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Switch in post-translational modifications upon Influenza virus infection

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DOCTORAL THESIS

SWITCH IN POST-TRANSLATIONAL MODIFICATIONS UPON INFLUENZA VIRUS INFECTION

Rocío Seoane Abelenda

INTERNATIONAL PHD SCHOOL OF THE UNIVERSITY OF SANTIAGO DE COMPOSTELA

PHD PROGRAMME IN MOLECULAR MEDICINE

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1. ABBREVIATIONS

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Asn (N)- Asparagine
ATF6- Activating transcription factor 6
ATP- Adenosine triphosphate
BAD- Bcl-2 associated agonist of cell death
BSA- Bovine serum albumin
CARD- Caspase activation and recruitment domains
cDNA- Complementary deoxyribonucleic acid
CHIKV- Chikungunya virus
CHX- Cycloheximide
CRL- Cullin-RING E3 ubiquitin ligase
CPSF- Cleavage and polyadenylation specific factor
CRM1- Chromosomal maintenance 1
cRNA- Complementary ribonucleic acid
cRNP- Complementary ribonucleoprotein
DAPI- 4',6'-diamidino-2-phenylindole
DHS- Deoxyhypusine synthase
DMEM- Dulbecco's Modified Eagle's Medium
DMSO- Dimethyl sulfoxide
DNA- Deoxyribonucleic acid
DOHH- Deoxyhypusine hydroxylase
dsRNA- Double-stranded RNA
EBOV- Ebola virus
EBV- Epstein-Barr virus
eIF2α- Eukaryotic translation initiation factor 2α
eIF4G1- Eukaryotic translation initiation factor 4 Gamma 1
eIF5A- Eukaryotic translation initiation factor 5A
ER- Endoplasmic reticulum
ERK1/2- Extracellular-signal regulated kinase 1 and 2
FBS- Fetal bovine serum
GAPDH- Glyceraldehyde-3-phosphate dehydrogenase
GC7- N1-guanyl-1,7-diaminoheptane
GFP- Green fluorescent protein
GSK-3 Glycogen synthase kinase 3
H2B- Histone 2 type B
HA- Hemagglutinin
HDAC6- Histone deacetylase 6

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HEK-293T- Human embryonic kidney 293T cells
His- Histidine
HIV- Human Immunodeficiency virus
HMCV- Human cytomegalovirus
HSP40- Heat shock protein 40
IAV- Influenza A virus
IBV- Influenza B virus
ICV- Influenza C virus
IDV- Influenza D virus
IFN- Interferon
IFNAR1- Interferon α and β receptor subunit 1
IP- Immunoprecipitation
IRE1- Inositol-required enzyme 1
IRF3- Interferon regulatory factor 3
IRF7- Interferon regulatory factor 7
ISG- Interferon stimulated gene
JNK- c-Jun N-terminal kinases
kDa- Kilodalton
LB- Luria Bertani
LRT- Lower respiratory tract
Lys (K)- Lysine
M1- Matrix protein 1
M2- Matrix protein 2
MAPK- Mitogen-activated protein kinase
MAVS- Mitochondrial antiviral-signaling protein
MAYV- Mayaro virus
MDCK- Madin-Darby canine kidney cells
MDM2- Murine double minute 2
MKK- MAP kinase kinase
moi- Multiplicity of infection
mRNA- Messenger ribonucleic acid
NA- Neuraminidase
NAE- NEDD8 activating enzyme
NEDD8- Neural precursor cell expressed, developmentally down-regulated 8
NEDP1- NEDD8-specific protease 1

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NEMO- NF-κB essential modulator
NEP- Nuclear export protein
NF-κB- Nuclear factor kappa B
NLS- Nuclear localization signal
NP- Nucleoprotein
NS1- Non-structural protein 1
NXF1-NXT1- Nuclear RNA export factor 1-nuclear transport factor 2-like export factor 1
ODC- Ornithine decarboxylase
PA- Polymerase acidic protein
PABP1- Poly(A)-binding protein 1
PAGE- Polyacrylamide gel electrophoresis
PAMP- Pathogen-associated molecular pattern
PB1- Polymerase basic protein 1
PB2- Polymerase basic protein 2
PBS- Phosphate-buffered saline
PEI- Polyethyleneimine
PERK- PKR-like endoplasmic reticulum kinase
PFU- Plaque-forming unit
PI3K- Phosphatidylinositol-3-kinase
PIP2- Phosphatidylinositol (4,5)-bisphosphate
PIP3- Phosphatidylinositol (3,4,5)-triphosphate
PKC- Protein kinase C
PKR- Protein kinase R
PRR- Pattern recognition receptors
PRRSV- Porcine reproductive and respiratory syndrome virus
PTM- Post-translational modification
PTV- Punta toro virus
qPCR- Quantitative polymerase chain reaction
qRT-PCR-Quantitative reverse transcription-polymerase chain reaction
RdRp- RNA-dependent RNA polymerase
RIG-I- Retinoic acid-inducible gene I
RING- Really interesting new gene
RNA-Ribonucleic acid
RNF135- RING finger protein 135

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RPL26- Ribosomal protein L26
RPS27A- Ribosomal protein S27a
SAE- SUMO activating enzyme
SAPK- stress-activated protein kinase
SDS- Sodium dodecyl sulfate
SENP1- SUMO-specific peptidase 1
Ser (S)- Serine
sgRNA- Single-guide ribonucleic acid
siRNA- Small interfering ribonucleic acid
SSAT- Spermidine/spermine-N(1)-acetyltransferase
STUB1- STIP1 homology and U-Box containing protein 1
SUBEs- SUMO-binding entities
SUMO- Small ubiquitin-like modifier
TBST- Tris-buffered saline Tween
TGEV- Transmissible gastroenteritis virus
Thr (T)- Threonine
TLR- Toll-like receptor
TRIM25- Tripartite interaction motif 25
TRIM40- Tripartite interaction motif 40
Trp (W)- Tryptophan
Tyr (Y)- Tyrosine
UB- Ubiquitin
UBA1- Ubiquitin activating enzyme 1
UBL- Ubiquitin-like protein
UFC1- UFM1-specific conjugase
UFM1- Ubiquitin fold modifier 1
UFSP- UFM1-specific cysteine protease
UNAV- Una virus
UPR- Unfolded protein response
UPS- Ubiquitin proteasome system
URT- Upper respiratory tract
UV- Ultraviolet
VDAC1- Voltage-dependent anion channel 1
vRNA- Viral ribonucleic acid
vRNP- Viral ribonucleoprotein
VSV- Vesicular stomatitis virus

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WB- Western blot
WCE- Whole cell extract
WHO- World Health Organization
WT- Wild type
XBP1- X-Box binding protein 1
ZAP- Zinc finger antiviral protein
ZIKV- Zika virus
β-GF- β grasp fold

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2. SUMMARY

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2.1 RESUMO

O virus influenza A (IAV) é o axente causal da gripe. Os síntomas da gripe, de manifestarse, adoitan ser de carácter leve incluíndo febre, tose, mocos e dor muscular. Sen embargo, en ciertos pacientes, especialmente en maiores, cativos e pacientes con patoloxías previas, pódese producir unha complicación da enfermidade, sobre todo se o virus consegue replicarse no tracto respiratorio inferior. Esta doenza afecta a millóns de persoas de todo o mundo causando máis de 600000 mortes anuais. A gripe é unha enfermidade estacionaria que aparece, nas zonas temperadas, principalmente no inverno. Este virus está asociado históricamente a varias pandemias, pero cabe remarcar a pandemia da gripe do 1918 coñecida como pandemia da "gripe española". Calcúlase que causou preto de 50 millóns de mortes e ata o momento é a pandemia máis grave da que se ten constancia. O virus da gripe pertence á familia *Orthomixoviridae* e a súa información xenética está contida en 8 fragmentos de ARN. A presenza dun xenoma segmentado, xunto co feito de que a polimerasa encargada de crear as copias do ARN é unha polimerasa que introduce gran cantidade de erros, fan que o xenoma do virus cambie constantemente e mesmo se produza un intercambio de fragmentos xénicos entre IAV de distinta orixe que estean coinfectando unha célula. É por iso que non existe una vacina universal contra o virus e segue a causar un problema grave de saúde en todo o mundo. Xa que a vacina contra a gripe é anual e está especificamente dirixida contra ás cepas do virus que están en circulación nese momento, considerase unha prioridade a xeración dunha vacina universal e a obtención de tratamentos máis eficaces contra o virus. Para acadar estes dous obxetivos, é fundamental coñecer todos os detalles moleculares da relación entre o virus e a célula infectada.

O virus da gripe, como todos os virus, depende en gran medida da maquinaria celular, que é capaz de inhibir, activar ou secuestrar no seu propio beneficio. Moitas das vías de sinalización celular son activadas en resposta á infección viral para impedir a replicación do virus nas

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células infectadas, avisar ás células veciñas da presenza dun patóxeno, ou promover a morte celular para así evitar que o virus complete o seu ciclo. Por outra banda, os virus teñen proteínas capaces de modular moitas destas vías de tal maneira que son capaces de activalas ou inactivalas en momentos precisos durante o proceso de infección co fin de completar con éxito a súa replicación. O coñecemento de todas as estratexias empregadas polo virus para favorecer a súa replicación é esencial para gañar a batalla contra o virus. Para ter éxito contra o virus a célula necesita poder responder rápidamente á infección. A célula conséguelo grazas entre outros, á regulación das proteínas mediante modificacións post-traducionais (PTM). Estas modificacións afectan a proteínas xa existentes na célula e polo tanto permiten unha resposta rápida ó estrés causado pola infección viral. No caso do virus da gripe sabemos que en resposta á infección celular co virus hai un aumento global de distintos tipos de modificacions post-traducionais coma as causadas polas proteínas da familia das ubiquitinas. Estas modificacions poden afectar a proteínas celulares e virais. Ademais, varias modificacións poden afectar a unha mesma proteína nun determinado momento ou poden competir entre elas, e a maioría destas modificacións son reversibles, o que pode levar a incrementar de maneira importante a versatilidade das proteínas. Isto é especialmente relevante no caso das proteínas víricas, xa que permite a estes patóxenos con poucas proteínas aumentar a súa versatilidade para contrarrestar a resposta antiviral da célula.

Por estes motivos, neste traballo puxémonos como obxetivo estudar a función que as modificacións post-traducionais teñen na relación entre o virus da gripe e a célula..

En primeiro lugar estudamos a posible relación entre o virus da gripe e a hipusinación. A hipusinación é unha modificación post-traducional que afecta soamente a unha proteína, o factor de tradución eIF5A. De feito considérase que é necesario que eIF5A estea hipusinado para que a proteína estea activa. O proceso de hipusinación é un proceso enzimático no que unha porción da espermidina, que é

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unha poliamina, se conxuga á lisina 50 de eIF5A mediante a acción do enzima DHS. A continuación, tras un paso de hidroxilación mediado polo enzima DOHH, finalízase a formación da hipusina necesaria para a actividade de eIF5A. eIF5A é un factor de elongamento e iniciación evolutivamente conservado. Unha das funcións máis relevantes deste factor é a liberación da pausa ribosomal que ocorre no proceso de tradución daquelas proteínas que posúen secuencias difíciles de traducir, como por exemplo rexións que posúan varias prolinas consecutivas. Altos niveis deste factor foron atopados en moitos tipos de tumores e adoitan correlacionar cun peor prognóstico da enfermidade. Ademais, fallos na regulación de eIF5A ou da súa modificación por hipusinación foron atopados en enfermidades neuronais e tamén en enfermidades cardiovasculares. Neste traballo demostramos que a infección polo virus da gripe, así como a infección polo virus da estomatite vesicular ou simplemente o tratamento das células cun axente sintético que simula o ARN de dobre cadea que se produce coma consecuencia da infección viral, son estímulos capaces de activar eIF5A. Además propoñemos que esta activación está mediada pola vía do NF-κB. A activación desta vía induciría un aumento dos niveis de ODC, un enzima implicado no metabolismo das poliaminas, que favorece a formación da hipusina. Actualmente existe un fármaco capaz de inhibir a activación de eIF5A (capaz de inhibir polo tanto a hipusinación da proteína). Anteriormente demostrouse que este fármaco, GC7, é capaz de inhibir a replicación de diversos virus como o ébola ou o VIH. Neste traballo describimos que tanto o tratamento das células con este fármaco, coma a reducción nos niveis de eIF5A, teñen un efecto negativo sobre a replicación de virus de ARN tan diversos como o virus da gripe, o virus da estomatite vesicular, ou os virus emerxentes zika, una, chikungunya, punta toro ou mayaro. Ademais postulamos que o mecanismo polo que o fai é a través da ruta NF-κB, xa que a inhibición de eIF5A promove un estado antiviral na célula que disminúe na presenza de inhibidores da vía NF-κB. Ademais propoñemos á proteína p85β humana, unha proteína chave na ruta PI3K/AKT e que ten un papel moi importante na replicación do virus,

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coma unha diana de eIF5A posto que o GC7 modula os niveis da mesma.

Polo tanto, eIF5A xoga un papel importante na replicación dunha gran variedade de virus de ARN. Sen embargo, o mecanismo polo cal se regula esta proteína é algo que non se coñece con profundidade máis alá da súa hipusinación. Aquí demostramos que a proteína da familia da ubiquitina SUMO é capaz de modificar eIF5A tanto *in vitro* como en células humanas. Tamén demostramos que esta modificación non depende da hipusinación da proteína e identificamos 5 lisinas implicadas na modificacion por SUMO. A conxugación con SUMO regula a localización subcelular de eIF5A. Así, cando estas lisinas están mutadas, eIF5A localízase maiormente no núcleo celular, a diferencia da localización no núcleo e no citoplasma celular da proteína eIF5A silvestre. Ademáis, a proteína mutada nos sitios de SUMOilación é moito menos estable ca proteína silvestre e interacciona máis forte coa ligasa da ubiquitina STUB1. Por outra banda, estudamos como afectan diversos tipos de estrés á SUMOilación de eIF5A e vemos que esta modificación parece xogar un papel moi relevante en resposta a estrés. Así, mentras que o tratamento das células con radiación ultravioleta ou con hipoxia diminúe a SUMOilación da proteína, a infección viral, o estrés proteotóxico e o estrés térmico incrementan considerablemente esta modificación. Dada a pouca estabilidade do mutante de eIF5A que non se pode SUMOilar, estudamos a funcionalidade desta noutro modelo máis sinxelo, en lévedos. Demostramos que en lévedos o mutante en SUMOilación é estable pero non consegue rescatar o crecemento de un lévedo no que impedimos a expresión da proteína eIF5A endóxena, mentres que a introdución da proteina silvestre sí que é capaz de rescatala. Ademáis podemos observar que en lévedos sometidos a estrés térmico, a parada da tradución mediada por eIF5A está comprometida cando expresan o mutante en SUMOilación. Estos resultados reforzan a teoría de que a SUMOilación de eIF5A xoga un papel moi relevante en condicións de estrés.

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Estudos previos de proteómica identificaron a eIF5A como candidata a ser SUMOilada pero tamén a identificaron como posible substrato de NEDD8, outro modificador de proteínas da familia da ubiquitina. Neste traballo demostramos que eIF5A se NEDDila e, a pesares de non identificar as lisinas que funcionan como aceptoras de NEDD8, demostramos que existe unha interrelación entre a SUMoilación e a NEDDilación para controlar eIF5A. Desta forma, demostramos que os mutantes en SUMOilación se NEDDilan máis, que o tratamento das células con inhibidor de SUMO aumenta a NEDDilación da proteína e que o tratamento cun inhibidor de NEDDilación aumenta a súa SUMOilación. Ademais, demostramos que a NEDDilación de eIF5A regula a súa estabilidade favorecendo a súa degradación, coma indican os resultados acadados co inhibidor de NEDDilación MLN4924. Finalmente, propoñemos como posible ligasa de NEDD8 a STUB1. A identificación de ligasas é un punto moi relevante unha vez identificado un substrato de NEDD8 ou de calquera outro membro da familia das ubiquitinas. O proceso de NEDDilación, ao igual que o de ubiquitinación ou SUMOilación entre outros, é un proceso ATP-dependente que require da actuación de tres enzimas, o E1, E2 e E3. Xeralmente os enzimas E1 e E2 son específicos para cada unha das modificacións e son comúns para a maioría de sustratos, polo tanto hai un número reducido delas, mentres que no caso das E3, as ligasas, existe multitude delas descritas e son as que marcan en certa medida a especificidade da reacción.

Unha vez demostrada e caracterizada a relevancia da hipusinación na infección co virus da gripe, continuamos co estudio de outras modificacións post-traducionais no contexto da infección co virus da gripe. Anteriormente demostrouse que o tratamento das células co inhibidor de NEDDilación inhibe a replicación do virus da gripe e que a infección co virus da gripe aumenta os niveis de NEDDilación das cullin, unha familia de ligasas de ubiquitina que está considerada como o principal substrato de NEDD8. Nestre traballo ampliamos esa información mediante o estudo das proteínas NEDDiladas en células

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infectadas co virus da gripe e comparándolas coas proteínas NEDDiladas nas células non infectadas. Identificamos certos substratos, algúns que xa aforan identificados como substratos de NEDDilación que son regulados pola infección. Tamén identificamos novos substratos de NEDD8 como pode ser a proteína VDAC1. Esta proteína, involucrada no funcionamento da mitocondria, modifícase por NEDD8 na súa lisina K110, e esta modificación parece ser relevante para a infección co virus da gripe posto que sobreexpresar a proteína mutada afecta negativamente á replicación do virus mentres que a sobreexpresión da proteína silvestre non ten ningún efecto.

Ademais, ata o momento poucas proteínas virais foron propostas como substratos de NEDD8. Dúas delas son as proteínas M1 e PB2 do virus da gripe. Segundo a literatura, a NEDDilación destas proteínas induce a súa degradación, e polo tanto unha inhibición da NEDDilación afectaría positivamente á replicación do virus. Sen embargo, o efecto do inhibidor da NEDDilación demostrou ter efectos negativos para o virus, e por iso hipotetizamos que podería haber outras proteínas virais modificadas por NEDD8. NS1 é a proteína do virus da gripe máis relevante na inhibición do interferón e dos mecanismos antivirais desatados na célula infectada. Neste traballo demostramos que NS1 se modifica por NEDD8 *in vitro* e *in vivo*. Ademais, analizamos a NEDDilación durante a infección, nun contexto non que non soamente sobreexpresamos a proteína NS1 senón que todas as proteínas virais están presentes, e observamos que efectivamente a NEDDilación de NS1 ocorre de maneira fisiolóxica en células infectadas. Para analizar o rol da NEDDilación sobre NS1 tentamos xerar mutantes en diferentes lisinas pero sen conseguir unha baixada clara na súa NEDDilación. Por ese motivo, mutamos todas as lisinas presentes en NS1 e reintroducímolas de novo unha a unha para ver cal delas permitía recuperar a NEDDilación. Mediante este método identificamos 4 lisinas de NS1 como as aceptoras de NEDD8. A continuación demostramos que unha proteína coas 4 lisinas mutadas non se pode NEDDilar. Este resultado foi ademais comprobado no

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contexto da infección viral. Para iso xeramos un virus recombinante no que a proteína NS1 ten as 4 lisinas importantes para a NEDDilación mutadas e analizamos a súa NEDDilación. Desta maneira demostramos que as lisinas 108, 110, 126 e 131 de NS1 son as aceptoras de NEDD8. Por outra banda, estudamos o efecto da falta de NEDDilación de NS1 sobre a replicación viral. Para iso comparamos o crecemento do virus silvestre co do virus recombinante que expresa una proteína NS1 que non se pode NEDDilar, e observamos que a inhibición da NEDDilación de NS1 afecta negativamente á replicación do virus. Este dato está en liña co xa descrito papel negativo do inhibidor de NEDDilación sobre a replicación viral. Finalmente, para coñecer máis especificamente o papel da NEDDilación sobre NS1 estudamos algunhas características da proteína viral. En primeiro lugar estudamos a súa estabilidade, posto que as proteínas M1 e PB2, anteriormente descritas como dianas de NEDD8, ven afectada a súa estabilidade por NEDD8. No caso de NS1 non detectamos ningunha variación na vida media da proteína mutante en comparación coa silvestre, descartando polo tanto un papel desta modificación na estabilidade da proteína viral. Unha das características máis importantes de NS1 e a súa habilidade para inhibir a síntese e a sinalización do interferón. Levando a cabo un ensaio de luciferasa vemos que as células que foron previamente transfectadas con NS1 silvestre son capaces de inhibir a actividade dun xene reporteiro do interferón, ISG54-luc, mentres que as células transfectadas coa versión mutante de NS1 non son capaces de inhibir esta sinalización. Un dos mecanismos polos cales NS1 inhibe o interferón é a unión ao ARN de dobre cadea. O ARN de dobre cadea non se presenta de maneira abundante nas células non infectadas, e xeralmente prodúcese durante o proceso de infección do virus. É por iso que a célula ten mecanismos para detectar este ARN e desencadear unha resposta antiviral. A unión de NS1 a esta molécula impide que sexa detectada pola célula e así atenuar a resposta antiviral. Neste estudo demostramos que o mutante de NS1 que non se pode NEDDilar presenta unha unión a ARN de dobre cadea máis débil que a proteína silvestre. O mesmo sucede cando analizamos a unión de NS1 silvestre a ARN bicatenario en presenza do

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inhibidor de NEDDilación. Con isto concluímos que a proteína NS1 do virus influenza A se NEDDila e que a súa NEDDilación é relevante para o seu papel inhibidor da resposta antiviral, posto que se une con pouca afinidade ao ARN de dobre cadea e non é quen de inhibir o interferón. Ademais da agora proposta NEDDilación, NS1 demostrouse que pode ser modificada por outras modificación post-traducionais como SUMOilación, ISGilación, acetilación ou fosforilación. Además algúns dos residuos descritos poden ser modificados por máis dun mecanismo. Isto pon de relevancia o papel tan importante de NS1 na replicación do virus e o nivel tan alto de complexidade deste proceso. Así, todas estas modificacions permiten que unha proteína relativamente pequena, de aproximadamente 26 kDa, sexa capaz de realizar multitude de funcións e dunha maneira dinámica gracias a súa capacidade de interaccionar con diversos modificadores de proteínas.

Finalmente, decidimos estudar o posible papel da UFMilación no contexto da infección polo virus da gripe. UFM1 é un modificador de proteínas similar á ubiquitina. O seu descubrimento é relativamente recente polo que descoñecemos moitos aspectos desta modificación como poden ser os mecanismos que o activan, a súa relevancia e moitas das súas dianas. As súas dianas máis salientables son a proteína ribosomal RPL26 e a proteína supresora de tumores p53. Ademais UFM1 e a UFMilación parecen ter un papel moi importante na resposta a estrés do retículo endoplasmico. A maioría dos enzimas implicados na ruta de UFMilación localízanse nas inmediacións do retículo endoplasmático e unha alteración do mesmo provoca un aumento de UFM1 ou das enzimas relacionadas. Dado que existe unha interconexión entre a infección co virus da gripe e o estrés do retículo endoplásmico, decidimos evaluar se UFM1 podía tamén estar alterado trala infección.

Poidemos observar que existe un aumento na conxugación de UFM1 aos seus substratos tras a infección có virus da gripe e que este aumento non vai paralelo cunha maior expresión do xene que codifica para UFM1 ou para dous dos enzimas implicados na UFMilación, o que

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suxire que posiblemente esta regulación sexa tamén a nivel post-traducional. A inhibición da NEDDilación ou da SUMOilación afectan claramente á replicación do virus da gripe. Aquí demostramos que células con niveis reducidos de UFM1 son menos favorables para a replicación viral, o cal suxire que a UFMilación pode ter un papel importante sobre o virus da gripe. Con isto demostramos por primeira vez a relación desta nova modificación post-traducional, a UFMilación, coa replicación viral, neste caso co virus da gripe. A pesares de non coñecer o papel específico desta modificación no contexto viral, supón a apertura dun novo campo de coñecemento no que afondar na relación virus-célula. Por outra banda, o feito de que trala infección viral a UFMilación aumente pero que non o faga a nivel transcripcional suxire, entre outras causas, que isto poida ser debido a unha regulación post-traducional. Dado o seu recente descubrimento, non existe moita información sobre a regulación de UFM1 pero a interrelación entre os membros da familia da ubiquitina é un feito que ocorre moi frecuentemente e que engade aínda máis complexidade ó estudo das modificacións post-traducionais. Por iso estudamos se UFM1 pode ser modificado por dous dos membros da familia da ubiquitina, SUMO2 e NEDD8. Para iso foi imprescindible traballar sobre unha versión mutada de UFM1 que non sexa capaz de conxugarse a unha proteína substrato. Usando esta proteína mutante, fomos capaces de demostrar que UFM1 se modifica tanto por SUMO2 como por NEDD8. Ademais, para comprobar a hipótese de que a regulación que o virus da gripe exerce sobre a UFMilación é a nivel post-traduccional, estudamos se estas modificacións se ven alteradas trala infección co virus influenza A. As células infectadas mostraron un maior nivel de UFM1 NEDDilado mentres que o nivel de SUMOilación de UFM1 vese claramente diminuído, o cal suxire que as modificación post-traducionais de UFM1 teñen un papel relevante no contexto da infección polo virus influenza A.

En resumo, neste traballo ofrecemos unha visión detallada da complexa relación entre o virus influenza A e distintas modificacións

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post-traducionais. Describimos o rol do virus no control da hipusinación, da NEDDilación e da UFMilación, propoñemos mecanismos de activación destas modificacions e identificamos dianas destos modificadores que poden ser de grande importancia para a replicación viral. Ademais propoñemos por primeira vez a UFM1 como unha proteína importante na replicación viral e afondamos nos mecanismos que a regulan, poñendo de relevancia a complexa interconexión entre os membros da familia da ubiquitina.

2.2 SUMMARY

Influenza is an acute respiratory disease caused by influenza virus that affects millions of individuals and causes the death of more than 300,000 people every year. It is well known that the severity of the disease depends on both virus and host factors. The host response to influenza virus infection is necessary for viral clearance but influenza virus manipulates the machinery of the infected cells to improve virus replication. The outcome of this battle determines the evolution and severity of the disease. Here we have studied influenza virus-host cell interactions in order to decipher the molecular mechanisms of the disease. We have focussed on the role that post-translational modifications play in these interactions. Our data revealed that activation of eIF5A plays is key for influenza A virus replication (as well as in replication of other viruses). We identified both SUMOylation and NEDDylation as novel mechanisms to control eIF5A activity, and demonstrated the critical role of eIF5A SUMOylation on the cellular response to different types of stress. Furthermore, we identified the influenza virus NS1 protein as well as several cellular proteins as targets for NEDD8 conjugation upon influenza virus infection, and deciphered the role of NS1 NEDDylation in the antiviral activity mediated by the viral protein. Finally, we demonstrated, for the first time, an interplay between UFMylation and influenza virus replication. Our data revealed that UFM1 is a key factor for influenza virus replication. In summary, this work demonstrates the importance of post-translational modifications in influenza virus-host cell interplay.

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Influenza A virus causes a respiratory disease that affects millions of individuals every year. The host response to IAV is necessary for viral clearance, but the virus manipulates cellular machinery to improve its replication. Here we studied the virus-cell interactions to decipher the molecular mechanisms of the disease. Our data revealed that activation of eIF5A is key for IAV and we described SUMOylation and NEDDylation as novel mechanisms to control eIF5A. Also, we identified NS1 and several cellular proteins as targets for NEDD8 upon IAV, and deciphered the role of NS1 NEDDylation. Finally, we demonstrated, for the first time, an interplay between UFMylation and IAV. In summary, this work demonstrates the importance of post-translational modifications in influenza virus-cell interplay.

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3. INTRODUCTION

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3.1 INFLUENZA VIRUS

3.1.1 Influenza virus, pathogenesis, and transmission

Influenza viruses are enveloped negative-sense single-stranded ribonucleic acid (RNA) viruses that belong to the family *Orthomixoviridae* (Fields et al., 2013; Palese and Shaw, 2006). This family comprises 4 genera: Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus and Deltainfluenzavirus, each containing only a single specie: Influenza A virus (IAV), Influenza B virus (IBV), Influenza C virus (ICV) and Influenza D virus (IDV), respectively. IBV, ICV and IDV are present almost exclusively in humans. Influenza A virus has a broad host range, although the natural reservoir are wild birds (Webster et al., 1992). The genome of influenza virus is segmented into 7-8 fragments that encode the different proteins of the virus. The segmented nature of the viral genome allows for an exchange of genome segments when two different influenza viruses co-infect a cell, by a process called reassortment, a major mechanism of influenza virus evolution (Fields et al., 2013). A reassortment of two or more influenza viruses within the same cell or a direct jump from an animal to humans can result in an antigenic shift, defined as the generation of a new virus with antigenic properties extremely different from those of the circulating strains (Bouvier and Palese, 2008; Krammer et al., 2018; Lowen, 2017; Palese and Shaw, 2006). In addition, the influenza RNA-dependent RNA polymerase (RdRp) lacks proofreading activity and exhibits high mutation rates (Steinhauer et al., 1992). Accumulation of point mutations in the genes encoding antigens that are recognized by the immune system (antigenic drift) can reduce or inhibit the binding of neutralizing antibodies, and consequently, the virus may evade the immune system and cause severe disease (Hay et al., 2001; Kim et al., 2018; Nachbagauer and Krammer, 2017; Webster and Govorkova, 2014) (Figure 1).

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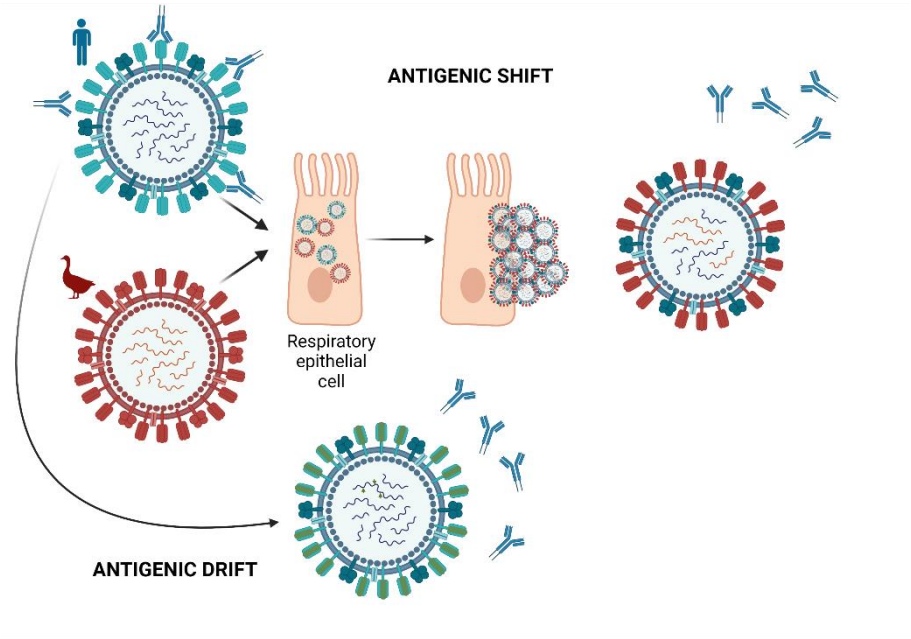


Figure 1. Antigenic shift and antigenic drift in IAV. Schematic representation of the process of antigenic shift, by which two or more different strains of IAV combine to form a virus with different antigenic properties, and of antigenic drift, by which mutations in the genes encoding surface proteins produce viruses with new antigenic determinants. (Figure designed with Biorender).

Influenza virus is the causative agent of seasonal influenza, an acute respiratory disease that causes severe illness and death in high-risk populations. Influenza virus typically causes an acute infection of the upper respiratory tract (URT). However, in severe cases, it can infect the lower respiratory tract (LRT) triggering severe pulmonary inflammation and inducing acute lung injury. Infection with influenza virus results in relevant host cell death, which can intensify inflammation and compromise the integrity of the epithelial cell barrier leading to respiratory failure (Fields et al., 2013).

The symptoms of influenza include fever, headache, cough, muscle and body pain, runny nose, and fatigue, and usually appear within one to four days after infection. The disease typically resolves after 1-2

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weeks without the need for medical attention or further complications, but it can take a severe and even fatal course, especially in immunocompromised individuals, infants and the elderly (Fields et al., 2013).

Influenza is a highly contagious airborne disease. Influenza A virus is believed to transmit through aerosols or respiratory droplets (Lakdawala and Subbarao, 2012; Lindsley et al., 2016, 2015; Milton et al., 2013). Influenza infections in humans exhibit a clear seasonal cycle in temperate regions where outbreaks usually occur in the winter season. However, in tropic areas, a flu outbreak may occur throughout the year. Reports from the World Health Organization (WHO) estimate that influenza virus infection results in around 1 billion illnesses and 290,000-650,000 deaths annually, although the overall impact of influenza varies from year to year (Iuliano et al., 2018; Thompson et al., 2009). So far, the 1918 pandemic, caused by influenza A H1N1, has remained as the most devastating pandemic in the recorded history. It infected almost 50% of the world's population and killed an estimated 20-50 million people (Crosby, 2003; Garcia-Sastre and Whitley, 2006; Johnson and Mueller, 2002; Taubenberger et al., 2001).

3.1.2 Influenza virus structure

Influenza virus is an enveloped virus with a size of 80-120 nm (Fujiyoshi et al., 1994; Noda, 2012). They are highly pleiomorphic, exhibiting spherical, filamentous, elliptical or even irregular shapes (Chu et al., 1949; Kilbourne and Murphy, 1960; Stevenson and Biddle, 1966).

The envelope of influenza virus is derived from the host plasma membrane and contains multiple copies of the viral proteins hemagglutinin (HA), ion channel (M2), and neuraminidase (NA) (Fields et al., 2013; Nayak et al., 2009; Scheiffele et al., 1999). Right underneath the membrane, the matrix protein 1 (M1) forms an

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oligomeric layer critical for particle stability and pH-dependent RNA genome release. The membrane of influenza viruses contains embedded the glycoproteins HA and NA, which are the major antigenic components of the virus, and they are essential for virion binding to cellular receptors (Wiley and Skehel, 1987) and for the release of the progeny virus after completing the viral cycle. So far, there are at least 18 known HA subtypes and 11 known NA subtypes (Kosik and Yewdell, 2019), and the combination of HA and NA determines the influenza A subtype (e.g., H1N1, responsible for the 2009 influenza pandemic, or the highly pathogenic H5N1 (Gamblin and Skehel, 2010; Park and Ryu, 2018; Sullivan et al., 2010). Influenza virus genome is divided into 7 (ICV and IDV) or 8 segments (IAV and IBV) that encode the different proteins of the virus. The terminal regions of each segment are associated with the polymerase complex consisting of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA), and is covered by multiple copies of the viral nucleoprotein (NP), forming the viral ribonucleoprotein (vRNP) complex (Arranz et al., 2012; Klumpp, 1997; Ye et al., 2006).

3.1.3 Influenza virus cell cycle

3.1.3.1 Viral entry

In humans, influenza virus replication takes place mainly in epithelial cells of the respiratory tract. The first step of influenza virus entry is the recognition of the terminal alpha sialic acid (the host cell receptor molecule) by HA (Dou et al., 2018; Sakai et al., 2017; Weis et al., 1988). Human IAV binds preferentially to sialic acid linked by an α2,6 linkage to the rest of the oligosaccharide (Fields et al., 2013). When HA binds to a non-optimal receptor, NA protein removes this interaction (Sakai et al., 2017). The interaction of HA with the receptors triggers the endocytosis of the virus. The endosome containing the virus is then translocated near the nucleus. There, acidification of the endosome induces a conformational change in HA that exposes its

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fusion peptide (Cross et al., 2009; Martin and Heleniust, 1991; Pinto et al., 1992; Skehel and Wiley, 2000). As a consequence, the viral membrane fuses with the endosomal membrane and the vRNPs dissociate from M1 allowing them to interact with importin α/β and enter the nucleus (Boulo et al., 2007; Cros et al., 2005; Martin and Helenius, 1991; O'Neill et al., 1995) (Figure 2).

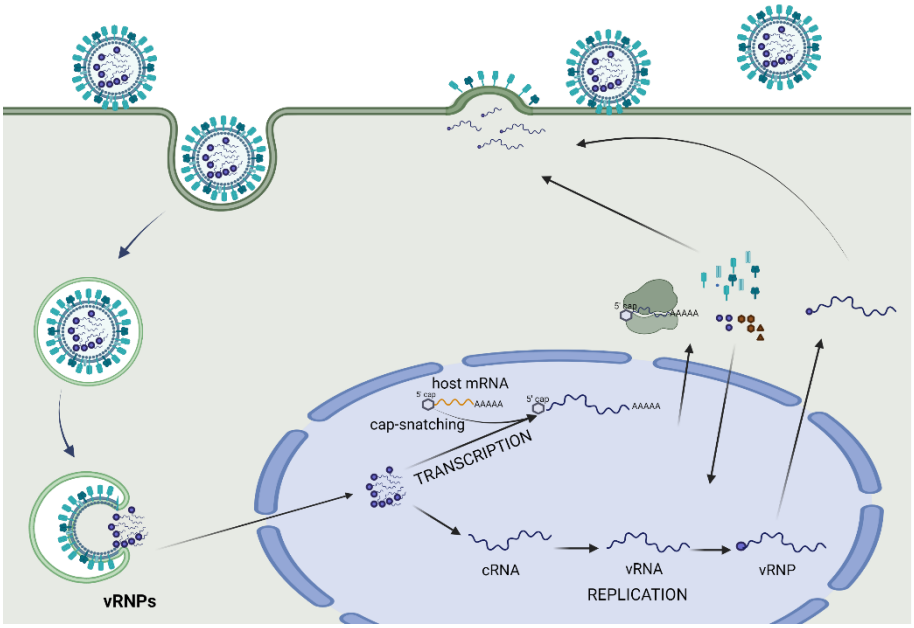


Figure 2. Life cycle of Influenza A virus. Schematic diagram of the life cycle of influenza A virus in the host cell. Influenza virus binds to the host receptors and upon binding the virus becomes endocytosed. Then, viral and endosome membranes fuse, and the viral genomes are released in the cytoplasm. The vRNPs are transported to the nucleus where transcription and replication of viral RNA occurs. The mRNAs are transported to the cytoplasm where the translation of viral proteins occurs through host machinery. Some of these proteins enter the nucleus where they assemble with viral RNAs. Progeny vRNPs are exported from the nucleus towards the cytoplasmic membrane with viral components to be packaged into new infectious particles. Finally, the virus buds off from the host cell. (Figure designed with biorender).

