 (L72A, S83A and L85F) as required for its release from the cell (Swaim et al. 2020). Interestingly, the non-structural protein 1 of Influenza B virus (NS1B) has been demonstrated to bind to ISG15 and to hinder the L72 residue, inhibiting the release of ISG15 from the cell, supporting the relevance of this amino acid for ISG15 cellular release (Yuan and Krug R M. 2001; Zhao et al. 2016).

3.2 FUNCTIONS OF ISG15

The functional outcome of ISG15 conjugation to its substrates is still poorly characterized for many reasons. Until now, hundreds of proteins have been identified by mass spectrometry as putative candidates to be ISGylated but only a few have been tested and validated (Giannakopoulos et al. 2005a; Zhao et al. 2005). In addition, only a limited portion of the complete protein pool is affected by ISG15 modification, which poses a challenge in comprehending the broader influence of ISGylation on a protein's overall functionality.

3.2.1 ISG15 and virus

3.2.1.1 Regulation of IFN signaling by ISG15

ISGylation plays a dual role by both enhancing and negatively regulating antiviral signaling pathways. Conjugation of ISG15 to its substrates increases the production of type I IFN and interferon-stimulated genes (ISGs) by extending the activation of signaling proteins like IRF3 and STAT-1, thereby reinforcing antiviral responses. By reversing protein ISGylation, USP18 acts as a critical modulator in the regulation of immune responses (Figure 3) (Ganesan et al. 2016; Shi et al. 2010). However, to prevent excessive immune reactions, ISG15 simultaneously exerts negative feedback on type I IFN signaling through various mechanisms. One mechanism involves the ISGylation of RIG-I, leading to reduced IFN promoter activity (Kim et al. 2008). Additionally, non-covalent binding of ISG15 to RIG-I-LRRC25-p62 targets RIG-I for autophagic degradation (Du et al. 2018). Moreover, ISG15 directly inhibits type I IFN receptor signaling by binding to the subunit 2 of the receptor through STAT-2. This impedes IFNAR subunit dimerization and prevent JAK1 recruitment, crucial for the phosphorylation and activation of STAT1 (Arimoto et al. 2017; Malakhova et al. 2003). ISG15 can also downmodulate IFN signaling by interacting with the deISGylase USP18 in a non-covalent manner. In human cells, the binding of ISG15 to USP18 increases USP18 levels (Zhang et al. 2015) by preventing its degradation mediated by SKP2-dependent ubiquitination (Kaufmann et al. 2018). USP18 inhibits IFN signaling by directly binding to the intracellular portion of IFNAR2 inhibiting the binding of JAK1 to the IFN receptor (Malakhova et al. 2006). Consequently, human ISG15 interaction with USP18, reinforces the inhibition of IFN receptor signaling. Therefore, depletion of USP18 and/or ISG15 in human cells results in prolonged type I IFN signaling and higher ISG levels (Speer et al., 2016). Importantly, although murine USP18 similarly downmodulates IFN signaling, mouse USP18 is not stabilized by mouse or human ISG15, indicating that ISG15 functions may vary between organisms (Speer et al. 2016).

In agreement with the divergent functions of ISG15 between species, it has been observed that while ISG15 knockout mice display increased susceptibility to various viruses (Morales

and Lenschow 2013), ISG15-deficient human patients have a type I IFN autoinflammatory condition and display enhanced resistance to viral infection. This divergence between human and mouse, underscores the complex and species-specific differences in the ISG15 pathway and the necessity of studying the different forms of ISG15 during innate immune signaling.

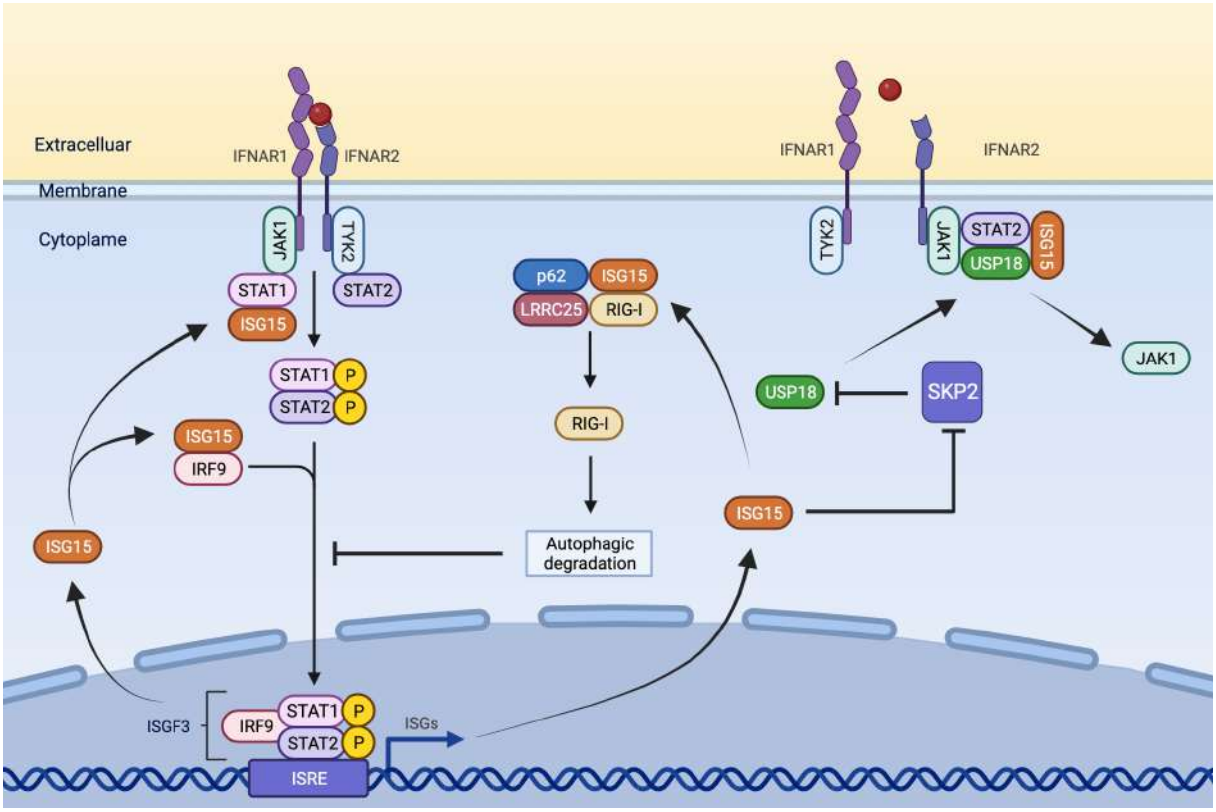


Figure 3. Regulation of IFN signaling by ISG15

ISG15 regulates positively and negatively the IFN signaling pathway using different ways of action: (i) Conjugation of ISG15 to STAT-1 and IRF9 enhances the production of type I IFN and ISGs (ii) USP18 inhibits IFN signaling by directly binding to the intracellular portion of IFNAR2 and inhibiting the binding of JAK1 to the IFN receptor. (iii) The binding of ISG15 to USP18 stabilizes USP18 by preventing its ubiquitin-mediated degradation by SKP2, reinforcing the inhibition of IFN receptor signaling. (iv) ISG15 non-covalent binding to RIG-I-LRRC25-p62 targets RIG-I for autophagic degradation. (v) ISG15 directly inhibits type I IFN receptor signaling by binding to the subunit 2 of the receptor through STAT-2. This impedes IFNAR subunit dimerization and prevent JAK1 recruitment, crucial for the phosphorylation and activation of STAT1. (Figure designed with Biorender).

3.2.1.2 Antiviral and proviral activities of ISG15

ISG15 and components of the ISG15 conjugation machinery are strongly and rapidly induced by type I IFNs. Consequently, ISG15 was considered as a central player in the host antiviral response (Lenschow et al. 2005). Indeed, ISG15 was found to exert antiviral effect on many viruses by acting on various actors and steps during virus replication. However, the antiviral functions of ISG15 depend on whether ISG15 interacts in a covalent or non-covalent manner with viral or cellular proteins. In addition, the role of ISG15 on virus replication and the mechanisms of action of ISG15 differ among mammals.

3.2.1.2.1 Downmodulation of virus replication through ISG15 interaction with viral proteins

ISG15 has been reported to target nascent proteins. Therefore, it has been proposed that viral proteins are among the primary candidates for such targeting (Durfee et al. 2010). The first viral protein that was found to be modified by ISG15 was the non-structural protein 1 of Influenza A virus (NS1/A) (Tang et al. 2010; Zhao et al. 2010). NS1/A is a multifunctional protein that inhibits the induction of type I IFN (Wang et al. 2000), prevents the activation of protein kinase R (PKR) (Bergmann et al. 2000), selectively enhances viral mRNA translation (de la Luna et al. 1995), and interferes with cellular mRNA processing (Fortes, Beloso, and Ortín 1994; Nemeroff et al. 1998), being crucial to viral replication. ISG15 conjugates at lysine 41 (K41) of NS1/A, inhibiting its nuclear translocation through the disruption of its interaction with importin-α (Zhao et al. 2010), and rendering the virus vulnerable to interferon-mediated inhibition. The protease 2A (2APro) of the coxsackievirus B3 (CVB3) is another example of viral protein modified by ISG15. This protein is responsible for cleaving the mammalian eukaryotic translation initiation factor 4γ1 (eIF4G1), leading to the suppression of host cell protein synthesis, ultimately favoring viral replication. ISGylation of 2APro impedes the cleavage of eIF4G1 during CVB3 infection, mitigating host cell shut-off and restricting CVB3 replication (Rahnefeld et al. 2014). Further insights from the research on influenza B virus (IBV) and human papilloma virus (HPV) demonstrate that ISGylation can disrupt the function and structure of viral complexes. The formation of IBV nucleoprotein (NP) oligomers is essential for the assembly of the viral ribonucleoprotein (vRNP), a process critical for viral RNA synthesis. ISGylated IBV NP functions as a dominant-negative inhibitor, disrupting the oligomerization of unmodified NP, and consequently limiting viral RNA synthesis and diminishing IBV replication (Zhao et al. 2016). In the case of HPV, the ISGylation of the capsid protein L1 does not abolish its integration into viral particles. However, both the quantity and infectivity of particles containing ISGylated L1 protein are reduced, potentially due to modifications in the geometry of the viral capsid (Durfee et al. 2010). ISGylation has also been shown to inhibit human cytomegalovirus (HCMV) gene expression and the release of virions. HCMV pUL26 is recognized for its capacity to suppress tumor necrosis factor-α (TNFα)-induced nuclear factor-κB (NF-κB) activation (Mathers et al. 2014). It has been demonstrated that ISG15 engages in both covalent and non-covalent interactions with HCMV pUL26 protein. The ISGylation of pUL26 results in alterations in pUL26 stability and in the inhibition of its ability to suppress NF-κB signaling (Kim et al. 2016).

3.2.1.2.2 Modulation of virus replication through ISG15-mediated regulation of signaling proteins

ISG15 has been proposed to be conjugated to more than 160 host cell proteins, as identified by mass spectrometry. Among these target proteins, some are interferon-stimulated genes (ISGs) involved in the regulation of antiviral innate immune response, including PKR, MxA, Hup56, and RIG-I (Zhao et al. 2005). Additionally, other target proteins play a role in the regulation of type I IFN signaling, such as PLCγ1, JAK1, ERK1, and STAT1(Malakhova et al. 2003)**.** Moreover, viruses can be impaired by the ISGylation of host cell proteins involved in different steps of the viral cycle such as the viral release. The initial indication of ISG15 blocking virus release emerged from investigations on HIV-1 replication. Expression of a plasmid encoding ISG15 together with the HIV-1 proviral DNA, has been reported to inhibit the release of HIV-1 without affecting the production of HIV-1 proteins (Okumura et al. 2006). This inhibition was attributed to the interference with the mono-ubiquitination of the HIV-1

Gag polyprotein by ISG15, and to the disruption of its interaction with the host tumor susceptibility gene 101 (TSG101) protein, both essential processes for HIV-1 budding and release. These are just some examples of the capability of ISGylation to reduce the efficiency and quality of viral progeny production and to limit the control of the viral proteins over the host immune response.

3.2.2 Modulation of ISG15 by viruses

Consistent with a role of ISG15 in antiviral defense, numerous virus express proteins capable of antagonizing ISGylation (Perng and Lenschow 2018). Thus, some viral proteins are able to sequester ISG15 or ISGylated proteins, as demonstrated for Vaccinia virus E3 protein or Influenza B NS1 protein. Both proteins interact with ISG15 inhibiting the ISG15-mediated stimulation of the immune response (Guerra et al. 2011; Sridharan, Zhao, and Krug 2010; Versteeg et al. 2010). Other viruses encode proteases that remove ISG15 from its target proteins such as virus belonging to the Nairovirus genus (e.g., Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus, and Erve virus), arteriviruses (e.g., equine arteritis virus and porcine reproductive and respiratory syndrome virus), picornaviruses (e.g., foot and mouth disease virus), and coronaviruses (e.g., severe acute respiratory syndrome-related coronavirus, Middle East respiratory syndrome-related coronavirus, and mouse hepatitis virus) (Mielech et al. 2014). These proteases, often termed viral deubiquitinating proteins (DUBs), are cysteine proteases with substantial activity in reversing ubiquitin (Ub) conjugation. They are generally classified into three different types: ovarian tumor domain proteases (OTUs), papain-like proteases (PLPs), or leader proteases (Lbpro) (Frias-Staheli et al. 2007; Lindner et al. 2005; Swatek et al. 2018). Although the precise function of viral deISGylases is still unclear it has beeen speculated that it may be critical.

3.2.3 Exploitation of the ISGylation machinery by viruses

In addition to develop methods to counteract the antiviral effects of ISG15 conjugation, viruses have also developed strategies to exploit the ISGylation machinery in their own benefit. Recent findings have revealed that ISG15 conjugation to viral proteins, such as the hepatitis C virus (HCV) non-structural 5A (NS5A) protein or the hepatitis B virus (HBV) X protein (HBx) exert proviral effects (Gao et al. 2020; Iglesias-Guimarais et al. 2020; dos Santos et al. 2018). ISGylation of NS5A protein from HCV facilitates the recruitment of host proteins that enhance viral RNA replication (dos Santos et al. 2018). Similarly, in the context of Zika virus infection, ISG15 conjugation amplifies viral protein expression in human cell lines (Swaim et al. 2017).

3.3 ISG15 AND CANCER

The role of ISG15 in cancer is controversial, likely due to the different types of cancers and to the potential different functions of conjugated, intracellular free and extracellular free ISG15. Thus, although ISG15 gene expression is highly upregulated in various types of cancer (Desai et al. 2012; Kiessling et al. 2009; Li et al. 2014; Wood et al. 2012) and it has been shown to be a prognostic marker in patients with different malignancies (Qiu X et al. 2015), it can have both protumor and immunomodulatory anti-tumor functions, and the consequences of the ISG15 expression on cancer patients' survival depends on the cancer type (Figure 4).

3.3.1 Pro-tumoral activity of ISG15

ISG15 has been identified as a protein promoting cancer cell migration and metastasis through the interaction with or modification of certain oncogenic proteins. ISG15 is also among the genes that are found in cancer patients showing resistance to DNA damage-inducing

therapies (Weichselbaum et al., 2008; Fornai et al., 2001; Sirota et al., 1996) and its downmodulation has been reported to re-sensitize cancer cells to chemo- and radiotherapy (Boelens et al., 2014).

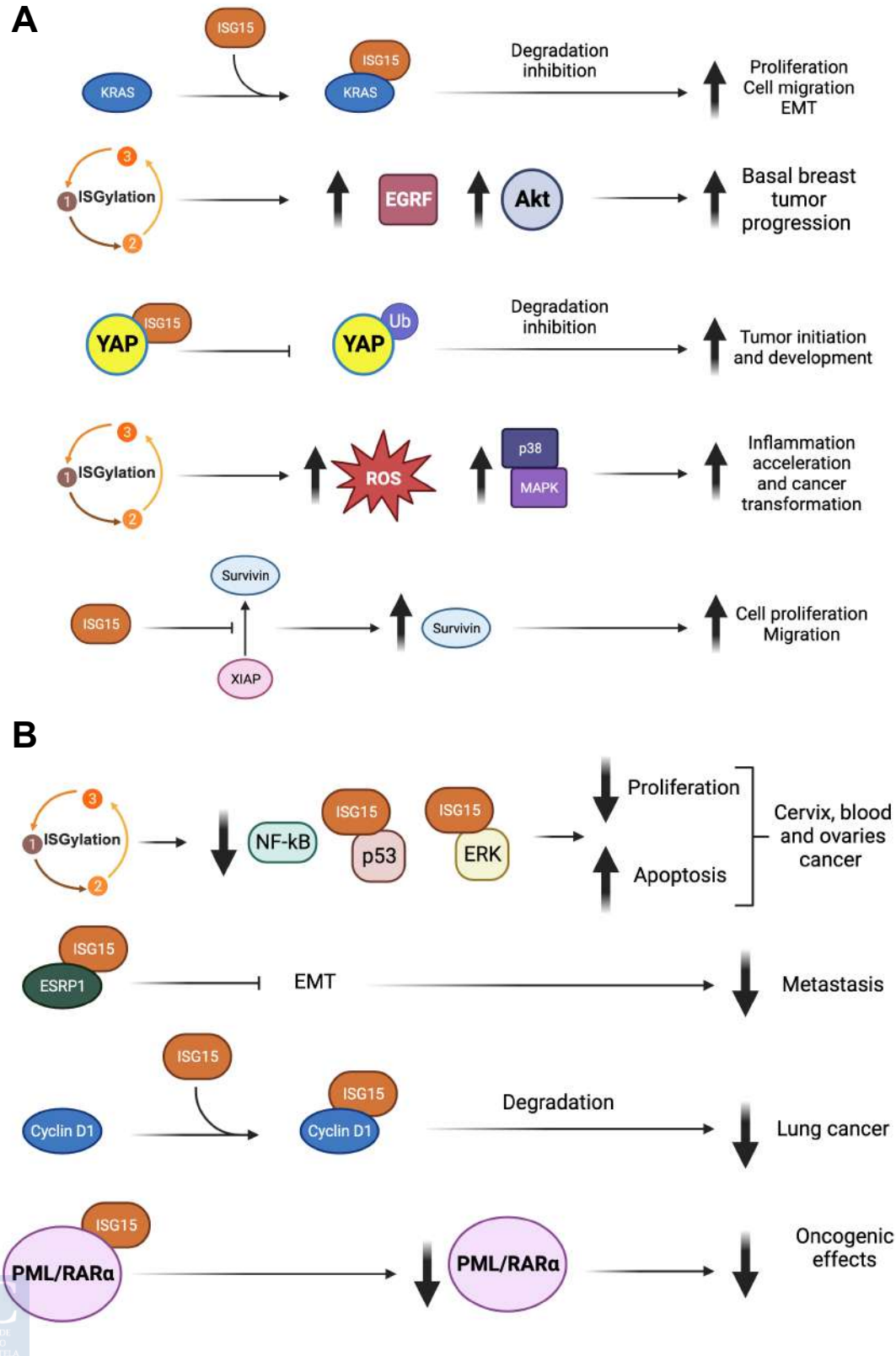


Figure 4. Pro and anti-tumoral activity of ISG15

A Schematic representation of some tumor-promoting activities of ISG15: (i) ISGylation, activated in response to oncogenic KRas, inhibit KRas lysosomal degradation, promoting cell proliferation, migration and epithelial mesenchymal transition (EMT) in breast cancer cells. (ii) ISGylation facilitates EGFR recycling and Akt signaling in basal breast tumor progression. (iii) ISGylation of YAP inhibits its ubiquitination and proteasomal degradation, which contributes to tumor initiation and development in lung cancer. (iv) Protein ISGylation has a negative impact on the ubiquitin-proteasome system which results in elevated IFN-induced ROS leading to enhanced activation of p38/MAPK and the expression of inflammation-related cytokines in macrophages, accelerating inflammation and cancer transformation. (v) In HCC, ISG15 interferes with the interaction between survivin and X-linked inhibitor of apoptosis protein (XIAP), thereby stabilizing survivin and facilitating cell proliferation and migration. **B** Schematic representation of some anti-tumoral activities of ISG15: (i) ISGylation of p53, suppression of the NF-kB signal transduction or the ISGylation of ERK decreases proliferation and increases apoptosis in cervix, blood and ovaries cancer. (ii) ISGylation of ESRP1 leads to its stabilization, inhibiting EMT and decreasing metastasis. (iii) ISGylation of cyclin D1 facilitates its degradation in human bronchial epithelial cells and lung cancer cells promoting lung cancer growth suppression. (iv) ISGylation of PML/RARα reduces its levels diminishing the oncogenic effect of the fusion protein. (Figure designed with Biorender).

Among the cancers in which ISG15 was found upregulated are melanoma, lung, breast, prostate, nasopharyngeal, oral cancers and hepatocellular carcinoma (HCC) (Bektas et al. 2008; Burks, Reed, and Desai 2014; Y.-L. Chen et al. 2019; Desai et al. 2012; Tecalco Cruz and Mejía-Barreto 2017; Tecalco-Cruz and Cruz-Ramos 2018). In addition, a correlation between elevated levels of ISG15 and STAT1 and reduced levels of p53 and ARF are frequently observed in triple-negative breast cancers (TNBCs) (Bektas et al. 2008; Forys et al. 2014). The mechanisms by which ISG15 and ISGylation could promote cancer growth or metastasis are diverse (Figure 4). Thus, ISGylation of non-muscle myosin II A (NMIIA) and Ras GTPase-activating-like protein 1 (IQGAP1) has been shown to be linked to the reorganization of the cytoskeleton (Cerikan and Schiebel 2017; Cruz-Ramos et al. 2019; Giannakopoulos et al. 2005) and the impact of ISG15 on the cytoskeleton has been proposed as a mechanism contributing to the invasive and metastatic behavior of breast cancer cells (Cruz-Ramos et al. 2019; Cui et al. 2004; Tecalco-Cruz et al. 2019). Additionally, ISG15 and ISGylation, activated in response to oncogenic Kirsten-Ras (KRas), has been shown to inhibit the lysosomal degradation of KRas and this mechanism has been shown to promote migration and epithelial-mesenchymal transition (EMT) of breast cancer cells (Burks et al. 2014). Furthermore, ISGylation has been implicated in basal breast tumor progression by facilitating EGFR recycling and Akt signaling (Bolado-Carrancio et al. 2021). In lung cancer cell lines, ISGylation of Yes-associated protein (YAP) inhibits YAP ubiquitination, and proteasomal degradation, thereby promoting YAP stability and contributing to tumor initiation and development (Xue et al. 2022). An increase in ISGylation also exerts a negative regulatory effect on the ubiquitin-protease system, leading to increased reactive oxygen species (ROS) generation. Elevated ROS levels enhance the enzymatic activity of p38/MAPK and the expression of inflammation-related cytokines in macrophages, thereby accelerating inflammation and cancer transformation (Fan, Miyauchi-Ishida, et al. 2015). ISG15 has also been proposed as a prognostic marker for predicting the overall survival of HBV-related HCC patients (Qiu X et al. 2015). In HCC, ISG15 interferes with the interaction between survivin and X-linked inhibitor of apoptosis protein (XIAP), thereby stabilizing survivin and facilitating cell proliferation and migration (B. Chen et al. 2019; Su 2016). Collectively, current knowledge suggests that the ISG15 pathway is dysregulated during tumorigenesis. Conjugated ISG15 may adversely impact patients by stabilizing cellular proteins that promote cancer, primarily through negative regulation of the ubiquitin/proteasome pathway and interference with protein polyubiquitination/degradation. However, ISG15 can

exerts pro-tumor effects through regulation of additional processes. Thus, recent results revealed that ISG15 and ISGylation are also essential for mitochondrial functionality and are required for maintaining the metabolic plasticity of pancreatic cancer stem cells (PaCSCs) (Alcalá et al., 2020).

3.3.2 Anti-tumoral activity of ISG15

Overexpression of ISG15 has been shown to decrease proliferation and increase apoptosis, resulting in tumor suppression in cervix, blood and ovaries cancer (Zhou et al., 2017; Mao et al., 2016; Yeung et al., 2018). Different mechanisms have been proposed to be involved in the antitumor activity of ISG15 in those cancers, including the ISGylation of p53, suppression of the NF-kB signal transduction or the ISGylation of ERK. Another study reported the ISG15-mediated suppression of lung adenocarcinoma (ADC) growth. In this research, ISG15 expression was observed to exhibit a positive correlation with the levels of epithelial splicing regulatory protein 1 (ESRP1), a molecule involved in the epithelial–mesenchymal transition (EMT) process (Owhashi et al. 2003). ESRP1 plays a regulatory role in the alternative splicing process and diminishes the conversion of CD44 and FGFR2 subtypes in ADC. This action inhibits epithelial–mesenchymal transition (EMT) and hampers metastasis (Owhashi et al. 2003). ISG15, in this context, promotes the ISGylation of ESRP1, leading to its stabilization and a deceleration in the molecule's degradation. Consequently, this process results in the suppression of the EMT process (Owhashi et al. 2003). The ISG15 E1 enzyme UBE1L has also been reported to suppress the growth of lung cancer. The overexpression of UBE1L results in the inhibition of both human bronchial epithelial cells and lung cancer cells by facilitating the ISGylation of cyclin D1, and its degradation (Feng et al. 2008; Kok et al. 1993; Liu, Li, and Hassel 2003). ISG15 has been reported to have anti-tumor functions in acute promyelocytic leukemia (APL). APL is characterized by the presence of the oncogenic PML/RARα fusion protein (Kakizuka et al. 1991; Xiao et al. 1993). Treatment with retinoic acid induces UBE1L-mediated ISGylation of PML/RARα, resulting in a reduction in the levels of PML/RARα. This, in turn, counteracts the oncogenic effects of the fusion protein (Kitareewan et al. 2002; Pitha-Rowe et al. 2004; Shah et al. 2008).

Some researchers propose that while conjugated ISG15 has protumor functions, free ISG15 has antitumor functions and that tumor cells may inhibit secretion of ISG15 by conjugating it to proteins and consequently escaping immune surveillance. Thus, in contrast to the activity of conjugated ISG15 enhancing breast cancer growth (Bolado-Carrancio et al. 2021; Burks, Reed, and Desai 2014; Desai et al. 2012; Forys et al. 2014), free ISG15 has been reported to inhibit breast cancer growth (Burks, Reed, and Desai 2015). The molecular mechanisms involved in its antitumor activity are an enhancement of NK infiltration into tumors (Burks et al., 2015), the establishment of IFN gamma-mediated antitumor innate response (D'acunha et al., 1996) and the enhancement of MHC-class I antigen presentation (Burks et al. 2015). However, clear evidence supporting this hypothesis is still lacking.

