

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA



TESIS DOCTORAL

**Función y mecanismos moleculares de las proteínas
inducidas por interferón IFI27 e IFI6 en la replicación viral e
inducción de respuestas inmunitarias innatas frente a las
infecciones por virus ARN**

**Molecular function and mechanisms of interferon-induced
proteins IFI27 and IFI6 in viral replication and induction of
innate immune responses against RNA virus infections.**

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Darío López García

DIRECTORA

Marta López de Diego

Madrid



UNIVERSIDAD
COMPLUTENSE
MADRID

**FUNCIÓN Y MECANISMOS MOLECULARES DE LAS PROTEÍNAS
INDUCIDAS POR INTERFERÓN IFI27 E IFI6 EN LA REPLICACIÓN
VIRAL E INDUCCIÓN DE RESPUESTAS INMUNITARIAS INNATAS
FRENTE A LAS INFECCIONES POR VIRUS ARN**

**MOLECULAR FUNCTION AND MECHANISMS OF INTERFERON-
INDUCED PROTEINS IFI27 AND IFI6 IN VIRAL REPLICATION AND
INDUCTION OF INNATE IMMUNE RESPONSES AGAINST RNA
VIRUS INFECTIONS**

DARÍO LÓPEZ GARCÍA

DIRECTORA: DRA. MARTA LÓPEZ DE DIEGO

**CENTRO NACIONAL DE BIOTECNOLOGÍA
(CNB-CSIC)**

**FACULTAD DE MEDICINA
PROGRAMA DE DOCTORADO EN INVESTIGACIÓN BIOMÉDICA
UNIVERSIDAD COMPLUTENSE DE MADRID**

TESIS DOCTORAL, MADRID 2026

ACKNOWLEDGMENTS

Cuando era pequeño me imaginaba a mí mismo descubriendo nuevos tiburones, jugando en la NBA, siendo piloto de fórmula 1 o incluso siendo Spider-Man... Todo eso sigue sonando muy bien, pero puedo decir con alegría, que este “espíritu aventurero” ha conducido al desarrollo de una tesis doctoral. Estos años han sido toda una aventura, una aventura muy bonita, que me ha mostrado la importancia de la paciencia, la perseverancia, de perseguir tus objetivos y a confiar en tu intuición, aunque parezca la opción más difícil. Todo esto ha sido posible, en primer lugar, gracias a mi directora de tesis, Marta. Tú me diste la oportunidad y la confianza desde el primer momento, y eso es algo que siempre te agradeceré. Tu disciplina de trabajo y tu perseverancia son admirables, y estoy muy feliz de haber hecho la tesis contigo, eres una científica increíble, pero sobre todo una muy buena persona. Muchas gracias por tu guía y tu apoyo.

Agradecer a todo el personal del Centro Nacional de Biotecnología (CNB-CSIC), al servicio de proteómica, genómica, microscopía óptica avanzada, al servicio de bioseguridad, cocinas, almacén, a Santa, etc., todos ellos esenciales para el desarrollo de esta tesis doctoral; también, agradecer a la fundación “la Caixa”, por haberme financiado todos estos años tan cruciales en mi formación. Gracias a la asociación CSIC-Fulbright por financiar mi estancia de investigación en el Icahn School of Medicine at Mount Sinai, Nueva York. Gracias a todos los colaboradores por su ayuda y enseñanzas.

Mi agradecimiento a la coordinadora del programa de doctorado en Investigación Biomédica, la Dra. Ángeles Vicente López, a la comisión académica, por su constante ayuda y guía durante toda la tesis doctoral. Asimismo, quiero agradecer la ayuda de mi tutor académico, el Dr. Eduardo Martínez Naves.

Asimismo, agradecer enormemente a la Dra. Ana Fernández Sesma, mi supervisora durante mi estancia en Nueva York, así como a todas las personas de su laboratorio, que hicieron de mi estancia una experiencia increíble e inolvidable. Thank you, Ana, Dabeiba, Rafa, Victoria, Jeury, Eva, Dani, and Tarrell for making me feel at home, let's go Mets!

También quiero tener unas palabras de dedicatoria y agradecimiento, para aquellas personas que me han acompañado estos años, ayudándome a disfrutar del camino y a crecer. Toda tesis se hace gracias a un equipo y gente que te apoya, te ayuda, y con la que compartes los buenos y los malos momentos. Por ello quiero agradecer tanto a la gente que estuvo como la que está en el laboratorio 16 (116 ahora). Laura, Sandra, y Raúl, por todo lo que aprendí de vosotros. A Vanessa, muchas gracias por toda tu ayuda y enseñanzas, y por ser la alegría del laboratorio, se te echa de menos. A Paula, compañera de batallas y de viajes desde el principio. A Julia, mi tocaya de cumple, que demuestra que la gente del 23 de septiembre es top. A Isa, que es como una más del (1)16, prometo que en el próximo viaje a Canarias te dejaré descansar en alguna playa más de 10 minutos. Gracias por vuestra amistad. Gracias David, te llevas un amigo para lo que necesites en tu amada ciudad de Madrid. A Noelia y Freya, mis compañeras de piso que te sacan una risa cuando lo necesitas, ha sido muy bonito que hayáis estado presentes desde el principio de esta aventura, gracias. A Andrés, por sus buenos consejos y su ayuda siempre dispuesta. A María y Elena por los buenos momentos compartidos, y a Alberto, que, aunque no pudimos acabar compartiendo equipo de baloncesto, lo que habría sido... A Yadi, Sonia y Domingo, por su colaboración y ayuda cuando la he necesitado. El laboratorio 16 no se puede entender sin su otra mitad, la gente más divertida del CNB, el 15. Josema, me llevo de aquí a un amigo de por vida, gracias por ser mi “gymbro” preferido, mi compi de misiones secundarias, te queremos todos mucho. Jose, un amigo excepcional y con un corazón tremendo, gracias por acogerme cuando llegué y por todo lo que hemos vivido juntos. A Sara, un ejemplo de lo que es ser una profesional y una científica, y sobre todo una bellísima persona que sé que llegará a

donde se proponga. A Darío, por hacer mejores las comidas con sus espectaculares chistes y su alegría contagiosa; a Edu, nuestro guionista preferido y un amigo increíble; a Esther, por compartir más congresos y menos eventos apocalípticos; a Uxía, te echamos de menos.

A Juanma, el cocinero de las mejores hamburguesas de Madrid, y a mis nuevos vecinos preferidos, Ricardo, Quique y Carlos, muchas gracias por vuestra amistad, los viernes y las escapadas con vosotros dan mucha vida.

Agradecer a todas aquellas personas que han crecido conmigo, incluso antes de comenzar la aventura de la tesis doctoral. A Rubén, ese amigo que es más bien un hermano, te quiero. A Marc, mi compañero de aventuras, eres una persona muy especial y muestra de ello es que tienes que volver para que todos podamos juntarnos. A Erik, echo de menos que me lleves a una ruta de montaña donde tema por mi integridad. A Fiestas, la persona que más honor hace a su nombre. A Plo, nuestro italiano favorito, te echamos de menos en Madrid. A María, por aquellas tardes de piscina en el desierto madrileño de agosto. Y a todas aquellas personas que me vieron crecer y con los que compartí una infancia y adolescencia inmejorable, gracias.

A Pablo Carr y a Elena, gracias, sois amigos muy especiales, no os hagáis tanto de rogar para veros, se os echa de menos.

A toda mi familia de la universidad, a Dani y Laurington, unos compañeros y amigos insuperables, sois hogar; a Del Amo, que aunque te cayera mal al principio, sé que al final me quieres porque yo a ti también; JJavi, un auténtico máquina, el corazón de grupinchi, conseguirás todo lo que te propongas; Valeria, me gustaría que viajases menos para poder verte más... pero siempre vale la pena verte ni que sea un ratito; a Paula Hermenegildo, por tu risa contagiosa y tu enorme corazón, y a Raúl, que ya eres un bioquímico más, te toca empezar la tesis; a Laura por tantos bonitos momentos y crecimiento compartidos, y gracias a toda la familia MartínRo.

A Patri, por ser una compañera increíble en nuestra aventura por el Upper West Side, echo de menos el tiki chick. A Estrella, por convertirse en una hermana mayor, y en una amiga esencial. A Yako, por alegrarme las vueltas a casa con sus charlas. El piso sin vosotros no es lo mismo.

A toda mi familia, una familia increíble y llena de amor. A mi familia Granaina, a mi querida tita Yoli, a Mario, por sus lecciones de baloncesto, y a mi profe preferida, Alba; a toda mi familia de Maracena, a mis abuelos, mis tios, mis primos, y en especial a mis primillos Alejandra y Jose. A mi familia Malagueña, siempre estoy desando ir a veros, me llenáis de alegría (y de café, que para mí es casi lo mismo), a mi tito Alfredo y mi tita Anabel; a mi prima Ana, la persona más perseverante y valiente, y Alfredo, la persona a la que le debo haber cumplido un sueño que ahora es un lugar de felicidad, el buceo.

Y, por último, agradecer y dedicar este trabajo a las personas más esenciales en mi vida. Neus, eres luz y felicidad en mi día a día, la mejor persona que he conocido, por seguir luchando y creciendo juntos. Estos años de tesis han sido mucho más bonitos gracias a ti. Timus molt. Gràcies a la Núria, l'Agustí i el Marc, per acollir-me. L'Empordà se sent com una segona casa gràcies a vosaltres.

A mis Padres, Antonio y Silvia, mis referentes, las personas que me lo han dado todo y me han hecho ser quien soy hoy. Espero que estéis orgullosos y contentos, porque este trabajo es vuestro; trabajo que también dedico a Morita, que sigue buscando la sombra del romero allá donde esté. A mi hermano, mi Dr. preferido, ídolo, la persona más honesta, leal, inteligente, el mejor amigo y hermano que se puede imaginar, que siempre ha estado ahí, firme y cercano para ayudar y acompañar cuando lo necesitas. Eres todo un ejemplo, nos haces feliz a todos. Gracias.

Funding

This work was supported by: the “Atracción de Talento Investigador” programme (2017-T1/BMD-5155) funded by “Comunidad de Madrid” to Marta López de Diego (MLD). The European Commission – NextGenerationEU (Regulation EU 2020/2094), through Spanish National Research Council (CSIC)’s Global Health Platform (PTI Salud Global, CSIC-COV19-012/012202020E086), to MLD; by grant RTI-2018-094213-A-I00, funded by MCIN/ AEI /10.13039/501100011033, to MLD; by grant PID-2021-123810OB-I00, funded by MCIN/ AEI /10.13039/501100011033 and by FEDER, to MLD; by grant PID2024-155405OB-I00 funded by MCIN/AEI/10.13039/501100011033 and by FEDER, UE, to MLD; and by grant CNS2022-135276, funded by MCIN/AEI/10.13039/501100011033 and the European Union NextGenerationEU/PRTR, to MLD.

The project that gave rise to these results received the support of a fellowship from “la Caixa” Foundation (ID 100010434). The fellowship code is LCF/BQ/DR22/11950020. “la Caixa” foundation funded my PhD salary with a INPhINIT Retaining fellowship.

During the development of the doctoral thesis, a research stay was conducted at the Icahn School of Medicine at Mount Sinai, New York, United States of America. This stay was performed under the supervision of Dr. Ana Fernández-Sesma (15/08/2024 – 15/12/2024), and thanks to CSIC-Fulbright funding. Fellowship codes: FULBR23035; PS00372239.

Licenses: The illustrations in this doctoral thesis were created using BioRender (<https://www.biorender.com/>). Graphics were represented with GraphPad Prism (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

TABLE OF CONTENTS

TABLE OF CONTENTS – FIGURES AND TABLES	I
TABLE OF CONTENTS - LIST OF ABBREVIATIONS	V
RESUMEN	1
ABSTRACT	3
1. INTRODUCTION	7
1.1. PUBLIC HEALTH AND SOCIAL CHALLENGES POSED BY RNA VIRUSES	7
1.1.1. SARS-CoV-2	7
1.1.2. Influenza A virus (IAV)	9
1.1.3. Dengue virus (DENV)	12
1.2. INNATE IMMUNE RESPONSES AFTER RNA VIRUS INFECTIONS	14
1.2.1. Viral RNA specific PRRs	15
1.2.1.1. RIG-I-Like Receptors (RLRs) and the initiation of host innate immune responses	16
1.2.2. Viral RNA-dependent effectors	19
1.2.2.1. PKR effector activity and Stress-Granules (SGs) generation	20
1.3. IN SEARCH OF NOVEL FUNCTIONS OF IFI27 AND IFI6 PROTEINS	24
1.3.1. Genetic and molecular characteristics of IFI27 and IFI6	24
1.3.2. Importance of IFI27 and IFI6 in viral infections of major concern: SARS-CoV-2, IAV, and DENV	26
2. OBJECTIVES AND HYPOTHESIS	31
3. MATERIALS AND METHODS	35
3.1. CELL CULTURES	35
3.1.1. Cell lines and cell culture conditions	35
3.1.2. Generation of hACE2-expressing A549 and HEK-293T cells	36
3.1.3. Generation of A549 and A549-hACE2 cells KO for IFI27 or IFI6	38
3.1.4. Generation of IFI27-HA-stably expressing cell lines	39
3.1.5. Generation of monocyte-derived dendritic cells (MoDCs)	40
3.2. VIRUSES	40
3.2.1. Influenza A virus (IAV)	40
3.2.2. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)	41
3.2.3. Dengue virus 2 (DENV2)	41
3.2.4. Sendai virus (SeV)	41
3.2.5. Recombinant Vesicular Stomatitis virus (rVSV), encoding the green fluorescent protein (GFP) (rVSV-GFP)	41
3.3. VIRUS TITRATIONS	41
3.3.1. Immunofocus assay for IAV and DENV2	41
3.3.2. Plaque assays for SARS-CoV-2, DENV2, and rVSV-GFP	43

3.4. DNA MANIPULATION AND ANALYSIS -----	44
3.4.1. Polymerase chain reaction (PCR) -----	44
3.4.2. DNA electrophoresis in agarose gels -----	44
3.4.3. DNA fragment's purification-----	45
3.4.4. DNA cloning: restriction enzymes, phosphorylation, dephosphorylation, and ligation of DNA. -----	45
3.5. PLASMIDS AND BACTERIAL STRAINS-----	45
3.5.1. Plasmids used for transfection assays-----	45
3.5.2. Plasmids used for the generation of KO cells -----	49
3.5.3. Plasmids used to generate recombinant IAVs (rIAVs)-----	50
3.5.4. Bacterial strains and growth-----	51
3.5.5. Bacterial transformation and plasmid purification-----	52
3.6. VIRUS RESCUE -----	52
3.7. STRATEGIES FOR THE MODULATION OF GENETIC EXPRESSION: PROTEIN OVEREXPRESSION (PLASMID TRANSFECTION) AND GENE KNOCK-DOWN (SMALL-INTERFERING RNA (siRNA) TRANSFECTION) -----	53
3.7.1. Protein overexpression in cells by transfecting protein-encoding plasmids -----	53
3.7.2. Protein knock-down in cells by transfecting small-interfering RNAs (siRNAs) (gene silencing)-----	53
3.7.3. Protein knock-down by using short hairpin RNA (shRNA)-coding lentiviruses ----	54
3.8. RNA MANIPULATION AND ANALYSIS OF mRNA LEVELS -----	56
3.8.1. Total RNA extraction and purification -----	56
3.8.2. cDNAs synthesis by retrotranscriptase (RT) reactions-----	56
3.8.3. Quantitative PCR (qPCR) -----	56
3.8.4. RNAseq-----	57
3.9. ANALYSIS OF PROTEIN EXPRESSION-----	58
3.9.1. Western blot -----	58
3.9.2. Translation shut-down assay -----	59
3.9.3. Fluorescence optical microscopy -----	60
3.9.4. Indirect immunofluorescence -----	61
3.9.5. Colocalization analysis-----	63
3.10. ANALYSIS OF PROTEIN-RNA INTERACTIONS -----	65
3.10.1. RNABindRPlus bioinformatic prediction -----	65
3.10.2. Binding of proteins to poly(I:C)-----	65
3.10.2.1. Binding of proteins to poly(I:C)-agarose -----	65
3.10.2.2. Binding of proteins to biotinylated poly(I:C)-----	66
3.10.3. RIG-I binding to SARS-CoV-2 RNA -----	67
3.11. ANALYSIS OF PROTEIN-PROTEIN INTERACTIONS-----	68

3.11.1. Co-IPs -----	68
3.11.1.1. Co-IPs with anti-HA or anti-FLAG-conjugated agarose beads -----	68
3.11.1.2. Co-IPs with protein A-conjugated sepharose beads -----	69
3.11.2. Mass-spectrometry -----	70
3.12. IFN RESPONSE ASSAYS: POLY(I:C) TREATMENT AND VIRUS INFECTIONS	71
3.13. <i>IN VIVO</i> EXPERIMENTS IN MICE -----	72
4. RESULTS -----	75
PART I. STUDY OF THE IMPORTANCE OF IFI27 AND IFI6 ON THE REPLICATION OF RNA VIRUSES: IAV, SARS-CoV-2, AND DENV2 -----	75
4.1.1. <i>IFI27</i> and <i>IFI6</i> are ISGs induced after poly(I:C) and IFN treatments, and after IAV, SARS-CoV-2, and DENV infections -----	75
4.1.2. <i>IFI27</i> and <i>IFI6</i> absence negatively affects IAV, SARS-CoV-2, and DENV2 replication -----	77
PART II: EFFECT OF IFI27 AND IFI6 ON ANTIVIRAL RESPONSES AFTER RNA VIRUS INFECTIONS -----	83
4.2.1. <i>IFI27</i> and <i>IFI6</i> negatively affect the induction of innate immune responses: A549 and HEK-293T cell systems -----	83
4.2.2. <i>IFI6</i> negatively affects the induction of antiviral responses in different cell systems: HAP-1 and BEAS-2B cell lines -----	90
4.2.3. <i>IFI27</i> and <i>IFI6</i> counteract the induction of IAV-induced innate immune responses <i>in vivo</i> -----	96
PART III: MOLECULAR MECHANISMS UNDERLYING THE MODULATION OF INNATE IMMUNE RESPONSES BY IFI27 AND IFI6 -----	100
4.3.1 Elucidating the molecular mechanisms of <i>IFI27</i> and <i>IFI6</i> on innate immunity modulation -----	100
4.3.1.1. <i>IFI27</i> and <i>IFI6</i> are RNA-binding proteins (RBPs) -----	100
4.3.1.2. <i>IFI27</i> and <i>IFI6</i> interact with RIG-I-like receptors and negatively modulate their activation -----	103
4.3.1.2.1. <i>IFI27</i> and <i>IFI6</i> interact with RIG-I through RNA binding -----	103
4.3.1.2.2. <i>IFI27</i> and <i>IFI6</i> partially co-localize with RIG-I in the cell cytoplasm -----	105
4.3.1.2.3. <i>IFI27</i> and <i>IFI6</i> negatively affect RIG-I activation -----	106
4.3.1.2.3.1 <i>IFI27</i> negatively affects RIG-I ability to interact with viral RNAs -----	108
4.3.1.2.3.2. Ubiquitination levels of RIG-I are reduced due to <i>IFI6</i> -mediated negative regulation -----	110
4.3.1.2.4. <i>IFI27</i> negatively regulates MDA5 activation -----	111
4.3.1.2.4.1. <i>IFI27</i> interacts with MDA5 through RNA binding -----	111
4.3.1.2.4.2. <i>IFI27</i> partially colocalizes with MDA5 at the cell cytoplasm -----	114
4.3.1.2.4.3. <i>IFI27</i> negatively affects MDA5 oligomerization and activation, as both proteins compete for RNA binding -----	116
4.3.1.3. <i>IFI27</i> affects protein translation by interacting with PACT and PKR -----	120
4.3.1.3.1. <i>IFI27</i> interacts with PACT and PKR proteins -----	120

4.3.1.3.2. IFI27 partially colocalizes with PACT and PKR in the cell cytoplasm----	125
4.3.1.3.3. IFI27 enhances PKR activation through a PACT-dependent mechanism -127	
4.3.1.3.3.1 IFI27 overexpression promotes PKR activation and consequently suppresses <i>de novo</i> protein synthesis-----	127
4.3.1.3.3.2. KO and knock-down of IFI27 reduce PKR activation and increases <i>de novo</i> protein synthesis -----	130
4.3.1.3.3.3. PACT is essential for IFI27-mediated PKR activation-----	133
4.3.1.3.4. IFI27 overexpression potentiates stress granules (SGs) formation in a PKR and PACT-dependent manner -----	136
4.3.1.3.5. PACT is a critical factor in IFI27-PKR interaction -----	140
5. DISCUSSION-----	145
5.1. STUDY OF THE IMPORTANCE OF IFI27 AND IFI6 ON THE REPLICATION OF RNA VIRUSES: IAV, SARS-CoV-2, AND DENV2 -----	145
5.2. EFFECT OF IFI27 AND IFI6 ON ANTIVIRAL RESPONSES AFTER RNA VIRUS INFECTIONS -----	149
5.3. MOLECULAR MECHANISMS UNDERLYING THE MODULATION OF INNATE IMMUNE RESPONSES BY IFI27 AND IFI6 -----	151
5.3.1. IFI27 and IFI6 are RBPs-----	151
5.3.2. IFI27 and IFI6 interact with RLRs and negatively modulate their function -----	152
5.3.3. IFI27-protein interactome: describing novel protein partners, such as PACT, PKR, and others -----	155
5.3.3.1. IFI27 affects protein translation by interacting with PACT and PKR-----	158
5.3.4. Integration of IFI27-mediated modulation of RLRs and PKR: two arms of innate immune responses -----	160
6. CONCLUSIONS -----	167
7. BIBLIOGRAPHY -----	171
8. APPENDIX -----	197
8.1. APPENDIX TABLES -----	197
8.2. RESEARCH ARTICLES IN WHICH I HAVE PARTICIPATED DURING THIS PHD THESIS -----	218

TABLE OF CONTENTS – FIGURES AND TABLES

Figure I1. Coronavirus (CoV) taxonomy with a particular emphasis on human CoVs (HCoVs).	7
Figure I2. SARS-CoV-2 structure-----	9
Figure I3. IAV virion structure and genome organization -----	11
Figure I4. Structure of DENV immature and mature particles -----	13
Figure I5. RLRs structure and conformational changes during activation-----	18
Figure I6. Schematic representation of RIG-I and MDA5 activation and transduction of antiviral signaling to surrounding cells-----	19
Figure I7. Structure, activation, and function of PKR -----	21
Figure I8. Schema of SGs composition -----	23
Table I1. Molecular characteristics of canonical IFI27 and IFI6 proteins -----	25
Table I2. IFI27 and IFI6 known importance on SARS-CoV-2, IAV, DENV, and other RNA viruses -----	27
Figure M1. Generation of hACE2-expressing retroviral vectors and cell transduction -----	37
Table M1. sgRNAs selected for IFI6 and IFI27 using the webpages of ATUM and CRISPOR.org -----	38
Figure M2. A549 IFI6 and IFI27 KO cells generation-----	39
Table M2. Primers used to perform the RT-PCR and to genotype target sequences in IFI6 and IFI27 KO clones -----	39
Figure M3. Monocyte-derived DCs generation from buffy coats-----	40
Figure M4. Immunofocus assay for IAV titration -----	42
Figure M5. Immunofocus assay for DENV2 titration -----	43
Figure M6. Plaque assay titrations for SARS-CoV-2, rVSV-GFP, and DENV2 -----	44
Table M3. Primers used for cloning of different pCAGGS-coding plasmids -----	46
Figure M7. Overlapping PCR to achieve single nucleotide mutagenesis of IFI27 cDNA-coding sequence-----	48
Table M4. Plasmids used in transfection experiments -----	49
Table M5. Primers used to generate complementary sequence to the selected sgRNA for KO generation -----	49
Table M6. Primers used to introduce TAV-2A autoproteolytic site in pDZ-NS-2xBsmBI plasmid -----	50
Table M7. Primers used to amplify IFI27 and IFI6 human sequences and clone them into the pDZ-NSsplit-2xBsmBI-2A plasmid -----	51
Figure M8. Schema of modified NS segment -----	51
Table M8. siRNAs from ThermoFisher Scientific, used for gene knock-down -----	54
Figure M9. shRNA-coding lentiviruses generation -----	55
Figure M10. CD14+ cells transduction using shRNA-coding lentiviruses and differentiation to DCs.-----	55
Table M9. Primers used for qPCR -----	56

Table M10. Taqman probes for qPCR-----	57
Table M11. Antibodies used in Western blot detection-----	59
Figure M11. Graphical representation of translation shut-down assay-----	60
Table M12. Primary antibodies used for immunofluorescence detection-----	62
Table M13. Secondary antibodies used for immunofluorescence detection, and DAPI (used for nuclear staining) -----	62
Figure M12. Indirect immunofluorescence and colocalization analysis -----	64
Figure M13. Analysis of the binding of proteins to poly(I:C) -----	66
Figure M14. Analysis of the binding of IFI27 to biotinylated-poly(I:C) -----	66
Figure M15. RIP assay of SARS-CoV-2 RNA binding to RIG-I protein-----	68
Figure M16. Graphical representation of co-IPs-----	70
Figure M17. Mass-spectrometry analysis of IFI27-bound proteins-----	71
Figure R1. Induction of IFI27 and IFI6 expression after poly(I:C), IFN- α , IAV, SARS-CoV-2, and DENV2 infections -----	76
Figure R2. IFI6 and IFI27 silencing negatively affects IAV, SARS-CoV-2, and DENV2 replication-----	78
Figure R3. DENV2 replication in MoDCs transduced with IFI6 shRNA-coding lentiviruses --	80
Figure R4. IFI27 or IFI6-KO affects IAV, SARS-CoV-2, and DENV2 replication -----	82
Figure R5. IFI27-knock-down upregulates the mRNA expression levels of IFN-response genes and proinflammatory cytokines-----	84
Figure R6. IFI27 and IFI6 KO upregulate the mRNA expression levels of IFN response genes and proinflammatory cytokines-----	85
Figure R7. IFI27 impairs IFN-induced responses after IAV and SARS-CoV-2 infections -----	86
Figure R8. Effect of IFI6 absence on the induction antiviral responses after IAV or SARS-CoV-2 infections in A549 cell line -----	87
Figure R9. IFI27 or IFI6 absence strongly potentiates the establishment of an antiviral state in cells after poly(I:C) treatment -----	88
Figure R10. IFI27 and IFI6 impair antiviral responses following SeV infection-----	89
Figure R11. Bubble plot from RNAseq data -----	91
Figure R12. Analysis of IFI6 effect on innate immune responses by RNAseq -----	92
Figure R13. Effect of IFI6 on the induction of innate immune responses after poly(I:C) treatments and SeV infection in HAP-1 cells -----	93
Figure R14. Effect of IFI6 knock-down in antiviral responses in HAP-1 cells -----	94
Figure R15. Effect of IFI6 on the modulation of innate immune responses induced in non-tumorigenic epithelial cells-----	95
Figure R16. Generation of rIAV-PR8 expressing IFI27, IFI6 or mCherry proteins -----	97
Figure R17. IFI27 or IFI6 expression negatively modulates the induction of innate immune responses after IAV infection in mice -----	99
Figure R18. IFI27 and IFI6 bind to poly(I:C) -----	101
Figure R19. Binding of IFI27-WT and IFI27 variants S63L and V82A to poly(I:C)-----	103

Figure R20. IFI27 and IFI6 interact with RIG-I through RNA binding-----	104
Figure R21. IFI27 and IFI6 partially colocalize with RIG-I in the cell cytoplasm -----	106
Figure R22. IFI27 and IFI6 negatively modulate RIG-I activation -----	107
Figure R23. IFI27-V82 variant partially loses its ability to negatively modulate the innate immune responses -----	109
Figure R24. IFI6 negatively affects RIG-I ubiquitination-----	111
Figure R25. IFI27 interacts with RIG-I and MDA5 through RNA binding -----	112
Figure R26. IFI27 co-immunoprecipitates with MDA5 after SARS-CoV-2 infection -----	113
Figure R27. IFI27 binds MDA5 in A549-hACE2 cells stably overexpressing IFI27-HA -----	114
Figure R28. IFI27 partially colocalizes with MDA5 at the cell cytoplasm -----	115
Figure R29. IFI27 negatively affects MDA5 oligomerization and function, hampering the induction of innate immune responses -----	117
Figure R30. KO and knock-down of IF27 leads to a higher MDA5 activation -----	118
Figure R31. MDA5 and IFI27 compete for poly(I:C) binding -----	119
Figure R32. Bubble-plots of IFI27-interacting proteins categorized by biological processes and molecular functions-----	121
Figure R33. IFI27 interacts with PACT and PKR proteins through an interaction mediated by RNA -----	122
Figure R34. IFI27 co-immunoprecipitates with PACT after SARS-CoV-2 infection -----	123
Figure R35. IFI27 variants S63L and V82A do not co-immunoprecipitate with PACT -----	124
Figure R36. IFI27 and PKR co-immunoprecipitate together -----	125
Figure R37. IFI27 partially colocalizes with PACT and PKR in the cell cytoplasm-----	126
Figure R38. Schema of the experimental concept for analyzing PKR activation levels and the overall state of cellular translation by Western blot and RLuc luminescence-----	128
Figure R39. IFI27 overexpression enhances PKR activation and function -----	129
Figure R40. IFI27 KO leads to a phenotype of lower PKR activation, which is rescued by restoring IFI27 expression-----	131
Figure R41. IFI27 KO leads to a lower PKR and eIF2 α phosphorylation levels after SARS-CoV-2 infection -----	132
Figure R42. IFI27 knock-down hampers PKR activation both after SARS-CoV-2 infection and poly(I:C) treatment -----	133
Figure R43. PACT deficiency impairs the activation of PKR by IFI27-----	135
Figure R44. IFI27 overexpression promotes SGs generation in a PACT-dependent manner --	137
Figure R45. SGs generation in IFI27-overexpressing cells is reduced in the absence of PACT-----	138
Figure R46. PKR overexpression promotes its own activation, which is further enhanced by IFI27 overexpression in a PACT-dependent manner-----	139
Figure R47. The interaction between IFI27 and PKR is facilitated by PACT -----	141
Figure D1. The expression of IFI27 and IFI6 is strongly induced after the treatments with poly(I:C) or IFN, and after the infections with IAV, SARS-CoV-2, and DENV2 -----	146

Figure D2. Graphical schema of IFI27 and IFI6 effect on the induction of innate immune responses -----	154
Figure D3. Schematic representation of IFI27-PACT-mediated modulation of PKR activity in response to RNA viruses -----	160
Figure D4. Graphical schema of the integration of the different IFI27 and IFI6 findings of this doctoral thesis -----	164
Table A1. RNAseq study comparing poly(I:C)-treated HAP-1 WT versus mock-treated HAP-1 WT cells-----	197
Table A2. RNAseq study comparing HAP-1 WT and HAP-1 IFI6 KO cells, both treated with poly(I:C)-----	199
Table A3. IFI27-HA-bound proteins -----	203
Table A4. Biological process organization of mass spectrometry data -----	214
Table A5. Molecular functions organization of mass spectrometry data -----	215

TABLE OF CONTENTS - LIST OF ABBREVIATIONS

14q32: chromosome 14, long arm, band 32 region	CRISPR: clustered regularly interspaced short palindromic repeats
1p35: chromosome 1, short arm, band 35 region	CTD: carboxy-terminal domain
3'UTR: 3'untranslated region	CXCL: chemokine (C-X-C motif) ligand
A549: human lung epithelial carcinoma A549 cell line	CXCL10: C-X-C motif chemokine ligand 10
ACE2: Angiotensin-Converting Enzyme 2	CXXC4: CXXC-type zinc finger protein 4
ADAR1: adenosine deaminase acting on RNA 1	DAMPs: damage associated molecular patterns
ADE: antibody dependent-enhancement	DAPI: 4',6-diamidino-2-phenylindole
AGC: automatic gain control	DCs: dendritic cells
AIV: avian influenza virus	DDX: DExD/H-box helicase
APOBEC: apolipoprotein B mRNA editing catalytic polypeptide-like	DEG: differentially expressed genes
ARDS: acute respiratory distress syndrome	DENV: dengue virus
ART: antiretroviral therapy	DENV2: dengue virus 2
ASC: caspase recruitment domain	DF: dengue fever
avSGs: antiviral stress granules	DHF: dengue hemorrhagic fever
Bax: Bcl2 associated X	DHX: DEAH-box helicase
Bcl2: B cell leukemia/lymphoma 2	DKO: double knock-out
BEAS-2B: non-tumorigenic human bronchial epithelial cell line	DMEM: Dulbecco's modified Eagle's medium
BH: Bcl2-homology	DMSO: dimethylsulfoxide
BHK-21: baby hamster kidney-21	dpi: days post-infection
bp: base pair	ds: double-stranded
BSA: bovine serum albumin	dsRBD: double-stranded RNA binding domain
BSL: biosafety level	dsRBM: double-stranded RNA binding motif
C: capsid protein	dsRNA: double-stranded ribonucleic acid
CAG: cytomegalovirus enhancer/chicken β -actin promoter	DSS: dengue shock syndrome
CARD: caspase activation and recruitment domain	DTT: dithiothreitol
CAS9: CRISPR-associated protein 9	DVuniv: DENV universal primers
CCL: chemokine (C-C motif) ligand	<i>E. coli</i> : <i>Escherichia coli</i>
CD14+: cluster of differentiation 14 +	E: envelope protein
CD81: cluster of differentiation 81	EboV: Ebola virus
cDNA: complementary DNA	EDTA: Ethylenediaminetetraacetic acid
cGAS-STING: cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING)	EEO: electroendosmosis
CHIKV: Chikungunya virus	EGFR: epidermal growth factor receptor
CIP: Quick calf intestinal alkaline phosphatase	EIF2K2: eukaryotic translation initiation factor 2 α kinase 2
CLDN1: Claudin-1	eIF2 α : eukaryotic initiation factor 2 α
co-IPs: co-immunoprecipitations	eIF2 α -P: S52-phosphorylated eIF2 α
COVID-19: coronavirus disease 2019	eIF3: eukaryotic initiation factor 3
CoV: coronavirus	eIF3 η : eukaryotic initiation factor 3 subunit η
	EMCV: encephalomyocarditis virus
	emPAI: exponentially modified protein abundance index

ER: endoplasmic reticulum	hpt: hours post-transfection/treatment
ERK: extracellular signal regulated kinases	HRI: heme-regulated inhibitor
FBS: fetal bovine serum	HRP: horseradish peroxidase
FcγR: fragment crystallizable gamma receptor	HSV-1: herpes simplex virus type 1
FDR: false discovery rate	IAV H1N1 PR8: influenza A virus A/Puerto Rico/8/1934 H1N1 strain
FFU: fluorescent/focus forming units	IAV: influenza A virus
FS: forward sequence	IBV: influenza B virus
G3BP1: ras GTPase-activating protein-binding protein 1	ICTV: international committee on taxonomy of viruses
GAPDH: glyceraldehyde 3-phosphate dehydrogenase	ICU: intensive care unit
GCN2: general control nonderepressible 2	ICV: influenza C virus
GDP: guanosine diphosphate	IDV: influenza D virus
GEF: guanine exchange factor	IFI27: interferon alpha-inducible protein 27
GFP: green fluorescent protein	IFI44: interferon-induced protein 44
GM-CSF: granulocyte-macrophage colony-stimulating factor	IFI44L: interferon-induced protein 44-like
GO terms: gene ontology terms	IFI6: interferon alpha-inducible protein 6
GSEA: gene set enrichment analyses	IFIH1: interferon-induced with helicase C domain 1
GST: glutathion S-transferase	IFIT: interferon-induced protein with tetratricopeptide repeats
HA: hemagglutinin	IFIT3: interferon-induced protein with tetratricopeptide repeats 3
hACE2: human angiotensin converting enzyme 2	IFITM: interferon-induced transmembrane proteins
HAP-1: human near-haploid	IFN: interferon
HBV: hepatitis B virus	IFNAR: IFN α/β receptor
HCD: higher-energy collisional dissociation	IFNL1: IFN lambda 1
HCl: hydrochloric acid	IFNL3: IFN lambda 3
HCMV: human cytomegalovirus	IFNLR1: IFN lambda receptor 1
HCoV: human coronavirus	IgG: immunoglobulin G
HCoV-229E: human coronavirus 229E	IKK: I κ B kinase
HCoV-HKU1: human coronavirus HKU1	IL: interleukin
HCoV-NL63: human coronavirus NL63	IL-10R2: interleukin 10 receptor subunit β
HCoV-OC43: human coronavirus OC43	IL1R1: interleukin 1 receptor-like 1
HCV: hepatitis C virus	ILF2: interleukin enhancer binding factor 2
HDAC: histone deacetylase	ILF3: interleukin enhancer binding factor 3
HEK-293T: human embryonic kidney 293T cell line	ISG15: interferon-stimulated gene 15
HeLa: immortalized cell lines from cervical cancer – Henrietta Lacks	IP: immunoprecipitation
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid	IRES: internal ribosome entry site
HERC: HECT and RLD domain containing E3 ubiquitin protein ligase family	IRF: interferon-regulatory factor
HGF: hepatocyte growth factor	ISG: interferon-stimulated gene
6xHis: 6xHistidine	ISGF3: interferon-stimulated gene factor 3
HIV-1: human immunodeficiency virus 1	ISRE: interferon-stimulated response element
HPAIV: highly pathogenic avian influenza virus	JACoP: just another colocalization plug-in
hpi: hours post-infection	JAK-STAT: Janus kinase -signal transducer and activator of transcription
	K48 or K63: lysine 48 or lysine 63

Ka: association rate constant	NCR: non-coding regions
kb: kilobase	NEK7: never in mitosis gene A (NIMA)-related kinase 7
KCl: potassium chloride	NEP: nuclear export protein
KH: K homology domains	NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells
LB: Luria-Betani	NLRP3: NOD-like receptor family, pyrin domain-containing 3
LCMV: lymphocytic choriomeningitis virus	NLRs: nucleotide-binding oligomerization domain-containing (NOD)-like receptors
LDLR: low-density lipoprotein receptor	NONO: non-POU domain-containing octamer-binding protein
LGP2: laboratory of genetics and physiology 2	NoV: Nodamura virus
LiCl: lithium chloride	NP: nucleoprotein
LRRs: leucine-rich repeats	NP-40: non-ionic detergent (nonidet) P-40
LTR: long terminal repeat	NR4A1: nuclear receptor subfamily 4 group A member 1
LY6E: lymphocyte antigen 6 complex, locus E	NS1: non-structural protein 1
M: membrane (SARS-CoV-2, DENV2) or matrix protein (IAV)	NT: non-targeting
m7G: 7-methylguanosine	nTPM: normalised transcripts per million
mA: milliampere	O.E.: overexpression
mAb: monoclonal antibody	OAS: 2',5'-oligoadenylate synthetase
MACS: magnetic-activated cell sorting	Opti-MEM: Opti-minimal essential medium
MAM: mitochondrial-associated ER membranes	ORF: open reading frame
MAP: mitogen-activated protein	ORFV: orf virus
MAVS: mitochondrial antiviral-signaling	PA: acidic protein
MDA-5: melanoma differentiation-associated gene 5	pAb: polyclonal antibody
MDCK: Madin-Darby canine kidney epithelial cell line	PACT or PRKRA: PKR-activating protein
MERS-CoV: Middle East respiratory syndrome coronavirus	PAMPs: pathogen-associated molecular patterns
Mex3C: Mex-3 RNA binding family member C	PB1: polymerase basic 1
mgf: mascot general file	PB2: polymerase basic 2
MHV: mouse hepatitis virus	PBS: phosphate-buffered saline
MIF: macrophage migration inhibitory factor	PCBP2: poly(rC)-binding protein 2
miRNA: micro RNA	PCR: polymerase chain reaction
MMTS: S-methyl methanethiosulphonate	PD: pull-down
MoDCs: monocyte-derived dendritic cells	pDCs: plasmacytoid DCs
MOI: multiplicity of infection	PEI: polyethylenimine
mRNAs: messenger ribonucleic acid	PERK: protein kinase RNA-like endoplasmic reticulum kinase
mRNPs: messenger ribonucleoproteins	PFU: plaque forming units
MS: mass spectrometry	PKR: protein kinase R
Mx: Myxovirus resistance protein	PKR-P: phosphorylated PKR
N: nucleocapsid protein (SARS-CoV-2) or neuraminidase (IAV)	poly(A): poly-adenosine
NA: neuraminidase	poly(C): polycytidylic acid
Na ₂ HPO ₄ : sodium phosphate	poly(I:C): polyinosinic:polycytidylic acid
NaCl: sodium chloride	pp: diphosphate
NaOH: sodium hydroxide	PP1: protein phosphatase 1

ppp: triphosphate	shRNA: short-hairpin RNA
PPP1CC: protein phosphatase 1C	SINV: Sindbis virus
PPP1R12C: protein phosphatase 1 regulatory subunit 12C	siRNA: small-interfering RNA
prM: pre-membrane protein	SP: severe pneumonia
PRRs: pattern recognition receptors	ss: single stranded
PTV: porcine teschovirus	ssRNA: single-stranded ribonucleic acid
qPCR: quantitative PCR	STAU: Staufen homolog
qRT-PCR: reverse transcription and quantitative PCR	STING: stimulator of interferon genes
RaTG13: bat coronavirus RaTG13	SV40: simian virus 40
RBP: RNA binding proteins	T446: threonine position 446 of PKR
RdRp: RNA dependent RNA polymerase	TANK: TRAF family member associated NF- κ B activator
rIAVs: recombinant IAVs	TAV: Thosa assigna virus
RIG-I: retinoic acid-inducible gene I	TBK1: TANK-binding kinase 1
RIP: RNA Immunoprecipitation	TBS: tris-buffered saline
RISC: RNA-silencing inducing complex	TCEP: tris(2-carboxyethyl)phosphine
RLR: RIG-I-like receptors	TLRs: toll-like receptors
RLuc: renilla luciferase	TNF: tumor necrosis factor
RNAi: RNA interference	TNFRSF: tumor necrosis factor receptor superfamily member
RNAse: ribonuclease	TPCK: tosylsulfonyl phenylalanyl chloromethyl ketone
RNAseq: RNA sequencing	TRAF: tumor necrosis factor receptor-related factor
RNF6: ring finger protein 6	TRAIL: TNF-related apoptosis-inducing ligand
RNP: ribonucleoprotein	TRBP/TARBP2: transactivation response element (TAR)-RNA-binding protein 2; subunit of RISC loading complex
RPMI: Rosewell Park Memorial Institute	TRIM: tripartite motif
RRMs: RNA recognition motifs	tRNA ^{Met} : methionyl-initiator transfer RNA
RS: reverse sequence	VEGF: vascular endothelial growth factor
RSAD2: radical S-adenosyl methionine domain containing 2 / viperin	Vero E6: African green monkey kidney epithelial cell line
RSV: respiratory syncytial virus	vRNA: viral RNA / viral segments
RT: retrotranscriptase/retrotranscription or room temperature	vRNPs: viral ribonucleoprotein
rVSV-GFP: recombinant VSV-GFP	RO: replication organelle
S: spike protein	VSV: vesicular stomatitis virus
S52: serine position 52	VSV-G: VSV glycoprotein
SARS-CoV: severe acute respiratory syndrome coronavirus	WNV: West Nile virus
SARS-CoV-2: severe acute respiratory syndrome coronavirus 2	WT: wild-type
SDD-PAGE: semi-denaturing detergent-polyacrilamide gel electrophoresis	YFV: yellow fever virus
SDS-PAGE: sodium dodecyl sulfate-polyacrilamide gel electrophoresis	ZCCHC3: zinc finger CCHC-type containing 3
SeV: Sendai virus	ZIKV: Zika virus
sgRNAs: single-guide RNAs	ZNF: zinc-finger domain
SGs: stress-granules	Ψ: packaging signal

RESUMEN

Función y mecanismos moleculares de las proteínas inducidas por interferón IFI27 e IFI6 en la replicación viral e inducción de respuestas inmunitarias innatas frente a las infecciones por virus ARN.

Los virus ARN constituyen una de las mayores amenazas globales a nivel socio-sanitario, debido a su elevada variabilidad y su capacidad para adaptarse y superar diferentes barreras selectivas como son la respuesta inmunitaria de los hospedadores, el empleo de vacunas y tratamientos antivirales, o barreras externas como la adaptación a nuevos ambientes, o la adaptación a nuevas especies hospedadoras. La primera barrera contra las infecciones virales es la inmunidad innata, que reconoce patrones moleculares asociados a patógenos (PAMPs, del inglés *pathogen-associated molecular patterns*) tales como los ARNs virales (ARNv), mediante receptores especializados para ello, los PRRs (del inglés *pattern recognition receptors*). Estos ARNv son reconocidos por los PRRs citosólicos *retinoic acid-inducible gene 1* (RIG-I)-like receptors (RLRs), los cuales iniciarán vías de señalización que conducen a la producción de interferones (IFN) y citoquinas proinflamatorias, estableciendo un estado antiviral, para detener la propagación y replicación del virus invasor. También existen proteínas efectoras que detectarán la presencia de ARNv y pondrán en marcha mecanismos antivirales, como la proteína quinasa R (PKR), cuya activación en respuesta a dichos ARNv inhibirá la traducción, para evitar que los virus generen sus proteínas. Los IFNs producidos, a su vez, de manera autocrina y paracrina activarán programas de expresión génica dirigidos a la producción de los genes inducidos por IFN (ISGs, del inglés *IFN-stimulated genes*). Gran parte de estos ISGs presentan una enorme variedad de funciones antivirales dirigidas a detener la replicación y propagación viral. No obstante, muchos otros ISGs presentan funciones moduladoras, dirigidas a controlar el nivel de activación inmune, ya sea potenciando la respuesta antiviral, o ejerciendo mecanismos de retroalimentación negativa enfocados a detener la producción de IFNs y citoquinas proinflamatorias, para recuperar la homeostasis celular y evitar una respuesta excesiva, que puede ser perjudicial para el hospedador. Además, la función de muchos ISGs es aún desconocida.