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3.1.3.2 Transcription and replication of the viral RNA (vRNA)

First, vRNA is transcribed into positive-strand viral messenger RNAs (mRNAs). Synthesis of viral mRNAs depends on short, capped primers derived from host RNA polymerase II transcripts. During a process known as cap-snatching, PB2 binds to the cap structure of cellular RNAs through a cap-binding domain and the PA protein subsequently cleaves the host's pre-mRNA 10-15 nucleotides downstream of the cap structure (Dias et al., 2009; Engelhardt et al., 2005; Martínez-Alonso et al., 2016; Plotch et al., 1981). This capped oligonucleotide primer is then used by the RNA-dependent RNA polymerase (RdRp) to initiate transcription. After elongation, the poly (A) tail is generated through stuttering of the polymerase on a short Poly-U sequence proximal to the template 5' end (Poon et al., 1999). This process is different from cellular polyadenylation, where the polyadenylation signal is cleaved by the cleavage and polyadenylation specific factor (CPSF) and then a poly(A) tail is added to the mRNA. Interestingly, the viral non-structural protein 1 (NS1) is able to bind CPSF and inhibit its cleavage activity, impairing host mRNA maturation and inducing host gene shutoff (Nemeroff et al., 1998; Shimizu et al., 1999) (Figure 3). In addition, NS1 prevents the binding of the mRNA export receptor complex NXF1-NXT1 (nuclear RNA export factor 1-nuclear transport factor 2-related export protein 1) to nucleoporins, causing an accumulation of mRNA in the nucleus and preventing host gene expression (Satterly et al., 2007; Zhang et al., 2019) (Figure 3).

Another mechanism by which NS1 contributes to IAV replication is the increase in the rate of translation of viral mRNAs but not of cellular mRNAs (de la Luna et al., 1995). NS1 binds to translation initiation factor eIF4G1 (eukaryotic translation initiation factor 4 Gamma) and poly(A)-binding protein 1 (PABP1) and enhances the initiation of translation of viral mRNAs (Aragón et al., 2000; Arias-Mireles et al., 2018; Burgui et al., 2003) (Figure 3).

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Influenza A virus has developed several strategies to optimize the coding potential of its segmented genome (Dubois et al., 2014). Thus, in addition to the classical HA, NA, PB1, PB2, PA, M1, NP, and NS1 proteins, PB1-F2, PB1-N40, PA-N155, and PA-N182 are generated by using alternative translation initiation sites (W. Chen et al., 2001; Muramoto et al., 2013; Wise et al., 2009), PA-X is generated by a ribosomal frameshift (Jagger et al., 2012), and M2 and NEP are generated by alternative splicing (Lamb and Choppin, 1981, 1979). Noteworthy, NS1 interacts with components of the splicing machinery and modulates both cellular and viral mRNA splicing (Fortes et al., 1995). NS1 negatively modulates host splicing processes but promotes splicing of the viral mRNAs (Dubois et al., 2014; Fortes et al., 1995; Qiu et al., 1995; Zhang et al., 2019) (Figure 3).

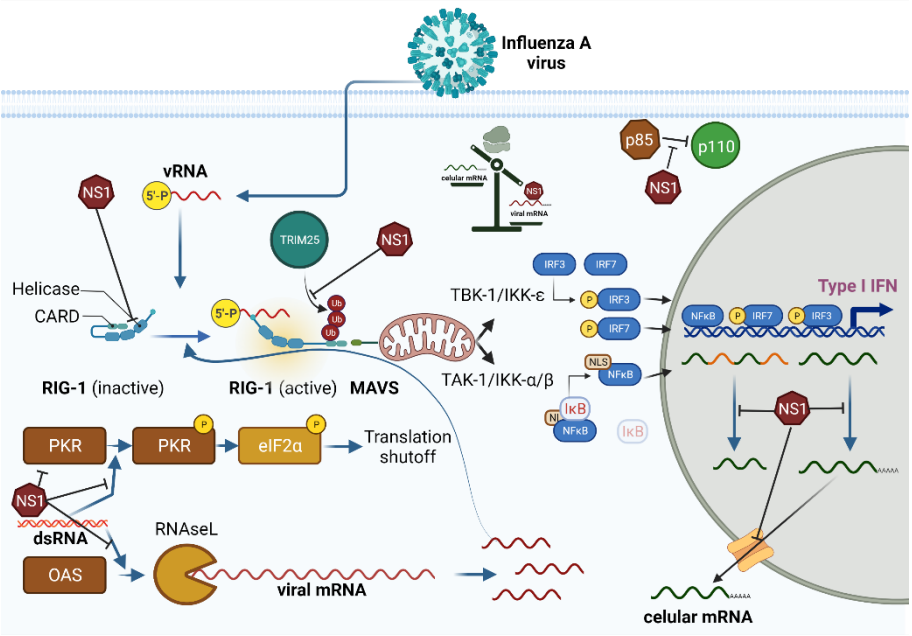


Figure 3. Functions of NS1 in the life cycle of influenza virus. NS1 interferes with three steps in RIG-I activation: binds Trim25 to prevent the ubiquitination of RIG-I, directly interacts with the second CARD of RIG-I, and interacts with dsRNA preventing the activation of RIG-I.

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Interaction with dsRNA also inhibits the OAS/RNase pathway. Binding of NS1 to PKR prevents PKR activation, and interaction between NS1 and p85β activates the PI3K pathway. NS1 also hijacks the host translation machinery to increase the rate of translation of viral mRNAs but not of cellular mRNAs. NS1 prevents polyadenylation of host mRNAs, the nuclear export and the splicing of host pre-mRNA. (Figure designed with Biorender).

The transcribed mRNAs are translated into *the novo* viral proteins in cytosolic ribosomes (PB1, PB2, PA, NS1, NEP, and M1) or in endoplasmic reticulum-associated ribosomes (HA, NA, and M2) (York and Fodor, 2013). Then, the newly synthesized proteins can enter the nucleus and assist in viral genome replication and mRNA transcription, or suppress the host antiviral response (Figure 2).

The replication of IAV genome involves two steps: first, the RdRp initiates RNA synthesis in a prime-independent manner generating a complementary RNA (cRNA) that binds to viral proteins generating complementary ribonucleoproteins (cRNPs). Then, the heterotrimeric polymerase assembles and binds to the newly formed cRNPs to transcribe new vRNA copies (Figure 2).

3.1.3.3 Assembly and budding

After assembly of progeny vRNPs in the nucleus, these are exported from the nucleus to the cytoplasm. This process is mediated by the cellular exportin CRM1 (Elton et al., 2001; Watanabe et al., 2001), and requires the participation of several viral proteins. The two major proteins that mediate this process are M1 and NEP, especially the latter, which is able to bind chromosomal maintenance 1 (CRM1) and hydrolyze RanGTP (Boulo et al., 2007; Bui et al., 2000; Neumann, 2000; O'Neill, 1998). Once in the cytoplasm, vRNPs, as well as viral proteins HA, NA, and M2, are transported to the apical plasma membrane. The mechanism that explains the packing of all the eight vRNPs into the virion is still not very well understood. Evidence suggests that each of the viral segments contains specific signals that would orchestrate the proper incorporation into the virion (Li et al., 2021; Liang et al., 2008; Marsh et al., 2008, 2007). Budding of IAV requires the alteration of membrane curvature, a complex multiple-step

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mechanism mediated by HA and NA proteins (Chen et al., 2007; Chlanda et al., 2015). After budding, NA catalyzes the hydrolysis of sialic acid residue from glycoproteins and glycolipids, preventing the binding of HA to the cell surface and facilitating the release of the virus (Dou et al., 2018; Palese et al., 1974) (Figure 2). N-mediated cleavage of sialic acid also contributes to the viral movement through mucus and respiratory epithelial cells (Cohen et al., 2013; Yang et al., 2014).

3.1.4 Influenza A virus-host cell interaction

Viral infections trigger the induction of signaling cascades in the infected cells to control virus replication, and some viruses are able to hijack cellular pathways to promote their propagation. Infection with influenza A virus modulates different essential cellular signaling pathways such as the TLR/RIG-I, PI3K/AKT, MAPK, PKR, and post-translational modification signaling pathways.

3.1.4.1 TRL/RIG-I pathway and influenza A virus infection

Innate immunity is the first line of defense against invading viruses. When host pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RIG-I, MDA5 and LGP2) recognize pathogen-associated molecular patterns (PAMPs), they trigger the activation of signaling pathways leading to the production of type-I interferon (IFN) and other-proinflammatory cytokines. RIG-I senses the 5'-triphosphate of viral single-stranded or short double-stranded RNA as a pathogen-associated molecular pattern (Hornung et al., 2006) (Figure 4). Structurally, RIG-I and MDA5 have two N-terminal caspase activation and recruitment domains (CARD), an RNA helicase domain, and a C-terminal regulatory domain (Kolakofsky et al., 2012). Under normal conditions, RIG-I is in an inactive, closed conformation. The PAMP recognition provokes a conformational change, the exposure of the N-terminal CARDs of RIG-I, and its ubiquitination by tripartite interaction motif 25 (TRIM25) and RING (really interesting new gene) finger protein

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135 (RNF135/RIPLET) (Gack et al., 2007; Oshiumi et al., 2010). Ubiquitinated CARDs allow the interaction of RIG-I with the mitochondrial antiviral-signaling protein (MAVS) and the consequent activation of the downstream signaling for phosphorylation and nuclear translocation of interferon regulatory factor 3 and 7 (IRF3 and IRF7), the phosphorylation of NF-κB by the IKKalpha/beta/gamma complex, and type I interferon (IFN) production (Chattopadhyay and Sen, 2017; García-Sastre, 2017; Rehwinkel and Gack, 2020; Wu and Hur, 2015).

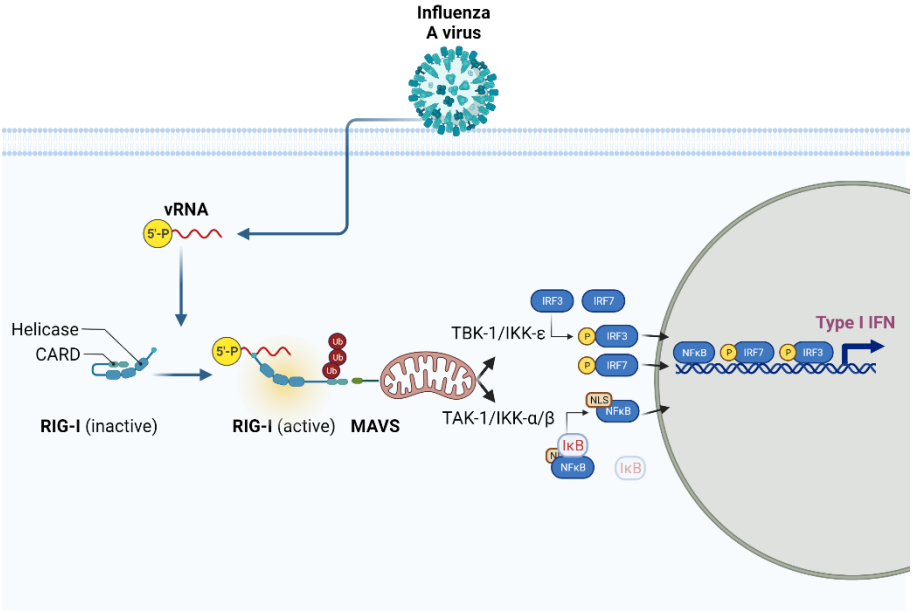


Figure 4. Activation of the RIG-I pathway by IAV. 5'-triphosphate of vRNAs are recognised as a pathogen-associated molecular pattern that induces a conformational change in RIG-I, that can be then be ubiquitinated and consequently activate MAVS, which trigger the signaling cascades that promote the phosphorylation of IRF3 and IRF7, as well as the nuclear translocation of NF-κB, all of them promoting the translation of type I IFN as well as proinflammatory cytokines. (Figure designed with Biorender).

Influenza A virus has developed many strategies to counteract the TLR/RIG-I pathway. For example, the virus performs its transcription inside the nucleus, which keeps its genome inaccessible for the cytoplasmic PAMP sensors, such as RIG-I (Dou et al., 2018; Fields et

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al., 2013). In addition, influenza A virus encodes for proteins that counteract this antiviral signaling pathway. Thus, the viral proteins PB1-F2 and PB2 can bind MAVS, block MAVS-mediated signaling, and inhibit MAVS-mediated IFN production (Dudek et al., 2011; Varga et al., 2011). Another mechanism to evade the innate immune responses involves the synthesis of the viral protein PA-X which targets for degradation the mRNAs for early type-I IFN signaling (Rigby et al., 2019). Another mechanism is the synthesis of the viral protein NS1, that binds to TRIM25 preventing the TRIM25-mediated ubiquitination of RIG-I (Gack et al., 2009), or directly binds to RIG-I with similar consequences (Jureka et al., 2020) (Figure 3).

3.1.4.2 NF-κB pathway

The nuclear factor kappa B (NF-κB) family of transcription factors constitute a key pathway that regulates more than 150 genes involved in several cellular processes including inflammation, immune response, proliferation, and apoptosis (F. Chen et al., 2001; Hayden and Ghosh, 2011; Zinatizadeh et al., 2021). This family is composed of five structurally related members, including p50, p52, p65, RelB and c-Rel (Hayden and Ghosh, 2008). These proteins can homodimerize or heterodimerize through their conserved Rel homology domain to mediate gene transcription. Without stimuli, NF-κB is present as a cytoplasmic heterodimer bound to an inhibitory protein from the IκB family, that masks the nuclear localization signal (NLS) of the NF-κB complex (Hayden and Ghosh, 2008). NF-κB activation following the canonical pathway occurs in response to different stimuli including PPRs, and the mechanism of activation is mediated by the degradation of IkBa triggered through its site-specific phosphorylation by IKK. IKK is constituted by IKKa and IKKb and a regulatory subunit called NEMO (NF-κB essential modulator) (Antonia et al., 2021; Mulero et al., 2019). Activated IKK phosphorylates and consequently degrades IκB, releasing its inhibitory control over NF-κB that now has its NLS exposed and can be translocated to the nucleus (Antonia et al., 2021; Mulero et al., 2019). Following entry into the nucleus, NF-κB binds to

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cis-regulatory elements at promoters promoting the expression of the target genes such as IFN-β, different interleukins, or cell cycle regulators (Bonizzi and Karin, 2004; Ghosh, 1999; Israël, 2010; Karin and Ben-Neriah, 2000).

The NF-κB pathway has classically been considered an antiviral pathway due to the upregulation of IFN (Oeckinghaus and Ghosh, 2009; Pahl, 1999; Schmolke et al., 2009; Viemann et al., 2011). However, several pieces of evidence revealed the importance of a proper activation of the pathway for an efficient viral replication (Nimmerjahn et al., 2004; Wurzer et al., 2004). Altogether these reports highlight that the effects of the NF-κB pathway activation on virus replication depend on the stage of viral replication.

IAV infection activates NF-κB and numerous reports demonstrate that this process facilitates virus replication and spreading (Ehrhardt et al., 2013; Kumar et al., 2008; Mazur et al., 2007; Pinto et al., 2011). However, overactivation of NF-κB has also a negative effect on IAV, likely due to the induction of the expression of inflammatory mediators that help to clear the infection. In agreement with the antiviral function of NF-κB in the early phase of IAV infection, the expression of NS1 has been shown to bind to IKKa and IKKb and to antagonize IAV-induced NF-κB activation and subsequent IFN synthesis (Gao et al., 2012).

3.1.4.3 PI3K-AKT pathway

IAV infection triggers the activation of the PI3K/AKT pathway. The family of PI3Ks (Phosphatidylinositol-3-kinase) comprises three main classes (I, II, and III). The class I PI3Ks are heterodimeric enzymes consisting of a regulatory subunit (p50α, p55α, p55γ, p85α, or p85β) and a catalytic subunit p110α, p110β, or p110δ (Vanhaesebroeck et al., 2010; Vanhaesebroeck and Waterfield, 1999). The regulatory subunits inhibit and stabilize the catalytic subunits. PI3K is an evolutionarily conserved kinase that is activated in response to receptor tyrosine kinase activation and is responsible for

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phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) (Cantley, 2002). The resulting phosphatidylinositol (3,4,5)-triphosphate (PIP3) acts as a second messenger and recruits the serine-threonine kinase AKT to the membrane, facilitating its phosphorylation and consequent activation (Burgering and Coffer, 1995; Dhand et al., 1994; Franke et al., 1995). AKT is a central effector in the cell, mediating key processes such as apoptosis, cellular proliferation or glycolysis (Burgering and Coffer, 1995; Deprez et al., 1997; Dudek et al., 1997). Dephosphorylation of PIP3 is carried out by the phosphatase PTEN, thus leading to the inhibition of the pathway (Cantley, 2002; Carracedo and Pandolfi, 2008; Maehama and Dixon, 1998; Vanhaesebroeck and Waterfield, 1999). AKT is involved in the TBK-1-independent phosphorylation of IRF-3, which is one of the major regulators of the IFN-β enhanceosome and consequently, upregulates IFN-β levels (Sarkar et al., 2004). In agreement with that, different inhibitors of the PI3K pathway decrease the levels of influenza virus-induced phosphorylated IRF-3. According to that, the inhibition of the pathway and the concomitant blockade of the phosphorylation of IRF-3 would be beneficial for influenza A virus replication and thus, PI3K would be classified as an antiviral pathway. However, chemical inhibition of the pathway resulted in impaired viral replication (Ehrhardt et al., 2006), suggesting that the pathway may have pro and antiviral activities. But influenza virus is not an exemption, and many other viruses have developed the ability to take advantage of the activation of the PI3K for their own benefit. Activation of the PI3K/AKT pathway has been proposed to control apoptosis, maintain viral latency or favor viral reactivation from latency (Cooray, 2004; Darr et al., 2001; Shih et al., 2003; Thomas et al., 2002; Whitman et al., 1985; Zhang et al., 2003). Influenza A virus activates AKT at different points after infection: immediately after the attachment of the viral particle to the cell membrane and at a later point of infection (Ehrhardt et al., 2006). Chemical inhibitors of the PI3K pathway impair the virus uptake, probably due to the involvement of the PI3K signaling in clathrin-independent endocytosis, explaining its pro-influenza virus

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activity. However, 5'-triphosphate RNA also activates the pathway in a RIG-I-dependent manner (Hrincius et al., 2011) highlighting the key and dynamic role of PI3K upon IAV infection. Binding of the IAV NS1 protein to the p85β regulatory subunit of PI3K has been also found to activate AKT (Figure 3) and to inhibit caspase 9, glycogen synthase kinase-3 (GSK-3) and the Bcl-2 associated agonist of cell death (BAD), which are mediators of the apoptotic cascade. Inhibition of apoptosis at early times after infection allows the virus to complete its viral cycle (Ehrhardt et al., 2007, 2006; Hale et al., 2006; Herold et al., 2012; Li et al., 2008).

3.1.4.4 MAPK pathway

Mitogen-activated protein kinases (MAPK) are a family of ubiquitous proline-directed, protein-serine/threonine kinases that regulate different cellular programs including cellular proliferation, motility, differentiation, survival, and immune responses by relaying extracellular signals to intracellular responses (Pearson et al., 2001). So far, four members of the family that are organized in different cascades have been identified: extracellular-signal regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), p38 and ERK5. These kinases are activated by dual phosphorylation on tyrosine and threonine mediated by MAP kinases kinases (MEKs or MKKs). ERK is activated by MEK that is activated by the serine threonine kinase Raf. P38 and JNK are activated by MKK3/6 and MKK4/7, respectively, and ERK5 is activated by MEKK5 (Boulton et al., 1991; Kholodenko and Birtwistle, 2009; Kondoh and Nishida, 2007). Different reports demonstrate that the four MAPK family members are activated by IAV infection (Cannon et al., 2014; Kujime et al., 2000; Pleschka et al., 2001). Different mechanisms trigger the activation of the MAPK pathway upon IAV infection. The binding of HA to the host cell surface receptor activates the protein kinase C (PKC), an upstream molecule of Raf leading to the activation of the Raf/MEK/ERK pathway (Arora and Gasse, 1998; Marjuki et al., 2006; Pleschka, 2008). In addition, the accumulation of RNA produced

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by the viral polymerase has been proposed as a mechanism mediating the activation of JNK or the ERK5 MAPK pathway (Yu et al., 2020). The consequences of MAPK activation on IAV replication depend on the specific MAPK signaling pathway activated. Thus, whereas the JNK pathway plays a critical role in the antiviral response to IAV infection by co-modulating IFN-β expression, activation of the ERK pathway was not found to interfere with the outcome of virus propagation (Yu et al., 2020). In contrast, activation of Raf/MEK/ERK is required for both influenza virus production and RNP export (Arora and Gasse, 1998; Ludwig et al., 2004; Marjuki et al., 2006; Pleschka, 2008; Pleschka et al., 2001).

3.1.4.5 Protein Kinase R

Eukaryotic translation initiation factor 2 alpha kinase 2, also known as PKR, is a critical player in the antiviral response. PKR senses double-stranded RNAs (dsRNA) molecules (Meurs et al., 1990) triggering the autophosphorylation of the kinase and the phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2α). This phosphorylation leads to a conformational change in eIF2α that blocks the nucleotide exchange activity of the translation complex inhibiting mRNA translation (Kashiwagi et al., 2019). Prolonged PKR activation has been shown to induce apoptosis. In addition, PKR also contributes to amplifying the IFN response by triggering the NF-κB pathway through the activation of NIK and IKKB, and the stabilization of IFN-β mRNA (Schulz et al., 2010).

Viruses have developed different mechanisms to counteract PKR activation. Regarding IAV, the interaction of NP with the heat shock protein 40 (HSP40) leads to its dissociation of the cellular inhibitor of the PKR p58IPK. P58IPK can then interact with and inhibit PKR (Sharma et al., 2011). In addition, different mechanisms contribute to the inhibition of PKR by the NS1 protein (Figure 3). Influenza virus NS1 contains two domains, the N-terminal domain with an RNA binding motif, and the C-terminal domain involved in the interaction with host proteins (Hale et al., 2008). It has been postulated that NS1

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sequesters dsRNA avoiding the dsRNA-mediated activation of PKR (Dauber et al., 2006; Yin et al., 2007). In addition, NS1 also directly binds PKR inhibiting its autophosphorylation and the concomitant phosphorylation of eIF2α (Bergmann et al., 2000; Li et al., 2006; Lu et al., 1995).

3.2 POST-TRANSLATIONAL MODIFICATION SIGNALING PATHWAYS AND IAV INFECTION

Although the human genome contains an estimate of around 25,000 genes, the proteomic complexity within an organism is increased by different mechanisms such as alternative splicing, alternative promoters and mRNA editing (Manzoni et al., 2016). However, the critical mechanisms that increase the functional diversity of the proteome and facilitate a rapid and dynamic signaling in a cell are post-translational modifications (PTM). PTM are covalent modifications of proteins after their biosynthesis that have a significant impact on the structure and/or function of the proteins (del Monte and Agnetti, 2014; Marshall, 1993; Ramazi and Zahiri, 2021; Schaefer, 2021). PTM constitute highly versatile systems allowing cells to respond to internal or external signals. Importantly, several modifications can occur at the same time on a protein and modifiers can themselves be modified while they are modifying a target protein, thus increasing the complexity of PTM. More than 400 different types of PTM have been described (Khoury et al., 2011) and phosphorylation, glycosylation, acetylation, and ubiquitylation (Karve and Cheema, 2011; Ramazi and Zahiri, 2021) are among the more frequently found and more studied PTM.

The viral infection causes a stress in host cells that triggers a response to fight against the pathogen. This response includes a change in cellular signaling pathways or the reprogramming of gene expression but, interestingly, most of these changes are preceded by a reprogramming of host PTM. Many viruses have evolved strategies to modulate the PTM of cellular proteins. In addition, PTM of viral

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proteins increases their already multifunctional nature or can act as a mechanism to sequester the modifiers and therefore interfere with the host response. However, the functions of many of these modifications are still unknown (Kumar et al., 2020).

IAV infection induces global changes in PTM signaling pathways and the virus employs PTM to modulate different steps through the viral replication cycle. In addition, IAV infection induces the modification of viral proteins by different PTMs such as phosphorylation, SUMOylation, NEDDylation, Ubiquitination, ISGylation, glycosylation, acetylation, ADP-ribosylation, and palmitoylation. Some of the PTMs that occur in response to IAV infection contribute to virus replication. Other PTMs are part of the cellular antiviral response (Dawson and Mehle, 2018; Hu et al., 2020). One example is the NS1 protein, the most relevant protein of IAV involved in the attenuation of the host-immune response, and in many other aspects of the replication of the virus. This protein harbors many different post-translational modifications, some contribute to improve NS1 activities, and others are part of the host cellular antiviral response to block NS1 functions (Ji et al., 2021; Lamotte and Tafforeau, 2021) (Figure 5).

3.2.1 Phosphorylation

Phosphorylation is the most studied PTM and consists on a reversible enzymatic process where a phosphate group from adenosine triphosphate (ATP) is transferred to a target protein. This modification can occur in different amino acids although serine (Ser), tyrosine (Tyr), and threonine (Thr) are the amino acids most commonly modified by phosphorylation (McKay and Johnson, 2010; Ramazi and Zahiri, 2021). Phosphorylation has a pivotal role in most of the cellular processes, especially in response to stress (Hunter, 1995; Karve and Cheema, 2011), and alterations in phosphorylation/dephosphorylation processes have been related to many pathological conditions such as metabolic or neurological disease and cancer (Anderson et al., 2006;

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Harsha and Pandey, 2010; Haystead et al., 1989; Hernandez-Aya and Gonzalez-Angulo, 2011; Levine and Puzio-Kuter, 2010; Maclaine and Hupp, 2011; Zhao et al., 2009).

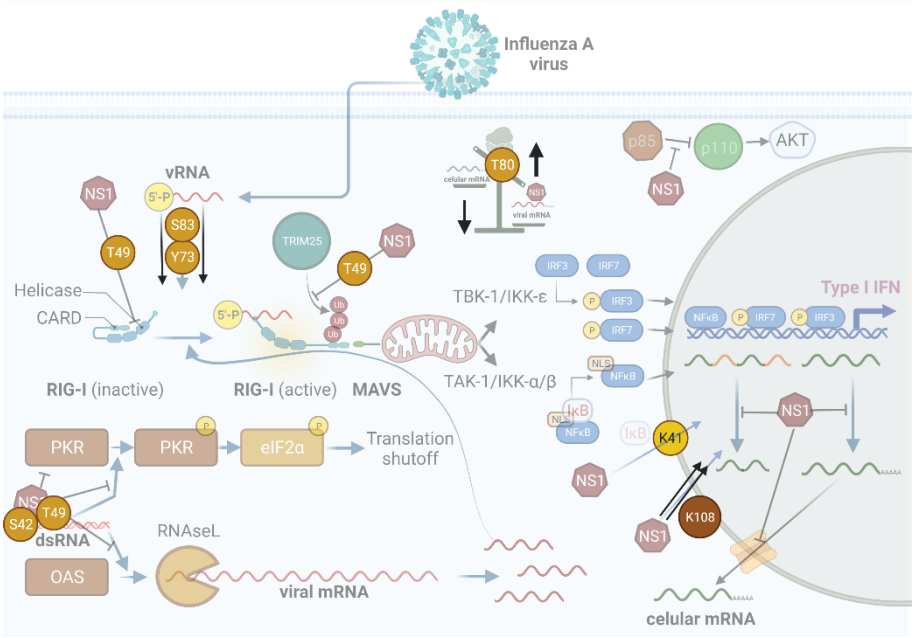


Figure 5. Role of PTM modifications in NS1 activity. Phosphorylation of S42, T49 and T80 attenuates the IFN inhibition whereas phosphorylation of S83 and Y83 favors the RIG-I activation. Acetylation of K108 is relevant for nuclear translocation of NS1. ISGylation of K41 impairs its nuclear import. (Figure designed with Biorender).

As mentioned above, IAV infection induces the activation of kinase cascades during the course of the infection. Some of these pathways such as the Ras/MEK/ERK signaling pathway, NF-κB signaling, PI3K/AKT, and PKC signaling cascade are essential for IAV replication and their inhibition has been proposed as a potential strategy to control IAV. Furthermore, several IAV proteins are regulated by phosphorylation and at least 36 phosphorylation sites in IAV proteins have been identified (Hutchinson et al., 2012). NP protein is phosphorylated at different residues and this modification has different consequences. Phosphorylation of NP at the N-terminal nuclear

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localization signal inhibits its interaction with the importin machinery whereas its phosphorylation at the internal region inhibits the interaction with nuclear export factors (Cui et al., 2019). In addition to modulate NP subcellular localization, phosphorylation also modulates protein-protein interaction. Phosphorylation of NP protein at the homotypic interface modulates NP oligomerization and inhibition of phosphorylation causes defects in RNP assembly, function, and viral replication (Cui et al., 2019). Other IAV proteins that are regulated by phosphorylation are the polymerase subunits PB1, PB2, and PA, although the functionality of these modifications is unknown. M1 is another phosphorylation substrate. M1 phosphorylation promotes its nuclear import and mutants with altered M1 phosphorylation exhibit replication defects (Wang et al., 2013). However, sometimes phosphorylation of viral proteins are cellular strategies to disarm viral countermeasures. One example is the NS1 protein whose activity can be negatively modulated by phosphorylation. NS1 is phosphorylated in different residues (Hutchinson et al., 2012), and some of these modifications disrupt protein-RNA and protein-protein interactions. Thus, phosphorylation of influenza virus A/Udorn/72 (Ud) NS1 at serine 42 has been shown to decrease the RNA binding capacity of the protein and to attenuate viral replication (Hsiang et al., 2012). Similarly, phosphorylation of NS1 at threonine 49 also impairs the binding of NS1 to dsRNA, TRIM25 and RIG-I, affecting significantly its IFN-antagonism activity. In addition, phosphorylation of NS1 at threonine 80 has been reported to difficult the growth of influenza virus A/WSN/1933 probably due to the reduced translation of viral mRNAs (Zheng et al., 2017). NS1 can be phosphorylated at other residues such as serine 48 (Hsiang et al., 2012) or threonine 215 (Hale et al., 2009), but with no significant consequences on viral replication. In addition, NS1 phosphorylation can be also beneficial for IAV replication as shown by Chen (Cheng et al., 2019). These authors demonstrated that phosphorylation of NS1 at both serine 83 and tyrosine 73 in A/swine/Shanghai/3/2014 favors the RIG-I-mediated inhibition of IFN (Figure 5).

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3.2.2 Glycosylation

Glycosylation is a PTM that involves the transference of a sugar moiety to a target molecule (proteins or lipids) by a covalent bond. Protein glycosylation can occur in a wide variety of amino acids but especially in side chains of serine (Ser), threonine (Thr), aspargine (Asn) and tryptophan (Trp) (Ohtsubo and Marth, 2006). There are different classes of protein glycosylation, the major ones representing the N-linked (the sugar moiety is attached to the oxygen molecule of a threonine or serine) and the O-linked (the sugar is bound to a nitrogen atom or the asparagine residue) glycosylation. Most of the target proteins for this modification are membrane proteins and this modification regulates protein solubility, folding, thermostability, catalytic efficiency, antigenicity, etc. Glycosylation plays a major role in cell adhesion, cellular proliferation, differentiation, migration, invasion, and cell cycle control, among others, being critical for physiological and pathological functions (Goulabchand et al., 2014; Karve and Cheema, 2011; Lauc et al., 2013; Ohtsubo and Marth, 2006).

Glycosylation of viral envelope proteins is the most well-known example of glycosylation in the virus and it has been identified as a mechanism of immune evasion. In IAV infected cells, modulation of N-linked glycosylation of HA has been shown to protect the protein from antibody recognition and neutralization (Skehel et al., 1984). This process is exploited by the virus to increase viral spread virulence and fitness (Kosik et al., 2018; Zhao et al., 2017). In addition, glycosylation of HA has been also reported to modulate the affinity of HA for sialic acid receptor (de Vries et al., 2010).

3.2.3 Acetylation

Acetylation is the transfer of an acetyl group from acetyl-CoA to lysine residues (Lys) of a target protein. It is one of the most common

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PTMs in histones. It plays a critical role in global regulation of gene expression.

Acetylation has been demonstrated to regulate different stages of virus cycle including virus entry, replication, fusion, transport, and release by modulating cellular and viral proteins. One example is the p53 protein, whose acetylation during infection induces the transactivation of pro-apoptotic and IFN-stimulated genes (Muñoz-Fontela et al., 2011). However, acetylation of cellular proteins can also favor virus replication. Thus, C-terminal deacetylation of RIG-I has been demonstrated to enhance RNA sensing (Choi et al., 2016). Moreover, HDAC6-mediated deacetylation of microtubules has been shown to prevent the traffic of IAV to the membrane (Husain and Cheung, 2014). Many different influenza virus proteins are also targeted by acetylation such as PB1, PA, and NP (Hutchinson et al., 2012). Acetylation of some residues may have a positive impact on IAV(Chen et al., 2019). Thus, deacetylation of PA by HDAC6 promotes its degradation suppressing RNA polymerase activity, and consequently, restricting RNA transcription and replication (Chen et al., 2019). In contrast, acetylation of NP protein on several internal lysine residues abolishes the stabilization of replication intermediates induced by the viral protein, and thereby inhibits virus replication (Giese et al., 2017; Hatakeyama et al., 2018). Interestingly, NP can be modified by both acetyl groups and ubiquitin at the same lysine residue, suggesting a competition for occupancy between these two modifiers. Acetylation of IAV NS1 plays a positive role in virus replication by increasing the activity of the protein and enhancing NS1 mediated IFN antagonism. Thus, inhibition of K108 acetylation in NS1 from influenza virus A/WSN/1933 attenuates viral replication due to an impaired IFN inhibition and the retention of the viral protein in the cytoplasm (Ma et al., 2020) (Figure 5).

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3.2.4 Ubiquitination

Ubiquitin (UB) is an ubiquitous small protein (76 amino acids), (Goldstein et al., 1975) highly conserved among species that in humans is encoded by four different genes, UBB, UBC, RPS27A, and UBA52. Ubiquitin belongs to a family of ubiquitin-like (UBL) proteins that are covalently attached to target proteins to modify their function. All UBL proteins possess a beta grasp-fold (β-GF) composed of a five-stranded beta-sheet that partially wraps around a central alpha-helix. The ATP-dependent degradation of ubiquitin-protein conjugates was described for the first time in 1978 (Ciehanover et al., 1978) and since then an uncountable number of proteins have been identified as ubiquitin substrates. Ubiquitin is attached to substrates by a 3-step ATP-dependent enzymatic cascade. First, ubiquitin-specific proteases process the immature UB, which can then be activated by forming a thioester bond with E1 ubiquitin-activating enzyme through its carboxyl terminus. Then, the activated UB is transferred to an E2 ubiquitin conjugating enzyme. Finally, a variety of E3 ubiquitin ligases transfer UB from E2 to substrates to facilitate formation of an isopeptide bond between the C-terminal carboxyl of UB and the e-amino group of a substrate lysine sidechain of free N-terminal amino group (Ciechanover and Schwartz, 1998). This process could be reversed by the action of a specialized family of proteases, the deubiquitinating enzymes, which remove conjugated ubiquitin molecules from target substrates (Wilkinson, 1997). Importantly, although under non-stress conditions, each UBL has unique and dedicated E1 and E2 enzymes, under stress conditions or a reduction in the levels of free ubiquitin, the ubiquitin E1 enzyme Ube1 can mediate conjugation of the ubiquitin-like protein NEDD8 (neural precursor cell expressed, developmentally down-regulated 8) (Leidecker et al., 2012). Moreover, it has been found that ubiquitin E3 enzymes can also function as NEDD8 E3 enzymes (Noguchi et al., 2011; Xirodimas et al., 2004). Proteomic studies identified thousands of ubiquitinated proteins and ubiquitination has emerged as a key post-translational

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modification for the maintenance of homeostasis. When a single ubiquitin molecule is attached to a substrate lysine residue is called monoubiquitination. But ubiquitin itself can be modified by different PTM including ubiquitination, increasing the complexity. Although the most common function attributed to UB modification is the proteasome-mediated degradation (usually linked to K48 poly-ubiquitin chains), ubiquitination can also modulate the subcellular localization, protein-protein interaction, or activity of the target proteins (Flotho and Melchior, 2013; Mayer et al., 1991).

Ubiquitination plays an important role in virus infection. In the case of IAV, it has been demonstrated that components of the ubiquitin proteasome system (UPS) are upregulated in IAV infected cells, and that proteasome activity is required for entry and replication of the virus (Kroeker et al., 2013). IAV employs different strategies to modulate ubiquitination in order to promote replication. Thus, whereas IAV HA protein induces the ubiquitination and degradation of the Interferon-α/β receptor 1 IFNAR1) in order to attenuate IFN signaling (Xia et al., 2015), NS1 inhibits the E3 ubiquitin ligase Trim25, a key mediator in antiviral RIG-I signaling pathway (Gack et al., 2009). In addition, IAV proteins themselves can be directly modified by UB. Thus, the antiviral protein ZAPL can induce the ubiquitination and degradation of PA and PB2 (Liu et al., 2015). In addition, different proteins belonging to the RING family of E3 ubiquitin ligases can ubiquitinate NP and PB1 targeting them for degradation (Fu et al., 2015; Liao et al., 2010; Patil et al., 2018). But conjugation of ubiquitin to IAV proteins not necessarily leads to protein degradation. Conjugation of monoubiquitin to lysine K184 in NP has been reported to regulate its interaction with genomic RNA to facilitate replication (Lin et al., 2017). Ubiquitination of M2 plays a role in particle assembly and release (Liu et al., 2021; Su et al., 2018), and ubiquitination of M1 facilitates the release of the incoming virion (Mahesutihan et al., 2018). Furthermore, ubiquitin proteins have been detected incorporated in IAV virions although its biological significance is still unclear (Hutchinson et al., 2014).

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3.2.5 NEDDylation

Among the members of the UBL protein family, NEDD8 is the closest in sequence to ubiquitin, with around 60% sequence identity (Kumar et al., 1993; Whitby et al., 1998). This protein is ubiquitously expressed in all cells in the body and is essential for viability, growth and development. NEDD8 precursor is cleaved by the NEDD8-specific protease 1 NEDP1 (Gan-Erdene et al., 2003) to expose the C-terminal di-glycine domain. The mature NEDD8 is then activated by the E1 NAE (NEDD8-activating enzyme) in an ATP-dependent manner and transferred between the active cysteine residue of the NEDD8 activating enzyme (NAE) to an E2-conjugating enzyme, UBE2M or EBE2F. Finally, NEDD8 E3 ligases catalyze the transfer of NEDD8 from the E2 onto the target protein. Importantly, so far, all the reported NEDD8 E3 enzymes like MDM2 (murine double minute 2) or tripartite interaction motif 40 (TRIM40) can also function as ubiquitin E3 enzymes. Conjugation of NEDD8 is reversed by the action of NEDP1, that deconjugates NEDD8 from the substrate (Abidi and Xirodimas, 2015; Lu and Yeh, 2013; Soucy et al., 2010) (Figure 6). However, and as mentioned above, this scenario corresponds to canonical NEDDylation, under homeostasis conditions. Upon stress conditions or a reduction in free ubiquitin levels, NEDD8 can be additionally activated by the ubiquitin activating enzyme E1, UBA1, and the global NEDDylation pattern changes. Thus, some proteins or even specific lysine residues in a target protein are only NEDDylated upon stress conditions (Lobato-Gil et al., 2021). The most well-known targets for NEDDylation are the members of the cullin protein family (Pan et al., 2004). Cullin proteins are molecular scaffolds for ubiquitin ligases. The members of the cullin protein family assemble multi-subunit Cullin-RING E3 ubiquitin ligases (CRL) complexes constituting the largest family of E3 ubiquitin ligases. The core CRL consists of four components: a cullin protein that serves as the scaffold, a RING finger protein that binds to an E2 ubiquitin, a substrate receptor that recognizes the target protein, and adaptor proteins that bridge the substrate receptor

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to the cullin (Sarikas et al., 2011). NEDDylation of cullins occurs in their C-terminal domain and leads to a conformational change that facilitates the interaction of the RING finger protein with the ubiquitin substrate (Jones et al., 2008; Ohh et al., 2002; Pan et al., 2004). Thus, cullin NEDDylation results in the activation of cullin-RING ligases and, concomitantly, in the ubiquitination of their targets. In addition to cullins, an increasing list of proteins has been reported to be NEDDylated, such as the tumour suppressor p53, MDM2, most of the components of the NEDDylation machinery, and a relevant list of ribosomal proteins (Abida et al., 2007; Enchev et al., 2015; Jones et al., 2008; Xirodimas, 2008; Xirodimas et al., 2008, 2004). Different evidence support a relationship between deregulation of the NEDDylation pathway and pathogenic processes. Cancer is one of the pathological conditions associated with an alteration in NEDDylation. Components of the NEDDylation machinery have been found overexpressed or hyperactivated in different types of cancer (Abidi and Xirodimas, 2015; Chairatvit and Ngamkitidechakul, 2007; Gai et al., 2021; Salon et al., 2007; Soucy et al., 2010), and NEDDylation is considered as a therapeutic target for many of them (Soucy et al., 2009). The NEDDylation inhibitor MLN4924, also known as pevonedistat, is a potent and selective inhibitor of NAE that has been shown to induce autophagy, apoptosis and senescence, impairing tumor growth in cancer cell lines and xenografts (Luo et al., 2012; Soucy et al., 2009). Apart from its role in tumor progression, NEDD8 and NEDDylation inhibition have been shown relevant in other pathological conditions such as neurological disorders (Dil Kuazi et al., 2003; Mori et al., 2005) or in viral infections. Treatment with MLN4924 has been reported to inhibit the replication of different viral agents such as hepatitis B virus, cytomegalovirus, or influenza virus (Abounouh et al., 2022; Flores-Martínez et al., 2021; Sun et al., 2018).