3.4 UBIQUITIN AND UBIQUITIN-LIKE PROTEINS' INTERPLAY

The maintenance of cellular homeostasis demands meticulous control over protein activities, stability, and intracellular localization. This intricate regulation is frequently achieved through an array of posttranslational modifications (PTM) on proteins. These modifications often encompass the addition of small chemical substituent, such as phosphorylation, acetylation, and methylation. Alternatively, protein substrates can undergo covalent conjugation with additional polypeptides, including ubiquitin and ubiquitin-like proteins, further expanding the repertoire of regulatory mechanisms (Hochstrasser 2009). Despite its relatively small molecular weight

of approximately 8.6 kDa, ubiquitin exerts a potent influence on cellular processes, such as cell signaling, DNA repair, membrane-protein trafficking, and endocytosis (Oh, Akopian, and Rape 2018; Varshavsky 2017). Since its discovery in 1975, several ubiquitin-like proteins (Ubls) have been identified. These Ubls, such as interferon-stimulated gene 15 (ISG15), various paralogs of small ubiquitin-like modifier (SUMO), neural precursor cell expressed and developmentally downregulated 8 (NEDD8), human leukocyte antigen F locus 10 (FAT10), or ubiquitin-fold modifier 1 (UFM1), exhibit structural and evolutionary similarities to ubiquitin (Hochstrasser 2009; van der Veen and Ploegh 2012) and function as critical modulators of many cellular processes such as cell cycle control, DNA repair, autophagy, signal transduction, transcription, etc. Ubiquitin and Ubls covalently modify protein substrates through an enzymatic cascade involving E1, E2, and E3 enzymes. While the majority of ubiquitinated proteins are targeted for degradation by the 26S proteasome, ubiquitination is also involved in a variety of nonproteolytic cellular functions (Oh et al. 2018; van der Veen and Ploegh 2012). Similar to ubiquitin, the Ubls control an array of biological processes. Sometimes different Ubls can bind at the same lysine residue in substrates, competing for the same attachment site to trigger different reactions (Cuijpers, Willemstein, and Vertegaal 2017; Desterro, Rodriguez, and Hay 1998). For example, in the context of NF-κB activation, IκBα undergoes ubiquitination and subsequent degradation to release its inhibitory control over NF-κB (Chen and Chen 2013). In contrast, SUMOylation at the same site interferes with the ubiquitination process and degradation of IκBα (Desterro et al. 1998), thereby suppressing NF-κB activation. Additionally, Ubls can enhance the activation of ubiquitin ligases. Thus, in the case of Cullin-RING ubiquitin ligases (CRLs), the conjugation of NEDD8 to the Cullin scaffold proteins becomes imperative for achieving their maximal catalytic activity (Lydeard, Schulman, and Harper 2013).

3.5 ISG15 AND UBIQUITIN INTERPLAY

Although it has been stablished that ISG15 does not form polymeric chains, ISG15-ubiquitin mixed chains, formed by the conjugation of ISG15 to lysine K29 and K48 of ubiquitin, have been identified and an interplay between these two post-translational modifications has been reported (Fan, Arimoto, et al. 2015). Conjugation of ISG15 to ubiquitin is believed to counteract the ubiquitin pathway, preventing the degradation of proteins targeted for degradation by the ubiquitin-proteasome pathway. The precise mechanism by which ISGylation or ISG15-ubiquitin chains antagonizes ubiquitination is not fully elucidated, but competition for enzymes shared between the two processes and for substrate binding sites are proposed theories.

3.6 SUMO

The conjugation of small-ubiquitin-related modifier (SUMO) to its substrates or SUMOylation often orchestrates intermolecular interactions and conformational changes, affecting its substrates at the molecular level (Gareau and Lima 2010; Wilkinson and Henley 2010). By interacting with thousands of substrates, SUMO governs numerous cellular processes encompassing nuclear transport, transcription, chromatin remodeling, DNA repair, and ribosomal biogenesis.

The molecular weight of SUMO proteins is approximately 11 kDa and although these proteins are very similar to ubiquitin structurally, show less than 20% amino-acid sequence identity and distinct overall surface-charge distribution compared to ubiquitin (Bayer et al. 1998; Bernier-Villamor et al. 2002; Mossessova and Lima 2000). Similarly to ISG15, SUMO proteins are initially expressed in an immature pro-form, featuring a variable-length C-terminal extension (ranging from 2 to 11 amino acids) beyond the invariant Gly-Gly motif. The removal of this C-terminal extension by SUMO-specific proteases is essential for the conjugation of SUMO to its

targets. There are five SUMO paralogs in humans: SUMO1 to SUMO5. SUMO2 and SUMO3 mature forms exhibit a 97% identity in amino acid sequence and are usually denoted SUMO2/3, while they share only 50% sequence identity with SUMO1. SUMO2/3 is more abundant than SUMO1. SUMO2 and SUMO3 contain internal lysines that serve as SUMO acceptor sites, resulting in the formation of SUMO chains, a process referred to as polySUMOylation whereas SUMO1 does not form chains as readily as SUMO2/3 (Jansen and Vertegaal 2021). SUMO enzymes play a crucial role in efficiently catalyzing the generation of SUMO polymers. It is though that SUMO1 and SUMO2/3 may have distinct roles, since different studies implicate SUMO2/3 and not SUMO1 in global SUMOylation of cellular proteins in response to stress and they conjugate to different target proteins *in vivo* (Rosas-Acosta et al. 2005; Saitoh and Hinchey 2000; Vertegaal et al. 2006). SUMO4 and SUMO5 are the less well understood but it has been proposed that their expression is limited to specific tissues (Guo et al. 2004; Mattoscio, Medda, and Chiocca 2020; Owerbach et al. 2005). As said above, the SUMO conjugation cascade flows similarity to ISGylation cascade (Figure 5). First, mature SUMO is activated at its C-terminus by the SUMO-specific E1 activating enzyme SAE1/SAE2 (Desterro et al. 1999; Gong et al. 1999; Johnson 1997; Okuma et al. 1999). This step employs ATP to form a SUMO-adenylate conjugate, serving as an intermediate for the thioester bond formation between the C-terminal carboxy group of SUMO and the catalytic cysteine residue of E1. Subsequently, SUMO is transferred from E1 to the unique E2 conjugating enzyme, Ubc9, establishing a thioester linkage between the catalytic cysteine residue of Ubc9 and the C-terminal carboxy group of SUMO. Finally, Ubc9 transfers SUMO to the substrate, forming an isopeptide bond between the C-terminal Gly residue of SUMO and a lysine side chain on the target. SUMO E3 ligases, which catalyze the transfer of SUMO from Ubc9 to the substrate, typically facilitate this process (Desterro, Thomson, and Hay 1997; Johnson and Blobel 1997; Lee et al. 1998; Saitoh et al. 1998). SUMOylation is a reversible covalent enzymatic reaction due to the activity of specific proteases. In humans, the family of SUMO-specific cysteine proteases, known as sentrin-specific proteases (SENP1-3 and SENP5-7), has been identified. Importantly, SENP proteins also possess C-terminal hydrolase activity, which is essential for the maturation of newly synthesized SUMO proteins.

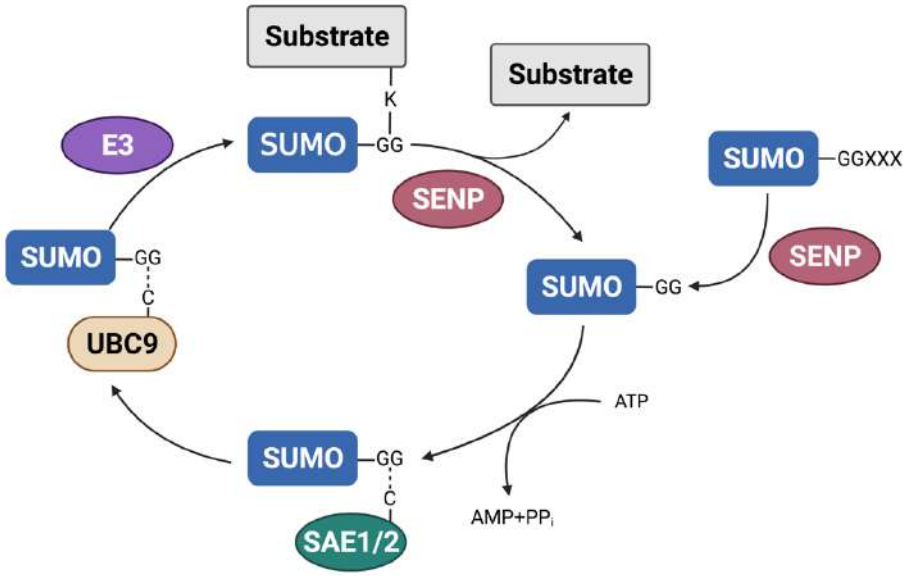


Figure 5. SUMO and the SUMOylation machinery
Mature SUMO protein can conjugate to other proteins through a three steps enzymatic process called SUMOylation: (i) The activation of SUMO by SAE1/SAE2 leading to the formation of an thioester bond with SUMO in an ATP-dependent manner (ii) SUMO is then transferred to the catalytic cysteine of the E2 conjugating enzyme Ubc9 (iii) The E2 enzyme alone or in concert with an E3 ligase catalyzes the formation of an isopeptide linkage between the C-terminal carboxyl group of SUMO and the ε-amino group of specific lysine residues of substrates. As a dynamic process, SUMOylation is reversible thanks to the action of a SUMO-specific proteases (SENP). (Figure designed with Biorender).

3.6.1 SUMO interaction motif

The effects of SUMO conjugation often depend on non-covalent protein-protein interactions between SUMO or SUMOylated proteins and proteins containing SUMO interaction motif (SIM) (Cappadocia and Lima 2018). SIMs typically consist of a hydrophobic core consisting in 3-4 aliphatic residues juxtaposed to a negatively charged cluster of amino acids (Song et al. 2004). SIMs have been proposed to facilitate the SUMOylation of the substrate. Interestingly, a recent study suggests that nearly 90% of SUMO-binding proteins containing SIMs are also SUMOylation substrates, indicative of a strong functional association between covalent and non-covalent SUMO interaction on protein targets (González-Prieto et al. 2021). The promyelocytic leukemia (PML) protein is one example of a SUMOylation substrate containing numerous SUMO interaction motifs (SIMs) (Borden 2002). The multiple SIMs in PML are required for facilitating the recruitment of SUMO and SUMOylated proteins within PML-NBs, modulating their assembly dynamics (Shen et al. 2006). In addition, SUMOylation is also essential for PML-NB function. SUMO/SIM interactions play a critical role in the formation of other membraneless organelles such as stress granules (Keiten-Schmitz et al. 2021). Other SUMO substrates containing SIMs are some deubiquitinating enzymes such as USP25. The SIM in USP25 is essential for its SUMOylation, a modification that negatively regulates its deubiquitinating activity (Meulmeester et al. 2008).

SIMs are also involved in SUMO chain formation and SUMO-dependent ubiquitination. The interaction between E3 enzymes and the E2-bound SUMO on the opposite side appears to facilitate the formation of SUMO chains. Some E3 ligases require SIMs to catalyze the formation of SUMO polymers (Eisenhardt et al. 2015).

PolySUMOylation has profound molecular implications for target proteins. One important signaling process initiated by polySUMOylation is SUMO-targeted ubiquitin ligases (STUbLs) pathway (Sriramachandran and Dohmen 2014). STUbls contain several SIMs which are necessary to recognize SUMO chains. The best well known STUbLs are RNF4 and RNF111 and catalyze non-proteolytic or proteolytic ubiquitination of the polySUMOylated proteins. STUbLs can mediate K48- or K63- ubiquitination or it can ubiquitinate lysine residues in SUMO synthesizing mixed SUMO-ubiquitin chains. K48-linked ubiquitin chains designate proteins for proteasomal degradation, when K63-linked chains predominantly govern non-proteolytic signaling roles, commonly associated with the DNA damage response.

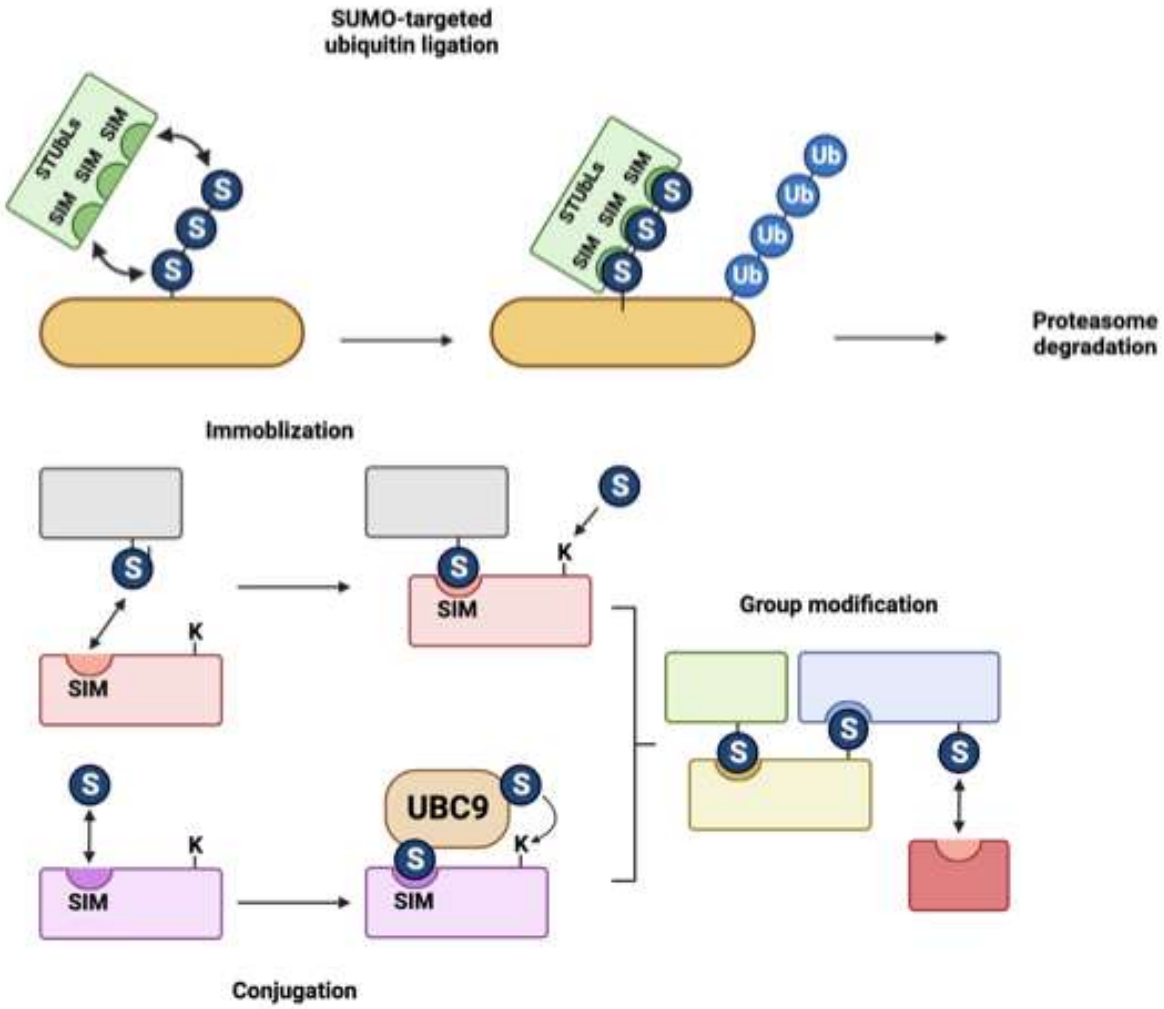


Figure 6. The SUMO Interaction Motif

SUMO interacting motifs (SIMs) in SUMO substrates can have a profound impact at different levels: **A** SUMO-targeted ubiquitin ligation: STUbLs is recruited to PoySUMOylated proteins and ubiquitinates them, leading to their proteasome degradation. **B** (i) Immobilization: proteins containing a SIM domain can be recruited to and immobilized on SUMOylated proteins, favoring their covalent interaction with SUMO. (ii) Conjugation: the SIM domain can facilitate the recruitment of SUMO-loaded Ubc9 to the protein, leading the covalent interaction of SUMO with a close lysine residue. (iii) SUMO/SIM interactions may facilitate the assembly and maintenance of cellular aggregates such as stress granules or other phase-separated membraneless organelles. (Figure designed with Biorender).

3.6.2 SUMO and viruses

SUMOylation have emerged as an important regulator of the type I IFN signaling pathway and the antiviral defense. Similarly to ISGylation, global SUMOylation is also induced by IFN but in this case through a mechanism mediated by the miRNA Lin28/Let7 axis (Sahin et al., 2014). SUMOylation then modulate innate and intrinsic immunity by acting at different levels of the IFN cascade. SUMO conjugates to STAT1 and inhibits viral-induced STAT1-phosphorylation (Begitt et al. 2011; Everett, Boutell, and Hale 2013). Moreover, SUMOylation of the transcription factors IRF3 and IRF7 inhibit virus-induced IFN synthesis (El-Asmi et al. 2020a). In contrast, the antiviral activity of different restriction factors such as PML, PKR and p53 require their SUMOylation (El-Asmi et al. 2020).

To neutralize the action of SUMOylation, viruses have developed different strategies to inhibit or exploit the SUMOylation pathway. Multiple investigations have underscored the significance of SUMOylation in facilitating viral infections (Gurer, Berthoux, and Luban 2005; Lamsoul et al. 2005; Yueh et al. 2006). This phenomenon can be generally divided into two categories: viruses that exploit the SUMOylation pathway to modify their own proteins and improve replication, and viruses that modulate the host SUMO pathway (Boggio and Chiocca 2006). Epstein-Barr virus (EBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and herpes simplex viruses (HSV) are just a few examples of viruses that exploit the host's SUMOylation machinery to enhance their replication (Brown et al. 2016; Conn et al. 2016; Li et al. 2012; Schaller et al. 2011; Sengupta et al. 2017). Thus, interactions between Ubc9 and the HIV-1 envelope protein gp120 lead to the stabilization of gp120, consequently enhancing the infectivity of HIV-1 (Jaber et al. 2009). Influenza virus type A (IAV) infection markedly augments the SUMO1 and SUMO2/3 modification of cellular substrates (Domingues et al. 2015) and specific viral proteins such as the EBV oncoprotein LMP1 acts to impede SUMO-protease activity, promoting the accumulation of SUMOylated proteins. Notably, LMP1 induces SUMOylation of the SUMO-protease SENP2 at specific lysine residues, K48 and K447, thereby diminishing SENP2 function, subcellular translocation, and overall stability (Selby et al. 2019). In contrast to the essential role of SUMO or the E2 SUMO-conjugating enzyme, Ubc9, in the replication of some viruses, SUMOylation of some cellular proteins have been identified as restriction factors against viral replication. Thus, the EBV protein kinase BGLF4 attenuates global cellular SUMOylation, thereby enhancing virus production (Schaller et al. 2011). In the context of HSV, the early viral protein ICP0 plays a pivotal role in initiating lytic infection. The expression of ICP0 in a stable cell line results in a reduction in overall SUMO conjugates, including SUMOylated PML forms, a mechanism to counteract the innate anti-HSV defense (Boutell et al. 2011).

3.6.3 SUMO and cancer

Changes in the expression or function of various components of the SUMO signaling pathway have a profound impact in the cell physiology (Bergink and Jentsch 2009) and the substantial involvement of SUMOylation in human tumorigenesis has become increasingly evident. SUMOylation has the capacity to modulate cell proliferation, confer resistance to apoptosis, and enhance metastatic potential by governing proteins implicated in carcinogenesis (Sarangi and Zhao 2015). Numerous investigations have reported an increased expression of SAE1/SAE2, Ubc9, and SUMO E3 ligases in different types of cancer (Chen et al. 2011; Hoellein et al. 2014; Zhang et al. 2013). Conversely, the expression of deSUMOylases (SENP) has been found upregulated in other cancers (Brems-Eskildsen et al. 2010; Han et al. 2010; SUN et al. 2013; Xiaojun Ding et al. 1994). Interestingly, in some cases, elevated levels of both SUMOylating and deSUMOylation enzymes have been detected (Zhu et al. 2022), which may indicate the necessity for an accelerated SUMO cycle, involving heightened SUMOylation, de-SUMOylation, and increased SUMO turnover. Prostate cancer, as an illustrative case, has been associated with elevated levels of both Ubc9 and the E3 SUMO-protein ligase PIAS1. Additionally, increased expression of SENP1 and SENP3 enzymes has been documented in this context (Bawa-Khalfe and Yeh 2010). One of the SUMO substrates commonly dysregulated in human cancers and influencing prognosis is the transcription factor c-Myc (Adler et al. 2006; Chen and Olopade 2008; Deming et al. 2000). The E1 SUMOylation enzyme SAE1/SAE2 has been demonstrated to be essential for Myc-driven tumorigenesis (Kessler et al. 2012). In contrast, SENP1, an enzyme responsible for c-Myc de-SUMOylation, significantly impacts

cancer cell growth and proliferation by preventing c-Myc proteasomal degradation, thereby enhancing its transcriptional activity and promoting tumorigenesis (Sun et al. 2018).

A study has identified a correlation between Ubc9 expression and cancer development in breast, lung, and prostate cancer. This study found that primary tumors tend to exhibit higher Ubc9 expression, whereas distant metastatic lesions often show lower or normal levels of Ubc9 expression (Moschos et al. 2010).

3.7 ISG15 AND SUMO

The innate immune response to viral infection is dependent on IFN synthesis and is often beneficial for virus clearance, but it can have pro- and antitumorigenic effects. IFNs exert their effects through autocrine and paracrine mechanisms, binding to their receptors and activating the JAK/STAT pathway, thereby inducing the expression of over a hundred ISGs, which include ISG15 (Ivashkiv and Donlin 2014; Schneider, Chevillotte, and Rice 2014; Wang et al. 2017). As explained above, some ISGs serve as restriction factors, mediating the IFN-induced antiviral state (Chelbi-Alix and Wietzerbin 2007). Notably, certain restriction factors are not only involved in the innate immune response but are also constitutively expressed, playing a role in intrinsic antiviral activity. Interestingly, several evidences suggest that SUMO can modulate both innate and intrinsic immunity by influencing IFN synthesis, JAK/STAT signaling, and the expression and function of certain ISG restriction factors (Crowl and Stetson 2018; Hannoun, Maarifi, and Chelbi-Alix 2016; Maarifi et al. 2015). Whether ISG15 is among the proteins regulated by SUMO is still unknown and is the main objective of this work.

4. OBJECTIVES

ISG15 is a ubiquitin-like protein that is strongly upregulated in response to type I interferon and different types of stress. An upregulation of ISG15 has also been found in different types of cancer. In cells, ISG15 is found conjugated or unconjugated to substrates. In addition, unconjugated ISG15 can be secreted from the cell. ISG15 is a multifunctional protein that impact a diversity of cellular processes such as immune modulation, apoptosis, autophagy, protein translation, and DNA damage response. The molecular consequences and underlying mechanisms of ISG15 are still unclear but it has been proposed that ISG15 functions may depend on the cell type, cell context, the specie, and ISG15 form (intracellular unconjugated, conjugated or extracellular free form). Thus, it has been suggested that ISG15 can exert pro-tumoral, anti-tumoral, pro-viral or antiviral activities depending on the cell context, the species, viral agent, and ISG15 form. Understanding the mechanisms of regulation of ISG15 activities could help to develop new strategies to intervene in the progression of many diseases. It has been reported the existence of an extensive crosstalk between ubiquitin-like proteins including the conjugation of ISG15 to ubiquitin, the conjugation of ubiquitin to SUMO or the SUMO conjugation to ubiquitin. We hypothesized that SUMO could also interact with ISG15 and that this interplay may impact ISG15 properties and/or activities. Therefore, the specific objectives of this project are:

Objective 1. To study the potential post-translational modification of ISG15 by SUMO.

Objective 2. To analyze the effect of SUMO conjugation on ISG15 properties and functions.

Objective 3. To evaluate the potential non-covalent interaction between ISG15 and SUMO.

Objective 4. To evaluate how the non-covalent interaction of SUMO with ISG15 impact on ISG15 and its functions.

Objective 4. To Study the regulation of the covalent interaction between ISG15 and SUMO.

5. MATERIALS AND METHODS

5.1 CELL CULTURE

5.1.1 Cell lines

In this study we have used human embryonic kidney 293 (HEK-293) cells, ISG15-wild type (WT) and ISG15-knockout (KO) human lung epithelial carcinoma A549 cells, human bone osteosarcoma epithelial U2OS cells, cells derived from the U2OS cell line that express an IPTG-inducible p14ARF (alternate reading frame) tumor suppressor gene (NARF2), African green monkey kidney epithelial BSC40 cells, and prostate cancer PC3 cells.

5.1.2 Cell culture media

All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Corning), 10 U/mL of penicillin-streptomycin (Gibco) and 2 mM of L-Glutamine (Gibco). Cells were passaged upon confluency.

5.1.3 Cell transfection

Cells were transfected with DNA plasmids using 1 mg/mL polyethylenimine (PEI) (Polysciences, Inc, Hirschberg an der Bergstrasse, Germany). The appropriate volume of PEI diluted in serum and antibiotic-free DMEM was added to a tube containing the appropriate amount of DNA plasmid diluted in serum and antibiotic-free DMEM (3:1 ratio PEI-DNA) and incubated for 20 min at room temperature. Then, the mix was added to the cells.

5.2 BACTERIAL TRANSFORMATION AND COMPETENT CELLS

5.2.1 Competent bacteria production

Escherichia coli DH5α or STBL3 were grown overnight at 37°C on Luria-Bertani (LB) agar plates. The next day a colony was picked and grown in 10 mL of LB broth overnight at 37°C with shaking (225 rpm). The next morning bacteria were refreshed into 1 L of LB and incubated at 37°C with shaking until reaching an optical density (OD) of 0.35-0.4 at 600 nm. The culture was chilled in ice for 20 min and bacteria were harvested by centrifugation at 3000 x g for 15 min at 4°C. The pellet was resuspended in 100 mM of $MgCl_2$ and centrifugated at 2000 x g for 15 min at 4°C. The pellet was resuspended in 85 mM of $CaCl_2$ and centrifugated at 1000 x g for 15 min at 4°C. Finally, the pellet was resuspended in 2 mL of 85 mM of $CaCl_2$ with 15% glycerol and aliquoted before being snap frozen in liquid nitrogen. Competent bacteria were stored at -80°C.