En esta tesis doctoral, nos hemos centrado en el estudio de dos ISGs cuya función no ha sido estudiada en profundidad: las proteínas inducidas por IFN, IFI27 e IFI6 (del inglés, *IFN α -inducible proteins 27 y 6*, respectivamente). El objetivo principal fue determinar el posible efecto de dichas proteínas en la replicación y respuestas antivirales frente a distintos virus ARN, así como describir los posibles mecanismos moleculares que pudiesen explicar los resultados. Se observó que la expresión de IFI27 e IFI6 en líneas celulares se indujo tras el tratamiento con IFN y con poly(I:C), así como después de las infecciones por tres de los virus de mayor importancia socio-sanitaria en la actualidad: *severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2), el virus de influenza tipo A (IAV, *influenza A virus*) y el virus del dengue, serotipo 2 (DENV2). El silenciamiento o *Knock-out* (KO) de los genes *IFI27* o *IFI6* en líneas celulares, disminuyó la replicación de los virus SARS-CoV-2, IAV y DENV2, lo cual correlacionó con una mayor inducción de respuestas antivirales. Por el contrario, la sobreexpresión de las proteínas IFI27 o IFI6 en líneas celulares, disminuyó la inducción de respuestas antivirales tras el tratamiento con poly(I:C), o infecciones por el virus Sendai (SeV) o SARS-CoV-2. Asimismo, la sobreexpresión de IFI27 o IFI6 en los pulmones de ratones infectados por IAV, condujo a una menor inducción de las respuestas antivirales y menores niveles de inflamación.

A continuación, se estudiaron los posibles mecanismos moleculares por los cuales IFI27 e IFI6 ejercen los efectos observados, demostrando que ambas proteínas son proteínas de unión a ARN.

Además, ambas proteínas interactúan con el RLR RIG-I, y adicionalmente IFI27 con el RLR MDA5 (del inglés, *melanoma differentiation-associated gene 5*), y dicha interacción está mediada por la presencia de ARN. La interacción de IFI27 con RIG-I y MDA5 inhibió la función de estos receptores, debido a una disminución de su capacidad de unir ARNv o poly(I:C), y por tanto una reducción de la inducción de las respuestas antivirales. De manera similar, se demostró que la interacción de IFI6 con RIG-I es inhibitoria para esta última.

Finalmente, en busca de nuevas funciones en las que IFI27 pudiese estar involucrado dentro de la respuesta antiviral, se analizó el interactoma proteico mediante espectrometría de masas. Gran parte de las proteínas que interactuaron con IFI27 eran proteínas de unión a ARN y proteínas involucradas en la traducción proteica. Presentes en ambos grupos, se identificaron las proteínas PKR y la proteína activadora de PKR (PACT, del inglés *PKR-activating protein*), y se observó que la interacción de IFI27 con PKR, está mediada por la presencia de PACT. Empleando células silenciadas o KO para IFI27, o complementariamente, células que sobreexpresan IFI27, se demostró que IFI27, de manera dependiente de PACT, actúa como activadora de la proteína PKR, y, por tanto, promueve una disminución de la traducción proteica. Esto se observó tras la infección con SARS-CoV-2 o el virus de la estomatitis vesicular (del inglés, *vesicular stomatitis virus*, VSV), y tras el tratamiento con poly(I:C), lo cual demuestra que es un efecto general para la infección con distintos virus ARN. Asimismo, se observó que la activación de PKR mediada por IFI27 aumentó la generación de unas estructuras esenciales en la respuesta inmunitaria innata frente a virus, los gránulos de estrés (SGs, del inglés *stress granules*).

En conclusión, durante esta tesis doctoral se ha descrito por primera vez que las proteínas IFI27 e IFI6, son proteínas de unión a ARN, que interactúan con los RLRs en respuesta a una infección por virus ARN y ejercen una modulación negativa de la función de los mismos, lo cual conduce a una menor inducción de las respuestas antivirales y un aumento de la replicación de virus ARN. Además, se ha descrito la interacción de IFI27 con PACT y PKR, actuando IFI27 como un activador de PKR y por tanto contribuyendo a la inhibición de la traducción. El conjunto de los resultados de esta tesis doctoral muestra que IFI27 e IFI6 modulan distintas vías de la respuesta inmunitaria innata, pudiendo tener un papel esencial en el control de la duración e intensidad de las mismas, evitando una respuesta excesiva que pueda tener efectos perjudiciales para el hospedador.

ABSTRACT

Molecular function and mechanisms of interferon-induced proteins IFI27 and IFI6 in viral replication and induction of innate immune responses against RNA virus infections.

RNA viruses constitute a major social and health threat worldwide, due to the high variability that these viruses present, and their ability to adapt and overcome selective internal constraints such as host immunity, vaccines, and antiviral treatments, or external constraints such as adaptation to new environments, or the adaptation to new host species. The first barrier against viral infections is the innate immune response, which recognizes pathogen-associated molecular patterns (PAMPs) thanks to specialized receptors, the pattern recognition receptors (PRRs). Within the different PAMPs that are recognized, viral RNAs (vRNAs) are one of the most relevant. vRNAs are recognized by the cytosolic PRRs, the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which after binding to vRNAs, initiate signaling pathways that drive the production of interferons (IFNs) and proinflammatory cytokines, establishing an antiviral state, with the aim of limiting viral replication and propagation. Additionally, there are effector proteins that also recognize and bind vRNAs, and respond by activating antiviral mechanisms, such as protein kinase R (PKR), whose activation leads to the inhibition of translation, in order to stop viruses from producing their own proteins. Meanwhile, IFNs will bind to their cellular surface receptors in an autocrine and paracrine way and subsequently activate gene expression programs that lead to the production of IFN-stimulated genes (ISGs). Many of these ISGs display antiviral functions aimed at restricting viral replication. However, recent studies have shown that many ISGs have modulatory roles in order to control the level of immune activation, either by enhancing antiviral responses, or by exerting negative feedback mechanisms that diminish the production of IFNs and cytokines in order to restore cellular homeostasis and avoid an excessive response, which can be deleterious to the host. Furthermore, the roles of many ISGs remain unknown.

In this doctoral thesis, we have focused on the study of two ISGs whose function has not been studied in depth: IFN alpha-inducible proteins 27 (IFI27) and 6 (IFI6). The main objective was to determine the possible roles that these proteins could present on viral replication and antiviral responses against different RNA viruses, as well as describing the possible molecular mechanisms that could explain the observed results. The expression of IFI27 and IFI6 was highly induced after the treatment with IFN and poly(I:C), as well as after the infections with three of the viruses causing a high social and health burden nowadays: severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), influenza A virus (IAV), and dengue virus serotype 2 (DENV2). Knocked-down and Knocked-out (KO) cell lines for either *IFI27* or *IFI6* genes, resulted in lower replication levels of SARS-CoV-2, IAV, and DENV2, which correlated with a stronger induction of antiviral responses. Conversely, overexpression of IFI27 or IFI6 proteins in cell lines, resulted in a reduction of the induction of antiviral responses after poly(I:C) treatment, or after Sendai virus (SeV) or SARS-CoV-2 infection. Also, overexpression of either IFI27 or IFI6 in the lungs of mice infected with IAV, led to a lower induction of antiviral responses and reduced inflammation.

Next, molecular mechanisms that could explain the observed results were studied, showing that both IFI27 and IFI6 proteins are RNA binding proteins (RBPs). Furthermore, both proteins were shown to interact with the RLR RIG-I, and additionally, IFI27 was shown to interact with the RLR melanoma differentiation-associated gene 5 (MDA5), being these molecular interactions dependent on the presence of RNA. The interactions of IFI27 with both RIG-I and MDA5 led to the negative modulation of the activation of both RLRs due to a reduction on their capacity to

bind to vRNAs and to poly(I:C), and thus, a reduction on the induction of antiviral responses. Similarly, IFI6 interaction with RIG-I led to a reduction of RIG-I activity.

Finally, in search of novel functions in which IFI27 could be involved within the antiviral responses, a mass spectrometry study of IFI27-protein interactome was performed. Two of the main molecular and biological functions shared in IFI27-interacting proteins were RNA-binding and protein translation. Within those proteins that were represented in both groups, we were particularly interested in PKR and PKR-activating protein (PACT), and interestingly, IFI27 interaction with PKR was shown to be mediated by PACT. By using Knock-down or KO cells for IFI27 and cells overexpressing IFI27, it was described that IFI27 acts as an enhancer of PKR function in a PACT-dependent manner, thus leading to an inhibition of protein translation. This was observed both after SARS-CoV-2 and vesicular stomatitis virus (VSV) infections, and after poly(I:C) treatment, suggesting a broad effect of IFI27 on PKR activation in response to different RNA viruses. Likewise, it was also observed that IFI27-mediated enhancement of PKR activity led to an increase of the generation of pivotal structures on innate immune responses against viral infections, stress granules (SGs).

In conclusion, in this doctoral thesis, we have described for the first time, that IFI27 and IFI6 proteins are RBPs that interact with RLRs in response to infections with RNA viruses, and negatively modulate their function. This leads to a lower induction of antiviral responses after RNA virus infection and to an increase in their replication. Also, IFI27 interacts with PACT and PKR, acting as a positive regulator of PKR function and inhibiting protein translation. All in all, results in this doctoral thesis show that IFI27 and IFI6 modulate different pathways within innate immune responses, suggesting that both proteins play a pivotal role on the modulation of duration and intensity of these responses, to prevent an excessive reaction that could have detrimental effects to the host.

INTRODUCTION

1. INTRODUCTION

1.1. PUBLIC HEALTH AND SOCIAL CHALLENGES POSED BY RNA VIRUSES

RNA viruses are viruses whose genetic material is RNA, and constitute a significant threat to public health and socio-economic stability due to different factors that lead to a persistent challenge: the elevated risk of cross-species transmission, the constant need of vaccines updates to properly respond against new viral strains, and their ability to expand into new geographical regions and adapt to different host species. All these characteristics come from the capacity of RNA viruses to rapidly adapt, change and overcome internal and external selective constraints such as the immune response, vaccines, or antiviral treatments [1]. In this regard, three different RNA viruses, which have been used in this PhD thesis, serve as great examples of these challenges nowadays: severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), influenza A virus (IAV), and dengue virus (DENV). To delve deeper into the study of these three RNA viruses, we will first review their taxonomy, origin, epidemiology, structure, and pathology.

1.1.1. SARS-CoV-2

SARS-CoV-2 belongs to the *Coronaviridae* family, a family of enveloped spherical viruses of 118-136 nm in diameter [2], with a non-segmented, positive-sense, single-stranded RNA genome (+ssRNA) of 22-36 kilobases (kb), which is 5'-m7G capped and 3'-polyadenylated (poly(A)) [3]. This family belongs to the order *Nidovirales* [4]. The *Nidovirales* order (from the latin word, *Nidus*, meaning nest) refers to the production of a 3' co-terminal nested set of subgenomic mRNAs during infection [5].

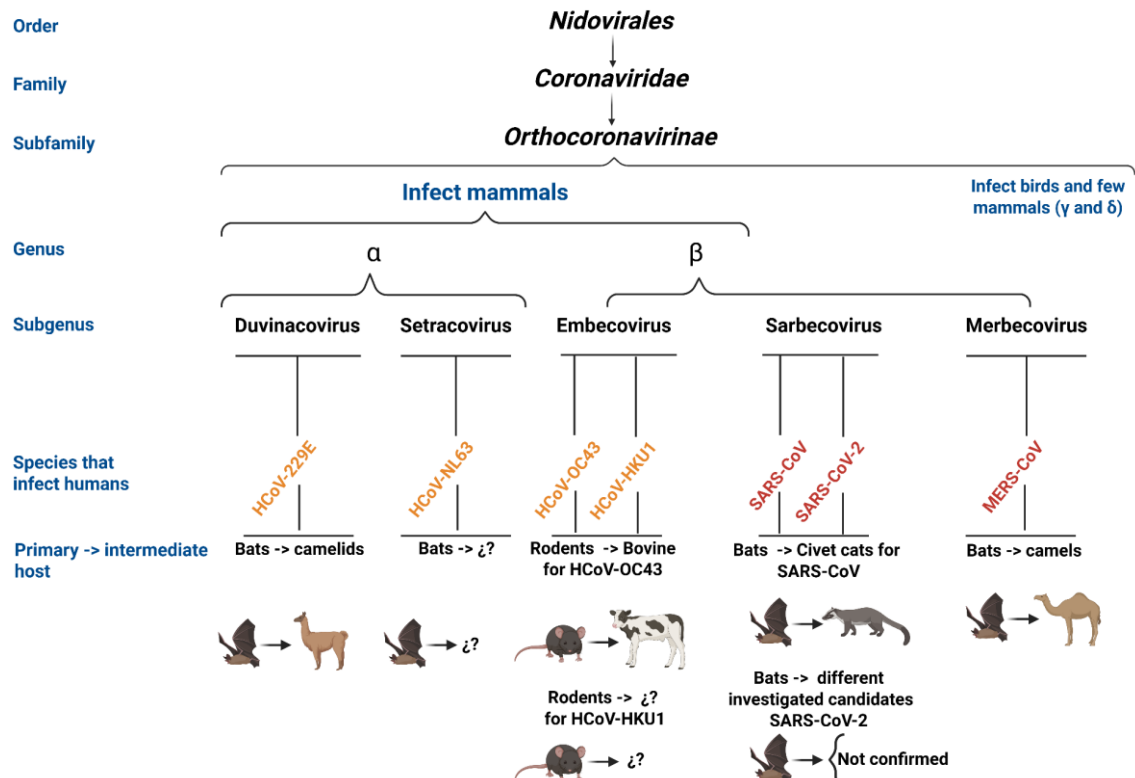


Figure 11. Coronavirus (CoV) taxonomy with a particular emphasis on human CoVs (HCoVs). Figure generated with information from [6,7]

The *Coronaviridae* family is composed of different subfamilies. One of these subfamilies is the *Orthocoronavirinae* subfamily, commonly referred to as coronavirus (CoV), which infect

mammals and birds, and it is composed of genus *Alpha*, *Beta*, *Gamma*, and *Deltacoronavirus* [3,8]. α and β CoVs infect humans and other mammals (cats, cows, dogs, horses, mice, pigs, etc.), whereas γ and δ are mostly found in birds (chicken, duck, goose, pigeon, sparrow, turkey, etc.) along with other mammals [9,10] (Fig. 11).

Seven different CoVs from α and β genera have been described to infect humans (HCoV) (Fig. 11): HCoV-NL63, HCoV-229E, HCoV-HKU1, and HCoV-OC43, which can be classified as low pathogenic; and severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2, which are classified as highly pathogenic CoVs [11]. On the one hand, low pathogenic CoVs cause self-limiting respiratory infections, resulting in 15-30% of the common colds in humans in a seasonal pattern, although sometimes life-threatening lower respiratory tract infections can occur in elderly people, infants, and in immunocompromised patients [11–16]. On the other hand, the first highly pathogenic HCoV to be identified was SARS-CoV, in China, in 2002. This virus caused a major epidemic and presented a high mortality rate, close to 10% of infected patients [11]. Later, in 2012, MERS-CoV emerged, and since then, 2,626 cases have been reported, displaying a particularly high mortality rate, close to 36% [11,17]. Most recently, in late 2019, SARS-CoV-2, a novel HCoV, emerged in Wuhan, Hubei Province in China. This virus caused the CoV disease 2019 (COVID-19) pandemic, which up to date, has caused more than 778 million reported infections with a mortality rate close to 1%, leading to more than 7 million deaths worldwide [18], although this mortality percentage could be overestimated as the number of SARS-CoV-2 infections could have been higher.

A common characteristic for HCoV is that bats are widely accepted as their primary original hosts, except for HCoV-OC43 and HCoV-HKU1, whose natural hosts are believed to be rodents [6,7] (Fig. 11). Coronaviruses present a great adaptive capacity and genetic variability driven by the accumulation of changes and mutations that enable them to jump from their original host to a different species, referred to as the intermediate host (Fig. 11). In the intermediate host, which is usually a different mammal species, the virus further accumulates adaptations and genetic changes that permit a second jump to humans, potentially leading to severe epidemics or pandemics due to the introduction of an emerging virus into the population [6,7]. Just in the past two decades, we have suffered the epidemics and pandemics caused by SARS-CoV, MERS-CoV, and SARS-CoV-2, all three viruses believed to infect bats as their primary hosts. This underscores the urgent need for surveillance following the One Health approach, which requires constant monitoring and identification of viruses in their natural reservoirs, in order to be better prepared for zoonotic spillover events.

As previously mentioned, SARS-CoV-2 is an enveloped virus, with a +ssRNA genome of 29.9 kb [19]. SARS-CoV-2 genome codifies for four structural proteins (S, E, M, and N), accessory proteins (ORF3a, ORF3b, p6, ORF7a, ORF7b, ORF8b, ORF9b, and ORF14), and sixteen non-structural proteins (nsp1–16). Nsp proteins are essential for viral replication and transcription, and include enzymes and factors that are necessary for the viral life cycle, but generally, they are not included in the mature virus particle [20–22] (Fig. 12). In addition, many of these nsps have different roles to counteract the host immune responses (in which we will focus later on).

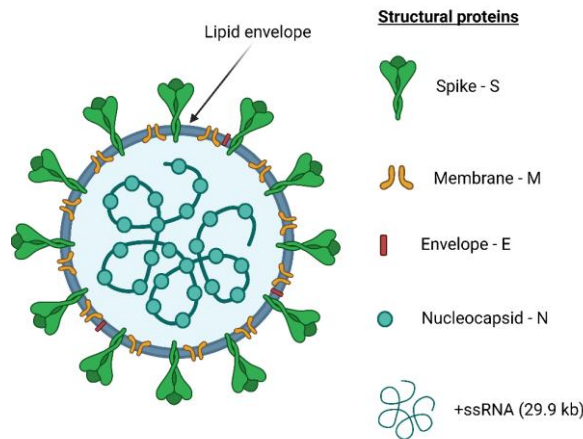


Figure 12. SARS-CoV-2 structure. The structural proteins common to all CoVs, envelope and genome are shown. SARS-CoV-2 is an enveloped +ssRNA virus of near 30 kb. Its genome codifies for four structural proteins: Spike glycoprotein (S), involved in viral entry, as it binds the ACE2 host receptor binding and membrane-fusion; nucleocapsid (N), essential in genome packaging and RNA synthesis during replication; membrane protein (M), which is the most abundant in the viral membrane and it maintains virion size and shape, as well as it facilitates the assembly of viral particles; and envelope protein (E), a small but versatile protein. It plays diverse roles such as particle assembly, virion budding from cells, envelope formation, facilitating ion transport across membranes as a viroporin and interaction with several host proteins. Also, SARS-CoV-2 genome codifies for non-structural proteins (nsp1-16) and accessory proteins (ORF 3a, ORF 3b, p6, ORF 7a, ORF 7b, ORF 8b, ORF 9b, and ORF 14), some of them forming part of the virion (not shown). Figure adapted from [20].

Respiratory droplets and aerosols are the main transmission route for SARS-CoV-2, and after infection, there is an incubation period of typically 4-5 days before symptoms onset. Most patients develop mild to moderate symptoms such as cough, fever, headache, myalgia, and diarrhea [23] and some remain asymptomatic. However, other patients can develop pneumonia, and of these, nearly 10% percent will require intensive care unit (ICU) admission. The clinical features of patients that develop a severe form of the disease, are characterized by dyspnea (short of breath), hypoxemia and progressive respiratory failure [23,24]. In severe cases, the immune responses are highly dysregulated and result in a clinical feature which is the development of systemic hyperinflammation, known as cytokine storm. The cytokine storm is characterized by the release of proinflammatory cytokines such as interleukin-1 β (IL-1 β), IL-6, IL-8, tumor necrosis factor (TNF) α , interferon (IFN) γ , and a reduction of anti-inflammatory cytokines such as IL-10, IL-4, the macrophage migration inhibitory factor (MIF), or the vascular endothelial growth factor (VEGF) [24,25]. This over-reactive response is highly deleterious to the host, and promotes an excessive immune cell infiltration in lungs and other organs, leading to destructive effects on tissues, acute respiratory distress syndrome (ARDS), multiple-organs failure, and ultimately death [24,26].

1.1.2. Influenza A virus (IAV)

Influenza viruses belongs to the *Orthomyxoviridae* family, where we find the genus *Alphainfluenzavirus* (generally named as type A), *Betainfluenzavirus* (type B), *Gammainfluenzavirus* (type C), and *Deltainfluenzavirus* (type D) [27,28], categorization that attends to the antigenic variations in viral nucleoprotein (NP) and matrix (M) proteins [29]. These enveloped viruses are spherical (~100 nm) or filamentous (~100 nm wide and 20 nm long) viruses, and present a segmented, negative sense, single-stranded RNA genome (-ssRNA) (Fig. 13) [30].

Type A influenza (IAV) and B (IBV) viruses have a widespread distribution and are the primary causes of seasonal flu epidemics, which result every year in significant socio-economic losses, with approximately 1 billion cases and 300,000-600,000 deaths [31]. IAV causes illness that ranges from mild to severe, both in humans and animals and has an enormous pandemic potential [30]. IBV infects mainly humans, although it has been isolated from other species such as seals, guinea pigs, ruminants, horses, gorillas, chimpanzees, zoo birds, and dogs [32]. IBV generally causes less severe illness, although it can also lead to severe cases and fatalities. IBV lacks the pandemic potential of IAV [32,33]. Influenza C virus (ICV) produces mild respiratory symptoms in humans and some animals such as swine, typically causing sporadic cases or minor outbreaks [34]. Type D influenza (IDV) viruses were recently discovered in Oklahoma [35], mainly affect swine, cattle and goats, but have not been described to infect humans to date [35–37].

IAVs, formally classified as the species *Alphainfluenzavirus influenzae* (following last taxonomy update on the 2024 release of the International Committee on Taxonomy of Viruses (ICTV) celebrated in Bari, Italy, August 2024) [8,27] are further categorized in different subtypes according to the antigenicity of two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). Currently, 18 different types of HA (H1-H18) and 11 types of NA (N1-N11) have been identified [38], and the combination of these gives rise to the different subtypes of IAV. The natural hosts of IAV are wild aquatic birds, but the virus has an enormous ability to adapt and spread to other species, breaking the host barrier and becoming a zoonotic infection. IAV has been observed to infect poultry and different mammals, including humans [29]. What drives the ability of IAV to cross species barriers is its remarkable capacity to adapt to new hosts through extensive variability [29] due to two key mechanisms: the antigenic drift, which is the accumulation of mutations in the HA and NA proteins, and the antigenic shift, led by the recombination and the reassortment of genome segments due to its segmented genome [39]. These processes allow IAV to rapidly generate novel variant viruses adapted to new hosts or that elude the immune responses generated against previous strains, making this virus an unpredictable and dangerous threat, as preexisting antibodies to previous IAV infections provide little to no protection [40]. Consequently, on an annual basis, vaccines against circulating strains of IAV have to be updated, due to the continual genetic and antigenic changes. A clear illustration of the risk of emergence of new IAV is evidenced by the fact that, in the 20th century, four global pandemics were caused by H1N1 (1918 and 2009), H2N2 (1957), and H3N2 (1968) IAVs [41]. Particularly devastating was the 1918 H1N1 pandemic, which caused 20-40 million deaths worldwide, and exhibited unusually high mortality rates among young, healthy adults [42]. Nowadays, we are under constant threat from the highly pathogenic avian influenza virus (HPAIV) H5N1. Avian influenza viruses (AIV) are a major cause of disease and mortality in wild birds and livestock, and spillover to mammals and humans have also been described, usually with fatal outcomes [43], with a mortality rate of 48% in confirmed human cases [44]. In the last years, increasing number of cases have been reported in a wide range of mammalian hosts throughout the Americas, Eurasia, and Africa (marine mammals, minks, dairy cattle, domestic cats, fur foxes, etc.), with multiorgan symptoms including respiratory, neurological, and systemic illness [43,45]. This situation is highly concerning and requires rigorous and constant surveillance as the risk of a pandemic is growing, and the disease caused by this virus is tremendously severe.

IAV are enveloped viruses, with a segmented -ssRNA genome, composed of 8 different segments (PB2, PB1, PA, HA, NP, NA, M, and NS) that encode the different viral proteins. The first three segments codify for the polymerase basic 2, 1 (PB2 and PB1), and the acidic protein (PA), which together form the polymerase complex. Next, the HA and NA segments codify these surface

glycoproteins that confer distinct antigenic properties to IAV. The HA protein attaches viral particles to host cells by binding to sialic acid-containing cellular receptors and induces viral particle internalization, whereas the NA protein enables the influenza viruses to be released from the host cell [46,47]. The NP segment codifies the nucleoprotein (NP), which binds to the genome to generate viral ribonucleoprotein complexes (vRNPs), as well as modulates its import into cell nucleus during infections. The M segment codifies at least two proteins: the matrix protein (M1), which supports vRNP interactions, and the M2 protein, encoding an ion channel activity, critical for uncoating and assembly of the virus particle. Finally, the NS segment codifies two viral proteins: the non-structural protein 1 (NS1), a virulence factor which antagonizes the innate immune responses and regulates processing of host mRNAs, and the nuclear export protein (NEP), which exports vRNPs from the nucleus [47] (Fig. I3). Additionally, other accessory proteins codified by IAV genome have been described. Some of these are PB1-F2, involved in apoptosis and modulation of host responses, PB1-N40, which could support IAV replication (both encoded in the PB1 segment), and PA-X, involved in host shut-off of protein translation and modulation of host responses (encoded in the PA segment). Also, different truncated versions of viral proteins have been described [48–50].

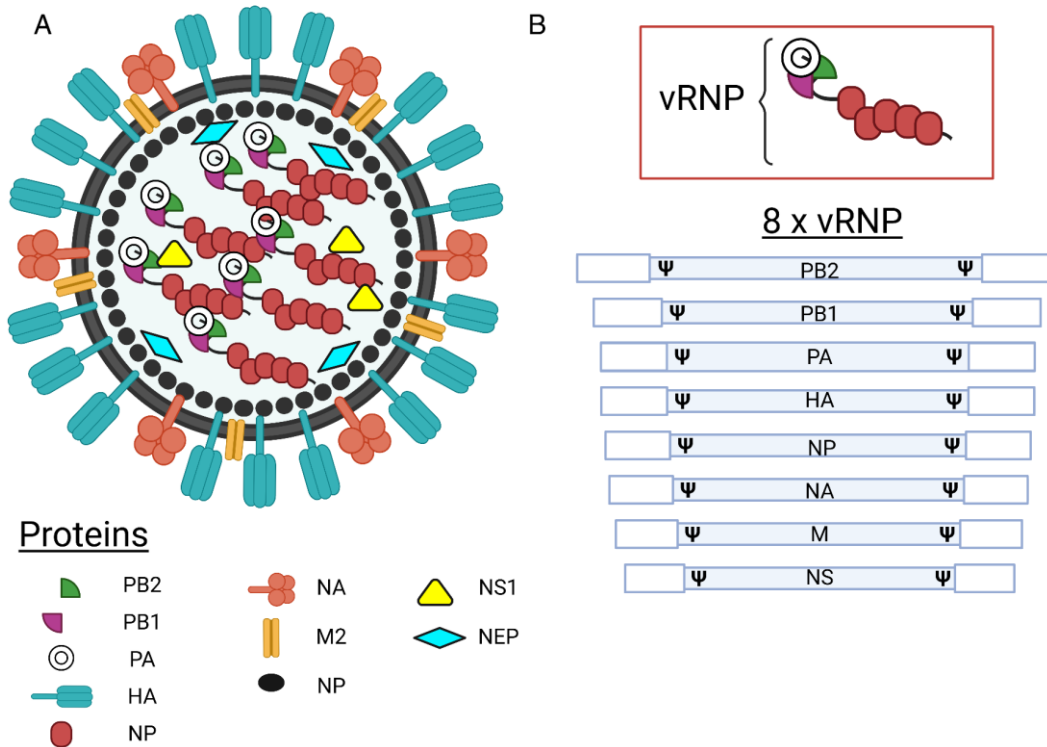


Figure I3. IAV virion structure and genome organization. (A) The structure of the virion is represented. IAV virions have a lipid envelope, where the surface glycoproteins are embedded: HA (hemagglutinin), NA (neuraminidase) and the M2 protein (ion channel). Under the lipid envelope, we find a protein layer, composed of several units of M1 and NEP proteins. Inside the virion, we find the 8 different viral ribonucleoproteins (vRNPs), composed of the viral -ssRNA (one of the 8 different segments of viral RNA), different coating subunits of NP protein, and one unit of PB2/PB1/PA proteins, which comprise the heterotrimeric polymerase complex, bound to each viral RNA. (B) Genome organization of IAV. The genome of IAV is composed of 8 different viral RNA segments (PB2, PB1, PA, HA, NP, NA, M, and NS). Each of these segments are -ssRNAs, flanked by non-coding regions (NCR) at 3' and 5' ends (white boxes). The light blue center of the representation of each viral RNA represents the coding region, which codifies for the different viral proteins. At the 3' and 5' ends of the coding region, we find a specific viral segment

packaging signal (Ψ), which allows an efficient encapsidation of the different vRNPs inside the virion. Adapted from [47].

IAV typically infects epithelial cells in the upper respiratory tract, as it is usually transmitted in respiratory droplets or aerosols. Mild infections in immune-competent hosts are limited to this upper region, however, with more virulent strains, or in elderly, immune-deficient, or patients with different health conditions, infection can progress to different lung cavities and produce a severe disease [51]. The initial response to infections determines the outcome. IAV infection leads to the induction of innate immune responses which results in the production of IFNs and proinflammatory cytokines [52,53]. These are essential for the control of viral replication and clearance of infection, but a dysregulated or excessive response causes the previously mentioned phenomena, the cytokine storm, which can lead to tissue damage, ARDS, systemic inflammation, multiorgan failure, and even death [51,53,54]. Furthermore, IAV is able to antagonize these responses by multiple mechanisms, utilizing both viral and host proteins [52]. Thus, responses must be well balanced between an effective and potent antiviral defense and avoiding an excessive and maintained response that could lead to the generation of immune-driven tissue damage [55].

1.1.3. Dengue virus (DENV)

DENV (formally named *Orthoflavivirus denguei*), belongs to the *Flaviviridae* family, *Orthoflavivirus* genus [8], a group of enveloped icosahedral viral particles of ~50 nm and a +ssRNA genome of ~11 kb, with a 5'-m7G cap at the 5' end, like those of cellular mRNAs, but lacking a 3' poly(A) tail. *Orthoflavivirus* genus includes some of the most relevant arboviruses (viruses transmitted by mosquitoes) to human health, such as *Orthoflavivirus flavi* (yellow fever virus, YFV) or *Orthoflavivirus zikaense* (Zika virus, ZIKV), apart from DENV. Within *Orthoflavivirus denguei*, we can differentiate 4 different serotypes: DENV1, DENV2, DENV3, and DENV4. DENV is fully adapted to humans, and its transmitted through the bites of the mosquitoes species *Aedes aegypti* and *Aedes albopictus* [56], usually found in tropical and subtropical regions. DENV exists in two distinct cycles, a sylvatic enzootic cycle where non-human primates act as reservoirs, with *Aedes* mosquitoes transmitting the infection, and an endemic urban cycle, where humans are the reservoir of the virus, which is found at high levels in blood, and transmitted by *Aedes* mosquitoes' bites to other humans. However, there is a constant risk of emergence or re-emergence of DENV viral strains, as these viruses could be potentially transferred from the sylvatic cycle to humans in rural areas, as for instance, in Africa and Asia, where the mosquito vectors reach high densities. Also, this represent the most probable scenario of how DENV was introduced to humans, with the adaptation of *Aedes* mosquitoes to urban environments [57]. DENV is mostly prevalent in tropical and subtropical urban and semi-urban settings, and it is estimated that half of world population are at risk of being infected by DENV. This is due to the fact that *Aedes* mosquitoes live in urban areas, and this mosquito is expanding to new geographical areas due to climate change with increasing temperatures and high rainfall and humidity, as well as due to several socioeconomical reasons [58,59]. In the year 2000, near 500,000 cases were reported, whereas in 2024, more than 14.6 million cases and more than 12,000 deaths were reported (being these numbers highly underestimated, as current models estimate up to 390 million infections every year), putting into perspective the enormous growth and burden of DENV infections [58]. All this makes DENV the arboviral disease that causes the greatest number of cases and deaths worldwide [56], and the situation is not expected to improve.

DENV presents extensive genetic variability, both among and within its four serotypes, primarily due to the error-prone nature of the RNA dependent RNA polymerase (RdRp). Aminoacidic

sequence variability across the four DENV serotypes is approximately 25–40%, while within each serotype it is near 3%. Moreover, significant intra- and inter-host genetic diversity in humans and mosquitoes has been reported [60]. The capacity of DENV to change and adapt has prevented the development of effective vaccines against all four serotypes, and represents a significant risk for the emergence of viral strains resistant to antiviral therapies [61,62]. A critical obstacle in vaccine development and dengue epidemic control is antibody-dependent enhancement (ADE), which increases disease severity in a secondary DENV infection with a serotype different to the first one. Immunoglobulin G (IgG) antibodies are generated from the first infection, but these are not able to neutralize a heterotypic infection with a different serotype of DENV. Instead, IgGs bind but do not neutralize DENV, forming virus-antibody complexes that are recognized by Fcγ receptors (FcγR), facilitating virus entry on target immune cells. This process triggers an immune cascade that leads to vascular leakage and is associated with the manifestation of severe forms of the disease such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [63].

As previously mentioned, DENV has a +ssRNA genome of ~11 kb that is directly translated upon entering host cells by host translation machinery. The DENV genome codifies for both structural and non-structural proteins [64]. The structural proteins are capsid protein (C), pre-membrane/membrane protein (prM/M), and envelope (E), while the non-structural (NS) proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [64] (Fig. I4).

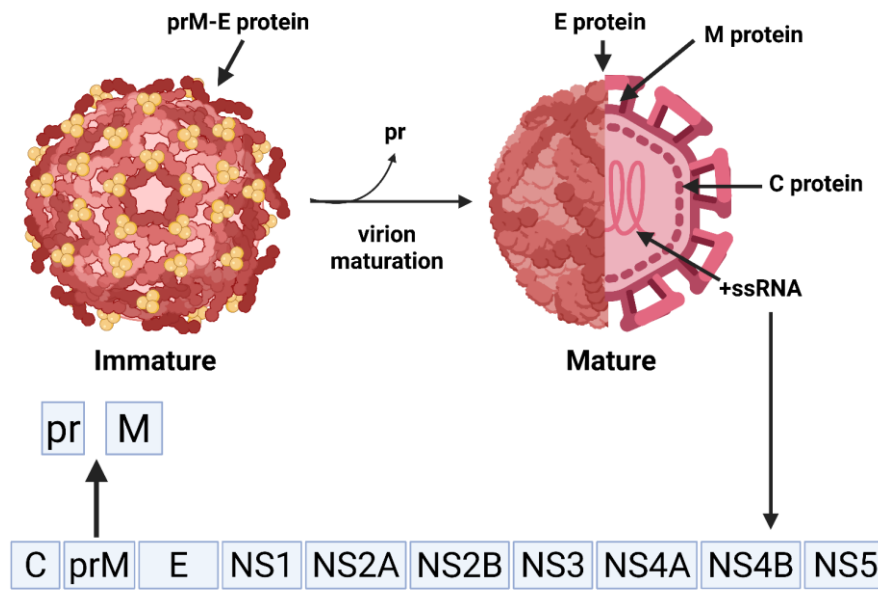


Figure I4. Structure of DENV immature and mature particles. Structural, non-structural (NS) proteins, and genome are indicated. The viral genome is a +ssRNA of ~11 kb. The capsid protein (C) contributes to nucleocapsid generation, that covers and protects the genetic material. The envelope (E) protein participates in receptor binding and fusion with cell membrane. In the immature virion, E protein forms a heterodimer with prM protein. During maturation, the virion transits through the trans-golgi, where thanks to the low pH and the protease furin, pre-membrane (prM) protein is cleaved in pr and membrane protein (M). pr stays attached to E protein (maintaining a position as a cap of E protein), while M protein lies embedded in the viral membrane, beneath the E protein shell. Following furin cleavage, the mature virion is secreted and the pr peptide is released from the mature particle. NS1 protein participates in replication, formation of viral particles, and inhibition of complement activation. NS2A is involved in replication, assembly, pathogenesis, immune evasion, and IFN resistance. NS2B is a protease cofactor of NS3. NS3 is a multifunctional protease and helicase that cleaves DENV polyprotein, and together with NS2B, generates mature protein C and prM, and participate in the cleavage of antiviral proteins. NS4A participates in IFN resistance and membrane induction, important for virus replication. NS4B is implicated in assisting viral

RNA replication through its interaction with NS3, and to block IFN-induced signaling. NS5 is a methyltransferase and has RdRp activity. Figure developed with information from [64,65].

Pathogenesis of DENV infections is highly complex, as we find asymptomatic infections, moderate febrile illness (dengue fever, DF), and in more severe cases, DHF and DSS. These severe forms of the disease are characterized by coagulation abnormalities, plasma leakage, and increased vascular fragility, which leads to fluid loss. This fluid loss can produce a hypovolemic shock and multi-organ failure [66]. Disease produced by DENV infection is highly complex, as various factors contribute to the progression of DENV infections towards either severe or non-severe forms of the disease. Correlating with what has been discussed for SARS-CoV-2 and IAV, one of the main factors determining prognosis is the dysregulation of innate immune responses. This dysregulation leads to an exacerbated immune activation and consequently to a cytokine storm that favors the development of DSS, multi-organ failure, and ultimately death [67,68].

Dayarathna *et al.* [69] analyzed the similarities and differences of cytokine storms induced in COVID-19 and DHF by comparing the induction of several cytokines in patients of varying severity. These authors found that patients that developed COVID-19 severe pneumonia (SP) and patients that developed DHF, showed high levels of IL-6, IL-10, and Chemokine (C-C motif) ligand 20 (CCL20) during early disease. Also, COVID-19 patients that developed SP, showed lower IFN γ levels during early illness than patients that had milder disease [69]. In addition, studies of cytokine inductions in severe *versus* non-severe IAV patients were performed. Xie *et al.* [70] showed that levels of IL-6, IFN, and IL-2 were incremented in patients with IAV SP (including 2009 H1N1, H1N1, H3N1, and H7N1) compared to patients with non-SP, while levels of IL-5, IL-25, IL-17A, and IL-22 decreased. In another study, Bradley-Stewart [71] *et al.* found that plasma concentration of IL-6, IL-10, IL-15, IFN γ -induced protein 10 (IP-10 or C-X-C motif chemokine ligand 10, CXCL10), IL-2R, hepatocyte growth factor (HGF), IL-1 receptor-like 1 (IL1R1), and chemokine (C-X-C motif) ligand 9 (CXCL9), were significantly incremented in patients with severe IAV infections (H1N1) compared to those with mild IAV.

All these data put into perspective the critical regulation of innate immune responses, as from the onset of the infection, these responses will determine the outcome and the severity of the disease that is produced. The final goal must be to comprehend better the molecular pathways that constitute antiviral responses, in order to be able to modulate them in a manner that allows for a better restriction of the infection of RNA viruses and to avoid immune-mediated damage. All in all, we seek to study host-factors that are implicated in these responses and whose functions and importance are still unknown.

1.2. INNATE IMMUNE RESPONSES AFTER RNA VIRUS INFECTIONS

We have already mentioned the critical role of the innate immune system as the host's first responder to viral infections. Thus, understanding its mechanisms is fundamental to decipher how viruses such as SARS-CoV-2, IAV, and DENV are sensed, how their replication is restricted or how these viruses are able to evade immune detection [72]. Therefore, this thesis will focus on studying novel players and functions within the innate immune responses induced after the infection with RNA viruses, giving special attention to molecular sensors and signaling pathways involved in the antiviral responses.

Innate immune responses act as the first line of defense against viral infections. The cellular recognition of certain molecules on pathogens, known as pathogen-associated molecular patterns (PAMPs), will initiate these responses. PAMPs include lipopolysaccharides, glycoproteins,

proteoglycans, and the most interesting for this PhD thesis, nucleic acid motifs, such as double-stranded RNAs (dsRNAs) and 5'phosphate ssRNAs [73]. Innate immunity is not completely nonspecific, as is able to discriminate between self- and pathogen-molecules, thanks to a limited number of germline-encoded cellular sensors that will detect PAMPs [74]. These are specialized receptors, known as pattern recognition receptors (PRRs) [73,74], which are constitutively expressed in host-cells and detect pathogens in any of their life-cycle stages, with the aim of initiating an effective response against them. PRRs are highly evolutionarily conserved among species, from plants to mammals [74]. Among the different PAMPs, the study of antiviral responses induced by viral RNA motifs is immensely broad, as a large number of sensors and effectors recognize these structures and activate different antiviral pathways [75]. In the response to RNA viruses, RNA binding proteins (RBPs) that recognize and bind viral RNAs are essential. We can differentiate, on the one hand, PRRs that are capable of recognizing RNA motifs of viruses and initiate signaling pathways to respond to the infection [73,75,76], and on the other hand, RNA-dependent effector proteins, which are also capable of recognizing viral RNA motifs or are activated downstream of sensor PRRs, and translate RNA detection into an effective antiviral response through their antiviral functions [73,76].

1.2.1. Viral RNA specific PRRs

PRRs that play an essential role in the recognition of RNA viral infections can be categorized in three different groups:

1. Toll-Like Receptors (TLRs).

TLRs are type I transmembrane proteins that contain three structural domains: leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain [77]. Since the first report of a Toll protein in the fruit fly *Drosophila melanogaster*, 10 TLRs have been identified in humans (TLR1-TLR10) [78]. TLRs recognize PAMPs within the extracellular and endosomal compartments. Of these, three TLRs play a critical role in recognition of the viral RNAs: TLR3 recognizes dsRNA, while TLR7 and TLR8 recognize ssRNAs [77].

2. Nucleotide-binding Oligomerization Domain-containing (NOD)-Like Receptors (NLRs).

NLRs are cytosolic regulators of inflammatory and apoptotic pathways in response to viral RNA. Of particular importance is the NOD-like receptor family, pyrin domain-containing 3 (NLRP3). This protein oligomerizes upon a two-step process that includes priming (transcriptional upregulation and post-translational modifications) and activation by different PAMPs or damage-associated molecular patterns (DAMPs). Activated NLRP3 recruits the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and the centrosomal NIMA-related kinase 7 (NEK7). This complex is known as the inflammasome. This active complex activates caspase-1, which cleaves pro-IL-1 β and pro-IL-18, leading to generation of the mature forms of these cytokines, which have a pro-inflammatory function. Also, caspase-1 cleaves a pore-forming protein known as gasdermin D, which mediates a type of cell death known as pyroptosis and the release of IL-1 β and IL-18 [73,79].

3. Retinoic Acid-Inducible Gene I (RIG-I)-Like Receptors (RLRs)

RLRs are a family of nucleic acid receptors capable of detecting the presence of viral RNA in the cellular cytoplasm that is introduced during infections or that accumulates during replication. This family of receptors is comprised of three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [80]. The three members

have a DExD/H box-containing RNA helicase domain and a so-called carboxy-terminal domain (CTD), while only RIG-I and MDA5 additionally have two tandem caspase activation and recruitment domain (CARD) at their amino-terminal end [81]. CARD domains are essential for downstream transduction of antiviral signaling, so LGP2 lacks functionality in this regard, but is widely believed to regulate RIG-I and MDA5 functions [82] (Fig. 15).

1.2.1.1. RIG-I-Like Receptors (RLRs) and the initiation of host innate immune responses

Even though both RIG-I and MDA5 are sensors of RNA viruses, their main substrates are different, primarily due to differences in their structures [83]. Firstly, the target ligands for RIG-I are short blunt-ended dsRNAs with 5'-triphosphate (5'ppp), 5'-diphosphate (5'pp), and 5'-m7G cap, as well as ssRNAs with 5'-triphosphate (5'ppp) [83,84]. These types of structures are typically generated in viral replication and not by host metabolism. RIG-I is able to efficiently recognize viruses such as DENV (+ssRNA genome), Sendai virus (SeV, -ssRNA genome), or IAV (-ssRNA genome) [83,84]. Recent studies show that RIG-I also recognizes and inhibit SARS-CoV-2 (+ssRNA genome) replication but in an IFN-independent manner [85]. Some of these -ssRNA viruses such as SeV [86,87] or IAV [87,88] produce low levels of dsRNA in their life cycle, which are the main substrates of RLRs. However, the ability to recognize 5'ppp allows RIG-I to respond to infections of -ssRNA viruses, as these viruses have a highly conserved secondary structure termed panhandle, which is a short dsRNA duplex with a 5'ppp terminus formed during replication and transcription [83]. Secondly, in contrast to RIG-I, MDA5 preferentially binds to long dsRNAs (>1000 bp) with no end specificity, generating a closed ring around the internal sites in the dsRNA instead of interacting with RNA ends [89,90]. This interaction leads to the generation of cooperative filaments of MDA5 along the dsRNA molecule [90]. MDA5 is essential in the recognition of SARS-CoV-2, as several studies show that MDA5 governs sensing of SARS-CoV-2 infections in lung cells [91,92], and it has an essential role in the responses against members of *Picornaviridae*, *Flaviviridae*, and *Caliciviridae* virus families, all of which are +ssRNA viruses [93–95]. Thus, MDA5 seems to be specialized in the recognition of +ssRNA and dsRNA viruses, while RIG-I is able to efficiently recognize -ssRNA and dsRNA viruses. However, recent studies show that even though RIG-I is the primary PRR for recognition of IAVs or paramyxoviruses, such as SeV, MDA5 is also a significant contributor to the cellular defense against these viruses [96,97]. Thus, there are still significant gaps in our knowledge about the function and modulation of both RIG-I and MDA5 proteins. We especially need to understand better how additional regulators may cooperate with RLRs, determining their functions and substrates.