IAV has been shown to promote the NEDDylation of cullins, and inhibition of NEDDylation has been demonstrated to decrease IAV replication (Sun et al., 2018). However, overexpression of NEDD8 and

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the E3 HDM2 has also been found to inhibit IAV replication (Zhang et al., 2017), suggesting that NEDD8 plays different roles in the IAV-host cell interplay. So far, there is little information about the potential modification of IAV proteins by NEDD8, and only two proteins of the virus, PB2 and M1, have been identified as NEDD8 substrates. Interestingly, NEDDylation of both proteins has been reported to negatively modulate IAV replication. Thus, conjugation of NEDD8 to PB2 or M1 destabilizes both proteins and inhibits virus replication (Li et al., 2020; Zhang et al., 2017).

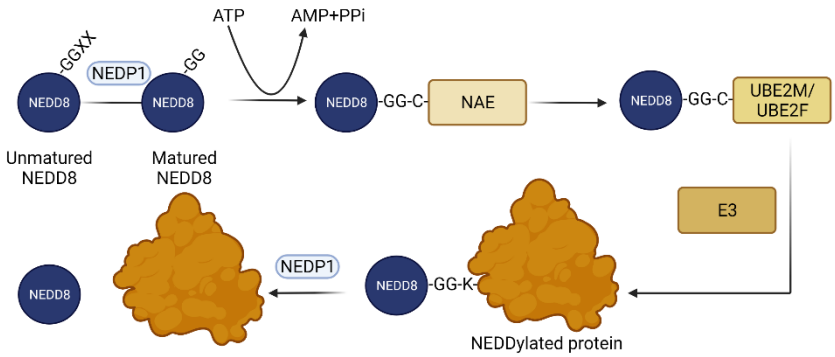


Figure 6. NEDDylation pathway. Schematic representation of the NEDDylation pathway. After maturation, NEDD8 is activated by forming a thioester linkage with NAE in an ATP dependent manner. Activated NEDD8 is transferred to the active cysteine site of E2 (UBE2M/UBE2F)). Finally, E3 catalyzes the transfer of NEDD8 from the E2 onto the target protein.The protease NEDP1 can revert this modification. (Figure designed with Biorender).

3.2.6 SUMOylation

SUMOylation plays a key role in influenza biology. IAV infection results in global changes in SUMOylation and most of the IAV proteins are SUMO substrates. SUMO conjugation to IAV NP protein is essential for its nuclear retention and virus expressing a SUMOylation-deficient NP mutant exhibits defects in replication (Han et al., 2014). SUMOylation of the IAV M1 protein has been shown to facilitate its

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interaction with RNPs and a virus carrying a SUMOylation-deficient M1 mutant exhibits defects in the nuclear export of vRNA and low viral titers (Wu et al., 2011). NS1 has also been identified as a SUMO substrate. Initially, the lysine residues K70 and K219 were proposed as the SUMOylation sites in NS1, and their mutation in a influenza A/PR8/1934 background delayed the viral growth (Santos et al., 2013). However, later on, other authors pointed to lysine residue K131 as the main SUMO acceptor in NS1, but again with similar effects on viral growth (Way et al., 2020).

3.2.7 UFMylation

Ubiquitin fold modifier 1 (UFM1) is a recently-discovered ubiquitin-like protein with low sequence homology to ubiquitin. UFM1 is ubiquitously expressed in many tissues, and highly conserved among most eukaryotes except in yeasts and other fungi. Similar to ubiquitination, UFMylation involves a three-step enzymatic cascade (Daniel and Liebau, 2014). First, the UFM1 precursor is maturated by a UFM1-specific cysteine protease 1 or 2 (UFSP1 or UFSP2), to expose a single glycine (instead of the di-glycine found in all others UBL modifiers) (Ha et al., 2011, 2008). Then, UFM1 is activated by the E1 UBA5 (ubiquitin-activating enzyme 5) resulting in a thioester bond formation between the E1 and UFM1. Then, UFM1 is transferred to the E2 UFM1-conjugase 1 (UFC1) (Liu et al., 2009) and finally, the E3 UFM1 ligase (UFL1) transfers UFM1 to the target protein (Tatsumi et al., 2010). Deconjugation of UFM1 is catalyzed by UFM1 specific peptidase 1 (UFSP1) or 2 (UFSP2). Most of the enzymes involved in UFMylation are located on the cytosolic side of the endoplasmic reticulum (ER) membrane. Interestingly, inhibition of the UFMylation pathway correlates with an increase in ER stress, and ER stress activation induces the expression of the UFMylation machinery (Cai et al., 2019; Zhang et al., 2012; Zhu et al., 2019). Upregulation of UFM1 has been observed in the hearts of mice with ischemic heart disease,

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correlating with the upregulation of genes from the unfolded protein response (UPR) such as ER stress markers (Azfer et al., 2006). UPR is a cellular stress response triggered in response to ER stress induced by a wide variety of insults. UPR signaling is detected by three sensors, PERK (PKR-like ER protein kinase-1), IRE1 (inositol-required enzyme 1) and ATF6 (activating transcription factor 6), and results in the regulation of the protein translation in order to restore homeostasis. Specifically, IRE1 activation catalyzes an alternative splicing of XBP1 (X-Box binding protein 1) generating a spliced form of XBP1 (XBP1s) that promotes the transcription of many genes related to protein quality control or chaperones including many genes of the UFMylation pathway (Zhang et al., 2012). The data published so far suggests that UFMylation plays a relevant role in the ER stress response. Although upregulation or downmodulation of UFMylation has been related with different pathological conditions, further studies are required to better understand this PTM and its role in cellular homeostasis (Gerakis et al., 2019; Witting and Mulder, 2021). So far only a handful of UFM1 substrates have been identified. Two of the identified substrates are the ribosomal protein 26 (RPL26) and p53. UFMylation induces the translocation of RPL26 to the ER (Walczak et al., 2019), whereas UFMylation competes with ubiquitination in modifying p53, and promotes its stability (Liu et al., 2020). Recently, a link between UFMylation and immune responses has been identified (Balce et al., 2021). However, so far, whether UFM1 plays a role in IAVor any other virus replication is unknown.

3.2.8 Hypusination

Hypusine is a basic amino acid discovered in 1971 in extracts of bovine brain (Shiba et al., 1971) and, to date, it has been found in only one cellular protein, the eukaryotic translation initiation factor 5A (eIF5A). Hypusine formation or hypusination involves two enzymatic steps. During the first step, the deoxyhypusine synthase (DHS) catalyzes the

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cleavage of spermidine and the subsequent transfer of the 4-aminobutyl moiety of spermidine to the epsilon amino group of lysine K50 of the eIF5A precursor protein to form an intermediate, deoxyhypusine. Finally, the deoxyhypusine hydroxylase (DOOH) hydroxylates the deoxyhypusine intermediate to form hypusine in eIF5A (Park et al., 1993; Schnier et al., 1991a) (Figure 7). Hypusination has been reported to be absolutely necessary for eIF5A activity. eIF5A is an essential translation factor involved in initiation, elongation, transcription, mRNA turnover, and nucleocytoplasmic transport. Initially, eIF5A was described as a translation initiation factor, necessary for the formation of the first peptide bond (Benne et al., 1978) but later on, it was demonstrated that depletion of eIF5A does not impair translation initiation but increases ribosome accumulation at start and stop codons. Thus, one of the functions of eIF5A is to suppress the ribosomal pausing at difficult to translate peptides (Doerfel et al., 2013; Gutierrez et al., 2013; Pelechano and Alepuz, 2017).

In humans eIF5A has two isoforms, eIF5A1 and eIF5A2, with high sequence identity (84%). eIF5A1 is ubiquitously expressed in almost all mammalian cells whereas eIF5A2 expression is restricted to some specific tissues like testis and brain. Although both isoforms have been found upregulated in different types of cancer (Cracchiolo et al., 2004), the association of eIF5A2 with cancer has received more attention (Guan et al., 2001). Importantly, abnormal upregulated levels of eIF5A2 correlate with aggressive characteristics of cancers and poor survival of patients (Chen et al., 2003, 2009; Fujimura et al., 2014; Guan et al., 2004; He et al., 2011; Luo et al., 2009; Meng et al., 2015; Xie et al., 2008; Yang et al., 2009). Accordingly, an inhibitor of hypusination, GC7 (N1-guanyl-1,7-diaminoheptane), has been reported to reduce tumorigenicity in different cancer models (Fujimura et al., 2014; Jasiulionis et al., 2007; Xu et al., 2014). Dysregulation of the hypusine cascade is not only important in cancer, but also in virus infections. Inhibition of hypusination has been demonstrated to exert antiviral activity against some viruses such as ebola (EBOV) virus or

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human immunodeficiency virus (HIV) (Hauber et al., 2005; Olsen et al., 2016; Ruhl et al., 1993; Schroeder et al., 2014). Whether eIF5A can be a therapeutic target for influenza A is still unknown.

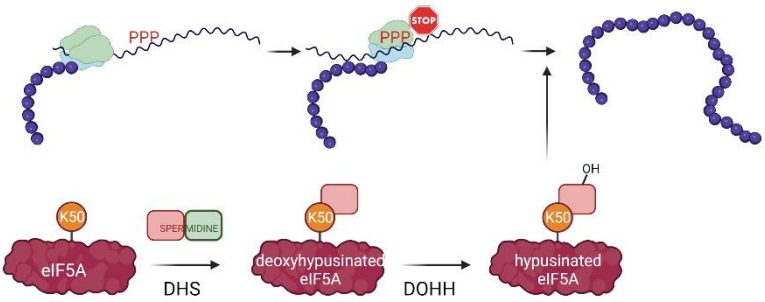


Figure 7. Schematic representation of eIF5A hypusination. Synthesis of hypusine involves two enzymatic steps: the deoxyhypusine synthase (DHS) catalyzes the transfer of 4-aminobutyl moiety from spermidine to the e-amino group of the lysine residue K50 of eIF5A precursor to form eIF5A-deoxyhypusine, which is deacetylated by deoxyhypusine hydrolase (DOHH) to form eIF5A-hypusine. eIF5A alleviates the ribosome stalling during translation of stretches of consecutive prolines (Figure designed with Biorender).

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4. OBJECTIVES

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Viruses adapt to the host cellular environment and manipulate cellular machinery and functions to perform virus replications tasks. A better understanding of virus-host cell interaction is essential to pinpoint critical virus and host factors to control virus pathogenesis. Post-translational modifications modulate protein functions and are critical for cellular responses to virus infection. PTM can be hijacked by viruses to increase the multifunctionality of their proteins, controlling key steps during virus replication. With the aim of understanding the role of different post-translational modifications in Influenza A virus infection, we propose the following objectives:

1.To determine the role of hypusination on Influenza A virus infection

1.1 To investigate the potential effects of Influenza A virus infection on eIF5A hypusination

1.2 To study the role of hypusination on Influenza A virus infection

2. To determine the role of NEDDylation on Influenza A virus infection

2.1 To investigate the potential effects of Influenza A virus infection on global NEDDylation

2.2 To study the NEDDylation of the viral protein NS1

3. To determine the role of UFMylation on Influenza A virus infection

3.1 To investigate the potential effects of Influenza A virus on global UFMylation

3.2 To study the role of UFMylation on Influenza A virus infection

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5. MATERIAL AND METHODS

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5.1 CELL CULTURE

The cell lines used in this studio were: A549 (human lung adenocarcinoma), SW480 (human colorectal cancer), A375 (human melanoma), HEK-293T (human embryonic kidney), HeLa (human cervical adenocarcinoma), MDCK (Madin-Darby canine kidney), Vero (african green monkey kidney), and BSC40 (african green monkey kidney). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (#35-079-CV, Corning), 10 U/mL penicillin/streptomycin (#P4333, Sigma-Aldrich), and 2 mM L-Glutamine (#G7513, Sigma-Aldrich), and in a humidified incubator at 37°C and 5% CO_2 (carbon dioxide). Cells were disgregated with Trypsin-EDTA (ethylenediaminetetraacetic acid) (#59418, Sigma-Aldrich).

5.2 TRANSFECTION

Cells were transfected using polyethilenimine (PEI) (#23966, CliniSciences), unless otherwise indicated, following manufacturer's instructions. Briefly, DNA or PEI were diluted in serum and antibiotic-free DMEM in a ratio of 6:1 PEI:DNA. After 5 min, both dilutions were mixed and incubated at room temperature for 20 min. Then, the mixture was added to the cells (70-80% confluency) dropwise. Cells were analyzed 24-48 h after transfection. Lipofectamine 2000 (#11668019, Thermofisher) was used to transfect RNA. Lipofectamine 2000 or RNA (Poly I:C (tlrl-picW, Invivogen)) were diluted in antibiotic and serum-free medium in a ratio of 3:1. 5 min later, the dilutions were mixed and incubated for 20-30 min at room temperature. The mixture was added to the cells dropwise.

5.3 siRNA TRANSFECTION

70-80% confluent cells were treated with siRNAs targeting eIF5A (#J-015739-06-0020, Horizon) or with non-targeting control siRNAs (#D-001810-01-20, Horizon). For a 24-well, siRNAs (5 pmol) were diluted in 25 µL of antibiotic and serum-free DMEM and 1.5 µL of

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Lipofectamine RNAiMAX (#13778-150, Invitrogen) were also diluted in the same volume of antibiotic and serum-free DMEM. Then, both preparations were mixed and incubated at room temperature for 15 min. After the incubation time, the mixture was added to the cells dropwise.

5.4 CELLULAR TREATMENTS

GC7 (#259545, Millipore) was added at a concentration of 20 µM unless otherwise indicated. MG132 (Z-Leu-Leu-al (#C2211, Sigma-Aldrich), was used at a final concentration of 20 µM). MLN4924 (#505477, Sigma-Aldrich) was added to a final concentration of 0.1 µM, and ML752 (#HY-108702, MedChemExpress was used at a final concentration of 0.1 µM. ML752 was kindly provided by Dr. Emilio Lecona (CBM, UAM). Cycloheximide was added at a final concentration of 50 µg/mL). BAY 11-7082 (#1956870, Sigma-Aldrich) was used at a final concentration of 2 mM and IKK16 (#sc-204009, Santa Cruz Biotechnology), was used at a final concentration of 10 mM.

5.5 PLASMIDS

The plasmids used in this work were:

pcDNA 3.1 empty vector (Invitrogen)

pcDNA-His6-NEDD8, pcDNA-His6-SUMO2, and pcDNA-ubc9-V5 were kindly provided by Dr. Manuel S Rodriguez (CNRS) (Rodriguez et al., 2001, 1999; Vertegaal et al., 2006). NS1-WT (PR8), pdZ-PA, pdZ-NA, pdZ-PB1, pdZ-PB2, pdZ-NS-split, pdZ-NP, pdZ-HA, and pdZ M1 were kindly provided by Dr. Adolfo Garcia-Sastre (Mount Sinai) (Martínez-Sobrido and García-Sastre, 2010). pCMV-Flag-STUB1 (#HG12496-NF, Sino Biological). PGK-H2BeGFP (#21210, Addgene). 3xFlagCMV-7-1/heIF5A, kindly provided by Dr. Myung Hee Park (NIDR, NIH) (Park et al., 2006). prK5-HA-UFM1 and UFM1 sg-RNA were kindly provided by Dr. Rosa Barrio (CICbioGUNE).

pcDNA-HA-eIF5A1, pcDNA-HA-NS1, HA-UFM-ΔG, pdZ-NS Split 4KR, pcDNA-HA-RPS27A, pcDNA-VDAC1 were generated by

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cloning. NS1-K0, the different mutants of NS1, pcDNA-VDAC1-K110R, HA-UFM-ΔG-K41R, and the mutants of HA-eIF5A1 were generated by site directed mutagenesis using the primers listed in Table 1.

5.6 ANTIBODIES

The primary antibodies used were: anti-HA (#901503, Biolegend), anti-eIF5A1 (#ab32443, Abcam), anti-phospho-eIF2a (#9721 Cell Signaling), anti-GAPDH (#sc-32233, Santa Cruz Biotechnology), anti-VSV G (a generous gift of Dr I Ventoso, CBMSO, Madrid), anti-actin (sc-4778, Santa Cruz Biotechnology), anti-influenza A virus M1 protein (#GTX127356, Gene Tex), anti-influenza A virus NS1 protein (#GTX125990, Gene Tex), anti-Alphavirus E1 and NSP1 (Llamas-González et al., 2019) , anti-PTV (mouse ascitic fluid kindly provided by Dr. Scott Weaver from World Reference Center for Emerging Arboviruses (WRCEVA) at University of Texas Medical Branch (UTNM), USA), anti-Hypusine antibody (#ABS1064-I, Millipore), anti-GFP (#902605, Biolegend), anti-Flag (OctA-probe) (#sc-166355, Santa Cruz Biotechnology), anti-NEDD8 (#ab81264, Abcam), anti-6x-His Tag (#MA1-21315, Thermo Fisher), anti-β-tubulin (#2146, Cell Signaling), anti-β-actin (#sc-47778, Santa Cruz Biotechnology), anti-UFM1 (#ab109305, Abcam), anti-p85β (#ab180967, Abcam), anti-Histone3 (#4499T, Cell Signaling), anti-STUB1 (#PA5-29024, Invitrogen), and anti-p65 (#3034, Cell Signaling). The secondary antibodies for Wester blot analysis were anti-rabbit (#a16035, Invitrogen) or anti-mouse (#a16072, Invitrogen) antibodies conjugated to horseradish peroxidase (HRP).

5.7 BACTERIAL TRANSFORMATION

Competent *Escherichia coli* DH5α are thawed on ice. Then, the DNA of interest is added to the bacteria. After 30 min of incubation on ice, the bacteria are heat shocked for 1 min at 42º C and cooled immediately on ice for 2-5 min. Then, 350 µL of Luria Bertani (LB) growth media with no antibiotics are added to the bacteria and the mixture is incubated

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for 1 h at 37º C with shaking (750 rpm). Finally, the culture is pelleted at 4000g for 5 min and 100 µL of the resuspended pellet are plated in an LB agar plate supplemented with the antibiotic of interest.

5.8 SITE-DIRECTED MUTAGENESIS

All the mutants used in this work were generated by site-directed mutagenesis using Phusion High Fidelity DNA polymerase and 100-300 ng of DNA. The primers used are listed in table 1. After the amplification, the template DNA was digested with DpnI (Thermo Fisher) for 2 h at 37º C and 1/5 of the total PCR volume was transformed into *Escherichia coli* DH5α and plated in an LB plate with the appropriate antibiotic. Colonies were then grown overnight in LB supplemented with antibiotic and the following day plasmidic DNA was purified using a commercial kit. The presence of the mutation and confirmation of no additional mutations were confirmed by Sanger sequencing.

5.9 CLONING

The gene of interest was amplified from a plasmid or from complementary DNA (cDNA) using Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase (#F-549S, Thermo Fisher) with the primers listed in Table 1. These primers include a region complementary to the 3' or 5' ends of the sequence targeted for amplification (the forward (F) and reverse (R) primers) together with sequences recognized by the restriction enzymes that will be used to cut the vector. After the amplification of the insert, vector and inserts were digested with the restriction enzymes for 30 minutes at 37º C. The resulting DNA was then loaded in an agarose gel and the bands corresponding with the vector or the insert were cut and the DNA was extracted from the agarose gel using a kit (#K0691, Thermofisher). Both purified vector and insert were incubated together in a ligation reaction in a ratio of 3:1 insert-vector using T4 DNA Ligase (#15224-025, Invitrogen). 20 min after incubation, 1/4 of the reaction was used

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to transform competent DH5α. The cloning was confirmed by digestion and Sanger sequencing.

5.10 WESTERN BLOT

Proteins were collected in Laemmli sample buffer and boiled for 5 minutes at 100º C. Then, proteins were separated by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a 0.45 μm nitrocellulose membrane. Membranes were then blocked by incubating in 5 % non-fat dry milk in tween tris-buffered saline (TBST). Blocked membranes were incubated with the primary antibody at 4º C overnight in a shaker. The following day, the membranes were washed in Tris buffered saline Tween20 (TBST) (3 washes, 10 minutes each) and incubated with the secondary antibody for 1 hour, at room temperature in a shaker. Finally, the membranes were washed in TBST (3 washes, 10 minutes each), incubated in ECL for 2 min and exposed to an X-ray film.

5.11 VIRAL INFECTION

Infections were carried out with the viruses: vesicular stomatitis virus (VSV) of indiana strain, recombinant VSV expressing GFP (rVSV-GFP), the mouse-adapted influenza A/PR/1934 (PR8) virus, mayaro virus (MAYV, AVR0565 strain, San Martin, Peru), una virus (UNAV, BT-1495-3, Bocas del Toro, Panama), chikungunya virus (CHIKV, Panama_256137_2014 strain, Panama), zika virus (ZIKV, 259249 strain, Panama), and punta toro virus (PTV, Adames strain, Panama). All arboviruses were a kindly gift of Dr. Scott Weaver from WRCEVA, UTMB, USA. For cellular infection, MAYV, UNAV, CHIKV, ZIKV, PTV and VSV were added to the cells resuspended in serum-free medium, whereas PR8 was resuspended in 0.3% bovine serum albumin (BSA) in phosphate buffer saline (PBS). One hour after incubation with the virus solution, the unbound virus was discarded and fresh medium was added to the cells. In the case of influenza virus, TPCK- treated trypsin (2.0 μg/mL) was added to the medium.

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5.12 RESCUE OF RECOMBINANT INFLUENZA VIRUS

A co-culture of HEK-293T and MDCK cells in a ratio of 3:1 was mixed with a transfection mixture of the pdZ plasmids encoding all eight segments of the viral genome . The co-culture is then transferred to a culture plate and cells are incubated for 30 h in incubator. Then, the incubation medium was replaced by DMEM supplemented with 1 µg/mL of TPCK-treated trypsin. 48 h later, supernatants were collected and virus yields were measured by plaque assay.

5.13 PLAQUE ASSAY

BSC40 (VSV), MDCK (IAV) or Vero cells (MAYV, UNAV, CHIKV, ZIKV, and PTV) were seeded in 12-well culture plates until a monolayer is formed. Then, the cells were washed with PBS and the serial dilutions of the virus, prepared in DMEM (for VSV, MAYV, UNAV, CHIKV, ZIKV, and PTV), or PBS-BSA 0.3% (for IAV), were added to the monolayer. After one h of incubation, rocking the plate every 5-10 min, the virus was aspirated from each well and 1 mL of the agar solution was added to the cells. Once the agar was solidified, the plates were incubated at 37º C and 5º CO_2 for 1-2 days. The agar solution was prepared by mixing 25 mL of oxoid agar 1.8 % and 25 mL of DMEM 2X. For influenza virus, TPCK-treated trypsin was added to the medium.

5.14 METABOLIC LABELING

Cells were infected at a multiplicity of infection (moi) of 5, washed twice with methionine-free DMEM and labeled by incubation in methionine-free DMEM supplemented with 35S-methionine (100 µCi/mL) for the times indicated in the figure. Whole protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis, fixed and dried. The radioactively labeled proteins were visualized by autoradiography.

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5.15 *IN VITRO* TRANSLATION

A plasmid containing the sequence of the gene of interest under the T7 promoter or the PCR product of the gene of interest generated using a primer containing the T7 promoter sequence were translated using TNT Quick Coupled Transcription/Translation System (#L1170, Promega) following manufacturer's instructions in the presence of ^{35}S-methionine. The reticulocytes, the DNA and the methionine were incubated at 30º C for 90 min.

5.16 *IN VITRO* SUMOYLATION AND DESUMOYLATION ASSAYS

For the SUMOylation reaction 0.3 µg of SAE1/2 (Biomol, Enzo Life Sciences), 2 mM adenosine triphosphate (ATP), 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 10 mM Creatine Phosphate, 3.5 U/mL of creatine kinase, 0.6 U/mL of inorganic pyrophosphate, 600 ng of UBC9, 10 µg of recombinant SUMO2, and the *in vitro* translated protein were incubated for 240 min at 37º C. The reaction was stopped by adding Laemmli sample buffer and boiling for 5 min at 100º C. For the deSUMOylation assay, after 180 min of the SUMOylation reaction, GST-SENP1 was added to the reaction, and 60 min later the reaction was stopped as described above.

5.17 *IN VITRO* NEDDYLATION ASSAY

NEDDylation assay was carried out following manufacturer's indications (#BML-UW0590-0001, Enzo Life Sciences). Briefly, the recombinant protein of interest together with NEDD8, the E1 and the E2 enzymes were mixed in the presence of ATP. The mixture was incubated at 37º C for 120 min and the reaction was stopped by adding Laemmli sample buffer and boiling at 100º C for 5 min.

5.18 PREDICTION OF SUMOYLATION SITES

The software used for the prediction of SUMOylation acceptor sites was: GPS-SUMO or JASSA (Joined advanced SUMOylation site and SIM analyzer).

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5.19 HISTIDINE TAG PURIFICATION

The cells seeded in 6-well plates and transfected with the indicated plasmids or treated with the indicated reagents were collected in PBS, and 10 % of the cellular resuspension was pelleted, resuspended in Laemmli sample buffer and boiled for 5 minutes at 100º C (whole cell extract (WCE)). The remaining 90% of cellular suspension was pelleted and lysed in denaturing buffer G (6 M guanidine HCl, 0.1 M Na_2HPO_4, 6.8 mM NaH_2PO_4, 25 mM Tris HCl pH 8). Then, to break the DNA, the samples were sonicated (amplitude 10%) for 15 seconds. 30-50 µL of equilibrated nickel beads (TALON® Nickel Affinity Resin (# 635503, Takara) were added to each sample and the mixture was incubated in rotation at room temperature for 90-120 min. Then, the samples were centrifuged (1000 xg for 1 min) and the beads were washed 4 times with buffer U (8 M Urea, 100 mM Tris HCl pH 8, 93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4). Finally, the beads were dried completely, and the histidine-tagged purified proteins were eluted in Laemmli sample buffer and boiled at 100º C for 5 minutes.

5.20 SUBEs

The cells grown in 15 cm plates were lysed (50mM Tris pH 8.5; 150 mM NaCl, 5 mM EDTA, 1% Igepal, supplemented with protease inhibitors) and sonicated for 15 seconds with 10 % amplitude. Lysates were then centrifuged at 14000 g for 10 minutes and clear lysate was incubated wit GST or GST-SUBEs and incubated for 2 hours at 4º C. Then the GST beads were washed with the lysis buffef, left dried, and resuspended in Laemmli buffer.

5.21 IMMUNOPRECIPITATION OF HA-TAGGED PROTEINS

The cells seeded in 15 cm plates and transfected with an HA-tagged plasmid were collected in lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) in the presence of a protease inhibitor cocktail (#78425, Thermo Fisher). The protein extracts were kept on ice for 30 min, passed through a needle and

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centrifugated at maximum speed for 10 min at 4º C. The supernatants were incubated with 25 µL of anti-HA beads (#88836, Thermo Fisher) in rotation overnight at 4º C. The following day, the HA beads were collected using a magnetic stand and the beads were washed with TBST 4 times and once with ultrapure water. Then, 100 µL of Laemmli buffer were used to elute the HA-tagged proteins.

5.22 LUCIFERASE ASSAY

HEK-293T cells were transfected with the IFN reporter ISG54-luc, the beta-galactosidase reporter (ratio 10:1) and the plasmids of interest. 24 h after transfection, IFN-β (500 U/mL) was added to the cells. 12 h later, the cells were lysed in 100 µL of lysis buffer and luciferase levels were measured using 20 µL of the lysed sample and the substrate provided by the Luciferase Reporter Gene Assay kit (#11814036001, Roche) using Berthold Mithras LB 940 luminometer. To measure the beta-galactosidase activity 70 µL of buffer Z (0.06M Na_2HPO_4, 0.04M NAH_2PO_4, 0.01M KCl, 0.001M $MgSO_4$, and 0.05M β-mercaptoethanol), 20 µL of ONPG (4 mg/mL) and 10 µL of the sample were mixed and incubated at 37º C for 30-60 min. Absorbance was measured at 420 nm and the levels were used to normalize the luciferase activity values.

5.23 IMMUNOPRECIPITATION IN DENATURING CONDITIONS

Cells seeded in 15 cm plates were collected and 10 % of the cell resuspension was centrifuged and resuspended in Laemmli buffer (WCE) whereas the 90 % remaining cells were resuspended in lysis buffer (50 mM Tris, pH 7.5, 70 mM β-mercaptoethanol) and boiled at 100º C for 10 minutes. Then, the protein extracts were diluted 4 times in BC100 lysis buffer (20 mM Tris, pH 8, 0.1 M KCl, 10% Glycerol, 1 mM EDTA, 1 mM DTT, 0.1 % NP-40) in the presence of protease inhibitors. Then, the protein extracts were kept on ice for 30 min, passed through a needle and centrifugated at maximum speed for 10 min at 4º C. The supernatants were then incubated with the antibodies of interest overnight at 4º C in rotation. The following day, the samples were

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incubated with 30 µL of the Rec-Protein G-Sepharose 4B (#101242, Invitrogen) for 120 min at 4º C in rotation. Then, the beads were centrifuged at 1000 xg for 1 min, washed 4 times and finally resuspended in 30-50 µL of Laemmli buffer.

5.24 RNA EXTRACTION AND QUANTITATIVE RT-PCR

For the RNA extraction the GenElute Mammalian Total RNA Miniprep Kit (#RTN10, Sigma-Aldrich) was used following manufacturer's protocol. Total RNA was quantified using Nanodrop and 1 µg of each sample was used as a template for the synthesis of cDNA using High Capacity cDNA Reverse Transcription Kit (#4368814, Applied Biosystems). The quantitative PCR was then performed using NZYSpeedy qPCR Green Master Mix (2x) Rox Plus, (#MB222, nzytech) and the primers described in Table 2.

5.25 IMMUNOFLUORESCENCE ASSAY

The cells were grown on coverslips and transfected or treated as indicated. Then, cells were fixed by incubating in 2 % paraformaldehyde in PBS for 20 min at room temperature. Next, after three washes with PBS, the cells were permeabilized by incubating them in a solution of Triton X-100 0.25 % in PBS for 20 min. Cells were washed three times in PBS and then non-specific interactions were prevented by incubating the cells in a solution of 2% BSA in PBS for 20 min. After washing the preparations in PBS, the cells were incubated with the indicated primary antibodies in a humid chamber at 4º C overnight. The cells were then washed three times with PBS and incubated with the secondary antibody (anti-mouse or anti-rabbit antibody conjugated to Alexa-488 or Alexa-594). After 1 h of incubation, cells were washed three times with PBS and then they were incubated with 4',6'-diamidino-2-phenylindole (DAPI) for 5 min to stain the nuclei. Finally, the cells were washed three times with PBS, the preparations were mounted using ProLong Gold antifade reagent (#P36934, Invitrogen) and the samples were analyzed using a Leica TCS SP5 confocal microscope.

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5.26 SUBCELLULAR FRACTIONATION

Cells grown in 10 cm plates were collected and centrifuged at 14,000 rpm for 10 s. The pellet was resuspended in 1 mL of 0.1% NP40 in PBS. The lysate was centrifuged at 14,000 rpm for 10 s. 300 µL of the supernatant were collected and mixed with 75 µL of 4X Laemmli buffer, boiled at 100º C for 5 min and kept at -20º C as the cytoplasmic fraction. The pellet, containing the nuclear fraction, was resuspended in 1 mL of 0.1% NP40 in PBS. After 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.

5.27 DSRNA BINDING ASSAY

The cells grown in 6 well plates were collected in PBS. 10 % of the cell suspension were centrifuged and the pellet was resuspended in 4xLaemmli buffer and boiled at 100C for 5 min and it was used as a WCE. The 90 % remaining was pelleted, resuspended in binding buffer (20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 5 mM MgCl2,1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 % NP-40, 10 % glycerol, supplemented with protease inhibitors), and incubated with 45-180 µL of Poly I:C beads overnight under rotation. The following day, the beads were washed 4 times with binding buffer, resuspended in 4xLaemmli buffer and boiled at 100C for 5 min.

5.28 MASS SPECTROMETRY ANALYSIS

A549 cells stably expressing His6-NEDD8-R74K (generated by transduction with lentiviruses expressing the mutated protein kindly provided by Dr. Dimitris Xirodimas (CNRS), followed by incubating the cells in DMEM supplemented with puromycin) were infected with influenza A/PR8/1934 at an moi of 1 for 24 h or mock infected. Cells were collected and processed following the protocol designed by Vogl. et al. (Vogl et al., 2020). Cells were lysed in a strong denaturing buffer, reduced, alkylated and digested using Lys-C (#4987481427648, Fujifilm). Then, the peptides were purified and incubated with Di-

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glycine antibody (PTMScan, #5562, Cell Signaling) for 4 h at 4º C in rotation. Then, the preparation was washed and the peptides were eluted and desalted to be analyzed by mass spectrometry in collaboration with Dr. Jeffrey Johnson (Mount Sinai).

5.29 YEAST STRAINS AND VIABILITY ASSAYS

All S. cerevisiae strains employed are described in Table 3. For viability assays, cells were streaked onto SC-LEU plates and incubated at 25º C and 37º C respectively for 72 h. Then, they were imaged in a Gel Doc documentation system with a 0.5s exposure (Bio-Rad).

5.30 POLYSOME PROFILE ANALYSIS

Yeast were grown at 37º C and heat-shocked at 46º C for the indicated times. Then, the samples were analysed in collaboration with Paula Alepuz (Universidad de Valencia) (Romero et al., 2020). Data shows the ratio polysome/monosome (P/M) of one biological replicate.

5.31 IMAGE PROCESSING AND FIGURES

Intensity of the bands detected by Western blot analysis were quantified using ImageJ. Figures were created using Biorender.

5.32 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism software. At least three biological replicates were performed for each analysis.

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6. RESULTS

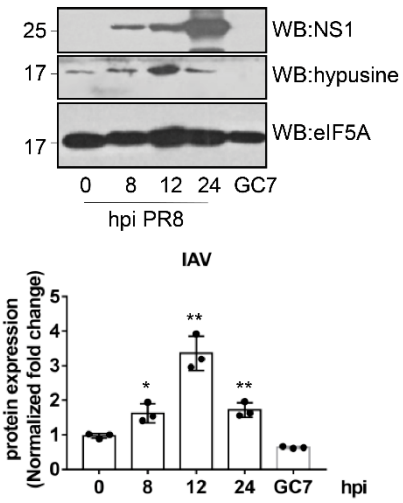
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6.1 INFLUENZA VIRUS AND EIF5A

6.1.1 Influenza A virus (IAV) infection induces hypusination

Several reports demonstrated that infection with different viruses such as human cytomegalovirus (HCMV) (Isom, 1979), herpes simplex virus type 1 (HSV-1) (Greco et al., 2005), or Epstein-Barr virus (EBV) (Shi et al., 2013) can alter the polyamine pathway at different levels. However, whether influenza virus infection modulates the polyamine pathway is still unknown. Therefore, we decided to study whether IAV can alter the hypusination levels, which is one of the downstream targets of the polyamine pathway. To study the putative modulation of eIF5A activation by IAV, we infected A549 cells with influenza virus A/PR8/1934 (PR8) strain at an moi of 5 and, at the indicated times after infection, cells were collected and protein extracts were analyzed by Western blot with anti-hypusine antibody. We observed an increase in the levels of hypusination from 0 to 12 h after IAV infection that decreased at longer times after infection (Figure 8). These results indicated that IAV infection promoted the activation of eIF5A.



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Figure 8.IAV induces eIF5A activation. A549 cells were infected with influenza virus A/PR8/1934 and at different times after infection protein extracts were analyzed by Western blot with the indicated antibodies (upper panel). Hypusinated eIF5A and eIF5A protein intensity bands from three biological replicates were quantified using Image J software. The hypusinated eIF5A/ total eIF5A ratios were plotted. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,$p<0.05$, **,$p<0.01$, ***,$p<0.005$.

6.1.2 Both infection with different RNA viruses and stimulation with dsRNA induce hypusination

Here we showed that infection with IAV infection induces hypusination. However, whether infection with other viruses triggers hypusination is unknown. To investigate this possibility, we infected A549 cells with vesicular stomatitis virus (VSV) and, at different times after infection, cells were collected and the protein extracts were analyzed by Western blot. Incubation with anti-hypusine antibody revealed an increase in hypusination levels from 0 to 5 h after VSV infection that decreased after longer times (Figure 9A), indicating that infection with VSV activates eIF5A. Then, we decided to test whether treatment with synthetic analogue double-stranded RNA (dsRNA) (Poly I:C) can also trigger the activation of eIF5A. For that, we transfected A549 or A375 cells with Poly I:C and, at different times after treatment, protein extracts were collected and analyzed by Western blot with anti-hypusine antibody. We observed that transfection with Poly I:C induced an increase in hypusination levels in both A549 (Figure 9B) and A375 (Figure 9C) cells. Altogether these results indicated that dsRNA promotes the activation of eIF5A.