5.2.2 Bacterial transformation

Competent bacteria were thawed on ice, the plasmid DNA was mixed with the chilled cells and incubated on ice for 30 min. The mixture was then exposed for 40 s at 42°C and cooled immediately on ice. The bacteria were grown for 1 h at 37°C with shaking (600 rpm) in 500 µL of LB broth. Finally, the cells were harvested by centrifugation at 4000 x g for 5 min and resuspended in 100 µL of LB broth before being plated on LB agar plates supplemented with the appropriate antibiotic.

5.2.3 DNA plasmid purification

DNA plasmids were purified following manufacturer's instructions using GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific) or DNA Purification Midiprep kit (Macherey-Nagel). Briefly, a colony was picked and grown in LB broth supplemented with the proper antibiotic

overnight at 37°C with shaking at 225 rpm. The next day, bacteria were harvested by centrifugation, resuspended in the resuspension buffer and lysed with the lysis buffer. After adding the neutralization buffer, the mixture was clarified by centrifugation and loaded onto a silica column. Columns were washed with the washing buffer and the DNA plasmid was eluted with the elution buffer before being precipitated with isopropanol. Finally, the DNA plasmid was washed with 70% ethanol and resuspended in sterile Milli-Q water.

5.3 PLASMIDS

pCAGGS-v5-hISG15-WT: Plasmid encoding for the mature human ISG15 WT protein, fused to V5 tag. Kindly provided by Susana Guerra (Eduardo-Correia et al. 2014).

pCAGGS-v5-hISG15-WT-AA: Plasmid encoding for the mature human ISG15 WT protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to V5 tag. Kindly provided by Susana Guerra (Eduardo-Correia et al. 2014).

pCAGGS-v5-mISG15-WT: Plasmid encoding for the mature mouse ISG15 WT protein, fused to V5 tag. Kindly provided by Susana Guerra (Eduardo-Correia et al. 2014).

pCAGGS-v5-mISG15-WT-AA: Plasmid encoding for the mature mouse ISG15 WT protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to V5 tag. Kindly provided by Susana Guerra (Eduardo-Correia et al. 2014).

HA-hISG15-WT: Plasmid encoding for the mature human ISG15 WT protein, fused to Hemagglutinin (HA) tag. It was obtained by subcloning ISG15 WT from pCAGGS-v5 vector into pCMV-HA using oligos listed in table 1.

HA-hISG15-WT-AA: Plasmid encoding for the mature human ISG15 WT protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to HA tag. It was obtained by subcloning hISG15-WT-AA from pCAGGS-v5 vector into pCMV-HA using oligos listed in table 1.

HA-hISG15-K: Plasmid encoding for the mature human ISG15 protein in which the lysine residues K8, K29, K35, K77, K90, K108, K129, and K143 were mutated to arginine, fused to HA tag. It was obtained by site directed mutagenesis of HA-hISG15-WT using oligos listed in table 1.

HA-hISG15-K-AA: Plasmid encoding for the mature human ISG15 protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), and the lysine residues K8, K29, K35, K77, K90, K108, K129, and K143 were mutated to arginine, fused to HA tag. It was obtained by site directed mutagenesis of HA-hISG15-K vector using oligos listed in table 1.

HA-hISG15-1xΔSIM: Plasmid encoding for the mature human ISG15 protein in which the SIM domain 82-LSILV-86 was mutated to 82-AAAAA-86, fused to HA tag. It was obtained by site directed mutagenesis of HA-hISG15-WT vector using oligos listed in table 1.

HA-hISG15-2xΔSIM: Plasmid encoding for the mature human ISG15 protein in which the SIM domains 82-LSILV-86 and 72-LLVVD-76 were mutated to 82-AAAAA-86 and 72-

AAAAA-76, respectively, fused to HA tag. It was obtained by site directed mutagenesis of HA-hISG15-1xΔSIM vector using oligos listed in table 1.

HA-hISG15-2xΔSIM-AA: Plasmid encoding for the mature human ISG15 protein in which the SIM domains 82-LSILV-86 and 72-LLVVD-76 were mutated to 82-AAAAA-86 and 72-AAAAA-76, respectively, and the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to HA tag. It was obtained by site directed mutagenesis of HA-hISG15-2xΔSIM vector using oligos listed in table 1.

His6-hISG15-WT: Plasmid encoding for the mature human ISG15 WT protein, fused to 6-Histidine (His6) tag. It was obtained by subcloning hISG15-WT from HA-hISG15-WT vector into pcDNA-His6 using oligos listed in table 1.

His6-hISG15-WT-AA: Plasmid encoding for the mature human ISG15 WT protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to His6 tag. It was obtained by subcloning hISG15-WT-AA from HA-hISG15-WT-AA vector into pcDNA-His6 using oligos listed in table 1.

His6-hISG15-K: Plasmid encoding for the mature human ISG15 protein in which the lysine residues K8, K29, K35, K77, K90, K108, K129, and K143 were mutated to arginine, fused to His6 tag. It was obtained by subcloning hISG15-K from HA-hISG15-K vector into pcDNA-His6 using oligos listed in table 1.

His6-hISG15-K-AA: Plasmid encoding for the mature human ISG15 protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), and the lysine residues K8, K29, K35, K77, K90, K108, K129, and K143 were mutated to arginine, fused to His6 tag. It was obtained by subcloning hISG15-K-AA from HA-hISG15-K-AA vector into pcDNA-His6 using oligos listed in table 1.

His6-hISG15-2xΔSIM: Plasmid encoding for the mature human ISG15 protein in which the SIM domains 82-LSILV-86 and 72-LLVVD-76 were mutated to 82-AAAAA-86 and 72-AAAAA-76, respectively, fused to His6 tag. It was obtained by subcloning hISG15-2xΔSIM from HA-hISG15-2xΔSIM vector into pcDNA-His6 using oligos listed in table 1.

His6-hISG15-2xΔSIM-AA: Plasmid encoding for the mature human ISG15 protein in which the SIM domains 82-LSILV-86 and 72-LLVVD-76 were mutated to 82-AAAAA-86 and 72-AAAAA-76, respectively, and the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to His6 tag. It was obtained by subcloning hISG15-2xΔSIM-AA from HA-hISG15-2xΔSIM-AA vector into pcDNA-His6 using oligos listed in table 1.

pInducer2.0: Tet-inducible lentiviral vector was obtained from Addgene (#44012) (Meerbrey et al. 2011).

pInducer2.0-HA-hISG15-WT: Tet-inducible lentiviral vector encoding for the mature human ISG15 WT protein, fused to HA tag. It was obtained by subcloning HA-hISG15-WT from HA-hISG15-WT vector into pInducer2.0 using oligos listed in table 1.

pInducer2.0-HA-hISG15-WT-AA: Tet-inducible lentiviral vector encoding for the mature human ISG15 WT protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to Hemagglutinin HA tag. It was obtained by subcloning HA-hISG15-WT-AA from HA-hISG15-WT-AA vector into pInducer2.0 using oligos listed in table 1.

pInducer2.0-HA-hISG15-K: Tet-inducible lentiviral vector encoding for the mature human ISG15 protein in which the lysine residues K8, K29, K35, K77, K90, K108, K129, and K143 were mutated to arginine, fused to HA tag. It was obtained by subcloning HA-hISG15-K from HA-hISG15-K vector into pInducer2.0 using oligos listed in table 1.

pInducer2.0-HA-hISG15-K-AA: Tet-inducible lentiviral vector encoding for the mature human ISG15 protein in which the C-terminal diglycine (GG) motif was replaced by alanine (AA), and the lysine residues K8, K29, K35, K77, K90, K108, K129, and K143 were mutated to arginine, fused to HA tag. It was obtained by subcloning HA-hISG15-K-AA from HA-hISG15-K-AA vector into pInducer2.0 using oligos listed in table 1.

pInducer2.0-HA-hISG15-2xΔSIM: Tet-inducible lentiviral vector encoding for the mature human ISG15 protein in which the SIM domains 82-LSILV-86 and 72-LLVVD-76 were mutated to 82-AAAAA-86 and 72-AAAAA-76, respectively, fused to HA tag. It was obtained by subcloning HA-hISG15-2xΔSIM from HA-hISG15-2xΔSIM vector into pInducer2.0 using oligos listed in table 1.

pInducer2.0-HA-hISG15-2xΔSIM-AA: Tet-inducible lentiviral vector encoding for the mature human ISG15 protein in which the SIM domains 82-LSILV-86 and 72-LLVVD-76 were mutated to 82-AAAAA-86 and 72-AAAAA-76, respectively, and the C-terminal diglycine (GG) motif was replaced by alanine (AA), fused to HA tag. It was obtained by subcloning HA-hISG15-2xΔSIM-AA from HA-hISG15-2xΔSIM-AA vector into pInducer2.0 using oligos listed in table 1.

pcDNA3.1: Empty vector (Invitrogen).

pcDNA3.1-v5-Ubc9: Expression plasmid encoding for the SUMO conjugation enzyme Ubc9 fused to V5 tag. Kindly provided by Manuel S. Rodríguez (Rodríguez et al., 2001).

pcDNA-His6-SUMO1: Expression plasmid encoding for His6-tagged SUMO1 protein. Kindly provided by Manuel S. Rodríguez (Rodríguez et al., 2001).

pcDNA3.1-His6-SUMO2: Expression plasmid encoding for His6-tagged SUMO2 protein. Kindly provided by Manuel S. Rodríguez (Rodríguez et al., 2001).

ISG15-E1: Expression plasmid encoding for the ISG15 activating enzyme UBE1L. Kindly provided by Adolfo García-Sastre (Versteeg et al. 2010).

ISG15-E2: Expression plasmid encoding for the ISG15 conjugation enzyme UbcH8. Kindly provided by Adolfo García-Sastre (Versteeg et al. 2010).

ISG15-E3: Expression plasmid encoding for the ISG15 ligase enzyme HERC5. Kindly provided by Adolfo García-Sastre (Versteeg et al. 2010b).

pcDNA-p14ARF: Plasmid encoding for p14ARF protein. Kindly provided by Susana Llanos (Kim, Mitchell, Fujii, Llanos, & Peters, 2003).

p14ARF-GFP: Plasmid encoding for p14ARF protein fused to green fluorescent protein (GFP). Kindly provided by Susana Llanos (Kim, Mitchell, Fujii, Llanos, & Peters, 2003).

pGFP: Plasmid encoding for GFP (Clontech).

5.4 CLONING

Coding sequence of the gene of interest was amplified by polymerase chain reaction (PCR) using appropriate primers (listed in table 1), containing sites for restriction enzymes, and a high-fidelity DNA Polymerase (Phusion High-Fidelity Polymerase II, Thermo Fisher Scientific), following the manufacturer's instructions. The PCR products and vectors were digested with adequate restriction enzymes following the indications of the manufacturer (FastDigest, restriction enzymes, Thermo Fisher Scientific) and purified from agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific). Purified inserts and vectors were ligated using T4 DNA ligase for 1 h at room temperature. Finally, the ligation product was transformed in competent bacteria. The resulting colonies were analyzed by restriction enzyme analysis and the positive ones were sent for sequencing.

5.5 MUTAGENESIS

Site-direct mutagenesis was carried out using Phusion High-Fidelity Polymerase II (Thermo Fisher Scientific) or the Quick-Change site-directed mutagenesis kit (Agilent) using primers listed in table 1, and following the manufacturer's instructions. Briefly, the PCR product was incubated with Dpn1 restriction enzyme for at least 1h at 37°C. The product was then transformed in competent bacteria and the resulting colonies were grown. Plasmid DNA was sent for sequencing.

5.6 ANTIBODIES

The primary antibodies used in this work were: anti-HA (#901503, Biolegend), anti-ISG15 (#2743, Cell Signaling), anti-SUMO1 (#4940, Cell Signaling), anti-GAPDH (#sc-32233, Santa Cruz Biotechnology), anti-Actin (sc-4778, Santa Cruz Biotechnology), anti-GFP (#902605, Biolegend), anti-Histidine (#MA1-21315, Thermo Fisher), anti-Histone3 (#4499T, Cell Signaling), anti-HA (#A190-138A, Bethyl Laboratories), anti-influenza A virus NS1 protein (#GTX125990, Gene Tex), anti- VSV G (a generous gift of Dr I Ventoso, CBMSO, Madrid) anti-PML (#377103, Santa Cruz Biotechnology), anti-Tom20 (#sc-17764, Santa Cruz Biotechnology) and anti-p14ARF (#2407, Cell Signaling). The secondary antibodies used in this work were anti-rabbit (#a16035, Invitrogen) or anti-mouse (#a16072, Invitrogen) antibodies, both conjugated to horseradish peroxidase (HRP) for Western blot and Donkey Anti-Rabbit IgG H&L, Donkey Anti-Goat IgG H&L and Donkey Anti-Mouse IgG H&L, conjugated to Alexa-488 or Alexa-594 for immunostaining (#ab150073, #ab150129, #ab150105, #ab150108, #ab150076 and # ab150132, Abcam)

5.7 *In vitro* SUMOYLATION ASSAY

A mixture of 50 mM of Tris pH 7.5, 5 mM $MgCl_2$, 10 mM of Creatine Phosphate, 3.5 U/mL of Creatine Kinase, 0.6 U/mL of inorganic pyrophosphate, 0.3 µg of SAE1/2 (Biomol, Enzo Life Sciences), 600 ng of Ubc9, 10 µg of SUMO1 or SUMO2 and the recombinant inactive ISG15 protein (Abcam #ab173044 and Enzo Life science #BML-UW9230-0500), was incubated for

90 min at 37°C. The reaction was stopped by adding Laemmli buffer and boiling for 5 min at 100°C. Samples were loaded on an SDS-PAGE and analyzed by Western-blot.

5.8 *in vitro* DESUMOYLATION ASSAY

The SUMOylated protein was incubated with 2 µg of GST-SENP1 (Biomol) for 1 h at 37°C. The reaction was stopped by adding Laemmli buffer and boiling for 5 min at 100°C. Samples were loaded on an SDS-PAGE and analyzed by Western-blot.

5.9 PROTEIN ELECTROPHORESIS AND WESTERN BLOT

Purified proteins, recombinant proteins or whole cell extracts were resuspended in Laemmli buffer, boiled for 5 min at 100°C and loaded on SDS-PAGE gels. After separation, the proteins were transferred to a nitrocellulose membrane and blocked in 5% non-fat dry milk in Tween20 Tris-buffered saline (TTBS) buffer (150 mM NaCl, 10 mM Tris pH 8.0, 0.1% v/v Tween 20) for 20 min at room temperature. The blocked membranes were incubated with the primary antibody diluted in 5% non-fat dry milk or 2% bovine serum albumin (BSA) in TTBS at 4°C with shaking overnight. The next day, membranes were washed 3 times (10 min per wash) with TTBS and then incubated with the secondary antibody diluted in 5% non-fat dry milk in TTBS at room temperature with shaking for 1 h. The membranes were washed 4 times with TTBS (10 min per wash). Finally, membranes were incubated with enhanced chemiluminescence (ECL) solution and exposed to X-ray films.

5.10 HISTIDINE PURIFICATION

Cells were washed with phosphate-buffered saline (PBS) and recovered by scraping in PBS. 10% of the cell suspension was collected to a tube and cells were pelleted by centrifugation at 1000 x g for 5 min. The pellet was resuspended in SDS-sample buffer and boiled for 5 min at 100°C (input). The remaining cell suspension was centrifuged at 1000 x g for 5 min and cells were lysed in buffer G (6 M guanidine HCl, 0.1 M Na_2HPO_4, 6.8 mM NaH_2PO_4, 25 mM Tris-HCl pH 8). Cell lysate was subjected to sonication (3 times, 15 sec at 10%) and lysates were then incubated with TALON® Nickel Affinity Resin beads (Clontech) on a rotating wheel for 2 h at room temperature. Finally, the nickel beads were washed 4 times with 1 mL of buffer U (8 M Urea, 100 mM Tris-HCl pH 8, 93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4) and the Histidine-tagged purified proteins were resuspended in Laemmli buffer and boiled for 5 min at 100°C.

5.11 SUBCELLULAR FRACTIONATION

Cells cultured in 10 cm plates were harvested and centrifuged at 14,000 rpm for 10 s. The resulting pellet was resuspended in 1 mL of 0.1% NP40 in PBS. After centrifugation at 14,000 rpm for 10 s, 300 µL of the supernatant were collected as the cytoplasmic fraction, supplemented with 75 µL of 4X Laemmli buffer, and boiled at 100º C for 5 min. The pellet was resuspended in 1 mL of 0.1% Nonidet-P-40 (NP40) in PBS. Following centrifugation (14,000 rpm, 10 s), the supernatant was discarded, and the pelleted nuclei were resuspended in 30 µL of Laemmli buffer and boiled at 100º C for 5 min.

5.12 GST-PULL DOWN

GST-pulldown experiments were conducted using either the recombinant human ISG15 protein (ab268685, Abcam) or protein extracts obtained from cells transfected with HA-hISG15-WT, HA-hISG15-1xΔSIM or HA-hISG15-2xΔSIM. Recombinant proteins or protein extracts were

pre-incubated in GST-binding buffer (20 mM Tris-HCl pH 8.8, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5% NP40) with GST for 1 h at 4ºC. A second incubated in GST-binding buffer (20 mM Tris-HCl pH 8.8, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5% NP40) was performed with either GST or GST-SUMO1 proteins immobilized on glutathione sepharose beads (GE Healthcare, #17-0756-01), for 2 h at 4ºC. Following the incubation, the resin was washed four times with the same buffer. Subsequently, sepharose beads were resuspended in Laemmli buffer and boiled for 5 min at 100ºC. The proteins bound to GST were then evaluated by Western-blot analysis.

5.13 MTT ASSAY

1000 A549 WT, A549 ISG15-KO or A549 ISG15-KO stably expressing inducible HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA cells were seed in 96-well plates. A549 WT or A549 ISG15-KO cells were treated or not with 1000 U/mL of IFN-α. Cells stably expressing ISG15 in an inducible manner were treated with 1µg/ml doxycycline (DOXY) for 24 h before their incubation in presence or not of 1000 U/mL of IFN-α. A solution of 2 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was added to each well prior to an incubation at 37º C and 5 % CO2 for 2 h. After removing the medium, plates were stored at -20°C until further processing. Finally, the precipitate was resuspended in 100 µL of DMSO and the absorbance of the solution was measured (at 530 nm) using a Berthold microplate reader.

5.14 COLONY FORMATION ASSAY

200 cells from A549 WT, A549 ISG15-KO were seed in P6-well plates. Cells were treated with 1000 U/mL of IFN-α and left at 37º C and 5 % CO2 for 6 days. After removing the medium, cells were fixed with 2% paraformaldehyde solution in PBS for 20 min. The fixation solution was removed and cells were stained using crystal violet staining solution.

5.15 APOPTOSIS ASSAY

The percentage of apoptotic cells was measured using FITC Annexin V Apoptosis Detection Kit (ImmunoStep) following manufacturer's instructions. Briefly, A549 WT or A549 ISG15-KO cells treated or not with 1000 U/mL of IFN-α were harvested, washed with PBS and resuspended in Annexin-binding buffer containing Annexin V-FITC and Propidium iodide (PI). Cell suspension was incubated for 15 min at room temperature in the dark. Finally, cells were analyzed by flow cytometry.

5.16 VIRAL INFECTION

Cells were infected with vesicular stomatitis virus (VSV) Indiana strain, recombinant VSV expressing GFP (VSV-GFP), or the mouse-adapted influenza A/PR/1934 (PR8) virus. Infections with VSV were carried out in FBS-free medium. PR8 infections were carried out in 0.3% bovine serum albumin (BSA) in PBS. One hour after incubation with the virus, unbound viral particles were removed, and fresh FBS-free medium was added to the cells. In the case of the influenza virus, FBS-free medium was supplemented with TPCK-treated trypsin (2 µg/mL).

5.17 VIRAL TITRATION

BSC40 cells were cultured in 12-well plates until a monolayer was formed. Subsequently, cells were washed with PBS, and virus serial dilutions were added to the monolayer. After 1 h incubation with periodic rocking, virus was aspirated, and 1 mL of agar solution (1.8 %) in 2x DMEM was added to each well. Following agar solidification, plates were incubated at 37º C

and 5 % CO2 for 1-2 days. The agar was removed and cells were stained using crystal violet staining solution.

5.18 GENERATION OF STABLE CELL LINES

A549 ISG15-KO cells were transfected with His6-hISG15-WT, His6-hISG15-AA, His6-hISG15-K, His6-hISG15-K-AA, His6-hISG15-2xΔSIM or His6-hISG15-2xΔSIM-AA and 48 h after transfection, cells were tripsinized and treated with G418 (1000 μg/mL) for 15 days. Resistant cells were amplified and evaluated for the expression of the transfected plasmids. In addition, A549 ISG15-KO cells were transduced with lentiviral particles expressing HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM, HA-hISG15-2xΔSIM-AA or pInducer2.0 in an inducible manner. First, HEK-293 cells were co-transfected with PLP1, PLP2, VSV-G and pInducer2.0-HA-hISG15-WT, pInducer2.0-HA-hISG15- WT-AA, pInducer2.0-HA-hISG15-K, pInducer2.0-HA-hISG15-K-AA, pInducer2.0-HA-hISG15-2xΔSIM, pInducer2.0-HA-hISG15-2xΔSIM-AA or pInducer2.0. 36 h after transfection, cell supernatant was collected, filtered (through a 0.45 μm filter), supplemented with polybrene and added to A549 ISG15-KO cells. The process was repeated three times (each 12 h). Transduced cells were tripsinized and incubated with G418 (600 μg/mL) for 15 days. Resistant cells were amplified, treated with 1μg/ml DOXY for 24 h and evaluated for the expression of the plasmids using Western blot.

5.19 IMMUNOFLUORESCENCE ASSAY

Cells were grown on coverslips and transfected as mentioned in each experiment. Cells were fixed in 2 % paraformaldehyde solution in PBS for 20 min, and permeabilized in 0.25 % Triton X-100 solution in PBS for 20 min. The non-specific binding sites were blocked with 2 % BSA in PBS for 20 min. Cells were then incubated with the proper primary antibody overnight at 4°C. The next day, cells were washed with PBS (3 times, 5 min each) and incubated with the appropriate Alexa-conjugated secondary antibody for 1 h at room temperature. After three washes with PBS, the nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI), and washed again with PBS. Coverslips were finally mounted using ProLong Diamond antifade mounting medium (P36970, Thermo Fisher Scientific) and preparations were analyzed by confocal microscopy (Leica SP5).

5.20 IMAGE PROCESSING

Confocal images were processed using Adobe Photoshop CC 2019.

5.21 *IN SILICO* ANALYSIS OF SUMOYLATION SITES AND SIMs

SUMOylation sites and SUMO interaction motifs were predicted using SUMOsp software (GPS-SUMO) (**http://sumosp.biocuckoo.org/online.php**).

5.22 CREATION OF FIGURES

Figures were created using Biorender software.

5.23 STATISTICAL ANALYSIS

Statistical analysis was performed with GraphPad Prism software using Student's t-test or ANOVA-one way. At least 3 biological replicates were analyzed for each condition.

6 RESULTS

6.1 ISG15 IS MODIFIED BY SUMO

6.1.1 ISG15 is modified by SUMO *in vitro*

To evaluate whether ISG15 is modified by SUMO, we performed an *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2 and using recombinant Histidine (His)-tagged human (h) ISG15 protein as a substrate. Western-blot analysis of the *in vitro* SUMOylation reactions with anti-His antibody revealed the unmodified His-hISG15 protein as a band of around 17 kDa molecular weight, as expected. We observed the appearance of at least two additional higher molecular weight bands (of around 36 kDa and 55 kDa molecular weight) when SUMO1 or SUMO2 were added to the reaction (Figure 7A). To further demonstrate that the higher molecular weight bands correspond to ISG15-SUMO conjugates, we performed a deSUMOylation assay. The ISG15-SUMO2 protein resulted from an *in vitro* SUMOylation assay was incubated with the recombinant SUMO-specific protease SENP1. The higher molecular weight bands observed when ISG15 was incubated with SUMO2 were almost undetectable after incubation with SENP1 (Figure 7B). Altogether, these results indicated that ISG15 is modified by SUMO1 and SUMO2 *in vitro*.

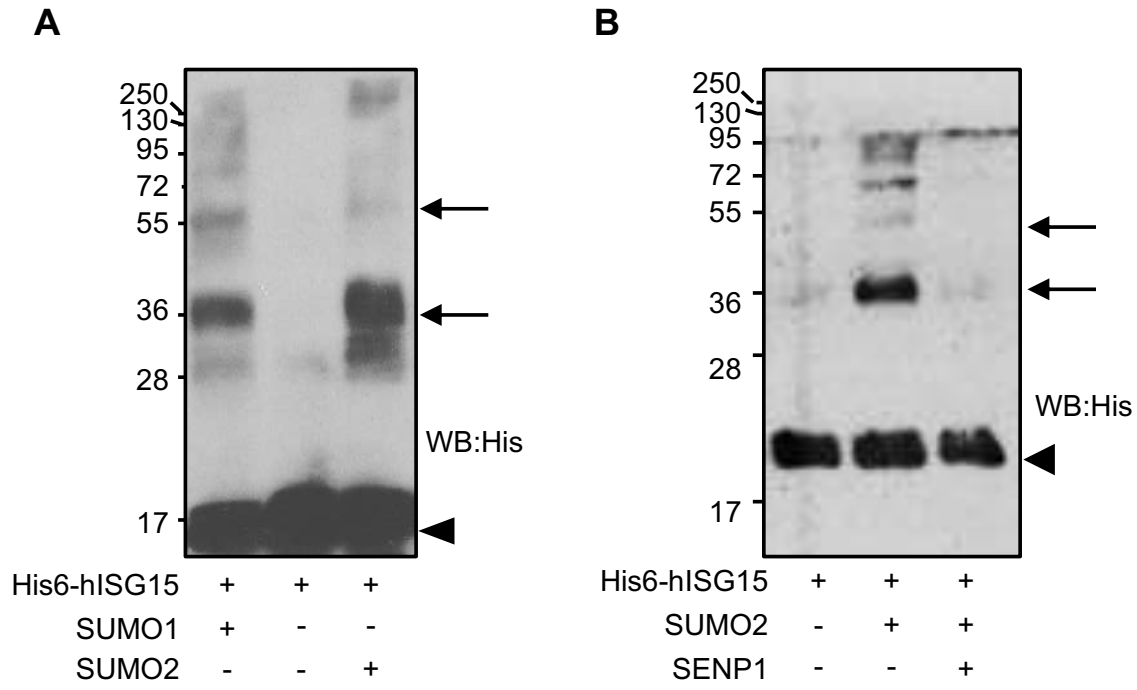


Figure 7. Human ISG15 is modified by SUMO1 and SUMO2 *in vitro*.