In a basal state in the absence of viral RNAs, both RIG-I and MDA5 are in closed conformations in which the N-terminal tandem CARD domains are masked by their interaction with the helicase domain, and post-translational modifications help to keep this unactive conformation [98]. When RIG-I and MDA5 interact with stimulating RNAs, this leads to a conformational change that exposes CARD domains, thanks to a flexible hinge between CARD domains and the helicase domain and between the helicase domain and CTD (Fig. 15). Next, several post-translational modifications further promote the activation of RIG-I and MDA5 [98](Fig. 15):

- The protein phosphatases 1 (PP1) α and γ participate in the dephosphorylation of CARD domains of RIG-I and MDA5, promoting their activation.
- Lysine (K)63 polyubiquitination is critical for the activation of RIG-I and MDA5. Several E3 ligases like tripartite motif (TRIM)25 and Riplet add K63-ubiquitin chains to RIG-I in different domains enabling the generation of RIG-I 2CARD tetramers [99–101]. While

these mechanisms are better characterized for RIG-I, some studies show that MDA5 activation is also dependent on K63-linked polyubiquitination, exerted by several E3-ubiquitin ligases [98] promoting its oligomerization along the dsRNAs.

- Reversible acetylation on the CTD of RIG-I has been shown to prevent RIG-I from binding to viral RNA, while the deacetylation by histone deacetylase 6 (HDAC6), allows for an efficient sensing of RNA and activation of RIG-I [80].
- Deamination by the adenosine deaminase acting on RNA 1 (ADAR1) catalyzes conversion of adenosines to inosines in dsRNAs substrates, thereby preventing recognition by various PRRs and the subsequent initiation of distinct signaling pathways [102].

In addition, there is a higher level of complexity to this regulation, as different proteins also modulate RIG-I and MDA5 function by affecting TRIM25-mediated activation, subcellular location, their ability to bind to RNA (co-receptor proteins) or their ability to oligomerize. Many of these protein regulators are dsRNA-binding proteins (dsRBPs), involved in many aspects of RNA biology [80]. Here, we present examples illustrating some of these mechanisms:

- **PKR-activating protein (PACT):** PACT is an dsRBP that interacts with the CTD of RIG-I, positively regulating its ATPase activity [103]. Recently, PACT has also been shown to positively modulate MDA5 function by facilitating its oligomerization, both in response to polyinosinic:polycytidylic acid (poly(I:C)) which is an analog of the dsRNAs that are generated after the infection with many RNA viruses [104], and viral infection [105].
- **Zinc finger CCHC-type containing 3 (ZCCHC3):** This protein functions as a co-receptor and recruits TRIM25 to RIG-I and MDA5, promoting their activity [106].
- **LGP2:** several studies have described that LGP2, the third member of the RLR family, have both positive and negative regulatory effects on RIG-I and MDA5 functions through different mechanisms [80]. LGP2 can function as a feedback inhibitor of RIG-I signaling by various mechanisms:
 - LGP2 negatively modulates RIG-I activity by interacting with TRIM25 and inhibiting the TRIM25-mediated K63-specific ubiquitination of the RIG-I N-terminal [107]. This prevents RIG-I binding to the mitochondrial antiviral signaling (MAVS) protein and subsequently, the transduction of the antiviral signal.
 - LGP2 acts as a negative feedback regulator by sequestering the immunomodulatory dsRNA [108]. Contrary, LGP2 has been shown to promote MDA5 function by facilitating its oligomerization, as mice lacking LGP2 were more susceptible to infections with the encephalomyocarditis virus (EMCV) and poliovirus, mainly recognized by MDA5 [109].
- **RNA helicases:** DEAH-box helicase 15 (DHX15), 16 (DHX16), and DExD/H-box helicase 60 (DDX60) and 6 (DDX6), facilitate RIG-I binding to dsRNA, promoting its activation. In contrast DDX21 negatively regulates RIG-I signaling by competing for RNA binding [81,110].

Nonetheless, even though these regulators and many others have been described, there is still a big need for a better understanding of the molecular mechanisms regulating RLR, as these responses are essential to respond to viral infections, but their dysregulation and overactivation can lead to an immune-mediated damage and the cytokine storm [110]. Thus, negative feedback mechanisms that stop RLRs signaling are essential and not completely understood.

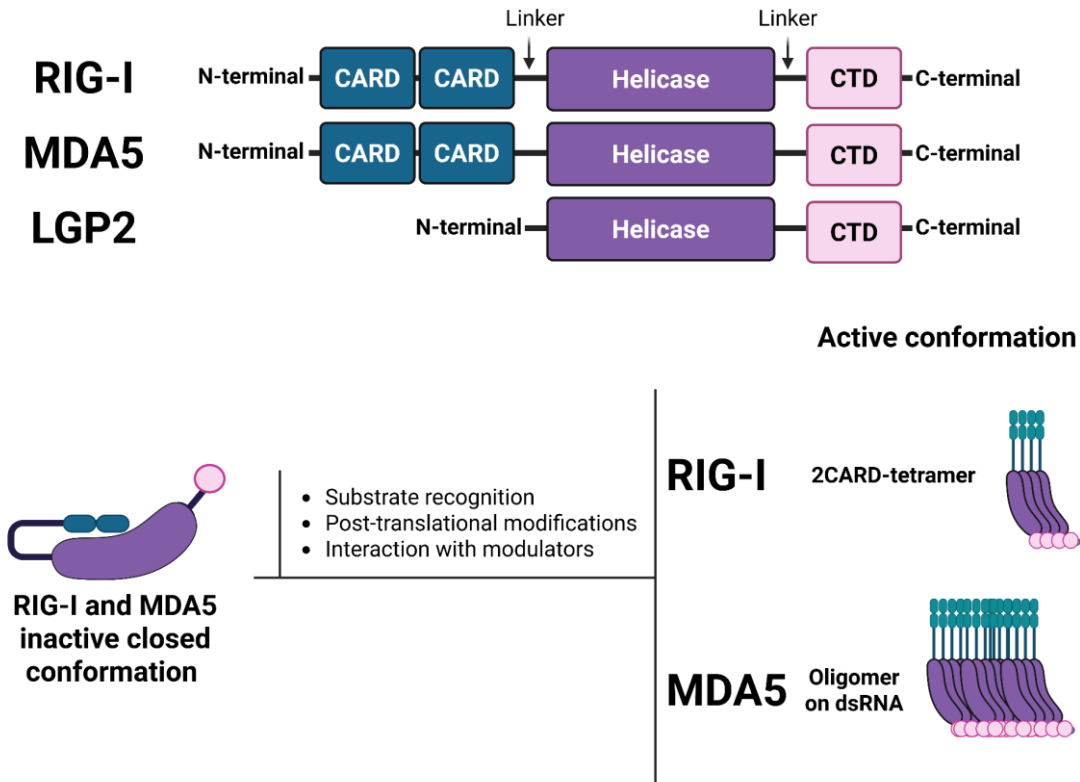


Figure 15. RLRs structure and conformational changes during activation. RLR family of receptors is composed of RIG-I, MDA5, and LGP2. LGP2 lacks the ability to interact with MAVS protein and promote the antiviral signaling pathways as it does not present functional CARD domains at the N-terminal. In basal conditions, RIG-I and MDA5 are autoinhibited due to a close conformation in which the CARD domains are blocked by the helicase domain. Upon substrate binding (short ds and ssRNAs with 5'ppp for RIG-I and long dsRNAs for MDA5), together with post-translational modifications and the interaction with different regulators, RIG-I and MDA5 can change their conformation and get activated. RIG-I generates a functional structure known as 2CARD tetramer while MDA5 generates active oligomers along the dsRNA substrate. Figure developed with information from [80].

Active RIG-I generates the 2CARD tetramer structure while activated MDA5 is oligomerized into filamentous structures along the longer dsRNA substrates [83,111] (Fig. 15). In both cases, CARD domains interact with the CARD domain of MAVS protein. MAVS is anchored at the mitochondrial membrane, peroxisomes, and mitochondrial-associated endoplasmic reticulum (ER) membranes (MAM) [112–114]. These CARD-CARD interactions promote MAVS oligomerization and filament formation into prion-like aggregates, which are essential for the transduction of antiviral signaling [115]. Activated MAVS is the essential mediator of the transduction of signaling. MAVS binds the tumor necrosis factor receptor-related factor (TRAF) members TRAF2, TRAF3, TRAF5, and TRAF6, which promote the activation of TANK (TRAF family member-associated NF- κ B activator)-binding kinase 1 (TBK1) complex and the I κ B kinase (IKK) complex [115]. TBK1 and IKK ϵ promote the phosphorylation and homodimerization of interferon-regulatory factor (IRF) 3 and/or IRF-7 transcription factors, which translocate to the nucleus as dimers and bind to the IFN-stimulated response elements, inducing the transcription of genes such as type I or type III IFNs [115,116]. On the other hand, the IKK complex activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) transcription factor, which promotes the transcription of proinflammatory cytokines [115,117] (Fig. 16).

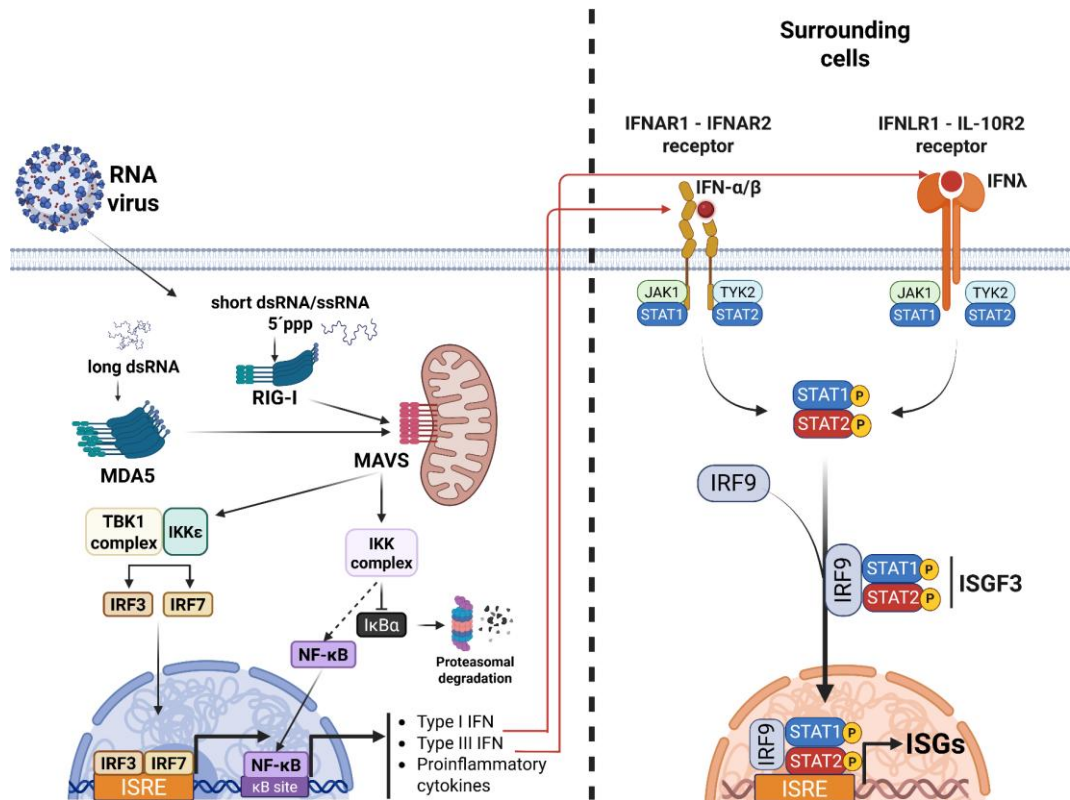


Figure 16. Schematic representation of RIG-I and MDA5 activation and transduction of antiviral signaling to surrounding cells. After an RNA virus enters cells, cytoplasmic RLRs recognize their PAMP substrates (MDA5 is primarily activated by long dsRNA, while RIG-I efficiently recognizes short ds and ssRNAs with a 5'ppp). MDA5 oligomerizes and RIG-I forms the 2CARD tetramer structure; these conformational changes lead to the binding of RLRs to the adaptor MAVS through CARD-CARD interactions. MAVS is inserted mainly at the mitochondrial membrane but can also be inserted at the membrane of peroxisomes and mitochondrial-associated ER membranes (MAM). MAVS is the responsible for the transduction of this signaling pathway that promotes the activation of the TBK1 complex and the IKK complex. TBK1 complex and IKK ϵ phosphorylate the transcription factors IRF-3 and IRF-7, leading to their translocation to the nucleus. In contrast, the IKK complex phosphorylates I κ B α , targeting it for degradation by the proteasome. I κ B α degradation liberates NF- κ B, which translocates to the nucleus. IRF-3, IRF-7, and NF- κ B, induce the transcription of type I and III IFNs and different proinflammatory cytokines. Type I and III IFNs act in surrounding cells binding their receptors IFNAR1-IFNAR2 and IFNLR1-IL-10R2, respectively. This binding activates a signaling pathway mediated through the Janus kinase and signal transducer and activator of transcription (JAK-STAT), generating the ISGF3 complex, comprising phospho-STAT1, phospho-STAT2, and IRF-9. This complex translocates to the nucleus and binds IFN-stimulated response elements (ISRE) in gene promoters, leading to the production of a plethora of genes known as interferon-stimulated genes (ISGs). Adapted from [98,118].

1.2.2. Viral RNA-dependent effectors

After the generation of IFNs and proinflammatory cytokines, IFN molecules act in an autocrine and paracrine way, binding their receptors: dimers of IFNAR1 and IFNAR2 proteins for type I IFNs or dimers of IFNLR1 and IL10RB for type III IFNs. This binding activates a signaling cascade through the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway [118]. This signaling leads to infected and surrounding cells to activate anti-viral programs by inducing the transcription of a plethora of genes known as interferon-stimulated genes (ISGs). These ISGs encode antiviral activities mediated by different molecular mechanisms

[118], for instance, translation arrest mediated by protein kinase R (PKR) [119], 2',5'-oligoadenylate synthetase (OAS)-RNaseL degradation of viral RNAs [120] or the negative effect on viral transcription, replication, translation, and trafficking by IFN-induced GTP-binding protein Myxovirus resistance (Mx)1 protein [121]. Furthermore, many of these ISGs are not only antiviral effectors, but also important regulators of the induction of antiviral responses, both positive and negative. On the one hand, some E3 ubiquitin ligases are encoded by ISGs, such as TRIM25, which modulate the activation of RIG-I [122], or ring finger protein (RNF)6, which further promote the induction of ISGs [123]. On the other hand, some ISGs are negative regulators of the innate immune responses, in order to avoid an excessive and sustained response, which, as previously explained with the cytokine storm concept, can be deleterious to the host [124]. For example, ISG15 protein has been described to exert a negative feedback regulation of RIG-I mediated antiviral signaling [125]. ADAR1, as previously mentioned, catalyzes conversion of adenosines to inosines in dsRNAs substrates preventing recognition by various PRRs [102]. Also, DeDiego *et al.* [126,127] described that IFN-induced protein 44 (IFI44) and 44-like (IFI44L) are negative regulators of innate immune responses by decreasing the ability of IKK β to phosphorylate the NF- κ B inhibitor I κ B α and the ability of IKK ϵ to phosphorylate IRF-3, thus reducing the production of IFNs and proinflammatory cytokines. Furthermore, some ISGs are specially interesting due to their ability to act as pivotal regulators of antiviral responses and cell homeostasis, such as PKR.

1.2.2.1. PKR effector activity and Stress-Granules (SGs) generation

Human PKR, also known as Eukaryotic Translation Initiation Factor 2 α Kinase 2 (EIF2K2), is codified by *EIF2K2* gene, located in chromosome 2. This protein is a 551-aminoacid long dsRBP, with serine-threonine kinase activity [128]. *EIF2K2* has an IFN-stimulated response element (ISRE) sequence in its promoter, so it is considered an ISG, being its expression induced after IFN stimulation [129]. However, this kinase is also constitutively and ubiquitously expressed on mammalian cells. PKR structure consists of two N-terminal tandem dsRNA binding motifs (dsRBMs), which are able to interact with viral dsRNA, and a C-terminal catalytic kinase domain [130]. In the basal state, in the absence of any infection, PKR is an inactive monomer, as its C-terminal kinase domain remains autoinhibited by the intramolecular interaction with the N-terminal dsRBMs. Upon recognition and binding of viral dsRNAs or RNA duplexes by the dsRBMs, PKR conformation changes and the kinase domain is freed from the autoinhibition, enabling its activation. Then, PKR molecules dimerize and undergo trans-autophosphorylation on serine and threonine residues, which stabilizes the active conformation and amplifies their activation level [131]. Next, several molecules of PKR can interact, and these oligomerized and active PKR molecules phosphorylate the eukaryotic Initiation Factor 2 α (eIF2 α) at the Ser52 residue. In normal homeostatic conditions, the delivery of methionyl-initiator transfer RNA (tRNA^{Met}) to the 40S ribosomal subunit is mediated by GTP-bound eIF2 complex, consisting on α , β , and γ subunits [119]. GTP hydrolysis to guanosine diphosphate (GDP) favors the binding of tRNA^{Met} to the 40S subunit. In order to start a new translation cycle and to incorporate new tRNA^{Met}, GDP must be exchanged for a new GTP molecule. GTP binding to eIF2 is the limiting process, and it is mediated by eIF2B, a guanine exchange factor (GEF). Thus, when the α subunit of eIF2 is phosphorylated by PKR, eIF2B is sequestered, generating an eIF2-GDP-eIF2B complex that can no longer initiate new translation cycles, leading to the suppression of *de novo* protein synthesis [119]. This is not unique to viral infections, as PKR dysregulation has been linked to inflammatory processes, metabolic disorders, and diseases such as cancer and neurodegeneration

[128,129,132]. Also, there are other similar kinases able to induce translation stalling in response to other types of cellular stress [119]:

- Heme-regulated inhibitor, HRI (EIF2AK1), in heme deprivation, oxidative stress or heat shock.
- Protein kinase RNA-like ER kinase, PERK (EIF2K3), in ER stress or glucose deprivation.
- General control nonderepressible 2, GCN2 (EIF2K4), in nutrient deprivation or UV irradiation conditions.

Translation stalling in response to viral infection, also known as host shut-off, is well studied antiviral mechanism, positioning PKR as one of the central players in innate immune responses. The inhibition of new rounds of translation prevents infecting viruses from synthesizing their own proteins, thereby inhibiting viral replication and the production of viral particles [128]. However, this process can also affect the translation of host proteins. Some studies show that PKR activation after hepatitis C virus (HCV), ZIKV, or vesicular stomatitis virus (VSV) infection, leads to the reduction of translation of certain ISGs and IFNs [133–136], adding more complexity to PKR function.

One clear demonstration of PKR's critical role in the antiviral responses is the extensive number of strategies that different viruses have developed to overcome or antagonize PKR pathway: PKR degradation, dsRNA sequestration, RNA degradation, interference through cellular partners, inhibitory interactions with PKR, small viral RNAs, pseudo substrates, eIF2 α dephosphorylation, the recovery of eIF2B activity, or different translation systems independent of eIF2 α , such as internal ribosome entry site (IRES) [129,137,138].

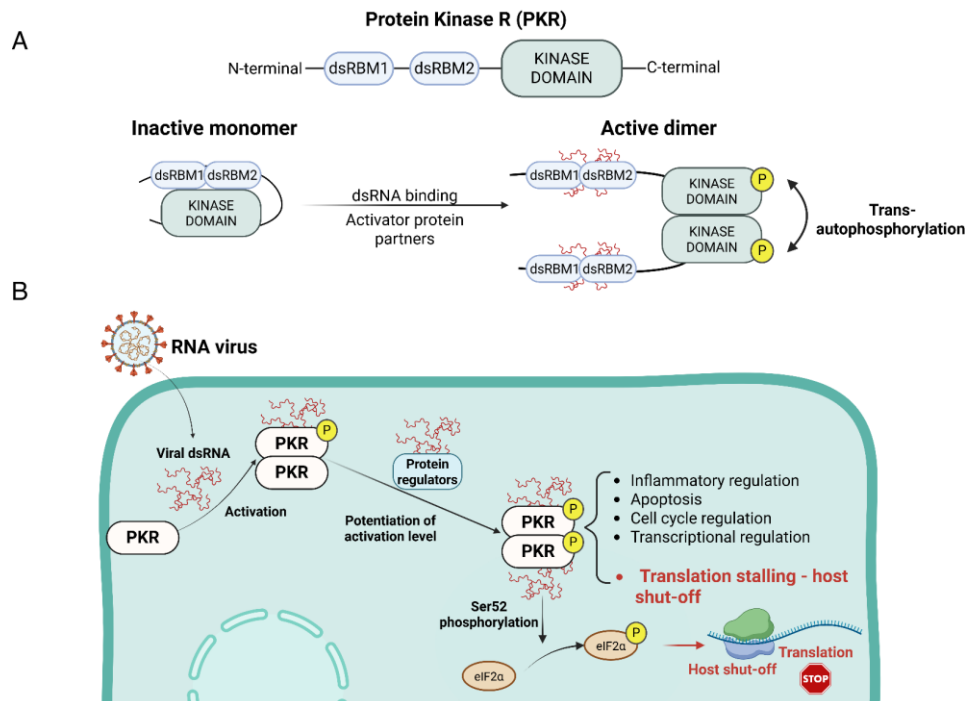


Figure 17. Structure, activation, and function of PKR. (A) PKR structure and activation mechanism. In basal conditions, PKR is auto repressed, as the catalytic domain is blocked by the intramolecular interaction with the dsRBMs. Upon recognition and binding of dsRNAs and/or interaction with positive regulators of PKR-activity, PKR conformation changes, and kinase domains are released from their interaction with the dsRBMs. This allows PKR to form homodimers through the interaction between the

C-terminal kinase domains, followed by trans-autophosphorylation that activate the kinase activity. Structure adapted from [139]. **(B) PKR induced host shut-off.** Active PKR phosphorylates eIF2 α , leading to a translational stalling or host shut-off of translation. Active PKR have other functions such as the regulation of inflammatory responses, apoptosis, cell cycle and transcription.

Different cellular protein partners have been described to modulate PKR function, and of these, three dsRBPs are specially interesting: PACT [132,140], transactivation response element RNA-binding protein 2 (TRBP or TARBP2) [141], and ADAR1 [130], being PACT the most extensively studied. PACT is a dsRBP composed of three dsRNA-binding domains (dsRBDs). The first two dsRBDs mediate RNA binding, while the third motif is involved in protein-protein interactions and is critical for activating PKR, as it associates with the kinase CTD promoting its activity [130,142]. PACT is an essential modulator of PKR under different types of cellular stress, such as ER perturbations, oxidative stress in mitochondria, or infection conditions [132,143], and can activate PKR even on absence of a dsRNA substrate [140,144]. Conversely, TARBP2 acts as an inhibitor of dsRNA-dependent activation of PKR function [141,145]. Similar to PACT, TARBP2 is also composed of three dsRBDs, two of which interact with the dsRNA while the third one, the C-terminal copy, is involved in protein-protein interactions [142]. PACT and TARBP2 modulation of PKR are connected. The main difference between both proteins resides within the C-terminal dsRNA-binding domain (Ct-dsRBD). In response to certain types of cellular stressors, PACT Ct-dsRBD is phosphorylated at the Ser246 and Ser287 by an unknown kinase, and it interacts with PKR [146], leading to the homodimerization and activation of the last [142]. In contrast TARBP2 C-terminal dsRBD, is inhibitory for PKR activity, as it interacts with PKR promoting the generation of heterodimers of PKR-TARBP2 [141], instead of promoting PKR homodimers, which are required for PKR activation. Interestingly, when PACT is phosphorylated, it reduces its affinity to interact with TARBP2, and increases its affinity for PKR, promoting its activation [147]. The importance of PACT as a regulator of PKR is illustrated by the existence of viral proteins capable of preventing their interaction. For instance, Us11 protein of the herpes simplex virus type 1 (HSV-1) binds to PKR to prevent its interaction with PACT [148]. In addition, OV20.0 protein, encoded by the member of *Poxviridae* family, the orf virus (ORFV), interacts with PACT, impeding its interaction with PKR [149].

However, other studies have shown that PACT-dependent activation of PKR is conditional and much more complex as initially thought. One example of this complexity was described by Clerzius *et al.* [150], as they observed that during human immunodeficiency virus 1 (HIV-1) infection, PACT behaves as an inhibitor of eIF2 α phosphorylation mediated by PKR, instead of an activator, leading to an increment of HIV-1 protein expression and virion production. Additionally, they observed that TARBP2 and ADAR1, also inhibited PKR and eIF2 α phosphorylation and interacted with PACT. Their hypothesis is that HIV-1 has adapted to change PACT function to that of an inhibitor of PKR, probably due to a combinatory activity with TARBP2 and ADAR1 [150,151]. As we previously mentioned, the phosphorylation of PACT on the Ser246 and Ser287 residues are hallmarks of PACT-mediated activation of PKR. So, one possibility is that different post-translational modifications in PACT could explain this switch to become an inhibitor [132]. In line with this, Manjunath *et al.* recently described that in the absence of infection, PACT cooperates with ADAR1 to suppress PKR function, avoiding a PKR activation by self-dsRNAs in uninfected cells [152]. Also, seeing how PACT interaction with TARBP2 and ADAR1 modifies its function as a PKR activator, it would be of particular interest to identify regulatory proteins with similar roles, capable of modulating the balance towards either enhanced or reduced activation of the PKR pathway.

Host shut-off of translation induced by PKR activity is the main driver of viral-induced stress granules (SGs) [153,154]. SGs are membrane-less cytoplasmic aggregates, that are generated in response to different types of cellular stressors, such as oxidative stress, heat shock, nutrient deprivation, ultraviolet radiation, proteotoxic stress, and viral infections. These cellular foci derive from stalled pre-initiation complexes and are mainly composed by messenger ribonucleoprotein (mRNPs) complexes, 40S ribosomal subunits, RBPs such as eIF2 α , eukaryotic initiation factor 3 (eIF3), ras GTPase-activating protein-binding protein 1 (G3BP1), and many other proteins such as PKR, RIG-I, or MDA5 [154–157]. Translation arrest induced by PKR-mediated phosphorylation of eIF2 α leads to the dissociation of translation initiation complexes, so that the different components of SGs are recruited and aggregated. When the stressor conditions disappear, SGs can be either disassembled by chaperones or cleared by autophagy, followed by a recovery of protein translation. Thus, the generation and clearance of these structures is a highly dynamic process in constant exchange with the cytosol, in which mRNAs and proteins can shuttle in and out of the SGs in the seconds to minute range [154,156–158](Fig. 18). SGs are not only structures to store mRNPs when translation is stalled, as increasing evidence highlights their role as central hubs for antiviral signaling pathways. PKR function and the generation of SGs are coordinated processes [153] in which PKR not only mediates translational stalling, but also plays a not totally understood role on modulating other components of innate immune responses, such as RIG-I induction of IFN production [159], MDA5 [160], MAVS [161], cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) [162], as well as immune transcription factors such as NF- κ B [163], and STAT1 or STAT3 [164]. Furthermore, PKR has been described to regulate other cellular processes such as mitosis or apoptosis [165,166].

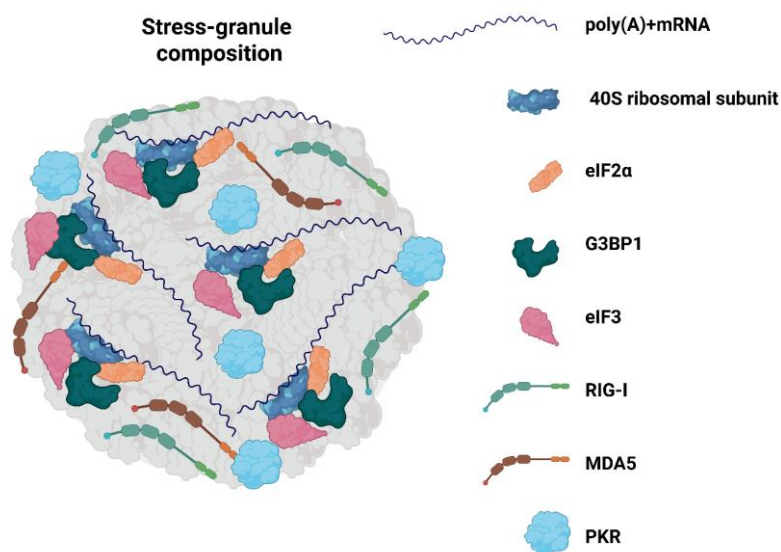


Figure 18. Schema of SGs composition. Figure adapted from [157].

Many viruses present different mechanisms to counteract the generation of SGs [167]. Nonetheless, there is still much to learn about the regulation of antiviral responses, as SGs are also preventors of excessive and uncontrolled innate immune responses. Paget *et al.* [168] proposed that SGs have a dual role: on the one hand, restricting viral replication by limiting translation, promoting the activation of RLRs and also by different RLR-independent processes; and on the other hand, acting as preventors of excessive activation of RLR signaling and immune-mediated cell death, allowing cells to restore homeostasis and survive. These findings show the controversial and highly complex role of all the players we have described.

1.3. IN SEARCH OF NOVEL FUNCTIONS OF IFI27 AND IFI6 PROTEINS

As previously highlighted, the biological functions and molecular mechanisms of many ISGs remain poorly characterized [169]. There is a great potential of finding novel functions and regulatory mechanisms within the wide genetic repertoire of ISGs that have not been studied in depth, as even new antiviral mechanisms of well characterized ISGs had come to light in recent years [169]. Describing these processes could explain how these responses amplify RLR signaling, or conversely, play negative feedback roles necessary to fine-tune or terminate the response and prevent the development of situations such as aberrant cytokine production and immune-mediated damage [81,98,110].

Considering this, the objective of this thesis is to explore novel functions and molecular mechanisms of ISGs that had not been characterized before, in order to improve our understanding of innate immune responses against RNA viruses of significant public health importance, such as SARS-CoV-2, IAV, or DENV. In addition, the identification of host factors affecting the induction of innate immune responses and viral replication guides the development of antiviral compounds directed against these factors. In this sense, the implementation of antiviral therapies targeting host factors or viral-host interactions could represent a better strategy to fight viral infections compared to drugs directed towards viral proteins, as the probability to generate resistant-viruses is negligible, and they may have also a broad spectrum against different viruses, opportunistic pathogens that become virulent in unhealthy individuals, and in case a new pandemic begins. Within this framework, special attention was directed towards two ISGs that had been scarcely studied before: IFN alpha-inducible proteins 27 (IFI27) and 6 (IFI6).

1.3.1. Genetic and molecular characteristics of IFI27 and IFI6

IFI6 (named also *GIP3*, *IFI6-16*, *ISG6-16*) was among the first ISGs to be ever identified by Friedman and others, and Kelly and others (in 1984, 1985-1986, respectively). These studies detected a 100-fold induction of its mRNA in HeLa cells after IFN- α and IFN- β stimulation [170–172]. *IFI27* (named also *ISG12*, *ISG12a*, *FAM14D*, and *P27*) mRNA was subsequently identified as an ISG related to *IFI6* by Rasmussen *et al.* in 1993 [173]. *IFI27* was initially described as an estrogen-inducible protein, and renamed later due to its strong induction after IFN- α stimulation [173,174]. Both proteins are highly induced after the stimulation with type I IFN with some reports highlighting that *IFI6* mRNA can constitute as much as the 0.1% of total mRNA after type I IFN stimulation [175]. Furthermore, *IFI27* mRNA levels continue to accumulate beyond 96 hours post-IFN treatment, even if the IFN is removed after 2 hours, while most of ISGs are maximally induced after 2-16 hours [176]. Thus, it is reasonable to think that these genes must play a critical role in IFN responses, although their precise roles, molecular mechanisms and implication in host-pathogen interactions have not been elucidated yet.

IFI27 and *IFI6*, as well as genes encoding similar proteins, are included in the *FAM14* gene family. *In silico* data identified 46 members of this family across 24 different organisms, from single-cell amoeba to mammals. In humans, this family comprises 4 members: *IFI6* (*IFI6-16*), *IFI27* (*ISG12a*), *ISG12b*, and *ISG12c*. Only *IFI6* and *IFI27* are IFN-inducible, even though *ISG12b* and *ISG12c* also present a putative ISRE in the promoter [175]. An alignment of the 46 putative amino-acid sequences of the different members of the family identified a common motif of 80 amino acids, named ISG12 motif. However, outside of this motif, sequences of the different members of the family are divergent [174,175]. Different studies propose that the presence of primordial ISG12-like sequences in species such as *Dictyostelium discoideum*, which lack an IFN system, may indicate that *FAM14* family members are components of the cellular machinery that

respond to environmental or cellular stressors. This is supported by the fact that in different types of cancer, *FAM14* ISGs are constitutively expressed. Later in the evolution, these genes could have been included into the IFN system, to participate in the response against different types of stressors such as viral infections and oxidative stress [174,175]. Both *IFI27* and *IFI6* genes encode small hydrophobic proteins being the canonical isoforms of 122 (11.5 kDa) and 130 (12.9 kDa) amino acids, respectively, and both proteins share a 36% amino-acid identity [175]. *IFI27* gene is located on the chromosome 14q32 [177], while *IFI6* is on chromosome 1p35 [174].

IFI27 has an N-terminal mitochondrial targeting sequence, and a transmembrane region [174]. Several studies show that *IFI27* is located in the inner mitochondrial membrane, due to its hydrophobic nature [177–180]. Also, other studies claim the localization of *IFI27* in the nuclear inner membrane, and on the ER [177,181]. *IFI6* is also a hydrophobic protein, which has putative transmembrane helices. *IFI6* has also been described to locate at the mitochondrial membrane [182]. However, recent studies show that *IFI6* is mainly located at the ER [183,184] and nucleus [185] (Table I1).

Table I1. Molecular characteristics of canonical *IFI27* and *IFI6* proteins.

Gene name	<i>IFI27</i>	<i>IFI6</i>
Family of genes	<i>FAM14</i>	<i>FAM14</i>
Chromosome	14q32 [177]	1p35 [174]
Codified protein characteristics	Hydrophobic, transmembrane region, 122 amino acid (11.5 kDa) [174,177]	Hydrophobic, transmembrane region, 130 amino acid (12.9 kDa) [174]
Subcellular location of protein	Inner mitochondrial membrane [177–180], inner nuclear membrane, and ER [177,181].	Inner mitochondrial membrane [182], ER [183,184], and nucleus [185].

The roles of *IFI27* and *IFI6* are not well-described and have been studied mainly in the context of several metabolic processes, cancer, and infections. Due to its mitochondrial localization, both *IFI27* and *IFI6* have been related to the regulation of apoptosis. Several studies support the role of *IFI27* as a pro-apoptotic factor, while *IFI6* has been described to play an anti-apoptotic role. Rosebeck *et al* [176] proposed that *IFI27* is an IFN-dependent apoptotic factor, that perturbs the normal mitochondrial function. They described that transient expression of *IFI27* led to a decreased number of viable cells, as well as to an enhanced sensitivity to DNA-damage induced apoptosis. *IFI27* promoted the etoposide-induced release of cytochrome c and activation of the proapoptotic factor Bax (B cell leukemia/lymphoma 2 (Bcl2)-associated X), leading to a loss of mitochondrial membrane potential. These effects were counteracted by the co-expression with the antiapoptotic protein Bcl2 or by treatment with a pan-caspase inhibitor [176]. *IFI27* has also been shown to interact with the nuclear receptor subfamily 4 group A member 1 (NR4A1), inducing TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through intrinsic apoptotic pathway [186]. Conversely to this, *IFI6* has been shown to antagonize TRAIL-induced apoptosis through the inhibition of the intrinsic apoptotic pathway, blocking the release of cytochrome C [187]. Additionally, Gytz *et al.* [188] found two putative Bcl2 homology (BH)3-like motifs, which may be decisive for the pro-apoptotic properties of *IFI27*. Also, they describe that both *IFI27* and *IFI6* sensitize HEK-293T cells to BH3 mimetic gossypol induced apoptosis [188]. Thus, there are different studies showing the importance of both genes in apoptotic processes, but their regulation and specific mechanisms still need better characterization. These apoptotic functions and their importance on mitochondrial metabolism [179,189] tightly associate

both *IFI27* and *IFI6* with different types of pathologies such as cancer, immune-mediated diseases like autoimmune processes, and viral infections.

1.3.2. Importance of *IFI27* and *IFI6* in viral infections of major concern: SARS-CoV-2, IAV, and DENV

Numerous studies, most of them related with gene expression analysis, have analyzed their importance in the context of various viral infections, showing both positive or negative effects on the infection progression depending on the study and the virus ([Table I2](#)).

- **SARS-CoV-2**

Several studies since the beginning of the COVID-19 pandemic have analyzed the potential of *IFI27* and *IFI6* as diagnostic and prognostic biomarkers of SARS-CoV-2 infections. The levels of expression of *IFI27* in blood of SARS-CoV-2 infected patients were found to be upregulated, allowing to distinguish between infected and non-infected subjects [190,191]. Similarly, on two different studies analyzing nasopharyngeal and/or oropharyngeal samples, *IFI6* was found to be significantly upregulated in COVID-19 patients compared to healthy controls and its expression levels positively correlated with viral load [192,193]. An analysis of upper airway gene expression in SARS-CoV-2 patients, showed a suppressed immune response against SARS-CoV-2 in comparison to patients infected with other respiratory viruses. A gene set enrichment analyses (GSEA) of differentially expressed genes (DEG) comparing SARS-CoV-2 infection with other respiratory viruses, suggested an attenuated IFN response in SARS-CoV-2 infections. Interestingly, even though the IFN responses were attenuated, they observed a significantly higher induction of *IFI27* and *IFI6* expression after SARS-CoV-2 infection compared to other respiratory viruses [194]. This finding is remarkable, as it shows that *IFI27* and *IFI6* induction positively correlates with attenuated IFN-mediated responses.

Using spatial transcriptomics, Tan *et al.* [195] showed that, in the lungs of SARS-CoV-2 patients that developed ARDS, M1-macrophages presented an elevated *IFI27* expression. In line with this observation, a multi-cohort study testing *IFI27* as a prognostic marker in COVID-19 patients, reported elevated *IFI27* levels in lower respiratory tract, correlating with a higher viral load [196]. Similarly, in blood samples, increased *IFI27* was associated with a systemic host response and severe outcomes, such as respiratory failure [196], associated with a cytokine storm and multiorgan failure.

- **IAV:**

Several studies have shown that *IFI27* is a reliable diagnostic marker for IAV infections [197–199], showing high accuracy and specificity to distinguish between IAV and bacterial respiratory infections [200]. Additionally, another study found that *IFI27* was significantly upregulated in IAV-infected individuals compared to healthy controls, especially in two cell populations that are essential for innate immune responses such as monocytes and conventional dendritic cells (DCs). This study also found an important upregulation of *IFI6* in patients with IAV compared to healthy controls [201].

- **DENV:**

Several studies have shown the potential antiviral role of IFI6 against several Flaviviruses such as DENV, West Nile virus (WNV), or YFV [183,202,203]. As previously mentioned, IFI6 localizes to the ER, an organelle with a crucial role on the generation of structures defined as replication organelles (ROs). These ROs are single-membrane vesicles that are generated by virus-induced invaginations of the ER membrane. Flaviviruses exploit these structures, where they efficiently synthesize viral RNA and assemble virions while hiding from innate immune sensors [204,205]. Richardson *et al.* [183], showed that IFI6 interacts with the ER-resident heat shock protein 70, chaperone BiP, and prophylactically protect from the infection of several Flaviviruses. On the other hand, several studies reported elevated *IFI27* expression in DENV patients, particularly in monocytes and plasmacytoid DCs (pDCs), two of the most essential populations in the immune responses against DENV [206,207]. Additionally, an integrative analysis of transcriptome data related to DENV infection showed that *IFI27* is a potential suitable biomarker for disease severity, as its levels of expression were found to be highly upregulated in the two more severe forms of the disease, DSS and DHF [208].

- **Other relevant viruses:**

Elevated *IFI27* levels have also been reported in blood of infants hospitalized with respiratory syncytial virus (RSV) [209], and associated with more severe cases, longer stays in hospital of preterm infants and a higher probability to require mechanical ventilation [210]. Also, *IFI27* was found to be upregulated in HIV-1 infected patients and reduced when these patients received antiretroviral therapy (ART) [211]. IFI6 has been described as an antiviral factor for HCV. HCV is internalized in hepatocytes by a clathrin-dependent endocytosis involving CD81 and Claudin-1 (CLDN1) [212]. The association of these two factors is required for HCV entry, and it is regulated by the epidermal growth factor receptor (EGFR) kinase. In this sense, Meyer *et al.* found that IFI6 inhibits the function of the EGFR thus impairing CD81-CLDN1 interactions and negatively affecting HCV entry [213].

Table 12. IFI27 and IFI6 known importance on SARS-CoV-2, IAV, DENV, and other RNA viruses.

Virus	IFI27	IFI6
SARS-CoV-2	• Upregulated in blood of SARS-CoV-2 patients [190,191]. • Higher induction of <i>IFI27</i> in SARS-CoV-2 compared to other respiratory viruses [194]. • High levels of <i>IFI27</i> in blood and lungs of patients that develop ARDS, systemic inflammation, and respiratory failure. This correlated with higher viral loads [195,196].	• Upregulated in nasopharyngeal and oropharyngeal samples of SARS-CoV-2 patients [192,193]. • Higher induction of <i>IFI6</i> in SARS-CoV-2 compared to other respiratory viruses, even though innate immune responses are suppressed [194]
IAV	• <i>IFI27</i> is a reliable diagnostic marker for IAV infections [197–199,201] and to compare IAV from bacterial respiratory infections [200].	• <i>IFI6</i> upregulation allows to distinguish IAV infected patients from healthy controls [201].
DENV	• Elevated <i>IFI27</i> expression in DENV patients, particularly in monocytes and pDCs [206,207].	• IFI6 exert prophylactic antiviral effects against DENV and other Flaviviruses by interfering with the

	IFI27 is a suitable biomarker for DENV disease progression, as elevated <i>IFI27</i> levels associate to the development of severe forms of the disease such as DHF and DSS [208].	generation of ROs in the ER [183,202,203].
Other RNA viruses	- Elevated IFI27 levels have also been reported in blood of infants hospitalized with RSV and associated with more severe cases [209,210]. <i>IFI27</i> was found to be upregulated in HIV-1 infected patients and reduced when these patients received ART. It was proposed as a therapeutic target [211].	IFI6 is an antiviral factor against HCV infection, as it negatively affects HCV entry by altering the proper regulation of CD81-CDLN1 interactions [213].

In summary, multiple evidences point to these genes as essential players in the innate immune responses, especially as possible modulators of fine-tuned responses aimed at avoiding excessive reactions that could lead to deleterious effects on hosts. However, these studies have not delved into the possible molecular mechanisms of IFI27 and IFI6.

OBJECTIVES AND HYPOTHESIS

2. OBJECTIVES AND HYPOTHESIS

Interferon-stimulated genes (ISGs) represent a plethora of different antiviral functions aimed at restricting viral replication. However, in recent years, many studies have found that some of these ISGs act differently, by modulating the innate immune responses. The magnitude and duration of antiviral IFN-responses are tightly related to different negative feedback modulatory mechanisms, as an excessive response can lead to inflammatory and immune damage of the host. Thus, deregulation on the expression of ISGs or proinflammatory cytokines is one of the determining factors that lead to the development of severe symptoms after the infections with SARS-CoV-2, IAV, or DENV. The function of many ISGs is still unknown, and in this work, we have focused on the study of two ISGs from the same family (*FAM14*): IFN alpha-inducible protein 27 (IFI27) and 6 (IFI6). These ISGs are among the first to be ever identified and have been associated to the infections with SARS-CoV-2, IAV, and DENV. *IFI27* and *IFI6* expression is strongly induced after the infections with different RNA viruses, which has led some authors to propose them as biomarkers in certain infections. Nonetheless, their effect on the replication of these viruses and molecular mechanisms have never been studied in depth.

General objective and hypothesis:

Considering the previously mentioned antecedents, the main objective of this work is to decipher the possible role of IFI27 and IFI6 on the infections with different RNA viruses, giving particular attention to SARS-CoV-2, IAV, and DENV2. We will focus particularly on the induction of innate immune responses, as our hypothesis is that IFI27 and IFI6 could have a modulatory effect on the induction of IFN and proinflammatory cytokines after the infection of RNA viruses, playing a yet undescribed essential role on antiviral responses.