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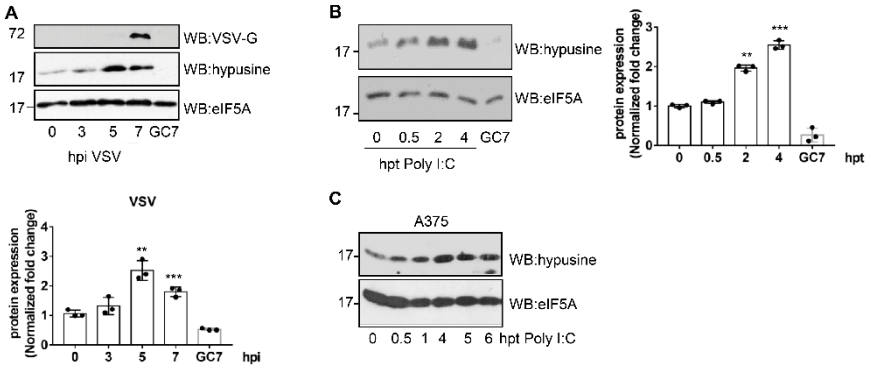


Figure 9. Both stomatitis vesicular virus (VSV) infection and dsRNA stimuli induce eIF5A activation. A549 cells were infected with VSV (A, upper panel) or transfected with Poly I:C (B, left panel) and, at different times after treatment, protein extracts were analyzed by Western blot with the indicated antibodies. Hypusinated eIF5A and eIF5A protein intensity bands from three biological replicates were quantified using Image J software. The hypusinated eIF5A/ total eIF5A ratios in cells infected with VSV (A, lower panel) or transfected with Poly I:C (B, right panel) were plotted. The hypusinated eIF5A/ total eIF5A ratios were plotted. Data represent the mean and error bars of three biological replicates. C, A375 cells were transfected with Poly I:C and, at different times after treatment, protein extracts were analyzed by Western blot with the indicated antibodies. Statistical analysis was assessed by a Student's t-test. *,$p<0.05$, **,$p<0.01$, ***,$p<0.005$.

6.1.3 Activation of eIF5A is modulated by NF-kB

In order to identify the mechanisms underlying the induction of hypusination in response to virus infection, we evaluated the transcription of different genes involved in the polyamine or hypusination pathways in cells infected with VSV or IAV using RT-qPCR. A significant increase in mRNAs for ornithine decarboxylase (ODC) and spermidine/spermine-N(1)-acetyltransferase (SSAT) were detected in both VSV and IAV infected cells (Figure 10A and Figure 10B). Different reports demonstrated that ODC and SSAT are targets of NF-κB (Casero and Pegg, 2009; Choi et al., 2006; Tacchini, 2004). Therefore, we hypothesized that the activation of eIF5A by virus

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infection could be mediated by the NF-κB pathway. In order to test this hypothesis, we first analyzed the hypusine levels in A549 cells treated with the NF-κB inhibitor BAY 11-7082 or after p65 depletion, by Western blot analysis. The levels of hypusine in cells treated with the NF-κB inhibitor or depleted of p65 were lower than in control cells

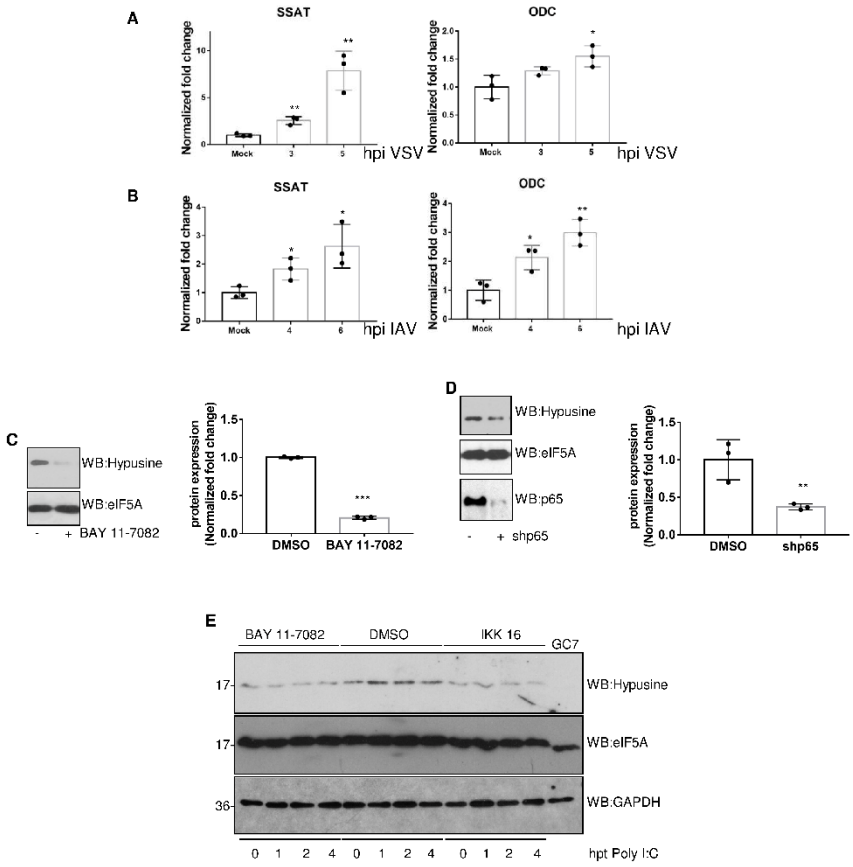


Figure 10. Modulation of the hypusination pathway by NF-κB. A, A549 cells were infected with VSV (A) or IAV (B) and, at the indicated times after infection, RNA was analyzed by Q RT-PCR. Data represents the normalized fold change of three replicates of infected cells in comparison with the mock-infected cells. C, A549 cells were treated or not with the NF-kB inhibitor BAY 11-7082 and at 12 h after treatment, protein extracts were analyzed by Western blot with anti-hypusine antibody (left panel). Hypusinated eIF5A and eIF5A protein intensity

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bands from three biological replicates were quantified using Image J software. The hypusinated eIF5A/ total eIF5A ratios in cells treated with BAY 11-7082 were plotted (right panel). D, A549 cells were transduced with sh-p65 and after 48 h transduced cells were selected by incubating with puromycin for 3 days. Then, protein extracts were analyzed by Western blot with anti-hypusine antibody (left panel). Hypusinated eIF5A and eIF5A protein intensity bands from three biological replicates were quantified using Image J software. The hypusinated eIF5A/ total eIF5A ratios in the cells with downmodulated p65 were plotted (right panel). Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,$p<0.05$, **,$p<0.01$, ***,$p<0.005$. D, A549 cells were treated or not with the NF-kB inhibitor BAY 11-7082 or IKK16 for x h and then, cells were transfected with Poly I:C. At the indicated times after treatment, protein extracts were analyzed by Western blot with anti-hypusine antibody.

(Figure 10C and 10D), indicating that the NF-κB pathway modulates hypusination in unstimulated cells. Finally, we analyzed the hypusine levels in cells treated or not with the NF-κB inhibitors IKK16 or BAY 11-7082 and then transfected with Poly I:C for the indicated times. The increase in hypusine levels observed after poly I:C treatment in cells untreated with NF-κB inhibitors, was not detected in those cells treated with IKK16 or BAY 11-7082 (Figure 10E). Altogether these results suggest that NF-κB is a positive regulator of eIF5A hypusination under both basal and dsRNA-stimulated conditions.

6.1.4 Inhibition of eIF5A hypusination impairs the replication of IAV

It has been previously demonstrated that the activation of eIF5A is essential for the replication of ebola virus, marburg virus, and human immunodeficiency virus type 1 (HIV-1) (Hauber et al., 2005; Olsen et al., 2016; Ruhl et al., 1993; Schroeder et al., 2014). In addition, here we demonstrated that IAV induces hypusination. Therefore, we hypothesized that eIF5A can play a role on IAV. To evaluate this hypothesis, we first studied whether the treatment with the spermidine analogue and competitive deoxyhypusine synthase (DHS) inhibitor N1-guanyl-1,7-diamine-heptane (GC7), which inhibits the activation of eIF5A, had an impact on IAV replication. A549 cells were incubated with GC7 20μM or dimethylsulfoxide (DMSO) overnight and then the

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cells were infected with IAV at an moi of 5. At 24 h after infection cellular supernatants were collected for titration of infectious virus by plaque assay. Treatment with GC7 resulted in a significant decrease in the viral titer (Figure 11A). Then, A549 cells treated with DMSO or GC7 20μM overnight were infected with IAV at an moi of 5 and, at the indicated times after infection, protein extracts were collected and analyzed by Western blot. We observed a clear decrease in the M1 protein levels in those cells incubated with GC7 in comparison with the DMSO-treated cells (Figure 11B). To analyze whether GC7 treatment also decreased the IAV RNA transcription, we infected cells previously treated or not with GC7 and, at the indicated times after infection, cells were collected, total RNA was isolated, and mRNA of IAV transcripts was quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). We observed a significant reduction in IAV transcripts in the cells treated with GC7 (Figure 11C). Finally, we evaluated the impact of eIF5A depletion, by using small interfering RNA (siRNA) targeting eIF5A (sieIF5A) or negative control siRNAs (siControl), on both the synthesis of IAV proteins and IAV titers. We detected a clear reduction in the levels of the IAV NS1 protein in the cells depleted from eIF5A in comparison with the siC transfected cells (Figure 11D). A significant decrease in the viral titer was also observed in those cells depleted from eIF5A compared to control cells (Figure 11E). Altogether these results demonstrated that activation of eIF5A is essential for efficient IAV replication.

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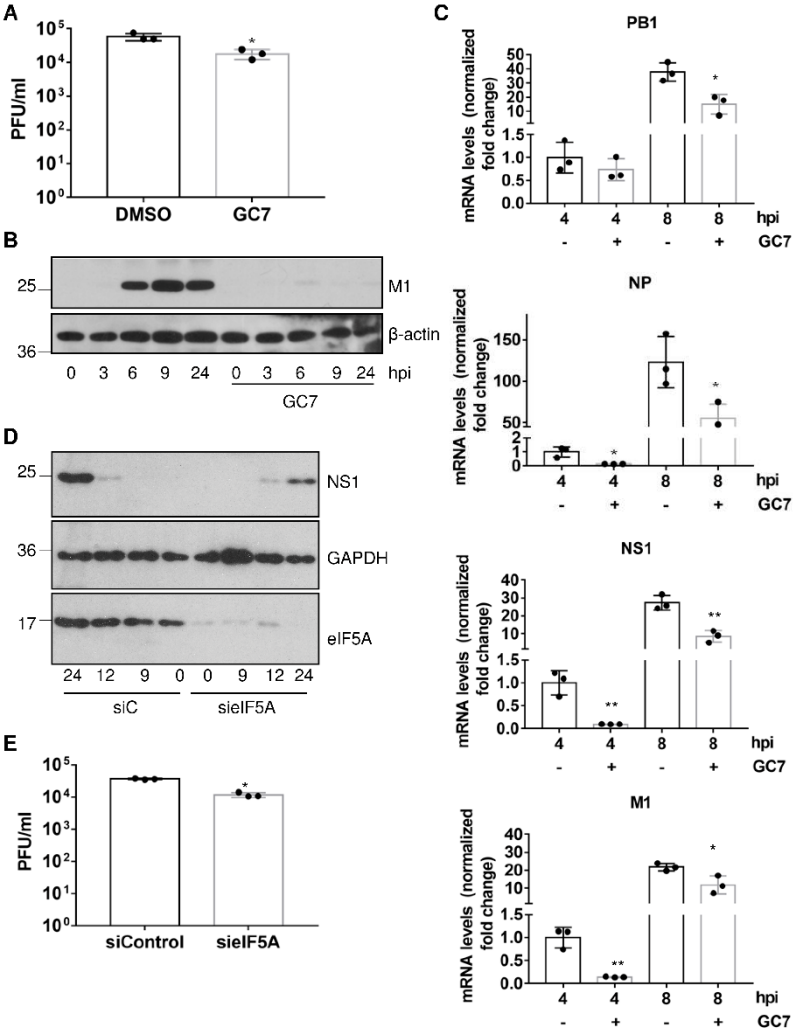


Figure 11. Effect of eIF5A inhibition on influenza virus replication. A, Effect of hypusine inhibition on IAV replication. A549 cells treated with GC7 or DMSO were infected with IAV and 24 h after infection viral titers in the supernatants were analyzed by plaque assay. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,p<0.05, **,p<0.01, ***,p<0.005. B, Consequences of GC7 treatment on IAV protein synthesis. A549 cells treated with GC7 or DMSO were infected with IAV and, at

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the indicated times after infection viral synthesis of viral proteins were analyzed by Western blot. C, Consequences of hypusine inhibition on IAV gene transcription. A549 cells treated with DMSO or GC7 were infected with IAV and at the indicated times after infection total RNA was purified and mRNA levels of viral genes were analyzed using qRT-PCR. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,p<0.05, **,p<0.01, ***,p<0.005. D, Effect of eIF5A downmodulation on NS1 IAV protein expression. A549 cells were transfected with siRNA against eIF5A (sieIF5A) or siRNA control (siC) and 72 h after transfection, cells were infected with IAV and at different times after infection the synthesis of viral proteins was analyzed by Western blot. F, Effect of eIF5A downmodulation on IAV replication. A549 cells transfected with siRNA against eIF5A (sieIF5A) or siRNA control (siC) were infected with IAV and 24 h after infection the viral titers in the supernatants were quantified by plaque assay. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,p<0.05, **,p<0.01, ***,p<0.005.

6.1.5 Inhibition of eIF5A hypusination impairs the replication of VSV

Here we showed that eIF5A activation is essential for IAV replication. We then decided to evaluate whether eIF5A is also essential for the replication of VSV. First, A549 treated with GC7 or DMSO were infected with VSV at an moi of 1 and 24 h after infection, cellular supernatants were collected and analyzed by plaque assay. A significant reduction in the viral titer (up to 2-log decrease) was detected in the supernatant from GC7 treated cells (Figure 12A), indicating that hypusination is essential for efficient VSV replication. Then, A549 treated with GC7 or DMSO were infected with a recombinant VSV expressing green fluorescent protein (GFP) at an moi of 1 or 0.1 and 12 h after infection cells were fixed, analyzed by fluorescence microscopy, photographed, and then collected for fluorescence-activated cell sorting analysis. A picture of the infected cells can be seen in figure 12B (upper panel). The percentage of GFP-positive cells in GC7 treated cells was significantly lower than in DMSO treated cells (Figure 12B, lower panel). Metabolic labeling with ^{35}S-methionine of A549 cells treated or not with GC7 and infected with VSV at an moi of 5 was then carried out. For that, cells were washed and incubated with methionine-free DMEM for 1 hour. Then, cells were infected with VSV and ^{35}S-

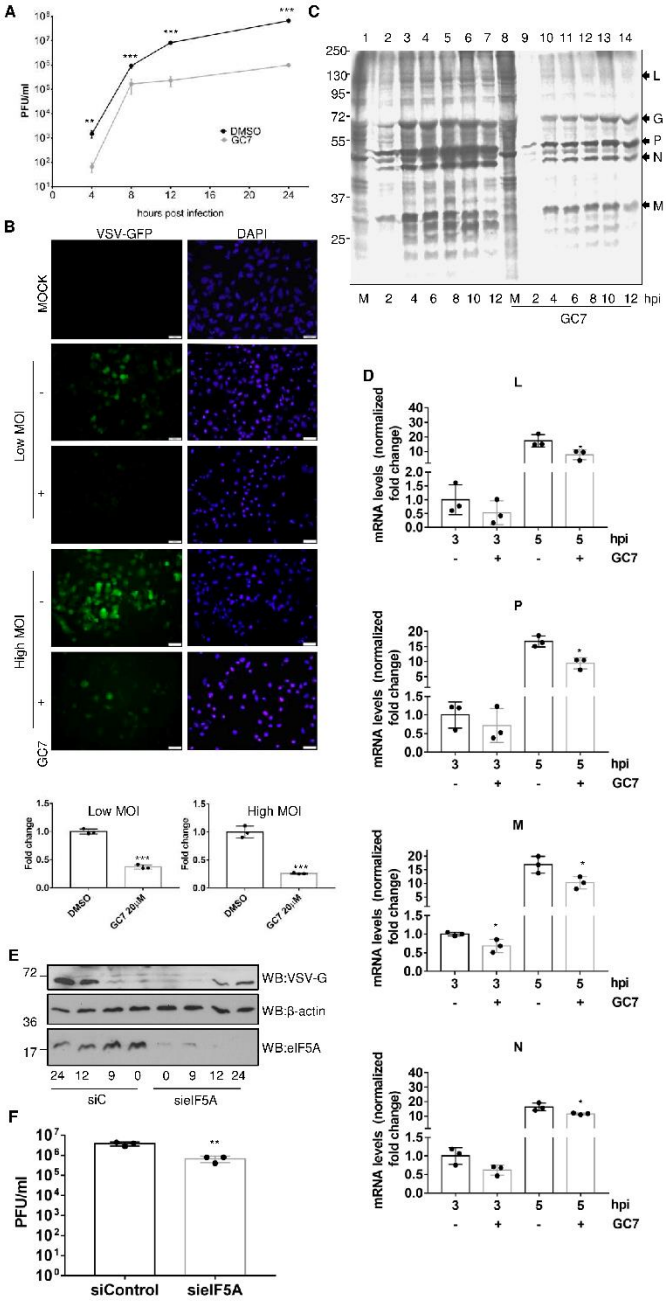
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methionine (100 μCi/mL) was added to the media. At the indicated times after infection, cell extracts were collected in SDS-loading buffer, proteins were analyzed by electrophoresis in an SDS-polyacrylamide gel, and the dried gel was exposed to an X-ray film. Analysis of the ^{35}S-methionine labeled proteins revealed a reduction in the levels of both viral and cellular proteins upon GC7 treatment (Figure 12C). Furthermore, we quantified the mRNA levels of VSV genes in A549 cells treated or not with GC7 and infected with VSV at an moi of 5 by qRT-PCR. We observed a significant reduction in VSV transcripts after inhibition of eIF5A hypusination (Figure 12D). Finally, we evaluated the consequences of eIF5A depletion on VSV protein synthesis or titer. A clear reduction in the levels of VSV-G protein (Figure 12E) and a significant decrease in the viral titer (Figure 12F) were detected upon depletion of eIF5A. Altogether these results indicated that eIF5A activation is essential for VSV replication.

6.1.6 Inhibition of eIF5A hypusination impairs the replication of mayaro virus, una virus, chikungunya virus, punta toro virus and zika virus

We decided to evaluate the potential effect of the eIF5A inhibitor GC7 on the protein synthesis or titers of the emerging mayaro, una, chikungunya, punta toro, and zika viruses, in collaboration with José González Santamaría lab (Instituto Conmemorativo Gorjas de Estudios de la Salud, Panama). Our collaborators treated HeLa cells with GC7 or DMSO and 24 h after treatment, they infected the cells with the different viruses. At different times after infection, cells were collected and protein extracts were analyzed by Western blot with the indicated antibodies. In addition, cellular supernatants of infected cells were recovered at 24 h after infection and virus titers were quantified by plaque assay.

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Figure 12. Inhibition of eIF5A impaires VSV replication. A, Effect of hypusine inhibition on VSV replication. A549 cells treated with GC7 or DMSO were infected with VSV and 24 h after infection viral titers in the supernatants were analyzed by plaque assay. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,p<0.05, **,p<0.01, ***,p<0.005. B, Consequences of hypusine inhibition on VSV-GFP. A549 cells treated with GC7 or DMSO were infected with VSV and 24 h after infection, we visualized the VSV-GFP positive cells by immunofluorescence microscopy (upper panel) and the percentage of VSV-GFP positive cells was quantified by flow cytometry (lower panel). Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,p<0.05, **,p<0.01, ***,p<0.005. C, Metabolic labeling of cells treated or not with GC7 and infected or not with VSV for the indicated times. D, Consequences of hypusine inhibition on VSVgene transcription. A549 cells treated with DMSO or GC7 were infected with VSV and at the indicated times after infection total RNA was purified and mRNA levels of viral genes were analyzed using qRT-PCR. Statistical analysis was assessed by a Student's t-test. *,p<0.05, **,p<0.01, ***,p<0.005. E, Effect of eIF5A downmodulation on VSV protein G expression. A549 cells were transfected with siRNA against eIF5A (sieIF5A) or siRNA control (siC) and 72 h after transfection, cells were infected with VSV and at different times after infection the synthesis of viral proteins was analyzed by Western blot. F, Effect of eIF5A downmodulation on VSV replication. A549 cells transfected with siRNA against eIF5A (sieIF5A) or siRNA control (siC) were infected with VSV and 24 h after infection the viral titers in the supernatants were quantified by plaque assay. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,p<0.05, **,p<0.01, ***,p<0.005.

We observed a clear reduction in the levels of the proteins and/or titers of mayaro virus (MAYV) (Figure 13A), una virus (UNAV) (Figure 13B), chikungunya virus (CHIKV) (Figure 13C), punta toro virus (PTV) (Figure 13D), and zika virus (ZKV) (Figure 13E) in the cells treated with GC7, indicating that hypusination is essential for the replication of these emerging viruses.

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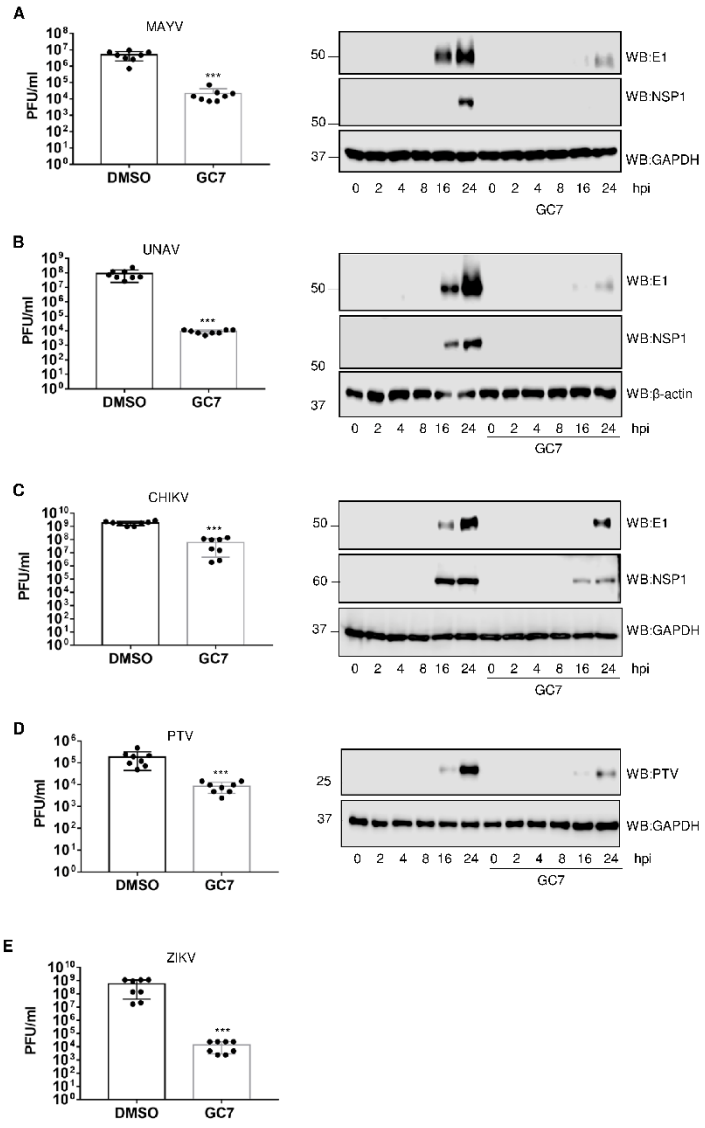


Figure 13. Inhibition of eIF5A hypusination reduces Alphavirus, Flavivirus and Phlebovirus replication. Cells treated with GC7 or DMSO were infected with the indicated viruses. Cells were recovered at the indicated times after infection and proteins were analyzed by Western blot. In addition, cell supernatants were recovered at 24 h after infection and titers

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were quantified by plaque assay. Titers (A, left panel) and NSP1 or E1 viral protein synthesis (A, right panel) of Mayaro virus (MAYV). (Titers (B, left panel) and NSP1 or E1 viral protein synthesis (B, right panel) of Una virus (UNAV). Titers (C, left panel) and NSP1 or E1 viral protein synthesis (C, right panel) of chikungunya virus (CHIKV). Titers (D, left panel) and PTV viral protein synthesis (D, right panel) of Punta toro virus (PTV). E, Titers of zika virus (ZIKV). Bar graphs represent the mean and error bars of eight biological replicates (the experiment was repeated twice and in each experiment, 4 independent samples per treatment type were measured). Statistical analysis was assessed by a Student's t-test. *,$p<0.05$, **,$p<0.01$, ***,$p<0.005$.

6.1.7 Inhibition of eIF5A activates transcription of both type I Interferon (IFN) and type I IFN-stimulated genes

The results shown here, as well as results previously published by other groups, indicated that inhibition of eIF5A modulates the replication of many different RNA viruses. Therefore, we speculated that inhibition of eIF5A may induce the activation of a broad-spectrum antiviral pathway. The type I IFN pathway is central to combating the replication of a broad range of viruses. In addition, it has been previously reported that inhibition of eIF5A induces the upregulation of markers of ER stress (Mandal et al., 2016; Robbins et al., 2010), a process that can lead to the upregulation of different pro-inflammatory cytokines, including IFN through NF-κB signaling pathway (Smith et al., 2008; Sprooten and Garg, 2020). We then wondered whether inhibition of eIF5A induced the IFN pathway. To test this hypothesis, we first evaluated the potential induction of ER stress by GC7. For that, we treated A549 cells with GC7 and at the indicated points protein extracts were collected and analyzed by Western blot with anti-P-PERK or anti-phosphorylated eIF2α antibodies. We observed an increase in the levels of phosphorylated PERK in a time-dependent manner (Figure 14A). In addition, we also detected an increase in the levels of phosphorylated eIF2α after treatment with GC7 in a time (Figure 14B) and a dose-dependent manner (Figure 14C). We then evaluated the poly I:C induced phosphorylated eIF2a levels in cells transfected with siC or siEIF5A. As shown in figure 14D, depletion of eIF5A also upregulated

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the basal and the Poly I:C induced phosphorylated eIF2α levels. Then, we evaluated the potential transcriptional transactivation of type I IFN and IFN-stimulated genes in response to GC7 treatment or after eIF5A depletion using qRT-PCR. As shown in Figure 14E, inhibition of hypusination induced the transcriptional transactivation of type I IFN and IFN-stimulated genes. Moreover, we observed that treatment with the NF-κB inhibitor IKK16 significantly reduced the transcriptional transactivation of IFN-β induced by GC7 (Figure 14F). Similarly, a transcriptional upregulation of IFN and IFN-induced genes was observed upon depletion of eIF5A (Figure 14G). Altogether these results indicate that inhibition of eIF5A induces ER stress and activates transcription of type I Interferon (IFN) and type I IFN-stimulated genes through NF-κB signaling.

6.1.8 Identification of human p85β as a potential target of eIF5A

The activation of eIF5A in an NF-κB dependent manner in response to dsRNA suggests that hypusination can play a role in innate immune responses. It is known that activation of eIF5A is required for the efficient translation of mRNAs encoding proteins with polyproline tracts. There are a plethora of proteins containing polyproline tracts but, so far, only a few of them have been demonstrated to be regulated by eIF5A (Coni et al., 2020; Lubas et al., 2018; Mandal et al., 2014). The PI3K regulatory subunit p85β is among the cellular proteins with a relevant role in IFN signaling (Guiducci et al., 2008; Kaur et al., 2008). Therefore, we decided to test whether p85β may be a target of eIF5A. First, we carried out an *in silico* analysis of the amino acid sequence of human p85β. We observed that this protein contains 3 clusters with 3 consecutive prolines (Figure 15A). Interestingly, the mouse version of this protein does not contain proline-rich domains (Figure 15A).

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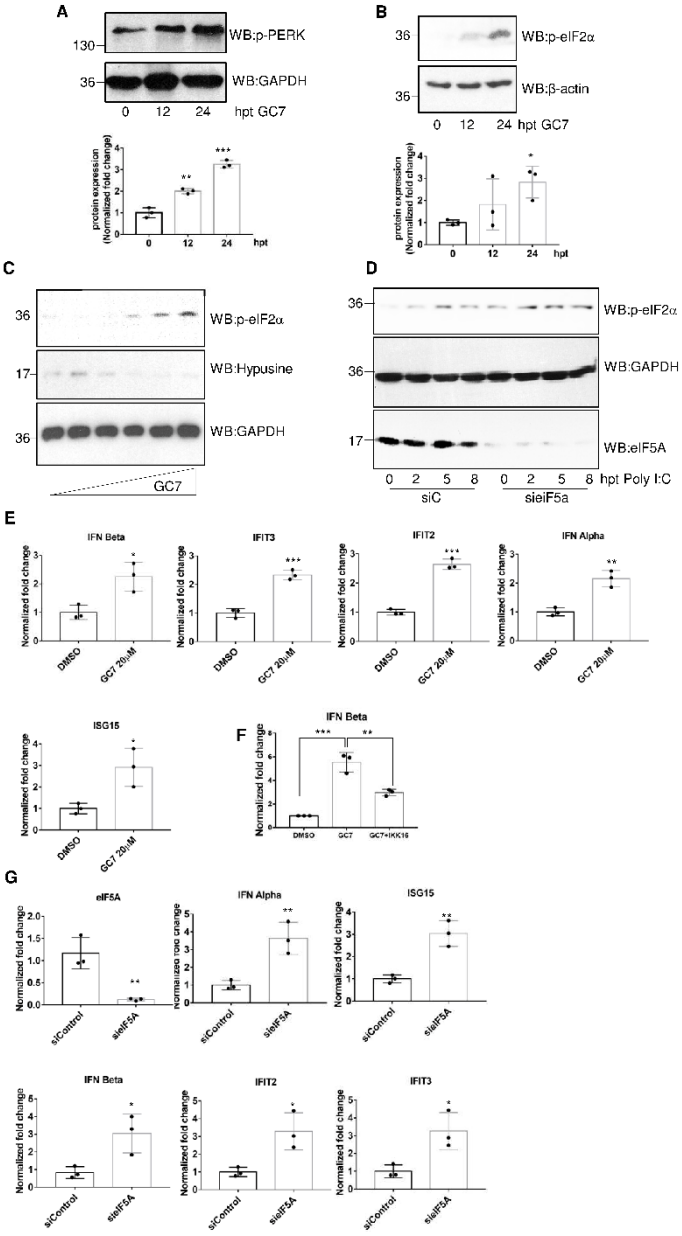

A
130
WB:p-PERK
36
WB:GAPDH
0 12 24 hpt GC7
protein expression (Normalized fold change)
hpt
B
36
WB:p-eIF2α
WB:β-actin
C
17
WB:Hypusine
GC7
D
WB:eIF5A
0 2 5 8 0 2 5 8 hpt Poly I:C
siC
sieIF5a
E
IFN Beta
IFIT3
IFIT2
IFN Alpha
ISG15
Normalized fold change
DMSO
GC7 20μM
F
GC7
GC7+IKK16
G
eIF5A
siControl
sieIF5A

Figure 14. Inhibition of eIF5A induces both ER stress and transcriptional transactivation of IFN and IFN-stimulated genes. A549 cells were treated with GC7 and at different times after treatment protein extracts were analyzed by Western blot with anti-phosphorylated ERK (A) or anti-phosphorylated eIF2α (B) antibodies. C, A549 cells were treated with increasing doses of GC7 and 24 h after treatment protein extracts were analyzed by Western blot with anti-phosphorylated eIF2α antibody. D, A549 cells were transfected with smart-pool small interfering RNAs (siRNAs) against eIF5A1 (sieIF5A) or non-targeting siRNAs (siC). 72 h after transfection, cells were transfected with Poly I:C and at different times after transfection protein extracts were analyzed by Western blot with anti-phosphorylated eIF2α antibody. Total RNA purified from A549 cells treated with GC7 for 24 h (E) or transfected with siC or sieIF5A for 72 h (G) were analyzed by qRT-PCR. and represented as fold induction over untreated cells. F, Total A549 cells were treated with IKK16 for 16 h and then treated with GC7 or DMSO for 24 h. Total RNA was purified and was quantified by real-time RT-PCR and represented as fold induction over untreated cells. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *,$p<0.05$, **,$p<0.01$, ***,$p<0.005$.

To test whether eIF5a modulates the levels of p85β, we carried out a Western blot analysis of the human A549 or SW480 cells treated with GC7 or DMSO using anti-hypusine antibody. We observed a clear downmodulation in p85β protein levels after GC7 treatment (Figure 15B). We then analyzed the transcription of p85β in A549 cells treated with GC7 or DMSO by qRT-PCR. We did not observe significant different differences in the levels of the mRNA for p85β (Figure 15C). Altogether these results suggest that p85β can be a target of eIF5A. We then decided to study whether mouse p85β protein levels are also modulated by eIF5A. HEK-293 cells were transfected with a plasmid encoding HA-tagged mouse p85β and 24h after transfection cells were treated with DMSO or GC7, and 24 h after treatment we analyzed the protein extracts by Western blot with anti-HA antibody. We did not observe differences in the levels of HA-p85β protein between DMSO and GC7 treated cells (Figure 15D). Additional experiments are required in order to confirm the regulation of human p85β by eIF5A in the context of virus infection.

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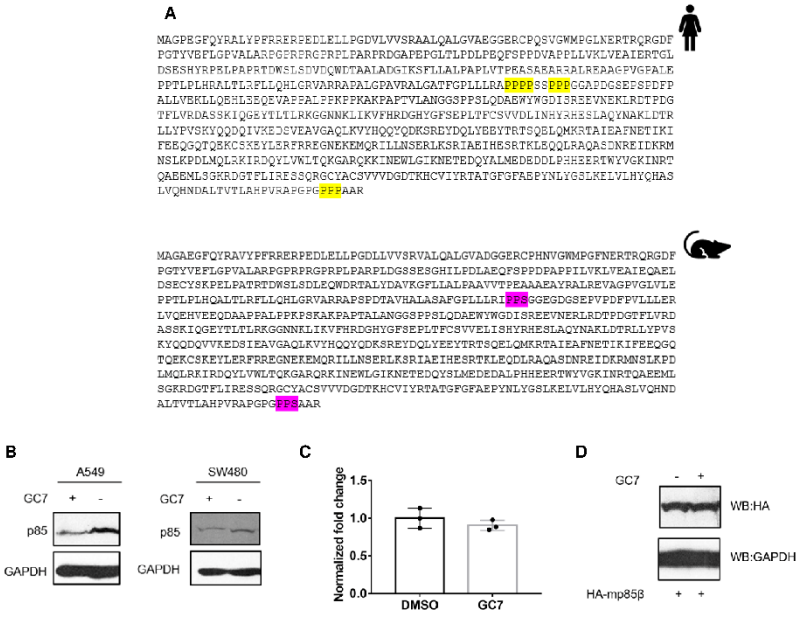


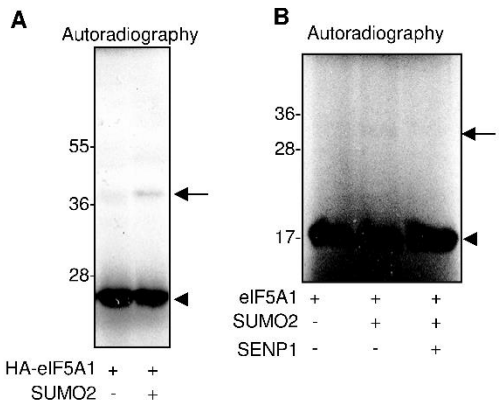
Figure 15. Human p85β is a potential target of eIF5A. A, *In silico* analysis of the amino acid sequence of human and mouse p85β, B, Human A549 or SW480 cells were treated with DMSO or GC7 and 24 h after treatment protein extracts were analyzed by Western blot with anti-p85β antibody. C, A549 cells were treated with DMSO or GC7 and 24 h after treatment mRNA levels of p85β were analyzed by qRT-PCR. D, HEK-293 cells were transfected with a plasmid encoding HA-tagged mouse p85b and 24 h after transfection cells were treated with DMSO or GC7. 24 h after treatment protein extracts were analyzed by Western blot with anti-HA antibody.

6.1.9 eIF5A1 is modified by SUMO2 *in vitro*

The expression of eIF5A has been found altered in different pathological conditions (Chen et al., 2003, 2009; Fujimura et al., 2014; Guan et al., 2004; He et al., 2011; Luo et al., 2009; Meng et al., 2015; Xie et al., 2008; Yang et al., 2009), and a correlation between the protein and the mRNA expression levels has not been always found. Therefore, it has been proposed that eIF5A can be regulated by additional mechanisms. We hypothesized that eIF5A may be post-

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translationally regulated by ubiquitin-like proteins. Firstly, we tested whether eIF5A1 can be modified by SUMO *in vitro*. For that, we used ^{35}S-methionine labeled *in vitro* translated HA-eIF5A1. The protein was incubated in a SUMOylation reaction with recombinant SUMO2 protein, the E1 SUMO-activating enzyme 1 (Sae1), and the E2 Ubc9. 3 h later, the reaction was stopped, the products were run in an SDS-PAGE and the dried gel was then exposed to a X-ray film. We observed a band of around 20 kDa molecular weight corresponding to unmodified HA-eIF5A1, and the appearance of a band of the expected eIF5A1-SUMO2 molecular weight only when SUMO2 was added to the reaction, indicating that eIF5A1 can be modified by SUMO2 *in vitro* (Figure 16A). To further demonstrate that the higher molecular weight band correspond with SUMOylated eIF5A1, we carried out a deSUMOylation assay. eIF5A1 subjected to an *in vitro* SUMOylation assay was incubated with SUMO specific peptidase 1 (SENP1), an enzyme that catalyzes the de-conjugation of SUMO to the target proteins, and the samples were then processed as described above. As shown in Figure 16B, the band with the expected eIF5A1-SUMO2 molecular weight disappeared after incubation with SENP1, confirming that eIF5A1 can be modified by SUMO2 *in vitro*.



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Figure 16. *In vitro* SUMOylation of eIF5A1. A, *In vitro* SUMOylation assay with SUMO2 using ^{35}S-methionine labeled *in vitro* translated HA-eIF5A1 protein as a substrate. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates the SUMO2-conjugated eIF5A1 protein. B, *In vitro* deSUMOylation assay using recombinant SENP1. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates the SUMO2-conjugated eIF5A1 protein.

6.1.10 eIF5A1 is modified by SUMO2 *in vivo*

To determine whether eIF5A1 can be modified by SUMO2 in cells, we transfected HEK-293T cells with HA-eIF5A1 together with pcDNA or pCDNA-His6-SUMO2 and pCDNA-Ubc9-V5. 36 h after transfection, cells were collected, lysed under denaturing conditions, and subjected to histidine-purification assays. Analysis of the purified histidine-tagged proteins by Western blot with anti-HA antibody showed a band corresponding with the unmodified HA-eIF5A1 protein in both lanes. In addition, we observed a strong band of around 40 kDa as well as a fainter 60 kDa molecular weight band in the cells co-transfected with the SUMO machinery, indicating that eIF5A1 is modified by SUMO2 in cells (Figure 17A). Similar results were observed when the experiment was carried out using Flag-eIF5A1 plasmid (Figure 17B). We then studied the SUMOylation of eIF5A1 in endogenous conditions. SUMOylation is a reversible and very dynamic process, and the levels of modified substrate at a given time is limited. Therefore, we used SUMO-traps (SUBES) to capture endogenous SUMOylated proteins. HEK-293T cells were lysed in the presence of ubiquitin-like isopeptidases inhibitors. Lysates were clarified by centrifugation and the supernatants were incubated with GST-agarose beads or GST-SUBES-agarose beads for 2 h at 4º C. Then, the beads were washed and the bound proteins were eluted using Laemmli buffer. Samples were then analyzed by Western blot with anti-eIF5A1 or anti-SUMO2 antibodies. Western blot analysis using anti-eIF5A1 antibody revealed the presence of two bands of around 35 and 55 kDa only in the sample incubated with SUBES (Figure 17C). Altogether these results demonstrate that eIF5A1 is modified by SUMO2 in cells.