A. Histidine (His) tagged recombinant human (h) ISG15 protein was subjected to an *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2, as indicated. **B**. Recombinant hISG15 protein was subjected to an *in vitro* SUMOylation assay in the presence of SUMO2 and the product of the SUMOylation reaction was then subjected to a deSUMOylation assay in the presence of SENP1. Proteins were evaluated by SDS-PAGE and Western-blot analysis using anti-His antibody. Arrowhead and arrows indicate the unmodified and the SUMO-conjugated ISG15 protein, respectively.

6.1.2 ISG15 is modified by SUMO *in vivo*

In order to evaluate if ISG15 is also SUMOylated *in vivo*, we co-transfected HEK-293 cells with HA-hISG15-WT together with pcDNA, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2. At 48 h after transfection, cells were harvested, and the Histidine-tagged proteins were purified under denaturing conditions using nickel affinity beads. The whole cell lysates and the Histidine-tagged purified proteins were then analyzed by Western-blot using anti-HA antibody. Analysis of the Histidine-tagged purified proteins revealed bands of the expected molecular weight corresponding to ISG15-SUMO1 or ISG15-SUMO2 proteins only in those cells co-transfected with His6-SUMO1 and His6-SUMO2, respectively (Figure 8A). To discard that the high molecular bands detected in the cells co-transfected with SUMO1 or SUMO2 correspond to ISGylated proteins, we repeated the experiment using a plasmid encoding for a mutant of ISG15 in which the C-terminal diglycine (GG) motif was replaced by alanine (AA) (HA-hISG15-WT-AA), which prevents its conjugation to substrates, fused to HA tag. Co-transfection with SUMO1 or SUMO2 led to the appearance of higher molecular weight bands similar to those observed in the experiment carried out with HA-hISG15-WT (Figure 8B). These results indicated that human ISG15 is modified by SUMO1 and SUMO2 in transfected cells.

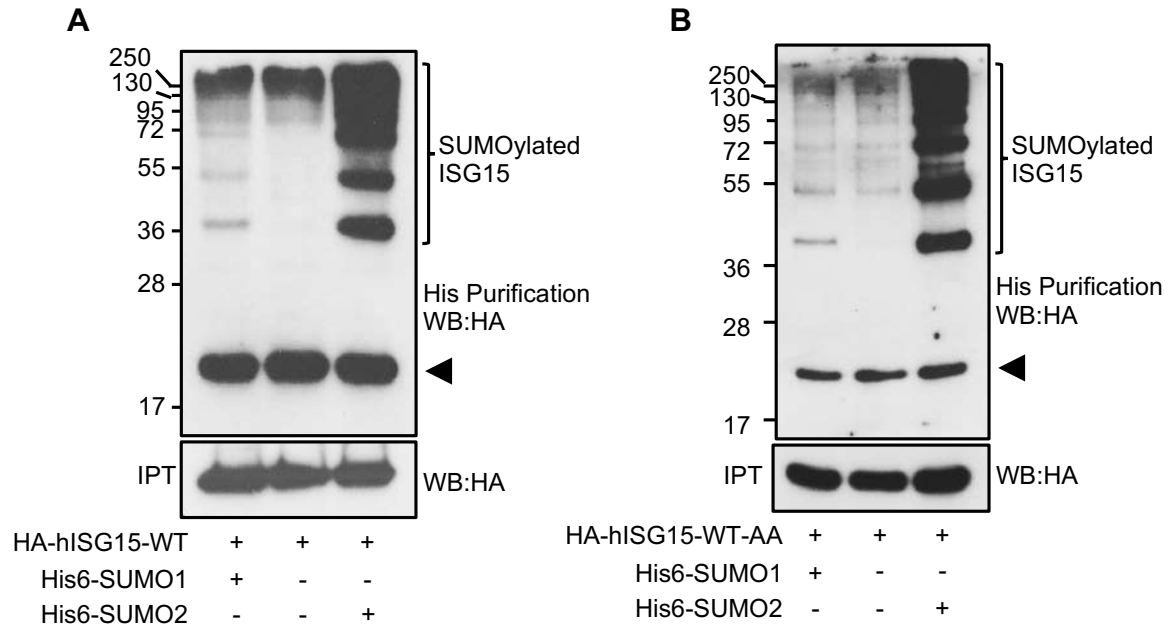


Figure 8. Human ISG15 is modified by SUMO1 and SUMO2 *in vivo*.
HEK-239 cells were co-transfected with HA-hISG15-WT (**A**) or a mutant of hISG15 unable to conjugate to its substrates (HA-hISG15-WT-AA) (**B**) together with pcDNA, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2. The whole cell extract (IPT) and the Histidine-tagged purified proteins were analyzed by Western-blot (WB) using anti-HA antibody. Arrowhead and arrows indicate the unmodified and the SUMO-conjugated ISG15 protein, respectively.

6.1.3 SUMOylation of ISG15 is not human-specific

ISG15 primary sequence is poorly conserved across mammalian species, with human and murine ISG15 sharing approximately 63% sequence identity at protein level (Kang et al., 2022) and potential divergent functions. Therefore, we decided to investigate whether SUMOylation of ISG15 is restricted to human or if SUMO could also conjugate to mouse ISG15. We then carried out an *in vivo* SUMOylation assay, similar to the one described in section 6.1.2, using a plasmid encoding a mutant of mouse (m) ISG15 in which the C-terminal diglycine (GG) motif was replaced by alanine (AA) (HA-mISG15-WT-AA), which prevents its conjugation to substrates, fused to an HA-tag. Western-blot analysis of the Histidine-tagged purified proteins revealed the presence of several higher molecular weight bands indicative of mouse ISG15 SUMOylation exclusively in the cells co-transfected with His6-SUMO1 or His6-SUMO2 (Figure 9). These results indicated that the SUMOylation of ISG15 is not human-specific.

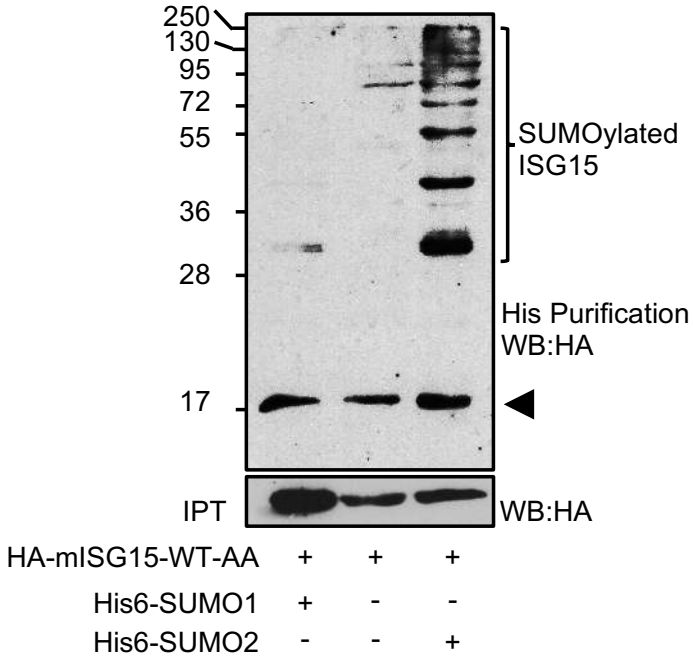


Figure 9. SUMOylation of mouse ISG15 in transfected cells.
HEK-239 cells were co-transfected with a mutant of mouse (m)ISG15 unable to conjugate to its substrates (HA-mISG15-WT-AA) fused to HA-tag together with pcDNA, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2, as indicated. The total protein extract (IPT) and the Histidine-tagged purified proteins were analyzed by Western-blot (WB) using anti-HA antibody. Arrowhead indicates the unmodified ISG15 protein. Bracket indicates ISG15-SUMO2 conjugates.

6.1.4 Endogenous ISG15 is SUMOylated in untransfected cells

To determine whether ISG15 is SUMOylated in endogenous conditions, A549 cells were treated with IFN-α (1000 U/mL) for 16 h and then subjected to heat shock stress by incubating them for 2 h at 42 °C (a condition that induces an upregulation in global SUMOylation (Golebiowski et al. 2009)) or left untreated. After the treatment, cells were lysed in the presence of proteases and deSUMOylases inhibitors, and protein extracts were incubated with GST- or GST-SUBES-agarose beads for 2 h at 4 °C. After washing, bound proteins were eluted using

Laemmli buffer. Then, protein extracts were subjected to Western blot analysis with anti-ISG15 or anti-SUMO2 antibodies. The Western-blot analysis using the anti-ISG15 antibody revealed the appearance of bands of the expected molecular weight corresponding to ISG15-SUMO conjugated proteins exclusively in the samples incubated with SUBES (Figure 10), indicating that endogenous ISG15 can be modified by SUMO. Western-blot analysis with anti-SUMO2 antibody demonstrated the purification of SUMO2-modified proteins by SUBES (Figure 10). To note, we observed a strong smear of high molecular weight in the Western-blot with anti-ISG15 antibody in those cells subjected to heat stress, likely corresponding to SUMO chains conjugated to ISG15 or mixed SUMO-ISG15 chains. Altogether these results demonstrated that ISG15 can be modified by SUMO1 and SUMO2 *in vitro*, in transfected cells and under totally endogenous conditions.

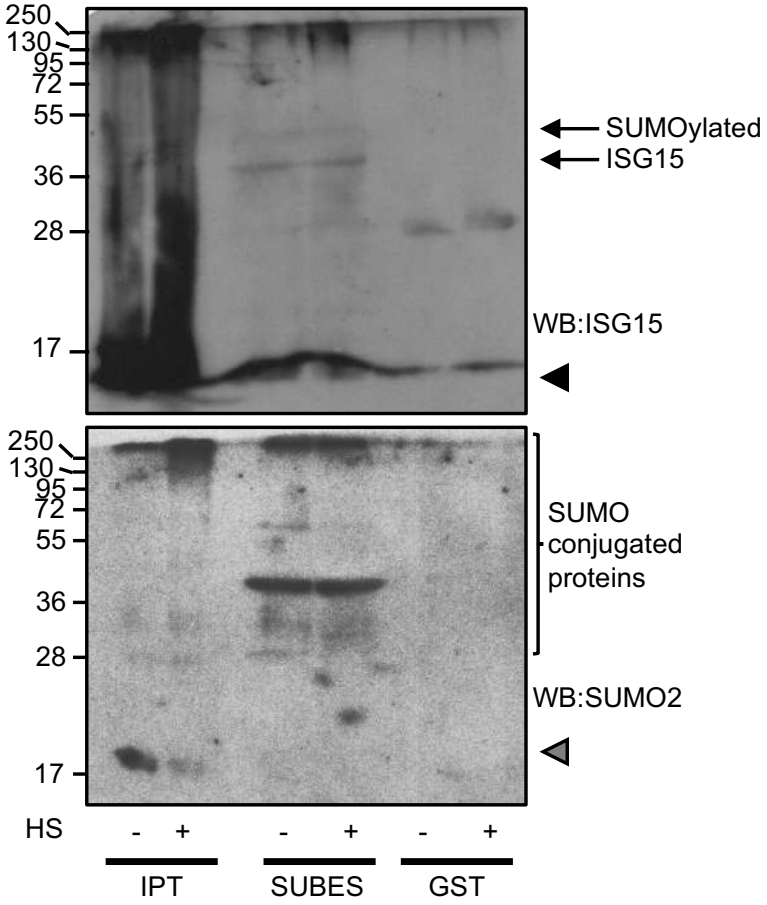


Figure 10. Endogenous SUMOylation of ISG15

A549 cells were treated with IFN-α (1000 U/mL) for 16 h and then incubated for 2 h at 42 °C or left untreated. Protein extracts were incubated with GST- or GST-SUBES-agarose beads. Total protein extracts (IPT) or interacting proteins were subjected to Western blot analysis with anti-ISG15 or anti-SUMO2 antibodies. Black and grey arrowhead indicate the unmodified ISG15 and SUMO proteins, respectively. Brackets correspond to ISG15-SUMO or SUMO conjugates, as indicated.

6.2 SUMOYLATION OF ISG15 IS INDUCED UPON STRESS

It has been previously demonstrated that SUMOylation, mainly by SUMO2/3, plays a crucial role in the cellular response to stress. Therefore, we decided to investigate whether the SUMOylation of ISG15 is modulated in response to different stress conditions. HEK-293 cells were co-transfected with HA-hISG15-WT-AA together with pcDNA or Ubc9 and His6-SUMO2. After 36 h of transfection, cells were exposed to UV light (20 J/m^2) followed by 12 h rest, incubated at 42 °C for 2 h, or left untreated. Histidine-tagged proteins were then purified under denaturing conditions. Total protein extracts and purified proteins were analyzed by Western blot using an anti-HA antibody. Analysis of the Histidine-tagged proteins revealed bands corresponding to ISG15-SUMO2 protein in the cells co-transfected with His6-SUMO2 (Figure 11). We also observed that the intensity of the ISG15-SUMO2 bands were higher in those cells treated with UV light or subjected to heat shock than in untreated cells, indicating that both UV and heat stress induce the SUMOylation of ISG15 (Figure 11). It has been reported that virus infection can induce SUMOylation. Therefore, we decided to explore whether virus infection influenced ISG15 SUMOylation. HEK-293 cells were transfected with HA-hISG15-WT-AA and pcDNA or Ubc9 and His6-SUMO2, and 36 h after transfection, cells were left uninfected (Mock) or were infected with vesicular stomatitis virus (VSV) or Influenza A virus (IAV) at a multiplicity of infection (MOI) of 5 PFU/mL. At different times after infection (3 or 6 h with VSV, and 3, 8 or 12 h with IAV), cells were collected and analyzed as described above. Western blot analysis of the Histidine-tagged purified proteins with anti-HA antibody demonstrated that the intensity of the ISG15-SUMO2 bands detected in the cells co-transfected with His6-SUMO2 increased upon infection with VSV or IAV, indicating that virus infection induces the SUMOylation of ISG15 (Figure 12). Taken together, these findings indicate that the SUMOylation of ISG15 is triggered in response to various types of stress.

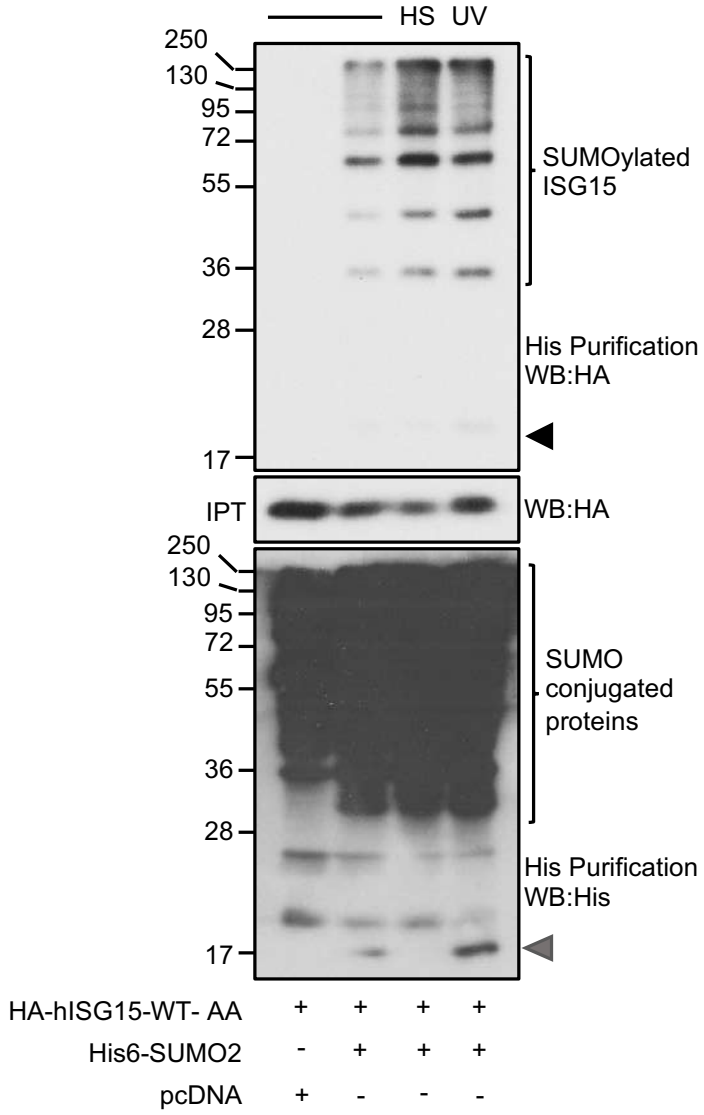


Figure 11. Induction of ISG15 SUMOylation in response to UV light or heat shock stress. HEK-239 cells were co-transfected with HA-hISG15-WT-AA together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, cells were exposed to UV light (20 J/m^2) followed by 12 h rest, heat shock stress (42°C, 2 h) or left untreated. The total protein extract (IPT) or the Histidine-tagged purified proteins were analyzed by Western-blot (WB) using anti-HA antibody. Black arrowhead and grey arrowhead indicate the unmodified ISG15 and SUMO proteins, respectively. Brackets indicate ISG15-SUMO2 or SUMO2 conjugates, as indicated.

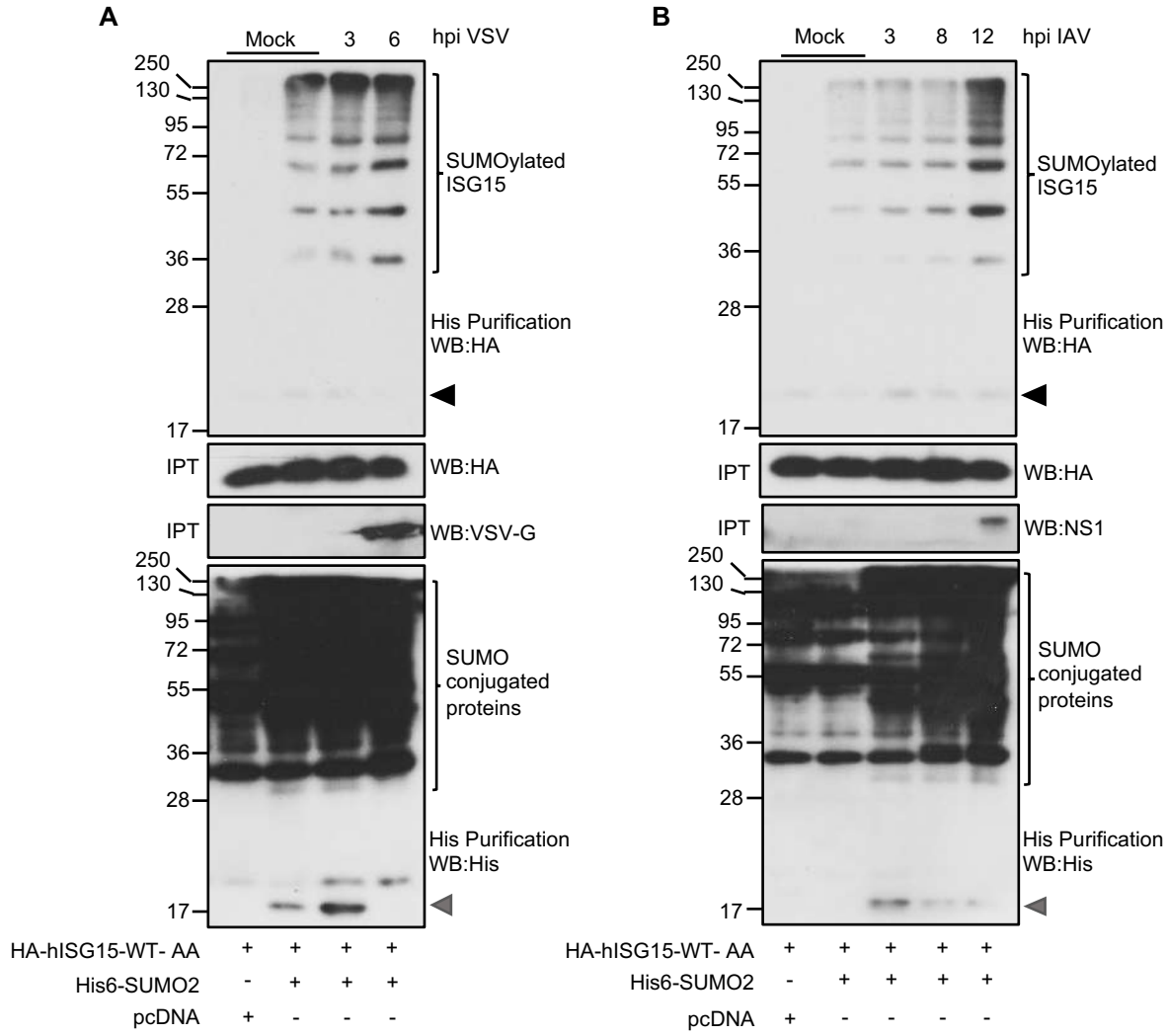


Figure 12. Induction of ISG15 SUMOylation in response to VSV or IAV infection.
HEK-239 cells were co-transfected with HA-hISG15-WT-AA together with pcDNA or Ubc9 and His6-SUMO2, and 36 h after transfection, cells were infected with VSV (**A**) or IAV (**B**) at a MOI of 5 PFU/mL. At the indicated times after infection, total protein extracts (IPT) and Histidine-tagged purified proteins were analyzed by Western-blot (WB) using the indicated antibodies. Black and grey arrowheads indicate the unmodified ISG15 and SUMO proteins, respectively. Brackets indicate ISG15-SUMO2 or SUMO2 conjugates, as indicated.

6.3 THE TUMOR SUPPRESSOR p14ARF INCREASES ISG15 SUMOYLATION

Recent data demonstrate that the tumor suppressor p14ARF protein stimulate immune activation in the tumor microenvironment (Alagu et al., 2018; Cerqueira et al., 2020). In addition, despite not being a SUMO E3 ligase, p14ARF has been demonstrated to enhance the SUMOylation of its interaction partners, such as p53 and MDM2 (Ivanschitz et al. 2015a; Xirodimas et al. 2002). Based on these findings, we hypothesized that p14ARF may induce ISG15 SUMOylation. To explore this hypothesis, we co-transfected HEK-293 cells with HA-hISG15-WT or HA-hISG15-WT-AA together with pcDNA, Ubc9 and His6-SUMO2, or Ubc9, His6-SUMO2 and pcDNA-p14ARF. 36 h after transfection, total protein extracts and Histidine-tagged purified proteins were analyzed by Western blot with the indicated antibodies. Western blot analysis of the purified proteins using anti-HA antibody revealed the appearance of ISG15-SUMO2 bands only in those cells co-transfected with His6-SUMO2 (Figure 13A, 13B). The intensity of bands corresponding to SUMO2-conjugated ISG15 protein clearly increased in those cells co-transfected with p14ARF (Figure 13A, 13B), indicating that p14ARF increases the SUMOylation of ISG15. To note, we observed an increase in the levels of global SUMOylation in those cells expressing p14ARF, as expected (Figure 13A, 13B). Finally, we decided to evaluate the SUMOylation of ISG15 in NARF2 cells, a cell line with inducible expression of p14ARF. NARF2 cells were co-transfected with HA-hISG15-WT or HA-hISG15-WT-AA together with Ubc9 and His6-SUMO2. 24 h post transfection, cells were treated with IPTG for 16 h to induce the expression of p14ARF. Cells were then harvested and the total protein extracts and Histidine-tagged purified proteins were analyzed by Western blot using anti-HA antibodies. We observed that IPTG treatment led to the expression of p14ARF, as expected (Figure 13C). We also observed that cells expressing p14ARF showed higher levels of SUMOylated ISG15 protein than the untreated cells (Figure 13C), indicating that p14ARF increases the SUMOylation of ISG15.

The tumor suppressor p14ARF has been reported to be modified by ubiquitin at the N-terminus. Therefore, we decided to evaluate whether ISG15 could also conjugate to p14ARF. To test that, HEK-293 cells were co-transfected with GFP or ARF-GFP together with pcDNA or His6-hISG15-WT and its E1, E2 and E3. 36 h after transfection, cells were recovered and the Histidine-tagged proteins were purified under denaturing conditions. Western blot analysis of the purified proteins using anti-GFP antibody revealed the appearance of a band of the expected ARF-GFP-ISG15 molecular weight exclusively in those cells co-transfected with both ARF-GFP and the ISGylation machinery (Figure 14) whereas no bands corresponding to GFP-ISG15 protein were detected (Figure 14), indicating that p14ARF is post-translationally modified by ISG15.