- **Specific objectives**

To answer our questions, in this PhD thesis, we have set the following specific objectives:

- 1. Study the importance of IFI27 and IFI6 on the replication of RNA viruses: IAV, SARS-CoV-2, and DENV2.**
 - A. Analysis of the induction of IFI27 and IFI6 after the treatment with IFN or poly(I:C) or after the infection with SARS-CoV-2, IAV, and DENV2.
 - B. Analysis of the effect of IFI27 and IFI6 on the replication of SARS-CoV-2, IAV, and DENV2.
- 2. Analyze the effect of IFI27 and IFI6 on antiviral responses after RNA virus infections.**
 - A. Study the effect of IFI27 and IFI6 on the induction of antiviral responses after poly(I:C) treatment or infections with RNA viruses *in vitro*
 - B. Study the effect of IFI27 and IFI6 on the induction of antiviral responses after IAV infection *in vivo*
- 3. Determination of the molecular mechanisms underlying the modulation of innate immune responses by IFI27 and IFI6**
 - A. Analyze IFI27 and IFI6 molecular characteristics and mechanisms, to explain the observed effects on viral replication and on the induction of antiviral responses.
 - B. Study of IFI27-protein interactome, to find novel protein partners that help us to infer new molecular mechanisms of IFI27.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. CELL CULTURES

3.1.1. Cell lines and cell culture conditions

Different cell lines were used during the development of this doctoral thesis. These cells were cultured at 37°C in an atmosphere of 5% CO₂ and 90% humidity, using their respective culture medium as specified below for each cell line.

Human embryonic kidney 293T (HEK-293T) (ATCC CRL-11268), human lung epithelial carcinoma A549 (ATCC CCL-185), and African green monkey kidney epithelial (Vero E6) (ATCC CRL-1586) cells were kindly provided by Prof. Luis Enjuanes (Centro Nacional de Biotecnología, CNB-CSIC, Spain). Madin-Darby canine kidney epithelial (MDCK; ATCC CCL-34) cells were obtained from Prof. Luis Martínez-Sobrido (Texas Biomedical Research Institute, San Antonio, TX, US). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), and 50 µg/mL gentamicin (Gibco). A549 and HEK-293T cells overexpressing human angiotensin converting enzyme 2 (hACE-2) (A549-hACE2 and HEK-293T-hACE2) were generated as described below (section 3.1.2) and cultured in the same medium additionally supplemented with 2.5 or 5 µg/mL of blasticidin (ThermoFisher Scientific), respectively. Also, IFI6 and IFI27 Knock-Out (KO) A549-hACE2 cells were generated as described below (section 3.1.3) and grown in the same medium supplemented with 2.5 µg/mL of blasticidin. A549-hACE2 cell lines stably expressing IFI27 tagged with an HA tag (A549-hACE2-IFI27-HA), or the control cells transfected with a linearized empty plasmid (A549-hACE2-empty), were generated as described below (section 3.1.4), and cultured in the same medium supplemented with 400 µg/mL of hygromycin (ThermoFisher Scientific).

Human near-haploid wild-type (WT) HAP-1 cells, which are derived from leukemic cells, and IFI6 KO HAP-1 cells, generated using the clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/CAS9) technology, were obtained from Horizon Discovery, Inc. Both cell lines were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS (Gibco), and 50 µg/mL gentamicin (Gibco).

The non-tumorigenic human bronchial epithelial cell line BEAS-2B (CRL-9609) was kindly provided by Prof. Carlos López-Larrea (Instituto de Investigación Sanitaria del Hospital Universitario Central de Asturias, Spain) and maintained in LHC-9 medium (Gibco) supplemented with 10% FBS (Gibco), and 50 µg/mL gentamicin (Gibco).

HeLa M WT, HeLa M PACT-KO, HeLa M PKR-KO and HeLa M PACT and PKR double KO (DKO) cells were kindly provided by Thomas Michiels (Université Catholique de Louvain, Brussels, Belgium) [131]. These cell lines were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 50 µg/mL gentamicin (Gibco).

Baby hamster kidney-21 (BHK-21) cells were kindly provided by Dr. Pablo Gastaminza (CNB-CSIC, Spain) and maintained in DMEM (Gibco), supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (ThermoFisher Scientific).

For storage, cells were resuspended at a density of $1-2 \times 10^6$ cells per mL in FBS containing 10% dimethylsulfoxide (DMSO, Sigma) and frozen in liquid nitrogen.

3.1.2. Generation of hACE2-expressing A549 and HEK-293T cells

SARS-CoV-2 utilizes ACE2 as the main cell-receptor for entry [214,215]. A549 and HEK-293T cell lines are highly valuable immortalized cell lines to study viral infections. Their rapid growth and their permissiveness to different techniques and manipulations (transfections, treatments, knock-downs) make them a very suitable model to study viral-host interactions. However, both cell lines lack significant levels of endogenous ACE2 expression, rendering them non-permissive to SARS-CoV-2 infections [216,217]. In order to study SARS-CoV-2 replication in these cell lines, we generated A549 and HEK-293T lines stably expressing hACE2. A widely used method to achieve this objective is retroviral gene transfer [218], which allows for efficient transduction of genetic material to many cell lines (primary cell lines or established immortalized cell lines, including hard-to transfect-cells). The recombinant retrovirus vector genome integrates in the cellular genome leading to a stable and long-term transgene expression [218]. To this end, we produced hACE2-encoding retroviral particles and used these to transduce A549 and HEK-293T cells, resulting in the stable integration of hACE2 gene into their genome and its sustained expression. This protocol was performed in biosafety level (BSL) 2 conditions.

Retroviral vectors were generated by the co-transfection of three different DNA plasmids into HEK-293T cells (Fig M1):

- **gag-pol** (packaging unit, obtained from addgene): this plasmid contains the packaging proteins needed to produce the viral particles, as well as necessary enzymes for the particle construction of murine leukemia virus (MLV). In this packaging system, several viral proteins considered virulence factors were removed [219]. The deletion of the genes encoding for these proteins eliminates the risk of a WT virus reconstruction through recombination during the vector's production.
- **VSV-G** (obtained from addgene): this plasmid encodes the VSV glycoprotein (G). As the retroviral envelope (Env) protein has been deleted, the recombinant retroviral vectors will be pseudotyped with the VSV-G protein. This modification increases stability of the viral particles and broadens their tropism to different cell types, as VSV-G-pseudotyped viral vectors have a quasi-universal tropism and high efficiency of infection [220,221].
- **Plasmid encoding hACE2 and a blasticidin-resistance gene** (kindly provided by Dr. Pablo Gastaminza, CNB-CSIC, Madrid, Spain): This plasmid codifies the transgene we want to transduce (in this case hACE2) and a blasticidin-resistance gene. This plasmid carries a deletion in the U3 region of the viral long terminal repeat (LTR) at the 3' end, which abolishes its promotor activity leading to self-inactivating viruses. This mutation minimizes the risk of the generation of replication-competent viruses both in the producer and target cells [218,219]. This plasmid drives the expression of the transgenes and the blasticidin-resistance gene, producing RNA transcripts that contain a packaging sequence that facilitates their incorporation into virions generated in the infectious cycle. These RNA sequences are the only sequences incorporated into the virions. This specificity ensures the safety of the system [218].

Complete virions will consist of the structural retroviral proteins, the VSV-G protein in the envelope, and the RNAs of the transgenes (genes of interest). These virions will infect target cells, and gag-pol polyprotein transcripts will encode the enzymatic functions that allow reverse transcription (RT) of the RNA vector and the integration of proviral DNA in the cellular genome. Thus, these retroviral particles are able to infect and transduce the gene of interest and the

blasticidin-resistance gene into dividing and non-dividing cells, but not to produce new generations of infectious viruses, as they are not replication-competent [218].

To produce retroviruses expressing hACE2 and the blasticidin-resistance protein, 2.5 million HEK-293T cells were plated in 100 mm dishes (to achieve a 50% confluency the day of transfection) and grown at 37°C in a humidified atmosphere of 5% CO₂ and 90% humidity using DMEM (Gibco) supplemented with 10% FBS (Gibco), and 50 µg/mL gentamicin (Gibco) (growth medium). Next day, the medium was replaced with antibiotic-free growth medium 4 hours prior to transfection (Fig. M1). Then, transfection was performed by using, per 100 mm dish, 500 µL of solution A (containing 250 mM CaCl₂, 8 µg of gag-pol plasmid, 3 µg of VSV-G plasmid, and 5 µg of hACE2 and blasticidin-resistance protein-encoding plasmid), and 500 µL of solution B (containing 50 mM HEPES, 1.5 mM Na₂HPO₄, and 140 mM NaCl in H₂O ultrapure, pH 7.05 adjusted with 1M NaOH or 1M HCl). Solutions A and B were mixed and incubated for 20 minutes prior to their dropwise addition to the cells. At 24 hours post-transfection (hpt), the medium was removed and replaced with fresh complete supplemented growth medium. At 48 hpt, cell supernatants (containing viral particles) were collected, filtered using 0.45 µm filters, and 1 mL was used to transduce A549 or HEK-293T cells previously seeded in a 100 mm dish. Subsequently, transduced cells were selected by culturing them in medium containing gradually increasing concentrations of blasticidin (from 2.5 µg/mL to 10 µg/mL) to eliminate the risk of selecting non-transduced cells (Fig. M1). Once the transduced cells were selected and non-transduced cells were eliminated, HEK-293T-hACE2 and A549-hACE2 were grown using DMEM (Gibco) supplemented with 10% FBS (Gibco), 50 µg/mL gentamicin (Gibco), and 5 or 2.5 µg/mL of blasticidin (ThermoFisher Scientific), respectively, at 37°C in air enriched with 5% CO₂ and 90% humidity. Correct and stably expression of hACE2 was confirmed by immunofluorescence (Fig. R28B).

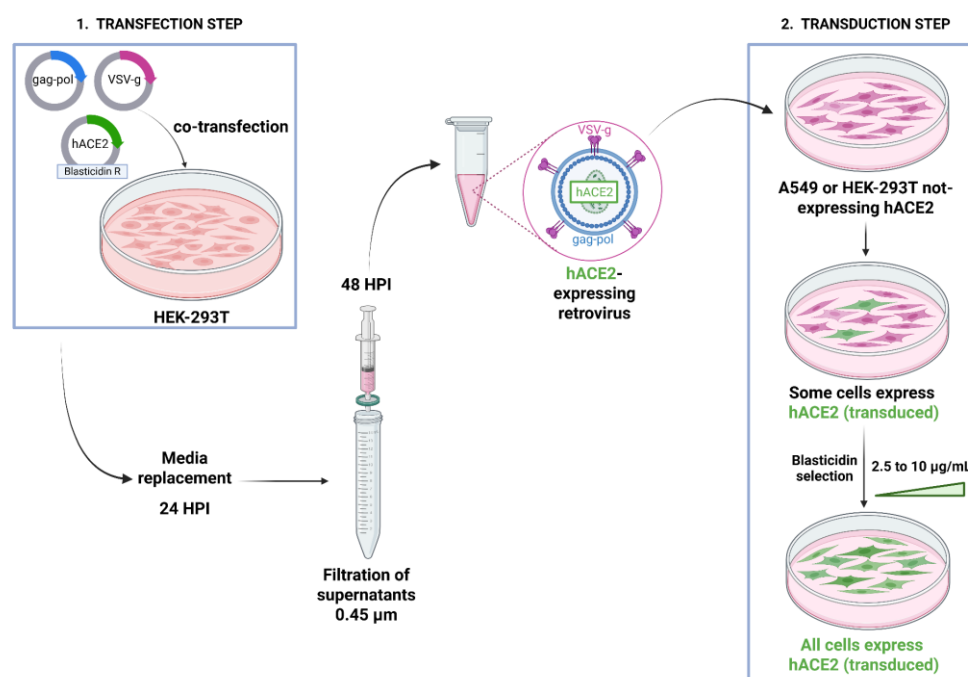


Figure M1. Generation of hACE2-expressing retroviral vectors and cell transduction. 1. Transfection step. 2. Transduction step.

3.1.3. Generation of A549 and A549-hACE2 cells KO for IFI27 or IFI6

To study the importance of IFI6 and IFI27 in innate immune responses against several RNA viruses, KO cells for either IFI6 or IFI27 were generated. By doing this, we can evaluate the effect of the complete absence of protein expression on viral replication and innate immune responses.

To generate KO cells for IFI6 or IFI27, we used the CRISPR/CAS9 system. The first step was to decide on the single-guide RNA (sgRNA) to use for knocking-out IFI6 and IFI27. To do this selection, we used two different webpages, ATUM (<https://www.atum.bio/eCommerce/cas9/input>) and CRISPOR.org (<https://crispor.gi.ucsc.edu/>), that offer different tools for selecting sgRNAs (Table M1).

Table M1. sgRNAs selected for IFI6 and IFI27 using the webpages of ATUM and CRISPOR.org

Target gene	sgRNA sequence (5' - 3')
IFI6 (2 different sgRNAs)	GCCCUGACCUUCAUGGCCGU
	GCUGACCUUCAUGGCCGUCGG
IFI27 (1 unique sgRNA)	GUGCCAUGGGCUUCACUGCGG

In order to generate IFI6 and IFI27-KO cells, A549 cells were seeded on 12-well plates at a 70% confluency and transfected with pX300 plasmids (1250 ng/well) expressing sgRNAs (Table M1) for either IFI6 or IFI27 (obtained as explained in the section plasmids, materials and methods section 3.5.2), and employing lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), following manufacturer's instructions. The sgRNA together with CAS9 will be expressed in transfected cells, so that the genomic DNA is modified (Fig. M2). In order to select transfected cells, at 24 hpt, cells were treated with 1 µg/mL of puromycin (InVivoGen). Transfected cells are resistant to puromycin as pX330 plasmids were modified to codify a puromycin-resistance gene (kindly provided by Pedro A. Mateos-Gomez, Universidad Alcalá de Henares, Madrid, Spain), so the cells that have not been transfected will not survive. At 48 hours after puromycin treatment, medium was changed to fresh supplemented medium without puromycin, and 24 hours later, surviving cells were detached using Trypsin-ethylenediaminetetraacetic acid (EDTA) (ThermoFisher Scientific) and cloned by limiting dilution up to three times, to avoid possible contamination with WT parental cells. After the last round of cloning, total RNA of the different clones was extracted, purified, retrotranscribed and amplified by polymerase chain reaction (PCR) (according to protocols detailed in subsequent sections 3.4.1, 3.8.1, and 3.8.2 using the appropriate primers (Table M2)). The PCR products were separated on an agarose gel electrophoresis (see materials and methods section 3.4.2) and the desired fragment was excised and purified using kit E.Z.N.A[®] Gel Extraction Kit (Omega Bio-tek). Subsequently, the purified DNA fragment, together with the specific appropriate primers (Table M2), was sent for sequencing by the Sanger method (Macrogen), to confirm the KO of the desired target gene. Sequences were visualized and analyzed using the Serial Cloner software. For SARS-CoV-2 studies, parental A549, A549-IFI6-KO and A549-IFI27-KO cells were transduced with retroviruses expressing hACE2 and a blasticidin-resistance gene (as explained before in section 3.1.2), in order to make these cells susceptible to SARS-CoV-2 infections.

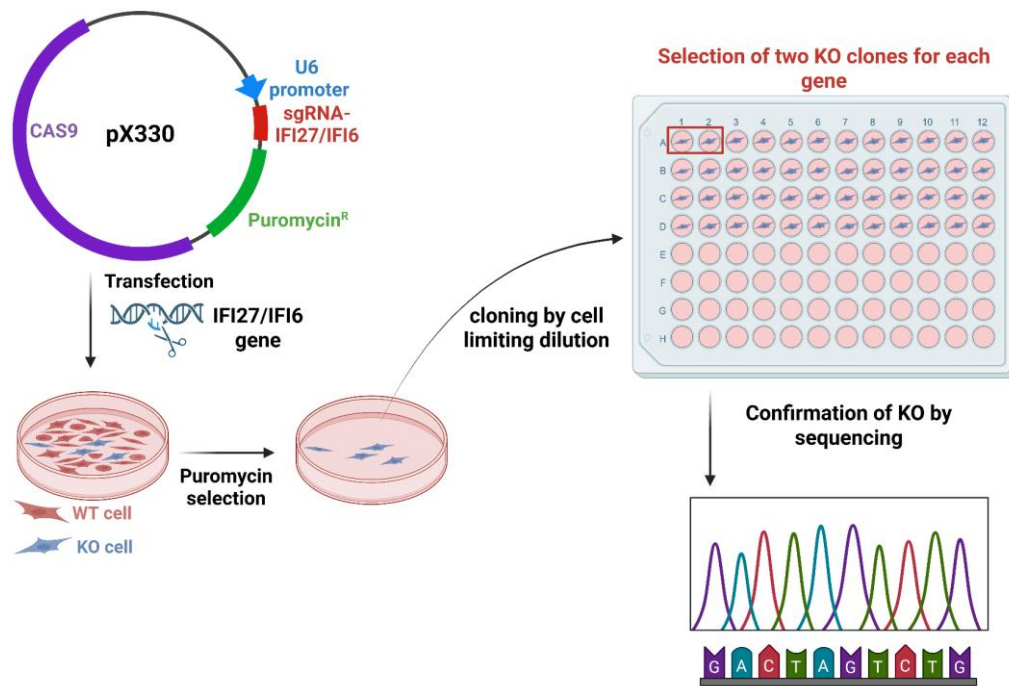


Figure M2. A549 IFI6 and IFI27 KO cells generation

Table M2. Primers used to perform the RT-PCR and to genotype target sequences in IFI6 and IFI27 KO clones. FS: forward sequence; RS: reverse sequence

Primer	Sequence (5'- 3')
<i>IFI6</i> -mRNA genotyping-32-FS	GCTACCTGCTGCTCTCACTTGC
<i>IFI6</i> -mRNA genotyping-291-RS	CTGCAGCGTGGCCACTAGC
<i>IFI27</i> -mRNA genotyping-118 FS	GGAGTTGTGGCCATGGCGG
<i>IFI27</i> -mRNA genotyping-302-RS	CCGGAGAGTCCAGTTCCC

3.1.4. Generation of IFI27-HA-stably expressing cell lines

To facilitate the study of IFI27, we developed a pCAGGS plasmid expressing IFI27 C-terminally tagged with an exogenous hemagglutinin (HA)-epitope tag [222] (pCAGGS-IFI27-HA, see plasmid section 3.5.1), in order to detect IFI27 expression and to affinity-purify the IFI27 protein, by using anti-HA antibodies. Thus, we generated cell lines stably expressing IFI27 tagged with HA (A549-hACE2-IFI27-HA), in order to facilitate our protocols and detection of IFI27.

The cells A549-hACE2 IFI27-KO were employed to obtain a cell line stably overexpressing the HA-tagged IFI27 protein, and not endogenous IFI27. pCAGGS-IFI27-HA plasmid or pCAGGS-empty plasmid, both encoding for a hygromycin-resistance gene, were linearized by incubation at 37 °C with the restriction enzyme *Sall* (New England Biolabs), following manufacturer's instructions. Enzyme activity was inactivated by a following incubation at 65°C for 20 minutes. To purify linearized plasmids, an agarose gel electrophoresis was performed to analyze the product of the previous reaction, and the fragment of interest (linearized plasmid) was excised,

taking into account the molecular size of the bands. Once excised, the E.Z.N.A.[®] Gel Extraction Kit (Omega Bio-tek) was used, following the manufacturer's instructions, to obtain purified linearized pCAGGS-IFI27-HA and pCAGGS-empty plasmids. Then, we transfected these plasmids in A549-hACE2 IFI27-KO cells by using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), to obtain the A549-hACE2-IFI27-HA, and A549-hACE2-empty cell lines. At 48 hpt, cells were treated with 400 µg/mL of hygromycin (ThermoFisher Scientific) in order to positively select transfected cells and avoid contamination with non-transfected cells. Cells were grown in the following passages with the same concentration of hygromycin.

3.1.5. Generation of monocyte-derived dendritic cells (MoDCs)

Human MoDCs were kindly provided by Dr. Ana Fernandez-Sesma (Icahn School of Medicine at Mount Sinai (ISMMS), New York, U.S.A). Dr. Fernandez-Sesma's laboratory obtained these cells from healthy human blood donors (Mount Sinai Blood Center and New York Blood Center), as previously described [223]. Buffy coats from healthy donors were used to isolate peripheral blood mononuclear cells, using a Ficoll-Hypaque density gradient centrifugation (Histopaque; Sigma Aldrich). Then, cluster of differentiation (CD)14⁺ cells were isolated from the mononuclear fraction using a magnetic-activated cell sorting (MACS) CD14 isolation kit (Milteny Biotec), based on CD14 antibody-labeled magnetic beads and iron-based Midimacs LS columns [223,224]. Once isolated, CD14⁺ cells were plated in 24-well plates (at a concentration of 7×10^5 cells/mL). Next, cells were differentiated to naïve DCs by incubation in DC medium containing Rosewell Park Memorial Institute (RPMI) 1640 medium (Invitrogen), 10% fetal calf serum (FCS, HyClone), 100 U/mL L-glutamine, 100 g/mL penicillin-streptomycin, and 1 mM sodium pyruvate, supplemented with 500 U/mL of human granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech) and 1,000 U/mL of human IL-4 (PeproTech)[223,224]. Cells were incubated in this DC-differentiation media for 5 to 6 days at 37°C in air enriched with 5% CO₂ and 90% humidity (Fig. M3).

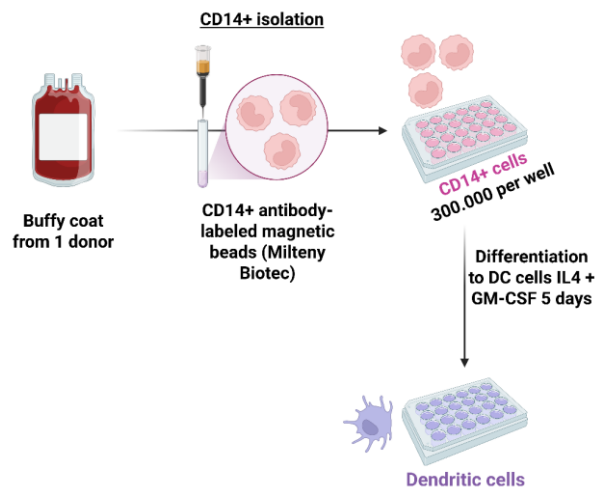


Figure M3. Monocyte-derived DCs generation from buffy coats.

3.2. VIRUSES

3.2.1. Influenza A virus (IAV)

MDCK cells were used to grow viral stocks of IAV A/Puerto Rico/8/1934 (PR8) H1N1 strain, both WT and recombinant viruses [225], under BSL2 conditions. IAV infections were performed

in the presence of different concentrations of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) at 1 µg/mL for MDCK cells, or at 100 ng/mL for A549 cells. Titration was performed by immunofocus assay, as indicated below (section 3.3.1) [226].

3.2.2. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

SARS-CoV-2 was kindly provided by Dr. Luis Enjuanes (Centro Nacional de Biotecnología, CNB-CSIC, Madrid, Spain). This SARS-CoV-2 isolate was obtained from a patient in Madrid at the beginning of the COVID-19 pandemic, in 2020 [227]. SARS-CoV-2 was grown in Vero E6 cells under BSL3 conditions and titrated by plaque assay, as indicated in section 3.3.2.

3.2.3. Dengue virus 2 (DENV2)

DENV2 (Thailand/166811/1964), was kindly provided by Dr. Pablo Gastaminza (CNB-CSIC, Madrid, Spain) and Dr. Ana Fernández-Sesma (ISMMS, New York, U.S.A). This isolate was originally isolated from a patient in Thailand in 1964 with DSS [228]. DENV2 was grown in BHK-21 cells for 4 days. DENV2 was handled in BSL3 facilities at CNB-CSIC and in BSL2 facilities at ISMMS, New York, and titrated by plaque assay in BHK-21 for viral stock preparation (section 3.3.2.), or by immunofocus assay in Vero E6 cells (section 3.3.1.).

3.2.4. Sendai virus (SeV)

SeV Cantell strain was grown in embryonated chicken eggs at BSL2 conditions [229] and provided by Prof. Luis Martínez-Sobrido (Texas Biomedical Research Institute, San Antonio, TX, US).

3.2.5. Recombinant Vesicular Stomatitis virus (rVSV), encoding the green fluorescent protein (GFP) (rVSV-GFP)

rVSV-GFP (Indiana strain) [230], provided by Prof. Luis Martínez-Sobrido (Texas Biomedical Research Institute, San Antonio, TX, US), was grown in Vero E6 cells under BSL2 conditions. rVSV-GFP was titrated by plaque assay, as indicated in section 3.3.2).

3.3. VIRUS TITRATIONS

Viral infections and titrations were carried out in the appropriate biosafety level laboratories for each virus: BSL2 for IAV PR8, rVSV-GFP and SeV, and BSL3 for SARS-CoV-2. DENV2 was handled in BSL3 facilities at CNB-CSIC and in BSL2 facilities at ISMMS, New York, due to different legislation between Europe and the US. The laboratories at the CNB-CSIC and ISMMS are equipped with all the necessary infrastructure and equipment. BSL3 at CNB-CSIC facilities use an air treatment system and a biological inactivation system for liquid effluents. The operator is also protected with an air filtration system (HEPA AirMate; 3, Saint Paul, MN) and appropriate clothing.

3.3.1. Immunofocus assay for IAV and DENV2

For IAV titrations, confluent monolayers of MDCK cells were seeded on 96-well plates, until reaching confluency. Then, 10-fold serial dilutions of infected-cell supernatants (from 10^{-1} to 10^{-7}) were made. Subsequently, these diluted supernatants were used to infect MDCK monolayers in a low volume (50 µL) of phosphate-buffered saline (PBS) supplemented with 0.3% of bovine serum albumin (BSA), to achieve adsorption for 1 hour at 37°C. 1 hour later, infection medium was retired, growth medium was added (DMEM containing 0.3% BSA and 1 µg/mL of TPCK-trypsin), and cells were incubated for 13 hours at 33°C in air enriched with 5% CO₂ and 90%

humidity. At 13 hours post-infection (hpi), medium was retired, cells were washed with PBS and fixed and permeabilized with 10% formaldehyde and 0.1% of Triton X-100 for 20 minutes at room temperature (RT). Infection was performed only for 13 hours to achieve single cell infection, avoiding the formation of infection foci (after an entire generation of infection, adjacent cells are also infected). Next, cells were incubated for an hour at RT with blocking buffer containing 2.5% BSA, 0.1% Tween-20, and 0.1% Triton X-100 in PBS. Then, an immunofluorescence was performed by incubating cells at 4°C overnight with a 1:1,000 dilution of a mouse antibody specific for the IAV NP (mAb HB-65, ATCC H16-L10-4R, [table M12](#)), diluted in PBS containing 0.1% Tween-20. Next day, cells were washed 4 times with PBS and incubated with an anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen) for 45 minutes at RT. Finally, after 4 washes with PBS, cells were observed on a fluorescence microscope, and positive-signal cells (infected with IAV) were counted. Each infected cell presumably corresponds to a cell infected by a single infectious virus, so by reversing the dilution factor we can provide the number of fluorescent forming units (FFU) per mL, which counts for the number of infectious viral particles per mL ([Fig. M4](#)).

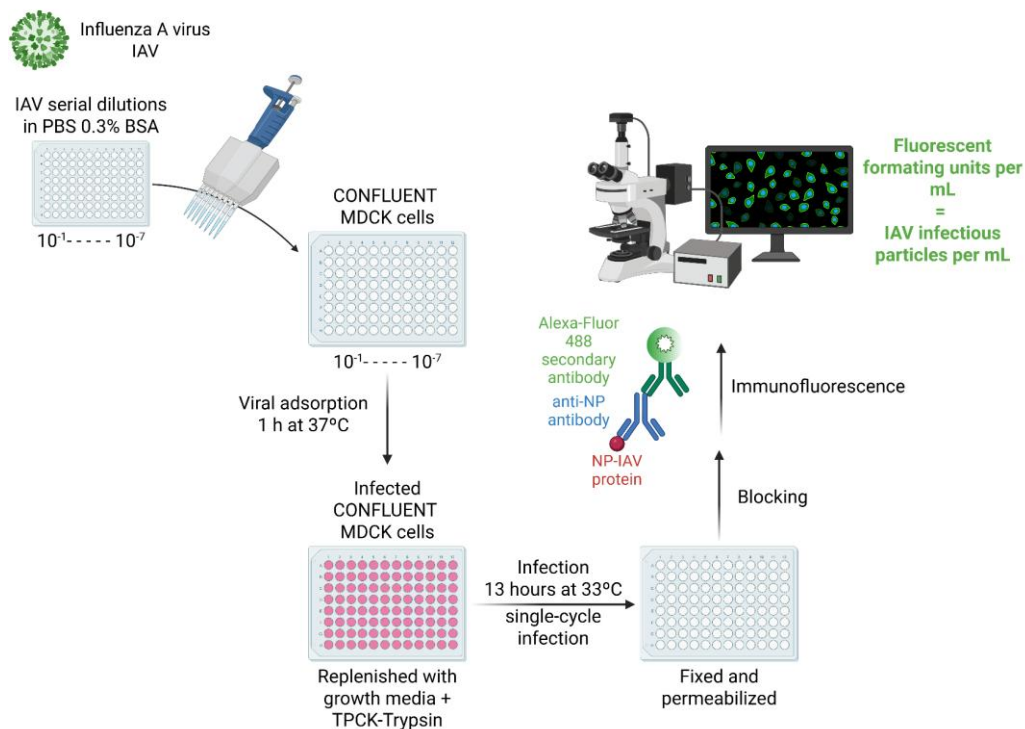


Figure M4. Immunofocus assay for IAV titration.

Alternatively, for DENV2, a similar experimental procedure was performed. First, one day before starting titrations, Vero E6 cells were seeded in 96-well plates to achieve confluency the day of infection. 10-fold serial dilutions of infected-cell supernatants (from 10⁻¹ to 10⁻⁷) were prepared in normal growth medium. Subsequently, these dilutions were used to infect Vero E6 cells monolayers in growth medium, and cells were incubated for 3 to 4 days at 37°C in air enriched with 5% CO₂ and 90% humidity. 3 to 4 days post-infection (dpi), medium was retired, cells were washed with PBS and fixed with 10% formaldehyde for 1 hour at RT. Next, cells were incubated for an hour at RT with blocking buffer containing 10% FBS, 0.1% Tween-20, and 0.1% Triton X-100 in PBS. Then, an immunofluorescence was performed by incubating cells at 4°C overnight with a 1:1,000 dilution of a mouse antibody specific for a conserved epitope of E protein of several flaviviruses, including DENV (4G2, BeiResources, NR-50327, [table M12](#)), diluted in PBS containing 0.1% Tween-20. Next day, cells were washed 4 times with PBS and incubated with an

anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen) for 45 minutes at RT. Finally, after 4 washes with PBS, cells were observed on a fluorescence microscope, and foci of infection, formed by several infected cells (infected with DENV) were counted. Each focus presumably corresponds to a single infectious virus that directly infects a cell, and the progeny viruses infect neighbor cells, so by reversing the dilution factor we can provide the number of fluorescent focus units (FFU) per mL (Fig. M5).

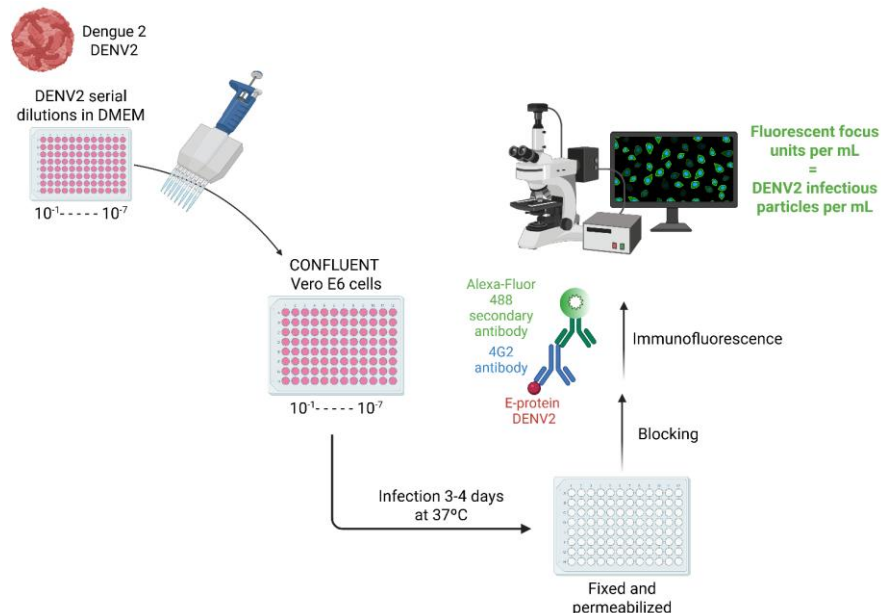


Figure M5. Immunofocus assay for DENV2 titration.

3.3.2. Plaque assays for SARS-CoV-2, DENV2, and rVSV-GFP

SARS-CoV-2, rVSV-GFP, and DENV2 viruses were titrated by plaque assay (plaque forming units per mL, PFU/mL) in Vero E6 cells for SARS-CoV-2 and rVSV-GFP, and in BHK-21 cells for DENV2. One day before infections, Vero E6 or BHK-21 cells were seeded in 24-well or 12-well format plates, respectively, in order to reach approximately a 90% confluency, homogeneous monolayer in each well. Next day, cells were infected with 10-fold serial dilutions of supernatants collected from infected cells (from 10^{-1} to 10^{-7}) in PBS or DMEM, for 1 hour at 37°C for SARS-CoV-2 and rVSV-GFP, and for 2 hours at 33°C for DENV. This incubation was performed in a low volume to facilitate viral-cell adsorption (300 μL or 600 μL per well for 24-well (Vero E6) or 12-well (BHK-21) plates, respectively). After adsorption, the infection media was retired, and cells were overlaid with a semi-solid media containing: a 48% 2X DMEM (Sigma-Aldrich), 50% agarose D1 low electroendosmosis (EEO) diluted in distilled water (Condalab), and 2% FBS for Vero E6 cells titrations (rVSV-GFP, SARS-CoV-2); or 45% 2X DMEM (Sigma-Aldrich), 50% agarose D1 low EEO diluted in distilled water (Condalab), and 5% FBS for BHK-21 cells titrations (DENV2).

Once this medium had solidified, cells were incubated at 37°C in air enriched with 5% CO_2 and 90% humidity, during 1, 3, or 7 days for rVSV-GFP, SARS-CoV-2, and DENV2 titrations, respectively, to achieve the generation of visible lysis plaques. After incubation, cells were fixed with 10% formaldehyde for 30-60 minutes at RT, and then, the semi-solid medium was removed, and cells were dyed using crystal violet for 20 minutes, which stains viable cells in a purple color, while unattached, dead cells (infected cells, which are going to generate foci of infection) will

remain unstained. Once the crystal violet was removed, we appreciated undyed plaques, which correspond to viral plaques (Fig. M6). Viral plaques were counted, and the dilution factor was corrected to determine the viral titer expressed as PFU/mL. Each plaque corresponds to the infection by one individual infectious viral particle.

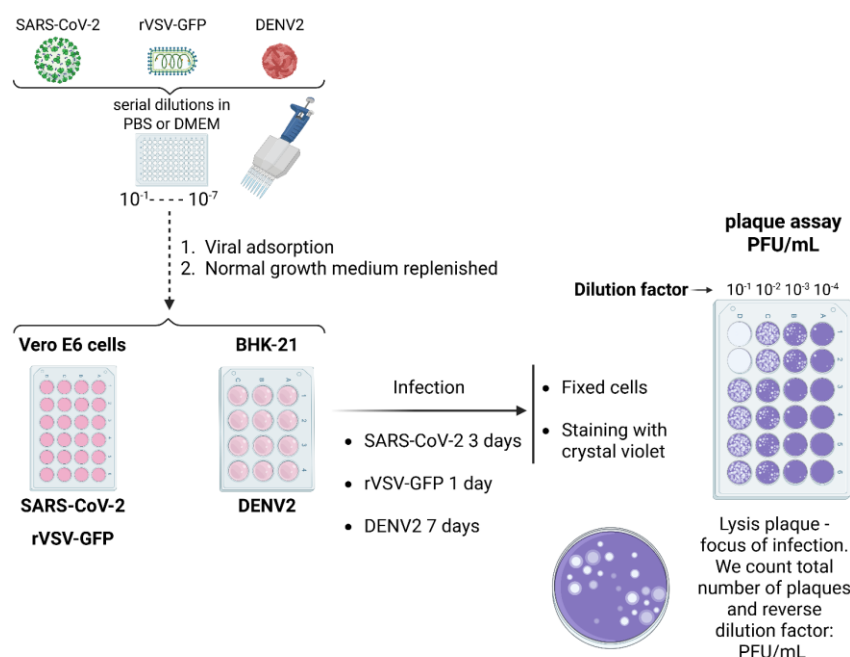


Figure M6. Plaque assay titrations for SARS-CoV-2, rVSV-GFP, and DENV2.

3.4. DNA MANIPULATION AND ANALYSIS

3.4.1. Polymerase chain reaction (PCR)

PCR was performed to amplify DNA, using 25 to 50 ng of DNA template per reaction. The specific primers for each amplification (indicated in each protocol) were obtained from Integrated DNA Technology (IDT) and used at a concentration of 100 nM. In addition, the following reagents were added: deoxynucleotides triphosphate, ATP, TTP, GTP, CTP (50 nM, ThermoFisher); the concentration of magnesium recommended by the manufacturer; the specific buffer of the enzyme, and 0.5 U of *Taq* DNA polymerase (Roche) for analytic PCRs or 1 U of PhusionTM High-Fidelity DNA polymerase (New England Biolabs) for PCRs of DNA fragments used for cloning or DNA fragments that were going to be sequenced, as this enzyme has a higher fidelity (lower error rate) than the *Taq* Polymerase. PhusionTM High-Fidelity DNA polymerase reactions were supplemented with DMSO following manufacturer's instructions. The annealing temperatures were adjusted depending on the sequence of the primers, as well as the time of the elongation step, which was adjusted depending on the PCR product and the enzyme elongation speed at the optimum temperature of elongation, which is 72°C in both cases. In every case, 25-35 cycles of elongation were performed. PCR reactions were performed on a T100 Thermal Cycler (Bio-Rad).

3.4.2. DNA electrophoresis in agarose gels

To achieve separation of DNA fragments both for analytical studies and to purify DNA bands, agarose gels of medium electroendosmosis (EEO) (Condalab, Spain) were used in concentrations varying from 0.6 to 1.2% (depending on the size of the DNA fragments) in TAE buffer (Tris-acetate 40 mM, EDTA 1mM). DNA was stained with SYBRTM Safe (ThermoFisher Scientific)

included in the agarose gels. Electrophoresis reactions were performed at 120 V. PCR products were observed in a GelDoc 2000 (BioRad).

3.4.3. DNA fragment's purification

To purify DNA fragments amplified by PCR or digested plasmids, as mentioned above, agarose gel electrophoresis were carried out to separate the DNA fragments by their molecular size. To select the appropriate fragments, *Quick-Load*® 1 kilobase (kb) DNA ladder or 100 base pair (bp) DNA ladder (New England Biolabs) were loaded on the gels. The DNA fragments were visualized under UV light using the SYBRTM Safe (ThermoFisher Scientific) staining and subsequently cut and purified using E.Z.N.A® Gel Extraction Kit (Omega Bio-tek) following manufacturer's instructions. For the purification of DNA fragments in solution, E.Z.N.A® Cycle Pure Kit (Omega Bio-tek) was used following manufacturer's instructions. When needed, once purified, DNA was sent together with the appropriate primers to Macrogen for sequencing by the Sanger method.

3.4.4. DNA cloning: restriction enzymes, phosphorylation, dephosphorylation, and ligation of DNA.

The different restriction enzymes used to cleave DNA at sequence-specific sites were obtained from New England Biolabs, and used with the indicated buffer, temperature, and times of incubation. Dephosphorylation of the 5'- and 3'-ends of linear DNA fragments (to avoid recircularization and non-desired ligations) was performed with the Quick calf intestinal alkaline phosphatase (CIP) (New England Biolabs), following manufacturer's instructions. Additionally, 5' phosphorylation reactions to achieve primer annealing, were achieved by using T4 Polynucleotide Kinase (New England Biolabs), following manufacturer's instructions. For DNA ligation, the T4 phage DNA ligase (New England Biolabs) was used following manufacturer's instructions. Every cloned product construct was analyzed by sequencing by Sanger method (Macrogen). All these reactions were performed following manufacturer's instructions and previously described protocols [231].

3.5. PLASMIDS AND BACTERIAL STRAINS

3.5.1. Plasmids used for transfection assays

The polymerase II expression pCAGGS plasmid is a mammalian expression vector which was used to clone the coding sequences of IFI27, IFI6, PKR, and PACT, under control of the cytomegalovirus enhancer/chicken β -actin promoter (CAG promoter) [232]. This plasmid is a high-copy plasmid, with a length of 4,800 bp [233]. It has a simian virus (SV)40 poly(A) signal under the control of SV40 promoter, to efficiently terminate transcription and confer a poly(A) tail to the synthesized mRNA. It has a variety of restriction sites for cloning and it contains a chimeric intron that enhances the efficiency of expression, the compatibility in different cell types and species, and promotes an efficient gene regulation [233]. In addition, this plasmid was modified to encode for a hygromycin-resistance gene.

The different pCAGGS plasmids were obtained by standard cloning techniques (as described below), by reverse transcription (RT)-PCR using total purified RNA (obtained as explained below) from human epithelial A549 cells: pCAGGS plasmids encoding the longest transcript variant 3 of IFI6 (GenBank accession number NM_022873, encoding a 138-amino acid protein) and the longest transcript variant 1 of IFI27 (GenBank accession number NM_001130080.3, encoding a 122-amino acid protein), both C-terminally fused to an HA epitope tag (pCAGGS-

IFI6-HA and pCAGGS-IFI27-HA, respectively); pCAGGS plasmid encoding PACT (GenBank accession number NM_003690.5), C-terminally fused to a FLAG epitope tag (pCAGGS-PACT-FLAG); and pCAGGS plasmid encoding PKR (GenBank accession number NM_001135651.3) fused to a N-terminal myc epitope tag (pCAGGS-PKR-myc). Primers used in the PCRs performed to generate the DNA fragments are indicated, as well as the restriction enzymes used for digesting the DNA sequences and inserting the fragments into the pCAGGS plasmid (Table M3).