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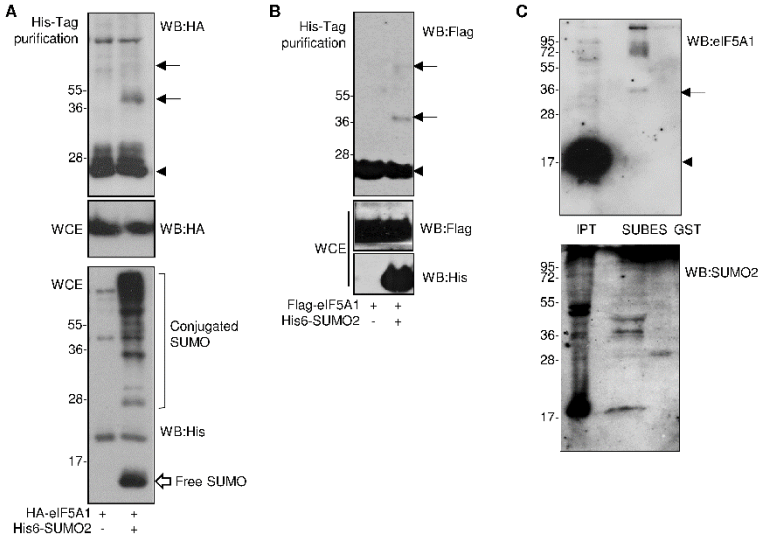


Figure 17. SUMO2 modification of eIF5A1 in cells. A, HEK-293T cells were co-transfected with a plasmid encoding HA- eIF5A1 together with pcDNA, Ubc9 and His6-SUMO2 and 36 h after transfection whole protein extracts or histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrows indicate SUMO2-conjugated eIF5A1 protein. B, HEK-293T cells were co-transfected with a plasmid encoding Flag-eIF5A1 together with pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection whole protein extracts or histidine-tagged purified proteins were analyzed by Western blot with anti-Flag antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrows indicate SUMO2-conjugated eIF5A1 protein. C, Analysis of the endogenous SUMOylation of eIF5A1. SUMOylated proteins in HEK-293 cells were captured using SUBES and analyzed by Western blot using anti-eIF5A1 antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrows indicate SUMO2-conjugated eIF5A1 protein.

6.1.11 eIF5A2 is modified by SUMO2

eIF5A1 and eIF5A2 share large sequence homology, and most of the lysine residues are conserved between the two isoforms (Figure 18A). Therefore, we speculated that eIF5A2 may be also modulated by SUMO2. To test this hypothesis, we carried out an *in vitro* and *in vivo* SUMOylation assays using 35S-methionine labeled *in vitro* translated Flag-eIF5A2 protein and Flag-eIFA2 expression plasmid, respectively.

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We observed that eIF5A2 was modified by SUMO2 *in vitro* (Figure 18B) and *in vivo* (Figure 18C). ^{35}S-methionine labeled *in vitro* translated Flag-eIF5A2 protein was incubated in a SUMOylation reaction with recombinant SUMO2 protein, the E1 SUMO-activating enzyme 1 (Sae1), and the E2 Ubc9. 3 h later, the reaction was stopped, the products were run in an SDS-PAGE and the dried gel was then exposed to a X-ray film. We observed a band of around 20 kDa molecular weight corresponding to unmodified Flag-eIF5A2, and the appearance of a band of the expected eIF5A1-SUMO2 molecular weight only when SUMO2 was added to the reaction, indicating that eIF5A1 can be modified by SUMO2 *in vitro* (Figure 18B). To test the result in mammalian cells, HEK-293T were transfected cells with Flag-eIF5A2 together with pcDNA or pCDNA-His6-SUMO2 and pCDNA-Ubc9-V5. 36 h after transfection, cells were collected, lysed under denaturing conditions, and subjected to histidine-purification assays. Analysis of the purified histidine-tagged proteins by Western blot with anti-Flag antibody shows a band corresponding with eIF5A2-SUMO2 (Figure 18C).

6.1.12 eIF5A1 is modified by SUMO2 in a hypusine-independent manner

eIF5A1 protein is modified by hypusine at lysine K50 readily after translation. Therefore, we decided to analyze whether lysine K50 is required for eIF5A1 SUMOylation. We first analyzed whether a mutant of eIF5A1 in lysine 50 (eIF5A-K50R) was still SUMOylated. We co-transfected HEK-293T cells with HA-eIF5A1-K50R together with pcDNA or pCDNA-His6-SUMO2 and pCDNA-Ubc9-V5. 36 h after transfection, cells were collected, lysed under denaturing conditions and subjected to histidine-purification assays.

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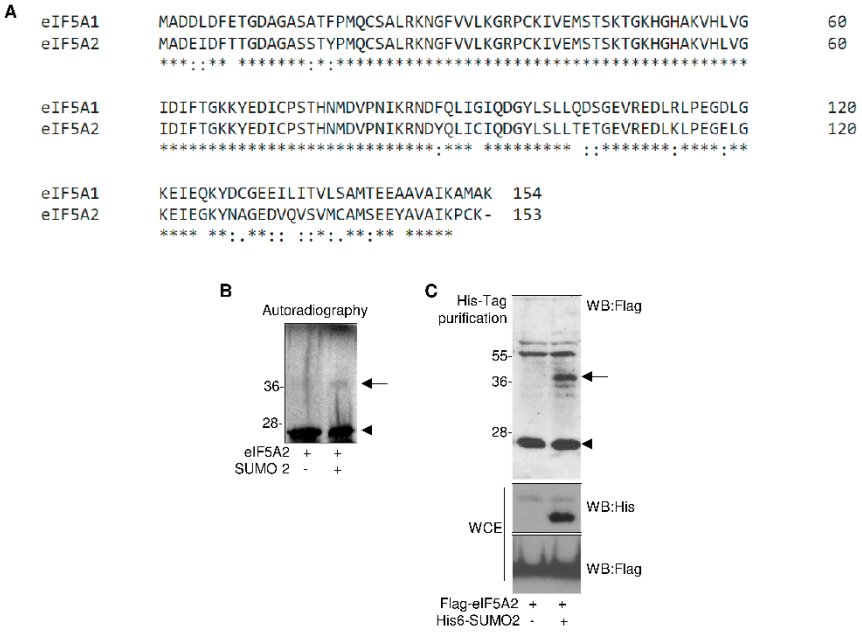


Figure 18. eIF5A2 is modified by SUMO2. A, Alignment of the amino acid sequences of the two isoforms of eIF5A using CLUSTAL O(1.2.4). B, *In vitro* SUMOylation assay with SUMO2 using ^{35}S-methionine labeled *in vitro* translated eIF5A2 protein. Arrowhead indicates unconjugated eIF5A2 protein. Arrow indicates SUMO2-conjugated eIF5A2 protein. B, HEK-293T cells were transfected with a plasmid encoding Flag-eIF5A2 together with pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-Flag antibody. Arrowhead indicates unconjugated eIF5A2 protein. Arrow indicates SUMO2-conjugated eIF5A2 protein.

Analysis of the purified proteins by Western blot with anti-HA antibody revealed a band of around 40 kDa molecular weight corresponding with the SUMO2 modified eIF5A1-K50R protein only in the cells co-transfected with the SUMOylation machinery (Figure 19A), indicating that the eIF5A-K50R hypusination mutant is modified by SUMO2 in cells. We then analyzed the potential SUMO2 modification of eIF5A1-K50R *in vitro*. 35S-methionine labeled *in vitro* translated eIF5A1-WT or eIF5A-K50R proteins were subjected to an *in vitro* SUMOylation

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assay in the presence of SUMO2. The resulting products were analyzed by SDS-PAGE and the dried gel was exposed to a X-ray film. Incubation with the SUMOylation machinery led to the appearance of a 40 kDa molecular weight band corresponding with SUMO2 modified eIF5A1 or eIF5A1-K50R protein (Figure 19B). Altogether these results indicate that eIF5A1 is SUMOylated in a hypusination-independent manner.

6.1.13 SUMO2 can be conjugated to different lysine residues in eIF5A1

To understand the relevance of SUMO conjugation to a specific target protein, a straightforward approach is to identify the lysine residue(s) involved in SUMO conjugation and then to analyze the properties of the SUMOylation mutant in comparison with the wild type protein. Proteomic data previously published pointed to lysine residues K27, K34, K39, K67, K85, and K126 as probably SUMOylation sites in eIF5A1 (Hendriks and Vertegaal, 2016). In addition, using GPS-SUMO software (Zhao et al., 2014), we identified three 3 highly scored potential SUMOylation sites (K126, K68, and K85). Next, we generated mutants of eIF5A1 in the lysine residues with higher probability to be SUMOylation sites, and then we evaluated their SUMOylation in cells.

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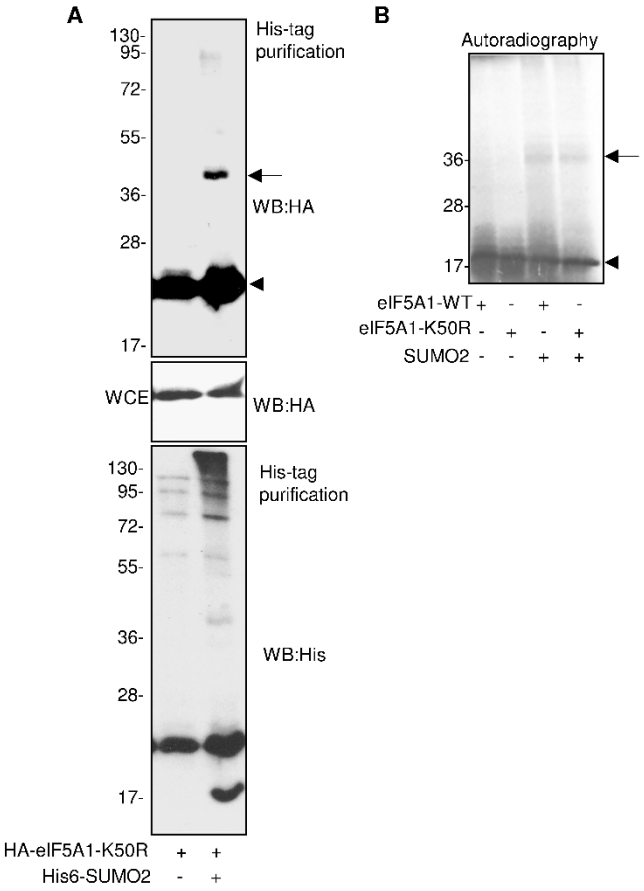


Figure 19. SUMOylation of the non hypusinable mutant of eIF5A1, eIF5A1-K50R. A, HEK-293T cells were co-transfected with a plasmid encoding HA-eIF5A1-K50R together with pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates SUMO2-conjugated eIF5A1 protein. B, *In vitro* SUMOylation assay with SUMO2 using 35S-methionine labeled *in vitro* translated eIF5A1-K50R or eIF5A-WT protein as a substrate. Arrowhead indicates unconjugated eIF5A1-K50R or eIF5A1-WT protein. Arrow indicates SUMO2-conjugated eIF5A1 or eIF5A-K50R protein.

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SUMOylation of the single mutants was slightly reduced relative to the WT protein (Figure 20A). We then generated triple (eIF5A1-K68AK85AK126A (eIF5A1-3KA) or quintuple eIF5A-K68AK85AK126AK27AK34A (eIF5A1-5KA) mutants and we evaluated their SUMOylation *in vitro*. We observed a reduction in the SUMOylated band in the 3KA mutant that is even more accentuated in the 5KA mutant by *in vitro* SUMOylation assays (Figure 20B). We then decided to evaluate their SUMOylation *in vivo*. However, Western blot analysis revealed that the mutant proteins were almost undetectable unless we inhibited the proteasome (Figure 20C). Therefore, we carried out *in vivo* SUMOylation assays with eIF5A-3KA and eIF5A-5KA in the presence of the proteasome inhibitor MG132. HEK-293T co-transfected with HA-eIF5A1-WT, HA-eIF5A1-3KA or HA-eIF5A1-5KA, and pcDNA or pcDNA-Ubc9-V5 and His6-SUMO2, and treated with MG132 were lysed under denaturing conditions and histidine-tagged purified proteins were analyzed by Western blot using HA antibody. SUMOylation of eIF5A-3KA was reduced relative to the WT protein (Figure 20C), and an even more pronounced reduction in SUMOylation was observed in eIF5A1-5KA mutant. These results suggested that SUMO can conjugate to different lysine residues in eIF5A1 and pointed to K27, K34, K68, K84, and K126 as potential SUMO acceptor sites in eIF5A1.

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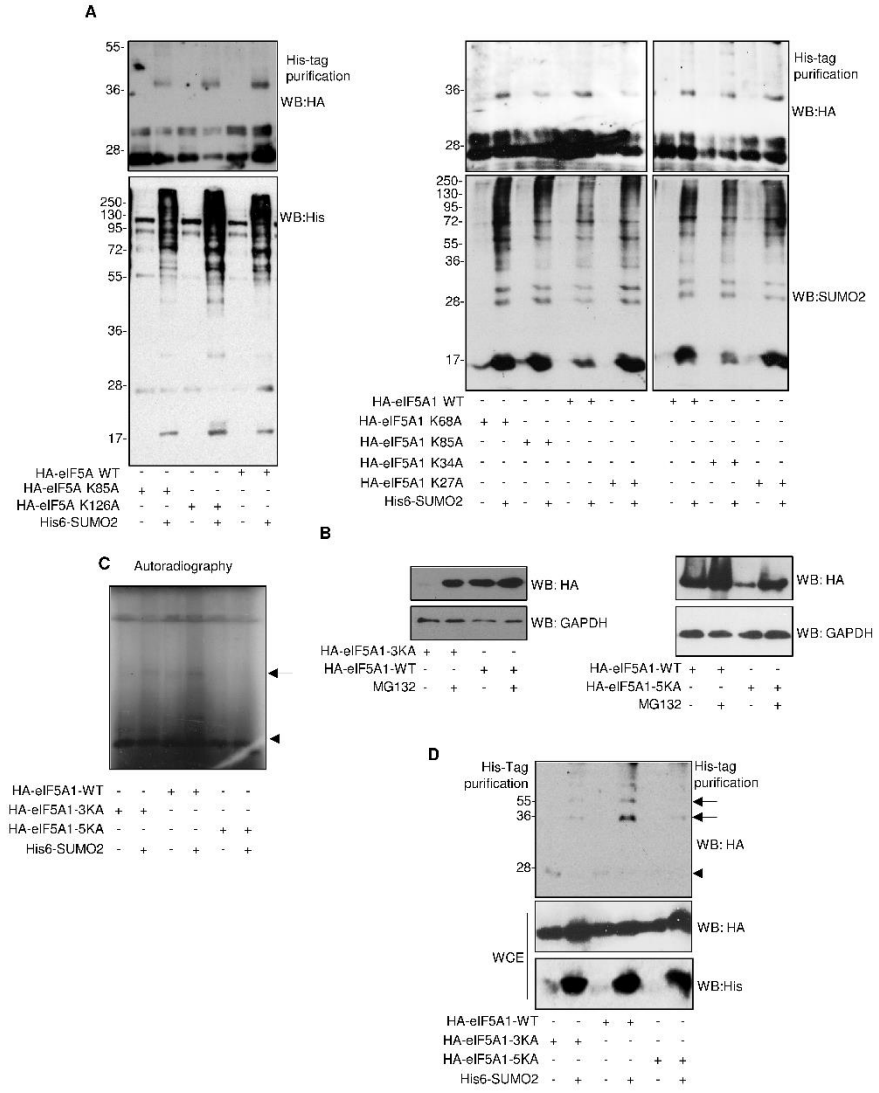


Figure 20. Identification of SUMO conjugation sites in eIF5A1. A, HEK-293T cells were transfected with a plasmid encoding HA-tagged eIF5A1-WT or the indicated mutants of eIF5A1 and 36 h after transfection whole protein extracts or histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. B, HEK-293T were transfected with HA-eIF5A-WT,HA-eIF5A1-3KA or HA-eIF5A1-5KA. 36 h after transfection, cells were treated with MG132 and 12 h after treatment protein extracts were collected and analyzed by

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Western blot using anti-HA antibody. C, *In vitro* SUMOylation assay with SUMO2 using ^{35}S-methionine labeled *in vitro* translated HA-eIF5A1, HA-eIF5A1-3KA or HA-eIF5A1-5KA proteins as a substrate. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates SUMO2-conjugated eIF5A1 protein. C, HEK-293T cells were co-transfected with a plasmid encoding HA-tagged eIF5A1-WT, eIF5A1-3KA or eIF5A1-5KA together with pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection cells were treated with MG132 for 16 h. Then, whole protein extracts or histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates SUMO2-conjugated eIF5A1 protein.

6.1.14 Mutation of the SUMOylation sites in eIF5A1 reduces its stability

As mentioned above, the levels of the eIF5A1-3KA and eIF5A1-5KA mutants were clearly lower than those of the wild type, suggesting that mutation of the SUMOylation sites in eIF5A decreases its stability. To evaluate this hypothesis, we carried out stability assays. We transfected HEK-293T with HA-eIF5A1-WT , or the different mutants of eIF5A, and 36 h after transfection, cells were treated with cycloheximide. At different times after treatment, cells were collected and protein extracts were analyzed by Western blot with anti-HA antibody. As shown in Figure 21A, the individual mutants of eIF5A1 are less stable than the wild type protein. Similarly, the stability of eIF5A1-3KA or eIF5A1-5KA was significantly reduced compared with the stability of the WT protein (Figure 21B). These results suggested that the conjugation of SUMO to eIF5A1 contributes to its stability. To determine whether the eIF5A1-3KA or eIF5A1-5KA mutants were degraded by the proteasome, we carried out the stability assays in the presence of the proteasome inhibitor MG132. Western blot analysis showed that the stability of both mutants increased in the presence of MG132, indicating that the mutation of the SUMOylation sites in eIF5A1 promoted its proteasome-dependent degradation (Figure 21C).

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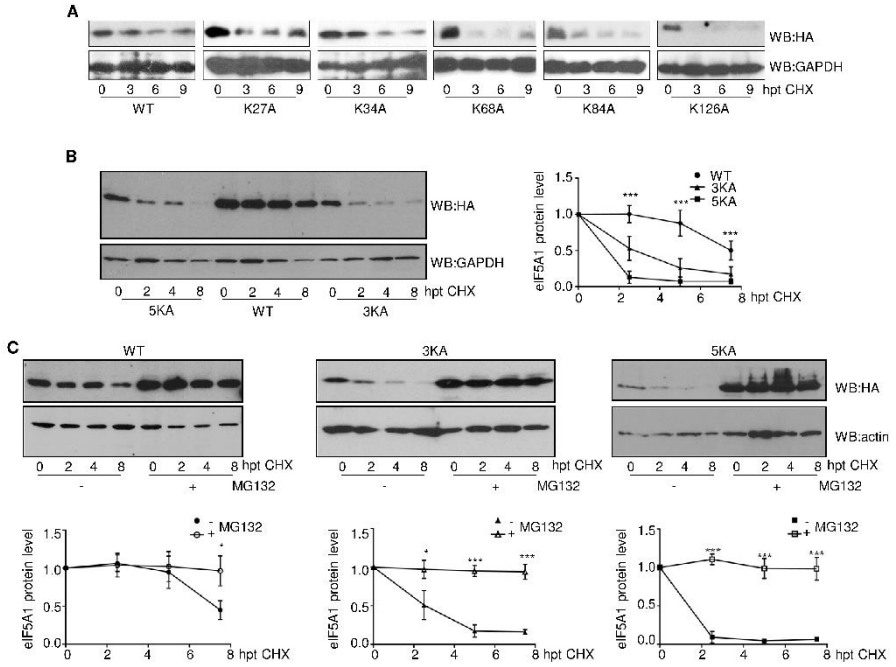


Figure 21. SUMOylation of eIF5A1 modulates its stability. A, HEK-293T cells were transfected with HA-eIF5A1-WT or the indicated mutants of eIF5A1, and 24 h after transfection cells were treated with cycloheximide (CHX). At the indicated h after CHX treatment, protein extracts were analyzed by Western blot with anti-HA antibody. B, HEK-293T cells were transfected with HA-eIF5A1-WT , HA-eIF5A1-3KA, or HA-eIF5A1-5KA, and 24 h after transfection cells were treated with CHX. At the indicated h after CHX treatment, protein extracts were analyzed by Western blot with anti-HA antibody (left panel). eIF5A1 protein intensity bands were quantified using ImageJ software. eIF5A bands intensity was normalized to GAPDH from each respective time and plotted (right panel). Data represent the mean and error bars of 3 biological replicates. Statistical analysis was assessed by a Student's t-test. C, HEK-293T cells were transfected and treated as indicated in B but in presence or absence of MG132 treatment. At the indicated times after CHX treatment protein extracts were analyzed by Western blot with the indicated antibodies (upper panels). Stability of the protein in presence or absence of MG132 from three independent experiments is shown (lower panels). Statistical analysis was assessed by a Student's t-test.

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6.1.15 Mutation of the SUMOylation sites in eIF5A1 modulates its subcellular localization

To decipher if SUMO can modulate the subcellular localization of eIF5A1, we studied the subcellular localization of the eIF5A1-3KA and eIF5A1-5KA mutants in comparison with the WT protein. For that, we transfected A549 cells with HA-eIF5A1-WT, HA-eIF5A1-3KA or HA-eIF5A1-5KA. At 24 h after transfection, cells were fixed and analyzed by immunofluorescence assay with anti-HA antibody. Confocal analysis revealed that the wild type protein localized both at the nucleus and cytoplasm of the transfected cells (Figure 22A). However, we observed that both the eIF5A1-3KA and eIF5A1-5KA mutants displayed a more nuclear localization (Figure 22A), indicating that mutation of the SUMOylation sites in eIF5A1 modulates its subcellular localization. To confirm this result, we transfected HEK-293T cells with HA-eIF5A1-WT, HA-eIF5A1-3KA or HA-eIF5A1-5KA, and carried out a subcellular fractionation assay. We detected a more nuclear localization of the eIF5A1-3KA and eIF5A1-5KA mutants than the wild type protein (Figure 22B). Finally, to confirm that SUMOylation modulates the subcellular localization of eIF5A1, we carried out a subcellular fractionation assay of untransfected HEK-293T cells treated with DMSO or the SUMOylation inhibitor ML752. eF5A1 protein displayed a more nuclear localization in cells treated with the SUMOylation inhibitor than in DMSO treated cells (Figure 12C). Altogether these results indicated that SUMO modulates the subcellular localization of eIF5A1.

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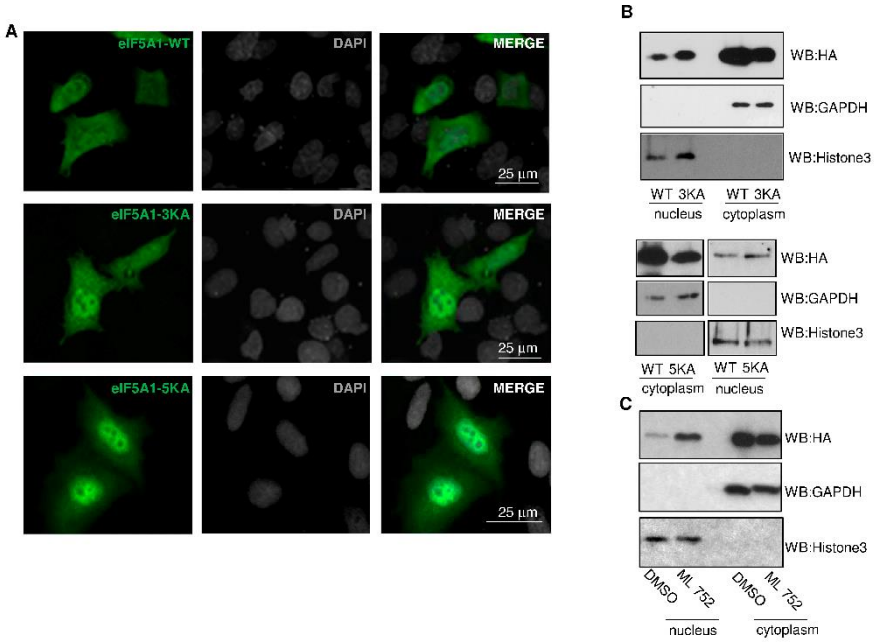


Figure 22. SUMOylation of eIF5A1 modulates its subcellular localization. A, A549 cells were transfected with HA-eIF5A1-WT, HA-eIF5A1-3KA, or HA-eIF5A1-5KA and 36 h after transfection we evaluated the subcellular localization of eIF5A using immunofluorescence assays with anti-HA antibody. B, HEK-293T cells were transfected with HA-eIF5A1-WT, HA-eIF5A1-3KA or HA-eIF5A1-5KA, and 36 h after transfection, cells were subjected to subcellular fractionation assays. Then, the nuclear and cytoplasmic protein extracts were analyzed by Western blot with anti-HA antibody. C, HEK-293T cells were treated with the SUMOylation inhibitor ML752 or DMSO. 24 h after treatment, cells were subjected to subcellular fractionation assays and then the levels of eIF5A in the nucleus or cytoplasm were analyzed by Western blot with anti-eIF5A1 antibody.

6.1.16 Modulation of eIF5A1 SUMOylation by stress

As shown in Figure 20D, treatment with the proteasome inhibitor MG321 led to the appearance of multiple eIF5A1-SUMO2 bands, suggesting that the inhibition of the proteasome may induce eIF5A1 SUMOylation. To test this hypothesis, we first analyzed the SUMOylation of eIF5A1-WT protein in cells treated or untreated with MG132. HEK-293T cells were co-transfected with HA-eIF5A1 and

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pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection cells were treated with DMSO or MG132 for 4 h. Then, cells were lysed and histidine-tagged proteins were purified under denaturing conditions. Western blot analysis of the purified proteins with antibody against HA revealed the appearance of a band or around 40 kDa, corresponding with eIF5A1-SUMO2 protein in the cells co-transfected with the SUMOylation machinery and treated with DMSO (Figure 23A). Higher levels of the 40 kDa protein and additional higher molecular weight bands corresponding with eIF5A-SUMO2 protein were observed in those cells treated with MG132 (Figure 23A). It has been previously reported that inhibition of the proteasome induces the accumulation of SUMO2/3 conjugates *in vivo* (Schimmel et al., 2008; Uzunova et al., 2007) and part of these conjugates can represent ubiquitin associated SUMO2/3 conjugates. Therefore, we speculated that MG132 may induce the formation of heterologous SUMO2/3-ubiquitin chains. To explore this hypothesis, we then generated a mutant of SUMO2 in all the lysine residues (SUMO2-K0) and then we evaluated the SUMOylation of eIF5A1 in cells transfected with HA-eIF5A1 and pcDNA or Ubc9 and His6-SUMO2-K0 and treated or not with MG132. Western blot analysis of the histidine-tagged proteins purified under denaturing conditions using anti-HA antibody revealed the appearance of two bands of around 40 kDa and 80 kDa molecular weight in the untreated cells co-transfected with the SUMOylation machinery (Figure 23B). A clear increase in the levels of the 40 kDa band as well additional bands were detected in those cells treated with MG132 (Figure 23B). Altogether these results indicated that inhibition of proteasome promoted the SUMOylation of eIF5A1 independently of the formation of SUMO2 chains or of hybrid chains composed of SUMO2 and ubiquitin.

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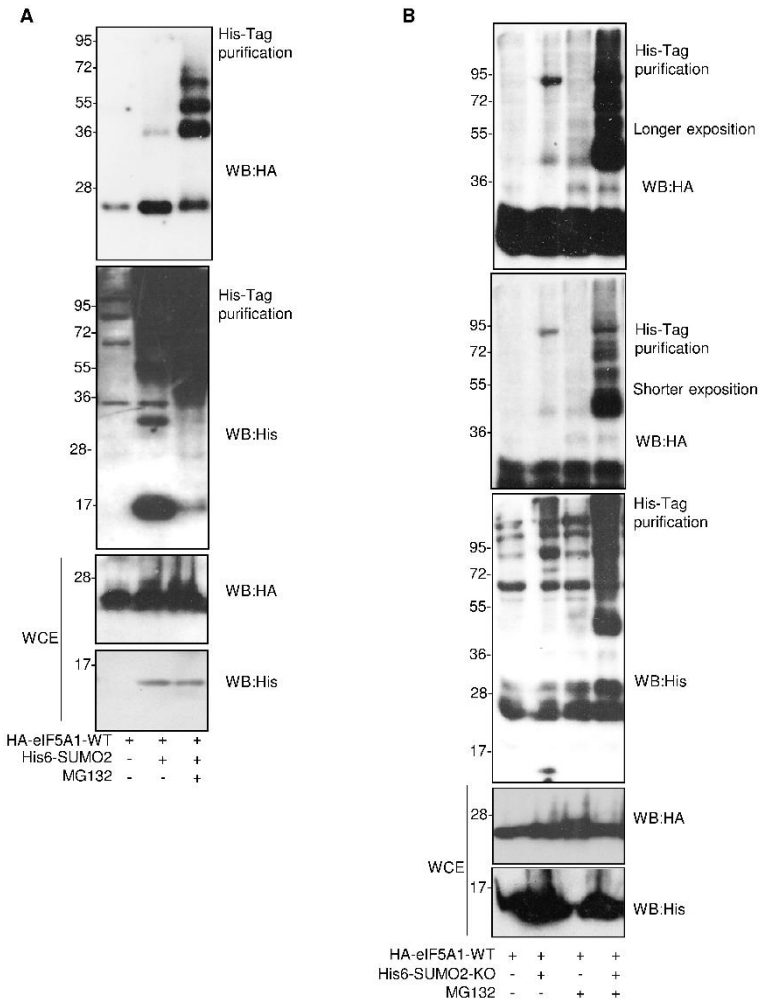


Figure 23. Proteasome inhibition induces eIF5A1 SUMOylation. A, HEK-293T cells were transfected with a plasmid encoding HA-eIF5A1 together with pcDNA orUbc9 and His6-SUMO2, and 36 h after transfection cells were treated with MG132 for 4 hours. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. B, HEK-293T cells were transfected with a plasmid encoding HA- eIF5A1 together with pcDNA, Ubc9 and His6-SUMO2 or Ubc9 and His6-SUMO2-K0 and 36 h after

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transfection cells were treated with MG132 for 4 hours. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody.

The regulation of protein translation allows cellular adaptation during stress conditions by adjusting the proteome without the necessity of changes in RNA synthesis. Although eIF5A has been reported to only marginally affect protein synthesis under normal conditions, it is essential for the onset of stress-induced translational repression. In addition, SUMOylation status of different substrate proteins has been reported to play a crucial role in cellular responses to stress. We then decided to study whether the SUMOylation of eIF5A1 is modulated in response to other types of stress. First, we analyzed the effect of UV light on eIF5A1 SUMOylation. HEK-293T cells were transfected with HA-eIF5A1 and pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection cells were subjected to irradiation of UV light and cells were collected at two times after irradiation. Western blot analysis of the histidine-tagged proteins purified under denaturing conditions using anti-HA antibody revealed that the 40 kDa band corresponding with eIF5A1-SUMO2 protein detected in the untreated cells disappeared after UV light irradiation (Figure 24A). Then, we analyzed the potential modulation of eIF5A1 SUMOylation in response to hypoxic or adriamycin treatment. HEK-293T cells were transfected as described above and at 36 h after transfection cells were subjected to hypoxic conditions or treated with adriamycin. Finally, the cells were recovered and the histidine-tagged proteins were purified under denaturing conditions. Analysis by Western blot of the purified proteins revealed similar levels of eIF5A-SUMO2 protein in untreated cells and in cells subjected to hypoxic stress (Figure 24B). We also observed a reduction in the levels of the eIF5A-SUMO2 band in those cells treated with adriamycin (Figure 24B). Altogether these results indicated that treatment with DNA-damage agents downmodulated eIF5A SUMOylation.

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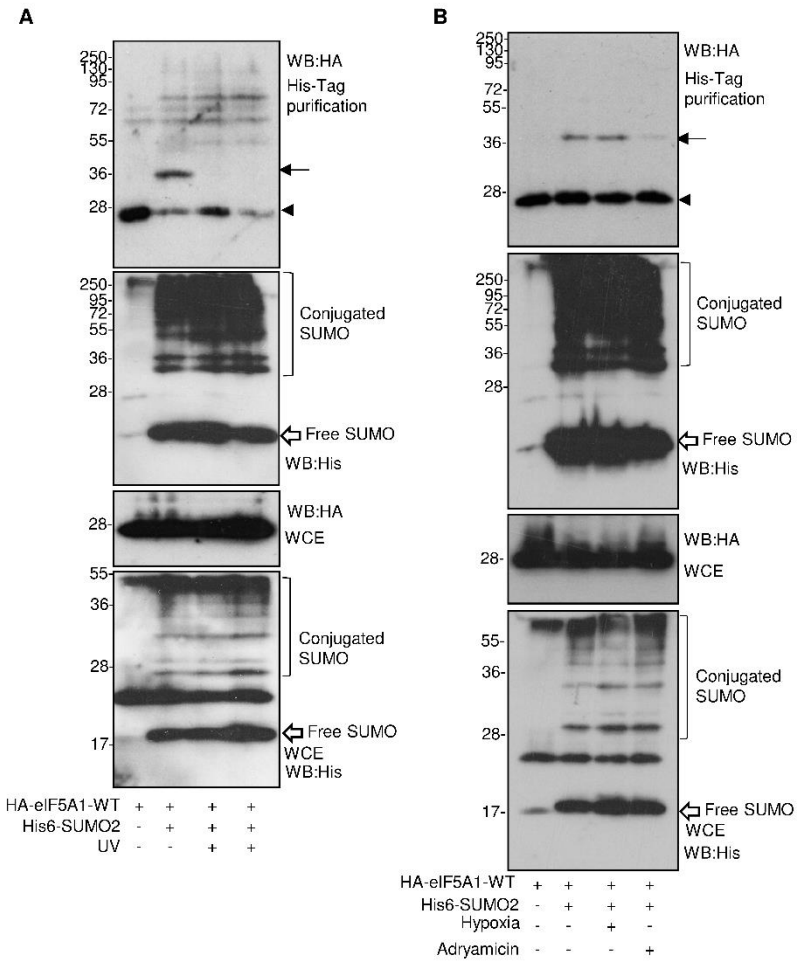


Figure 24. SUMOylation of eIF5A in response to UV light irradiation, adriamycin, or hypoxic conditions. A, HEK-293T cells were transfected with a plasmid encoding HA-eIF5A1 together with pcDNA or Ubc9 and His6-SUMO2, and 36 h after transfection cells were subjected to UV light irradiation. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates SUMO2-conjugated eIF5A1. B, HEK-293T cells were transfected with a plasmid encoding HA-eIF5A1 together with pcDNA or Ubc9 and His6-SUMO2, and 36 h after transfection the cells were subjected to hypoxic conditions or treated with adriamycin. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates SUMO2-conjugated eIF5A1.

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Next, we decided to evaluate whether virus infection had an impact on eIF5A SUMOylation. For that, we carried out similar experiments to the above described, but instead of treating the cells with cytotoxic agents, we infected the cells with VSV (Figure 25A) or IAV (Figure 25B) for 8 h. Western blot analysis with anti-HA antibody of the histidine-tagged purified proteins showed an increase in the levels of the eIF5A-SUMO2 protein in the infected cells, indicating that virus infection induces eIF5A SUMOylation.

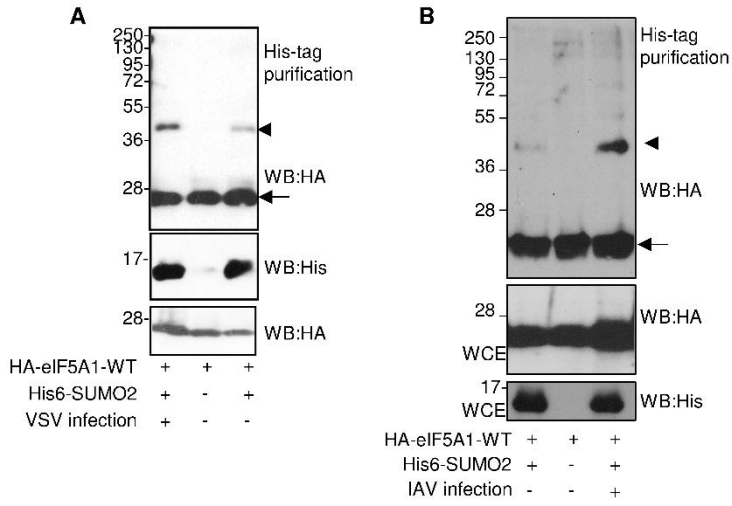


Figure 25. Modulation of eIF5A1 SUMOylation by viral infection. HEK-293T cells were were co-transfected with a plasmid encoding HA-eIF5A1 together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, cells were infected with VSV (A) or IAV (B) for 8 h. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates SUMO2-conjugated eIF5A1.

Finally, we evaluated the potential modulation of eIF5A1 SUMOylation in response to heat shock. Cells were transfected as described above and 48 h after transfection, cells were subjected or not to heat shock stress. Whole cell extracts and histidine-tagged purified proteins were then analyzed by Western blot with anti-HA antibody.

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We observed a clear increase in the intensity of the 40 kDa SUMOylation band in addition to the appearance of additional SUMOylation bands in those cells subjected to heat shock stress (Figure 26A), indicating that heat stress induces the SUMOylation of eIF5A1. It has been reported that eIF5A is required for stress granules assembly in cells subjected to adverse environmental conditions and for the rapid onset of stress-induced translational repression. We then evaluated the dynamics of eIF5A-SUMO2 conjugation after heat shock. Cells were transfected as above described and at 36 h after transfection, we subjected the cells to heat shock. Cells were processed immediately after heat shock or they were incubated at 37º C for 30 min or 2 h. Then, we collected the cells and analyzed them as above indicated. Analysis by Western blot of the histidine-tagged purified proteins using anti-HA antibody showed increased levels of eIF5A-SUMO2 protein immediately and 30 minutes post-heat shock, and a complete return to basal SUMOylation levels was observed at 2 h of recovery at 37º C (Figure 26B). Altogether these results indicated that eIF5A SUMOylation is modulated in response to different types of stress. In agreement with our results, different proteomic data previously reported have identified eIF5A as a SUMO target which SUMOylation increases following proteasome inhibition or heat shock stress (Golebiowski et al., 2009; Hendriks et al., 2014). These results also suggested that SUMOylation sites in eIF5A may be modulated by stress conditions. To test this hypothesis, we evaluated the SUMOylation of the eIF5A-3KA mutant in cells subjected to heat shock. HEK-293T cells were co-transfected with HA-eIF5A1-3KA and pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection, the cells were incubated at 43º C or left at 37º C for 2 h. Then, cells were collected and the histidine-tagged proteins were purified under denaturing conditions. Western blot analysis of the purified proteins using anti-HA antibody did not reveal a band corresponding to eIF5A-3KR-SUMO2 protein in the untreated cells (Figure 27).