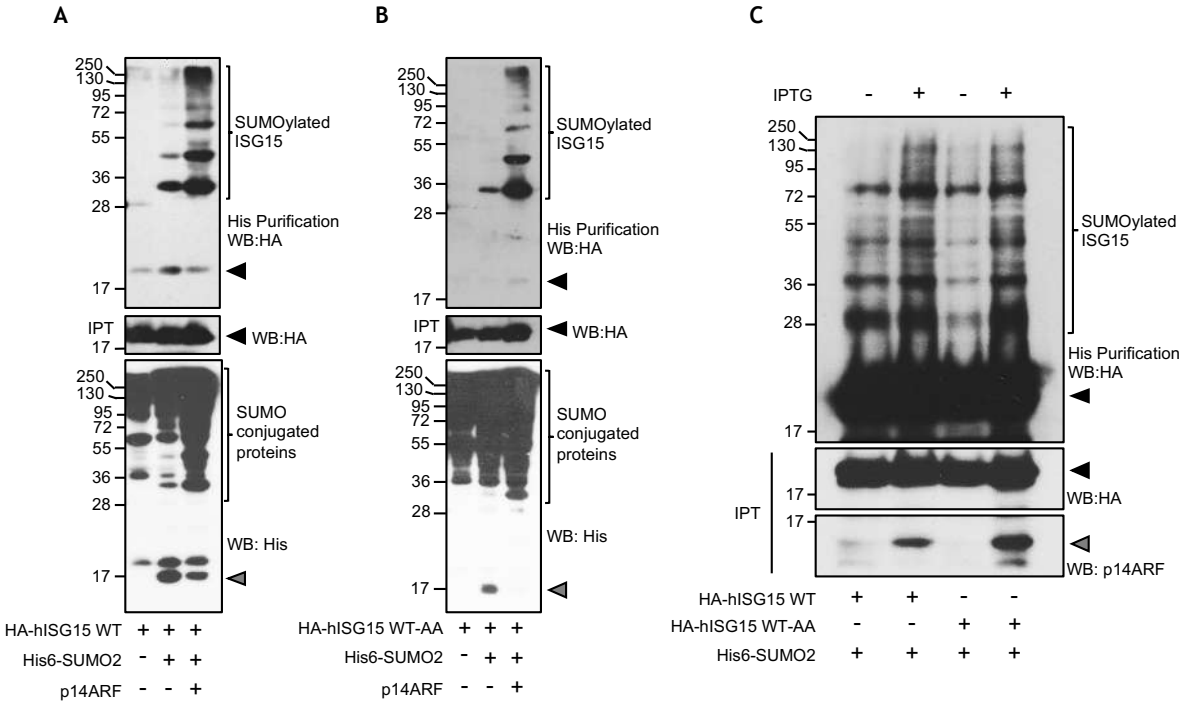


Figure 13. Impact of p14ARF on ISG15 SUMOylation

HEK-293 cells were co-transfected with HA-hISG15-WT **(A)** or HA-hISG15-WT-AA **(B)** together with pcDNA, Ubc9 and His6-SUMO2, or Ubc9, His6-SUMO2 and p14ARF. 36 h after transfection, the total protein extract (IPT) and the Histidine-tagged purified proteins were analyzed by Western-blot (WB) using the indicated antibodies. Black arrowheads and grey arrowheads indicate the unmodified ISG15 and SUMO2, respectively. Brackets indicate ISG15-SUMO or SUMO conjugates, as indicated. **C** NARF2 cells were co-transfected with HA-hISG15-WT or HA-hISG15-WT-AA together with Ubc9 and His6-SUMO2, and were treated or not with IPTG (1 mM). 16 h after treatment, total protein extracts (IPT) and Histidine-tagged purified proteins were analyzed by Western-blot (WB) with the indicated antibodies. Black arrowheads indicate the unmodified ISG15 protein. Grey arrowhead indicates p14ARF. Bracket indicates ISG15-SUMO2 conjugates.

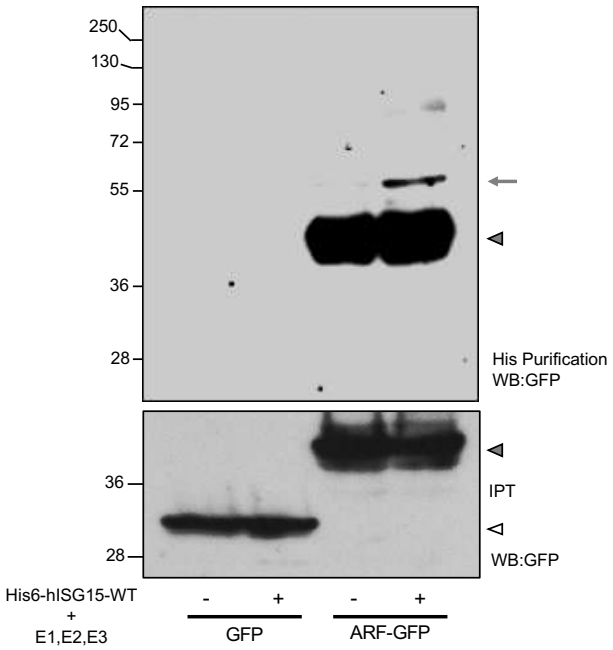


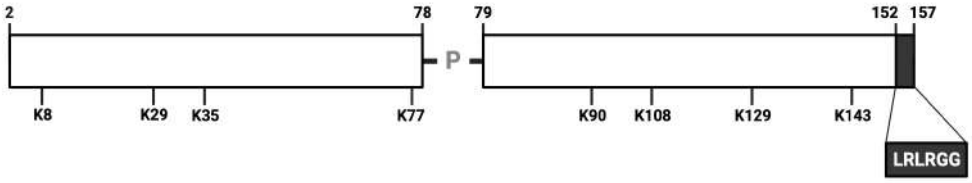
Figure 14. p14ARF is modified by ISG15

HEK-239 cells were co-transfected with GFP or p14ARF-GFP together with pcDNA or His6-hISG15-WT and the ISGylation enzymes (E1, E2 and E3). 36 h after transfection, the total protein extracts (IPT) and the Histidine-tagged purified proteins were analyzed by Western-blot using anti-GFP antibody. Grey arrowheads and grey arrow indicate the unmodified and the ISG15-conjugated p14ARF protein, respectively. White arrowhead indicates the unmodified GFP.

6.4 SUMO can be conjugated to different lysine residues in ISG15

To elucidate the functional impact of SUMO conjugation to a particular substrate, a direct approach involves identifying the lysine residue(s) crucial for SUMO conjugation and subsequently analyzing the properties of the SUMOylation mutants in comparison to the wild type protein. *In silico* analysis employing GPS-SUMO software identified four potential SUMOylation sites in each ubiquitin-like domain of ISG15: K8, K29, K35, and K77 (located in the first ubiquitin-like domain), and K90, K108, K129, and K143 (located at the second ubiquitin-like domain) (Figure 15).

A



B

SUMOylation Sites

Position	Score	Position	Score
77	2.488	35	0.743
8	1.687	143	0.59
129	1.552	90	0.372
29	0.755	108	0.296

C

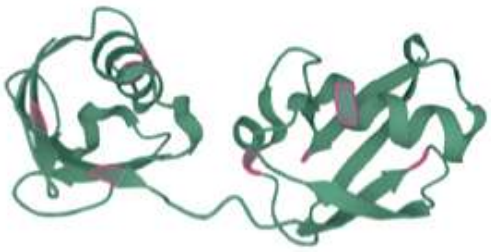


Figure 15. SUMOylation sites in ISG15

After *in silico* analysis of the amino acid sequence of the human ISG15 protein using GPS-SUMO we draw a schematic representation of the localization of the putative SUMOylation sites in ISG15 (**A**). **B**, Result of the GPS-SUMO analysis indicating the score for each putative SUMOylation site in ISG15. **C**, 3D representation of the structure of human ISG15 protein indicating the localization of the potential SUMOylation sites.

We then generated mutants of ISG15 in those lysine residues with the highest probability of being SUMO acceptor sites, and assessed their SUMOylation in cells. Single mutants did not show reduced SUMOylation in comparison with the WT protein (Figure 16A). We then generated ISG15 constructs containing mutations in several putative SUMOylation sites and evaluated their SUMOylation in cells. Mutation of the lysine residues K8, K77, K35, and K129 clearly reduced the SUMOylation of ISG15 (Figure 16B). Additional mutation of the lysine residues K90, K29, and K109 further decreased the SUMOylation of ISG15 (Figure 16B) and the additional mutation of K143 totally inhibited ISG15 SUMOylation (Figure 16C, 16D). We called this SUMOylation mutant, ISG15-K. Interestingly, Western-blot analysis of the Histidine-tagged purified proteins using anti-Histidine antibody revealed that expression of the SUMOylation mutant of ISG15 altered the global SUMOylation pattern independently of the presence of the diglycine motif in ISG15 (Figure 16C, 16D), suggesting that some of the SUMOylated proteins correspond to SUMOylated ISG15. To further evaluate this hypothesis, we generated A549 cells depleted from ISG15 (ISG15-KO) stably expressing HA-hISG15-WT or HA-hISG15-WT-AA and then, the stable cells were treated or not with 20 μM of the SUMOylation inhibitor ML792. At 16 h after treatment, cells were analyzed by Western blot with anti-HA antibody. We observed a change in the ISGylation pattern of the cells treated with the SUMOylation inhibitor relative to the observed in the untreated cells (Figure 17), suggesting that SUMOylation alters the ISGylation pattern.

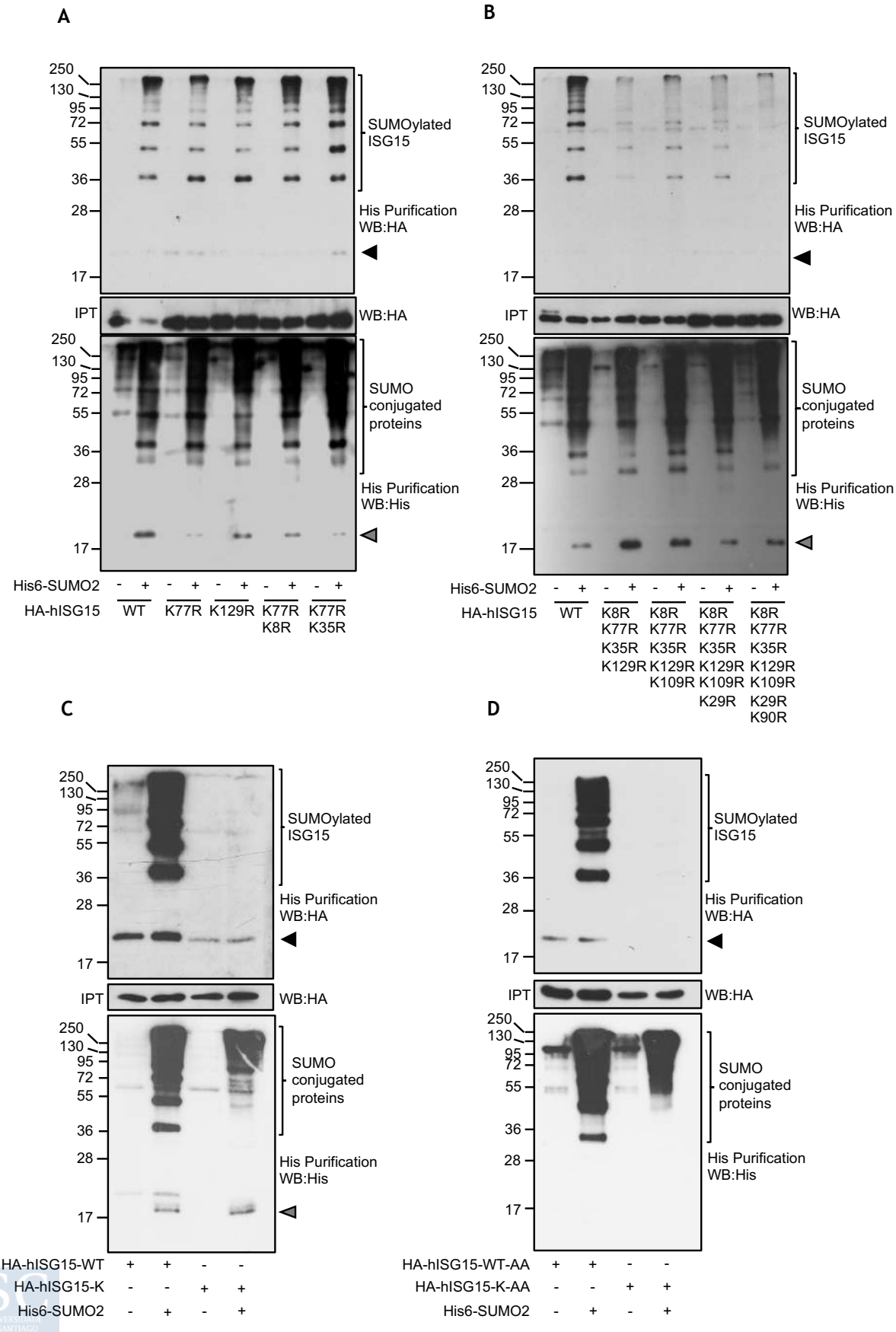


Figure 16. Identification of the SUMOylation sites in ISG15

(A and B) HEK-239 cells were co-transfected with HA-hISG15-WT or the mutants in the indicated lysine residues together with pcDNA or Ubc9 and His6-SUMO2, and 36 h after transfection, the total protein extracts (IPT) and the Histidine-tagged purified proteins were analyzed by Western-blot (WB) using the indicated antibodies. Black and grey arrowheads indicate the unmodified ISG15 and SUMO proteins, respectively. Brackets indicate ISG15-SUMO2 or SUMO2 conjugates, as indicated. **C** HEK-293 cells were co-transfected with HA-hISG15-WT or HA-hISG15-K (K77R/K129R/K8R/K35R/K109R/K29R/K90R/K143R) together with pcDNA or Ubc9 and His6-SUMO2. At 36 h after transfection, total protein extracts (IPT) and Histidine-tagged purified proteins were analyzed by Western blot with the indicated antibodies. Black and grey arrowheads indicate the unmodified ISG15 and SUMO proteins, respectively. Brackets indicate ISG15-SUMO2 or SUMO2 conjugates, as indicated. **D** HA-hISG15-WT-AA or HA-hISG15-K-AA together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, the total protein extracts (IPT) and the Histidine-tagged purified proteins were analyzed by Western-blot (WB) using anti-HA antibody. Black arrowhead indicates the unmodified ISG15 proteins. Brackets indicate ISG15-SUMO2 or SUMO2 conjugates, as indicated.

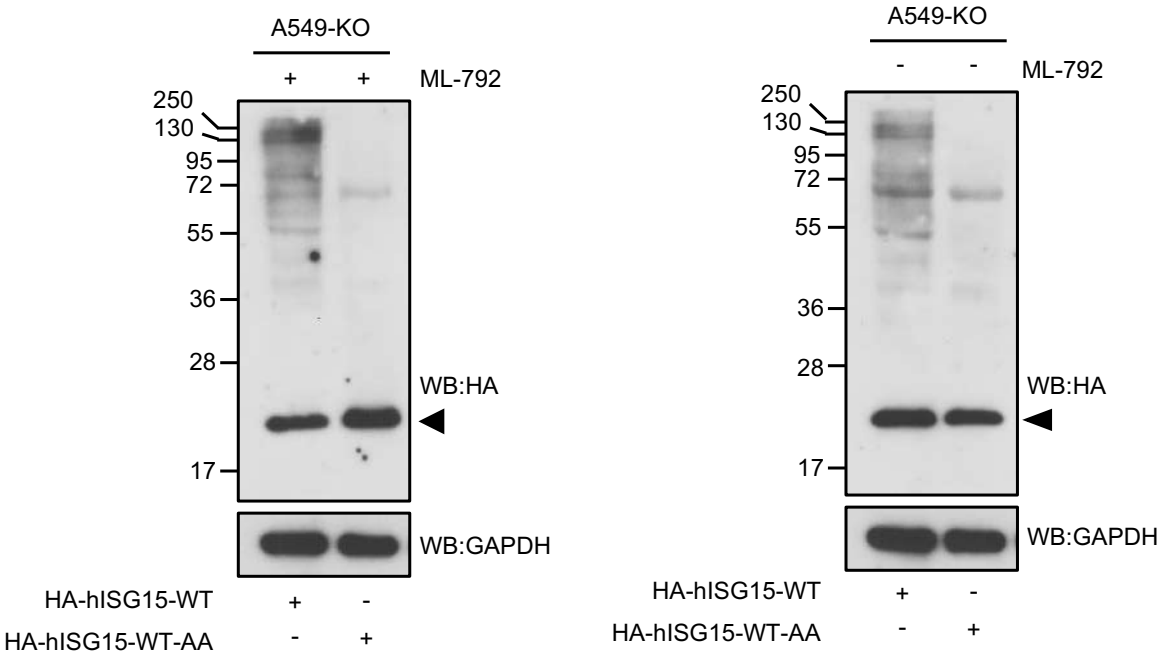


Figure 17. Analysis of the ISGylation pattern in cells treated or not with the SUMOylation inhibitor ML-792.

A549 ISG15-KO stably expressing HA-hISG15-WT or HA-hISG15-WT-AA were treated or not with 20 μM of ML-792. 24 h after treatment, cells were recovered and analyzed by Western-blot (WB) using anti-HA antibody. Arrowheads indicate unmodified ISG15 protein.

6.5 ISG15 can interact with SUMO in a non-covalent manner through its SIM domains

Many SUMOylated proteins can interact with SUMO in a non-covalent manner through a SUMO interacting motif (SIM). In order to test whether ISG15 could also interact non-covalently with SUMO, we performed GST-pulldown assays using protein extracts derived from cells expressing HA-hISG15-WT protein and GST or GST-SUMO1 recombinant protein. As shown in Figure 18, we detected ISG15 protein interacting with GST-SUMO1 but not with GST, indicating that ISG15 can interact in a non-covalent manner with SUMO1. An *in silico* analysis of the amino acid sequence of ISG15 using GPS-SUMO software revealed the presence of two potential SIM domains (72LLVVD76 and 82LSILV86) in ISG15 (Figure 19).

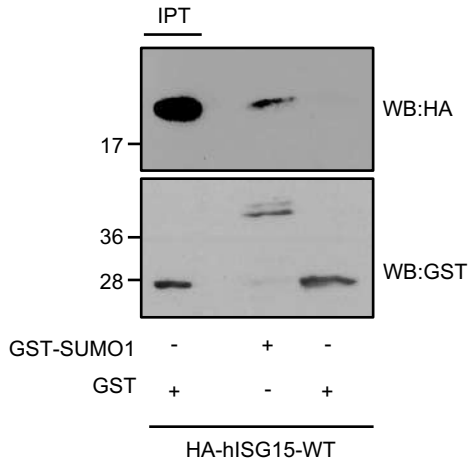


Figure 18. Non-covalent interaction of ISG15 with SUMO1

HEK-239 cells were transfected with HA-hISG15-WT and 36 h after transfection, cells were lysed and protein extracts were incubated with GST or GST-SUMO1 recombinant protein immobilized on glutathione sepharose beads. The total protein extracts (IPT) and the interacting proteins were analyzed by Western-blot (WB) using anti-HA antibody.

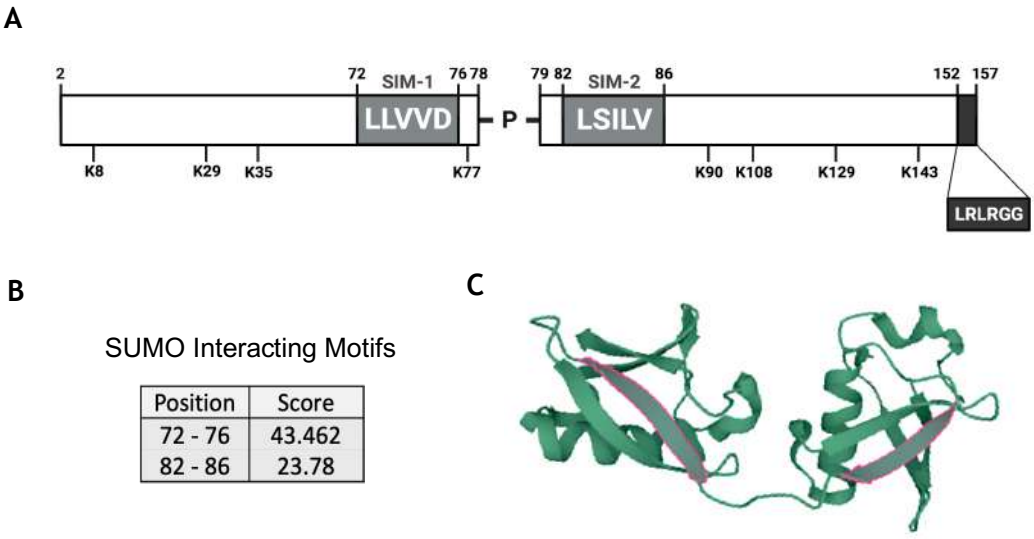


Figure 19. *In silico* analysis of potential SIM domains in ISG15

In silico analysis of the amino acid sequence of the human ISG15 protein using GPS-SUMO software. **A** Schematic representation of the localization of the potential SIM domains in ISG15. **B** Results of the GPS-SUMO analysis indicating the score for each putative SIM domain. **C** 3D representation of the structure of human ISG15 protein indicating the localization of the potential SIM domains.

We then generated mutants of ISG15 in the putative SIM domains and analyzed their non-covalent interaction with SUMO1 using GST-pulldown assays. The mutation of the residues 72LVVD76 to 72AAAA76 (ISG15-1xΔSIM) totally inhibited the non-covalent interaction of ISG15 with SUMO1 (Figure 20A). Similar results were observed with the double mutant 72LVVD76 to 72AAAA76 and 82LSILV86 to 82AAAA86, (ISG15-2xΔSIM) (Figure 20A), indicating that ISG15 requires its SIM domains to interact with SUMO1 in a non-covalent manner.

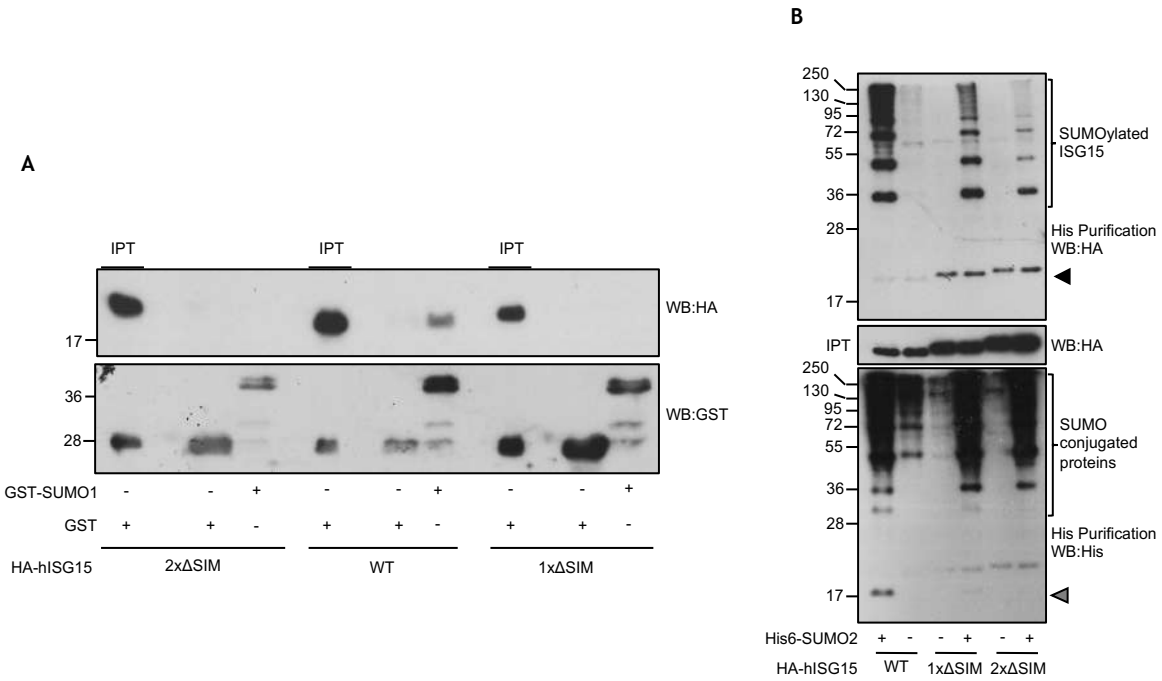


Figure 20. Identification of SIM domains in ISG15

A HEK-239 cells were transfected with HA-hISG15-WT, HA-hISG15-1xΔSIM or HA-hISG15-2ΔxSIM and 36 h after transfection, cells were lysed and protein extracts were incubated with GST or GST-SUMO1 proteins immobilized on glutathione sepharose beads. Interacting proteins were analyzed by Western-blot using anti-HA antibody. **B** HEK-239 cells were co-transfected with HA-hISG15-WT, HA-hISG15-1xΔSIM or HA-hISG15-2ΔxSIM together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, the total protein extract (IPT) and the Histidine-tagged purified proteins were analysed by Western-blot using the indicated antibodies. Black and grey arrowhead indicate the unmodified ISG15 and SUMO proteins, respectively. Brackets indicate ISG15-SUMO2 or SUMO2 conjugates, as indicated.

It is well known that SIM domains can contribute to SUMO conjugation (Lascorz et al. 2022). To evaluate whether the SIM domains in ISG15 facilitates ISG15 SUMOylation, we co-transfected HEK-293 cells with HA-hISG15-WT, HA-ISG15-1xΔSIM or HA-ISG15-2xΔSIM together with pcDNA, or His6-SUMO2 and Ubc9. 36 h after transfection cells were lysed and the whole cell extracts and Histidine-tagged purified proteins were analyzed by Western blot using the indicated antibodies. A reduction in the SUMOylation of ISG15 was observed after mutation of the SIM domain 72LVVD76 (Figure 20B). This reduction was clearly stronger after mutation of the two SIM domains (Figure 20B). These results suggested that the non-covalent conjugation of ISG15 with SUMO or SUMOylated proteins facilitate its SUMOylation.

6.6 SUMO INTERACTION EFFECTS ON ISG15

6.6.1 Impact of SUMO interaction on the secretion of ISG15

One of the characteristics of ISG15 is its secretion, through still unclear mechanisms, from cells. We decided to evaluate whether mutation of the SUMO interaction domains in ISG15 had an impact on its secretion. We co-transfected HEK-293 cells with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA together with its E1, E2 and E3 enzymes. 48 h after transfection cells and cell supernatants were collected and resuspended in Laemmli buffer supplemented or not with DTT (10 mM). Samples were then analyzed by Western blot using anti-HA antibody. We detected a band corresponding to free ISG15 protein exclusively in the supernatant of those cells transfected with ISG15-WT (Figure 21), suggesting that mutation of the SIMs or of the SUMOylation sites in ISG15 reduces its secretion. However, we cannot discard that one of the causes of the non-detection of the SUMOylation mutant in the supernatant is not caused by the lower levels of the protein inside the cells.

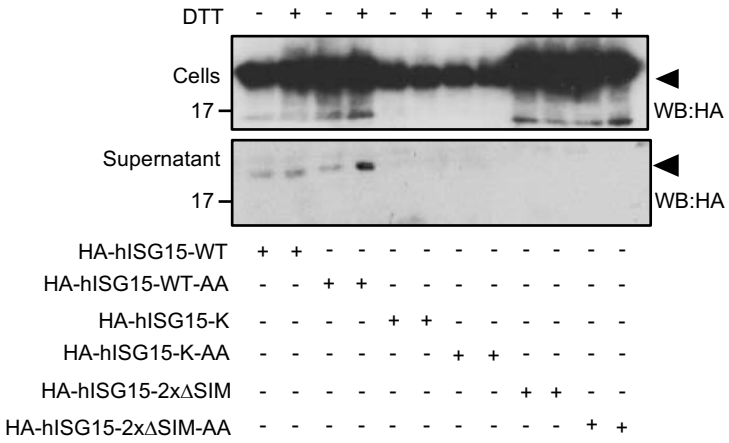


Figure 21. Impact of mutating the SUMOylation sites or the SIM domains in ISG15 on its secretion.