Table M3. Primers used for cloning of different pCAGGS-coding plasmids. FS: forward sequence; RS: reverse sequence. Highlights represent restriction enzyme sites or nucleotide substitutions (in red), HA, myc, or FLAG tag sequences (in green) and gene sequence open reading frame (ORF, in purple).

cDNA product	Primers used for PCRs Sequence 5' - 3' Sequence highlights	
IFI6-HA cDNA	SphI-IFI6-FS GAGGCCTGCATGCCACCATGCGGCAGAAGGCGGTATCGC In red: <i>SphI</i> restriction enzyme In purple: IFI6 ORF 5' sequence	
	IFI6-HA-NheI-RS CTGAGGAGCTAGCTCAGGCATAATCTGGCACATCATAAGGGTACTCCTC ATCCTCCTCACTATCGAGATAC In red: <i>NheI</i> restriction enzyme In green: HA tag sequence In purple: IFI6 ORF 3' sequence	
IFI27-HA cDNA	SphI-IFI27-FS GAGGCCTGCATGCCACCATGGAGGCCTCTGCTCTCACCTC In red: <i>SphI</i> restriction enzyme In purple: IFI27 ORF 5' sequence	
	IFI27-HA-NheI- RS CTGAGGAGCTAGCTCAGGCATAATCTGGCACATCATAAGGGTAGTAGA ACCTCGCAATGACAGCCGC In red: <i>NheI</i> restriction enzyme In green: HA tag sequence In purple: IFI27 ORF 3' sequence	
IFI27 S63L-HA cDNA (final DNA fragment by overlapping PCR)	PCR 1	Overlapping PCR
	SphI-IFI27-FS GAGGCCTGCATGCCACCATGGAGGCCTCTGCTCTCACCTC In red: <i>SphI</i> restriction enzyme In purple: IFI27 ORF 5' sequence	SphI-IFI27-FS GAGGCCTGCATGCCACCATGGAGGCCTCTGCTCTCACCTC In red: <i>SphI</i> restriction enzyme In purple: IFI27 ORF 5' sequence
	IFI27-S63L-RS CGGACATCATCTTGGCTGCTATGGAGGACAAGGCGATTCCCGCCGCA GTGAAGCC Nucleotide substitution in red	IFI27-HA-NheI- RS CTGAGGAGCTAGCTCAGGCATAA TCTGGCACATCATAAGGGTAGTA GAACCTCGCAATGACAGCCGC In red: <i>NheI</i> restriction enzyme In green: HA tag sequence In purple: IFI27 ORF 3' sequence
	PCR 2	
	IFI27-S63L-FS	

	GGCTTCACTGCGGCGGGAATCGC CTTGTCTCCATAGCAGCCAAGAT GATGTCCG Nucleotide substitution in red IFI27-HA-<i>NheI</i>- RS CTGAGGAGCTAGCTCAGGCATAA TCTGGCACATCATAAGGGTAGTA GAACCTCGCAATGACAGCCGC In red: <i>NheI</i> restriction enzyme In green: HA tag sequence In purple: IFI27 ORF 3' sequence	
IFI27 V82A-HA cDNA (Overlapping PCR)	PCR 1	Overlapping PCR
	<i>SphI</i>-IFI27-FS GAGGCCTGCATGCCACCATGGAG GCCTCTGCTCTCACCTC In red: <i>SphI</i> restriction enzyme In purple: IFI27 ORF 5' sequence IFI27-V82A-RS GCCACAAGGCTGCCCCGAGGCAGC TCCACCCCCATTGGCAATGG Nucleotide substitution in red	<i>SphI</i>-IFI27-FS GAGGCCTGCATGCCACCATGGAG GCCTCTGCTCTCACCTC In red: <i>SphI</i> restriction enzyme In purple: IFI27 ORF 5' sequence IFI27-HA-<i>NheI</i>- RS CTGAGGAGCTAGCTCAGGCATAA TCTGGCACATCATAAGGGTAGTA GAACCTCGCAATGACAGCCGC In red: <i>NheI</i> restriction enzyme In green: HA tag sequence In purple: IFI27 ORF 3' sequence
	PCR 2	
	IFI27-V82A-FS CCATTGCCAATGGGGGTGGAGCT GCCTCGGGCAGCCTTGTGGC Nucleotide substitution in red IFI27-HA-<i>NheI</i>- RS CTGAGGAGCTAGCTCAGGCATAA TCTGGCACATCATAAGGGTAGTA GAACCTCGCAATGACAGCCGC In red: <i>NheI</i> restriction enzyme In green: HA tag sequence In purple: IFI27 ORF 3' sequence	
PKR-myc cDNA	PKR-<i>SacI</i>-myc-FS CTGACGGGAGCTCACCATGGGAACAAAACTCATCTCAGAAGAGGATCT GGCTGGTGATCTTTCAGCAGGTTTCTTC In red: <i>SacI</i> restriction enzyme In green: myc tag sequence In purple: PKR ORF 5' sequence	
	PKR-<i>SmaI</i>-RS CCATCAGCCCGGGCTAACATGTGTGTCGTTCAATTTTCTCTGGG In red: <i>SmaI</i> restriction enzyme In purple: PKR ORF 3' sequence	
PACT-FLAG cDNA	PACT-<i>SacI</i>-FS GCCAGGCGAGCTCACCATGTCCCAGAGCAGGCAC In red: <i>SacI</i> restriction enzyme In purple: 5' PACT ORF sequence	
	PACT-<i>SmaI</i>-RS GCATCCGCCCGGGTTACTTGTGTCGTCATCGTCTTTGTAGTCCTTCTTTCT GCTATTATCTTTAAATACTGCAAG In red: <i>SmaI</i> restriction enzyme In green: FLAG sequence tag In purple: PACT ORF 3' sequence	

Also, we studied several variants of IFI27 protein that presented mutations in specific amino acid residues predicted as important residues for RNA binding by the RNABindRPlus software (see section 3.10.1). Thus, we decided to clone these IFI27 variants (S63L, V82A) in pCAGGS plasmids, which were constructed by cloning overlapping PCR products containing the IFI27 open reading frames into the pCAGGS vector (Fig. M7). The first two PCRs were generated using the pCAGGS-IFI27-HA plasmid as a template and primers introducing the desired amino acid substitutions (PCR1 and PCR2 in Table M3, and Fig. M7). Then, an overlapping PCR using as DNA templates the two overlapping DNA fragments obtained in the first PCRs, and the flanking primers containing the sequences for the *SphI* and *NheI* restriction sites were performed (overlapping PCRs in Table M3, and Fig. M7).

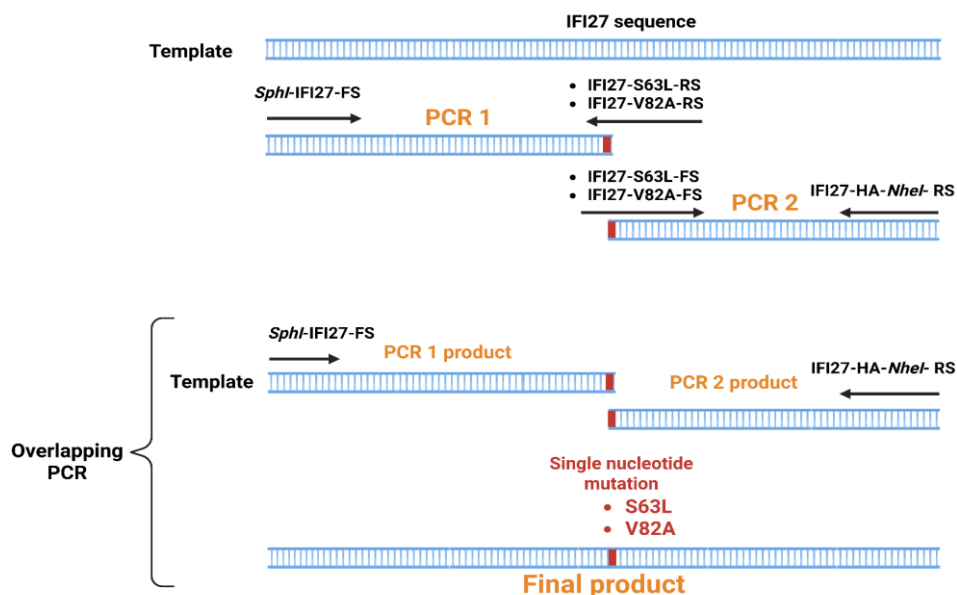


Figure M7. Overlapping PCR to achieve single nucleotide mutagenesis of IFI27 cDNA-coding sequence. pCAGGS-IFI27-HA plasmid is used as a template. In a first round of PCRs, on the one hand, *SphI*-IFI27-FS primer and the RS primers coding the specific mutation are used. On the other hand, the forward sequence primers coding the mutation and the reverse IFI27-HA-*NheI*-RS primer are used. This generates two different PCR products (PCR1 and PCR2). These products are used as templates in a second round of PCRs (overlapping PCR), using the *SphI*-IFI27-FS and IFI27-HA-*NheI*-RS primers, constructing the full-length cDNA sequence of IFI27, but with a single nucleotide substitution which constitutes the desired mutation (either S63L or V82A).

Polymerase II expression pCAGGS plasmid encoding IFI44 protein (GenBank accession number NM_006417.4) C-terminally fused to a HA epitope tag was obtained by RT-PCR using total isolated and purified RNA from human epithelial A549 cells as previously described [127]. Polymerase II expression pCAGGS plasmid encoding RIG-I protein (GenBank accession number AF038963.1) N-terminally fused to a FLAG epitope tag (pCAGGS-RIG-I-FLAG), pCAGGS plasmid expressing MDA5 protein (GenBank accession number NM_022168.4) N-terminally fused to a FLAG epitope tag (pCAGGS-MDA5-FLAG) and a pCAGGS plasmid expressing GFP (pCAGGS-GFP) were kindly provided by Luis Martínez-Sobrido (Texas Biomedical Research Institute, San Antonio, Texas, US), and the generation protocol was previously described [234].

The pRL plasmid expressing the renilla luciferase (RLuc) under the early SV40 enhancer/promoter region (pRL-RLuc) was obtained from Promega. This vector has a size of 3,705 bp, multiple restriction sites, SV40 early enhancer/promoter, a T7 promoter, an SV40 poly(A) signal, and an ampicillin-resistance gene. RLuc is cloned from the sequence of the

anthozoan coelenterate *Renilla reniformis* [235]. All plasmids used for transient transfections are indicated in [Table M4](#).

Table M4. Plasmids used in transfection experiments. The name of the plasmid and the encoded protein are indicated.

Plasmid	Encoded protein (-tag)
pCAGGS-IFI27-HA	IFI27-HA
pCAGGS-IFI6-HA	IFI6-HA
pCAGGS-IFI27-S63L-HA	IFI27-S63L-HA
pCAGGS-IFI27-V82A-HA	IFI27-V82A-HA
pCAGGS-PACT-FLAG	PACT-FLAG
pCAGGS-PKR-myc	PKR-myc
pCAGGS-RIG-I-FLAG	RIG-I-FLAG
pCAGGS-MDA5-FLAG	MDA5-FLAG
pCAGGS-GFP	GFP
pCAGGS-IFI44-HA	IFI44-HA
pRL-RLuc	RLuc

3.5.2. Plasmids used for the generation of KO cells

cDNAs complementary to the sgRNAs sequences selected using ATUM and CRISPOR.org, as previously mentioned (section 3.1.3, [Table M1](#)) were generated by annealing and phosphorylation of a pair of forward and reverse oligonucleotides partially overlapping ([Table M5](#)), synthesized by IDT. To this end, oligonucleotides were incubated with T4 polynucleotide kinase (New England Biolabs) during 30 minutes at 37°C, followed by 5 minutes at 95°C incubation, and then ramp down to 25°C at a rate of 5°C reduction per minute. Once annealed and phosphorylated, these cDNA sequences were inserted by ligation between *BbsI* restriction sites in a pX330 plasmid, digested with the *BbsI* restriction enzyme (New England Biolabs) and expressed under the U6 promoter. The original pX330 plasmid encoding the CAS9 gene, was modified to encode a puromycin-resistance gene (plasmid kindly provided by Dr. Pedro A. Mateos, Universidad Alcalá de Henares, Spain).

Table M5. Primers used to generate complementary sequence to the selected sgRNA for KO generation. Both the sequence of the primers used (5'-3') and the sgRNA sequence are shown. (Number) represents the size of the sequence in nucleotides.

Primer	Sequence (5'-3') (size nt)	Complementary sgRNA sequence (size nt)
IFI6-FS sgRNA 1	CACCGCCCTGACCTTCATGGCCGT (24)	GCCCUGACCUUCAUGGCCGU (20)
IFI6-RS sgRNA 1	AAACACGGCCATGAAGGTCAGGGC (24)	
IFI6-FS sgRNA 2	CACCGCTGACCTTCATGGCCGTCGG (25)	GCUGACCUUCAUGGCCGUCGG (21)
IFI6-RS sgRNA 2	AAACCCGACGGCCATGAAGGTCAG (24)	
IFI27-FS sgRNA	CACCGTGCCATGGGCTTCACTGCGG (25)	GUGCCAUGGGCUUCACUGCGG (21)
IFI27-RS sgRNA	AAACCCGCAGTGAAGCCCATGGCAC (25)	

3.5.3. Plasmids used to generate recombinant IAVs (rIAVs)

To study the possible effect of IFI27 and IFI6 on antiviral responses after IAV infections *in vivo* (see results section 4.2.3.), we generated rIAVs, based on the PR8 strain, encoding the human IFI27 or IFI6 proteins. These viruses overexpress these proteins in the cells infected with IAV.

In order to produce rIAVs expressing IFI27, IFI6, or mCherry (see virus rescue, section 3.6), NS viral segments of IAV were modified to encode NS1 and NEP viral proteins plus the sequence of interest (either IFI27, IFI6, or mCherry) (Fig. M8). To this end, we modified the previously described plasmid pDZ-NS-2xBsmBI [225,236]. The PDZ ambisense plasmid is a high-copy plasmid, with a length of 4,947 bp, and a multicloning site. This plasmid encodes an ampicillin-resistance gene, the promoter for the chicken β -actin in the 5'-3' sense, to drive the synthesis of viral mRNAs, and the promoter for the human RNA polymerase I in the 3'-5' sense, to drive the synthesis of viral genomic RNAs. The NS segment of IAV codifies for NS1 and NEP protein by a mechanism involving alternative splicing [237]. Therefore, the objective was to obtain an IAV NS segment that produces both proteins in separated open reading frames (ORFs) and clone another sequence of interest between NS1 and NEP proteins, so that the NS segment will encode viral NS1, our protein of interest (IFI27, IFI6, or mCherry), and viral NEP, all derived from the same polyprotein (Fig. M8). To do this, using pDZ-NS-2xBsmBI plasmid as a template, inverse PCRs to introduce the *Thosea asigna* virus (TAV)-2A autoproteolytic cleavage site (EGRGSLTTCGDVEENPGP) were performed using the primers indicated in Table M6.

Table M6. Primers used to introduce TAV-2A autoproteolytic site in pDZ-NS-2xBsmBI plasmid.

Primer	Sequence (5' - 3')
TAV 2A-FS	AATTACGCGTGGAGAGGGCAGAGGAAGTCTGCTAACATGCGGTG ACGTCGAGGAGAATCCTGGACCTGGGTCCGGCTGAGACGAGATC TC
TAV 2A RS	AATTACGCGTTCCAACCTCGCTTCTAATTGTTCCCGCCATTCTCG

Once TAV 2A sequence was introduced, the plasmid (pDZ-NSsplit2xBsmBI-2A) contains the following elements: 5'-non-coding region (NCR), NS1 sequence, link, TAV-2A autoproteolytic cleavage site, GSG-*BsmBI* restriction site, *BglIII* restriction site, *BsmBI* restriction site-GSG, link, porcine teschovirus (PTV)-1 2A autoproteolytic cleavage site, NEP sequence, and the 3'-NCR. Thus, NS1 and NEP do not overlap and are translated as two separated, independent ORFs. Subsequently, the sequence of interest was cloned between the NS1 and NEP segments, flanked by the autoproteolytic sequences. To this end, after generating the plasmid pDZ-NSsplit2xBsmBI-2A, the plasmids pDZ-NSsplit-2xBsmBI-2A-IFI27-HA, or IFI6-HA, or mCherry were generated. Briefly, the sequence of the gene of interest (human IFI27 C-terminally tagged with HA, and human IFI6 C-terminally tagged with HA) was amplified by PCR using specific primers (Table M7) flanked by *BsmBI* restriction sites, using as template the plasmids pCAGGS-IFI27-HA and pCAGGS-IFI6-HA, respectively. The amplified PCR products and the pDZ-NSsplit-2xBsmBI-2A plasmid were digested with *BsmBI* restriction enzymes (New England Biolabs) and subsequently ligated, using the T4 phage DNA ligase (New England Biolabs) obtaining the final plasmid. The plasmid pDZ-NSsplit-2xBsmBI-2A-mCherry was kindly provided by Dr. Aitor Nogales (CISA-INIA/CSIC). In this way, we had in the same segment the sequences coding for 3 proteins, in independent ORFs: NS1, NEP, and a protein of interest such as IFI27, IFI6, or mCherry.

Table M7. Primers used to amplify IFI27 and IFI6 human sequences and clone them into the pDZ-NSsplit-2xBsmBI-2A plasmid. By using these primers, a C-terminal HA-tag and flanking *BsmBI* restriction sites are introduced into IFI27 and IFI6 amplified sequences. FS: forward sequence; RS: reverse sequence. Highlights represent *BsmBI* restriction enzyme sites (in red), HA tag sequence (in green) and gene sequence open reading frames (ORF, in purple).

cDNA product	Primers used for PCRs Sequence 5' - 3' Sequence highlights
IFI27-HA	IFI27/<i>BsmBI</i>-FS AATT CGTCTCT CGGCATGGAGGCTCTGCTCTC In red: <i>BsmBI</i> restriction enzyme In purple: 5'IFI27 ORF sequence
	HA tag/<i>BsmBI</i>-RS AATT CGTCTC ATGCCGGCATAATCTGGCACATCATAAGGGTA In red: <i>BsmBI</i> restriction enzyme In green: HA tag sequence
IFI6-HA	IFI6/<i>BsmBI</i>-FS AATT CGTCTCT CGGCATGCCGGCAGAAGGCGGTA In red: <i>BsmBI</i> restriction enzyme In purple: 5'IFI6 ORF sequence
	HA tag/<i>BsmBI</i>-RS AATT CGTCTC ATGCCGGCATAATCTGGCACATCATAAGGGTA In red: <i>BsmBI</i> restriction enzyme In green: HA tag sequence

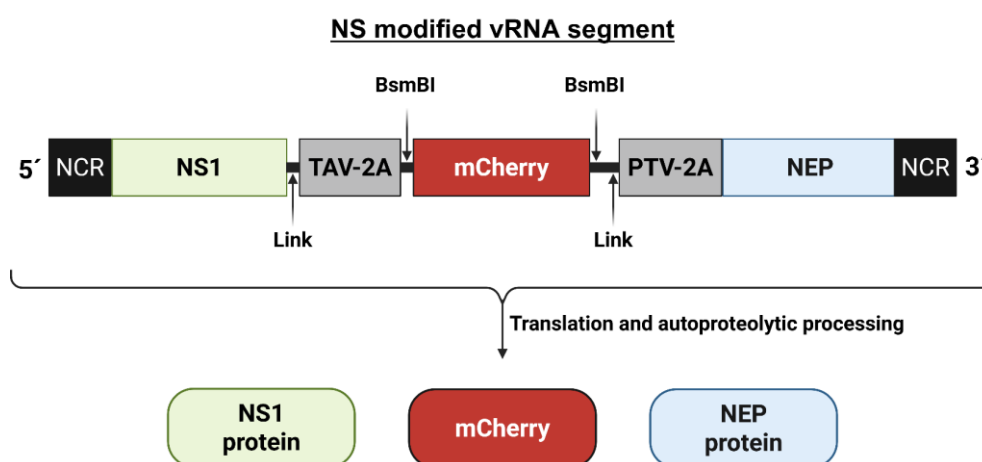


Figure M8. Schema of modified NS segment. Representation of the NS segment and codified proteins. NCR: non-coding region; NS1: non-structural protein 1; TAV-2A: Thosea asigna virus (TAV)-2A autoproteolytic cleavage site; mCherry protein (used as an example, with IFI27-HA and IFI6-HA segments it would apply identically); PTV-2A: porcine teschovirus-1 (PTV-1) 2A autoproteolytic cleavage site; NEP: Nuclear-export protein. 5' and 3' represent nucleotide segment ends.

3.5.4. Bacterial strains and growth

Competent *Escherichia coli* (*E. coli*) DH5 α were obtained from Invitrogen. The different bacteria used for cloning were grown in Luria-Bertani (LB) medium, which contains 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of sodium chloride (NaCl), with pH adjusted to 7. This medium, either liquid or solid, was supplemented with 100 μ g/mL of ampicillin in order to select resistant

bacteria that contain the plasmid of interest. Bacteria were grown overnight at 37°C, either on LB-agar plates or in liquid cultures under agitation.

3.5.5. Bacterial transformation and plasmid purification

To generate new stocks of purified plasmids, the thermal shock procedure was followed to transform competent *E. coli* DH5 α bacteria with plasmidic DNA. To this end, 100 μ L of bacteria were incubated with 5 μ L of plasmid (after ligation) or 100 ng of purified plasmid for 20 minutes on ice. Mixes were then incubated at 42°C for 90 seconds, followed again by 2 minutes of incubation on ice. After that, 1 mL of LB medium was added to the mixture, which was then incubated at 37°C for 1 hour. Bacteria were pelleted by centrifugation at 600-800 $\times$ g for 5 minutes, these pellets were resuspended in 100 μ L of LB medium and plated in plates containing LB-agar supplemented with 100 μ g/mL of ampicillin, and the bacteria colonies were grown overnight at 37°C. The next day, the grown colonies contained bacteria transformed with the plasmid of interest, as the plasmid encoded an ampicillin-resistance gene. These individual colonies were selected and transferred to either 10 mL (for plasmid mini-preps) or 100-200 mL (for maxi-preps) of liquid LB medium supplemented with 100 μ g/mL of ampicillin and grown overnight at 37°C under agitation. The next day, bacteria were pelleted, and the plasmid was purified using E.Z.N.A.[®] Plasmid DNA Mini Kit II or E.Z.N.A.[®] Plasmid DNA Maxi Kit (Omega Bio-tek), following manufacturer's instructions. 1 mL of the liquid culture with overnight-grown transformed bacteria was mixed with 200 μ L of glycerol and stored at -80°C, in order to stably store the transformed bacteria-containing plasmids, for future plasmid purifications. Purified plasmids obtained by cloning techniques were sent to Macrogen together with specific primers, to confirm their sequences by Sanger sequencing.

3.6. VIRUS RESCUE

To generate recombinant viruses expressing human IFI27-HA, human IFI6-HA, or a negative control virus expressing a reporter gene (mCherry) (IAV-IFI27-HA, IAV-IFI6-HA, or IAV-mCherry, respectively), 1:1 co-cultures of HEK-293T and MDCK cells were seeded on 6-well plates. Then, cells were co-transfected with 1 μ g of each of the seven ambisense WT plasmids codifying for IAV-PR8 structural and non-structural proteins (pHW-PB2, -PB1, -PA, -HA, -NP, -NA, and -M), kindly provided by Dr. Aitor Nogales (CISA-INIA/CSIC), together with the modified NS segment, encoding non-overlapping NS1 and NEP viral products plus either IFI27-HA (pDZ-NSsplit-2x*BsmBI*-2A-IFI27-HA), IFI6-HA (pDZ-NSsplit-2x*BsmBI*-2A-IFI6-HA), or mCherry (pDZ-NSsplit-2x*BsmBI*-2A-mCherry), generated as described before (3.5.3 section), using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), following manufacturer's instructions. At 16 hpt, medium was replaced with DMEM containing 0.3% BSA, antibiotics, and 0.5 μ g/mL TPCK-trypsin (Sigma). At 48 hpt, the cell culture supernatants were collected, and used to infect fresh MDCK cells. At 3 dpi, recombinant viruses were plaque purified, and stocks were generated by infecting confluent 10-cm dishes of MDCK cells at a low multiplicity of infection (MOI) (0.001). Stocks were titrated by immunofocus assay (FFU per mL) on MDCK cells as described above (section 3.3.1). The correct composition of the recombinant viruses was confirmed by Sanger sequencing (Macrogen).

3.7. STRATEGIES FOR THE MODULATION OF GENETIC EXPRESSION: PROTEIN OVEREXPRESSION (PLASMID TRANSFECTION) AND GENE KNOCK-DOWN (SMALL-INTERFERING RNA (siRNA) TRANSFECTION)

Overexpression and knock-down of the expression of certain genes are essential tools for the functional study of proteins, as these strategies allow to modulate, in a controlled manner, the levels of a certain protein. By analyzing the phenotype derived from the gain- or loss-of-function, we can infer new biological functions and the interactome of the studied proteins.

3.7.1. Protein overexpression in cells by transfecting protein-encoding plasmids

Plasmid transfections were performed in different cell types and different formats of plates and dishes depending on the type of experiment. In general, dishes and multi-well plates were previously treated with Poly-L-Lysine (Sigma-Aldrich) for 20 minutes before seeding the cells, when the cells to be transfected were HEK-293T, to avoid detaching of cells during the experiment.

Transfection mixtures were prepared, consisting of low-serum medium Opti-minimal essential medium (Opti-MEM) (ThermoFisher Scientific), and lipofectamine 3000 and p3000 reagent (ThermoFisher Scientific) following manufacturer's instructions. After that, we added the plasmids of interest to this mixture. The quantity of plasmid varies between different experiments, but in general, in 24-well plates, we added 500 ng of each plasmid per well. This quantity was incremented or reduced proportionally to the increment or reduction of the area of the well or dish used. The mixture of plasmids with Opti-MEM, lipofectamine 3000, and p3000 was incubated at RT for 20-30 minutes. During this incubation time, the cells to be transfected were counted in a Neubauer counting chamber and seeded in the multi-well plates or dishes at a confluency of 70-80%. Once the incubation was finished and cells were seeded, the transfection mixture was gently added to the cells, dropwise. At 6 hpt, transfection medium was retired, and cells were replenished with complete growth medium. For every experiment, one negative control condition where we transfected only an empty pCAGGS plasmid was included. Furthermore, this empty pCAGGS plasmid was added when needed to equal the amount of total transfected plasmid in each well or dish, so that there were no differences due to the amount of added plasmid. The overexpression of the gene of interest was confirmed by different methods: at mRNA level by RT and quantitative PCR (qRT-PCR) and at protein level by Western blot or indirect immunofluorescence. Plasmids used for transfections are indicated in [Table M4](#).

3.7.2. Protein knock-down in cells by transfecting small-interfering RNAs (siRNAs) (gene silencing)

siRNA transfections were performed in different cell types and different format of plates and dishes depending on the type of experiment. In general, dishes and multi-well plates were previously treated with Poly-L-Lysine (Sigma-Aldrich) for 20 minutes before seeding the cells, when the cells to be transfected were HEK-293T, to avoid detaching of cells during the experiment.

Knock-downs were performed by using specific siRNAs for each gene (ThermoFisher Scientific, [Table M8](#)), Opti-MEM, and lipofectamine RNAiMax (ThermoFisher Scientific) following manufacturer's instructions, with quantities varying depending on the multi-well or dish to use. siRNAs were used at a final concentration of 20 nM. The mixture of siRNAs with Opti-MEM and lipofectamine RNAiMax was incubated at RT for 20-30 minutes. During this incubation time, the cells to be used in the experiment were counted in a Neubauer counting chamber and seeded in

multi-well plates or dishes at a confluency of 70-80%. Once the incubation was finished and cells were seeded, the transfection mixture was gently added to the cells, dropwise. At 6 hpt, transfection medium was retired, and cells were replenished with complete growth media. As indicated, siRNA transfections were performed twice for some experiments, 24 hours apart, to ensure an optimal knock-down. For every experiment, one negative control condition with a non-targeting (NT) siRNA (ThermoFisher Scientific) was included (Table M8).

Table M8. siRNAs from ThermoFisher Scientific, used for gene knock-down.

siRNA Target gene	Reference number
Human <i>IFI6</i>	s5441
	s5440
Human <i>IFI27</i>	s7140
	s7139
NT siRNA	AM4635

The proper knock-down of the gene of interest was confirmed by different methods: at mRNA level by qRT-PCR and at protein level by Western blot.

3.7.3. Protein knock-down by using short hairpin RNA (shRNA)-coding lentiviruses

Following the same procedure previously explained for the generation of hACE2 expressing retroviruses, we generated lentiviruses expressing specific shRNAs for either IFI27, IFI6, or a NT shRNA, as a negative control of knock-down. These shRNAs-coding lentiviruses allow for knock-down in cells that are not easily transfected with the lipofectamine RNAiMax (ThermoFisher Scientific) reagent, such as monocytes and DCs [238,239]. The plasmids codifying the shRNAs (SMARTvector™-shRNA) were purchased from horizon discovery (Smartvector Lentiviral). These plasmids have a constitutive expression under the hEF1 α promoter, and encode various functional elements, including, among others: the shRNA of interest, a puromycin-resistance gene, and the GFP protein for fluorescent detection of lentiviruses within cells. The selected clones were:

- 2 different clones of constitutive expression of IFI27 shRNA-coding plasmid: V3SH11240-225993286, V3SH11240-227753242.
- 3 different clones of constitutive expression of IFI6 shRNA-coding plasmid: V3SH11240-227055820, V3SH11240-228136504, V3SH11240-225025528.
- 1 negative control, NT shRNA coding plasmid: VSC11709.

In order to produce the lentiviral vectors expressing the shRNAs, 2.5 million HEK-293T cells were plated in 100 mm dishes (to achieve a 50% confluency the day of transfection) and grown at 37°C in air enriched with 5% CO₂ and 90% humidity using DMEM (Gibco) supplemented with 10% FBS (Gibco), and 50 μ g/mL gentamicin (Gibco) (growth medium). Next day, the medium was changed to growth medium without antibiotics 4 hours before transfection. 3 different plasmids were co-transfected in cells: gag-pol (packaging proteins, specific for HIV-1, obtained from addgene, plasmid psPAX2), VSV-G (envelope glycoprotein, addgene) and each of the different shRNA coding plasmids per reaction. Transfection was performed by using, per 100 mm dish, 500 μ L of solution A (containing 250 mM CaCl₂, 8 μ g of gag-pol plasmid, 3 μ g of VSV-G plasmid, and 5 μ g of shRNA coding plasmid), and 500 μ L of solution B (containing 50 mM HEPES, 1.5 mM Na₂HPO₄, 140 mM NaCl in H₂O ultrapure, pH 7.05 adjusted with 1M NaOH or 1M HCl). The solutions A and B were mixed and incubated for 20 minutes before adding it to

cells gently, dropwise. At 24 hpt, transfection medium was retired and plates were replenished with new fresh complete growth medium. At 48 hpt, cell supernatants (containing lentiviral particles), were collected and filtered using 0.45 μm filters and subsequently concentrated and purified using LentiX (Takara) following manufacturer's instructions (Fig. M9). Purified lentiviruses were used to transduce CD14⁺ cells previously isolated from buffy coats of healthy donors (Mount Sinai Blood Center and New York Blood Center). CD14⁺ isolation was performed as described before (in section 3.1.5), and once isolated, CD14⁺ cells were plated (7×10^5 cells/mL) and transduced using polybrene (Sigma-Aldrich) with 50 μL of purified concentrated lentivirus per reaction. 24 hours later, cells were centrifuged at $400 \times g$ for 5 minutes, supernatant was discarded and cell pellet was resuspended in 0.5 mL of DC medium (RPMI (Invitrogen), 10% FCS (HyClone), 100 U/mL L-glutamine, 100 g/mL penicillin-streptomycin, 1 mM sodium pyruvate) supplemented with 500 U/mL of GM-CSF (PeproTech) and 1,000 U/mL of IL-4 (PeproTech). Cells were incubated in this DC-differentiation media for 5 to 6 days at 37°C in air enriched with 5% CO₂ and 90% humidity. After 5 days, transduced naïve DCs were tested for proper knock-down of either IFI27 or IFI6 by analyzing the level of expression of the respective mRNAs by qRT-PCR (see next section), and the efficiency of transduction was analyzed by flow cytometry (by detecting GFP expressed in transduced cells) (Fig. M10).

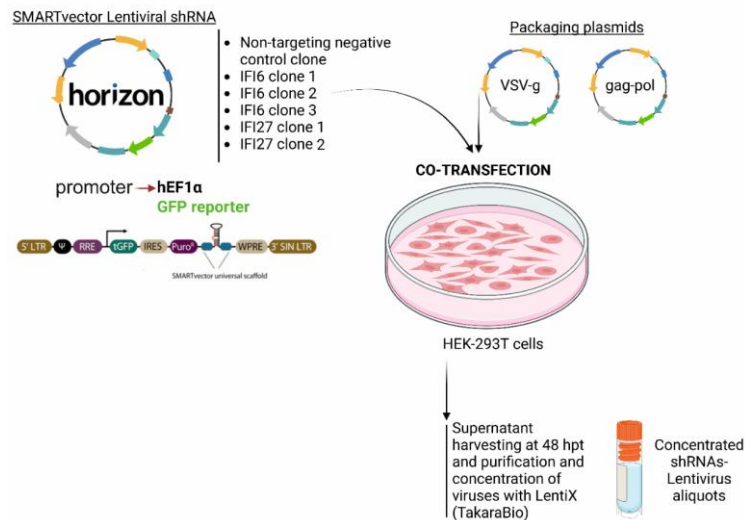


Figure M9. shRNA-coding lentiviruses generation.

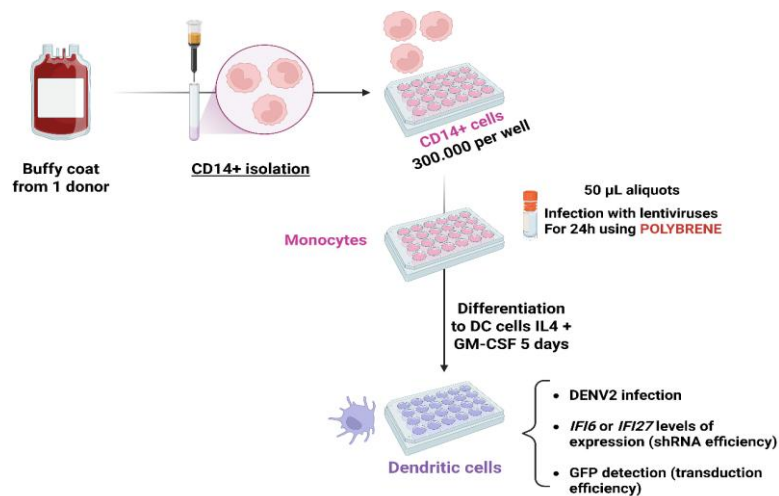


Figure M10. CD14⁺ cells transduction using shRNA-coding lentiviruses and differentiation to DCs.

3.8. RNA MANIPULATION AND ANALYSIS OF mRNA LEVELS

3.8.1. Total RNA extraction and purification

Total RNA from cell lines or homogenized lung-tissues from infected mice (see section 3.13) was obtained by lysis of the cells or tissue using the TRK lysis buffer (Omega Bio-tek) with added β -mercaptoethanol (Sigma-Aldrich) (20 μ L of β -mercaptoethanol per 1 mL of TRK buffer). Subsequently, RNA was purified using E.Z.N.A.[®] Total RNA Kit (Omega Bio-tek) following manufacturer's instructions. The purification and concentration yield of RNA purification was quantified with a *NanoDrop ND-1000* (ThermoFisher Scientific) spectrophotometer. Purified RNAs were stored at -80°C until their usage.

3.8.2. cDNAs synthesis by retrotranscriptase (RT) reactions

Purified RNAs were employed to synthesize cDNAs by using a retrotranscriptase (RT) enzyme [240]. To this end, we used the High-Capacity cDNA RT kit (Applied Biosystems, ThermoFisher Scientific) plus 40-50 units of RNasin[®] Ribonuclease Inhibitor (Promega) in a final volume of 20 μ L, using 250-500 ng of purified RNA as template and random primers (oligodeoxynucleotides with random bases, that are provided in the kit). The reactions were performed on a T100 Thermal Cycler (Bio-Rad). The reaction conditions used were: 10 minutes at 25°C, 120 minutes at 37°C and 5 minutes at 85°C to inactivate the RT enzyme. Obtained cDNAs were stored at -20°C until their usage.

3.8.3. Quantitative PCR (qPCR)

mRNA levels of genes were analyzed by reverse transcription and quantitative PCR (qRT-PCR). First, total RNAs from cells were extracted and retrotranscribed as described before using random primers. cDNAs were used to perform qPCRs, in a 7500 Real Time PCR System (Applied Biosystems). Samples were plated in MicroAmp[®] Optical 96-well Reaction Plates (Applied Biosystems), and the amplification detection was performed using either SYBR Green Reagent (Applied Biosystems) and specific primers for the genes of interest (Table M9), or TaqMan Gene Expression Assays (Applied Biosystem) using specific probes for the gene of interest (ThermoFisher Scientific) (Table M10). Quantification of qPCRs was performed using the threshold cycle ($2^{-\Delta\Delta CT}$) method [241] and normalized with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression levels.

Table M9. Primers used for qPCR. Name of target gene and sequences of both Forward Sequences (FS) and Reverse Sequences (RS) are indicated.

Target gene	Sequence (5'-3')
SARS-CoV-2 RdRp	FS: GTGAAATGGTCAGTGGCGG
	RS: CAAATGTAAAAACACTATTAGCATA
<i>Renilla</i>	FS: GCAGAAGTTGGTCGTGAGG
	RS: TCATCCGTTTCCTTTGTTCTG
<i>IFI27</i>	FS: TTGCCAATGGGGGTGGAGTTGC
	RS: CCGGAGAGTCCAGTTGCTCCCA
<i>IFI6</i>	FS: TGCCTCGCTGATGAGCTGGTCT
	RS: CTATGACGACGCTGCTGCCACC
<i>IFNL1</i>	FS: GGAGTAGGGCTCAGCGCATA
	RS: GCCTCCTCACGCGAGACCTC
<i>CXCL10</i>	FS: TCCCATCACTTCCCTACATG
	RS: TGAAGCAGGGTCAGAACATC

IFNA	FS: CTGAATGACTTGGAAGCCTG
	RS: ATTTCTGCTCTGACAACCTC
IL6	FS: AATGCCAGCCTGCTGACGAA
	RS: CTGAGGTGCCCATGCTACAT
GAPDH	FS: TGCACCACCAACTGCTTAGC
	RS: GGCATGGACTGTGGTCATGAG
DENV universal primers (DVuniv)	FS: GGTTAGAGGAGACCCCTCCC
	RS: GAGACAGCAGGATCTCTGGTCT

Table M10. Taqman probes for qPCR. Name of target gene and references (ThermoFisher Scientific) for the assays containing the primers and probes are indicated.

Target gene	Taqman probe reference
<i>Human IFI27</i>	Hs01086373_g1
<i>Human IFI6</i>	Hs00242571_m1
<i>Human IFNL1</i>	Hs00601677_g1
<i>Human IFN-induced protein with tetraatricopeptide repeats (IFIT)2</i>	Hs00533665_m1
<i>Human CXCL10</i>	Hs00171042_m1
<i>Human GAPDH</i>	Hs02786624_g1
<i>Mouse Ifnl3</i>	Mm00663660_g1
<i>Mouse Ifit2</i>	Mm00492606_m1
<i>Mouse Ccl2</i>	Mm00441242_m1
<i>Mouse Tnf</i>	Mm00443258_m1
<i>Mouse Gapdh</i>	Mm99999915_g1

3.8.4. RNAseq

Whole transcriptomes of WT and IFI6 KO HAP-1 cells were analyzed and compared by RNA sequencing (RNAseq). To do this, WT or IFI6 KO HAP-1 cells were seeded and either mock-transfected or transfected with poly(I:C) (Sigma) using polyethylenimine (PEI, Polysciences) for 16 hours. For poly(I:C) treatment of the cells, the poly(I:C) was always transfected either with PEI or with lipofectamine 3000 and p3000 (ThermoFisher Scientific) reagents, as indicated.

At 16 hpt, total RNA was harvested and purified as described before. RNA integrity was determined by the Genomics facility at the CNB-CSIC in an Agilent Bioanalyzer 2100 system. Next, RNAs were sent to Novogene Co, Ltd, where an RNAseq analysis was performed. mRNA was purified from total RNA by using polyT-oligo-attached magnetic beads. These purified RNAs, were fragmented with divalent cations under elevated temperatures in NEBNext First Strands Synthesis Reaction Buffer (5 ×). First, cDNA strand was synthesized using random hexamers and a M-MuLV Reverse Transcriptase (RNase H-), and the second cDNA strand was synthesized using DNA Polymerase I and RNase H. Overhangs were converted into blunt ends via exonuclease/polymerase activities, and 3' ends were adenylated. To prepare for hybridization, NEBNext Adaptor with hairpin loop structure were ligated. cDNAs of interest (fragments of 150-200 bp) were purified from the mix by using AMPure XP system (Beckman Coulter, Beverly, USA). Next, size-selected, adaptor-ligated cDNAs were processed by adding 3 µl of USER enzyme (NEB, USA) followed by an incubation at 37°C for 15 minutes and a step of denaturation at 95°C for 5 minutes. Subsequent amplification was performed using Phusion High-Fidelity DNA Polymerase (NewEngland Biolabs), Universal PCR primers and the appropriate Index Primer. Again, AMPure XP system was employed to purify PCR products, and next, the quality

of the library was assessed by using the Agilent 2100 Bioanalyzer. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) in accordance with the manufacturer's guidelines. Next, sequencing was performed with an Illumina HiSeq platform, which produced paired-end reads of 125-150 bp in length. This RNA-seq data is deposited in the NCBI's Sequence Read Archive (SRA) database, under the concept: BioProject PRJNA916250. To analyze the differences between the samples, a differential expression analysis was performed for two biological replicates per condition. To do this, DESeq E package (1.18.0) was used. The resulting p-values were adjusted with Benjamini and Hochberg's approach in order to reduce the False Discovery Rate (FDR), or type I errors. Genes with an adjusted p-value <0.05 found by DESeq were considered as DEG. These DEG were analyzed by a gene ontology (GO) enrichment using the webpage (<http://geneontology.org/>), to classify genes by their GO terms (functions or processes). GO terms that presented an FDR adjusted p-value lower than 0.05 were considered to be significantly enriched by DEGs (Tables A1 and A2, Appendix).

3.9. ANALYSIS OF PROTEIN EXPRESSION

3.9.1. Western blot

Cellular extracts were obtained from cell cultures using a protein lysis buffer containing 0.5% nonidet P-40 (NP-40) (Santa Cruz Biotechnology), 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM EDTA, 1 table of Pierce™ Protease Inhibitor (ThermoFisher Scientific), and 1 tablet of PhosSTOP phosphatase inhibitor cocktail (Roche) per 10 mL of buffer. These extracts were incubated on ice for 30 minutes, sonicated for 5 minutes in an ultrasonic bath sonicator (Fisherbrand™ FB15051), and cleared by centrifugation at $18,000 \times g$ during 30 minutes, at 4°C. Supernatants were transferred to a new Eppendorf tube, and pellets were discarded. These lysates were used directly or used to perform co-immunoprecipitations (co-IPs), in order to obtain immunoprecipitation (IP) eluates (see below in co-IP section 3.11.1), which were analyzed by Western blot.

Protein lysates or IP eluates were mixed with Laemmli sample buffer (Bio-Rad) containing 2.5% β -mercaptoethanol and heated at 95°C for 5 minutes, to achieve protein denaturation. These samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10 to 15% polyacrylamide-SDS gels, depending on the size of the proteins to study. For analyzing MDA5 oligomers, electrophoresis was performed under semi-denaturing detergent-polyacrylamide gel electrophoresis (SDD)-PAGE, and cell lysates were mixed with Laemmli sample buffer (Bio-Rad) without β -mercaptoethanol, and gels without SDS were used (4-20% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad). Electrophoresis reactions were performed at 100 to 120 V for 1 to 1.5 hours in a Mini-PROTEAN Tetra Cell System (Bio-Rad). Then, proteins were transferred to 0.45 μ m nitrocellulose membranes (Bio-Rad) with a Mini-PROTEAN Tetra Cell System (Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol) for 1 hour at 400 mA. Once transferred, membranes were incubated in blocking buffer containing either 5% nonfat dry milk or 5% BSA (for phosphorylated protein detection) and 0.1% Tween-20 (Sigma-Aldrich), in Tris-buffered saline (TBS, containing 25 mM Tris, 150 mM NaCl), during 1 hour at RT. After blocking, membranes were incubated overnight at 4°C with the specific primary antibodies diluted in blocking buffer, these antibodies being either monoclonal (mAb) or polyclonal (pAb) antibodies developed in mice or in rabbits (Table M11). Then, after three washes with TBS-0.1% Tween-20 (Sigma-Aldrich), membranes were incubated with 1:4,000 dilutions of goat anti-rabbit or anti-mouse IgG antibodies conjugated to the

horseradish peroxidase (HRP, Sigma-Aldrich) in blocking buffer. HRP allows for chemiluminescence detection using SuperSignal west femto maximum sensitivity substrate (ThermoFisher Scientific). Protein band intensity was quantified using ImageJ software.

Table M11. Antibodies used in Western blot detection. Target, host/source, clonality, dilution, manufacturer, and references of the antibodies are indicated.

Antibody target (Host/Source and clonality)	Dilution	Manufacturer	Reference
Anti-HA (rabbit pAb)	1:2,000	Sigma-Aldrich	H6908
Anti-IFI27 (rabbit pAb)	1:1,000	St John's laboratory	STJ190336
Anti-FLAG (mouse mAb)	1:2,000	Sigma-Aldrich	F3165
Anti-GFP (mouse mAb)	1:2,000	Merck	11814460001
Anti-glutathion S-transferase (GST)- peroxidase conjugate (rabbit pAb)	1:2,000	Sigma-Aldrich	A7340
Anti-6x-His (mouse mAb)	1:2,000	ThermoFisher Scientific	MA1-21315
Anti- β -actin (mouse mAb)	1:1,000	Sigma-Aldrich	A4700
Anti-ubiquitin (mouse mAb)	1:1,000	Santa Cruz Biotechnology	sc-166553
Anti-SARS-CoV-2-N protein (rabbit pAb)	1:4,000	GeneTex	GTX135357
Anti-MDA5 (rabbit pAb)	1:1,000	ThermoFisher Scientific	21775-1-AP
Anti-PACT (mouse mAb)	1:1,000	Santa Cruz Biotechnology	sc-377103
Anti-eIF2 α -P (rabbit pAb)	1:1,000	Cell Signaling Technology	9721
Anti-eIF2 α (rabbit pAb)	1:1,000	Cell Signaling Technology	9722
Anti-PKR-P (rabbit mAb)	1:1,000	Abcam	ab32036
Anti-PKR (mouse mAb)	1:1,000	Santa Cruz Biotechnology	sc-6282
Anti-GAPDH (mouse mAb)	1:1,000	Santa Cruz Biotechnology	sc-47724
Secondary antibody anti-rabbit (goat pAb)	1:4,000	Sigma-Aldrich	12-348
Secondary antibody anti-mouse (goat mAb)	1:4,000	Sigma-Aldrich	12-349

3.9.2. Translation shut-down assay

The level of *de novo* protein synthesis or translational state was studied within different cell lines: HEK-293T-hACE2, HeLa (WT, PACT KO, PKR KO, or DKO), A549-hACE2 (WT or IFI27

KO), A549 IFI27 KO stably transfected with an empty plasmid (A549-hACE2-empty) or A549 IFI27 KO stably expressing IFI27-HA by means of a linearized plasmid transfection (A549-hACE2-IFI27-HA). The different cell lines were transiently transfected with the previously described pRL-RLuc plasmid (Table M4), for measuring RLuc expression. In some cases, these transfections were performed in combination with other plasmids or siRNAs, as stated in each specific case. Following transfection procedure, cells were subjected to mock or poly(I:C) transfection using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), or to mock, SARS-CoV-2, or rVSV-GFP infections (at the MOIs and times specified for each assay).