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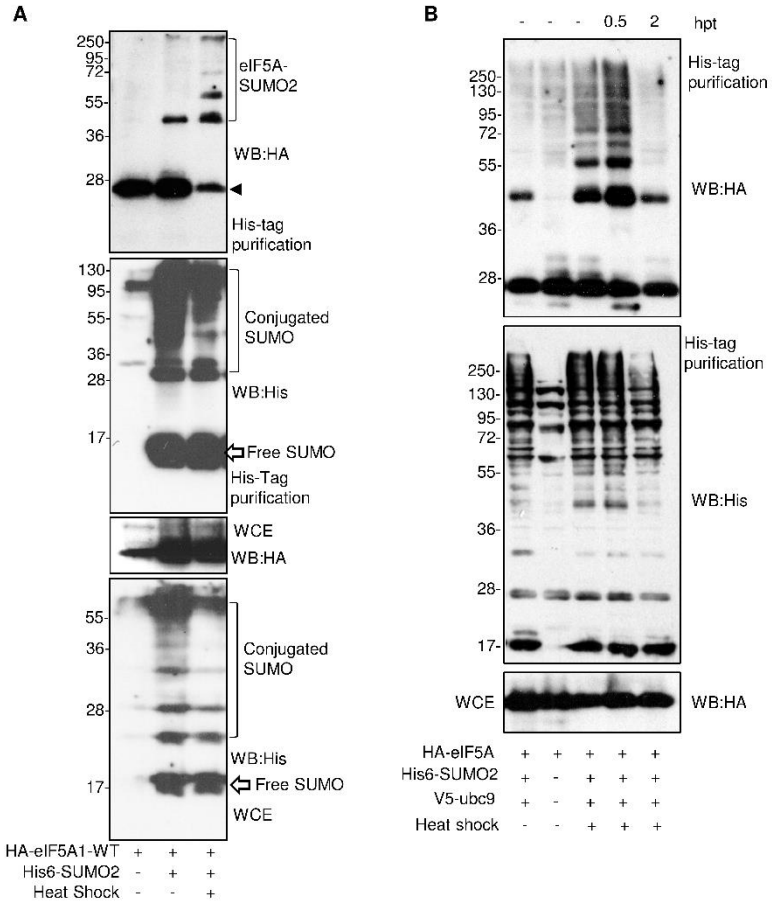


Figure 26. Induction of eIF5A1 SUMOylation by heat shock stress. A, HEK-293T cells were co-transfected with a plasmid encoding HA-eIF5A1 together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, the cells were incubated at 43º C for 2 h. Whole protein extracts and histidine-tagged purified proteins were then analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein.. B, HEK-293T cells were were transfected with a plasmid encoding HA-eIF5A1 together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, the cells were incubated at 43º C for 2 hours. Cells were analyzed immediately or incubated at 37º C for 30 min of 2 h. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein.

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However, we detected a 40 kDa band corresponding to the SUMOylated eIF5A-3KR protein in the cells subjected to heat shock, indicating that the SUMO acceptor sites on eIF5A are regulated by stress.

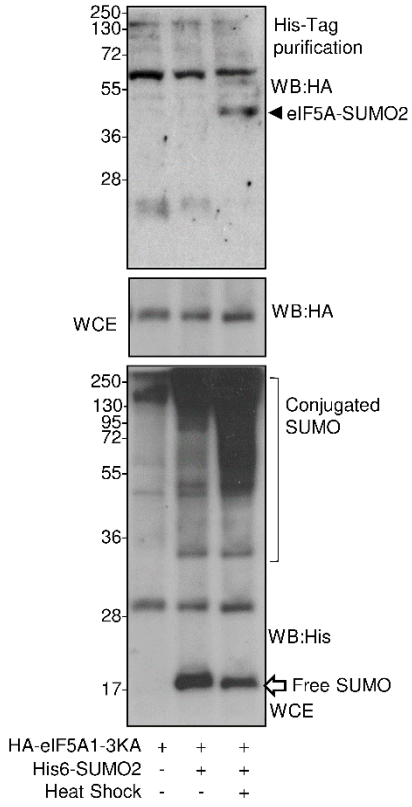


Figure 27. SUMO acceptor sites in eIF5A1 are regulated by heat shock stress. HEK-293T cells were co-transfected with a plasmid encoding HA-eIF5A1-3KA together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, the cells were incubated at 43º C for 2 hours. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates the SUMO2-conjugated eIF5A1-3KA.

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6.1.17 Conjugation of SUMO to lysine residues K68, K85, and K126 on eIF5A is essential for yeast survival

The reduced stability of eIF5A mutants in the SUMOylation sites in mammalian cells and the induction of eIF5A SUMOylation in response to proteasome inhibition difficult to evaluate the functionality of the SUMOylation mutants in mammalian cells. eIF5A is essential for cell growth in yeast and human eIF5A has been demonstrated to complement the inviability of a yeast strain in which the yeast eIF5A genes were disrupted (Schnier et al., 1991b). Therefore, we decided to study the functionality of a mutant of eIF5A1 in the SUMOylation sites in yeast (Saccharomyces cerevisiae) in collaboration with Dr. Miguel González Blanco lab (CiMUS). First, we analyzed the potential SUMOylation of eIF5A-WT in yeast. For that, yeast cells containing an empty vector (His-Hyp2-3-empty) or yeast cells with histidine-tagged human eIF5A1-WT (His-Hyp2-3 WT) were grown at 25º C and histidine-tagged proteins were purified under denaturing conditions. Western blot analysis of purified proteins using anti-SMT3 antibody revealed the presence of a band of around 40 kDa, likely corresponding with SUMO modified eIF5A1 protein (Figure 28A), indicating that eIF5A1 protein is modified by SUMO in yeast. Then, thermosensitive yeast mutant strains hyp2-3 (C39Y, G118D) and hyp2-1 (P83S) were transformed with plasmids encoding human eIF5A1-WT, eIF5A1-3KA or the hypusination deficient eIF5A-K50R, under the control of the constitutive GPD promoter and assessed their viability at 37º C. Both the WT and mutants of eIF5A exhibited normal growth at 25º C (Figure 28B). However, only the WT strain as well as the hyp2 strain harbouring the eIF5A-WT protein could proliferate at the restrictive temperature (Figure 28B), despite we detected expression of all the proteins at both permissive and restrictive temperatures (Figure 28C). These results suggested that SUMOylation of human eIF5A1 is essential for complementing HYP2 function in yeast. To further probe this hypothesis, we decided to evaluate the SUMOylation of eIF5A-3KA in yeast. Histidine-tagged proteins purified under denaturing

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conditions from yeast cells expressing histidine-tagged eIF5A-WT or eIF5A-3KA were analyzed by Western blot using anti-SMT3 antibody. We observed that the SUMOylation of eIF5A-3KA at 25º C was reduced in comparison with the WT protein (Figure 28D). Altogether these results suggest that SUMOylation of eIF5A at lysine residues K68, K85 and K126 is essential for complementing HYP2 function in yeast.

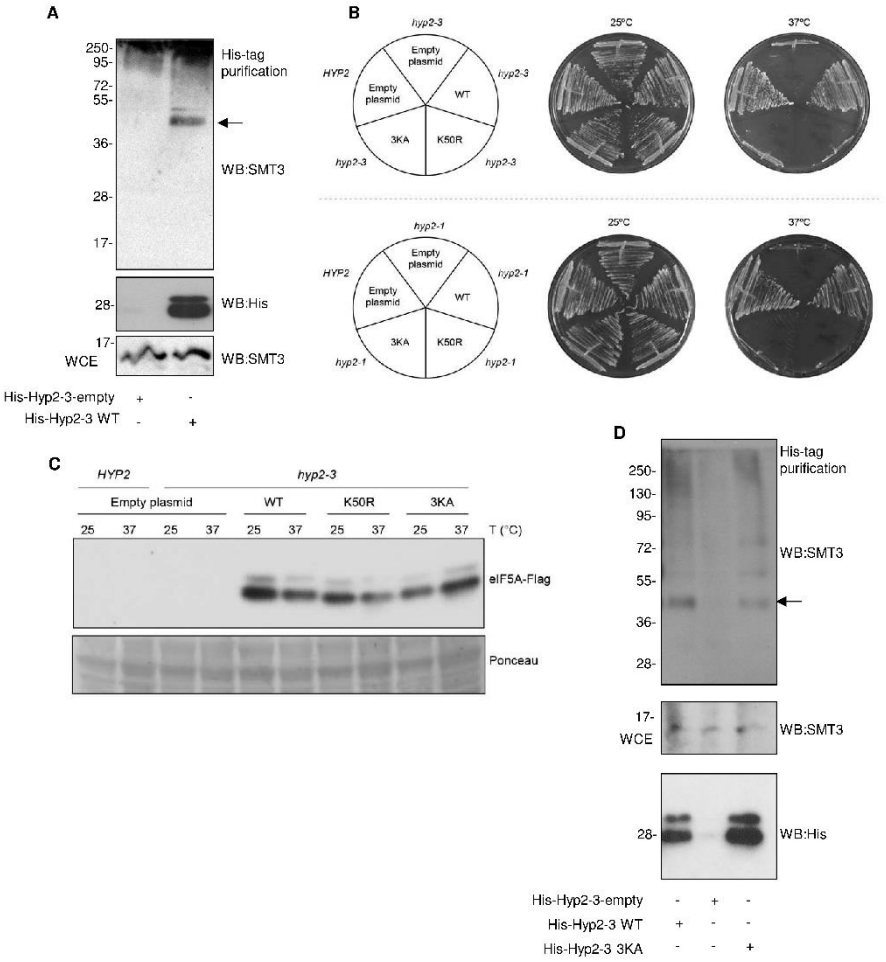


Figure 28. SUMOylation of eIF5A at lysine residues K68, K85 and K126 is essential for complementing HYP2 function in yeast. A, Yeast transformed with empty vector or histidine-tagged human eIF5A1 were grown at 25º C and histidine-tagged proteins were purified under denaturing conditions using nickel beads. Whole cell extracts and histidine-tagged proteins were analyzed by Western blot with anti-Smt3 antibody. Arrow shows Smt3-conjugated eIF5A1 protein. B, WT and *hyp2-3* (upper row) or *hyp2-1* (lower row) yeast strains were transformed with different alleles of Flag-eIF5A1 in a pAG425GPD vector or with the empty vector. The resulting strains were streaked on SC-Leu plates, incubated at 25º C or 37º C for 3 days and imaged in a GelDoc documentation system. C, Expression levels of the different versions of eIF5A in the indicated strains, growing at 25º C or after a 37º Cwere analyzed by Western blot with anti-Flag antibody. Ponceau staining of the membrane is shown as a loading control. D, Yeast cells transformed with His-eIF5A1-WT, His-eIF5A1-3KA, or with the empty vector were grown at 25º C. Whole cell extracts and histidine-tagged purified proteins were analyzed with anti-Smt3 antibody. Arrow shows Smt3-conjugated eIF5A1 protein.

Functionality assays in yeast pointed to eIF5A as an important protein in the regulation of the protein translation repression induced after stress due to polysome disassembly and the formation of stress granules (Li et al., 2010). Therefore, in collaboration with Paula Alepuz lab (Universitat de Valencia) we tested the putative role of SUMOylation in this function of eIF5A by analyzing the polysome profile of *hyp2-3* yeast strains transformed with Flag-eIF5A1-WT, Flag-eIF5A1-3KA, Flag-eIF5A1-K50R or with the empty vector and subjected to stress shock conditions. We observed that heat shock stress induced a reduction in the polysome fraction in the cells reconstituted with eIF5A1-WT(Figure 29A). Lower reductions were observed in those cells reconstituted with the empty vector, the hypusine mutant, or the SUMOylation mutant of eIF5A1 (Figures 29B, 29C and 29D).

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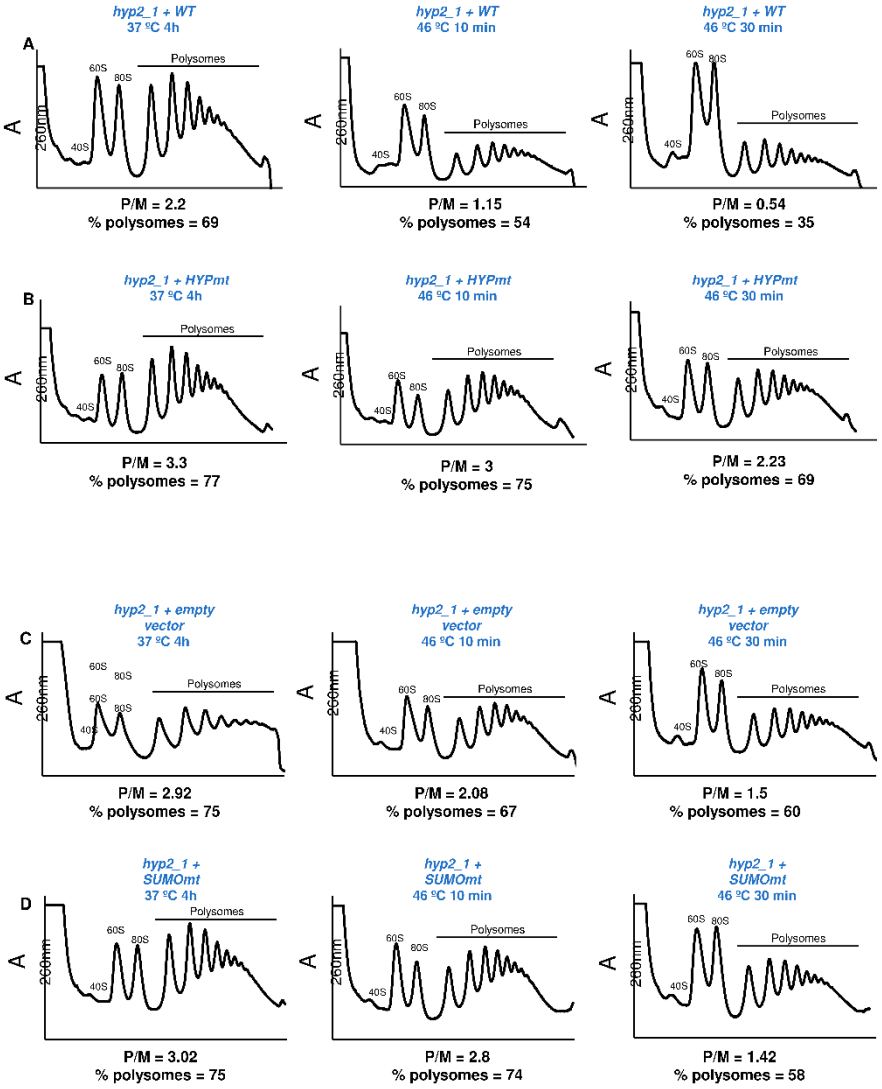


Figure 29. SUMOylation is necessary for eIF5A-mediated translation repression after heat shock stress. Polysome profiling of *HYP2_1 yeast cells* transformed with human eIF5A1 (A), eIF5A1-K50R (B), empty vector (C) or eIF5A1-3KA after incubation at 37º C for 4h, at 46º C for 10 min or at 46º C for 30 min, as indicated.

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6.1.18 Mutation of the SUMOylation sites in eIF5A1 favors its interaction with STUB1

So far, the stability of eIF5A has been reported to be regulated through its interaction with STUB1 which promotes its ubiquitination and degradation (Shang et al., 2014). In addition, proteomic approaches have suggested that the majority of the residues that are identified as potential SUMOylation sites in eIF5A (K27, K34, K85, K68, K39 and K67) are also potential ubiquitination sites, suggesting the existence of an interplay between both modifications. Since it has been found that the interaction of eIF5A with STUB1 promotes its ubiquitination and degradation, and the SUMOylation mutants were less stable we decided to study the interaction between eIF5A-WT or the SUMOylation mutant with STUB1. HEK-293T cells were co-transfected with HA-STUB1 and Flag-eIF5A1-WT or Flag-eIF5A-5KA expression plasmids and 24 h after transfection cells were treated with MG132. 36 h after transfection protein extracts were incubated with anti-HA or anti-Flag antibodies. Immunoprecipitated proteins were resolved using SDS-PAGE and analyzed by Western blot with the indicated antibodies. Western blot analysis of the anti-STUB1 immunoprecipitates using anti-Flag antibody revealed a band or around 10 kDa higher than unmodified eIF5A1-3KA likely corresponding with the ubiquitinated protein (Figure 30, left panel). In addition, we observed higher levels of STUB1 interacting with eIF5A-3KA than with eIF5A-WT protein (Figure 30, right panel), suggesting that mutation of the SUMOylation sites in eIF5A favours its interaction with STUB1 and its ubiquitination.

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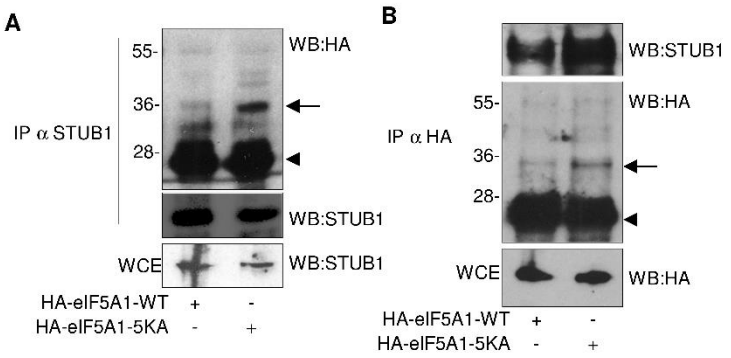


Figure 30. Mutation of the SUMOylation sites in eIF5A1 potentiates its interaction with STUB1. HEK-293 cells were transfected with HA-eIF5A1-WT or HA-eIF5A1-5KA and 24 h after transfection cells were treated with MG132. 16 h after treatment protein extracts were subjected to immunoprecipitation with anti-STUB1 (A) or anti-HA (B) antibodies. Finally, immunoprecipitated proteins were analyzed by Western blot with the same antibodies. Arrowheads indicate unconjugated eIF5A1 protein. A band likely corresponding to ubiquitinated eIF5A1 is indicated by an arrow.

6.1.19 eIF5A is modified by NEDD8

In addition to SUMO and ubiquitin, proteomic approaches have recently pointed to eIF5A as a potential NEDD8 substrate (Lobato-Gil et al., 2021). To verify whether eIF5A is a substrate of NEDD8, we first carried out a NEDDylation assay *in vitro*. 35S-methionine labeled *in vitro* translated eIF5A1 protein was incubated in the presence of NEDD8 together with the NEDDylation machinery. 2 h later, the reaction was stopped, the products were analyzed by SDS-PAGE and the dried gel was then exposed to a X-ray film. eIF5A1 protein was detected as a 17 kDa molecular weight band, as expected (Figure 31A). Incubation with the NEDDylation machinery led to the appearance of an additional higher molecular weight band, indicating that eIF5A1 can be modified by NEDD8 *in vitro* (Figure 31A). Then, we decided to evaluate the potential conjugation of eIF5A with NEDD8 in cells. HEK-293T cells were co-transfected with HA-eIF5A and pcDNA or His6-NEDD8 expression plasmids. At 48 h after transfection, cells were collected and histidine-tagged proteins were purified under denaturing

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conditions. Purified proteins were then analyzed by Western blot with anti-HA antibody. As shown in Figure 31B, a band of around 10 kDa higher than the unmodified eIF5A1 protein was detected exclusively in those cells expressing the NEDDylation machinery, indicating that eIF5A1 protein can be modified by NEDD8 in cells. Similarly, HEK-293T cells were co-transfected with Flag-eIF5A2 and pcDNA or His6-NEDD8 and 48 h after transfection cells were processed as described above. The detection of a band of the expected eIF5A2-NEDD8 protein exclusively in those cells expressing NEDD8 using Western-blot analysis with anti-Flag antibody (Figure 31C) indicated that eIF5A2 can also be modified by NEDD8. To further probe the modification of eIF5A1 by NEDD8, we evaluated the deNEDDylation of eIF5A1 by the NEDD8-specific protease NEDP1. HEK-293T cells were co-transfected with HEK-293T cells together with pcDNA, His6-NEDD8 or His6-NEDD8 and GFP-NEDP1. At 48 h after transfection cells were recovered and the histidine-tagged proteins were purified under denaturing conditions. Analysis of the purified proteins by Western blot using anti-HA antibody revealed that the band corresponding with eIF5A1-NEDD8 protein disappeared in those cells expressing NEDP1 (Figure 31D), confirming the identity of the NEDDylated band. Finally, we analyzed the NEDDylation of eIF5A in endogenous conditions. For that, protein extracts from HEK-293T cells were subjected to immunoprecipitation using anti-NEDD8 antibody and the immunoprecipitated proteins were analyzed using anti-eIF5A1 antibody. We detected a band of the expected eIF5A1-NEDD8 molecular weight (Figure 31E), indicating that eIF5A1 is modified by NEDD8 in endogenous conditions.

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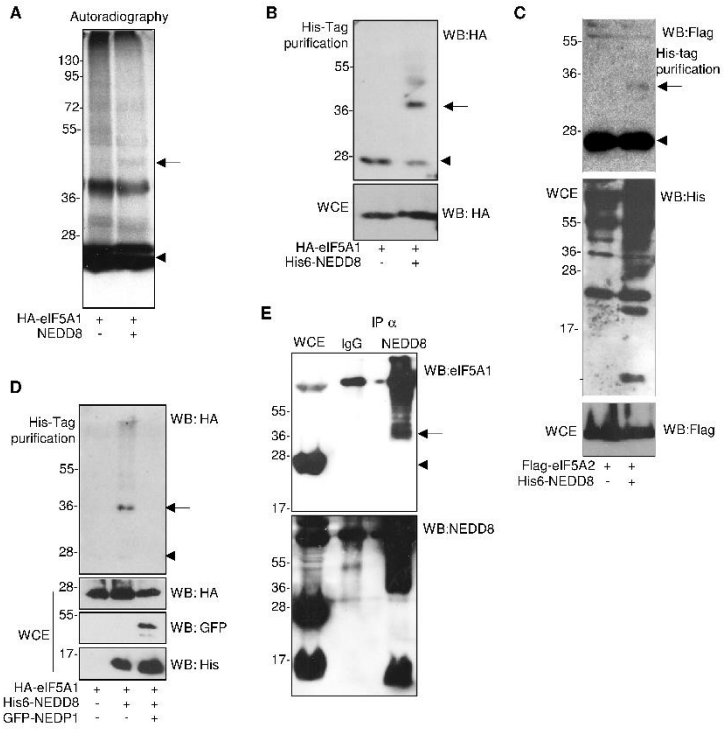


Figure 31. eIF5A is modified by NEDD8. A, *In vitro* NEDDylation assay using ^{35}S-methionine labeled *in vitro* translated HA-eIF5A1 protein as a substrate. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates NEDD8-conjugated eIF5A1 protein. B, HEK-293T cells were transfected with HA-eIF5A1 and His6-NEDD8. 36 h after transfection whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates NEDD8-conjugated eIF5A1 protein. C, HEK-293T cells were transfected with Flag-eIF5A2 and His6-NEDD8. 36 h after transfection whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A2 protein. Arrow indicates NEDD8-conjugated eIF5A1 protein. D, HEK-293T cells were transfected with HA-eIF5A1 and His6-NEDD8 in the presence or absence of the NEDD8-protease NEDP1. 36 h after transfection whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates NEDD8-conjugated eIF5A1 protein. E, Endogenous NEDDylation of eIF5A1. HEK-293T extracts were immunoprecipitated under denaturing conditions using anti-NEDD8 antibody or mouse IgG as a negative control. Immunoprecipitated proteins were analyzed by Western blot using anti-eIF5A1 antibody.

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6.1.20 Evaluation of potential NEDDylation sites in eIF5A.

In order to evaluate whether hypusination is required for NEDDylation of eIF5A, we evaluated the conjugation of the hypusination mutant HA-eIF5A-K50R to NEDD8 in cells. HEK-293T cells were co-transfected with HA-eIF5A-K50R and pcDNA or His6-NEDD8 and 48 h after transfection, histidine-tagged proteins were purified under denaturing conditions and analyzed by Western blot with anti-HA antibody. We observed the appearance of a band corresponding with NEDD8 conjugated to eIF5A-K50R in the cells expressing the NEDDylation machinery (Figure 32A), indicating that NEDDylation of eIF5A1 does not require its hypusination. Proteomic data pointed to lysine K27 as involved in NEDD8 conjugation. Therefore, we studied whether mutation of lysine K27 in eIF5A inhibits its SUMOylation. HEK-293T cells were co-transfected with HA-eIF5A-WT or HA-eIF5A-K27R and pcDNA or His6-NEDD8 and 48 h after transfection we evaluated the presence of NEDDylated eIF5A1 bands in the histidine-tagged purified extracts. The levels of the eIF5A1-WT-NEDD8 band were similar to the levels of the eIF5A-K27R-NEDD8 band (Figure 32B), indicating that other lysine residues in eIF5A can conjugate NEDD8. Finally, we decided to evaluate whether mutation of the lysine residues in eIF5A1 involved in SUMO conjugation altered its NEDDylation. HEK-293T cells were co-transfected with HA-eIF5A1-WT, HA-eIF5A1-3KA, or HA-eIF5A-5KA together with pcDNA or His6-NEDD8 and 36 h after transfection cells were treated with MG132. Cell extracts were recovered and lysed under denaturing conditions and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. The levels of the bands corresponding with NEDD8 conjugated to the eIF5A SUMOylation mutants were higher than the levels of the eIF5A1-WT-NEDD8 protein (Figure 32C), suggesting an interplay between NEDDylation and SUMOylation. To further evaluate this interplay, we studied the effect of inhibiting SUMOylation on eIF5A1 NEDDylation as well as the effect of inhibiting NEDDylation on eIF5A

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SUMOylation. For that, cells were co-transfected with HA-eIF5A1 and pcDNA, His6-NEDD8 or Ubc9 and His6-SUMO2 and 36 h after transfection cells were treated with the SUMOylation inhibitor ML792 or the NEDDylation inhibitor MLN4924, as indicated. At 48 h after transfection we recovered the cells, purified the histidine-tagged proteins and analyzed them by Western blot with anti-HA antibody. We observed a small increase in the levels of eIF5A SUMOylation after MLN4924 treatment (Figure 32D) and an increase in NEDDylation in those cells treated with the SUMOylation inhibitor ML792 (Figure 32E), confirming the existence of an interplay between SUMO and NEDD8 conjugation to eIF5A.

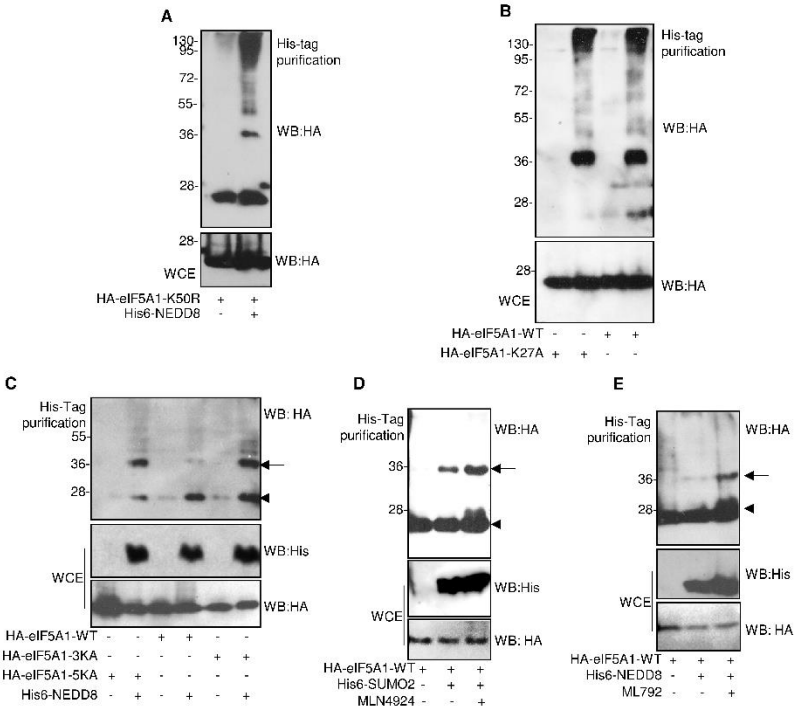


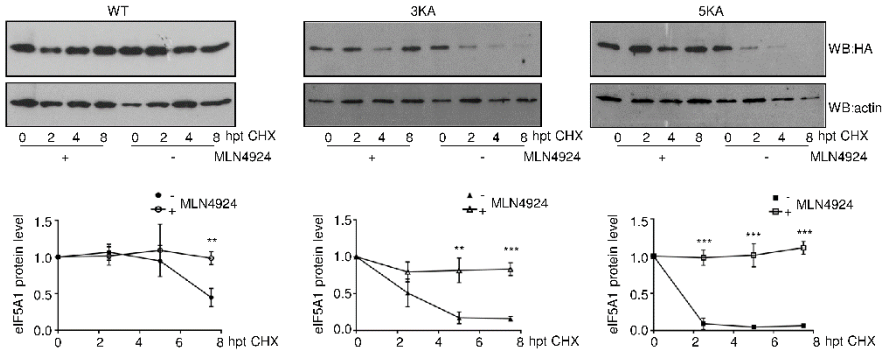
Figure 32. Interplay between NEDD8 and SUMO2 on eIF5A1. HEK-293T cells were transfected with HA-eIF5A1-50KR (A) or with HA-eIF5A1-WT or HA-eIF5A1-K27R (B) in the presence or absence of His6-NEDD8. 36 h after transfection whole cell extracts and histidine-tagged purified proteins were analyzed by Western blot using anti-HA antibody. C,

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HEK-293T cells were co-transfected with HA-eIF5A1-WT, HA-eIF5A1-3KA or HA-eIF5A1-5KA together with pcDNA or His6-NEDD8 and 24 h after transfection cells were treated with MG132. 16 h after treatment whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates NEDD8-conjugated eIF5A1 protein. D, HEK-293T cells were transfected with a plasmid encoding HA-eIF5A1-WT together with pcDNA or His6-SUMO2 and 24 h after transfection cells were treated or not with MLN4924. 16 h after treatment whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. E, HEK-293T cells were transfected with a plasmid encoding HA-eIF5A1-WT together with pcDNA or His6-NEDD8 and 24 h after transfection cells were treated or not with ML792 and 16 h after treatment whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody (right panel). Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates NEDD8-conjugated eIF5A1 protein.

6.1.21 Inhibition of NEDDylation stabilizes eIF5A

To determine the impact of NEDDylation on eIF5A1, we evaluated the effect of the NEDDylation inhibitor MLN4924 on eIF5A1 stability. HEK-293T cells were transfected with HA-eIF5A1-WT, HA-eIF5A1-3KA or HA-eIF5A1-5KA and 36 h after transfection, cells were treated with the NEDDylation inhibitor MLN4924 for 12 h. Then, cells were treated with cycloheximide and at different times after treatment the cells were collected and the protein extracts were analyzed by Western blot with anti-HA antibody. Treatment with MLN4924 significantly increased the stability of eIF5A1-WT as well as of the eIF5A-3KA and eIF5A-5KA mutants (Figure 33).



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Figure 33. NEDDylation modulates the stability of eIF5A1. A, HEK-293 cells were transfected with HA-eIF5A1-WT or the SUMOylation mutants, treated overnight with MLN4924 and then treated with CHX. At the indicated times after CHX treatment, protein extracts were analyzed by Western blot with the indicated antibodies (upper panels). Stability of the protein in presence or absence of MLN4924 from three independent experiments is shown (lower panels). Statistical analysis was assessed by a Student's t-test.

6.1.22 STUB1 promotes eIF5A1 NEDDylation

eIF5A can conjugate to different ubiquitin-like proteins, including Ubiquitin and NEDD8. It has been previously demonstrated that STUB1 is an E3 Ubiquitin ligase that mediates eIF5A ubiquitination and degradation (Shang et al., 2014). Interestingly, it has been reported that STUB1 can also work as an E3 NEDD8 ligase (Yoo et al., 2018). Therefore, we decided to evaluate the potential impact of STUB1 on NEDDylation. For that, we tested the NEDDylation of eIF5A1 in cells overexpressing STUB1. We observed that higher levels of STUB1 correlated with higher levels of eIF5A1 NEDDylation (Figure 34), indicating that STUB1 can promote eIF5A1 NEDDylation.

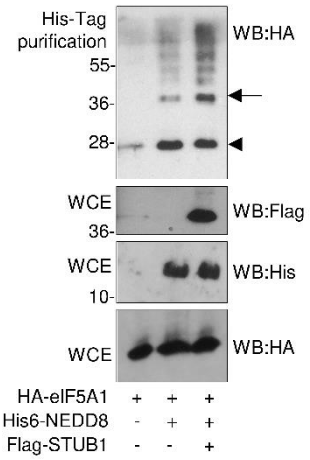


Figure 34. STUB1 promotes eIF5A1 NEDDylation. HEK-293T cells were transfected with HA-eIF5A1 and His6-NEDD8 in the presence or absence of Flag-STUB1. 36 h after transfection, whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrowhead indicates unconjugated eIF5A1 protein. Arrow indicates NEDD8-conjugated eIF5A1 protein.

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6.2 INFLUENZA VIRUS AND NEDD8

6.2.1 Influenza virus infection induces global reprogramming of host NEDDylation

IAV infection has been previously shown to induce the NEDDylation of cullins (Sun et al., 2018), probably the major NEDD8 substrate, suggesting that infection with IAV may alter NEDDylation. To evaluate this hypothesis, we infected A549 cells with influenza virus A/PR8/1934 at an moi of 5 and cells were recovered at different times after infection. Western blot analysis of the protein extracts using anti-NEDD8 antibody revealed dynamic NEDDylation changes during IAV infection (Figure 35A). Observed changes in NEDDylation could be due to transcriptional alterations in NEDDylation machinery genes. Therefore, we decided to analyze the transcript levels of NEDD8 as well as of the two NEDD8-conjugating enzymes E2 at different times of IAV infection. However, as shown in figure 35B, we did not observe significant changes in the mRNA expression levels of the analyzed genes.

6.2.2 Differentially NEDDylated proteins after IAV infection

The NEDDylation changes upon IAV infection as well as the previously reported upregulation in cullin NEDDylation in IAV infected cells (Sun et al., 2018), led us to study the NEDDylated proteome in IAV infected cells. Antibodies that recognize the Lys-ϵ-Gly-Gly (diGly) remnant on ubiquitinated proteins after trypsin digestion are used for the quantitation and identification of ubiquitinated proteins as well as for mapping of endogenously modified lysines by quantitative mass spectrometry (Xu et al., 2010). However, trypsin digestion generates a similar diGly remnant in NEDDylated, ubiquitinated and ISGylated proteins, therefore this approach cannot distinguish between these modifications (Wagner et al., 2011) (Figure 36A). To identify the NEDDylated proteome, and in collaboration with Dimitris Xirodimas (CNRS), we used a protocol developed by Vogl et al. based on the use of a NEDD8-R74K mutant together with the anti-

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diGly antibody, and using Lys-C protease, that cleaves at the carboxyl side of the lysine (Vogl et al., 2020).

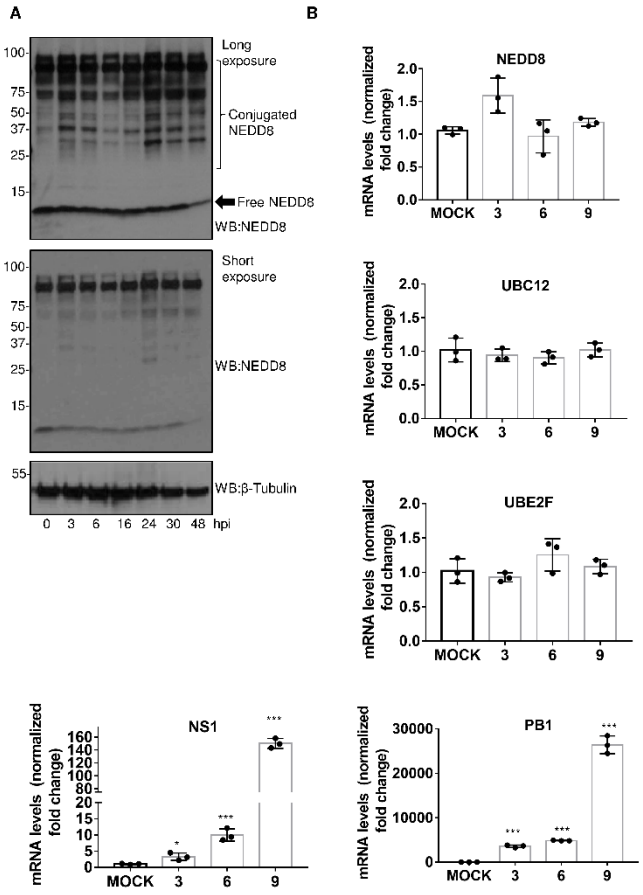


Figure 35. IAV induces global reprograming of host NEDDylation. A, A549 cells were infected with IAV (at an moi of 5), collected at the indicated times after infection and analyzed by Western blot using anti-NEDD8 antibody. B, A549 cells were infected with IAV as indicated above and at the indicated times after infection, the transcript levels of NEDD8, the two NEDD8-conjugating enzymes E2 and two different IAV genes were analyzed by Q RT-PCR and represented as fold induction over mock-infected cells. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *, P<0.05; **, P<0.005, ***P<0.001.

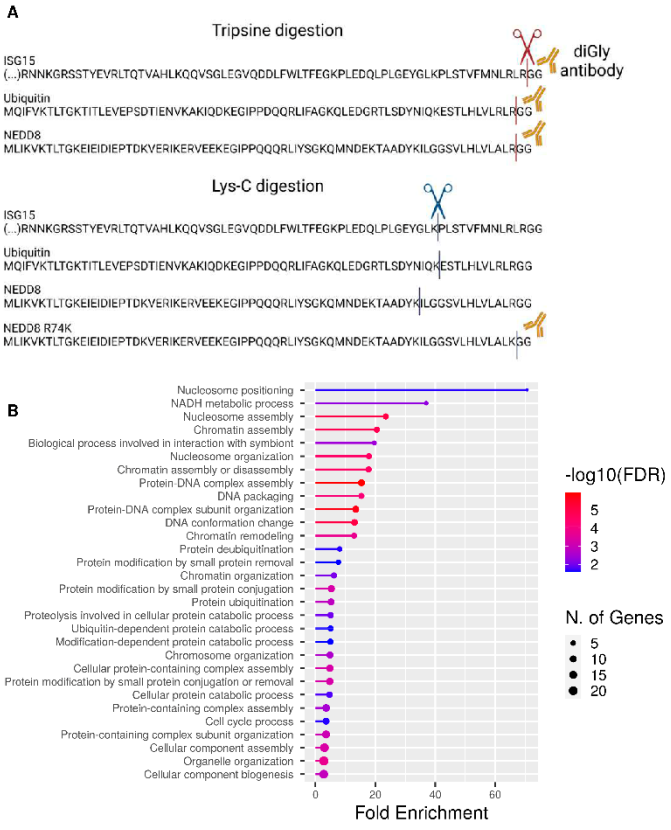
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Treatment with this protease will specifically generate diGly remnants only in NEDD8 modified proteins but not ISGylated or ubiquitylated proteins (Figure 37A) (Vogl et al., 2020). A549 were infected with lentivirus expressing His6-NEDD8-R74K protein and infected cells were then selected using puromycin. Immediately after selection, A549 cells stably expressing NEDD8-R74K were infected with IAV at an moi of 1. At 24 h after infection, cells were lysed under denaturing conditions and in collaboration with Dr. Jeffrey Johnson's laboratory at Icahn School of Medicine at Mount Sinai, proteins were analyzed by mass spectrometry. We observed changes in the NEDDylation of 55 putative NEDD8 substrates. Gene ontology enrichment analysis of the differentially modulated proteins revealed an enrichment for proteins involved in nucleosome assembly and chromatin-related biological processes (Figure 36B), including well known NEDD8 substrates such as histone proteins (Figure 36C). We identified 14 NEDDylated protein candidates in mock-infected cells that were not detected in IAV infected cells (Figure 36D) and 9 NEDDylated protein candidates that were detected only in the infected cells (Figure 36D).