HEK-239 cells were co-transfected with HA-hISG15-WT, HA-hISG15- WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA together with the E1, E2, and E3 ISGylation enzymes. 48 h after transfection, cells and supernatants were collected. The total protein extracts from cells and supernatants were recovered in Laemmli buffer supplemented or not with DTT (1 mM) and analyzed by Western-blot (WB) using anti-HA antibody. Arrowheads indicate ISG15 protein.

6.6.2 Impact of SUMO interaction on the subcellular localization of ISG15

6.6.2.1 Mutation of the SUMOylation sites or of the SIM domains in ISG15 alters its subcellular localization

SUMO interaction can regulate the subcellular localization of substrates (Zubiete-Franco et al. 2019). Therefore, we decided to evaluate whether SUMO could modulate the subcellular localization of ISG15. We transfected U2OS cells with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA constructs. 24 h after transfection, cells were fixed, permeabilized, blocked and stained using anti-HA antibody and DAPI. Finally, the cells were analyzed by confocal microscopy. We observed that the cells transfected with WT protein showed diffuse cytoplasmic and nuclear staining (Figure 22A), and that mutation of the diglycine GG motif

favored its presence in the cytoplasm (Figure 22A). Although the cells transfected with the SUMOylation mutant showed some diffuse cytoplasmic staining, a stronger signal was observed in nuclear aggregates in some cells (Figure 22A). Mutation of the GG motif did not affect to the distribution of the SUMOylation mutant (Figure 22A). Finally, those cells transfected with the SIM mutant showed also some diffuse cytoplasmic staining together with nuclear aggregates in some cells (Figure 22A) and its distribution was not affected by the mutation of the diglycine GG motif (Figure 22A). Altogether, these results suggested that the covalent and non-covalent interaction of SUMO with ISG15 regulate its subcellular distribution.

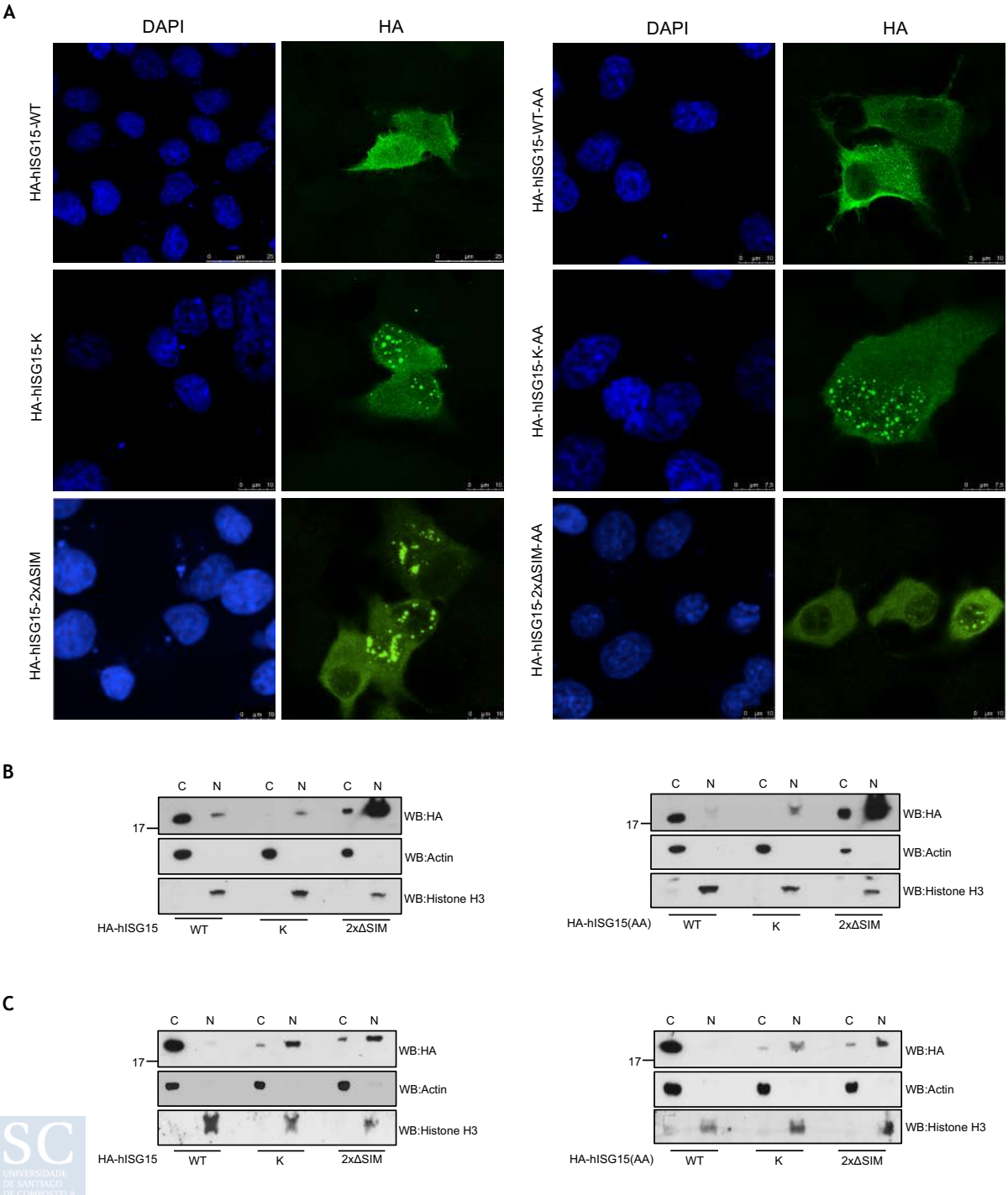


Figure 22. SUMO interaction modulates the subcellular distribution of ISG15

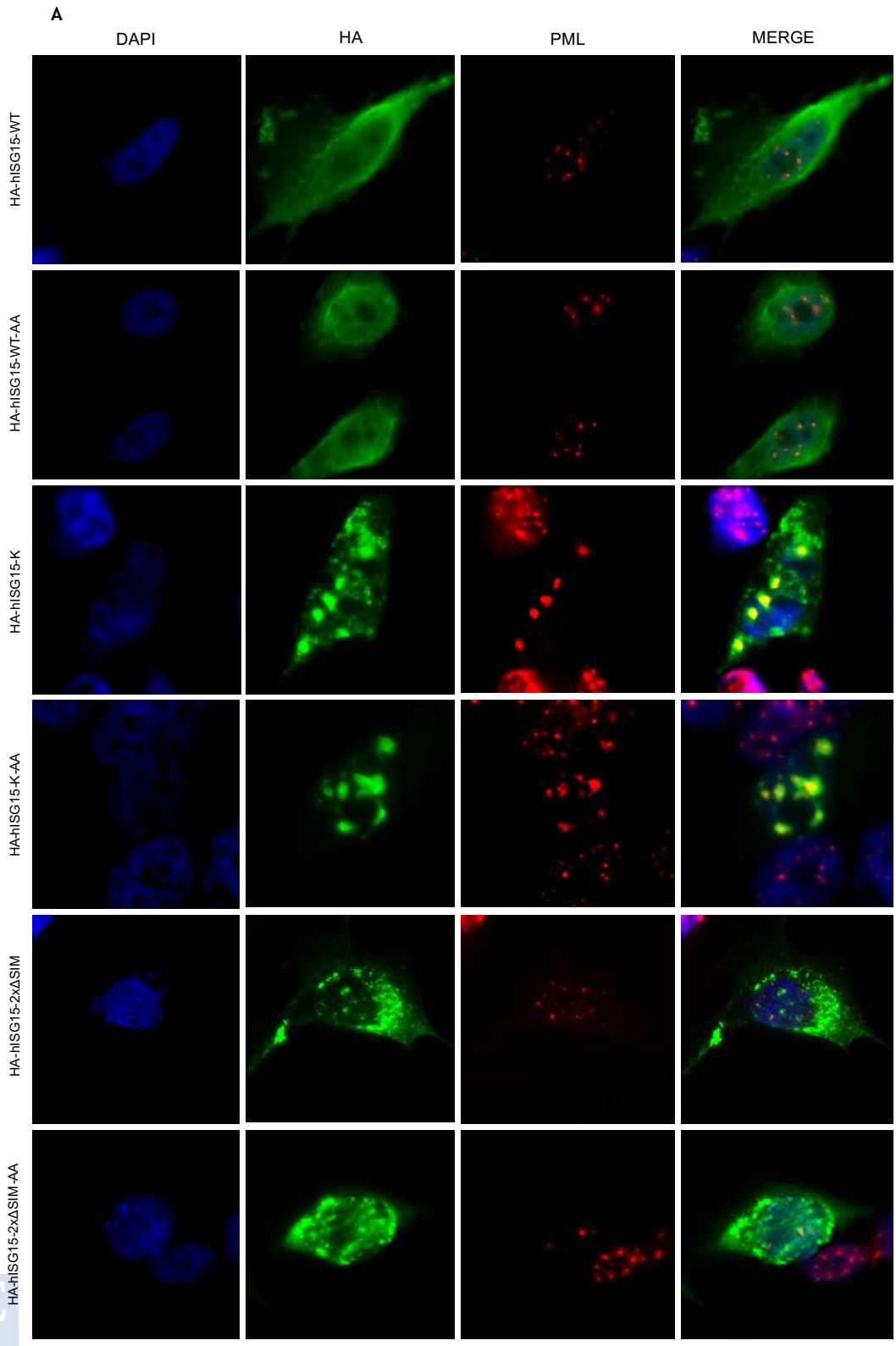
A U2OS cells were transfected with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA and 24h h after transfection, cells were immunostained with anti-HA and DAPI. **B** U2OS cells were transfected with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA and 24 h h after transfection, cells were subjected to a subcellular fractionation assay. The cytoplasmic (C) and nuclear (N) fractions were analysed by Western blot using the indicated antibodies. **C** PC3 cells were transfected with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA and 24 h after transfection, cells were subjected to a subcellular fractionation assay. The cytoplasmic (C) and nuclear (N) fractions were analysed by Western blot using the indicated antibodies.

To verify that mutation of the SUMO interaction sites had an impact on ISG15 subcellular localization, U2OS were transfected as described above and 24 h after transfection, we carried out subcellular fractionation assays. Western blot analysis of the protein extracts revealed that WT protein was mainly detected in the cell cytoplasm (Figure 22B) in comparison with the mutants in the SUMOylation sites or in the SIM motifs, detected mainly in the cell nucleus (Figure 22B). Similar results were observed when mutations in the diglycine GG motif were added to the ISG15 constructs (Figure 22B). To determine whether the subcellular distribution of ISG15-WT or the mutants of ISG15 is cell type-dependent, we transfected PC3 cells as described above and carried out a subcellular fractionation assay. Western blot analysis of the protein extracts revealed that WT protein was mainly detected in the cell cytoplasm (Figure 22C) in comparison with the mutants in the SUMOylation sites or in the SIM motifs, detected mainly in the cell nucleus (Figure 22C), and independently of the presence of the diglycine GG motif in ISG15 (Figure 22C). Altogether these results suggested that the covalent and non-covalent interaction of SUMO with ISG15 modulate its subcellular distribution.

6.6.2.2 Co-localization of ISG15 with PML

Although the mechanism by which p14ARF induces the SUMOylation of its interactors is still unclear, one of the hypotheses is that p14ARF interacts with the promyelocytic leukemia protein (PML) and this interaction serves to stabilize the SUMO1-conjugating enzyme Ubc9 within nuclear bodies (NB), thereby facilitating the SUMOylation of its interactors (Ivanschitz et al. 2015). In addition, PML-NBs has been proposed to work as sites specialized to recognize and sequester foreign proteins within the nucleus to facilitate their clearance (Fu et al., 2005). Analysis of the subcellular distribution of the ISG15 mutants revealed the formation of nuclear aggregates in some cells. Therefore, we hypothesized that mutation of the SUMO interaction domains in ISG15 may promote the formation of aggresomes. To evaluate this hypothesis, we decided to evaluate the potential co-localization between ISG15 and PML-NB as well as the potential alteration in PML-NBs distribution. PC3 cells were transfected with HA-hISG15-WT, HA-hISG15-K or HA-hISG15-2xΔSIM constructs containing the diglycine GG motif or with the GG motif mutated to AA. 24 h after transfection, cells were fixed, permeabilized, blocked and stained using anti-HA and anti-PML antibodies and DAPI. Cells were then analyzed by confocal microscopy. The ISG15-WT protein was detected both in nucleus and cytoplasm, independently of whether the protein contained or not the GG motif (Figure 23A). Numerous small dots corresponding with PML-NBs were detected in the cells expressing the WT protein and a partial co-localization between PML and ISG15 could be observed (Figure 23A). A reduced number of bigger dots

corresponding to PML-NBs were observed in those cells expressing the ISG15 mutants (Figure 23A) and a stronger co-localization between PML and SIM or SUMOylation mutants of ISG15 was observed (Figure 23A). These results suggested that the interaction of SUMO with ISG15 avoided the aggregation and/or oligomerization of ISG15.



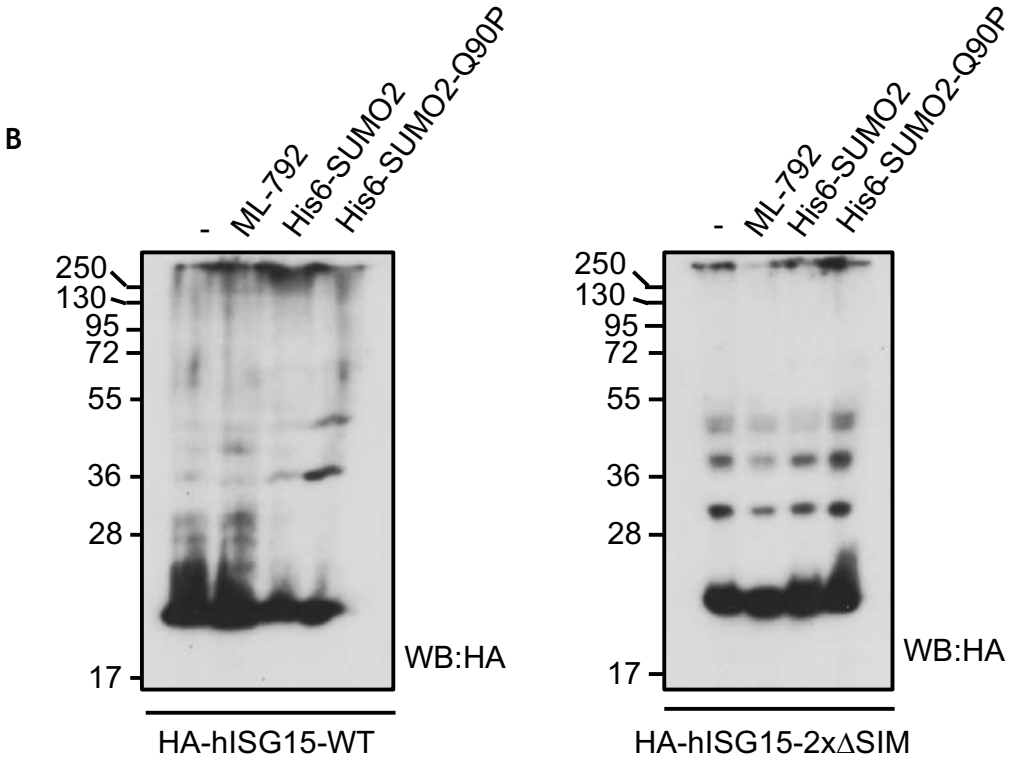


Figure 23. Colocalization of ISG15 with PML-NBs

A PC3 cells were transfected with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA and 24 h after transfection, cells were immunostained with anti-HA, anti-PML and DAPI. **B** HEK-239 cells were co-transfected with HA-hISG15-WT or HA-hISG15-2xΔSIM together with His6-SUMO2 or His6-SUMO-Q90P, treated with 20 μM of ML-792 or left untreated. 48 h after transfection, cells were collected. The total protein extracts were analyzed by Western-blot using anti-HA antibody.

To further test this hypothesis, we analyzed the ISG15 pattern in cells transfected with HA-hISG15-WT or HA-hISG15-2xΔSIM and treated or not with the SUMOylation inhibitor ML-792 or co-transfected with SUMO2 or a mutant of SUMO2 that cannot be deconjugated (SUMO2-Q90P). Western blot analysis using anti-HA antibody revealed a series of bands with different molecular weights in cells transfected with the WT protein (Figure 23B, left panel). A decrease in the intensity of the 36 kDa band and the appearance of a 50 kDa band was observed in the cells transfected with the WT protein and treated with ML-792. The 36 kDa band was predominant in those cells co-transfected with SUMO2 and its intensity clearly increased in the SUMO2-Q90P co-transfected cells (Figure 23B, left panel). A totally different ISG15 pattern was observed in the cells expressing HA-hISG15-2xΔSIM. Together with the unmodified protein, we detected the appearance of at least two bands of around 34 and 47 kDa, consistent with the hypothesis of ISG15 oligomerization (Figure 23B, right panel).

6.6.2.3 Overexpression of the tumor suppressor p14ARF promotes the recruitment of ISG15 to the nucleus

Our data indicated that there is an interplay between p14ARF and ISG15 and previously, it was reported that overexpression of p14ARF can recruit its interaction partners to the nucleus. Therefore, we decided to evaluate how p14ARF impact on the subcellular localization of ISG15. U2OS cells were co-transfected with HA-hISG15-WT together with pcDNA or ARF-GFP constructs. 24 h after transfection, cells were fixed, permeabilized, blocked and stained using anti-HA antibody and DAPI. Cells were then analyzed by confocal microscopy. ISG15 was detected in the cytoplasm and nucleus of those cells co-transfected with pcDNA (Figure 24). However, ISG15 was detected mainly in the nucleus in those cells overexpressing p14ARF (Figure 24), suggesting that the overexpression of the tumor suppressor p14ARF could promote the recruitment of ISG15 to the nucleus.

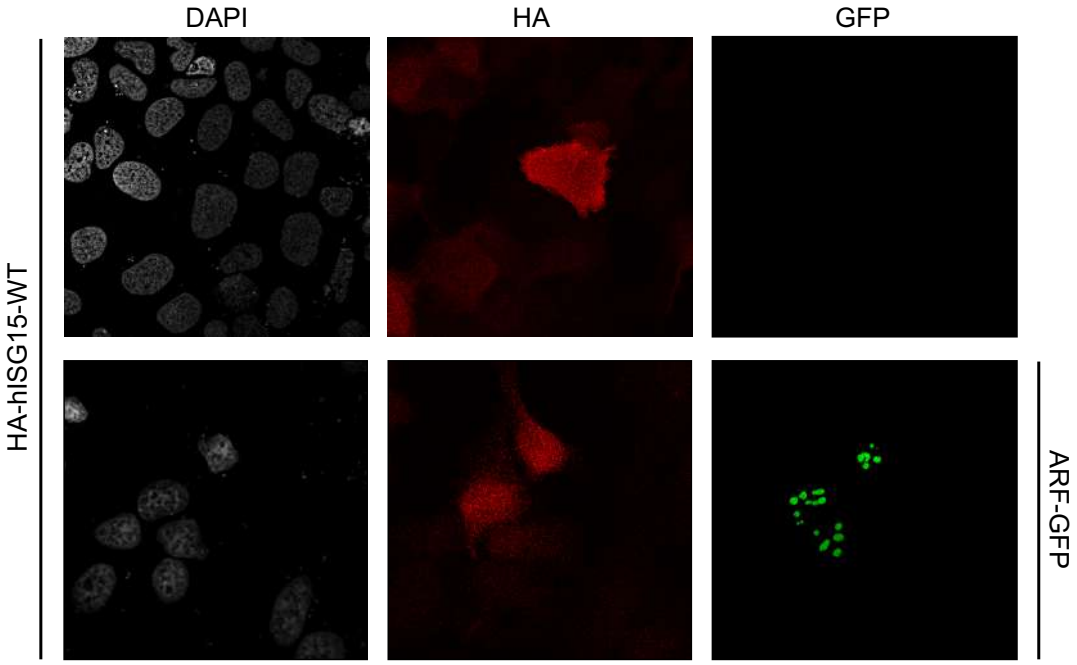


Figure 24. p14ARF overexpression induces the recruitment of ISG15 to the nucleus.

U2OS cells were co-transfected with HA-hISG15-WT together with pcDNA or ARF-GFP and 24 h after transfection, cells were immunostained with anti-HA antibody and DAPI.

6.7 SUMO CONTRIBUTES TO THE ANTI-APOPTOTIC ACTIVITY OF ISG15

It has been reported that ISG15 can exert pro-tumoral or anti-tumoral functions. We were interested in decipher whether the activity of ISG15 in cancer could be modulated by SUMOylation. We used A549 ISG15 WT and ISG15 depleted A549 cells (ISG15-KO) as a model. First, to evaluate the consequences of ISG15 upregulation in A549 cells, we treated the A549-WT or ISG15-KO cells with IFN-α (1000 U/mL) and we visualized the cells at the microscope after confirming that treatment with IFN-α triggered the upregulation of ISG15, as expected (Figure 25A). A549-WT cells continued proliferating and we did not observe a significant change in the cell morphology (Figure 25 B). In contrast, treatment with IFN-α led to a clear reduction in the number of ISG15-KO cells (Figure 25B), suggesting that ISG15 protects to A549 cells from IFN-α effects.

To further analyze the effect of IFN in both cell lines, we carried out a proliferation assay in presence or absence of IFN-α treatment, using MTT as well as a foci formation assay. Proliferation of A549 WT cells was not affected by IFN-α treatment (Figure 26A). In addition, we observed the formation of A549 WT foci in the presence of IFN-α (Figure 26B). In contrast, the proliferation of ISG15-KO cells was significantly reduced in response to IFN-α treatment (Figure 26A) and the formation of foci was totally inhibited (Figure 26B). A reduction in the number of cells in response to IFN-α treatment could result from the induction of apoptosis or from the induction of senescence. To evaluate whether the expression of ISG15 in A549 cells altered the ability of the cells to enter into senescence, we carried out a proliferation assay of A549-WT and ISG15-KO at different times after bleomycin (20 μM) treatment using MTT assay. The proliferation of both cell lines was similarly affected by bleomycin treatment (Figure 26C), suggesting that ISG15 does not alter the cellular capacity to enter into senescence in response to DNA damage agents. We then hypothesized that IFN-α treatment may induce apoptosis.

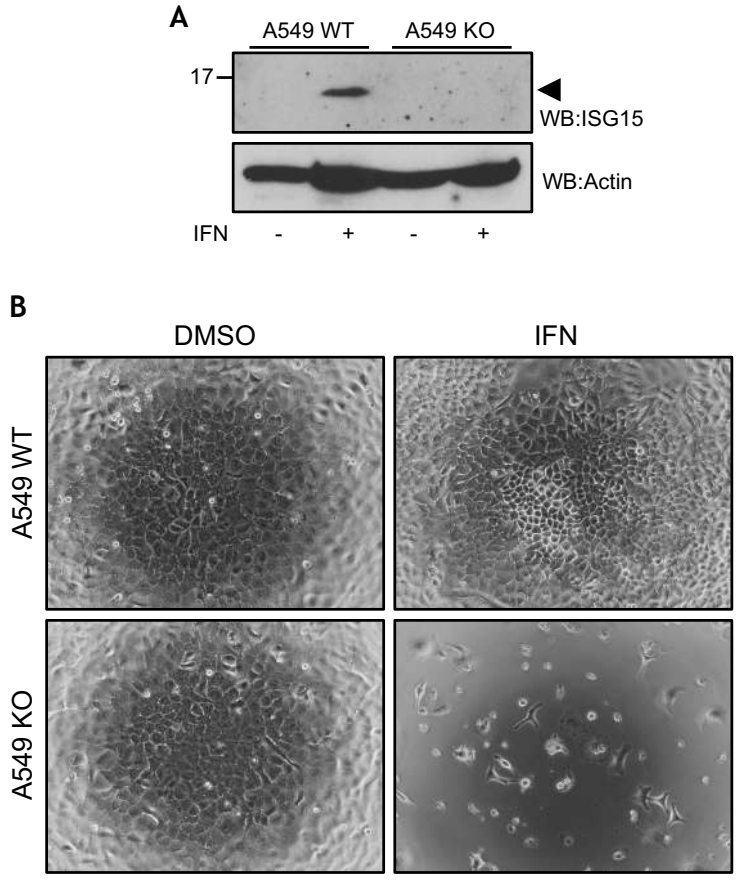


Figure 25. Effects of IFN-α treatment on A549 WT and ISG15-KO cells

A A549 WT and ISG15-KO cells were treated or not with 1000 U/mL of IFN-α for 12 h. Total protein extracts were analyzed by Western-blot using anti-ISG15 antibody. Arrowhead indicates ISG15 protein. **B** A549 WT or ISG15-KO cells were treated with 1000 U/mL IFN-α or DMSO. At 6 days after treatment, cells were photographed with an optical microscope.