For RLuc analysis, cells were lysed at the indicated time points with *Renilla Luciferase Assay Lysis Buffer* (Promega) supplemented with 1 tablet of Pierce™ Protease Inhibitor (ThermoFisher Scientific) and 1 tablet of PhosSTOP phosphatase inhibitor cocktail (Roche) per 10 mL of buffer. Cellular lysates were cleared by centrifugation and mixed in 96-well plates with the reaction substrate of the *Renilla Luciferase Assay System* (Promega), following manufacturer's instructions. This mix (1:1) was used to measure RLuc luminescence using a luminometer (Tecan infinite M200 pro) (Fig. M11). The mean values and standard deviations of luminescence values were calculated and represented with GraphPad Prism (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

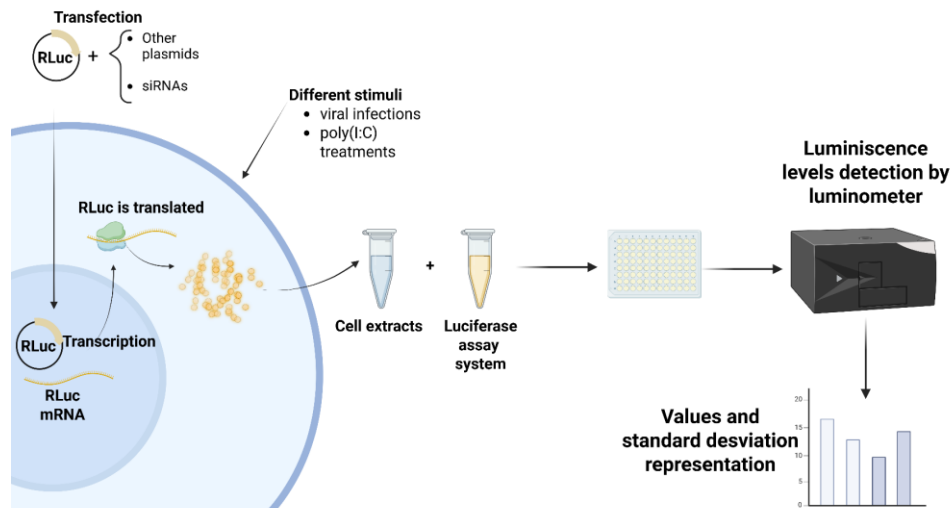


Figure M11. Graphical representation of translation shut-down assay. RLuc will be translated within cells, at higher or lower levels depending on the translational state of cells, allowing us to study it.

3.9.3. Fluorescence optical microscopy

Different types of microscopes were used to capture fluorescence and brightfield images of cells.

- Zeiss Axiovert 40 CFL (Zeiss): this brightfield microscope available at the CNB-CSIC was used for routine cell morphology and confluence observation during different experiments.
- Digital Fluorescence Cell Imager (Bio-Rad ZOE): available at the CNB-CSIC, employed to assess the efficiency of transfection or used in immunofluorescence experiments as it allows detection of blue, green, and red fluorescent signals, as well as brightfield detection.
- EVOS M5000 (ThermoFisher Scientific): fluorescence microscope available at ISMMS facilities. Used to assess transfection efficiency and lentiviral production and transduction

in cells by detecting GFP signal (as shRNA-coding plasmids contain GFP gene, as explained before in section 3.7.3).

- Leica Stellaris 5 confocal microscopy (Leica): available at the CNB-CSIC and used to obtain high-resolution images with the 63× oil immersion objective. Image acquisition was managed through the LAS X software suite, and the analysis of the images was done with ImageJ software.

3.9.4. Indirect immunofluorescence

Selected cell lines, as specified in each case, were seeded on sterile glass coverslips (24-well plates) in monolayers at a confluence of 70%. When indicated, cells were transiently transfected with the indicated plasmids using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), following manufacturer's instructions. Each plasmid was added at a specific concentration: pCAGGS-IFI27-HA and pCAGGS-IFI6-HA at 600 ng per well; pCAGGS-PACT-FLAG, pCAGGS-RIG-I-FLAG, or pCAGGS-MDA5-FLAG at 300 ng per well; pCAGGS-PKR-myc at 150 ng per well. Empty pCAGGS was transfected when needed to equal the total DNA amount for each well and as negative control. When indicated, at 24 hpt, cells were either mock- or poly(I:C)-transfected (Sigma) using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), at the specified concentration and duration (for HEK-293T cells, either 3 µg/mL, or 60 ng/mL (HEK-293T-hACE2) for 24 hours; for HeLa cells, 2 µg/mL for 7 hours); or mock- or SARS-CoV-2-infected (for HEK-293T-hACE2, MOI 1 for 24 hours). Alternatively, the A549-hACE2-IFI27-HA cells or the control cells A549-hACE2-empty, were mock-transfected, transfected with poly(I:C) (60 ng/mL) using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), or infected with SARS-CoV-2 (MOI 1) for 24 hours. Also, in MDCK cells, immunofluorescences of rIAV (MOI 0.1, 24 hours infections) (section 3.6) were performed.

After mock, poly(I:C) transfections, and IAV infections, cell monolayers were fixed and permeabilized with PBS containing 10% formaldehyde and 0.1% Triton-X100 for 20 min at RT. For SARS-CoV-2 infections, cells were fixed with PBS containing 10% formaldehyde for 1 hour at RT. Next, cells were blocked with immunofluorescence blocking buffer (PBS containing 10% FBS, 0.1% Triton-X100, and 0.1% Tween-20) for 1 hour at RT, and then, incubated overnight at 4°C with the specific primary antibodies for each assay. The primary antibodies were diluted in PBS containing 0.1% Tween-20 (see antibody [table M12](#) for references and dilutions). After overnight incubation, coverslips were washed 4 times with PBS and incubated 45 minutes at RT with the anti-mouse, anti-rabbit, or anti-rat secondary antibodies conjugated to either Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 (Invitrogen) depending on the biological source species of the primary antibodies (see antibody [table M13](#) for references and dilution). After the incubation with secondary antibodies, coverslips were washed again 4 times with PBS, and next, to achieve nuclei staining, coverslips were incubated 15 minutes at RT with 4',6-diamidino-2-phenylindole (DAPI, ThermoFisher Scientific) at a 1:500 dilution ([Table M13](#), [Fig. M12A](#)). Once this last incubation finished, cells were again washed 4 times with PBS and then coverslips were mounted on microscope slides using ProLong Gold antifade reagent (ThermoFisher Scientific, P36930). Mounted coverslips were visualized and analyzed on a Leica Stellaris 5 confocal microscope, using the 63× oil immersion objective. The photos were taken with different zoom levels, generally 1× and 3×, using the NAS X software. The same instrument settings were applied to all images, which were then analyzed using Fiji software ([Fig. M12B and M12C](#)).

Table M12. Primary antibodies used for immunofluorescence detection. Target, host/source, clonality, dilution, manufacturer, and references of the antibodies are indicated.

Antibody target (Host/Source and clonality)	Dilution	Manufacturer	Reference
Anti-HA (rabbit pAb)	1:1,000	Sigma-Aldrich	H6908
Anti-HA (mouse mAb)	1:500	Santa Cruz Biotechnology	sc-7392
Anti-FLAG (mouse mAb)	1:1,000	Sigma-Aldrich	F3165
Anti-NP protein of IAV (mouse mAb)	1:1,000	ATCC	ATCC H16-L10-4R5 HB- 65 (hybridoma)
4G2-Anti-E protein of DENV2 (mouse mAb)	1:1,000	BeiResources	NR-50327
Anti-MDA5 (rabbit pAb)	1:1,000	ThermoFisher Scientific	21775-1-AP
Anti-SARS-CoV-2-N protein (rabbit pAb)	1:2,000	GeneTex	GTX135357
Anti-SARS-CoV-2- affinity purified anti-N protein antibody generated in rat	1:1,000	Generated in rat, using His-tagged SARS-CoV-2 N protein as antigen	[242]
Anti-PKR (mouse mAb)	1:500	Santa Cruz Biotechnology	sc-6282
Anti-PKR (rabbit pAb)	1:500	Protein Tech	18244-1-AP
Anti-eIF3 η (mouse mAb)	1:500	Santa Cruz Biotechnology	sc-137214
Anti-G3BP1 (mouse mAb)	1:500	Santa Cruz Biotechnology	sc-365338

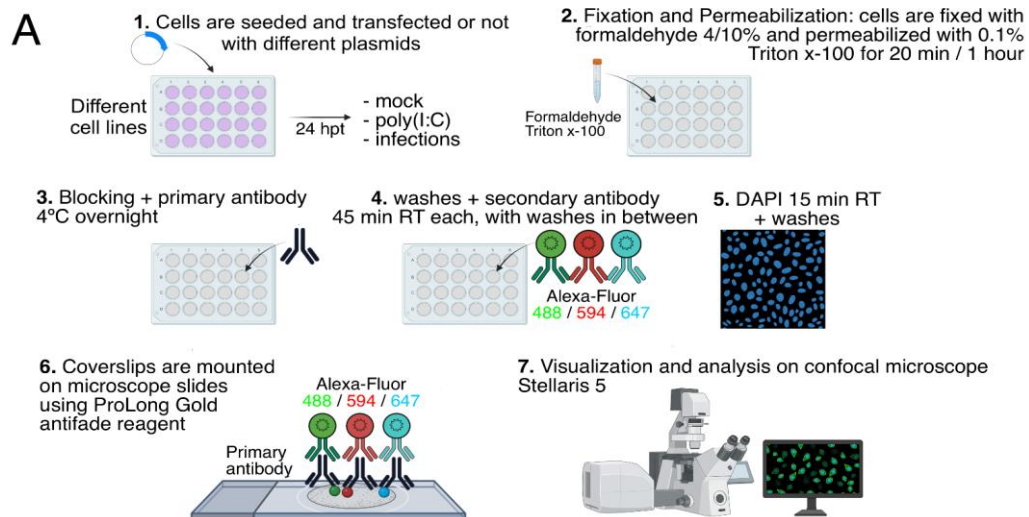
Table M13. Secondary antibodies used for immunofluorescence detection, and DAPI (used for nuclear staining). Target, host/source, clonality, dilution, manufacturer, and references of the antibodies are indicated.

Antibody target (Host/Source and clonality)	Dilution	Manufacturer	Reference
Anti-mouse (goat pAb) conjugated to Alexa Fluor 488	1:500	ThermoFisher Scientific	A11001
Anti-rabbit (goat pAb) conjugated to Alexa Fluor 594	1:500	ThermoFisher Scientific	A11037
Anti-mouse (goat pAb) conjugated to Alexa Fluor 594	1:500	ThermoFisher Scientific	A11032
Anti-rabbit (goat pAb) conjugated to Alexa Fluor 488	1:500	ThermoFisher Scientific	A11034
Anti-rat (goat pAb) conjugated to Alexa Fluor 647	1:500	ThermoFisher Scientific	A21247
DAPI (DNA)	1:500	Sigma-Aldrich	32670

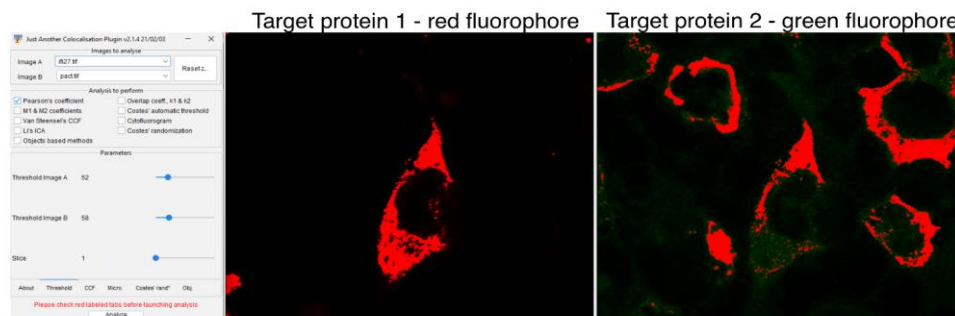
3.9.5. Colocalization analysis

Colocalization analyses of two different proteins in the cell cytoplasm were performed. This was done by following the same described procedure for indirect immunofluorescence (section 3.9.4). Studied proteins were labeled with Alexa Fluor 488 (green signal) and Alexa Fluor 549 (red signal), respectively, and then, the overlapping between both fluorescent signals was analyzed using the ImageJ software in order to provide a measurement for the colocalization between both proteins. Two different types of analyses were performed to measure the colocalization between two proteins:

1. **JACoP plug-in:** obtained images from Leica Stellaris 5 were opened in the ImageJ software by splitting the three channels: Alexa Fluor 488 (green), Alexa Fluor 594 (red), DAPI (UV excitation channel, around 405 nm, detected in blue). Green and red channels (staining both proteins of interest) were visualized in 8-bit. Next, JaCOP plug-in was opened, and the analysis of Pearson's coefficient was selected [243,244]. This coefficient provides the extent of overlap between two fluorophores, and it goes from -1 to 1. A value of 1 represents a total positive correlation between both fluorophores, while -1 represents a total negative correlation. Values close to 0 suggest little to no correlation [243,244]. Threshold of the signal of both channels was kept automatic if the intensity of both fluorophores was comparable. Next, we clicked on "Analyze", and the software provided us with the Pearson's coefficient value (Fig. M12B).
2. **Analysis of the area of colocalization of both fluorophores within the total cell area:** This alternative analysis is based on measuring the area within the cells where two different fluorophores are overlapping and dividing that area by the total cellular area. By doing this, we obtain a value which is next transformed to a percentage of colocalization of both fluorophores within cells. To do this, 8-bit images of the three channels (red, green, and blue) were merged. The resulting image was modified to RGB. As we wanted to analyze colocalization between red and green signal (our two proteins of interest), we selected the function "Image→Adjust→Color Threshold" and selected the values from 20 to 60 (merge between red and green, which are colors close to yellow). Next, we highlighted this selection in white and clicked on "select". Once selected, merge areas were highlighted, and we selected on "Analyze → Measure", obtaining a numerical value for that area of colocalization. This measurement provided us with a numerical value of the area where both studied fluorophores were colocalizing. Next, by selecting the "freehand selection" tool, we outlined the whole cell of interest and then clicked on "Analyze → Measure" to obtain the total area of that cell. Subsequently, we divided the previously obtained colocalization area by the total cell area. This result was converted into a percentage, and these percentages from a defined number of cells (n=29 or higher) were plotted, calculating the mean and standard deviation for each studied condition, allowing for their comparison (Fig. M12C).

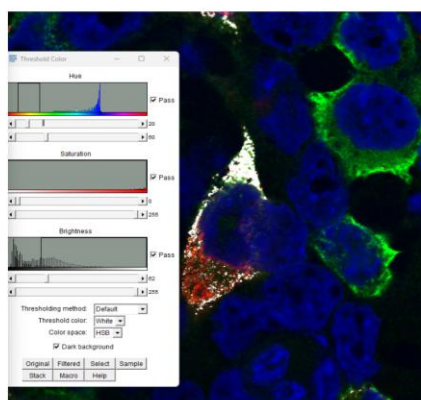


B Colocalization analysis by JaCOP plug-in - Pearson's coefficient



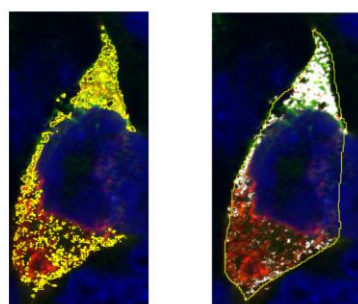
8-bit images: JaCOP plug-in selects an automatic threshold for both red and green signal and provides Pearson's coefficient, which gives the extent of overlap of two different fluorophores (red and green).

C Colocalization analysis by area of colocalization / total area of cell



Merge image (RGB):

1. Image -> Adjust -> Color threshold
2. Selection between 20-60, merge between red and green (yellow area).
3. Click select to highlight the area of colocalization of both fluorophores.



Merge area: 32.514 Total cell area: 204.404

	Area	Mean	Min	Max	%Area
1	32.514	83.463	33	190	0
2	204.404	65.093	6	255	0

- Merge area represents a 15.9% of the total cell area

- The percentages of different cells of the same condition are measured, obtaining then a mean and standard deviation

Figure M12. Indirect immunofluorescence and colocalization analysis.

3.10. ANALYSIS OF PROTEIN-RNA INTERACTIONS

3.10.1. RNABindRPlus bioinformatic prediction

To study if IFI6 and IFI27 proteins are able to bind to RNAs, as a first step, we performed a bioinformatic prediction using RNABindRPlus (Artificial Intelligence Research Laboratory, Penn State University) [245]. This method employs a homology-based approach (HomPRIP) to predict residues in a query protein that could bind to RNA, based on the information of RNA-binding residues of sequence homologs (if available), and combines it with a machine learning method (SVMOpt) that predicts RNA-binding residues only with features derived from the sequence of the query protein. Combining the two methods improves the reliability of predicted RNA-binding residues in proteins [246,247]. RNABindRPlus output consists of values for each amino acid residue from 0 to 1. These values represent probabilities, so if a residue has a value equal or greater than 0.5, it is predicted to bind RNA, while values lower than 0.5 mean that it is predicted that the given residue does not bind RNA.

3.10.2. Binding of proteins to poly(I:C)

3.10.2.1. Binding of proteins to poly(I:C)-agarose

HEK-293T cells were seeded at a 70-80% confluence (6-well plate format) and transiently transfected using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific) with the following plasmids (alone or in combinations):

- IFI27 protein: pCAGGS-IFI27-HA.
- IFI27 variants S63L and V82A: pCAGGS-IFI27-S63L-HA and pCAGGS-IFI27-V82A-HA.
- IFI6 protein: pCAGGS-IFI6-HA.
- Empty, as negative control: pCAGGS-Empty plasmid.
- MDA5 protein: pCAGGS-MDA5-FLAG.
- Positive control of RNA binding: we employed PACT protein (pCAGGS-PACT-FLAG), as it is a well-known RNA-binding protein [132,248].
- Negative control of RNA binding: we transfected pCAGGS-GFP, as GFP protein does not bind to RNAs.

At 24 hpt, cells were lysed in protein lysis buffer. These extracts were incubated on ice for 30 minutes, sonicated for 5 minutes in an ultrasonic bath sonicator (Fisherbrand™ FB15051), and cleared by centrifugation for 30 minutes at $18,000 \times g$ and 4°C . Supernatants were transferred to a new Eppendorf tube, and pellets were discarded. These lysates were incubated with poly(I:C)-conjugated agarose beads, previously prepared. Additionally, for IFI6, a purified recombinant IFI6 protein with N-terminal GST and C-terminal 6x-His tags, produced in *E. coli* (Origene) was also incubated with poly(I:C)-conjugated agarose beads, previously prepared. To prepare these beads, per sample, 6 mg of polycytidylic acid (poly(C))-conjugated agarose beads (Sigma) were washed five times with TBS buffer. After the last wash, beads were resuspended in buffer containing 50 mM Tris-HCl and 50 mM NaCl and incubated overnight at 4°C with 120 μg of inosinic acid (Sigma). After overnight incubation, beads were washed again three times with TBS, and then, resuspended in 200 μL of TBS buffer containing 1 mM EDTA and 0.5% Triton X-100. Subsequently, resuspended beads were incubated with 80 μL of protein extracts or with the purified IFI6 protein, and 50 units of RNasin® Ribonuclease Inhibitor (Promega) for 3 hours at 4°C . After incubation with extracts or the purified IFI6 protein, samples were washed 4 times with

TBS containing 1 mM EDTA and 0.1% Tween 20, and bound proteins were eluted by incubation with Laemmli sample buffer (Bio-Rad) containing 2.5% β -mercaptoethanol for 5 minutes at 95°C. Eluted proteins were detected by Western blot (Fig. M13) using specific antibodies (Table M11), as described in Western blot section (3.9.1).

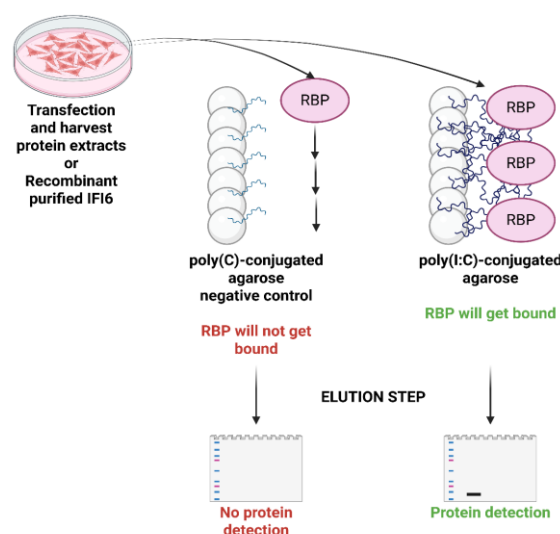


Figure M13. Analysis of the binding of proteins to poly(I:C).

3.10.2.2. Binding of proteins to biotinylated poly(I:C)

As an alternative to the pull-down with poly(I:C)-conjugated agarose beads, we also used biotinylated poly(I:C) and streptavidin-conjugated agarose beads. In this case, HEK-293T cells were transiently transfected with the pCAGGS plasmid expressing IFI27 or the empty pCAGGS plasmid, using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), following manufacturer's instructions. At 24 hpt, cells were transfected for 24 hours with 200 ng/mL of biotinylated poly(I:C) (InVivoGen), or non-biotinylated poly(I:C) (InVivoGen) as a control, using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific). Next, cells were lysed with protein lysis buffer and cleared, and these protein extracts were incubated with streptavidin-bound beads (ThermoFisher Scientific) for 4 hours at 4°C. After the incubation, the samples were washed 4 times with TBS buffer containing 1 mM EDTA and 0.1% Tween 20 and eluted by incubation with Laemmli sample buffer (Bio-Rad) containing 2.5% β -mercaptoethanol during 5 minutes at 95°C. Detection of bound proteins was performed by Western blot (Fig. M14), as described before.

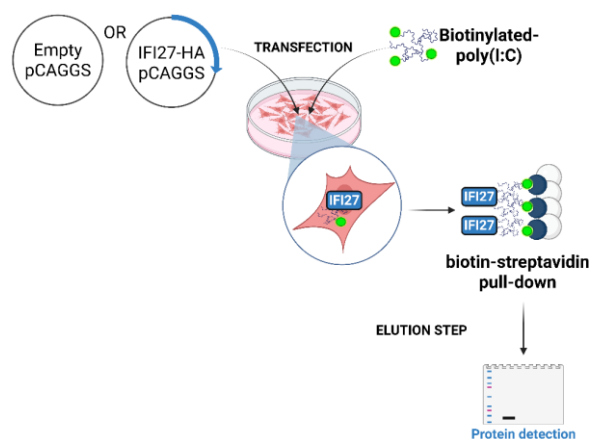


Figure M14. Analysis of the binding of IFI27 to biotinylated-poly(I:C).

3.10.3. RIG-I binding to SARS-CoV-2 RNA

To determine if RIG-I binds SARS-CoV-2 RNA, and whether IFI27 is able to modulate this interaction, we performed an RNA Immunoprecipitation (RIP) assay following the protocol described by Gagliardi and Matarazzo [249]. This protocol consists of IP of RNA-protein complexes using antibody-conjugated agarose beads. To this end, 3.5 million HEK-293T-hACE2 cells were seeded in 25 cm² flasks after treating the flasks with Poly-L-Lysine (Sigma-Aldrich) for 20 minutes. Next, using lipofectamine 3000 and p3000 reagents, and following the previously described protocol (section 3.7.1), these cells were transfected with either:

- 6.5 µg of pCAGGS-IFI27-HA and 6.5 µg of pCAGGS-empty.
- 6.5 µg of pCAGGS-IFI27-HA and 6.5 µg of pCAGGS-RIG-I-FLAG.
- 6.5 µg of pCAGGS-IFI27-V82A-HA and 6.5 µg of pCAGGS-RIG-I-FLAG.

Next day, cells were infected with SARS-CoV-2 (MOI 1) and 24 hpi, cells were detached using Trypsin-EDTA and collected by centrifugation at 1,000 × g for 5 minutes, at 4 °C. After the centrifugation, the supernatant was discarded and the cell pellet was washed twice with ice-cold PBS, each wash followed by a new centrifugation at 1,000 × g for 5 minutes, at 4 °C. Pellet was resuspended in polysome lysis buffer, in a volume equivalent to the pellet volume, gently pipetted up and down to disrupt cell aggregates, and incubated on ice for 5 minutes. Polysome lysis buffer was prepared at a 10× concentration (1,000 mM KCl, 50 mM MgCl₂, 100 mM HEPES-NaOH (pH 7.0), and 5% NP-40), and prior to use, the 10× buffer was diluted to 1× and supplemented with 1 mM dithiothreitol (DTT), 200 U/mL RNaseOUT (an RNase inhibitor), and an EDTA-free protease inhibitor cocktail. After resuspension in polysome lysis buffer, protein lysates were centrifuged at 20,000 × g for 10 minutes at 4°C, to discard pellets. 20 µL of protein lysates were stored at this point as “Input”.

Meanwhile, 100 µL per sample of anti-FLAG-conjugated agarose beads were washed twice with 250 µL of NT-2 buffer. This buffer was prepared as a 5× stock solution (250 mM Tris-HCl (pH 7.4), 750 mM NaCl, 5 mM MgCl₂, and 0.25% NP-40) and diluted to 1× working concentration prior to use. Each NT-2 wash was followed by centrifugation at 6,000 × g for 20 seconds to pellet the beads and remove the supernatant. After the second wash, beads were resuspended in 900 µL per sample of NET-2 buffer and mixed with 200 µL of the previously obtained protein lysates. This mixture was incubated overnight at 4°C in a rotator. NET-2 buffer consists of 1× NT-2 buffer supplemented with 20 mM EDTA pH 8.0, 1 mM DTT, and 200 units/mL of RNase OUT.

The next day, the RNA bound to these RNA-protein immunocomplexes was purified (Fig. M15). To do this:

1. IPs were centrifuged at 6,000 × g for 20 seconds to pellet the beads and remove the supernatants, and then, the pellets were resuspended in 150 µL of Proteinase K buffer. This buffer contains 1× NT-2 buffer supplemented with 1% SDS and 1.2 mg/mL of Proteinase K. To each stored lysate “Input”, 107 µL of NT-2 buffer, 15 µL of SDS 10%, and 18 µL of Proteinase K were added.
2. Every mix was incubated at 55°C for 30 minutes under shaking, in order to digest the proteins and preserve RNAs.
3. After the incubation, we spun down all tubes and transferred supernatants into new tubes, where we added 250 µL of NT-2 buffer. Next, 400 µL of phenol:chloroform:isoamyl

- alcohol (125:24:1) were added to each tube, and vortexed vigorously for 15 seconds. We proceeded to separate phases by centrifugation at $20,000 \times g$ for 10 minutes at RT.
4. We removed 350 μL of the aqueous phase without disturbing the protein interface. This aqueous phase was placed in a new tube, and 400 μL of chloroform were added. These mixes were vortexed for 15 seconds and centrifuged at $20,000 \times g$ for 10 minutes at RT, to separate phases again.
5. 300 μL of aqueous phase was placed on a new tube, where we added 50 μL of ammonium acetate 5M, 15 μL of LiCl 7.5 M, 5 μL glycogen (5 mg/mL), and 850 μL of absolute ethanol.
6. These mixtures were kept overnight at -80°C to precipitate RNA, and next day, centrifuged at $20,000 \times g$ for 30 minutes at 4°C . The supernatants were discarded.
7. The pellets were washed with 500 μL of cold 80% ethanol.
8. These samples were centrifuged at $20,000 \times g$ for 15 minutes at 4°C . Supernatants were discarded carefully, and the pellets were air-dried.
9. Air-dried pellets were resuspended in 10-20 μL of RNase-free water and placed on ice.
10. Finally, these purified RNAs were treated with DNase to remove residual contaminant DNA for further analysis.

Next step was to obtain cDNAs from purified RNAs by RT, and then, to perform a qPCR to detect the amount of SARS-CoV-2 RNA bound to each sample. To do this, we used SYBR-Green reagents and the primers indicated in [Table M9](#), to detect the SARS-CoV-2 RdRp gene.

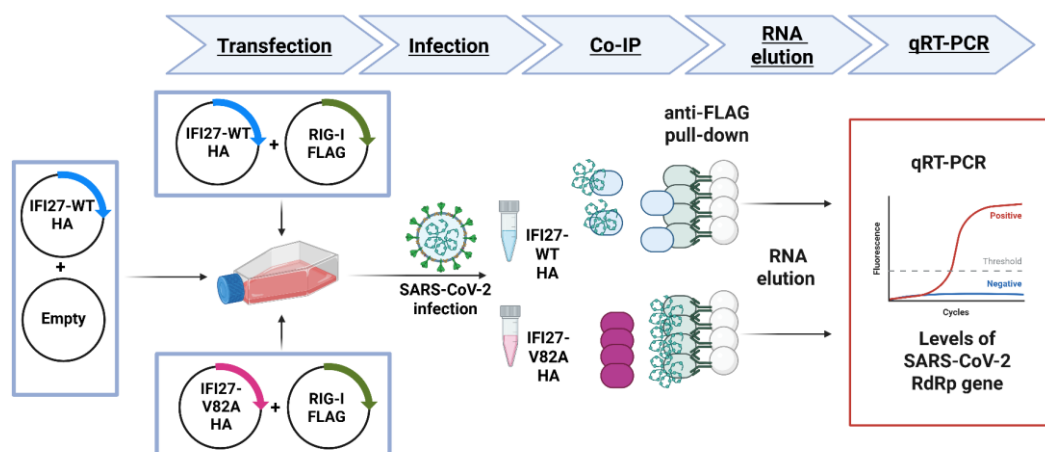


Figure M15. RIP assay of SARS-CoV-2 RNA binding to RIG-I protein.

3.11. ANALYSIS OF PROTEIN-PROTEIN INTERACTIONS

3.11.1. Co-IPs

3.11.1.1. Co-IPs with anti-HA or anti-FLAG-conjugated agarose beads

HEK-293T, HEK-293T-hACE2, HeLa WT, or HeLa KO PACT cells, as specified in each case, were seeded at a 70-80% confluency in 100 mm dishes, previously treated with Poly-L-Lysine (Sigma-Aldrich) for 20 minutes. Next, cells were transiently co-transfected with different combinations of plasmids, as specified in each case: 8.5 μg of either pCAGGS-IFI27-HA, pCAGGS-IFI27-S63L-HA, pCAGGS-IFI27-V82A-HA, or pCAGGS-IFI6-HA plasmid, alone or together with 8.5 μg of either pCAGGS-RIG-I-FLAG, pCAGGS-MDA-5-FLAG, or pCAGGS-PACT-FLAG plasmid. The empty pCAGGS plasmid was added to the transfection mixes when needed in order to equal the amount of DNA in each plate. To perform transfections,

lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific) were used following manufacturer's instructions. At 24 hpt, cells were left mock-transfected or mock-infected, or transfected using PEI (Polysciences) for an additional 24 hours with poly(I:C) at 3 µg/mL, or infected with SeV (MOI 3) or with SARS-CoV-2 (MOI 0.5), as specified in each case, for 24 hours. Cells were lysed using protein lysis buffer, incubated on ice for 30 minutes, and sonicated in an ultrasonic bath sonicator (Fisherbrand FB15051) for 5 minutes. Next, lysates were cleared by centrifugation. Where indicated, to analyze the importance of RNA presence on the interactions, lysates were treated with RNase A (10 U/mL), RNase T1 (400 U/mL), and RNase III (10 U/mL) at 37°C for 30 minutes. RNase-treated or untreated cleared lysates were incubated overnight at 4°C with anti-HA or anti-FLAG-conjugated agarose beads (Pierce, 26181 and Sigma-Aldrich, A2220, respectively), previously blocked by incubating the agarose beads during 1 hour at RT with blocking buffer (TBS containing 1% BSA). Next day, mixes were washed three times in TBS buffer containing 0.1% Tween-20 and proteins were eluted with 0.1M glycine buffer at pH 2.4, and then, we added Tris-HCl pH 8 buffer to neutralize the pH. Eluates were denatured in Laemmli sample buffer (Bio-Rad) containing 2.5% β-mercaptoethanol, during 5 minutes at 95°C. Proteins were detected by SDS-PAGE and Western blot, as described before, using anti-HA (to detect IFI27, IFI27-S63L, IFI27-V82A, and IFI6) and anti-FLAG (to detect RIG-I, MDA-5, and PACT) specific antibodies (see antibody [Table M11](#) for references and dilution) ([Fig. M16A](#)).

3.11.1.2. Co-IPs with protein A-conjugated sepharose beads

Additionally, protein A-conjugated sepharose beads were employed to analyze the binding of IFI27 to PKR or MDA5. To this end, HEK-293T, HeLa WT, and HeLa KO PACT cells were seeded at a 70-80% confluency in 100 mm dishes, previously treated with Poly-L-Lysine (Sigma-Aldrich) for 20 minutes. Next, these cells were transiently transfected with 8.5 µg of either an empty pCAGGS or the pCAGGS-IFI27-HA plasmids using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), following manufacturer's instructions. At 24 hpt, cells were either mock-transfected or transfected with 3 µg/mL of poly(I:C) for 16 or 24 hours, using PEI (Polysciences). Alternatively, A549-hACE2-empty or A549-hACE2-IFI27-HA cells were seeded at a 70-80% confluency in 100 mm dishes. Next, these cells were either mock-transfected or transfected with 60 ng/mL of poly(I:C) for 24 hours. After treatments, cells were lysed using protein lysis buffer, incubated on ice for 30 minutes, sonicated in an ultrasonic bath sonicator (Fisherbrand FB15051) for 5 minutes, and cleared by centrifugation. 100 µL of cleared lysates were incubated overnight with 4 µg of anti-PKR antibody (sc-6282, Santa Cruz Biotechnology) (for HEK-293T, and WT or KO PACT HeLa experiments) or 4 µg of anti-MDA5 antibody (21775-1-AP, ThermoFisher Scientific) (for experiments in A549-hACE2-empty and A549-hACE2-IFI27-HA cells). To prepare protein A-conjugated sepharose beads, 25 mg of protein A-conjugated sepharose beads (P3391, Merck) per sample were washed three times with TBS buffer, incubated with blocking buffer (TBS containing 0.1% BSA) for 1 hour at RT and washed again 3 times with TBS. Next, beads were resuspended in 100 µL of protein lysis buffer per sample and incubated 4 hours at 4°C with the previously overnight-incubated anti-PKR- or anti-MDA5-protein lysates. Next, mixes were washed three times with TBS containing 0.1% Tween-20 buffer, and proteins were eluted with 0.1M glycine buffer at pH 2.4, and then, we added Tris-HCl pH 8 buffer to neutralize the pH. Eluates were denatured in Laemmli sample buffer (Bio-Rad) containing 2.5% β-mercaptoethanol, during 5 minutes at 95°C. Proteins were detected by SDS-PAGE and Western blot as described before, using anti-HA (to detect IFI27), anti-PKR (PKR), and anti-MDA5 (MDA5) specific antibodies (see antibody [table M11](#) for references and dilution) ([Fig. M16B](#)).

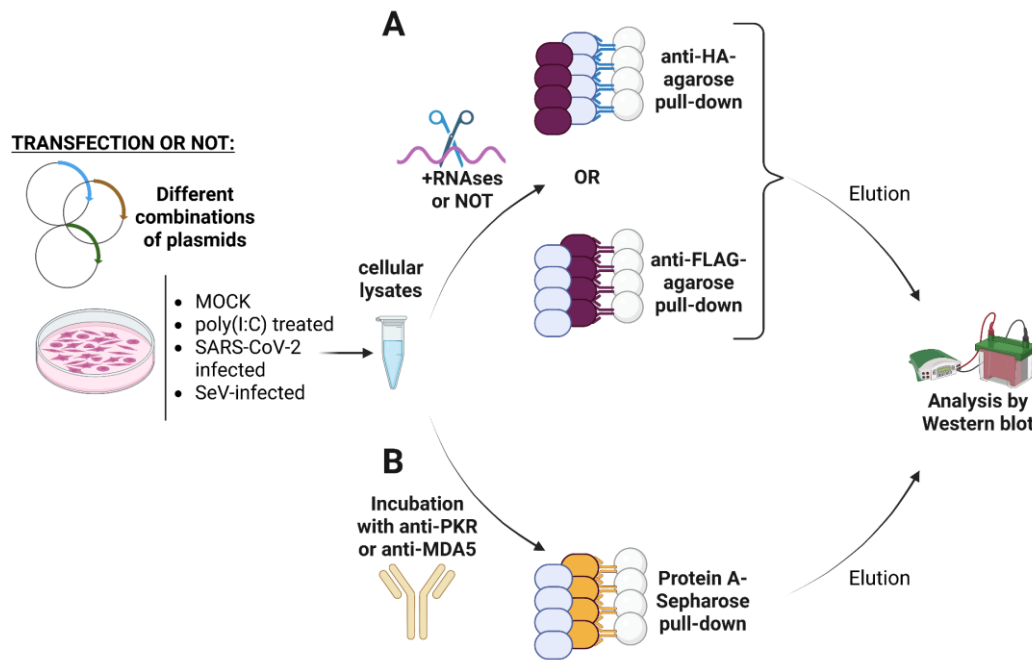


Figure M16. Graphical representation of co-IPs.

3.11.2. Mass-spectrometry

To study the protein interactome of IFI27 in order to find protein partners that help us infer new functions of IFI27, we performed a mass-spectrometry assay. To this end, HEK-293T cells were seeded at a 70-80% confluency in 100 mm dishes, previously treated with Poly-L-Lysine (Sigma-Aldrich) for 20 minutes. Next, cells were transiently transfected with a pCAGGS-IFI27-HA expressing plasmid or, as negative controls, a pCAGGS-IFI44-HA plasmid, expressing the protein IFI44 fused to an HA tag, to discard those proteins that bind to the HA tag, or an empty-pCAGGS plasmid to discard proteins that bind nonspecifically to the agarose matrix (as with the empty control, we will detect proteins binding to the anti-HA-agarose beads nonspecifically). Transfections were performed using lipofectamine 3000 and p3000 reagents (ThermoFisher Scientific), following manufacturer's instructions. 24 hours later, cells were treated with 3 $\mu\text{g}/\text{mL}$ of poly(I:C) using PEI (Polysciences) for 16 hours. After poly(I:C) treatment, cells were lysed using protein lysis buffer, incubated on ice for 30 minutes, sonicated in an ultrasonic bath sonicator (Fisherbrand FB15051) for 5 minutes, and cleared by centrifugation. These lysates were incubated overnight at 4°C with the anti-HA affinity resin (Pierce, 26181) to retain IFI27-HA and IFI27-bound proteins. Next day, mixes were washed three times with TBS buffer containing 0.1% Tween-20. To elute the proteins bound to the resin, we used 0.1 M glycine buffer at pH 2.4, and then, we added Tris-HCl pH 8 buffer to neutralize the pH. Samples were handled at the proteomics service at the National Centre for Biotechnology, where the IFI27 protein interactome was analyzed by mass-spectrometry and interpreted (Fig. M17). First, the eluted samples were reduced with 50 mM tris(2-carboxyethyl)phosphine (TCEP) (pH 8.0) for 60 min at 37°C and alkylated with 200 mM methyl methanethiosulphonate (MMTS, Pierce) for 10 min at RT. Subsequently, to improve the tryptic digestion yield of low-abundance samples, S-trap microcolumns (PROTIFI) were used, as previously reported [250]. For protein identification by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS Exploris 240), the peptide samples were analyzed on a nano liquid chromatography system (Ultimate 3000 nano HPLC system,

ThermoFisher Scientific) coupled to an Orbitrap Exploris 240 mass spectrometer (ThermoFisher Scientific). 5 μ L of each sample was injected on a C18 PepMap trap column (5 μ m, 100 μ m I.D. x 2 cm, Thermo Scientific) at 30 μ L/min, in 0.1% formic acid in water, and the trap column was switched on-line to a C18 PepMap Easy-spray analytical column (2 μ m, 100 $\text{\AA}$, 75 μ m I.D. x 50 cm, Thermo Scientific). Equilibration was done in mobile phase A (0.1% formic acid in water), and peptide elution was achieved in a 30 min gradient from 4% - 35% B (0.1% formic acid in 80% acetonitrile) at 300 nL/min. Data acquisition was performed using a data-dependent top-25 method, in full scan positive mode (range of 350 to 1200 m/z). Survey scans were acquired at a resolution of 60,000 at m/z 200, with normalized automatic gain control (AGC) target of 300 % and a maximum injection time = Auto. The top 25 most intense ions from each MS1 scan were selected and fragmented by Higher-energy collisional dissociation (HCD) of 30. Resolution for HCD spectra was set to 15,000 at m/z 200, with AGC target of 75 % and maximum ion injection time = Auto. Precursor ions with single, unassigned, or six and higher charge states from fragmentation selection were excluded.

MS and MS/MS raw data were translated to mascot general file (mgf) format and searched using an in-house Mascot Server v. 2.7 (Matrix Science, London, U.K.) against the human reference proteome from Uniprot Knowledgebase. Search parameters considered fixed MMTS alkylation of cysteine, and the following variable modifications: methionine oxidation, pyroglutamic acid from glutamine and glutamic acid at the peptide N-terminus, deamidation of asparagine/glutamine, and acetylation of the protein N-terminus. Peptide mass tolerance was set to 10 ppm and 0.02 Da, in MS and MS/MS mode, respectively, and 3 missed cleavages were allowed. The Mascot confidence interval for protein identification was set to $\geq 95\%$ ($P < 0.05$) and only peptides with a significant individual ion score of at least 40 were considered (Table A3).

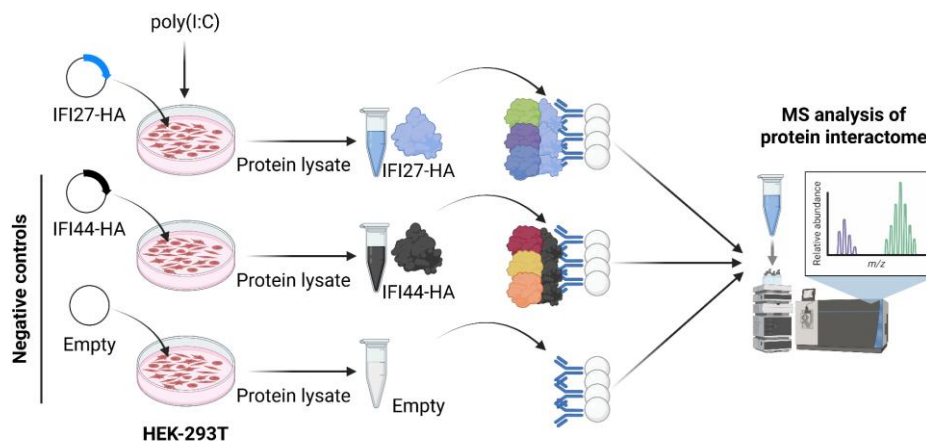


Figure M17. Mass-spectrometry analysis of IFI27-bound proteins.

3.12. IFN RESPONSE ASSAYS: POLY(I:C) TREATMENT AND VIRUS INFECTIONS

To study the possible effect that IFI27 or IFI6 could have on the induction of innate immune responses, knock-down or KO of these genes, or alternatively, protein overexpression experiments were performed, and the induction of IFNs, proinflammatory cytokines, and different ISGs was analyzed both after transfections with poly(I:C), or after infections with either IAV, SARS-CoV-2, or SeV.

First, for knocking-down the expression of IFI27 (A549 or HEK-293T cells) or IFI6 (HEK-293T, BEAS-2B, and HAP-1 cells), cells were transfected with the specific siRNAs for IFI27, IFI6, or the NT siRNA, as negative control, using lipofectamine RNAiMax (ThermoFisher Scientific)

following manufacturer's instructions, as explained before (see [Table M8](#)). Alternatively, A549 KO for IFI27 or IFI6 and their parental cells, and the stable A549-hACE2-IFI27-HA and A549-hACE2-empty cell lines, were used. Then, knocked-down, KO, or stably transfected cells were seeded and treated with different concentrations from 5 to 60 ng/mL of poly(I:C) (A549 cells) or 0.75, 1.5, 2, or 3 µg/mL (HAP-1 cells) using PEI (Polysciences), or treated with IFN- α hybrid protein (universal type I IFN, PBL assay Science, 600 and 2000 U/mL in A549 or HAP-1 cells, respectively) [251,252]. Additionally, to compare the antiviral state of either IFI27 or IFI6 KO and WT A549 cells, or IFI6 KO and WT HAP-1 cells, these cell lines were treated with poly(I:C) using PEI for 16 hours, and then, infected with rVSV-GFP for 24 hours. Viral titers were determined in Vero E6 cells as previously described (section 3.3.2) [253]. Alternatively to poly(I:C) treatment, cells were infected with either IAV (A549 WT and KO cells), SARS-CoV-2 (A549-hACE2 WT and KO cells), or SeV (HEK-293T, HAP-1, and BEAS-2B cells) during different time points and MOI, as specified in each case. Overexpression experiments were performed by transfecting HEK-293T cells with either pCAGGS-IFI27-HA, pCAGGS-IFI6-HA, or an empty pCAGGS plasmid using lipofectamine 3000 and p3000 reagents (following 3.7.1. section), and 24 hpt, cells were infected with SeV for 24 hours (MOI 3).