6.2.3 Validation of IAV-modulated NEDD8 targets

In order to validate the mass spectrometry results, we decided to study the conjugation to NEDD8 of several NEDDylation candidates in cells infected or not with IAV. For that, HEK-293T cells were co-transfected with the plasmid encoding the protein of interest together with pcDNA or His6-NEDD8 expression plasmids. At 24 h after transfection, cells were infected with IAV at an moi of 1 and 24 h after infection cells were collected and histidine-tagged purified proteins were analyzed with the indicated antibodies. First, we evaluated the NEDDylation of two well-known NEDD8 substrates, H2B and RPS27A. In cells transfected with H2B or RPS27A and co-transfected with NEDD8 we detected the appearance of bands of the expected H2B-NEDD8 and RPS27A-NEDD8 molecular weight, respectively,

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C

PROTEIN	log2FC
H2AX	2,931368778
Cofilin	0,984267201
VDAC-1	0,774043256
Tip39	0,473035077
EMAP-2	0,031646613
Gly-RS	-0,177001152
H2B.1	-0,324129354
VDAC-3	-0,523344706
RS27A	-0,684415973
RL40	-1,050260055

D

IAV ONLY	MOCK ONLY
eIF-5B	4F2hc
Aldo-keto reductase family 1 member C2	Histone H1.4
MICOS complex subunit MIC60	Histone H1.2
Germ cell-specific gene 1 protein	Parathymosin
AHNAK2	VCP
Abnormal spindle-like microcephaly-associated protein	Histone H2B type 1-D
[F-actin]-monooxygenase MICAL1	40S ribosomal protein S20
Serine/threonine-protein kinase TAO3	SUMO2
SMC-3	Histone H2B type 1-C
	NEDD8
	E3 ubiquitin-protein ligase RNF43
	NAPE-PLD
	Caveolae-associated protein 1
	Ssf-1

Figure 36. Differentialy NEDDylated proteins after IAV infection. A, Representation of different strategies to generate diGly remnants. B, Gene ontology analysis of the differentially NEDDylated proteins detected by MS after IAV infection. C, List of proteins with

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NEDDylation upregulated or downregulated after IAV infection. D, List of proteins whose NEDDylation is only detected in IAV or mock-infected cells.

indicating that both proteins can be conjugated to NEDD8 (Figure 37A left panel and 37B). Infection with IAV clearly decreased the NEDDylation of the proteins (Figure 37A right panel and 37B), indicative of the negative modulation of their NEDDylation upon IAV infection. We then studied the potential NEDDylation of one of the novel NEDDylation candidates identified in this study, the voltage-dependent anion channel 1 (VDAC1) protein, a regulator of mitochondrial function (and modulator of apoptosis, autophagy, and inflammation). Western-blot analysis of histidine-tagged purified proteins revealed the appearance of a band of the expected VDAC1-NEDD8 molecular weight in the cells transfected with His6-NEDD8, confirming that VDAC1 can be modified by NEDD8 (Figure 37C). An increase in the intensity of this band as well as additional bands were detected after IAV infection (Figure 37C), suggesting that VDAC1 NEDDylation increases upon IAV infection. The mass spectrometry data pointed to lysine residue K110 in VDAC1 as the acceptor site of NEDD8 in VDAC1. Therefore, we generated a mutant of VDAC1 in K110 (VDAC1-K110R). Then, we analyzed the NEDDylation of VDAC1-WT or VDAC1-K110R in HEK-293T cells transfected with His6-NEDD8. NEDDylation of VDAC1-K110R was reduced in comparison with the WT protein (Figure 37D), indicating that K110 in VDAC1 is involved in NEDD8 conjugation. Finally, we evaluated the potential impact of VDAC1 NEDDylation on IAV. For that, we transfected HEK-293T cells with VDAC1-WT or VDCA1-K110R expression plasmids. 36 h after transfection cells were infected with IAV, and at different times after infection, we analyzed the synthesis of the viral NS1 protein by Western blot analysis. Overexpression of VDAC1-WT did not alter the synthesis of NS1 in the IAV infected cells; however, we observed a clear reduction in the levels of the NS1 protein synthesized in the cells expressing VDAC1-K110R. These results suggested that NEDDylation of VDAC1 has a positive impact

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on virus replication (Figure 37E). However, an analysis of the impact of NEDDylation on VDAC1 properties and functions is required in order to confirm this data.

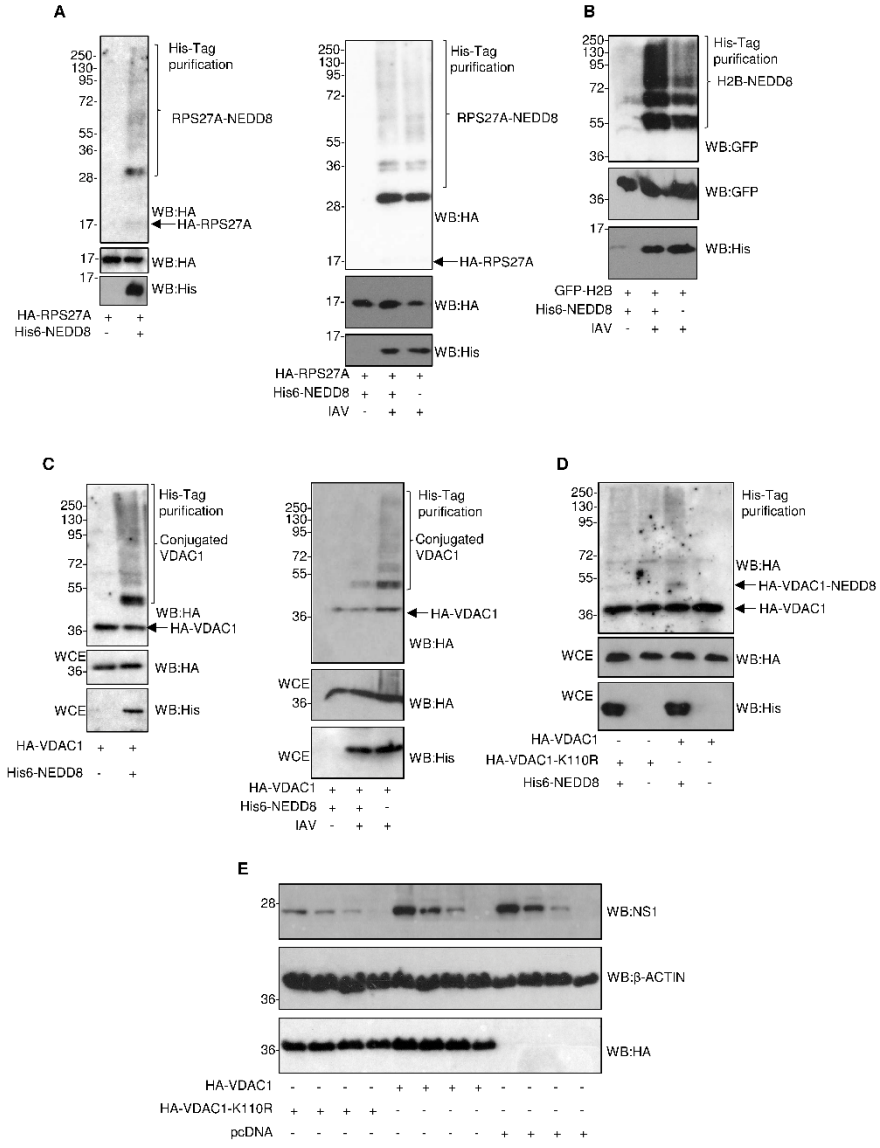


Figure 37. NEDDylation of RPS27A, H2B and VDAC1 after influenza virus infection. A, HEK-293T cells were transfected with HA-RPS27A and pcDNA or His6-NEDD8 (left panel) and infected or not with influenza A/PR8/1934 (right panel). Whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrow indicates the unmodified HA-RPS27A protein. B, HEK-293T cells were transfected with GFP-H2B and pcDNA or His6-NEDD8, and infected or not with influenza A/PR8/1934. Whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-GFP antibody. C, HEK-293T cells were transfected with HA-VDAC1 and pcDNA or His6-NEDD8 (left panel) and infected or not with influenza A/PR8/1934 (right panel). Whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrow indicates the unmodified HA-VDAC1 protein. D, HEK-293T cells were transfected with HA-VDAC1-WT or HA-VDAC1-K110R and pcDNA or His6-NEDD8. 36 h after transfection, whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. E, HEK-293T cells transfected with pcDNA, HA-VDAC1-WT or HA-VDAC1-K110R. 36 h after transfection, cells were infected with IAV and at the indicated times after infection cell extracts were analyzed by Western blot.

6.2.4 Influenza virus NS1 protein is modified by NEDD8

So far, only two proteins from IAV, PB2 and M1, have been reported to be NEDDylated. Conjugation of NEDD8 to these proteins diminishes their stability and recombinant viruses expressing PB2 or M1 NEDDylation mutants displayed improved growth compared with wild type control virus (Li et al., 2020; Zhang et al., 2017). However, it has been also reported that treatment of the cells with the inhibitor of NEDDylation MLN4924 has a negative impact on the replication of influenza virus (Sun et al., 2018). Therefore, we hypothesized that NEDD8 can modify other IAV proteins and that this modification may improve IAV replication. Among the IAV proteins, NS1 is known to be a multifunctional protein that modulates numerous cellular host processes acting as a promoter of IAV replication and an inhibitor of the host immune response. It is generally accepted that the multifunctional nature of viral proteins is mainly driven by PTMs. Therefore, we decided to evaluate the potential regulation of NS1 by NEDD8. First, we co-transfected cells with a NS1 expression plasmid and pcDNA or His6-NEDD8. 36 h after transfection, cell extracts were collected and histidine-tagged proteins were purified under denaturing conditions using Ni^{2+}columns. Western blot analysis of the purified proteins using anti-NS1 antibody showed the appearance of a band of

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the expected NS1-NEDD8 molecular weight (around 40 kDa) together with one additional lower molecular weight band (around 35 kDa) and several additional higher molecular weight bands (Figure 38A) only in those cells co-transfected with NEDD8. Since it is known that overexpression of NEDD8 can lead to activation of the ubiquitin pathway, we speculated that the 35 kDa band might correspond with NS1 conjugated to ubiquitin. To evaluate this hypothesis, we co-transfected HEK-293T cells with NS1 and pcDNA or His6-NEDD8 expression plasmids and 36 h after transfection cells were recovered and histidine-tagged proteins were purified under denaturing conditions. Then, purified proteins were analyzed by Western blot with anti-ubiquitin or anti-NEDD8 antibodies. Ubiquitin antibody recognized the 35 kDa molecular weight band as well as several higher molecular weight bands (labeled with an asterisk in figure 38B) but it did not recognise the 40 kDa band, suggesting that it corresponds with NS1 protein conjugated with NEDD8 (NS1-NEDD8). To verify the modification of NS1 by NEDD8 in cells without overexpression of NEDD8, we transfected HEK-293T cells with HA-NS1 and 36 h after transfection, cells were collected and protein extracts were immunoprecipitated using anti-HA antibody conjugated to magnetic beads. After Western blot analysis of the immunoprecipitated proteins using anti-NEDD8 antibody, we observed a band of around 40 kDa in those cells expressing the viral protein that likely corresponds with NS1-NEDD8 (Figure 38C). Altogether these results indicated that NS1 expressed from a plasmid is modified by NEDD8. To determine whether NS1 can be modified by NEDD8 when expressed from the virus, we transfected HEK-293T cells with His6-NEDD8. 24 h after transfection, cells were infected with influenza A/PR8/1934 at an moi of 5, and 12 and 24 h after infection cell extracts were collected and histidine-tagged proteins were purified under denaturing conditions. Western blot analysis of the histidine-tagged purified proteins using anti-NS1 antibody revealed a band of around 40 kDa molecular weight corresponding with NEDDylated NS1 in the cells transfected with His6-NEDD8 and infected with the virus, suggesting that NS1

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expressed from the virus can be modified by NEDD8 (Figure 38D). Finally, we analyzed the potential NEDDylation of NS1 expressed from the virus in cells without overexpression of NEDD8 using the protocol described by Barysch et al. for the identification of endogenous SUMOylated proteins (Barysch et al., 2014). For that, we infected A549 cells with IAV at an moi of 5. 12 h after infection, cell extracts were recovered, lysed in denaturing buffer and boiled for 10 minutes. Then cell extracts were diluted in a non-denaturing buffer and incubated with anti-NEDD8 antibody. Western blot analysis of the immunoprecipitated proteins using anti-NS1 antibody showed a band of the expected size of the NS1 protein conjugated to NEDD8, confirming the endogenous NEDDylation of NS1 in IAV-infected cells (Figure 38E).

6.2.5 NS1 is modified by NEDD8 at lysines K108, K110, K126, and K131

In order to identify the NEDD8 target lysine residues in NS1 we first studied the conservation of the different lysine residues in NS1 across different IAV strains (Figure 39A). All lysines are highly conserved among the 17 IAV strains (Figure 39A). Consequently, we generated single mutants in the different lysines of NS1 and evaluated their NEDDylation in cells. We did not observe a reduction in the NEDDylation of the NS1 mutants. Therefore, we then generated mutants of NS1 with different combinations of lysine residues mutated. However, evaluation of the NEDDylation of the different NS1 mutants did not reveal a clear reduction in the NEDDylation. Finally, we generated a mutant version of NS1 with all the lysines mutated to arginine (NS1-K0) and evaluated its NEDDyation in cells. HEK-293T cells were co-transfected with NS1-WT or NS1-K0 and pcDNA or His6-NEDD8 expression plasmid, and 36 h after transfection cells were collected and histidine-tagged proteins were purified under denaturing conditions. Western blot analysis of the purified proteins using anti-

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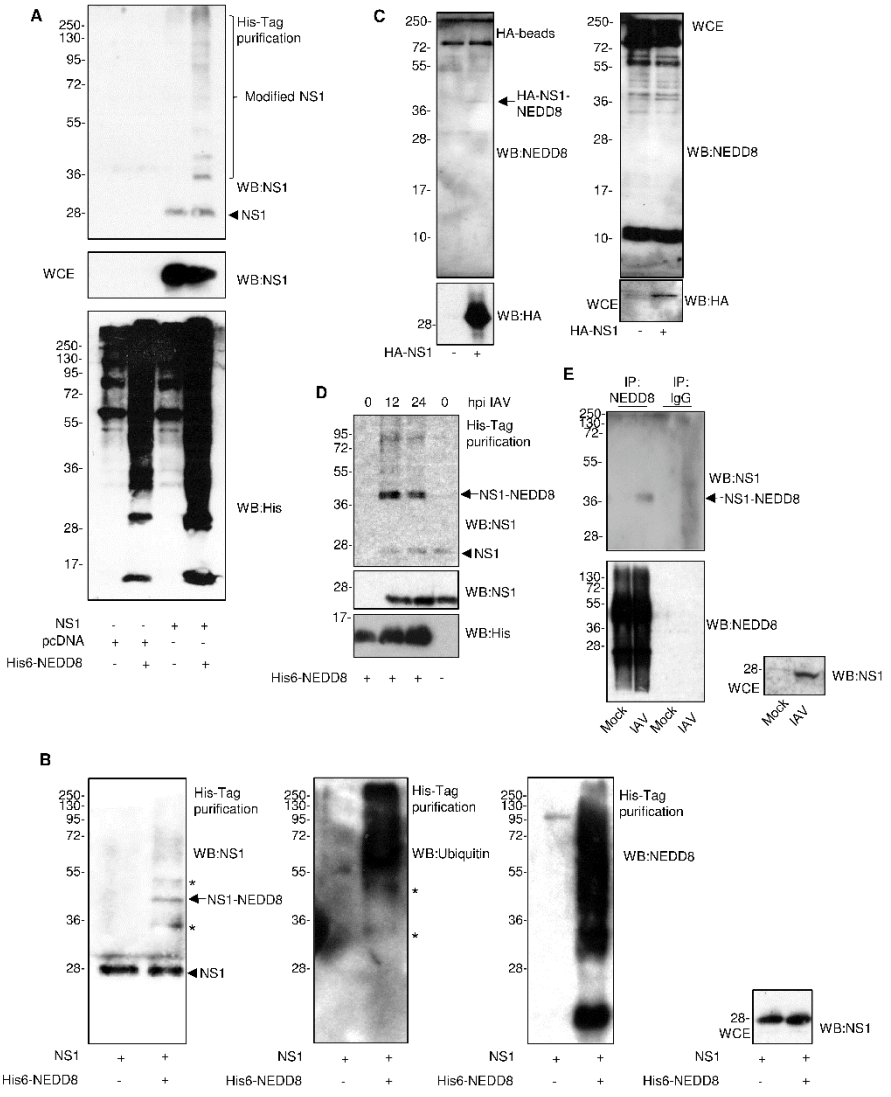


Figure 38. NS1 is modified by NEDD8. A, HEK-293T cells were transfected with NS1 and His6-NEDD8 or pcDNA and 36 h after transfection, whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-NS1 antibody. Arrowhead indicates unmodified NS1. B, HEK-293T cells were transfected with NS1 and His6-NEDD8 or pcDNA and 36 h after transfection, whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-ubiquitin antibody. Arrowhead indicates

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unmodified NS1. Asterisk indicates ubiquitinated NS1 whereas arrow indicates NEDDylated NS1. C, HEK-293T transfected with HA-NS1 were collected, lysed and incubated with anti-HA antibody conjugated to magnetic beads. Interacting proteins were analyzed by Western blot with anti-NEDD8 antibody. D, HEK-293T cells were transfected with pcDNA or His6-NEDD8 and infected with influenza A/PR8/1934 (at an moi of 5). At the indicated times after infection whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-NS1 antibody. Arrowhead indicates unmodified NS1 and arrow indicates NEDD8-modifed NS1. E, A549 cells were infected with IAV for 12 hours (at an moi of 5). Protein extracts were immunoprecipitated in denaturing conditions with anti-NEDD8 antibody and then NEDD8-interacting proteins were analyzed by Western blot with anti-NS1 antibody. Asterisks mark NS1-ubiquitin bands.

NS1 antibody revealed that mutation of all lysine residues in NS1 abolished NEDDylation (Figure 39B). Then we generated a series of NS1 mutants by reintroducing the different individual lysine residues into NS1-K0, and evaluated their NEDDylation in cells. HEK-293T cells were co-transfected with the different NS1 mutants together with His6-NEDD8 expression plasmids and at 36 h after transfection, we collected the cells and purified the histidine-tagged proteins under denaturing conditions. Analysis of the histidine-tagged proteins by Western blot using anti-NS1 antibody revealed that only the NS1 constructs in which we reintroduced K108, K110, K126 or K131, were NEDDylated (Figure 39C), indicating that lysine residues K108, K110, K126 and K131 are involved in NEDD8 conjugation. Therefore, we generated mutants of NS1 in which lysine residues K108, K110, K126 or K131 have been replaced by arginine as well as a mutant of NS1 with all four lysines mutated to arginine (NS1-4KR). We then analyzed the NEDDylation of these mutants in HEK-293T cells as described above. We did not observe a significant reduction in the NEDDylation of the single mutants (Figure 39D). However, NEDDylation was completely abolished in the NS1-4KR mutant (Figure 39E). Finally, by using reverse genetics approach, we generated a recombinant virus expressing NS1-4KR (PR8-4KR) and evaluated its NEDDylation when expressed from the virus. HEK-293T cells previously transfected with empty vector or with His6-NEDD8 were infected with PR8 or PR8-4KR. Analysis of the histidine-tagged proteins purified from the infected cells showed that mutation of the four lysine residues K108, K110, K126 and

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K131 abolished the NEDDylation of NS1 when expressed from the virus (Figure 39F).

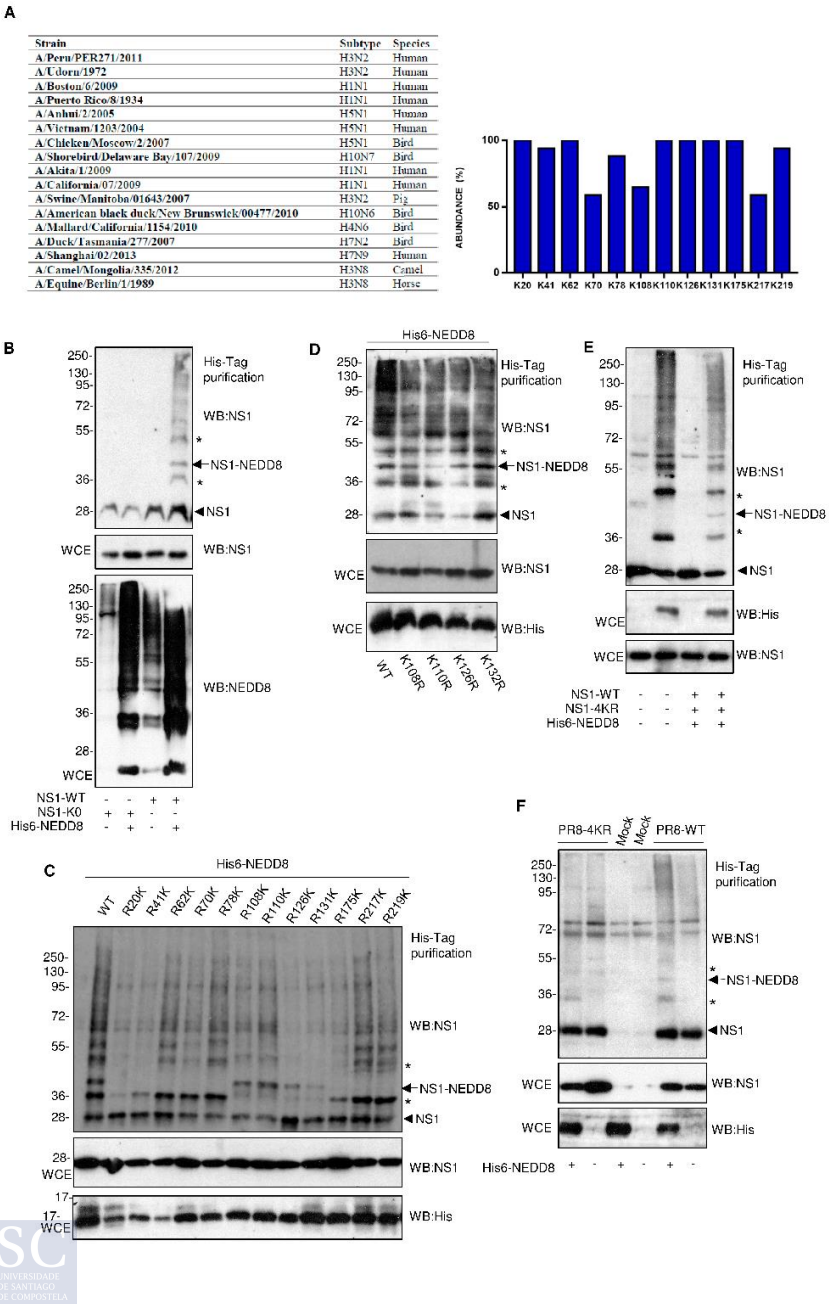


Figure 39. NS1 is modified by NEDD8 at lysines K108, K110, K126 and K131. A, List of a representative compilation of different IAV strains (left) and analysis of the presence of the different lysines in NS1 from the listed strains (right). B, HEK-293T cells were transfected with NS1-WT or NS1-K0 and pcDNA or His6-NEDD8. Whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-NS1 antibody. Arrowhead indicates unmodified NS1 and arrow indicates NEDDylated NS1. C, HEK-293T cells co-transfected with NS1-WT, NS1-K0, or mutants of NS1-K0 in which we reintroduced one by one the different lysines, and pcDNA or His6-NEDD8. Whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-NS1 antibody. Arrowhead indicates unmodified NS1 and arrow indicates NEDDylated NS1. D, HEK-293T cells were transfected with NS1-WT, NS1-K108R, NS1-K110R, NS1-K126-R, or NS1-K131R, and pcDNA or His6-NEDD8. 36 h after transfection whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-NS1 antibody. Arrowhead indicates unmodified NS1 and arrow indicates NEDDylated NS1. E, HEK-293T cells were transfected with NS1-WT or NS1-4KR and pcDNA or His6-NEDD8. 36 h after transfection whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-NS1 antibody. Arrowhead indicates unmodified NS1 and arrow indicates NEDDylated NS1. F, HEK-293T cells were transfected with pcDNA or His6-NEDD8 and infected with influenza A/PR8/1934 (PR8 WT) or PR8-4KR. 12 h after infection whole cell protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-NS1 antibody. Arrowhead indicates unmodified NS1 and arrow indicates NEDDylated NS1. Asterisks mark NS1-ubiquitin bands.

6.2.6 NS1 NEDDylation facilitates the replication of IAV

To evaluate the impact of NS1 NEDDylation on IAV replication, we infected A549 cells with PR8 or PR8-4KR at an moi of 0.1 or 1 PFU/cell. Cellular supernatants were collected 24 h after infection and virus titers were quantified by plaque assay. Mutation of the NEDDylation sites in NS1 significantly reduced the replication of the virus (Figure 40A). Similar results were observed in MDCK cells (Figure 40B).

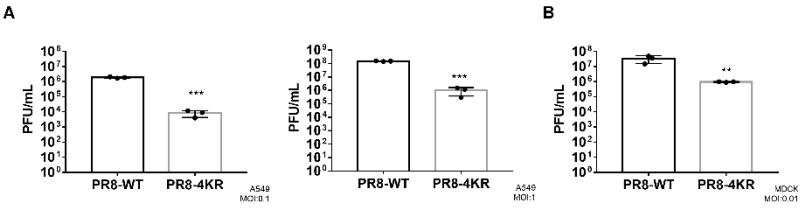


Figure 40. NEDDylation of NS1 facilitates IAV replication. A549 (A) or MDCK (B) cells were infected with PR8-WT or PR8-4KR at the indicated moi, supernatants were collected 24 h after infection and viral titers were analyzed by plaque assay. Data represent the mean and

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error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *, P<0.05; **, P<0.005

6.2.7 NEDDylation of NS1 is necessary for both interaction of NS1 with dsRNA and inhibition of IFN signaling.

In order to identify the molecular mechanisms that mediate the modulation of IAV replication by NS1 NEDDylation, we decided to study the consequences of NEDDylation on NS1 protein. Conjugation of NEDD8 can modulate the stability of the target proteins, as reported for M1 and PB2 (Li et al., 2020; Zhang et al., 2017). Therefore, we decided to analyze the stability of the NS1 NEDDylation mutant. HEK-293T cells were transfected with NS1-WT or NS1-4KR expression plasmids and 36 h after transfection, cells were treated with cycloheximide (CHX). At different times after treatment, cell extracts were lysed in Laemmli buffer, fractionated using SDS-PAGE and analyzed by Western blot with anti-NS1 antibody. We did not observe differences in the stability of NS1 after mutation of the NEDDylation sites (Figure 41A). One of the major NS1 functions is to modulate innate immune responses. Therefore, we decided to study the effect of mutation of the NEDDylation sites in NS1 on the ability of NS1 to block IFN signaling. HEK-293T cells were co-transfected with different amounts of NS1-WT or NS1-4KR expression plasmids together with a reporter plasmid containing the luciferase reporter under the control of an IFN-inducible gene (ISG54-luc), and pcDNA-beta-galactosidase. 24 h after transfection, cells were treated with IFN-β (500U/mL) and 12 h after treatment, cells were lysed and the luciferase levels were measured and normalized by beta-galactosidase activity values. As shown in figure 41B, NS1-4KR was unable to inhibit the interferon signaling in contrast to NS1-WT. One of the mechanisms mediating the inhibition of the IFN signaling by NS1 is the binding to dsRNA. To study whether NEDDylation impacts the interaction between NS1 with dsRNA, we first transfected HEK-293T cells with NS1-WT or NS1-4KR and 24 h after transfection, cells were lysed in RNA binding buffer and protein extracts were incubated overnight with dsRNA conjugated to agarose

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beads. Then, the beads were washed and resuspended in Laemmli buffer. Western blot analysis using anti-NS1 antibody showed a clear reduction in the levels of NS1-4KR interacting with dsRNA compared with the NS1-WT levels (Figure 41C). Then, we perform a similar assay using protein extracts from cells transfected with NS1-WT and treated or not with the NEDDylation inhibitor MLN4924. As shown in figure 41D, NS1 ability to bind dsRNA is attenuated in the presence of MLN4924.

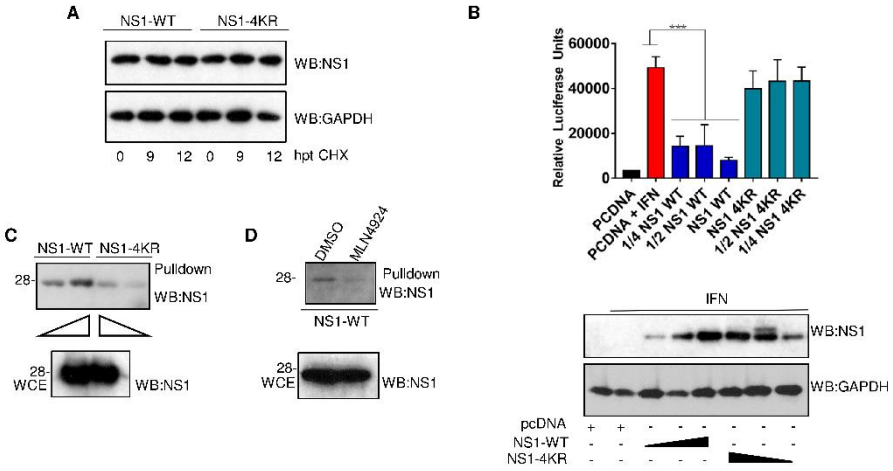


Figure 41. The NS1 NEDDylation mutant fails to inhibit IFN signaling. A, HEK-293T cells were transfected with the indicated plasmids and 36 h after transfection, the cells were treated with CHX and collected at the indicated times after treatment. Protein extracts were then analyzed by Western blot with anti-NS1 antibody. B, HEK-293T cells were co-transfected with ISG54-luc, pcDNA-beta-galactosidase and different amounts of the indicated plasmids. 24 h after transfection, cells were treated with IFN-β (500U/mL) and 12 h after treatment, cells were lysed and the luciferase levels were measured and normalized by beta-galactosidase activity values. Data represent the mean and error bars of three biological replicates normalized with beta-gal values. Statistical analysis was assessed by a Student's t-test. *, P<0.05; **, P<0.005. C, Pulldown assay of NS1-WT or NS1-4KR with dsRNA-agarose beads. D, Pulldown assays of NS1-WT with dsRNA-agarose beads in the presence of DMSO or MLN4924.

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6.3 INFLUENZA VIRUS AND UFM1

6.3.1 Influenza virus infection triggers UFMylation

Induction of ER stress triggers the upregulation of different components of the UFMylation machinery and it is known that IAV infection induces ER stress (Mazel-Sanchez et al., 2021). Therefore, we decided to study the UFMylation process after IAV infection. A549 cells were infected with influenza virus A/PR8/1934 at an moi of 5. At different times after infection cells were recovered and protein extracts were analyzed by Western blot using anti-UFM1 antibody. We detected a clear increase in the levels of conjugated UFM1 upon IAV infection (Figure 42A). To determine whether IAV infection modulates the transcription of UFMylation machinery components, A549 cells were infected with influenza virus A/PR8/1934 at an moi of 5 and, at different times after infection, cells were collected. Then, total RNA was extracted and analyzed by qRT-PCR. We did not observe significant changes in the mRNA levels of UFM1, UBA1 or UFC1 after IAV infection (Figure 42B).

6.3.2 UFMylation affects IAV replication

To decipher the role of UFMylation on IAV replication, we first transduced A549 cells with a lentiviral supernatant expressing Cas9 and single-guide RNA (sgRNA) targeting UMF1 (A549-sgUFM1) and 72 h after transduction cells were selected by incubation with puromycin. The downmodulation of UFM1 was confirmed by Western blot and qRT-PCR (Figure 43A). After cellular selection, we infected A549 or A549-sgUFM1 cells with influenza virus A/PR8/1934 at an moi of 1 and, at different times after infection, we collected the cells and analyzed the protein extracts by Western blot with anti-NS1 antibody. We only observed a slight reduction in the levels of the viral protein NS1 between both cells (Figure 43B). We then studied the potential effect of UFM1 depletion on the viral titer. For that, we infected A549

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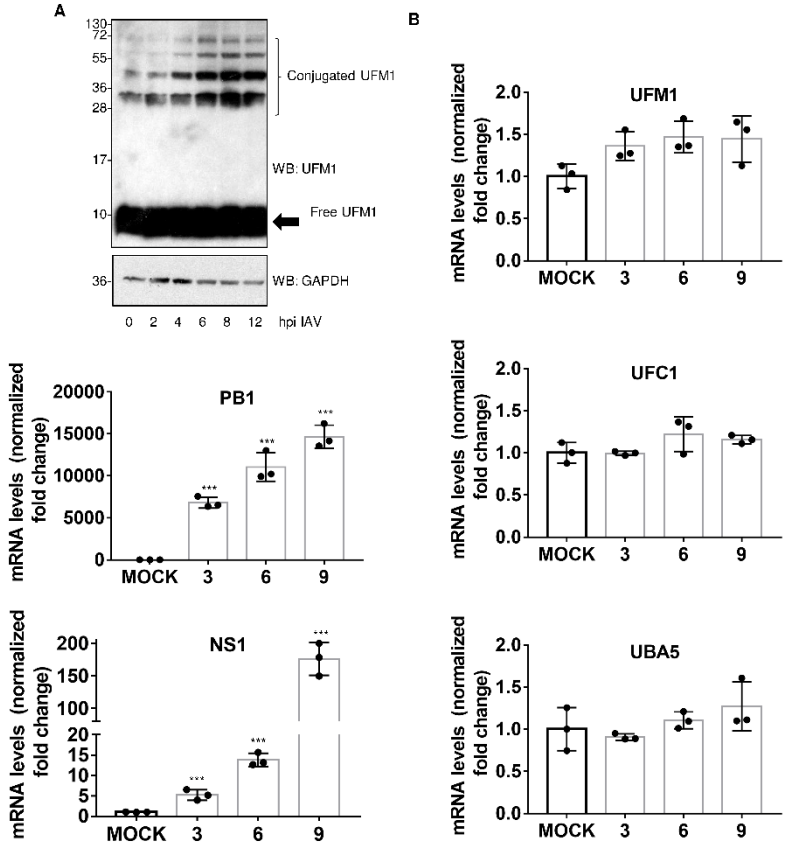


Figure 42. IAV infection modulates UFMylation. A, A549 cells were infected with IAV (at an moi of 5), collected at the indicated times after infection and analyzed by Western blot using anti-NEDD8 antibody. B, A549 cells were infected with IAV (at an moi of 5) and at the indicated times after infection, the transcript levels of the indicated genes were quantified by Q RT-PCR and represented as fold induction over mock-infected cells. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *, P<0.05; **, P<0.005, ***P<0.001.

or A549-sgUFM1 with IAV at an moi of 0.1 or 1, and 24 h after infection, supernatants were collected and analyzed by plaque assay.

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Importantly, we observed a significant decrease of the viral titer in cells with downmodulated UFM1 (Figure 43C).

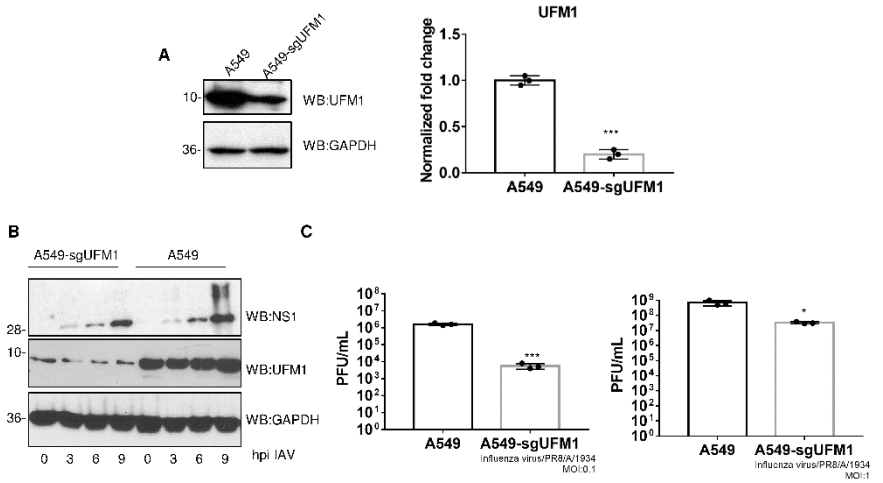


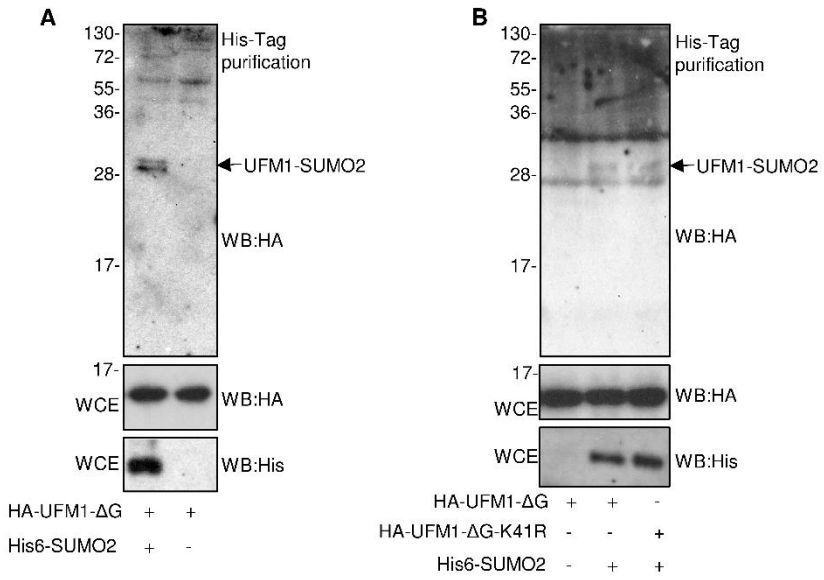
Figure 43. Effect of UFM1 downmodulation on IAV replication. A, Downmodulation of UFM1 by Western blot analysis and qRT-PCR. B, A549 and A549-sgUFM1 cells were infected with IAV and collected at the indicated times. Protein extracts were analyzed by Western blot using anti-NS1 antibody. C, Supernatants of A549 or A549-sgUFM1 collected 24 h after infection were analyzed for viral titers by plaque assay. Data represent the mean and error bars of three biological replicates. Statistical analysis was assessed by a Student's t-test. *, P<0.05; **, P<0.005, ***P<0.001.