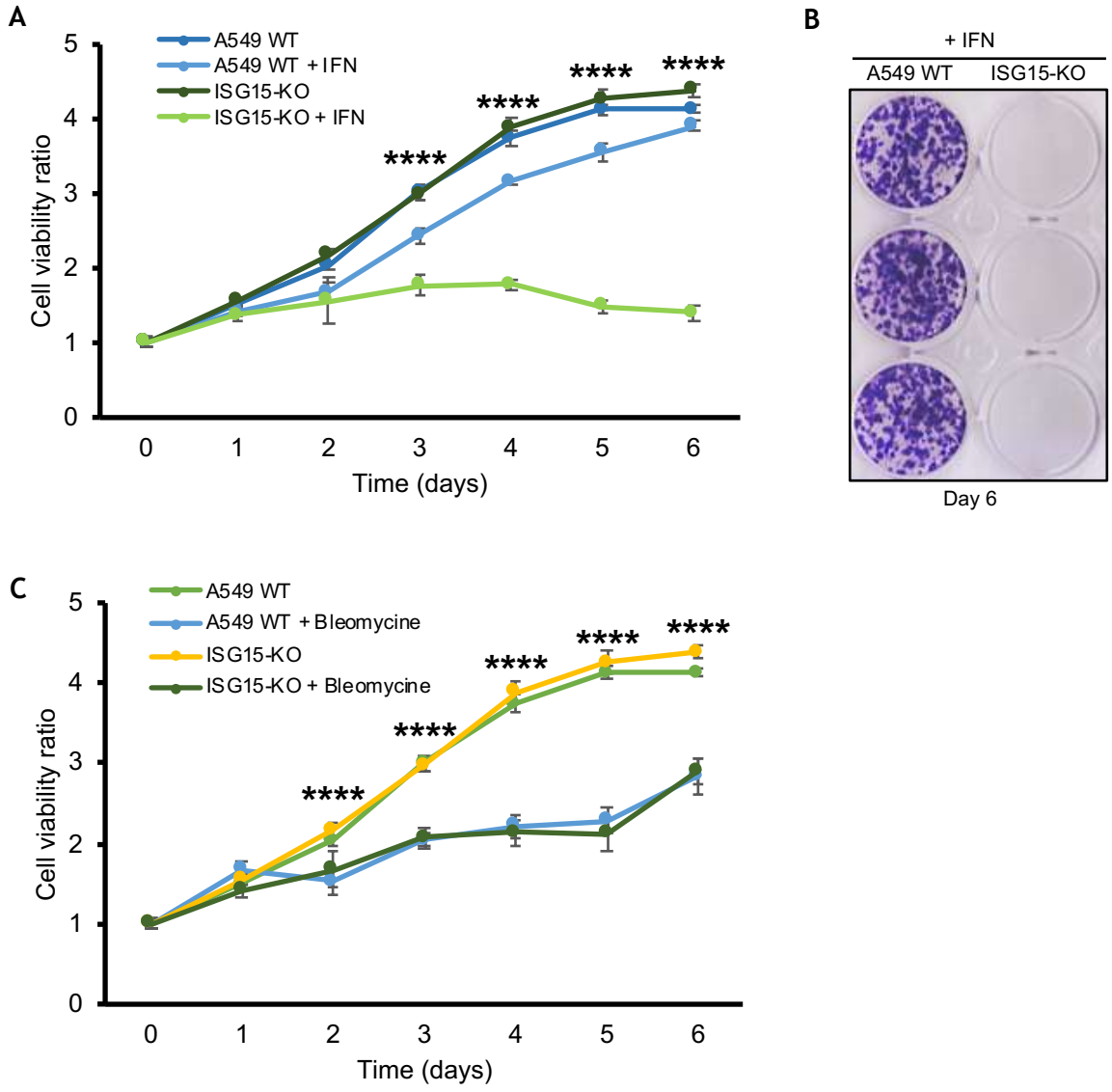


Figure 26. ISG15 protects A549 cells from the negative effect of with IFN-α treatment

A MTT assay of A549 WT and ISG15-KO cells treated or not with 1000 U/mL of IFN-α. Statistical analysis was assessed by One Way-ANOVA. ****, P<0.0001 comparing the cell viability of A549 WT and ISG15-KO cells treated with IFN-α. **B** Colony formation assay of A549 WT and ISG15-KO cells at 6 days after treatment with 1000 U/mL IFN-α. Colonies were stained with crystal violet. **C** MTT assay of A549 WT and ISG15-KO cells treated or not with 20 μM of bleomycin. Statistical analysis was assessed by One Way-ANOVA. ****, (P<0.0001) comparing A549 WT and ISG15-KO cells treated with IFN-α.

To evaluate this hypothesis, A549 WT and ISG15-KO cells were treated with IFN-α (1000 U/mL) and at different times after treatment we performed an annexin V assay to determine the percentage of apoptotic cells. IFN-α treatment of A549 WT cells did not result in the induction of apoptosis (Figure 27). In contrast, we observed a significant increase in the number of apoptotic ISG15-KO cells upon IFN-α treatment (Figure 27). Taken together, these results indicated that ISG15 prevents the induction of apoptosis in A549 cells upon IFN-α treatment.

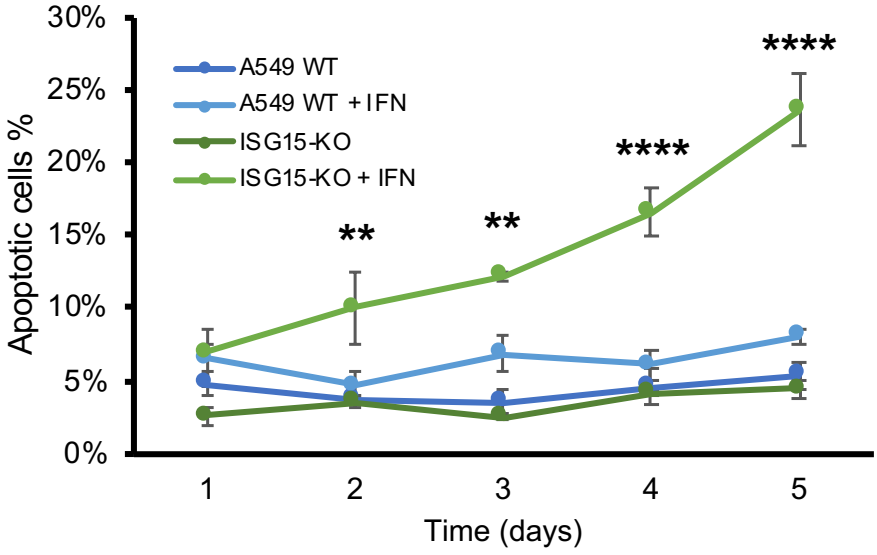


Figure 27. ISG15 prevents the induction of apoptosis in A549 cells upon IFN-α treatment. A549 WT and ISG15-KO were treated or not with 1000 U/mL of IFN-α and at the indicated times after treatment, cells were staining with annexin V and analyzed by cytometry to determine the percentage of apoptotic cells. Statistical analysis was assessed by One Way-ANOVA. **, (P<0.005); ****, (P<0.0001) comparing A549 WT and ISG15-KO cells treated with IFN-α.

To determine the potential role of SUMO interaction on the anti-apoptotic activity of ISG15, we generated A549 cell lines depleted from ISG15 and stably expressing His6-hISG15-WT, His6-hISG15- WT-AA, His6-hISG15-K, His6-hISG15-K-AA, His6-hISG15-2xΔSIM or His6-hISG15-2xΔSIM-AA. We then evaluated the expression levels of the transfected constructs. We observed that the levels of the transfected ISG15 WT and mutant constructs in the stable cells was too low to be detected by direct Western blot (Figure 28A). Only after Histidine purification we were able to detect the transfected constructs (Figure 28A). Therefore, we decided to generate cells stably expressing inducible lentiviral HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA constructs. After confirming that doxycycline treatment induced a good level of expression of the constructs in HEK-293 (Figure 28B) as well as in ISG15-KO cells (Figure 28C), we carried out an MTT assay with the A549 stable cell lines in presence or absence of IFN-α. We observed that IFN-α treatment did not have a significant effect on the proliferation of the ISG15-KO cells reconstituted with hISG15-WT protein (Figure 29). However, IFN-α treatment significantly reduced the proliferation of the cells reconstituted with hISG15-K or hISG15-2xΔSIM (Figure 29), suggesting that the interaction of SUMO with ISG15 has a positive impact on the anti-apoptotic activity of ISG15 in response to IFN-α treatment.

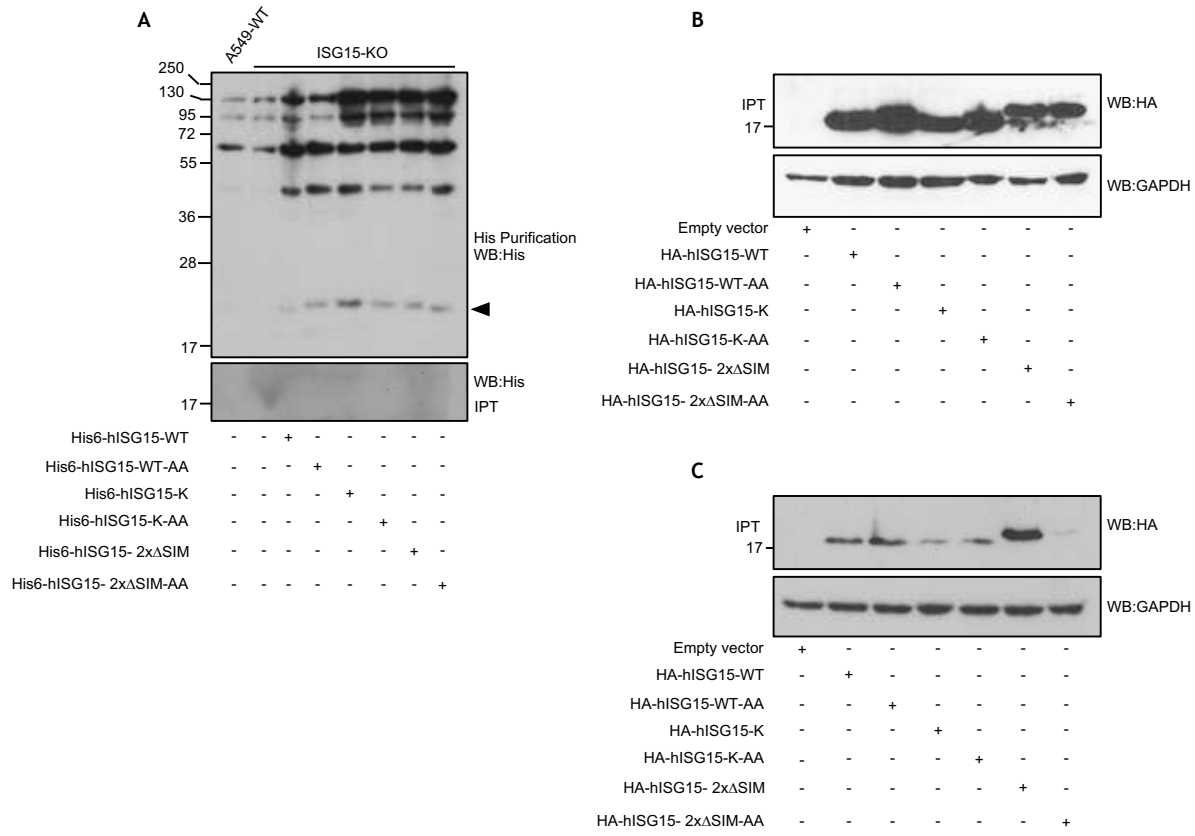


Figure 28. Generation of A549 cell lines depleted from ISG15 and stably expressing the indicated hISG15 constructs.

A A549 ISG15-KO cells were transfected with His6-hISG15-WT, His6-hISG15-WT-AA, His6-hISG15-K, His6-hISG15-K-AA, His6-hISG15-2xΔSIM or His6-hISG15-2xΔSIM-AA and 48 h after transfection, cells were splitted and treated with G418 (1000 µg/mL) for 15 days. Resistant cells were amplified. Total protein extracts and Histidine-tagged purified proteins were evaluated for the expression of the transfected plasmids using Western blot with the indicated antibodies. Arrowhead indicates the unmodified ISG15 protein. **B** HEK-293 cells were transfected with the inducible lentiviral constructs encoding for HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM, or HA-hISG15-2xΔSIM-AA, together with PLP1, PLP2 and VSV-G. 36 h after transfection, cells were treated with 1 µg/ml of doxycycline for 24 h and protein expression was analysed by Western blot using anti-HA antibody. **C** Supernatants recovered from HEK-293 cells transfected as indicated in B, were filtered and used to treat monolayers of A549 ISG15-KO, following the procedure indicated in materials and methods section. After transduction, cells were selected by incubating with G418 (600 µg/mL) for 15 days. Resistant cells were amplified, treated with 1µg/ml of doxycycline, and evaluated for the expression of the transduced plasmids by Western blot analysis using anti-HA antibody.

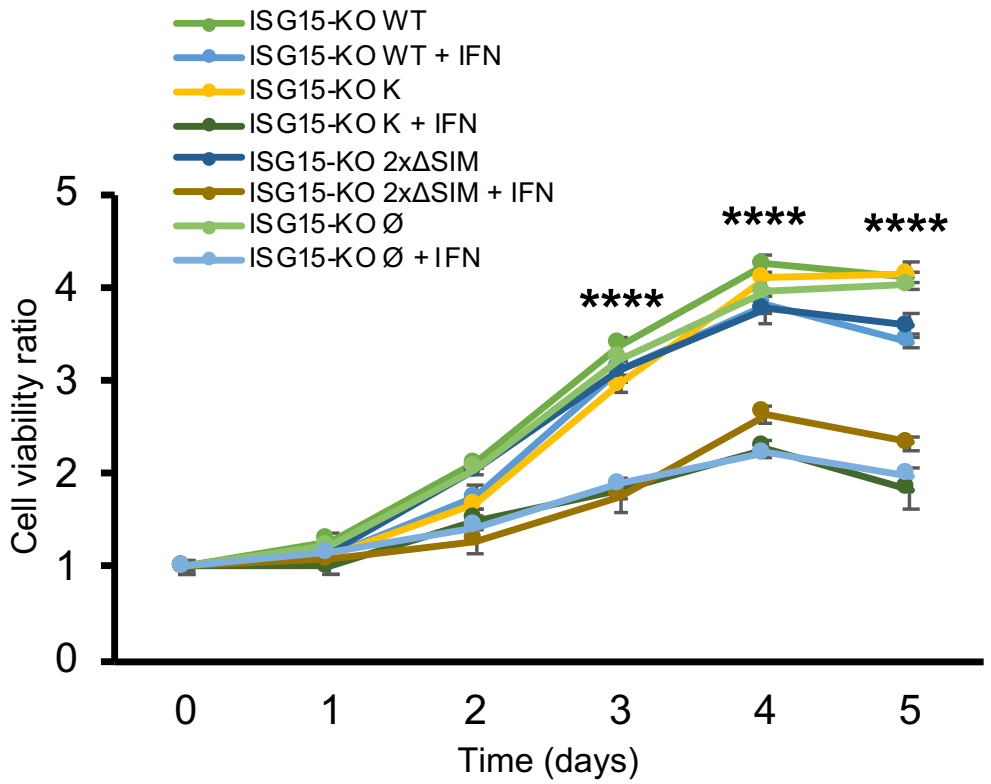


Figure 29. Reconstitution of A549 cells depleted of ISG15 with ISG15-K or ISG15-2xΔSIM expression constructs do not protect the cells from the negative effect of IFN-α treatment.

A549 cells depleted of ISG15 and stably transduced with an empty vector (ø), HA-hISG15-WT, HA-hISG15-K or HA-hISG15-2xΔSIM constructs were treated or not with 1000 U/mL of IFN-α. At the indicated times after treatment, cell viability was determined using an MTT assay. Statistical analysis was assessed by One Way-ANOVA. ****, P<0.0001, comparing cells transduced with empty vector, HA-hISG15-K or HA-hISG15-2xΔSIM with those expressing HA-hISG15-WT after IFN-α treatment.

6.8 SUMO CONTRIBUTES TO THE PRO-VIRAL ACTIVITY OF ISG15

ISG15 can exert proviral or antiviral actions. In order to determine whether human ISG15 contributes to the control or facilitates the replication of vesicular stomatitis virus (VSV) in A549 cells, we first infected A549 WT or ISG15-KO cells with a recombinant VSV expressing GFP (VSV-GFP) at a MOI of 1 PFU/cell. 24 h after infection, cell supernatants were recovered and viral titers were determined by plaque assay in BSC40 cells. The titer of VSV-GFP recovered from A549 WT cells was significantly higher than the titer of virus detected in ISG15-KO cells (Figure 30A), indicating that ISG15 facilitates the replication of VSV in A549 cells. To verify this result and evaluate how global SUMOylation impacts on the proviral activity of ISG15 on VSV in A549 cells, we repeated the assay but this time treating the cells with DMSO or ML-792 (20 μM) 12 h prior to VSV-GFP infection. Evaluation of the viral replication was assessed by quantification of the percentage of GFP positive cells. The percentage of GFP positive cells detected in A549 WT cells was significantly higher than the

one detected in ISG15-KO cells, confirming the proviral effect of ISG15 on VSV in A549 cells (Figure 30B). In addition, we observed that the percentage of GFP positive cells significantly increased after treating the cells with the SUMOylation inhibitor ML-792 (Figure 30B). This effect of ML-792 was independent of the expression or not of ISG15 (Figure 30B). These results suggested that SUMOylation plays a negative effect on VSV replication, but this effect does not require ISG15. Finally, we decided to evaluate the impact of SUMO on ISG15 proviral activity by analyzing the replication of VSV-GFP in cells ISG15-KO reconstituted with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA. Analysis of the viral titers recovered from the cells at 24 h after infection revealed that the amount of virus recovered from cells expressing ISG15-WT or ISG15-WT-AA was significantly higher than the virus recovered from HA-hISG15-K , HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA (Figure 31A), suggesting that the interaction of SUMO with ISG15 contributes to its proviral activity. To verify these results, we carried out similar experiments and we then evaluated the percentage of GFP positive cells at 24 h after infection. Again, we observed that the percentage of GFP positive cells was significantly higher in cells expressing hISG15-WT or hISG15-WT-AA than in HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA expressing cells (Figure 31B). No difference was observed between cells expressing hISG15-K, hISG15-K-AA, hISG15-2xΔSIM and ISG15-2xΔSIM-AA (Figure 31B). These results suggested that the interaction of SUMO with ISG15 facilitates the proviral activity of the ubiquitin-like protein.

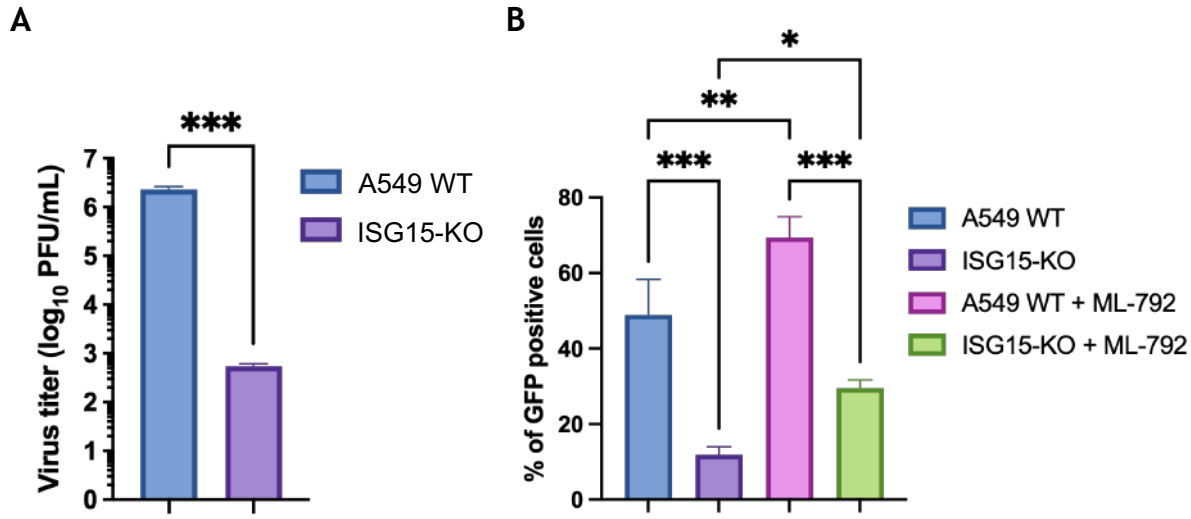


Figure 30. Proviral activity of ISG15 on VSV in A549 cells

A A549 WT or ISG15-KO cells were infected with VSV-GFP at a MOI of 1 PFU/cell and viral titer present in the cell supernatant at 24 h after infection was measured using plaque assay. Statistical analysis was assessed by a Student's t-test. ***, P<0.001. **B** A549 WT or ISG15-KO cells were treated or not with 20 μM of ML-792 12 h prior to VSV-GFP infection at a MOI of 1 PFU/cell, and the percentage of GFP positive cells at 24 h after infection was measured by cytometry. Statistical analysis was assessed by One Way-ANOVA. *, P<0.01 **, P<0.005; ***, P<0.001.

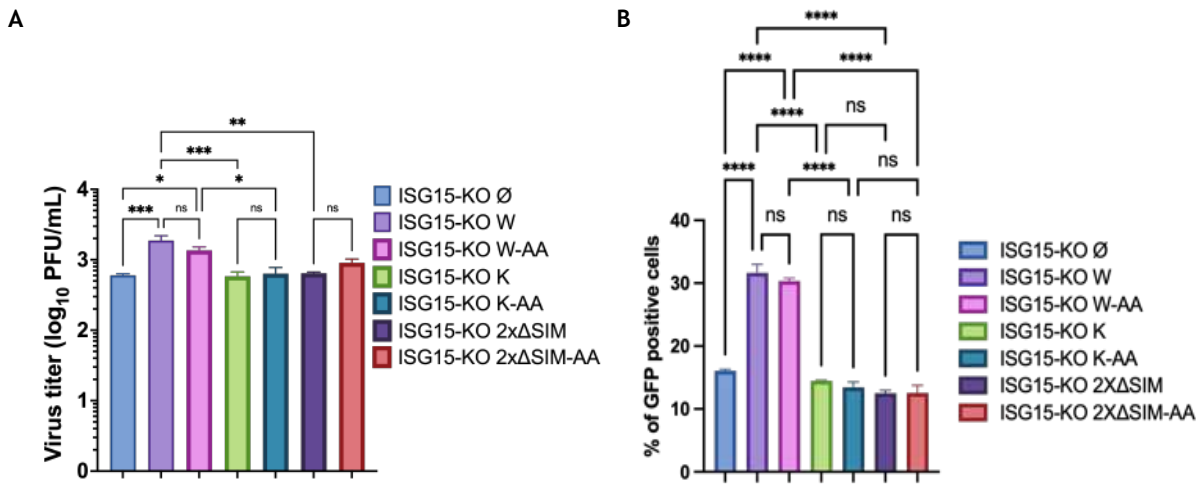


Figure 31. Mutation of the SUMO binding sites in ISG15 abolishes its proviral activity on VSV.

A A549 cells depleted of ISG15 and stably transduced with empty vector (ø), HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA constructs were infected with VSV-GFP at a MOI of 1 PFU/cell, and 24 h after infection, the viral titer in cell supernatants was measured using plaque assay. Statistical analysis was assessed by One way-ANOVA. *, P<0.05; **, P<0.01. **B** A549 cells depleted of ISG15 and stably transduced with empty vector (ø), HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K , HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA constructs were infected with VSV-GFP at a MOI of 1 PFU/cell, and at 24 h after infection, cells were analyzed by cytometry to quantify the percentage of GFP positive cells. Statistical analysis was assessed by One way-ANOVA. ****, P<0.01.

6.9 SUMO MODULATES THE ROLE OF ISG15 ON MITOCHONDRIA BIOGENESIS.

Recently, several studies have shown a connection between ISG15 and mitochondria, and its impact in cellular physiology, immune response and cellular metabolism (Alcalá et al. 2020; Baldanta et al. 2017; Zhang et al. 2019). SUMO is also involved in mitochondrial biogenesis (Cai et al. 2012; He, Cheng, and Wang 2020; Waters et al. 2022). Therefore, we decided to evaluate whether SUMO could modulate the role of ISG15 on mitochondria biogenesis.

First, we evaluated the impact of inhibiting global SUMOylation on mitochondria of A549 WT or ISG15-KO cells, by immunostaining with anti-TOM20 antibody. Both cell lines were treated with DMSO or ML-792 (20 μM) and at 12 h after treatment, cells were fixed, permeabilized, blocked and stained with anti-TOM20 antibody and DAPI. We observed an alteration in the TOM20 staining in cells lacking ISG15 (Figure 32). In contrast to the continuous network observed in WT cells, ISG15-KO cells showed a more fragmented and aggregated pattern (Figure 32), indicating that ISG15 plays a critical role in the biogenesis of the mitochondria. We did not observe differences in the mitochondria morphology between ISG15-KO cells treated with DMSO and ML-792 (Figure 32). In contrast, treatment with ML-792 triggered a fragmented and aggregated mitochondria pattern in A549 WT cells, similar to the observed in those cells lacking ISG15 (Figure 32). Then, we carried out immunostaining of TOM20 in A549 cells depleted of ISG15 and stably transduced with HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM, or HA-hISG15-2xΔSIM-AA. Cells expressing HA-hISG15-WT or HA-hISG15-WT-AA showed poorly fragmented mitochondria (Figure 33). In contrast, a strongly fragmented and aggregated pattern of TOM20 was observed

in those cells expressing the mutants of ISG15 for SUMO interaction (ISG15-K or ISG15-SIM), independently of the presence or not of the diglycine motif (Figure 33), suggesting that the interaction of ISG15 with SUMO is necessary for a proper mitochondria biogenesis.

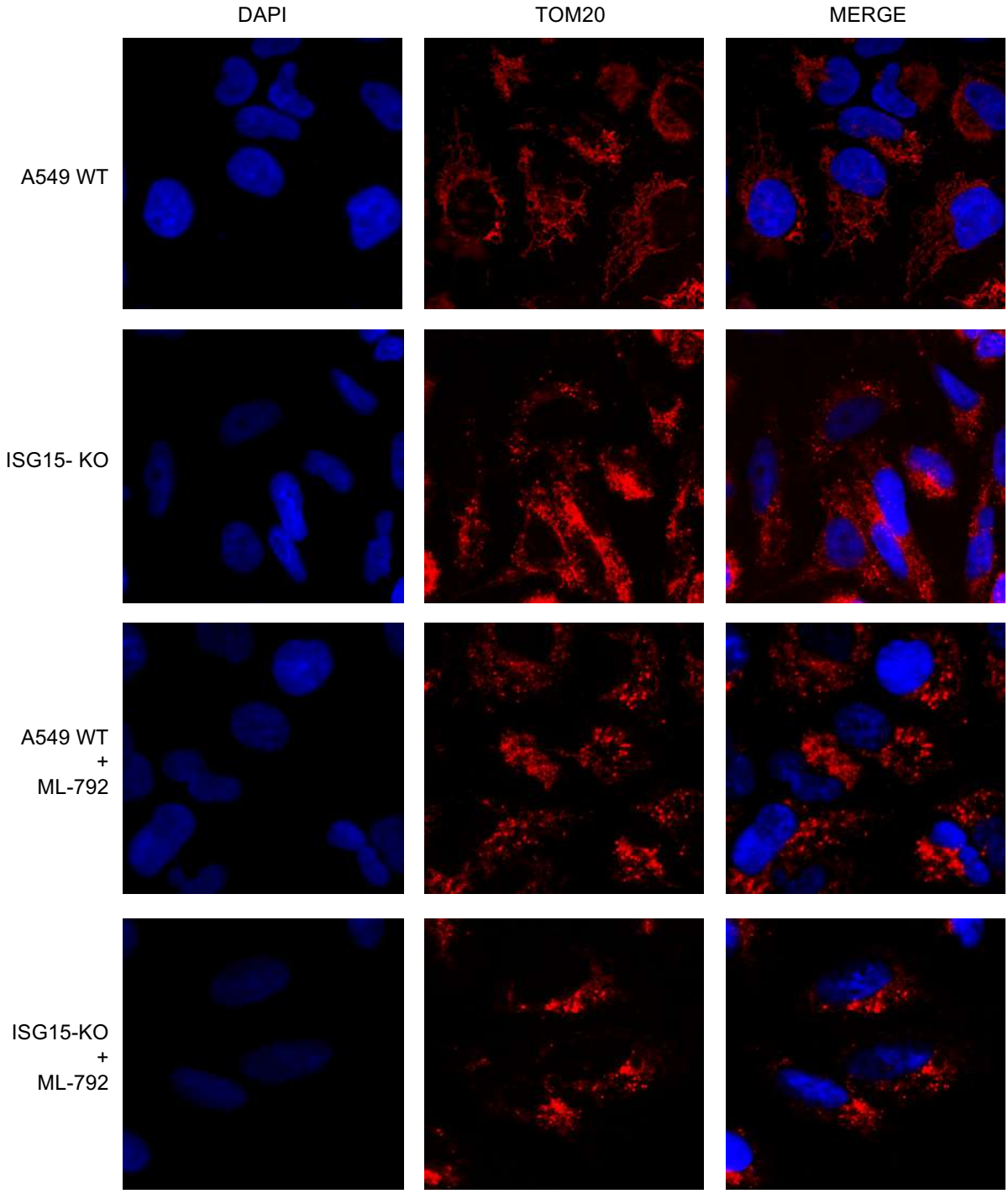


Figure 32. ISG15 and SUMOylation are necessary for mitochondria biogenesis.