At different time points after treatments and/or infections (as indicated in each specific case) both total RNAs and supernatants were harvested. RNAs were retrotranscribed and qPCRs were performed to analyze the level of expression of several genes of the IFN response, or the mRNA levels of *IFI27* and *IFI6* for IFN treatments (see [Table M9](#) and [M10](#)). Supernatants were used to determine the viral titers as explained before (section 3.3).

3.13. *IN VIVO* EXPERIMENTS IN MICE

To further study the ability of IFI27 or IFI6 to modulate innate immune responses against viral infections, we performed *in vivo* experiments with mice. To do this, rIAVs were generated as described before (sections 3.5.3 and 3.6). These rIAVs (IAV-IFI27-HA, IAV-IFI6-HA, or IAV-mCherry) codify sequences of the human IFI27, IFI6, or mCherry, respectively, on the NS segment of the genome, as previously explained. This allows for the overexpression of the protein of interest in every cell that is infected in the *in vivo* system. 6-week-old, C57BL/6 female mice were purchased from Envigo and maintained at the vivarium from the National Center for Biotechnology in a pathogen-free environment. Mice (n=4 per group) were slightly anesthetized using isoflurane and infected with the different rIAVs by intranasally inoculating 2,000 FFU/mice of the viruses, or the mice were left mock-infected, by inoculating PBS. On the one hand, to determine the viral titers, at 1 and 2 dpi, mice were sacrificed, the right lung lobules were extracted and homogenized in PBS using a dounce homogenizer, and the viral titers were determined by immunofocus assay in MDCK cells as explained before (section 3.3.1). On the other hand, to analyze ISGs mRNA expression, the left lung lobules were extracted and incubated for 24 hours at 4°C in RNAlater (Ambion). 24 hours later, RNA lysis buffer was added, and lobules were homogenized manually using a dounce homogenizer. Next, total RNA was extracted, purified and retrotranscribed to cDNA as explained before (sections 3.8.1-3.8.3). cDNAs were used to perform qPCRs (Taqman gene expression assays, Applied Biosystems) for mRNAs of ISGs (*Ifit2*), IFN (*Ifnl3*), inflammatory cytokines (*Ccl2*, and *Tnf*), and for *Gapdh* as a housekeeping gene ([Table M10](#)). qPCR data was analyzed following threshold cycle methodology ($2^{-\Delta\Delta CT}$) [241] and normalized with *Gapdh* expression levels.

RESULTS

4. RESULTS

PART I. STUDY OF THE IMPORTANCE OF IFI27 AND IFI6 ON THE REPLICATION OF RNA VIRUSES: IAV, SARS-CoV-2, AND DENV2

4.1.1. *IFI27* and *IFI6* are ISGs induced after poly(I:C) and IFN treatments, and after IAV, SARS-CoV-2, and DENV infections

IFI27 and *IFI6* contain ISRE sequences on their promoters, highlighting their importance in the IFN response triggered by viral infections [175]. In this context, with the aim of deciphering the functions and molecular mechanisms of *IFI27* and *IFI6*, our first step was to confirm that in the cell systems that we were going to use, the expression of these genes was induced after the treatment with recombinant IFN- α or with poly(I:C), which is an analog of the dsRNAs that are generated after the infection with many RNA viruses [104], as well as after the infection with IAV or SARS-CoV-2 viruses. First, human epithelial lung adenocarcinoma-derived A549 cells were transfected with poly(I:C) (60 ng/mL) or treated with recombinant IFN- α (600 units/mL) for 16 hours, and the levels of *IFI27* and *IFI6* were determined by qRT-PCR, and also, *IFI27* protein levels were assessed by Western Blot. We tried to show that *IFI6* is upregulated at the protein level by Western blot, but, unfortunately, we tried several commercial antibodies against *IFI6*, and we could not detect the *IFI6* protein, even in cell extracts overexpressing the *IFI6* protein (data not shown). mRNA levels of *IFI27* were induced approximately 4.5-fold and 3.7-fold, while *IFI6* mRNA levels were incremented 90- and 150-fold, after poly(I:C) and IFN- α treatments respectively, compared to mock-treated conditions (Fig. R1A, R1C). The upregulation of *IFI27* at the mRNA level, correlated with protein levels, which were incremented by 5.6-fold and 4.3-fold after poly(I:C) and IFN- α treatments respectively, compared to mock-treated conditions (Fig. R1B). Furthermore, *IFI6* induction was also studied in a different cell line, the human leukemia-derived HAP-1 cells. These cells were also treated with poly(I:C) at 2 μ g/mL or IFN- α at 2000 units/mL, and similarly, *IFI6* mRNA showed an increase of 28- and 284-fold by these treatments, respectively (Fig. R1C).

Next, A549 cells were infected with IAV at a MOI of 1 during 24 and 48 hours, and again, mRNA levels of *IFI27* and *IFI6*, and protein levels of *IFI27* were determined. *IFI27* mRNA expression was induced near to 60-fold both at 24 and 48 hpi compared to mock-infected cells (Fig. R1D), which correlated with a 5.5- and 5.1-fold induction at protein levels at 24 and 48 hpi, respectively (Fig. R1E). Similarly, *IFI6* mRNA expression was incremented by 50- and 550-fold at 24 and 48 hpi with IAV, respectively, compared to mock-infected cells (Fig. R1F). SARS-CoV-2 effect on *IFI27* and *IFI6* levels was also assessed in A549 cells overexpressing human ACE-2 protein receptor (A549-hACE2), so that these cells become susceptible to SARS-CoV-2 infection, at a MOI of 1. *IFI27* mRNA levels were induced 3- and 40-fold after 24 or 48 hpi with SARS-CoV-2 respectively (Fig. R1G), correlating with a 1.8- and 4.1-fold increment of *IFI27* protein at 24 and 48 hpi, respectively (Fig. R1H). Likewise, SARS-CoV-2 infection of A549-hACE2 cells induced *IFI6* mRNA levels by 80- and 50-fold at 24 and 48 hpi compared to mock-infected cells (Fig. R1I). Additionally, the induction of *IFI27* and *IFI6* mRNA levels was also assessed after infection with DENV2 (MOI 1) in A549 cells. *IFI27* mRNA levels were induced 3- and 1,308-fold at 24 and 48 hpi with DENV2, respectively, compared to mock-infected A549 cells (Fig. R1J). Similarly, *IFI6* mRNA levels were strongly induced after DENV2 infection, showing a 7- and 665-fold increment at 24 and 48 hpi, respectively (Fig. R1K). Thus, we could confirm that *IFI27* and *IFI6* act as ISGs in our cell systems after different types of stimuli and infections.

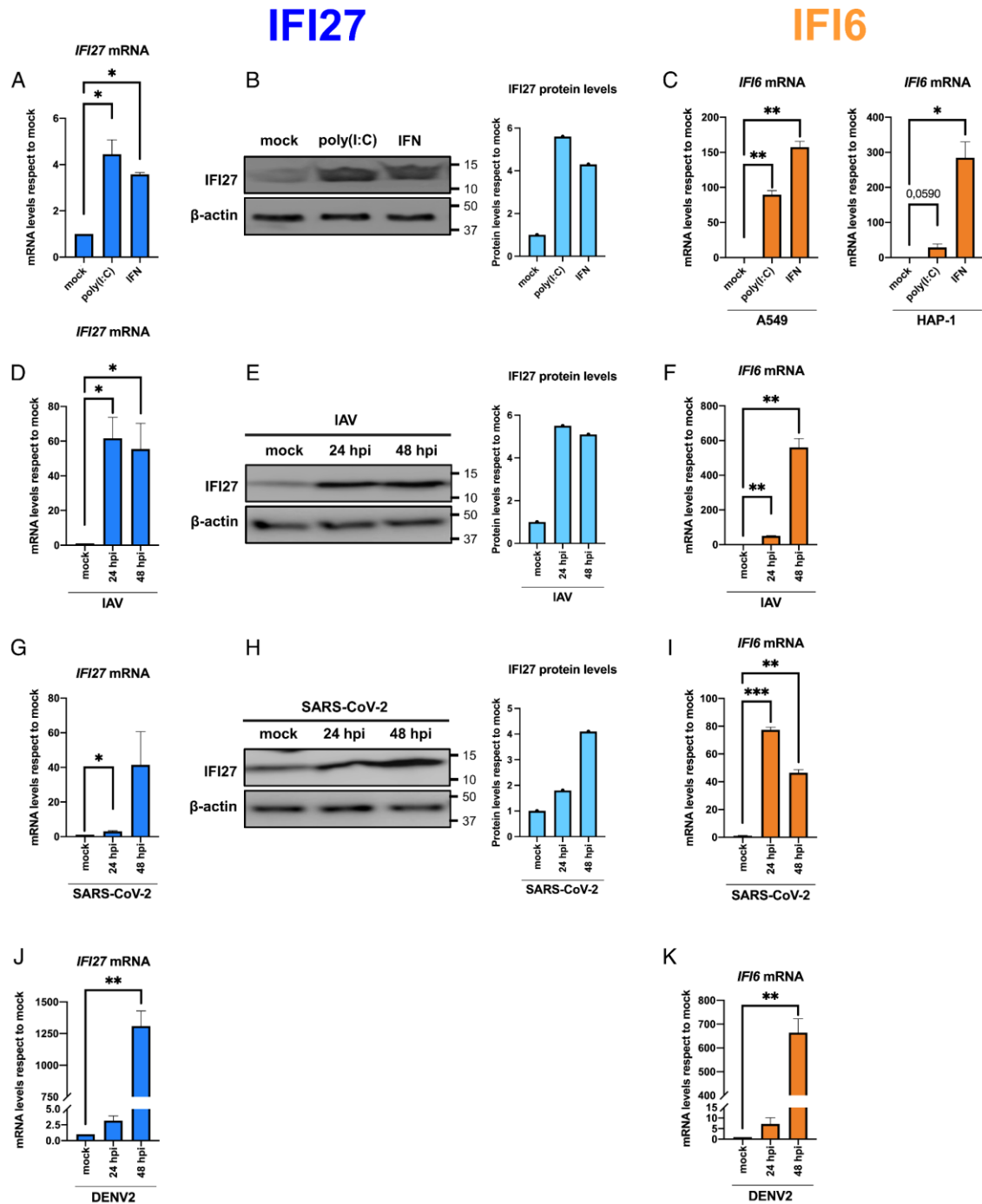


Figure R1. Induction of IFI27 and IFI6 expression after poly(I:C), IFN- α , IAV, SARS-CoV-2, and DENV2 infections. (A, B, and C) Human A549 cells were mock-treated or treated with poly(I:C) (60 ng/mL) or with IFN- α (600 units/mL) for 16 hours, and then, cellular extracts to analyze total RNAs (A, C) or proteins (B) were harvested. (C) Alternatively, HAP-1 cells were mock-treated or treated with either poly(I:C) (2 μ g/mL) or IFN- α (2000 units/mL) for 16 hours, and then, total RNA was harvested. (D-K) Human A549 cells were mock-infected or infected with IAV (MOI 1) (D, E, and F), A549-hACE2 cells were mock-infected or infected with SARS-CoV-2 (MOI 1) (G, H, and I) and A549 cells were mock-infected or infected with DENV2 (MOI 1) (J, K) for 24 or 48 hours, and then, cellular extracts to analyze total RNAs and proteins were harvested. (A, C, D, F, G, I, J, and K) Purified total RNA was retrotranscribed to cDNA and mRNA levels of *IFI27* (A, D, G, and J), *IFI6* (C, F, I, and K) and *GAPDH* (housekeeping gene) were measured by qRT-PCR. (B, E, and H) Protein extracts were used to measure the protein levels of IFI27 or β -actin (as a loading control) with their specific antibodies. Western blots

were quantified by densitometry using ImageJ software. The amount of IFI27 was normalized to the amount of β -actin. The IFI27 protein levels in mock-treated cells were expressed as 1, and the protein levels in the other conditions were normalized to this value (results of quantification showed in bars to the right of immunoblots). Molecular weight markers are indicated on the right of the blot (in kilodaltons, kDa). mRNA (A, C, D, F, G, I, J, and K) and protein levels (B, E, and H) are represented as fold change respect to values in mock-treated (A-C) or mock-infected (D-I) cells. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in A, C, D, F, G, I, J, and K). These measures were performed in two or more independent experiments, with similar results.

4.1.2. IFI27 and IFI6 absence negatively affects IAV, SARS-CoV-2, and DENV2 replication

In line with previous published studies, the expression of both IFI27 and IFI6 is strongly induced after the treatment with poly(I:C) and IFN- α , and after infections with IAV, SARS-CoV-2, and DENV2 (Fig. R1), confirming that these genes behave as important ISGs after the infection with RNA viruses in a broad way. Our next objective was to determine the functional significance of IFI27 and IFI6 in the context of viral replication. With this objective in mind, we performed targeted gene silencing by using siRNAs, and gene KO cells by using CRISPR/CAS9 technology, in order to follow a loss-of-function approach, to assess if these genes play an important role on viral replication. Contrary, we also performed IFI27 or IFI6-coding plasmid transfections, to study the effect of the overexpression of these genes on viral replication. Both proteins belong to the same family of genes and share many similarities [175], so this led us to think that both could have an analogous behavior and affect IAV, SARS-CoV-2, and DENV2 replication in a similar way.

To study the possible effect of IFI27 or IFI6 on the replication of different RNA viruses (i.e. IAV, SARS-CoV-2, and DENV2), we designed a loss-of-function approach by knocking-down the expression of these genes with siRNAs. First, human A549 cells were transfected with two different commercial siRNAs specific for IFI27 or IFI6, or with a non-targeting (NT) siRNA, as a negative control of siRNA transfection. At mRNA level, *IFI27* (Fig. R2A) and *IFI6* (Fig. R2C) expression was reduced by more than a 90% with both specific siRNAs for each gene, compared to the control cells transfected with the NT siRNA. To confirm that this reduction was also observed at protein levels, A549 cells were transfected with the IFI27 or IFI6 siRNAs, or the NT siRNA, and 24 hours later, cells were transfected with a pCAGGS plasmid expressing IFI27 or IFI6 tagged with an HA tag (pCAGGS-IFI27-HA or pCAGGS-IFI6-HA, respectively), to overexpress either IFI27 or IFI6 proteins, or with a pCAGGS plasmid not encoding any gene (empty plasmid), as a control of plasmid transfection. 24 hours later, protein extracts were obtained from the cells, and the level of expression of IFI27-HA (Fig. R2B) or IFI6-HA (Fig. R2D) were determined by Western blot, showing that only the NT siRNA-transfected cells showed expression of IFI27 or IFI6, while the transfection in cells of the respective specific siRNAs had reduced the expression of IFI27-HA (Fig. R2B) or IFI6-HA (Fig. R2D) to undetectable levels. These data indicated that the previous treatment with the IFI27 or IFI6 siRNAs avoided a proper expression of the transfected IFI27-HA or IFI6-HA, respectively. Therefore, siRNAs are able to reduce the expression of both IFI27 and IFI6, at mRNA and protein levels, validating both specific siRNAs. Once validated, we decided to test the possible effect of IFI27 or IFI6 knock-down on the replication of IAV. 24 hours after gene silencing, cells were infected with IAV and viral titers at 24 and 48 hpi were analyzed. IFI27 knock-down led to a reduction of 8-fold compared to the negative control cells (Fig. R2E). Similarly, IFI6 knock-down resulted in reproducible and significant 8-fold reduction at 24 hpi and 7-fold reduction at 48 hpi, compared to the NT siRNA-transfected cells (Fig. R2H). Thus, both genes had a similar effect on

IAV infection, with their absence negatively affecting IAV production. Additionally, we analyzed the effect of IFI27 knock-down in SARS-CoV-2 infections. To do this, 24 hours after IFI27 silencing, cells were infected with SARS-CoV-2 and viral titers were determined at 24 and 48 hpi. SARS-CoV-2 viral titers were reduced in IFI27 knocked-down cells approximately 2- and near to 4-fold at 24 and 48 hpi, respectively, compared to the negative control siRNA (Fig. R2F). Also, IFI27 knock-down led to a minor reduction of total DENV2 RNA levels at 48 hpi compared to the NT siRNA control (Fig. R2G). IFI6 knock-down, presented a similar effect on the replication of DENV2, showing a 1.3- and 3.5- fold reduction total DENV RNA levels at 24 and 48 hpi, respectively, compared to the NT siRNA control (Fig. R2I). Hence, the knock-down of IFI27 or IFI6 leads to a reduction of the replication of different RNA, non-related viruses, in epithelial cells, suggesting that this effect could be broad to many RNA viruses.

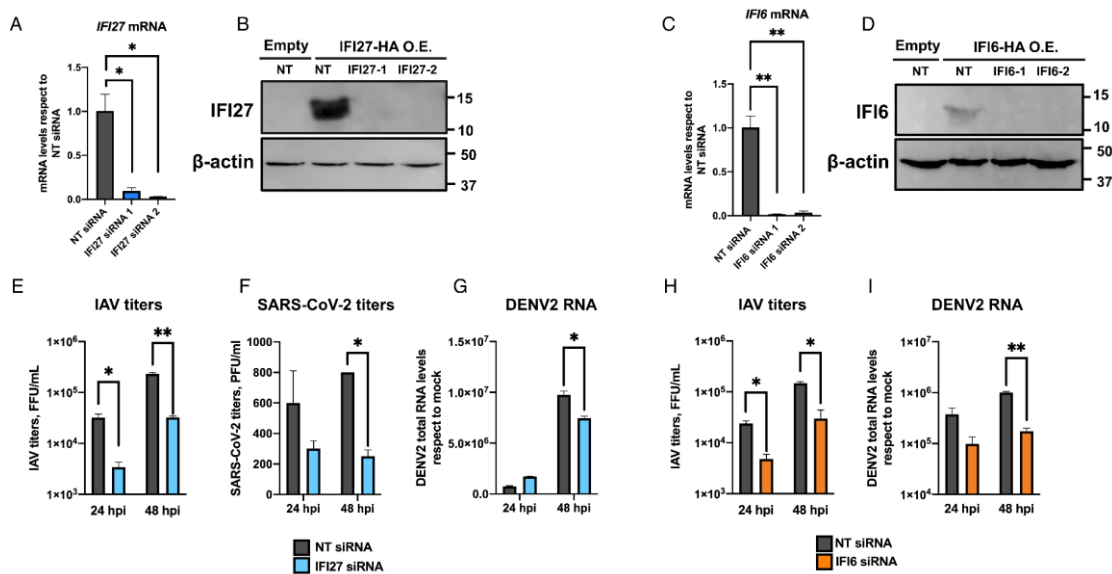


Figure R2. IFI6 and IFI27 silencing negatively affects IAV, SARS-CoV-2, and DENV2 replication. (A-D) *IFI27 and IFI6 silencing properly reduces mRNA and protein levels.* Human A549 cells were transfected with either a non-targeting (NT) siRNA as a negative control, or with two different siRNAs specific for IFI27 (A and B) or IFI6 (C and D) in order to test the knock-down of these genes. (A, C) 24 hours later, total RNA was extracted, purified and retrotranscribed to cDNAs, and qPCRs were performed to determine *IFI27* (A) or *IFI6* (C) mRNA levels of expression. mRNA levels are normalized to *GAPDH* mRNA levels and expressed as fold change respect to NT siRNA-transfected cells. (B and D) 24 hours after siRNA transfection (NT for non-targeting, IFI27-1 for IFI27 siRNA 1, IFI27-2 for IFI27 siRNA 2 (in B), IFI6-1 for IFI6 siRNA 1, and IFI6-2 for IFI6 siRNA 2 (in D)), cells were transfected with either an empty-pCAGGS plasmid (Empty) or a pCAGGS plasmid expressing IFI27 tagged with HA (IFI27-HA overexpression, O.E.) (B) or IFI6 tagged with HA (IFI6-HA O.E.) (D). 24 hours after this second transfection, protein extracts were harvested and protein levels of either IFI27-HA or IFI6-HA and β-actin (as loading control) were determined by Western blot using anti-HA and anti-β-actin specific antibodies. Molecular weight markers are indicated on the right of the blot (in kDa). These experiments were performed three times with similar results. (E and H) *IFI27 and IFI6 silencing negatively affects IAV replication.* A549 cells were transfected with either the NT siRNA or one of the IFI27- (E) or IFI6- (H) specific siRNAs. 24 hpt, cells were infected with IAV (MOI 3), and 24 and 48 hpi, viral titers on the supernatant were determined by immunofocus assay on canine MDCK cells. (F) *IFI27 silencing negatively affects SARS-CoV-2 replication.* A549-hACE2 cells were transfected with either the NT siRNA or an IFI27-specific siRNA. 24 hpt, cells were infected with SARS-CoV-2 (MOI 1), and 24 and 48 hpi, viral titers on the supernatant were determined by plaque assay on Vero E6 cells. (G and I) *IFI27 and IFI6 silencing*

negatively affects DENV2 replication. A549 cells were transfected with either the NT siRNA or one of the IFI27- (G) or IFI6- (I) specific siRNAs. 24 hpt, cells were infected with DENV2 (MOI 1), and 24 and 48 hpi, viral replication was determined by qRT-PCR. Total RNA was extracted, purified and retrotranscribed to cDNAs, and qPCRs were performed to determine the levels of DENV total RNA (*DV_{univ}* primers (table M9)) and *GAPDH* (housekeeping gene). Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in A, C, E-I). These experiments were performed three times with similar results.

The epithelial A549 cells are typically used in DENV infection studies as they are highly permissive for infection and appropriate for the study of the innate immune responses [254]. However, these cells do not represent a physiological target *in vivo*, limiting the importance of the obtained results. Thus, to further analyze the possible effect of IFI6 knock-down on DENV replication, we decided to extend this experiment to a more representative cell line, such as monocyte-derived dendritic cells (MoDCs). DCs are among the first cells to encounter DENV after infection [255], and one of the main targets of the virus. Especially, immature DCs, are one of the cell lines in which DENV replicates more efficiently and one of the most important cell lines in priming protective immune responses against DENV infections [256]. Human MoDCs generated *in vitro* support DENV infection [255] and represent one of the most useful available models for the study of DENV. Thus, we decided to assess the effect of IFI6 knock-down on DENV2 replication in MoDCs. Here, we show only data on shRNA for IFI6, as IFI27 specific shRNA did not work properly, and we did not achieve an efficient knock-down of the expression of IFI27 (data not shown). However, in future studies, IFI27 effect on DENV2 replication in DCs will also be assessed. To do this, we generated shRNA-coding lentiviruses encoding GFP as a reporter protein, as previously described (materials and methods section 3.7.3). Three of these lentiviral clones codified for shRNA targeting IFI6, and one codified for a NT shRNA as a negative control of transduction. MoDCs were generated by isolating CD14⁺ from buffy coats of healthy donors. CD14⁺ cells were transduced using polybrene with either one of the three clones of IFI6-shRNAs-coding lentivirus, or with the NT-shRNA-lentivirus as a control. At 24 hpt, transduced CD14⁺ cells were incubated for 5 days in DC differentiation media to achieve differentiation into naïve DCs (MoDCs) (materials and methods section 3.1.5). After 5 days, MoDCs were analyzed to confirm the proper transduction of IFI6 or NT shRNAs by analyzing the level of expression of the respective mRNAs by qRT-PCR, and the efficiency of transduction was analyzed by flow cytometry or by visualization with a EVOS M5000 microscope (by detecting GFP expressed in lentiviral-transduced cells) (data not shown). After this confirmation, transduced MoDCs were infected with DENV2 at a MOI of 0.5, and 48 hpi, total RNA was extracted to analyze the mRNA levels of expression of *IFI6* (to confirm that the knock-down is maintained throughout the experiment) (Fig. R3B), and the RNA levels of DENV2, to analyze the possible effect of IFI6 knock-down on DENV2 replication (Fig. R3C). The three different shRNA targeting IFI6 worked properly, as we could confirm a 10-, 31- and 40-fold reduction on *IFI6* expression in cells transduced with shRNA IFI6.1, IFI6.2, and IFI6.3, respectively, compared to the NT-shRNA control (Fig. R3B). The reduction on IFI6 expression correlated with a strong reduction ranging from ~30- to 60- fold of DENV total RNA compared to the NT control (Fig. R3C), further supporting the previous data obtained in the A549 cells (Fig. R2I), and suggesting that IFI6 absence negatively affects DENV2 replication.

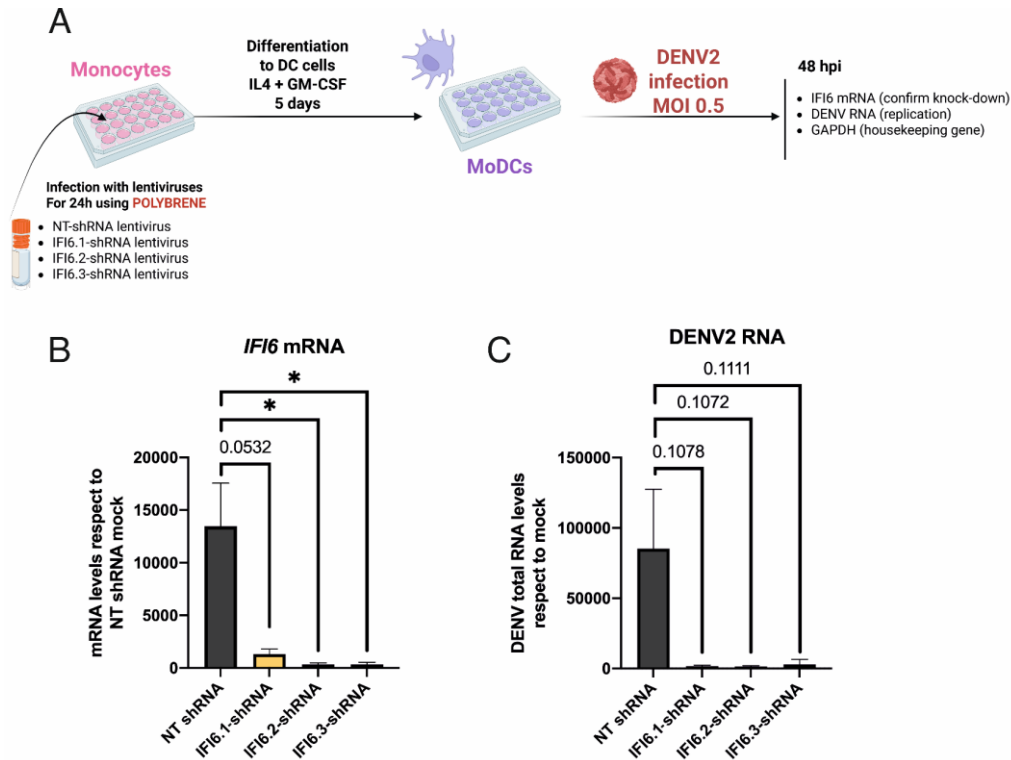


Figure R3. DENV2 replication in MoDCs transduced with IFI6 shRNA-coding lentiviruses. (A) Schema of the experimental timeline. (B and C) MoDCs were obtained from CD14⁺ cells isolated from buffy coats from healthy donors. CD14⁺ were transduced using polybrene with either a NT-shRNA lentivirus or one of the three IFI6-shRNA lentiviruses. 24 hpi, media was changed to DC differentiation media and cells were incubated for 5 days. Later, cells were infected with DENV2 (MOI 0.5) and 48 hpi with DENV2, total RNA was extracted, purified and retrotranscribed to cDNAs, and qPCRs were performed to determine *IFI6* (B) or DENV (C), and *GAPDH* (housekeeping expression control) (B and C) mRNA levels of expression. mRNA levels are expressed as fold change respect to NT shRNA-transduced and mock-infected cells. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or the exact p value (for comparisons using unpaired two-tailed Student's t test in B and C). This experiment was performed twice with similar results.

Knock-down approaches are easy and rapid methods to assess the loss-of-function of a specific gene, but may not completely inform about the effects of the gene of interest, as the suppression of the gene function is transitory, reversible, and uncomplete [257]. Generating KO cells achieves a complete and permanent elimination of the expression of the gene of interest [258], allowing for a clearer study of the effect of the absence of either IFI27 or IFI6 on viral replication. Thus, our next step to further support our data was to generate KO cell lines for the expression of IFI27 or IFI6 by using the CRISPR/CAS9 system (Fig. R4A, R4G). To this end, specific sgRNAs were selected using CRISPOR and ATUM webpages (see methods section 3.1.3), and we used a pX330 plasmid encoding these sgRNAs as well as the gene for CAS9 and a puromycin-resistance gene. Target cells were transfected with this plasmid and 24 hpt the medium was supplemented with 1 $\mu\text{g/mL}$ of puromycin to select the transfected cells in the presence of the antibiotic. After two days, medium was changed to normal growth medium, and 24 hours later, cells were detached and cloned by limiting dilution. Thereafter, different individual clones were grown, and we selected two for our studies. For IFI27-KO, selected clones were sequenced showing a 2- and 32-nucleotide deletion, respectively, leading to a frameshift change starting from amino acids 57 and 49 of the protein, respectively (Fig. R4A). Importantly, the two IFI27-KO clones and parental WT cells were treated with poly(I:C), and endogenous IFI27 protein levels were measured. The

two KO clones lacked IFI27 expression while parental cells did express IFI27, further confirming the proper KO (Fig. R4B). For IFI6, selected clones showed the same 1-nucleotide insertion, leading to a frameshift starting from amino acid 47 of the protein (Fig. R4G).

Once the KO cells were obtained, A549 parental WT cells and the selected A549 IFI27-KO or IFI6-KO clones were infected with IAV for 24 hours (MOI 1), and then, viral titers were determined in order to analyze the effect that the KO of either IFI27 or IFI6 had on viral replication. In IFI27-KO clones, IAV viral titers were reduced between 3- and 8-fold respect to parental A549 cells (Fig. R4C), hence supporting the results obtained with the siRNA knocked-down cells. In a similar manner, in IFI6-KO clones, viral titers were 2- to 3- fold lower than in the parental WT cells (Fig. R4H). Next, A549 parental cells and the IFI27- and IFI6-KO clones were transduced with a hACE2-expressing retrovirus to make these cells susceptible to SARS-CoV-2 infections. Then, parental A549-hACE2, and IFI27-KO and IFI6-KO clones of A549-hACE2 cells were infected with SARS-CoV-2 (MOI 1), and 48 hours later viral titers were determined. SARS-CoV-2 titers were reduced near to 3 times in the IFI27-KO clones (Fig. R4D), and 6- to 10-fold in IFI6-KO clones compared to the parental cells (Fig. R4I). Importantly, the reduction of viral titers of both IAV and SARS-CoV-2, was similar between the two different KO clones for each gene, further supporting that the observed result is specific of the absence of the expression of either IFI27 or IFI6 and not due to any other possible off-target effect. Also, to support siRNA data from DENV2 infections, one clone of IFI27-KO cells, one of IFI6-KO cells and the parental A549 cells were infected with DENV2 (MOI 0.5). At 48 hpi, viral replication was assessed by measuring the total RNA levels of DENV2 (Fig. R4E, R4J), and viral titers on supernatants were determined by a fluorescent focus assay (Fig. R4F, R4K). Correlating with the knock-down experiments (Fig. R2G), IFI27 KO led to a ~2-fold reduction both of total DENV2 RNA and DENV2 viral titers at 48 hpi compared to the WT parental cells (Fig. R4E, R4F). Similarly, at 48 hpi, IFI6-KO led to a 1.3- and 2.2-fold reduction of total DENV2 RNA and DENV2 viral titers, respectively, compared to the WT parental cells (Figs. R4J, R4K).

All in all, loss-of-function studies both with siRNA transfections and CRISPR/CAS9 KO cells show that diminishing IFI27 or IFI6 expression reduces the viral titers of three, non-related RNA viruses such as IAV, SARS-CoV-2, and DENV2 replication.

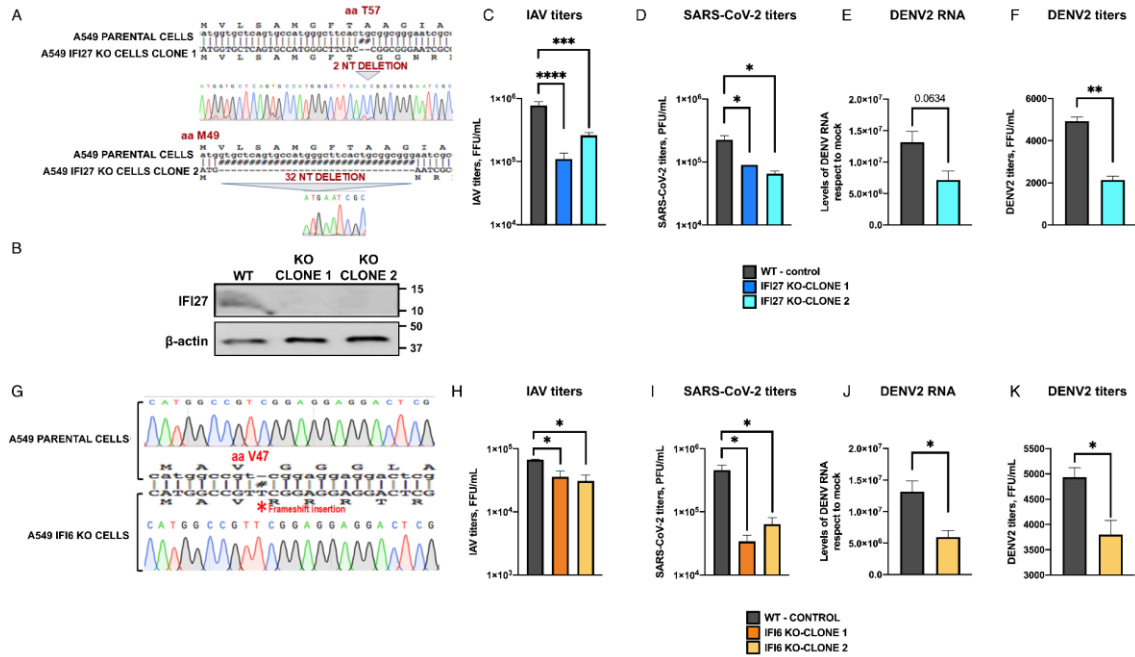


Figure R4. IFI27 or IFI6-KO affects IAV, SARS-CoV-2, and DENV2 replication. (A) Total RNA of IFI27-KO (A), IFI6-KO (G), and parental (A and G) A549 cells were harvested and retrotranscribed to cDNA. cDNAs were amplified by PCR and the PCR products were genotyped by the Sanger sequencing method (Macrogen). For IFI27-KO clones (A), we observed the deletion of 2 and 32 nucleotides within the open reading frame of IFI27 in KO clones 1 and 2 respectively (bottom line of each comparison) and comparing it to the WT sequence of parental A549 (top line of each comparison). The position of the amino acid at which the deletion occurred in each clone is indicated (Threonine 57 and Methionine 49 in KO clones 1 and 2, respectively). (G) For IFI6-KO clones, sequencing showed the insertion of 1 nucleotide within the open reading frame of IFI6 in both KO clones (upper line represents the parental WT sequence, and the bottom line represents KO clone's sequences). The position of the amino acid at which the insertion occurred is indicated (Valine 47). (B) Parental WT A549 and IFI27-KO clones 1 and 2 were treated with poly(I:C) at 60 ng/mL for 16 hours. Next, protein extracts were obtained and employed to measure protein levels of endogenous IFI27 and β-actin (as loading control) by Western blot using the specific antibodies. Molecular weight markers are indicated on the right of the blot (in kDa). (C and H) The two IFI27-KO clones and parental A549 WT cells (C), or alternatively, the two IFI6-KO clones and parental A549 WT cells (H), were infected with IAV at a MOI of 1. At 24 hpi, the viral titers in culture supernatants were analyzed by immunofocus assay, by measuring the fluorescent forming units per mL of supernatant (FFU/mL) on canine MDCK cells. The values in the KO cells were compared to the titers in the parental control cells. (D and I) The two IFI27-KO clones and parental A549-hACE2 cells (D) or alternatively, the two IFI6-KO clones and parental A549-hACE2 cells (I), were infected with SARS-CoV-2 at a MOI of 1. At 48 hpi, the viral titers in culture supernatants were analyzed by plaque assay, by measuring the plaque forming units per mL of supernatant (PFU/mL) on Vero E6 cells. The values in the KO cells were compared to the titers in the parental control cells. (E, F, J, and K) IFI27-KO (E, F), IFI6-KO (J, K) and WT parental A549 (E, F, J, and K) cells were infected with DENV2 at a MOI of 0.5 for 48 hours. (E and J) Total RNA was harvested, retrotranscribed, and a qPCR was performed to detect the RNA levels of DENV2 and *GAPDH* (housekeeping expression control). (F and K) Alternatively, the viral titers in culture supernatants were analyzed by fluorescent focus assay, by measuring the fluorescent focus units per mL of supernatant (FFU/mL) on Vero E6 cells. The values in the KO cells were compared to the titers in the parental control cells. Data is represented as the means and standard deviations of the different measures. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, or the exact p value (for comparisons using unpaired two-tailed Student's t test in C-F and H-K). These experiments were performed three times with similar results.

PART II: EFFECT OF IFI27 AND IFI6 ON ANTIVIRAL RESPONSES AFTER RNA VIRUS INFECTIONS

The impact of IFI27 and IFI6 on the replication of different RNA viruses (namely, IAV, SARS-CoV-2, and DENV2) was elucidated, showing that the absence of both genes led to a reduction on viral replication, presenting proviral-like behavior. While ISGs are typically considered antiviral effectors, a growing body of research has revealed that some of these ISGs, paradoxically, are able to function as proviral factors or as negative regulators of the innate immune responses, sometimes enhancing viral replication. As examples, it has been shown that IFIT3 is able to bind to RNA and act as a proviral factor for IAV infections [259]; ADAR1 catalyzes the conversion of adenosine (A) to inosine (I) in dsRNAs substrates, avoiding the activation of PRRs by endogenous substrates, thus acting as a negative regulator of innate immune responses [102]; and IFI44 and IFI44L knock-down negatively affects IAV and lymphocytic choriomeningitis virus (LCMV) replication while it increments antiviral responses, both acting as feedback negative regulators of the innate immunity [126,127]. Therefore, based on these previous works, and on the results observed for IFI27 and IFI6, we hypothesized that these two ISGs could act as negative regulators of innate immune responses. This hypothesis could explain the observed inverse correlation between IFI27 and IFI6 expression and RNA virus replication efficiency (Fig. R2-R4). Specifically, we suggest that IFI27 and IFI6 participate in a negative feedback mechanism dampening the innate immune responses and thereby facilitating viral replication. Thus, the subsequent aim of this part was to investigate the potential role of these genes in modulating the innate immune response to these viral infections.

4.2.1. IFI27 and IFI6 negatively affect the induction of innate immune responses: A549 and HEK-293T cell systems

To study the innate immune responses in our cell lines, we employed different stimuli, including poly(I:C) treatment and infections with various viruses such as IAV, SARS-CoV-2, and other RNA viruses like a recombinant VSV expressing GFP (rVSV-GFP) or SeV, which are widely used as model of infections of RNA viruses for different research purposes.

VSV belongs to the *Rhabdoviridae* family, Vesiculovirus genus. VSV has a negative-sense, ssRNA genome of 11 kb, which produces five distinct subgenomic mRNAs codifying the five major proteins: nucleoprotein (N) which coats the genome; matrix (M), essential for viral assembly and inhibition of host mRNA export; glycoprotein (G), which modulates viral entry, exit, and cellular recognition and fusion; the viral polymerase (L); and the phosphoprotein (P), an essential cofactor of L protein. VSV is a bullet-shaped virus which uses the low-density lipoprotein receptor (LDLR) to enter host cells. It is an arbovirus which infects insects, cattle, horses, and pigs [260–262]. On the other hand, SeV belongs to Respirivirus genus, within *Paramyxoviridae* family. This virus is also known as the murine parainfluenza virus type 1 and was first isolated in 1953 in Japan. It produces respiratory pathology in mice and is endemic in rodent colonies worldwide. It can also infect pigs, humans, non-human primates, and sheep, but without producing any disease. It has a negative-sense ssRNA genome of 15-16 kb which codifies for 6 structural genes: hemagglutinin-neuraminidase (HN) and fusion protein (F), surface glycoproteins involved in binding to cell surface and cell-membrane fusion, respectively; nucleocapsid-associated proteins nucleocapsid (N), phosphoprotein (P), and the polymerase L; and the matrix protein (M), which coordinates budding. Also, this virus produces several non-structural proteins (V, W, C', C, Y1, Y2, and X) [263,264].

Following poly(I:C) treatment, or infections with the different viruses, we analyzed the induction of key antiviral response genes by measuring their mRNA levels, including IFNs (e.g., *IFNL1*, a type III IFN [265]), IFN-stimulated genes (ISGs, e.g., *IFIT2* [266]), and IFN-induced proinflammatory cytokines (e.g., *CXCL10* [267]). Firstly, we started by transfecting A549 cells with poly(I:C) for 24 hours, to confirm that this treatment induced the expression of the IFN-innate immune responses, measured by mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10*. As expected [126,127], poly(I:C) treatment highly induced the expression levels of the three genes compared to mock-transfected A549 WT cells (Fig. R5A), confirming that the treatment with poly(I:C) induces the IFN-induced antiviral pathways. Next, to test our hypothesis, as a first approach, we performed knock-down experiments of IFI27 on A549 cells using the two previously described IFI27-specific siRNA or a NT siRNA, as a negative control (Fig. R2A, R2B). Once the cells were knocked-down for IFI27 expression by means of siRNA transfection, we treated them with poly(I:C) to induce the antiviral responses. At 24 hours after poly(I:C) treatment, as explained before, the mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10* were measured by qRT-PCR. Interestingly, after poly(I:C) treatment, the upregulation of the expression levels of *IFNL1*, *IFIT2*, and *CXCL10* was even higher in IFI27-knocked-down A549 cells compared to the NT siRNA-transfected control cells (Fig. R5B), suggesting that IFI27 could act as a negative regulator of the induction of IFN and proinflammatory responses.

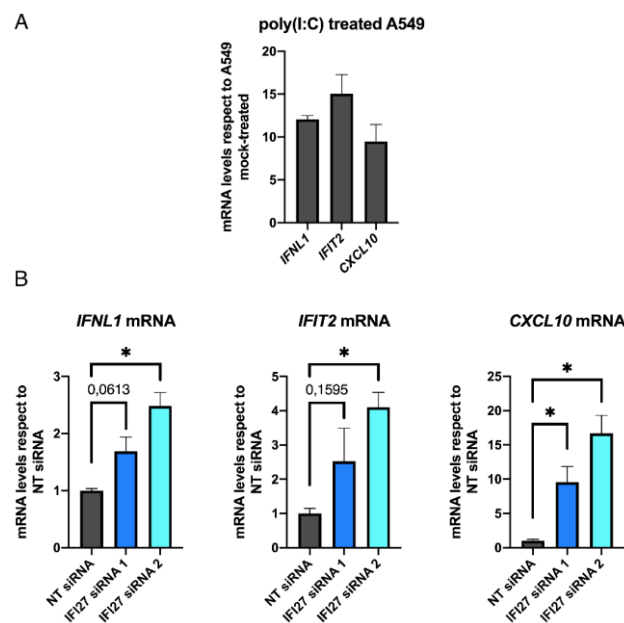


Figure R5. IFI27-knock-down upregulates the mRNA expression levels of IFN-response genes and proinflammatory cytokines. (A) Human A549 cells were transfected with poly(I:C) (60 ng/mL) for 24 hours, and then total RNA was harvested, isolated and retrotranscribed to cDNA. mRNA levels of *IFNL1*, *IFIT2*, *CXCL10*, and *GAPDH* (housekeeping expression control) were measured by qRT-PCR, and data shows fold-change respect to mock-transfected-A549 cells. (B) Human A549 cells were transfected with either a NT siRNA or with two different siRNAs specific to *IFI27* gene. At 24 hpt with siRNAs, cells were treated with poly(I:C) (60 ng/mL) for an additional 24 hours. Total RNA was extracted, isolated, and retrotranscribed to cDNA. The mRNA levels of *IFNL1*, *IFIT2*, *CXCL10*, and *GAPDH* (housekeeping expression control), were determined by qRT-PCR. mRNA levels were expressed as fold change (increases) in comparison to NT siRNA-transfected cells. Data was represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or the exact p value, is indicated (for comparisons using unpaired two-tailed Student's t test for B). Three different experiments were performed, with similar results.

Given that the effect of IFI27 and IFI6 absence on IAV, SARS-CoV-2, and DENV2 replication is very similar (Figs. R2, R4), and that the expression of both genes is induced in response to such infections (Fig. R1), it is a reasonable possibility that IFI6 may affect the induction of antiviral responses in a manner analogous to IFI27. Therefore, we decided to further test the possible modulation of these responses by IFI27 and IFI6. To further study this possibility, parental and the KO clones for IFI27 or IFI6 were first treated with poly(I:C) for 24 hours and then, total RNA was extracted, isolated and retrotranscribed to cDNA, in order to perform qPCRs of the genes of interest. Similar to what we observed with IFI27 knock-down (Fig. R5), IFI27-KO led to a strong and significative increment of the mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10* (Fig. R6A). Interestingly, the absence of IFI6 resulted in comparable results, with KO clones exhibiting a markedly and significantly higher expression of *IFNL1*, *IFIT2*, and *CXCL10* than in the parental A549 WT cells (Fig. R6B), supporting the idea of IFI27 and IFI6 as negative regulators of IFN and proinflammatory responses, as their absence leads to a higher induction in the expression of these genes.