6.3.3 UFM1 is regulated by SUMO

It is well known that there is an extensive interplay between the different ubiquitin-like proteins. However, so far, whether UFM1 can be post-translational modified by Ubiquitin-like proteins is still unknown. Therefore, we decided to evaluate the potential regulation of UFM1 by SUMO. To test this hypothesis we first generated a plasmid for the expression of an N-terminal HA-tagged UFM1 protein in which we deleted the C-terminal glycine required for its conjugation to target proteins (HA-UFM1-ΔG). Then, we co-transfected HEK-293T cells with HA-UFM1-ΔG together with pcDNA or Ubc9 and His6-SUMO2. 36 h after transfection, cells were lysed in denaturing buffer and

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histidine-tagged proteins were purified and analyzed by Western blot using anti-HA antibody. We observed the appearance of a band of the expected UFM1-SUMO2 molecular weight only in the cells expressing SUMO2, indicating that UFM1 can be modified by SUMO2 (Figure 44A). *In silico* analysis of UFM1 amino acid sequence using JASSA software pointed to lysine K41 as a potential SUMO acceptor site. Therefore, we generated a mutant of HA-UFM1-ΔG in which K41 was replaced by an arginine (HA-UFM1-ΔG-K41R) and we then analyzed its SUMOylation in cells. We co-transfected HEK-293T cells with HA-UFM1-ΔG or HA-UFM1-ΔG-K41R and pcDNA or Ubc9 and His6-SUMO2 and 36 h after transfection histidine-tagged proteins were purified and analyzed by Western blot with anti-HA antibody. Analysis of the purified proteins did not reveal differences in the conjugation to SUMO2 between HA-UFM1-ΔG and the HA-UFM1-ΔG-K41R mutant (Figure 44B), indicating that other lysine residues in UFM1 must be involved in SUMO2 conjugation.



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Figure 44. UFM1 is modified by SUMO2. HEK-293T cells were transfected with HA-UFM1-ΔG (A) or HA-UFM1-ΔG-K41R (B) and pcDNA or His6-SUMO2. 36 h after transfection whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrow indicates the SUMO2-modified UFM1 protein.

6.3.4 UFM1 is modified by NEDD8

To test whether UFM1 can be also modified by NEDD8, we co-transfected HEK-293T cells with HA-UFM1-ΔG and pcDNA or His6-NEDD8. 36 h after transfection, cells were lysed in denaturing buffer and histidine-tagged proteins were purified and analyzed by Western blot with anti-HA antibody. We observed a band of the expected UFM1-NEDD8 protein molecular weight as well as additional higher molecular weight bands only in those cells expressing His6-NEDD8 (Figure 45A), suggesting that UFM1 protein can be conjugated to NEDD8. Then, we decided to evaluate the potential involvement of K41 as a NEDD8 acceptor site in UFM1. For that, we co-transfected HEK-293T cells with HA-UFM1-ΔG or HA-UFM1-ΔG-K41R and pcDNA or pcDNA-His6-NEDD8. Western blot analysis of histidine-tagged proteins purified under denaturing conditions at 36 h after transfection did not reveal differences between NEDDylation of both proteins (Figure 45B), suggesting that other lysine residues in UFM1 can conjugate NEDD8.

6.3.5 IAV modulates the post-translational modification of UFM1 by both NEDD8 and SUMO2

So far, we still do not know what are the consequences of the conjugation of SUMO2 or NEDD8 to UFM1. However, we know that IAV infection led to reprogramming of both the SUMO2 and NEDD8 proteome. Therefore, we decided to evaluate the potential modulation of UFM1 SUMOylation or NEDDylation by IAV infection. We co-transfected HEK-293T cells with HA-UFM1-ΔG together with pcDNA, Ubc9, and His6-SUMO2 or His6-NEDD8. 24 h after transfection, cells were infected with influenza virus A/PR8/1934 and at 24 hours after infection, we lysed the cells in denaturing buffer and

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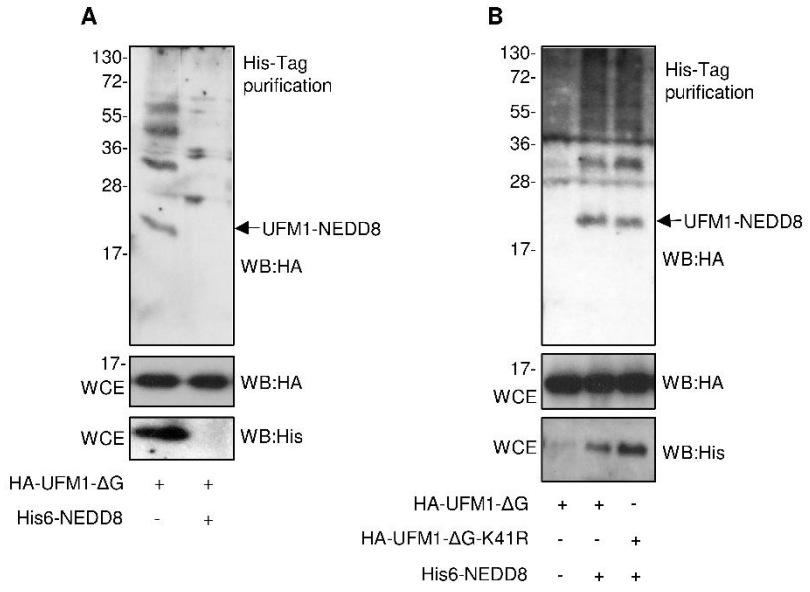


Figure 45. UFM1 is modified by NEDD8. HEK-293T cells were transfected with HA-UFM1-ΔG (A) or HA-UFM1-ΔG-K41R (B) and pcDNA or pcDNA-His6-NEDD8. 36 h after transfection whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrow indicates NEDD8-modified UFM1 protein.

purified the histidine-tagged proteins. Western blot analysis with anti-HA antibody of the purified proteins revealed that IAV infection increased SUMO2 modification (Figure 46A) whereas it decreased the NEDDylation (Figure 46B) of UFM1.

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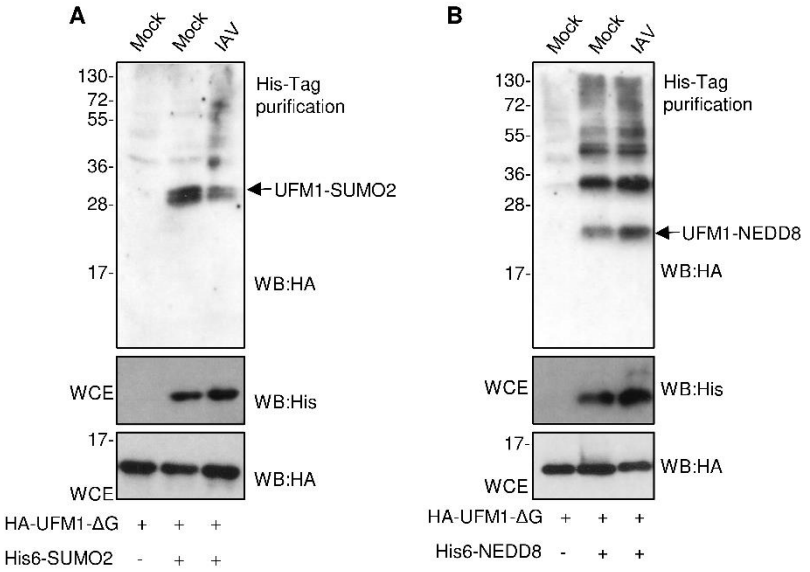


Figure 46. IAV modulates UFM1 SUMOylation and NEDDylation. HEK-293T cells were transfected with HA-UFM1-ΔG and His6-SUMO2 (A) or His6-NEDD8 (B). 24 h after transfection, cells were infected with IAV for 24 h (at an moi of 1) and after infection whole protein extracts and histidine-tagged purified proteins were analyzed by Western blot with anti-HA antibody. Arrow indicates the SUMO2-modified (A) or NEDD8-modified (B) UFM1.

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7. DISCUSSION

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Influenza virus is the causal agent of the seasonal acute respiratory disease commonly known as the flu. Influenza can cause mild to severe illness, and sometimes can lead to death, especially in people at high risk (such as the very young, the very old, and people with comorbid diseases). Thus, up to 650,000 deaths annually are associated with influenza. In addition, influenza viruses were responsible for several pandemics in the last centuries. However, there is no universal vaccine against the disease. Therefore, the need for new treatments is critical. Understanding the interactions between viral and host cell proteins is fundamental to the development of novel antiviral treatments or vaccines. Post-translational modifications are key mechanisms to control protein structure and function and represent a critical mechanism for diversifying the protein functions. Eukaryotic cells use post-translational modifications as economical responses to environmental changes including virus infections. In addition, viruses counteract or exploit host cell post-translational modifications system to establish an efficient infection (Dawson and Mehle, 2018; Gaur et al., 2011; Hu et al., 2020; Ludwig et al., 2021, 2003). Here, we studied the interplay between influenza A virus and four different post-translational modification systems: hypusination, SUMOylation, NEDDylation, and UFMylation.

First, we demonstrated that IAV infection, as well VSV infection or dsRNA viral mimic stimulation, induce the hypusination of eIF5A. So far, this is the first demonstration of hypusine induction in response to virus or dsRNA. Since dsRNA is a potent inducer of innate antiviral responses in vertebrates, these results suggest that eIF5A activation can be part of the cellular response to virus infection. Two of the innate immune mechanisms activated upon viral infection are the NF-κB and the type 1 IFN signaling pathway. Interferon has been reported to induce depletion of polyamines (Mounce et al., 2016) and a decrease in the hypusine synthesis required for the activation of eIF5A (Caraglia et al., 1997), suggesting that activation of eIF5A in response to virus infection was not mediated by interferon signaling. Hypusination

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occurs in two enzymatic steps catalyzed by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH). Recently, an upregulation of DHS and/or DOHH and the consequent activation of eIF5A has been found after the exposition of macrophages to bacterial pathogens (Gobert et al., 2020) or to IL-4 stimulation (Puleston et al., 2019). Hypusination uniquely depends on the polyamine spermidine as a selective substrate and the sentinel enzyme in polyamine biosynthesis is ODC. Transcriptional analysis of the genes from the polyamine pathway revealed the upregulation of both SSAT and ODC in infected cells. ODC has been proposed as a target gene of NF-κB (Tacchini, 2004; Zhang et al., 2021). Therefore, we hypothesized that induction of hypusination in response to virus infection or dsRNA stimuli may be mediated by NF-κB activation. We observed a reduction in the levels of hypusinated eIF5A after inhibition of NF-κB indicating that, indeed, dsRNA induces activation of eIF5A through the NF-κB pathway and reinforcing the hypothesis that the polyamine pathway plays an important role in the innate immune system. However, inhibition of hypusination or eIF5A downmodulation were negative for the replication of influenza virus and other viruses. Although NF-κB is generally considered an antiviral immune pathway, its activation is required for the replication of several viruses including influenza virus. Thus, treatment with NF-κB inhibitors has been reported to reduce influenza virus production, VSV replication and oncolysis (Shulak et al., 2014), and the induction of apoptosis by dengue virus (Marianneau et al., 1997) or mosquito-borne alphaviruses (Yeh et al., 2019) which may increase viral spread.

The polyamine pathway has been reported to be of extreme importance for a wide variety of infections (Firpo and Mounce, 2020; Li and MacDonald, 2016; Mounce et al., 2017) and the specific inhibition of hypusination has already been demonstrated to modulate the replication of the viral agents HIV, Ebola virus, and Marburg virus (Hauber et al., 2005; Olsen et al., 2016; Ruhl et al., 1993; Schroeder et al., 2014). Inhibition of hypusination has been proposed to suppress HIV gene

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expression at the level of transcription initiation (Hoque et al., 2009) and blocking hypusination has been suggested to limit the effective translation of mRNA transcribed by the ebolavirus (EBOV) polymerase (Olsen et al., 2018). Here we show that eIF5A activation is essential for the proper replication of viruses belonging to Rhabdoviridae, Orthomyxoviridae, Phenuiviridae, Flaviviridae and Togaviridae family, increasing the list of viruses able to exploit eIF5A for their replication, and supporting the proposal that targeting eIF5A can be a strategy for the control of virus replication (Olsen and Connor, 2017). The broad range of viruses that relies on eIF5A for proper replication suggests that a general function of eIF5A may be at work for these viruses.

Here we evaluated the hypothesis that blocking hypusination can trigger activation of the innate immune system. Hypusine or eIF5A are involved in protein translation initiation, elongation and termination. eIF5A alleviates stalling on different difficult to translate motifs (Schuller et al., 2017; Pelechano and Alepuz). Although it has been proposed that eIF5A is required for the synthesis of a specific subset of mRNAs (Gutierrez et al., 2013; Hanauske-Abel et al., 1995; Kang and Hershey, 1994), inhibition of eIF5A hypusination has been reported to block translation initiation through phosphorylation of eIF2α (Landau et al., 2010), and lead to global translation elongation and termination defects (Schuller et al., 2017). In addition, depletion of eIF5A or inhibition of hypusination has also been demonstrated to prevent arsenite-induced polysome disassembly and stress granule assembly (Li et al., 2010), indicating that eIF5A is required for the rapid onset of stress-induced translational repression. It agreement with these data, it has bfeen reported that depletion of eIF5A induces endoplasmic reticulum stress and an unfolded protein response (Mandal et al., 2016). In agreement with this data, we also observed an induction of the unfolded protein response (UPR) after eIF5A depletion or hypusine inhibition, as revealed by PERK activation and increased levels of phosphorylated eIF2α. It is known that induction of UPR suppresses the replication of transmissible gastroenteritis virus (TGEV) (Xue et al.,

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2018), porcine reproductive and respiratory syndrome virus (PRRSV) (Zhu et al., 2021), and different flaviviruses (Carletti et al., 2019). Interestingly, different pieces of evidence indicate that there is a direct interaction between UPR, early activation of innate antiviral responses, and cell-intrinsic inhibition of viral replication (Carletti et al., 2019; Xue et al., 2018; Zhu et al., 2021). Our results revealed that, indeed, inhibition of hypusination or eIF5A depletion induced an upregulation in the levels of IFN and IFN-response proteins through the NF-κB pathway. We then propose that influenza virus infection (or dsRNA stimuli) triggers the activation of the NF-κB pathway which induces the upregulation of hypusine and facilitates virus replication. In contrast, downmodulation or inhibition of eIF5A triggers an UPR response that leads to IFN production helping to control virus replication. In addition, eIF5A may favour virus replication through additional mechanisms. Here we propose that the PI3K regulatory subunit p85β may contain difficult to translate polyproline motifs and that eIF5A may contribute to its translation. Further studies are needed in order to elucidate whether this regulation plays a role in IAV replication.

As mentioned above, eIF5A has been reported to play an important role in stress granule formation. Stress granules are considered as regulatory components of the cellular stress response to virus infection. Interestingly, and similarly to eIF5A, SUMO has been reported to regulate stress granules formation and disassembly (Marmor-Kollet et al., 2020). Moreover, proteomic studies suggest that eIF5A interacts with SUMO (Smeltzer et al., 2021), and is post-translationally modified by SUMO (Becker et al., 2013; Hendriks and Vertegaal, 2016). Here we have demonstrated that eIF5A can be modulated by SUMO. Moreover, our data reveal that hypusination is not required for SUMO conjugation. In silico analysis pointed to different lysine residues in eIF5A as potential SUMO acceptors. In addition, previously reported proteomic studies suggested that some of these amino acids, as well as other lysine residues in eIF5A are conjugated to SUMO (Becker et al., 2013; Hendriks and Vertegaal, 2016; Lamoliatte et al., 2017). Our

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results revealed that K27, K34, K68, K85, and K126 lysine residues in eIF5A play a role in SUMO conjugation. SUMOylation can modulate subcellular localization, activity or stability. So far, no nuclear localization signal has been identified in eIF5A, and it has been suggested that its nuclear entry occurs through passive diffusion (Jao and Yu Chen, 2002), whereas its nuclear export may be mediated by exportin 4 in a hypusine-dependent manner (Lee et al., 2010). Here we show that mutation of the SUMOylation sites increased the nuclear localization of eIF5A1, raising the possibility that the altered localization of the SUMOylation mutants is due to a diminished interaction with exportin 4.

Evaluation of eIF5A SUMOylation under different stress conditions revealed an important increase in eIF5A SUMOylation after heat shock stress or MG132 treatment, in agreement with the reported proteomic data. We then hypothesized that SUMOylation of eIF5A may play a critical role in the cell response to these stressors. However, the instability of the SUMOylation mutants in mammalian cells makes them difficult to study. To solve this problem, we carried out experiments in yeast and found that the eIF5A SUMOylation mutant was unable to complement a yeast eIF5A knockdown even when expressed at levels comparable to the wild-type version. Therefore, we propose that SUMO has an impact on eIF5A activity independent of its stabilization. SUMOylation of eIF42 was previously reported to play a role is SG formation (Jongjitwimol et al., 2016) Here we demonstrate that SUMOylation of eIF5A is required for the disassembly of polysomes induced by heat shock stress.

Our results also revealed that mutation of the SUMOylation sites in eIF5A significantly diminishes its stability. Regulation of the stability of eIF5A has been reported to occur via STUB1-mediated ubiquitination and subsequent degradation (Shang et al., 2014). Our findings indicate that mutation of the SUMOylation sites in eIF5A1 potentiates its interaction with the ubiquitin ligase STUB1, suggesting that SUMO negatively modulates the eIF5A-STUB1 interaction.

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Interestingly, previously reported proteomic data indicate that most of the SUMOylation sites in eIF5A are also potential ubiquitin linkage sites, suggesting a complex interplay between both modifications. In addition to ubiquitin, the NEDD8 pathway can also facilitate the proteasome-mediated degradation of some proteins (Loftus et al., 2012; Vogl et al., 2020). Stabilization of the eIF5A SUMOylation mutants by treatment with the NEDDylation inhibitor MLN4924 led us to hypothesize that eIF5A may be a direct target of NEDD8. Interestingly, proteomic data recently identified eIF5A as a potential NEDDylation target and pointed to lysine k27 as a potential neddylation site (Lobato-Gil et al., 2021). Here we confirmed that eIF5A is indeed a NEDD8 substrate. In addition, the NEDDylation of a mutant of eIF5A in lysine K27 suggests that additional lysine residues in eiF5A must be involved in NEDD8 conjugation. It has been demonstrated that some ubiquitin ligases such as MDM2, c-Cbl, parkin or IAP can also function as NEDD8 ligases (Broemer et al., 2010; Embade et al., 2012; Watson et al., 2006; Xirodimas et al., 2004; Zuo et al., 2013). Our results revealed that STUB1 promotes eIF5A1 NEDDylation. Therefore, we propose that STUB1 may function as a NEDD8 ligase on eIF5A1. These results and the induction of eIF5A NEDDylation or SUMO2-conjugation in response to SUMOylation and NEDDylation inhibitors, respectively, lead us to propose that there is a negative interplay between SUMO2 and NEDD8 conjugation on eIF5A. However, whether the increased interaction with STUB1 of the SUMOylation mutants promotes their ubiquitination or NEDDylation is still unknown. Future studies will be needed to understand the function of NEDD8 conjugation on eIF5A.

In addition to eIF5A, STUB1 has been shown to promote the ubiquitination of c-myc or p21, among others (Biswas et al., 2017; Paul et al., 2013). Interestingly, treatment with the NEDDylation inhibitor MLN4924 has been also demonstrated to induce the upregulation of these STUB1 substrates, although no direct conjugation of NEDD8 to these proteins has been proved. Further studies will be necessary to

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evaluate the promotion of NEDD8 conjugation on these substrates by STUB1.

NEDDylation has emerged as a novel regulatory mechanism for the control of many different cellular processes and recently several reports support that the host exploits the NEDDylation pathway to fight against virus infection. Thus, NEDDylation has been reported to facilitate antiviral responses in zebrafish (Yu et al., 2019) and to be essential for RNA virus-triggered IFN expression or IFN promoter activation in different cell types (Zhao et al., 2021). However, the NEDD8 ultimate buster 1 (NUB1) has been identified as an interferon (IFN)-inducible protein that downregulates NEDD8 expression and its conjugated system (Kito et al., 2001). It has been reported that IAV infection induces the upregulation of Cullin NEDDylation (Sun et al., 2018). In addition, NEDD8 has been demonstrated to conjugate to two IAV proteins triggering their degradation. These data suggest that NEDDylation plays an important activity against influenza virus replication. However, treatment with the NEDDylation inhibitor MLN4924 has been reported to inhibit influenza virus replication, suggesting that NEDDD8 facilitates IAV replication (Li et al., 2020; Zhang et al., 2017).

Our data revealed changes in the NEDDylated proteome upon influenza virus infection. We detected changes in the NEDDylation of several proteins which were previously known to be NEDDylated such as RPS27A (Xiong et al., 2020), or histone proteins (Li et al., 2014), and we have identified some potential novel NEDD8 substrates. Interestingly, RPS27A is a host factor critical for IAV replication (Karlas et al., 2010) and NEDD8 has been shown to stabilize it (Xiong et al., 2020). NEDDylation of histones is involved in the DNA damage response, a pathway that is induced upon influenza virus infection and which activation is associated with the disease outcome (Li et al., 2018). NEDD8 has been proposed to negatively modulate the DNA damage repair process by inhibiting H2A ubiquitination (Li et al., 2014), and facilitate the DNA damage response through NEDDylation of H4 (Ma

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et al., 2013). Further studies are needed in order to both decipher whether a decrease in the NEDDylation of the RPS27A is a virus strategy to decrease its degradation and to discover the functions of NEDD8 conjugation on histones and on some of the putative new NEDD8 substrates.

One of these substrates is VDAC1, a mitochondrial protein that regulates mitophagy and apoptosis. VDAC1 can be regulated through both mono and polyubiquitination (Ham et al., 2020; Sun et al., 2012). Polyubiquitination of VDAC1 has been reported to occur in several lysine residues including K110, the lysine residue in VDAC1 that we identified as involved in NEDD8 conjugation. It has been reported that a VDAC1 protein deficient in polyubiquitination hampers mitophagy and that IAV induces mitophagy that is required for the clearance of damaged mitochondria. Although we do not know the consequences of NEDDylation on VDAC1 functionality, we observed that overexpression of a VDAC1 NEDDylation mutant has a negative impact on IAV replication. Interestingly, the IAV protein PB1-F2 has been shown to interact with VDAC1 to induce mitochondria permeabilization and apoptosis (Zamarin et al., 2005). It will be interesting to determine whether the binding of PB1-F2 to VDAC1 modulates its NEDDylation.

In addition to cellular proteins, viral proteins are also NEDD8 targets. Here we demonstrated that NEDD8 conjugates to IAV NS1 protein and we identified the lysine residues K108, K110, K126, and K131 in NEDD8 as the NEDD8 acceptors. NS1 protein has been proposed to be modulated through numerous post-translational modifications and some of them occur on the same lysine residue of NS1. Therefore, a potential rivalry between post-translational modifications could exist. Thus, it has been proposed that K131 is a SUMOylation site, K110 and K126 are ISGylation sites and K108 is acetylated (Ma et al., 2020, p. 1; Santos et al., 2013; Way et al., 2020; Zhao et al., 2010). Our data suggest that NEDD8 modulates the binding of NS1 to dsRNA and that NEDDylation is necessary for the NS1-mediated attenuation of the IFN

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signaling and for efficient IAV replication. These observations are in agreement with the inhibition of IAV replication upon blocking NEDDylation (Sun et al., 2018).

Recently, it has been found that the RIG-I signaling and the induction of IFN in response to RNA virus infection requires the E3 ligase for UFMylation UFL1 and the UFMylation process (Snider et al., 2022). Moreover, UFMylation has been found to regulate responses to IFN-ϒ and to be required for resistance to IAV in mice (Balce et al., 2021). Here, we demonstrated that IAV infection induces UFMylation, in agreement with the identification of UFMylation as a regulator of the antiviral innate immune response. However, we also observed that UFMylation is required for efficient influenza virus replication *in vitro*, suggesting that IAV can hijack the UFMylation pathway to its own advantage. Additionally, it is possible that UFM1 or UFMylation play additional roles in IAV replication. Thus, UFMylation of the ribosomal protein RPL26 has been reported to increase the translation of hepatitis A virus (HAV) proteins, and components of the UFMylation machinery have been identified as required for efficient HAV replication (Kulsuptrakul et al., 2021). Moreover, it has been proposed that UFMylation plays critical roles in the regulation of ER stress response. Several reports highlight the relevance of the ER stress response in IAV infection (Frabutt et al., 2018; Hassan et al., 2012). IAV infection triggers ER stress that is modulated by the IAV NS1 protein (Mazel-Sanchez et al., 2021). It will be of great interest to study whether UFM1 plays a role in this dynamic process.

Finally, our results demonstrated that UFM1 can be modified by both SUMO2 and NEDD8, suggesting the existence of an interplay between these post-translational modifications. Importantly, we have also shown that influenza virus infection modulates the UFM1 SUMOylation. Future studies will be designed in order to analyze the effect of SUMO conjugation on UFM1 activities and its potential impact on virus replication.

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In summary, our work provides a comprehensive study of the complex interplay between influenza A virus and PTM pathways, offering potential molecular mechanisms of action and regulation of some modifiers, and opening new questions about (i) the crosstalk between the different PTM, (ii) their impact on cellular and viral protein functions, (iii) their role during influenza virus infection, and (iv) the control of these PTM as therapeutic strategies against the virus.

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8. CONCLUSIONS

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- The translation factor eIF5A is activated by infection with IAV, VSV or dsRNA stimuli.
- Activation of eIF5A by dsRNA is mediated by NF-κB pathway.
- Activation of eIF5A is essential for the replication of different viruses including IAV, VSV, mayaro, una, chikungunya, punta toro and zika virus.
- Inhibition of eIF5A induces ER stress and IFN production that in turn induces an antiviral state.
- eIF5A1 and eIF5A2 are modified by SUMO2 *in vivo* and *in vitro*.
- SUMO can conjugate to multiple lysine residues in eIF5A1.
- Mutation of the SUMOylation sites in eIF5A1 modulates its localization and stability.
- SUMOylation of eIF5A1 is modulated by different types of stress.
- SUMOylation of eIF5A1 in lysine residues K68, K85, and K126 is essential for yeast survival
- SUMOylation of eIF5A in lysine residues K68, K85, and K126 is required for polysome disassembly in response to heat shock stress.
- eIF5A1 is modified by NEDD8 *in vitro* and *in vivo*
- Inhibition of NEDDylation stabilizes eIF5A1.
- Mutation of the SUMOylation sites in eIF5A1 facilitates its interaction with the E3 ubiquitin ligase STUB1.
- STUB1 regulates the NEDDylation of eIF5A1.
- Influenza virus infection induces global reprogramming of NEDDylation proteome
- NS1 is modified by NEDD8
- NEDD8 conjugates to lysine residues K108, K110, K126, and K131 in NS1.
- Mutation of the NEDDylation sites in NS1 abolishes the inhibitory activity of NS1 on the IFN signaling pathway
- Influenza virus infection triggers host cell UFMylation
- UFMylation is necessary for the replication of IAV.
- UFM1 can be modified by both SUMO2 and NEDD8
- Infection with influenza A virus modulates both UFM1 SUMOylation and NEDDylation

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9. TABLES

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Table 1. Primers used for cloning and mutagenesis

NAME	SEQUENCE
NS1-K20R-F	5'-CTTTGGCATGTCCGCAGACGAGTTGCAGACCAA-3'
NS1-K20R-R	5'-TTGGTCTGCAACTCGTCTGCGGACATGCCAAAG-3'
NS1-K41R-F	5'-CTTCGCCGAGATCAGAGATCCCTAAGAGGAAGG-3'
NS1-K41R-R	5'-CCTTCCTCTTAGGGATCTCTGATCTCGGCGAAG-3'
NS1-K62R-F	5'-GCCACACGTGCTGGAAGACAGATAGTGGAGCGG-3'
NS1-K62R-R	5'-CCGCTCCACTATCTGTCTTCCAGCACGTGTGGC-3'
NS1-K70R-F	5'-GTGGAGCGGATTCTGAGAGAAGAATCCGAT-3'
NS1-K70R-R	5'-ATCGGATTCTTCTCTCAGAATCCGCTCCAC-3'
NS1-K78R-F	5'-GATGAGGCACTTAGAATGACCATGGCC-3'
NS1-K78R-R	5'-GGCCATGGTCATTCTAAGTGCCTCATC-3'
NS1-K108R-F	5'-TCCATGCTCATACCCAGACAGAAAGTGGCAGGC-3'
NS1-K108R-R	5'-GCCTGCCACTTTCTGTCTGGGTATGAGCATGGA-3'
NS1-K110R-F	5'-CTCATACCCAAGCAGAGAGTGGCAGGCCCTCTT-3'

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NS1-K110R-R	5'-AAGAGGGCCTGCCACTCTCTGCTTGGGTATGAG-3'
NS1-K126R-F	5'-CAGGCGATCATGGATAGAAACATCATACTGAAA-3'
NS1-K126R-R	5'-TTTCAGTATGATGTTTCTATCCATGATCGCCTG-3'
NS1-K131R-F	5'-AAGAACATCATACTGAGAGCGAACTTCAGTGTG-3'
NS1-K131R-R	5'-CACACTGAAGTTCGCTCTCAGTATGATGTTCTT-3'
NS1-K175R-F	5'-ACTGCTGAGGATGTCAGAAATGCAGTTGGAGTC-3'
NS1-K175R-R	5'-GACTCCAACTGCATTTCTGACATCCTCAGCAGT-3'
NS1-K217R-F	5'-CCTCCACTCACTCCAAGACAGAAACGAGAAATG-3'
NS1-K217R-R	5'-CATTTCTCGTTTCTGTCTTGGAGTGAGTGGAGG-3'
NS1-K219R-F	5'-CTCACTCCAAAACAGAGACGAGAAATGGCGGGA-3'
NS1-K219R-R	5'-TCCCGCCATTTCTCGTCTCTGTTTTGGAGTGAG-3'
eIF5A-K27A-F	5'-TGCTCAGCATTACGTGCGAATGGCTTTGTGGTG-3'
eIF5A-K27A-R	5'-CACCACAAAGCCATTCGCACGTAATGCTGAGCA-3'
eIF5A-K34A-F	5'-GGGTTTGTGGTGCTCGCAGGCCGGCCATGTAAG-3'

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eIF5A-K34A-R	5’-CTTACATGGCCGGCCTGCGAGCACCACAAACCC-3’
eIF5A-K68A-F	5’-ATCTTTACCGGGAAGGCATATGAAGATATCTGC-3’
eIF5A-K68A-R	5’-GCAGATATCTTCATATGCCTTCCCGGTAAAGAT-3’
eIF5A-K85A-F	5’-GATGTCCCCAACATCGCAAGGAATGACTTCCAG-3’
eIF5A-K85A-R	5’-CTGGAAGTCATTCCTTGCGATGTTGGGGACATC-3’
eIF5A-K126A-F	5’-GGAGATTGAGCAGGCGTACGACTGTGGAGAAGA-3’
eIF5A-K126A-R	5’-TCTTCTCCACAGTCGTACGCCTGCTCAATCTCC-3’
HA-eIF5A-F	5’-GGGGAATTCAATGGCAGATGACT-3’
HA-eIF5A-R	5’-CCCGGGCCCTTATTTTGCCATG-3’
HA-NS1-F	5’-GGGCTCGAGGCCACCATGGATCCAAACACTGTGTCAAGCTTTC-3’
HA-NS1-R	5’-CGATGGGCCCTCAAACTTCTGACCTAATTGTTCCCGCC-3’
HA-VDAC1-F	5’-GGGGGATCCATGGCTGTGCCACCCACG-3’
HA-VDAC1-R	5’-CCGGGCCCTTATGCTTGAAATTCCAGTCCTAGACCAAGCTTG-3’
HA-RPS27A-F	5’-GGGGGATCCATGCAGATTTTCGTGAAAACCCTTACG-3’

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HA-RPS27A-R	5'-CCCGGGCCCTTACTTGTCTTCTGGTTTGTTGAAACAG-3'
T7-eIF5A-F	5'-GGATCCTAATACGACTCACTATAGGAGCCACCATGTGTGGAACTGGGGGG-3'
T7-eIF5A-R	5'-TTATTTTGCCATGGCCTTGATTGCAACAGC-3'
UFM1-DELTAG-F	5'-ATTCCTAGAGATCGTTGATGAAGTTGTTAAGCGGCC-3'
UFM1-DELTAG-F	5'-GGCCGCTTAACAACTTCATCAACGATCTCTAGGAAT-3'
pDZ-F	5'-CAGCCATTGCCTTTTATGGTAATCG-3'
pDZ-R	5'-CCCATCGATTCAAACTTCTGACCTAATTGTTCCCGC-3'
pDZ-NS1-F	5'-GACATAATGGATCCAAACACTGTGTCAA-3'
pDZ-NS1-R	5'-GTGTTTGGATCCATTATGTCTTTGTCAC-3'
VDAC-K110R-F	5'-CCTAACACTGGGAAAAGAAATGCTAAAATCAAG-3'
VDAC-K110R-R	5'-CTTGATTTTAGCATTTCTTTTCCCAGTGTTAGG-3'

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Table 2. Primers used for qRT-PCR

NAME	SEQUENCE
p85-F	5'-GTCCGAGATGCTTCTAGCAAG-3'
p85-R	5'-CAGCTTATTGTTCCCGCCTTT-3'
eIF5A-F	5'-TGAAGATATCTGCCCGTCAA-3'
eIF5A-R	5'-GCTGTCCTGGAGCAGTGATA-3'
ODC-F	5'-GTGCCACGCTCAGAACCAG-3'
ODC-R	5'-CCACCGCCAATATCAAGCAGA-3'
SSAT-F	5'-GGGAAGAAAAGCAAAAGACG-3'
SSAT-R	5'-AATGGAGGTTGTCATCTACAGC-3'
IFNBETA-F	5'-ATTGCCTCAAGGACAGGATG-3'
IFNBETA-R	5'-GGCCTTCAGGTAATGCAGAA-3'
IFNALPHA-F	5'-AAATACAGCCCTTGTGCCTGG-3'
IFNALPHA-R	5'-GGTGAGCTGGCATACGAATCA-3'
ISG15-F	5'-ACTCATCTTTGCCAGTACAGGAT-3'
ISG15-R	5'-CAGCATCTTCACCGTCAGGTC-3'
IFIT2-F	5'-CGAACAGCTGAGAATTGCAC-3'
IFIT2-R	5'-CAAGTTCCAGGTGAAATGGC-3'
IFIT3-F	5'-TGAGGAAGGGTGGACACAACTGAA-3'
IFIT3-R	5'-AGGAGAATTCTGGGTTGTTGGGCT-3'
NS1-F	5'-GGAAGGGGCAGTACTCTCGG-3'
NS1-R	5'-TTTCTGCTTGGGTATGAGCA-3'
M1-F	5'-CGCACAGAGACTTGAAGATG-3'
M1-R	5'-TGGATCCCCGTTCCCATTAA-3'

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NP-F	5’-GCGTCTCAAGGCACCAAAC-3’
NP-R	5’-AACCGTCCCTCATAATCAC-3’
PB1-F	5’-ACTTACTGGTGGGATGGT-3’
PB1-R	5’-CTGAAATTGGCAACAAAC-3’
VSV-N-F	5’-CGGATGCTTCAAGAACCAGCG-3’
VSV-N-R	5’-GTCACGACCTTCTGGCACAAGAG-3’
VSV-G-F	5’-GACCGAAGTATATAACACATTC-3’
VSV-G-R	5’-GAGTCACCTGGACAATCGCTGC-3’
VSV-L-F	5’-GCCAATCCCATCTCAACATCTC-3’
VSV-L-R	5’-GTATTCAATTGGTTTGTTGCCC-3’
VSV-M-F	5’-CCGTTCAGAACATACTCAGATG-3’
VSV-M-R	5’-GGTACATTGAGCATGGGAGGGG-3’
VSV-P-F	5’-CGGAGTGGAAGAGCATACTAG-3’
VSV-P-R	5’-CTCGTCGGATTCAAGCTCAGG-3’
NEDD8-F	5’-TTAAAGTGAAGACGCTGACC-3’
NEDD8-R	5’-TCATCATTCATCTGCTTGCC-3’
UBC12-F	5’-AGCAGAAGAAGGAGGAGGAG-3’
UBC12-R	5’-TCATCAGGACAGATGACCAG-3’
UBE2F-F	5’-ACCCCAACATCACAGAGAC-3’
UBE2F-R	5’-TTTAATCCCCAAACGACATCC-3’
UFM1-F	5’-GATCACGCTGACGTCGGACCC-3’
UFM1-R	5’-CCAACACGATCTCTAGGAATAATCCGC-3’
UFC1-F	5’-CGAGATCGTGAGTTGTGGGTGC-3’
UFC1-R	5’-CCAGCTCAGGAACTGCAATTTCTG

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UBA5-F	5'-GTGGTTGCTGGGATCTTAGTACAAAACG
UBA5-R	5'-CCTCAGATACCAGCTCAATACCCCATTC

Table 3. Yeast strains used in this work

Strain	Genotype*	Source
PAY723	*MATa BNI1-HA*	(Muñoz-Soriano et al., 2017)
PAY725	*MATa hyp2-1 BNI1-HA*	(Muñoz-Soriano et al., 2017)
YTL734	PAY725 *LEU2::pAG425GPD-ccdB*	This study
YTL736	PAY725 *LEU2::pAG425GPD-eIF5A1-WT-FTH*	This study
YTL738	PAY725 *LEU2::pAG425GPD-eIF5A1-K50R -FTH*	This study
YTL740	PAY725 *LEU2::pAG425GPD-eIF5A1-3KA -FTH*	This study
YTL669	*MATa*	Euroscarf (Brachmann et al., 1998)
PAY717	*MATa hyp2-3*	(Muñoz-Soriano et al., 2017)
YTL718	PAY717 *LEU2::pAG425GPD-ccdB*	This study
YTL720	PAY717 *LEU2::pAG425GPD-eIF5A1-WT-FTH*	This study
YTL722	PAY717 *LEU2::pAG425GPD-eIF5A1-K50R-FTH*	This study
YTL724	PAY717 *LEU2::pAG425GPD-eIF5A1-3KA-FTH*	This study

*All strains are haploid BY4741 derivatives (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*)

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Influenza A virus causes a respiratory disease that affects millions of individuals every year. The host response to IAV is necessary for viral clearance, but the virus manipulates cellular machinery to improve its replication. Here we studied the virus-cell interactions to decipher the molecular mechanisms of the disease. Our data revealed that activation of eIF5A is key for IAV and we described SUMOylation and NEDDylation as novel mechanisms to control eIF5A. Also, we identified NS1 and several cellular proteins as targets for NEDD8 upon IAV, and deciphered the role of NS1 NEDDylation. Finally, we demonstrated, for the first time, an interplay between UFMylation and IAV. In summary, this work demonstrates the importance of post-translational modifications in influenza virus-cell interplay.