A549 WT or ISG15 KO cells were treated or not with 20 μM of ML-792. At 12 h after treatment, cells were immunostained with anti-TOM20 antibody and DAPI.

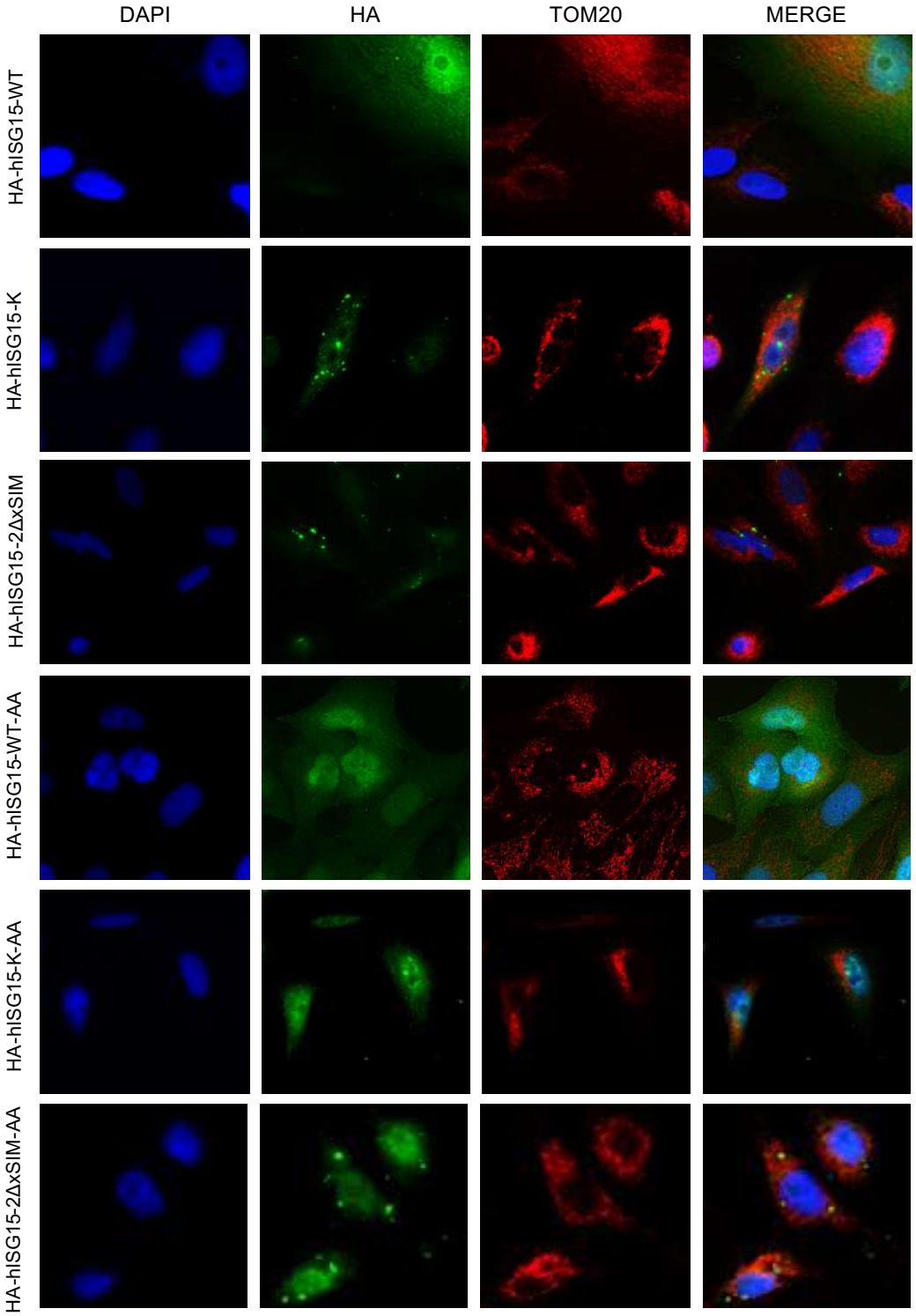


Figure 33. SUMO interaction with ISG15 is required for mitochondria biogenesis

A549 KO stably expressing HA-hISG15-WT, HA-hISG15-WT-AA, HA-hISG15-K, HA-hISG15-K-AA, HA-hISG15-2xΔSIM or HA-hISG15-2xΔSIM-AA constructs were treated with 1µg/ml doxycycline (DOX). 24h after induction, cells were immunostained with anti-HA, anti-Tom20 and DAPI.

7. DISCUSSION

ISG15 is a Ubl protein which expression is induced by type I IFN, DNA damage and other stresses (Mirzalieva et al. 2022). ISG15 can conjugate to its substrates through an enzymatic and reversible process called ISGylation. By conjugating to its substrates, ISG15 can modulate their subcellular localization, oligomerization, activity or stability. Although mass spectrometry has allowed the identification of hundreds of potential targets of ISGylation, only a small number of targets have been validated (Perng and Lenschow 2018). ISG15 frequently increases the stability of its substrates by competing with ubiquitin to bind to its substrates or through the formation of mixed ISG15-ubiquitin chains. ISG15 also exist as an unconjugated protein inside the cell and interacts with various intracellular proteins or it can be secreted from the cell by unclear mechanisms. Free extracellular ISG15 has been reported to function as a cytokine with immunomodulatory activities. Although ISG15 is minimally expressed under physiological conditions, its levels are abnormally elevated in different pathological conditions such as different types of cancer, neurodegenerative disorders (Mirzalieva et al. 2022). Contradictory results have been reported on the role of ISG15 protein on the pathogenesis of different diseases including cancer or virus replication. A potential explanation for these contradictory findings is that ISG15 can perform different functions depending on the cell type, the specie, physiological state, virus type or the ISG15 form (if ISG15 is conjugated to proteins or free unconjugated). How ISG15 functions are modulated is still unknown. Understanding the mechanisms of regulation of ISG15 activities could help to develop new strategies to intervene in the progression of many diseases. Increasing evidences indicate that the crosstalk between different Ubl proteins may a mechanism to fine-tune responses to different stresses. It has been reported the existence of an extensive crosstalk between ubiquitin-like proteins including the conjugation of ISG15 to ubiquitin, the conjugation of ubiquitin to SUMO or the SUMO conjugation to ubiquitin.

The attachment of SUMO to lysine residues in the target proteins or SUMOylation is considered as a key regulatory mechanism modulating the functional properties of a large number of proteins. Conjugation of SUMO to substrates regulates the interaction of the substrate with other proteins or nuclei acid affecting their stability, localization or activity, and influencing many different processes such as the cell cycle, DNA repair, tumor progression, and virus infection (Celen and Sahin 2020; Wilson 2017). The consequences of SUMO conjugation vary greatly depending on the substrate or the virus type. Thus, SUMO activity can promote or inhibit cellular proliferation, migration, oncogenic transformation or virus replication (El Motiam et al. 2020). In addition to ISG15 and ISGylation, IFN also enhances global levels of SUMOylation, and SUMO has been reported to interact with different intermediates along the signaling cascade modulating the transcriptional transactivation of type I IFN(El Motiam et al. 2020). The relevance of SUMO not only stand for their conjugation to target proteins. One characteristic of many SUMOylated proteins is to contain SIMs that mediate their non-covalent interaction with SUMO or with other SUMOylated proteins allowing the formation of big complexes with a function in many processes such as the tress response, virus-cell interplay or immune response (Lascorz et al. 2022).

Recent data support the existence of an interplay between SUMOylation and ISGylation. SUMO has been suggested to play a central role in the stabilization of IFN-stimulated genes products and in enhanced IFN-induced antiviral defense (Chelbi-Alix and Thibault 2021). In response to IFN, SUMO3 has been reported to upregulate ISG15 as well as the ISG15 E2 conjugating enzyme UBCH8 and the E3 ISG15 ligases Trim28 and HERC5 by still unknown

mechanisms. In contrast, downmodulation of SUMOylation through Ubc9 depletion has been shown to decrease the IFN-induced cellular ISGylation (El-Asmi et al. 2020).

We hypothesized that, similarly to the its interaction with ubiquitin, SUMO could also interact with ISG15 and that this interplay may impact ISG15 properties and/or activities. In this work we have study this putative interaction between SUMO and ISG15 and its impact on ISG15 properties and functions. This information may help us to clarify the role of the crosstalk between SUMOylation and ISGylation in innate immune responses and provide opportunities to identify potential therapeutic targets for different diseases.

First, we demonstrated the SUMOylation of human ISG15 by SUMO1 and SUMO2 *in vitro*, in transfected cells, and at totally endogenous levels. This modification was not exclusive of human ISG15, since we show that mouse ISG15 was also SUMOylated. *In vitro* SUMOylation assays using ISG15 and SUMO1 revealed the presence of different bands, suggesting that SUMO can conjugate to different lysine residues in ISG15. *In silico* analysis also identified various lysine residues in ISG15 as potential acceptors for SUMO modification. Finally, analysis of mutants of human ISG15 in potential SUMOylation sites confirmed that the lysine residues K8, K29, K35, K77, K90, K108, K129, and K143 in ISG15 are SUMO acceptors in ISG15. Most of the lysine residues identified as SUMOylation sites in human ISG15 are conserved in mammals (Kang et al. 2022), suggesting that SUMOylation of ISG15 may be conserved among mammals. Interestingly, three of the SUMOylation sites in ISG15 correspond with ubiquitin lysine residues that can be targeted for SUMOylation (Galisson et al. 2011; Hendriks et al. 2014; Lamoliatte et al. 2013), suggesting the idea that SUMO targeting sites may be influenced by some structural similarities.

It is well known that many SUMOylation substrates can also interact in a non-covalent manner with SUMO or other SUMOylated proteins through a SUMO-interacting motif (SIM). In this study, we demonstrated that ISG15 is one of this SUMOylation substrates. Our data revealed that ISG15 can interact with SUMO in a non-covalent manner trough the 72-LLVVD-76 and 82-LSILV-86 SIM domains. Importantly, both SIM domains are conserved among mammals (Kang et al. 2022), suggesting that the interaction between ISG15 and SUMO may be also conserved among mammals. So far, no SIMs have been identified in ubiquitin. However, one of the SIM domains in ISG15 is conserved in ubiquitin. Additional experiments to explore its functionality are required. Mutating the SIM domains in ISG15 not only disrupts its non-covalent interaction with SUMO but also inhibits its SUMOylation, indicating that the SUMO-SIM interaction contributes to the SUMOylation of ISG15, as reported for other SUMO substrates (Lin et al. 2006; Meulmeester et al. 2008). This result led us to visualize a complex regulation of ISG15 SUMOylation since an increase in the levels of free SUMO might compete with Ubc9-SUMO in the recognition of ISG15 inhibiting its SUMOylation, as reported for some SUMO substrates (Cong et al. 2011).

One of the proposed functions of SUMO, mainly SUMO2/3 is to participate in the cellular response to stress conditions (Enserink 2015). In agreement with this function, the SUMOylation of many proteins is triggered upon specific stresses. Here we showed that the SUMOylation of ISG15 is induced upon heat shock stress, UV irradiation, or viral infections, indicative of a physiological function. Interestingly, conjugation of SUMO to the lysine residues in ubiquitin which are conserved in ISG15, are also increased upon heat shock and proteasome inhibition (Hendriks et al. 2014). We then speculate that conjugation of SUMO to

ISG15 upon specific stresses may contribute to modulate innate immune response and inflammation.

The consequences of SUMOylation are substrate-dependent: SUMO conjugation can modulate the stability of the substrates, the activity of transcription factors, influence protein localization, or control the functions of enzymes, among others (Saitoh and Hinchey 2000). In this study, we were interested in evaluating whether SUMOylation plays a role in the modulation of ISG15 activity and functions. First, we evaluated the potential regulation of the subcellular distribution of ISG15 by SUMO interaction. Although ISG15 is detected mainly at the cell cytoplasm, it has been found across all cellular compartments (Albert et al. 2018). In our assays we found ISG15 WT diffusely distributed mainly at the cell cytoplasm, independently of its conjugation to substrates but we also found ISG15 in the nucleus, with a small portion co-localizing with PML-NBs, in agreement with its ability to interact with SUMO. Mutation of the SUMOylation sites or of the SIM domains in ISG15 led to its predominant nuclear localization, in a cell-type independent manner. These results suggested that the interaction of SUMO with ISG15 facilitates its cytoplasmic localization. Importantly, free ISG15 can also be found in the extracellular compartment as result of its secretion from the cytoplasm (D'Cunha et al. 1996). We found that mutation of the SUMOylation sites or of the SIM motifs in ISG15 was associated to the absence of ISG15 protein in the cell supernatant, independently of its conjugation to substrates This result is in agreement with recent findings identifying the amino acids L72, S83, and L85 in the ISG15 protein, contained inside the SIM domain, as required for its secretion (Swaim et al. 2017). A reduction in the levels of ISG15 in the supernatant of the cells expressing mutants of ISG15 for SUMO interaction, may suggest that SUMO, by interacting with ISG15, facilitates its secretion or it may be the result of its predominant nuclear localization. Since SUMOylation of ISG15 can be induced in response to different stresses, it would be interesting to evaluate if the secretion of ISG15 increases upon these stresses. In addition, it has been found that lack of deISGylation leads to an accumulation of extracellular ISG15 (Ketscher et al. 2015). Therefore, further experiments to evaluate the impact of USP18 downmodulation on the secretion of the mutants of ISG15 for SUMO interaction are needed.

Stress is not the unique condition that stimulates ISG15 SUMOylation. Our results revealed that the tumor suppressor p14ARF protein also triggers ISG15 SUMOylation. It is well known that p14ARF induces cell cycle arrest, senescence, or apoptosis in response to oncogenic stress (Cilluffo, Barra, and Di Leonardo 2020). In addition, p14ARF has also been proposed to contribute to immune stimulation (Mendonça et al. 2023). Although some of the activities of p14ARF occur through a p53-dependent pathway, p14ARF is capable of exert some of its activities in a p53-independent manner. Thus, p14ARF triggers SUMOylation of many interactors including p53 and MDM2 through a p53-independent manner (Ivanschitz et al. 2015b; Xirodimas et al. 2002). Our results point to ISG15 as an additional p14ARF interactor. Here we also observed that p14ARF overexpression induced the translocation of ISG15 to the nucleus. This phenomenon aligns with previous findings suggesting that p14ARF, when overexpressed, can sequester its interaction partners in the nucleus, thereby inducing their SUMOylation (Tago et al., 2005; Maggi et al. 2014; Weber et al. 1999).

Moreover, our data identified ISG15 as a new regulatory layer of p14ARF. It known that p14ARF, a protein lacking of lysine residues, can be polyubiquitinated at the N-terminus (Kuo et al. 2004). The impact of ISG15 conjugation on p14ARF remains unknown but the potential

competition between ISG15 and ubiquitin for interacting with p14ARF led us to speculate that it may serve as a stabilization mechanism for p14ARF.

Another tumor suppressor capable of inducing SUMOylation of its interactors is PML. PML-NBs serve as a primary hub for SUMO proteins and is recognized as a pivotal site for protein SUMOylation (Lallemand-Breitenbach and de Thé 2018). Interestingly, a role for p14ARF in the SUMOylation induced by PML has also been proposed (Ivanschitz et al. 2015). Our study indicated that mutation of the SUMOylation sites or of SIM domains in ISG15 enhanced the fraction of ISG15 co-localizing with the PML-NBs. The molecular mechanism of this phenomenon is unknown. One possible explanation is that the predominant nuclear localization of the SUMO interaction mutants facilitates their interaction with PML-NBs. In addition, it has also been reported that PML aggregates with misfolded proteins and components of the proteasome. Therefore, it is possible that the higher co-localization between PML-NBs and the SUMOylation mutants reflects the accumulation of the mutants of ISG15 together with PML at the proteasome. In addition, SUMO can attenuate intermolecular interactions and inhibit oligomerization (Zhao 2018), and the ladder-like pattern of the mutant of ISG15 for non-covalent interaction with SUMO is suggestive of its oligomerization. Additionally, since ISG15 has been reported to modify PML (Shah et al. 2008), it is also possible that abolishing the interaction of SUMO with ISG15 promotes PML accumulation and aggregation. The alteration in the number and size of PML-NBs observed in those cells expressing the SUMOylation mutants supports the formation of aggresomes containing PML. Further investigations are warranted to evaluate these hypotheses.

Here we show that ISG15 protect A549 cells from apoptosis induced in response to IFN treatment and that the interaction of SUMO with ISG15 contributes to its anti-apoptotic activity. However, it is important to notice that A549 cells are p53 WT cells and lack p14ARF. It will be important to evaluate the role of ISG15 and of ISG15-SUMO interaction in a p53 KO and p14ARF positive cancer cell line. The induction of SUMOylation upon p14ARF overexpression and the contribution of SUMO to the anti-apoptotic activity of ISG15 led us to hypothesize that SUMOylation of ISG15 may be one of the mechanisms by which p14ARF exerts its anti-tumoral activity.

Upon viral infection, host cells promote the expression of type I interferon (IFN) and proinflammatory cytokines, which represent the first line of host defense against viruses. Ubl proteins such as ISG15 and SUMO are central to the regulation of IFN production, IFN signaling, as well as the localization, stability and activity of many restriction factors (Liu et al. 2013; Perng and Lenschow 2018). ISG15 has been identified as a relevant player in the host antiviral response. However, the role of ISG15 is species-specific and depends on the virus (Eduardo-Correia et al. 2014; Morales and Lenschow 2013; Sengupta et al. 2017; Speer et al. 2016). Our experiments in A549 WT and ISG15-KO cells revealed that ISG15 exerts a proviral activity on VSV in these cells. In contrast SUMOylation inhibits VSV replication, as revealed by the increased viral replication upon treatment with the SUMOylation inhibitor, ML-792. However, this effect was independent of the expression or not of ISG15, indicating additional mechanisms of action. Analysis of A549 cells lacking ISG15 and reconstituted with ISG15 WT or the different ISG15 mutants revealed that the interaction of ISG15 with SUMO is required for its proviral activity on VSV in A549 cells. How SUMO interaction with ISG15 facilitates the replication of VSV is still under study. One possibility is that the interaction of SUMO with ISG15 is essential to stabilize USP18 and therefore to inhibit the type I interferon signaling.

Other putative explanation could be that the higher aggregates of PML resulting from the expression of the mutants of ISG15 for SUMO interaction are not functional against VSV.

Mitochondria are vital organelles responsible for processes such as energy production, calcium signaling, apoptosis, and immune system regulation (Garaude et al. 2016; Grasso et al. 2020; Osellame, Blacker, and Duchen 2012). Recent studies have unveiled a connection between ISG15 and mitochondria, emphasizing their pivotal role in cellular physiology, immune responses, and cellular metabolism (Alcalá et al. 2020; Baldanta et al. 2017; Zhang et al. 2019). SUMO has also been implicated in mitochondrial biogenesis (Cai et al. 2012; He et al. 2020; Waters et al. 2022). Accordingly, our data revealed increased mitochondrial fragmentation in A549 cells depleted of ISG15 or after inhibition of SUMOylation. In addition, our data suggested that the interaction of ISG15 with SUMO is essential for maintaining functional mitochondria. However, additional studies are warranted to confirm this hypothesis.

In summary, this study reveals a novel regulation of ISG15 through its covalent and non-covalent interaction with SUMO, the impact of these interactions on the ISG15 protein and their potential functional implications. This work contributes to advancing our understanding of the complex regulatory networks governing cellular responses and lays the groundwork for further investigations in the field.

8 CONCLUSIONS

1. ISG15 can be SUMOylated *in vitro*, in transfected cells, and under totally endogenous conditions.
2. SUMO can modify both human and mouse ISG15.
3. Lysine 8, 29, 35, 77, 90, 108, 129, and 143 are SUMO acceptor sites in human ISG15.
4. ISG15 interacts with SUMO in a non-covalent manner through the 72-LLVVD-76 and 82-LSILV-86 SIM domains.
5. SIM domains in ISG15 facilitate its covalent interaction with SUMO.
6. SUMOylation of ISG15 is triggered by different types of stress.
7. ISG15 secretion requires the covalent and non-covalent interaction of ISG15 with SUMO.
8. SUMO interaction with ISG15 modulates its subcellular distribution, promoting its cytoplasmic localization.
9. SUMO interaction with ISG15 may negatively modulate its oligomerization.
10. Overexpression of the tumor suppressor p14ARF induces the SUMOylation of ISG15.
11. p14ARF overexpression promotes the nuclear translocation of ISG15.
12. p14ARF can be ISGylated in transfected cells.
13. ISG15 partially co-localizes with PML-NBs.
14. ISG15 protects A549 cells from the pro-apoptotic activity of IFN.
15. Interaction of SUMO with ISG15 is required for the anti-apoptotic activity of ISG15 in A549 cells treated with IFN.
16. ISG15 facilitates the replication of VSV in A549 cells.
17. Interaction of SUMO with ISG15 is required to facilitate the replication of VSV in A549 cells.
18. Both ISG15 and SUMOylation are essential for proper mitochondria biogenesis.
19. Both covalent and non-covalent interaction of SUMO with ISG15 are required for proper mitochondria biogenesis.

9 TABLES

Table 1. Primers used for cloning and mutagenesis

NAME	SEQUENCE
hISG15-K8R-F	5' G GAC CTG ACG GTG AGG ATG CTG GCG GGC AA 3'
hISG15-K8R-R	3' TT GCC CGC CAG CAT CCT CAC CGT CAG GTC C 5'
hISG15-K29R-F	5' G GTG TCA GAG CTG AGG GCG CAG ATC ACC CA 3'
hISG15-K29R-R	3' TG GGT GAT CTG CGC CCT CAG CTC TGA CAC C 5'
hISG15-K35R-F	5' G CAG ATC ACC CAG AGG ATC GGC GTG CAC GC 3'
hISG15-K35R-R	3' GC GTG CAC GCC GAT CCT CTG GGT GAT CTG C 5'
hISG15-K77R-F	5' TG CTG GTG GTG GAC AGA TGC GAC GAA CCT CT 3'
hISG15-K77R-R	3' AG AGG TTC GTC GCA TCT GTC CAC CAC CAG CA 5'
hISG15-K90R-F	5' TG GTG AGG AAT AAC AGG GGC CGC AGC AGC AC 3'
hISG15-K90R-R	3' GT GCT GCT GCG GCC CCT GTT ATT CCT CAC CA 5'
hISG15-K109R-F	5' CC GTG GCC CAC CTG AGG CAG CAA GTG AGC GG 3'
hISG15-K109R-R	3' CC GCT CAC TTG CTG CCT CAG GTG GGC CAC GG 5'
hISG15-K129R-F	5' TG ACC TTC GAG GGG AGG CCC CTG GAG GAC CA 3'
hISG15-K129R-R	3' TG GTC CTC CAG GGG CCT CCC CTC GAA GGT CA 5'
hISG15-K143R-F	5' GG GAG TAC GGC CTC AGG CCC CTG AGC ACC GT 3'
hISG15-K143R-R	3' AC GGT GCT CAG GGG CCT GAG GCC GTA CTC CC 5'
hISG15-SIM1-72/76A-F	5' CCC GGC AGC ACG GTC GCG GCG GCG GTG GAC AAA TGC GA 3'
hISG15-SIM1-72/76A-R	3' TC GCA TTT GTC CAC CGC CGC CGC GAC CGT GCT GCC GGG 5'
hISG15-SIM1-82/86A-F	5' GAC GAA CCT CTG AGC GCC GCG GCG AGG AAT AAC AAG GG 3'
hISG15-SIM1-82/86A-R	3' CC CTT GTT ATT CCT CGC CGC GGC GCT CAG AGG TTC GTC 5'

HA-ISG15-F	5’ GG AGA TCT GGC TGG GAC CTG ACG 3’
HA-ISG15-R	3’ GG TCT AGA CTA TCA GCC ACC CCG 5’
HA-ISG15-AA-R	3’ GG TCT AGA CTA TCA GGC AGC CCG 5’
His6-ISG15-F	5’ GG GGA TCC GGC TGG GAC CTG ACG 3’
His6-ISG15-R	3’ GG TCT AGA TCA GCC ACC CCG CAG 5’
His6-ISG15-AA-R	3’ GG TCT AGA TCA GGC AGC CCG CAG 5’
His6-ISG15-Lenti-F	5’ G GGC GGC CGC ATG CAT CAT CAT CAT CAT 3’
His6-ISG15-Lenti-R	3’ GG TGG CCA TCA GCC ACC CCG CAG 5’
His6-ISG15-AA-Lenti-R	3’ GG TGG CCA TCA GGC AGC CCG CAG 5’

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ISG15 is a Ubiquitin-like protein that plays important functions in different pathologies but these functions depend on the specie, cell type, viral type and ISG15 form. How ISG15 activities are regulated is unclear. Here we show that ISG15 can be SUMOylated and interacts with SUMO in a non-covalent manner. SUMOylation of ISG15 is triggered by different types of stress as well as upon overexpression of the tumor suppressor p14ARF. We show that ISG15 can be detected at the PML-NBs and that mutation of the SUMO binding sites positively modulates its co-localization with PML-NBs. Finally, we show that the interaction of SUMO with ISG15 positively modulates the anti-apoptotic activity of ISG15 upon IFN treatment, the pro-viral activity of ISG15 during VSV infection, and is required for proper mitochondria biogenesis.