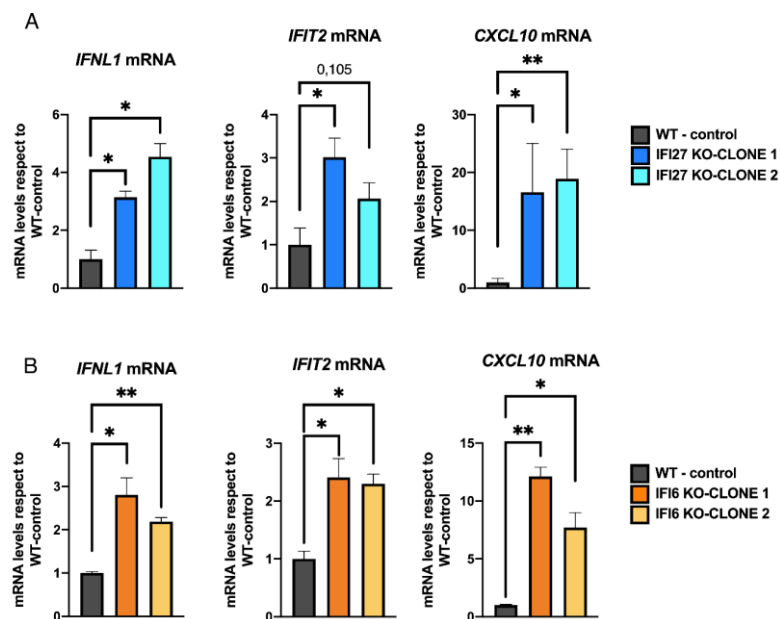


Figure R6. IFI27 and IFI6 KO upregulate the mRNA expression levels of IFN response genes and proinflammatory cytokines. (A and B) Human parental A549, two different clones of IFI27-KO cells (A) or two different clones of IFI6-KO cells (B) were treated with poly(I:C) (60 ng/mL) for 24 hours. Total RNA was extracted, isolated and retrotranscribed to cDNA. The mRNA levels of *IFNL1*, *IFIT2*, *CXCL10*, and *GAPDH* (housekeeping expression control) were determined by qRT-PCR. mRNA levels are expressed as fold change (increases) in comparison to WT control cells. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or the exact p value is indicated in one case (for comparisons using unpaired two-tailed Student's t test). Three different experiments were performed, with similar results.

Given that poly(I:C) is an analog of the dsRNAs generated during viral infections, we sought to confirm the effect of IFI27 and IFI6 on the innate immune responses induced after real viral infections (Fig. R7A). Firstly, parental A549 or A549-hACE2 WT cells and IFI27-KO cells were infected with either IAV (MOI 1) (Fig. R7B and R7D) or SARS-CoV-2 (MOI 1) (Fig. R7C and R7E). 24 or 48 hpi, total RNA was extracted from these infected cells, and mRNA expression levels of *IFNL1*, *IFIT2*, and *CXCL10* were quantified by qRT-PCR analysis (Fig. R7B-E). As expected [268,269], both IAV and SARS-CoV-2 infections highly induced the mRNA expression levels of these three genes compared to mock conditions (Fig. R7B, R7C, respectively). Similar

to the results obtained with poly(I:C) treatments (Fig. R6A), in the absence of IFI27, there was a significant 4-, 3- and near 6- fold increase on the induction of *IFNL1*, *IFIT2*, and *CXCL10*, respectively, compared to the parental WT cells, in cells infected with IAV during 24 h (Fig. R7D). Likewise, at 48 h after SARS-CoV-2 infection, the levels of *IFNL1*, *IFIT2*, and *CXCL10* were induced around 6-, 15-, and 12- fold higher in the IFI27-KO cells compared to the parental WT cells (Fig. R7E). Thus, IAV and SARS-CoV-2 infection strongly induce the expression of IFN-induced genes and proinflammatory cytokines in A549 cells (Fig. R7B, R7C, respectively), but this induction is even higher in the absence of IFI27 (Fig. R7D and R7E). All these evidences together point to IFI27 behaving as a negative regulator of the induction of IFN-induced antiviral responses.

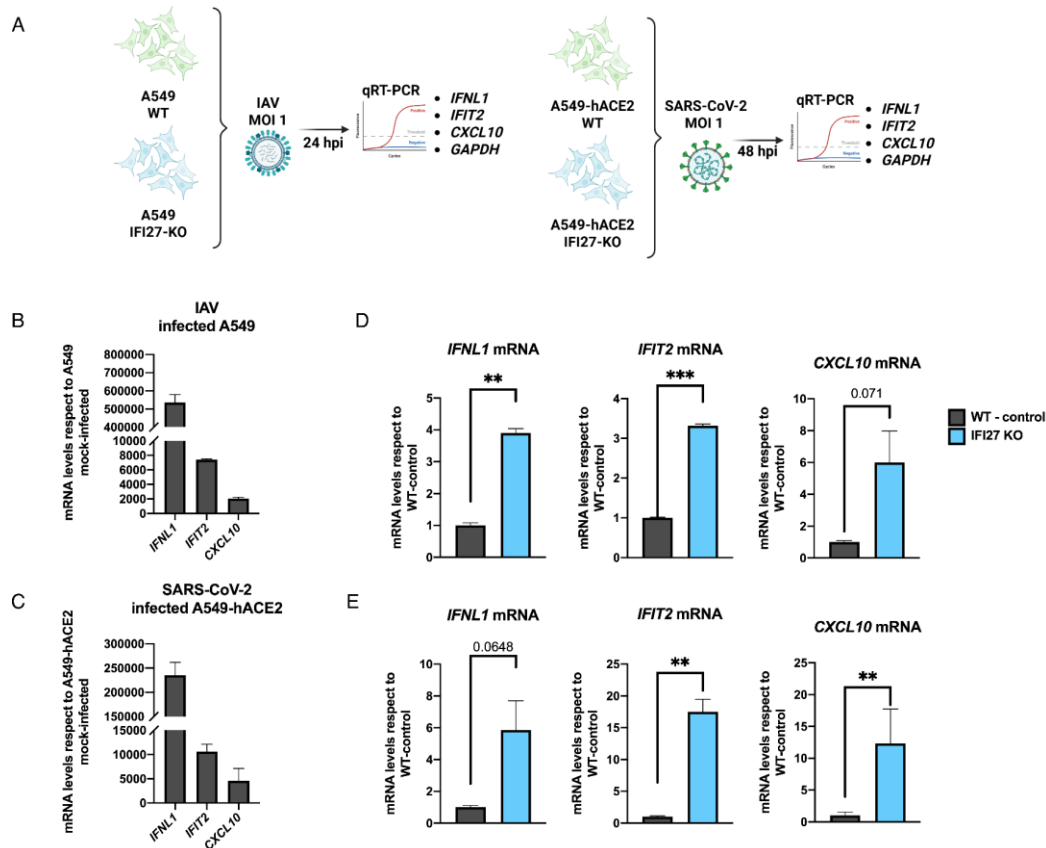


Figure R7. IFI27 impairs IFN-induced responses after IAV and SARS-CoV-2 infections. (A) Schema of the experimental timeline. Human parental A549 (IAV) or A549-hACE2 (SARS-CoV-2) cells, and IFI27-KO cells for each cell line, were infected with either IAV (MOI 1) for 24 h or SARS-CoV-2 (MOI 1) for 48 h. Then, total RNA was extracted, isolated and retrotranscribed to cDNA. The mRNA levels of *IFNL1*, *IFIT2*, *CXCL10*, and *GAPDH* (housekeeping expression control) were determined by qRT-PCR. (B, C) mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10* are expressed as fold of change (increases) in comparison to mock-infected A549 (IAV) (B) or A549-hACE2 (SARS-CoV-2) (C) parental WT cells. (D and E) mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10* are expressed as fold change (increases) in comparison to IAV- (D) or SARS-CoV-2- (E) infected parental WT cells. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or the exact p value in indicated in some cases (for comparisons using unpaired two-tailed Student's t test in D and E). Three different experiments were performed, with similar results.

Secondly, to ascertain whether IFI6 could have a similar effect to IFI27 on the induction of innate immune responses after IAV and SARS-CoV-2 infection, we repeated the same procedure but employing IFI6-KO instead of IFI27-KO cells. Correlating with the data for IFI27-KO clones

(Fig. R7D, R7E), in IFI6-KO cells, the induction of mRNA levels of *IFNL1* and *IFIT2* after IAV (Fig. R8B) and SARS-CoV-2 (Fig. 8C) infection, was significantly higher than in the WT control cells, indicating that IFI6 acts as a negative regulator of the induction of IFN-induced antiviral responses.

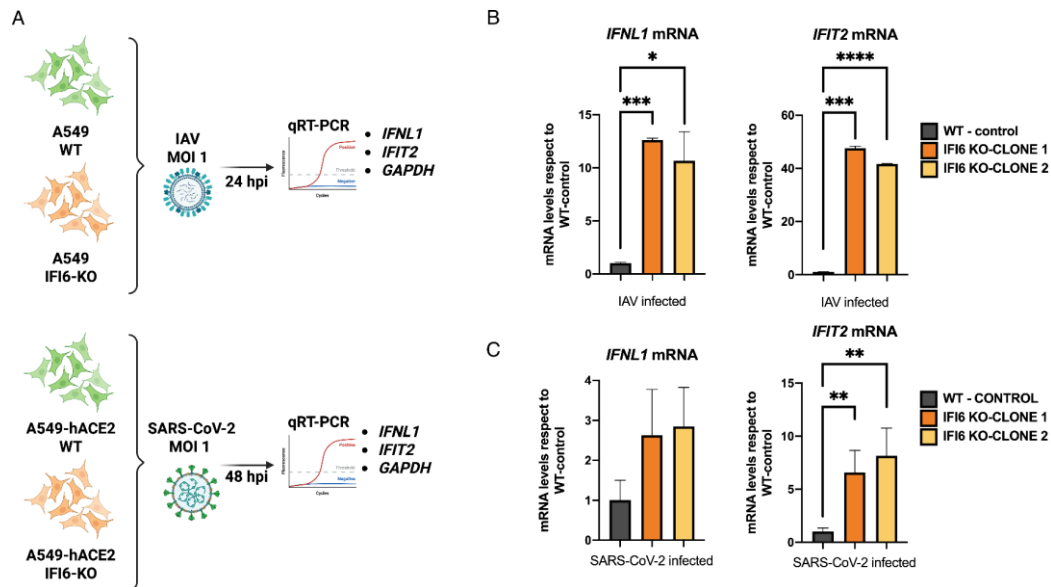


Figure R8. Effect of IFI6 absence on the induction antiviral responses after IAV or SARS-CoV-2 infections in A549 cell line. **(A)** Schema of the experimental timeline. **(B)** WT-control A549 cells or two clones of A549 specifically KO for IFI6 (IFI6-KO-clones 1 and 2), were infected with IAV (MOI 1) for 24 hours. **(C)** WT-control A549-hACE2 cells or two clones of A549-hACE2 specifically KO for IFI6 (IFI6-KO-clones 1 and 2), were infected with SARS-CoV-2 (MOI 1) for 48 hours. **(B and C)** After infections, total RNA was harvested, isolated, and retrotranscribed to cDNA. mRNA levels of *IFNL1*, *IFIT2*, and *GAPDH* (to normalize mRNA levels) were measured by qRT-PCR and expressed as fold change (increases) in comparison to WT-control cells. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in **B** and **C**). Three different experiments were performed, with similar results.

Next, to delve deeper, we investigated whether IFI27 and IFI6 ability to modulate IFN responses affects viral replication. To this end, we used the rVSV-GFP. This virus is a powerful tool to study the antiviral state of cells, as this virus is highly sensitive to IFN responses [253,270], and it is easily titrated either by plaque assay (see methods section 3.3.2) in Vero E6 cells, or by immunofluorescence, taking advantage of its GFP expression. Taking into account these results, parental WT A549 or either the two IFI27-KO clones or the two IFI6-KO clones, were first mock- or poly(I:C)-transfected, to activate the IFN-mediated antiviral responses. Then, these cells were infected with rVSV-GFP (MOI 0.1), and 24 hpi viral titers in the cell culture supernatants were analyzed in Vero E6 cells (Fig. R9A). rVSV-GFP grew with high titers, between $\sim 10^8$ PFU/mL and 10^9 PFU/mL, in mock-treated cells, both in the parental, IFI27-KO clones (Fig. R9B) and IFI6-KO clones (Fig. R9C) of A549 cells irrespective of whether they were knocked-out or not. Pre-treatment with poly(I:C) prior to the infection, reduced viral titers by more than 10-fold in A549 WT cells (Fig. R9B, R9C) due to the induction of a strong host antiviral-state in these cells, as shown before (Fig. R5), and as previously reported [126,127]. Interestingly, this reduction was even 10- to 100-fold higher, in both IFI27-KO clones (Fig. R9B), and IFI6-KO clones (Fig. R9C). Again, this correlated with the stronger induction of IFN, ISGs, and proinflammatory cytokines in IFI27-KO and IFI6-KO cells compared to the WT control cells after poly(I:C) treatment (Fig. R6) and after IAV and SARS-CoV-2 infections (Fig. R7, Fig. R8). These results provide

compelling evidence that IFI27 and IFI6 act as negative regulators of host antiviral response induction, as their absence led to a higher IFN-induced antiviral response and lower titers of rVSV-GFP virus.

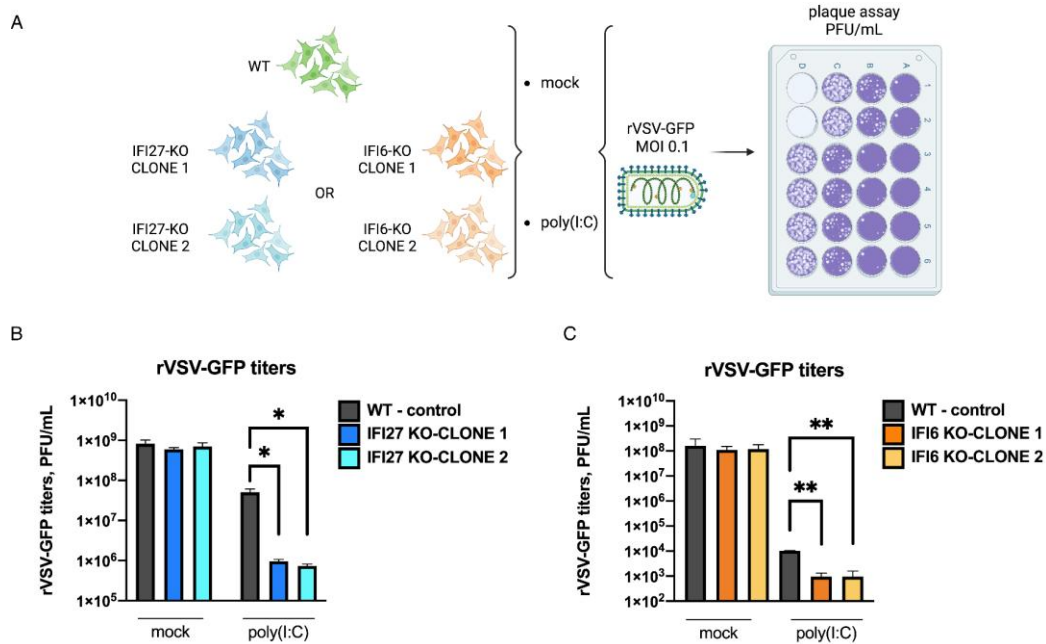


Figure R9. IFI27 or IFI6 absence strongly potentiates the establishment of an antiviral state in cells after poly(I:C) treatment. (A) Schema of the experimental timeline. (B and C) A549 WT cells and IFI27-KO (B) or IFI6-KO (C) A549 cells were mock- or poly(I:C)-treated (60 ng/mL) for 16 hours. At 24 hpt, cells were infected with rVSV-GFP (MOI 0.1) for an additional 24 hours. Finally, infected supernatants were titrated by plaque assay in Vero E6 cells, and the results of these titrations were represented (B and C). Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's *t* test in B and C). Three different experiments were performed, with similar results.

Prior experiments utilized loss-of-function approaches, so, in order to further support our findings, we followed the complementary approach by conducting gain-of-function studies. To this end, we assessed the level of induction of antiviral responses following transfection with pCAGGS plasmids overexpressing either IFI27 (pCAGGS-IFI27-HA), IFI6 (pCAGGS-IFI6-HA), or an empty plasmid (pCAGGS-empty) as a negative control for comparative analysis. To this end, HEK-293T cells were transiently transfected with either the empty plasmid or with the IFI27-HA or IFI6-HA overexpressing plasmids. Next, cells were infected with SeV (MOI 3). SeV is of great interest on the study of IFN pathways, as it has been widely used due to its ability to induce robust quantities of type I IFNs, and it is known that SeV PAMPs activate PRRs such as RIG-I and MDA5 [271–274]. At 24 h after SeV infection, mRNA levels of IFNs and proinflammatory cytokines were measured by qRT-PCR, to determine the antiviral state of cells. In HEK-293T cells, *IFNL1* and *CXCL10* levels were increased 450,000- and 110,000-fold, respectively, after SeV infection, respect to mock-infected cells, showing the efficiency of this virus to induce IFN responses (Fig. R10B). In line with our previous data, in SeV-infected cells, IFI27 overexpression attenuated *IFNL1* induction 9-fold and *CXCL10* induction by 2,750-fold, compared to the empty plasmid-transfected control cells (Fig. R10C). Similarly, IFI6 overexpression led to a 6-fold reduction of *IFNL1* mRNA levels compared to the empty plasmid-transfected control cells (Fig. R10D). These results show again how IFI27 and IFI6 negatively modulate antiviral responses. Conversely, and correlating with overexpression data, we performed knock-downs for either IFI27 or IFI6, followed by SeV infection. By doing this, we

observed that in SeV-infected HEK-293T cells, the induction of *IFNL1* and *CXCL10* was around 15- and 6-fold higher, respectively, in IFI27-knocked-down cells compared to the control cells, transfected with a NT siRNA (Fig. R10E). Likewise, *IFNL1* mRNA levels were 10-fold higher when IFI6 expression was knocked-down (Fig. R10F). All in all, either IFI27 or IFI6 overexpression strongly reduces IFN-induced responses after infection, while their knock-down strongly increases IFN-induced responses. Thus, both proteins act as negative regulators of antiviral host responses.

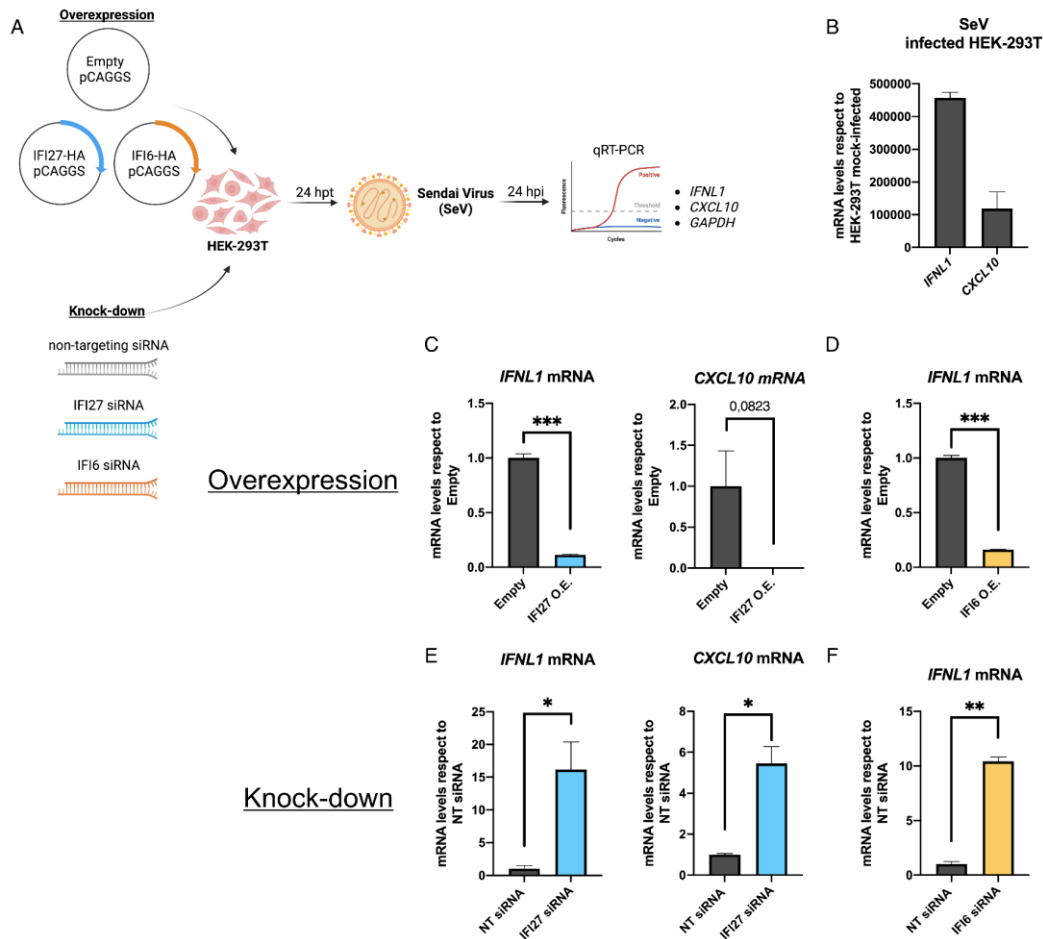


Figure R10. IFI27 and IFI6 impair antiviral responses following SeV infection. (A) Schema of the experimental timeline. (B) HEK-293T cells were infected with SeV (MOI 3) for 24 hours. Then, total RNA was harvested, isolated, and retrotranscribed to cDNA. mRNA levels of *IFNL1*, *CXCL10*, and *GAPDH* (housekeeping expression control) were determined by qRT-PCR compared to levels in mock-infected cells. (C and D) HEK-293T cells were transiently transfected with either an empty-pCAGGS plasmid as a negative control, or (C) an IFI27-HA overexpressing plasmid (pCAGGS-IFI27-HA) or (D) an IFI6-HA overexpressing plasmid (pCAGGS-IFI6-HA). At 24 hpt, cells were infected with SeV (MOI 3), and 24 hpi, total RNA was harvested, isolated, and retrotranscribed to cDNA. mRNA levels of *IFNL1* (C and D), *CXCL10* (C), and *GAPDH* (housekeeping expression control) were determined by qRT-PCR and compared to those of the empty plasmid-transfected control cells. (E and F) HEK-293T were transiently transfected with either a NT siRNA as a negative control, or (E) an IFI27-specific siRNA (to knock-down IFI27 expression) or (F) and IFI6-specific siRNA (to knock-down IFI6 expression). At 24 hpt, cells were SeV-infected (MOI 3), and at 24 hpi, total RNA was harvested, isolated, and retrotranscribed to cDNA. mRNA levels of *IFNL1* (E and F), *CXCL10* (E), and *GAPDH* (housekeeping expression control), were determined by qRT-PCR compared to those on control cells transfected with the NT siRNA. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****

$p < 0.0001$, or the exact p value in the indicated cases (for comparisons using unpaired two-tailed Student's t test in C-F). Three different experiments were performed, with similar results.

4.2.2. IFI6 negatively affects the induction of antiviral responses in different cell systems: HAP-1 and BEAS-2B cell lines

Extensive data indicate that IFI27 and IFI6 act as negative regulators of innate immune responses induced both by a viral dsRNA analog such as poly(I:C), and after infections by viruses such as IAV, SARS-CoV-2, or SeV (Figs. R6-R10). To further validate our findings, we sought to investigate the possible effect of IFI6 on antiviral responses in different cell lines: the human leukemia-derived HAP-1 cells, which are tumor-derived, have a myeloid origin, and are highly favored in genetic research due to their near-haploid genome, facilitating efficient gene editing and functional studies [275]; and the non-tumorigenic epithelial cell line derived from human bronchial epithelium (BEAS-2B) [276]. The objective is to validate our previous data in different and more physiological cell models to assess the feasibility of performing *in vivo* studies.

As a first step, we employed two cell lines which are the HAP-1 control cells and HAP-1 KO for IFI6, commercially available. We decided to perform an analysis of the whole transcriptome of both cell lines, which was then compared by RNAseq. This allows us to obtain more comprehensive data regarding the status of innate immune responses due to the absence of IFI6 expression. First, the whole transcriptome of mock-transfected, HAP-1 WT cells and poly(I:C)-transfected, HAP-1 WT cells, was obtained by RNAseq, and both transcriptomes were compared. Those genes that were differentially upregulated by at least 2-fold in poly(I:C)-transfected WT cells compared to mock-transfected WT cells, and with a false discovery rate (FDR) adjusted p -value lower than 0.05, were selected and classified by gene ontology according to the biological process in which they are involved (Table A1, Appendix). The top hits of biological process that were upregulated after poly(I:C) treatment included: “negative regulation of viral genome replication”, “positive regulation of IFN-beta production”, “interleukin 27-mediated signaling pathway”, “antiviral innate immune response”, “regulation of defense response to virus”, “cytoplasmic pattern recognition receptor signaling pathway in response to virus”, “positive regulation of RIG-I signaling pathway”, “cellular response to interferon-alpha”, “cellular response to exogenous dsRNA”, “response to bacterium”, “positive regulation of interferon-alpha production”, “positive regulation of tumor necrosis factor production”, “positive regulation of MDA5 signaling pathway”, “type I interferon signaling pathway”, “regulation of type III interferon production”, “ISG15-protein conjugation”, “regulation by virus of viral protein levels in host cell”, “negative regulation of cytokine-mediated signaling pathway”, and “negative regulation of innate immune response”. This was expected, as poly(I:C), as an analog of viral dsRNA, is known to induce the innate immune responses [104]. To simplify the interpretation of this data, we performed a further classification of the upregulated “biological processes” into 7 different categories, and the fold enrichment (x-axis, limited to a 100-fold enrichment) and $-\log_{10}$ of the FDR (y-axis) of those processes were represented to obtain a bubble plot diagram (Fig. R11A). This diagram shows the over-representation of the different innate immune processes after poly(I:C) treatment.

Furthermore, we compared the transcriptomes of HAP-1 WT cells and HAP-1 IFI6-KO cells, both transfected with poly(I:C), in order to analyze the possible effect of IFI6 absence on the induction of innate immune responses. Again, we selected all those genes that showed a 2-fold upregulation in IFI6-KO cells compared to WT cells, and with an adjusted p -value lower than 0.05, and we classified those genes attending to the biological process in which they participate

(Table A2). Following this criteria, the biological processes that were upregulated in IFI6-KO cells compared to WT cells were: “negative regulation of viral process”, “negative regulation of viral genome replication”, “defense response to virus”, “response to virus”, “regulation of viral genome replication”, “positive regulation of cell migration”, “negative regulation of cell migration”, “regulation of response to external stimulus”, “regulation of MAP kinase activity”, “response to interferon-alpha”, “response to type I interferon”, “positive regulation of interferon-beta production”, “response to interferon-gamma”, “interleukin-27-mediated signaling pathway”, “negative chemotaxis”, “regulation of viral entry into host cell”, “response to interferon-beta”, “regulation of ERK1 and ERK2 cascade”, “negative regulation of MAP kinase activity”, “negative regulation of viral life cycle”, “cellular response to type I interferon”, “regulation of immune system process”, “regulation of tumor necrosis factor production”, and “negative regulation of viral entry into host cells”. These results suggest that IFI6 counteracts the antiviral responses by negatively modulating IFN pathways and proinflammatory cytokines responses. Again, to simplify the interpretation of this data, we performed a further classification of the upregulated “biological processes” into 7 different categories, and the fold enrichment (x-axis) and $-\log_{10}$ of the FDR (y-axis) of those processes were represented to obtain a bubble plot diagram (Fig. R11B). This diagram shows the over-representation of the different processes in IFI6-KO cells compared to WT-cells, both of them transfected with poly(I:C).

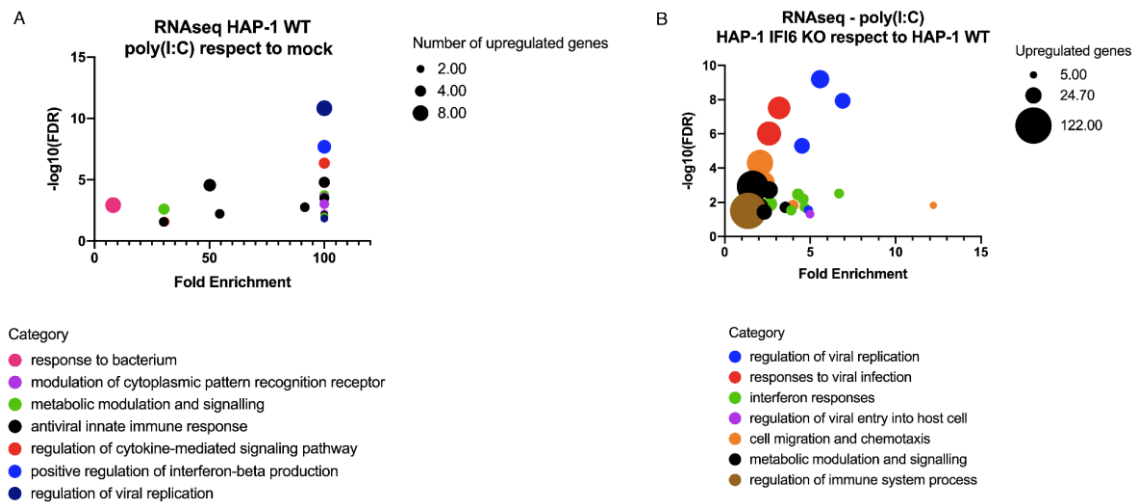


Figure R11. Bubble plot from RNAseq data. (A) HAP-1 WT parental cells were either mock- or poly(I:C)-treated (1.5 $\mu\text{g/mL}$) for 16 hours. Then, the whole transcriptome of these cells was determined, and the transcriptome of poly(I:C)-transfected cells was compared to that of the mock-transfected cells by RNAseq. (B) HAP-1 WT cells and HAP-1 IFI6-KO cells were transfected with poly(I:C) (1.5 $\mu\text{g/mL}$) for 16 hours. Then, the whole transcriptome of these cells was determined, and the transcriptome of IFI6-KO cells was compared to that of the WT-cells by RNAseq. (A and B) All those genes that were at least 2-fold upregulated in poly(I:C)-WT transfected cells, compared to WT mock-transfected cells (A) or in poly(I:C)-transfected IFI6-KO cells, compared to poly(I:C)-transfected WT cells (B), and showed an FDR adjusted p-value lower than 0.05, were classified attending to their “biological process” using gene ontology. (A and B) We performed a classification of the upregulated “biological processes” into 7 different categories (indicated below the graph). The fold enrichment (x-axis) and $-\log_{10}$ of the FDR (y-axis) of each set of genes within each “biological process” category were represented to obtain a bubble plot diagram. The color of the bubble represents the different “biological process category”, and the size of the bubble represent the number of upregulated genes within the set of genes included within one “biological process” category.

Among all upregulated genes in IFI6-KO transcriptome compared to WT cells transcriptome, both of them transfected with poly(I:C), we were particularly interested in those involved in IFN pathways, as well as proinflammatory and migratory cytokines. Therefore, genes within these groups that met the established criteria (2-fold upregulation and an FDR corrected p-value <0.05) were represented (Fig. R12A and R12B). We can highlight that the absence of the expression of IFI6 led to an upregulation of different ISGs (*IFI44*, *IFIT1*, *IFIT2*, *IFIT3*, *IFIT5*, *IFITM1*, *IFITM2*, *IFITM3*, *ISG15*, *ISG20*, *OAS1*, *OAS2*, *OASL*, *PKR*, *APOBEC 3C*, *APOBEC 3D*, *APOBEC 3G*, *RSAD2*, *HERC5*, *HERC6*, *TRIM5*, and *TRIM22*); type III IFN genes (*IFNL1* and *IFNL3*); transcription factors involved in the induction of IFN (*IRF-7*) and the expression of ISGs (*STAT1*, *STAT4*, and *STAT5A*); PRRs involved in dsRNA sensing after RNA viral infections (*MDA5* and *RIG-I*) (Fig. R12A); proinflammatory cytokines and cytokines involved in cell migration (*CXXC4*, *CXCL10*, *CXCL11*, *CXCL2*, *CXCL16*, *CCL2*, *CCL5*, *CX3CL1*); cytokine receptors (*CXCR5*); and TNF-superfamily genes (*TNFRSF1B*, *TNFRSF4*, *TNFRSF8*, *TNFRSF9*, *TNFRSF10D*, *TNFRSF12A*, and *TNFAIP3*) (Fig. R12B). To corroborate RNAseq data, the mRNA levels of some of these genes of interest, such as *IFNL1*, *IFIT2*, *CCL2*, and *CXCL10*, were measured in IFI6-KO cells respect to WT cells by qRT-PCR and compared to the RNAseq data (Fig. R12C). Similar results were obtained with both methods, validating RNAseq data.

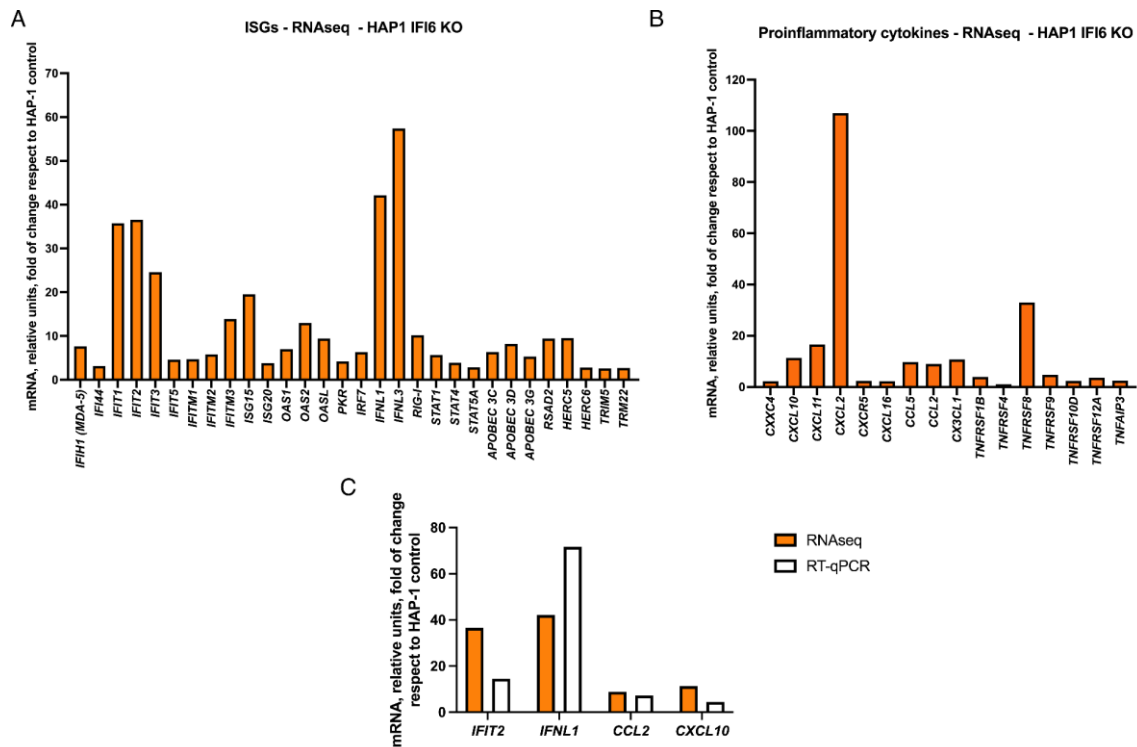


Figure R12. Analysis of IFI6 effect on innate immune responses by RNAseq. HAP-1 WT cells, as control, and HAP-1 IFI6-KO cells, were transfected with poly(I:C) (1.5 µg/mL) for 16 hours. Next, the total RNAs were extracted, and cellular transcriptomes of both cell lines were analyzed and compared by RNAseq. Representation of ISGs (A), and proinflammatory cytokines, receptors of proinflammatory cytokines, and genes from the TNF superfamily (B) that were upregulated in the IFI6-KO poly(I:C)-treated cells compared to the WT poly(I:C)-treated cells by at least 2-fold, and presented an FDR corrected p-value<0.05. The y-axis represents the fold of change for each gene in IFI6-KO cells compared to WT cells. (C) Comparison of measurement by RNAseq and qRT-PCR of mRNA levels of *IFIT2*, *IFNL1*, *CCL2*, and *CXCL10*.

Having described the effect of IFI6 on viral replication (Figs. R2H, R2I, R3C, and R4H-4K) and on innate immune responses in A549, and HEK-293T cells (Figs. R6B, R8, R9C, R10D, and R10F), we further investigated these findings in the HAP-1 cells. As we mentioned before, rVSV-GFP is an appropriate model of infection to test the antiviral state of the cells. Thus, both WT and IFI6-KO HAP-1 cells were transfected with different concentrations of poly(I:C) (0.75, 1.5, and 3 $\mu\text{g}/\text{mL}$), and then, at 24 hpt the cells were harvested to purify total RNAs to analyze mRNA levels of *IFNL1* and *IFIT2* (Fig. R13A), or at 16 hpt, infected with rVSV-GFP (MOI 0.1) during 24 hours (Fig. R13B). Interestingly, we observed a dose-dependent increment of both *IFNL1* and *IFIT2* mRNA levels with increasing concentrations of poly(I:C), both in WT and IFI6-KO cells (Fig. R13A). However, this effect was higher in the IFI6-KO cells compared to WT cells at every poly(I:C) concentration (Fig. R13A), showing again that IFI6 acts as a negative regulator of the induction of these antiviral IFN-induced genes. When analyzing rVSV-GFP viral titers under the same treatment conditions, we noticed that titers decreased with increasing poly(I:C) concentrations (Fig. R13B). In WT cells transfected with the highest poly(I:C) concentration before the infection, we observed a near 6-fold reduction of viral titers respect to viral titers in the mock-transfected cells before the infection. Remarkably, this reduction of viral titers was much more pronounced in IFI6-KO cells (Fig. R13B) after the treatment with every poly(I:C) concentration, especially at the highest poly(I:C) concentration, where we observed a near 150-fold reduction respect to WT cells transfected with the same poly(I:C) concentration (Fig. R13B). These results correlate with the stronger induction of *IFNL1* and *IFIT2* (Fig. R13A), confirming that the ability of IFI6 to negatively modulate antiviral responses positively affects viral replication.

Moreover, to test IFI6 effect on the replication of a different virus in HAP-1 cells, and since HAP-1 cells are not susceptible to IAV or SARS-CoV-2 infections (data not shown), we decided to test the effect of IFI6-KO on the induction of antiviral responses after SeV infection. Correlating with rVSV-GFP findings, mRNA levels of *IFNL1* and *IFIT2* were near 7- and 9-fold increased, respectively, in IFI6-KO cells compared to WT parental cells after SeV infection (Fig. R13C), indicating, again, that blocking IFI6 expression potentiates the induction of IFN-induced responses after infection.

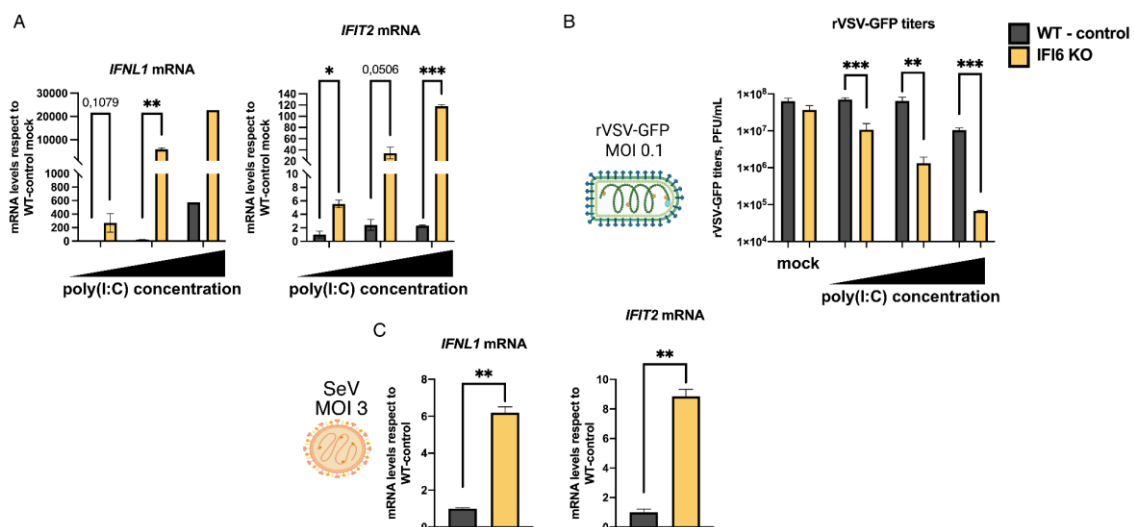


Figure R13. Effect of IFI6 on the induction of innate immune responses after poly(I:C) treatments and SeV infection in HAP-1 cells. (A and B) HAP-1 WT cells and HAP-1 IFI6-KO cells were either mock-transfected or transfected with increasing concentrations of poly(I:C) (0.75, 1.5, and 3 $\mu\text{g}/\text{mL}$). Next, at 24 hpt, (A) total RNA was harvested, purified, and retrotranscribed to cDNA. Alternatively, at 16 hpt,

(B) the cells were infected with rVSV-GFP (MOI 0.1). At 24 hpi, cell culture supernatants from infected cells were employed to measure viral titers in Vero E6 cells by plaque assay (PFU/mL). (C) HAP-1 WT control and HAP-1 IFI6-KO cells were infected with SeV (MOI 3) for 24 hours. Next, total RNA was harvested, purified, and retrotranscribed to cDNA. (A and C) mRNA levels of *IFNL1*, *IFIT2*, and *GAPDH* (as housekeeping expression control) were determined by qRT-PCR. Levels are expressed as fold change (increases) in comparison to WT control cells. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or the exact p value is indicated in some cases (for comparisons using unpaired two-tailed Student's t test in A, B, and C). Three different experiments were performed, with similar results.

To support these findings with a different approach, we tested rVSV-GFP viral titers in HAP-1 cells transfected with an IFI6-siRNA, to knock-down IFI6 expression, compared to control HAP-1 cells transfected with the NT siRNA. First, IFI6 siRNA efficiency was tested in the HAP-1 cell line, to confirm that the mRNA levels of *IFI6* were properly downregulated. We observed an 80% reduction of mRNA levels of *IFI6* in cells transfected with the IFI6 siRNA, compared to cells transfected with the NT siRNA (Fig. R14A). Next, HAP-1 cells were transfected with the IFI6 siRNA or the NT siRNA as a negative control. 24 hpt, cells were treated with poly(I:C) to induce the IFN responses, and 24 hours later, mRNA levels of *IFNL1* were measured to assess antiviral responses. We observed that *IFNL1* was induced ~50-fold after poly(I:C) treatment in the control cells compared to mock-treated cells. This increase was even higher in IFI6-siRNA-transfected cells, where we observed a ~6000-fold induction of *IFNL1* levels (Fig. R14B). In addition, viral titers of rVSV-GFP were measured in IFI6 siRNA and NT siRNA-transfected cells, both infected 16 hours post mock- or poly(I:C)-treatments. Correlating with the previous data of IFN-induced responses in knock-down cells, and with the data in the KO cells, rVSV-GFP titers in poly(I:C)-transfected cells were near 50-fold lower in IFI6 knocked-down cells than in control cells (Fig. R14C). These results strongly support previous data obtained from the titration of rVSV-GFP in IFI6-KO compared to WT cells (Fig. R13B).

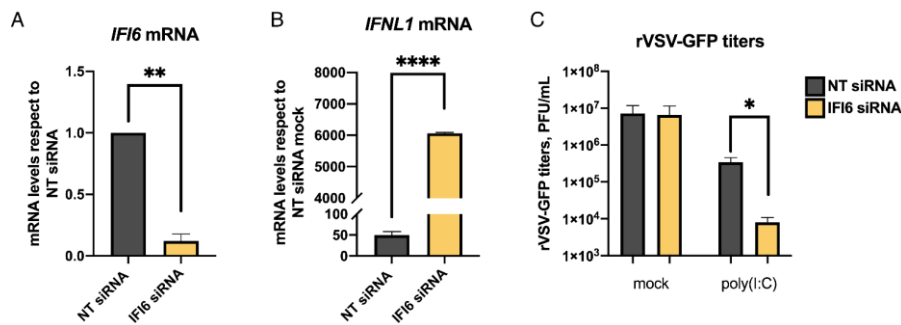


Figure R14. Effect of IFI6 knock-down in antiviral responses in HAP-1 cells. (A-C) HAP-1 cells were transfected with either a NT siRNA, as a negative control, or an IFI6-specific siRNA to knock-down the expression of IFI6. At 24 hpt, cells were treated with poly(I:C) (1.5 μ g/mL) for 24 hours. (A, B) Then, total RNA was harvested, isolated, and retrotranscribed to cDNA. mRNA levels of *IFI6* to confirm knock-down (A), and of *IFNL1* (B) were determined by qRT-PCR (*GAPDH* expression levels were used to normalize expression levels, A, B). mRNA levels are expressed as fold change respect to NT siRNA-transfected cells (A), or NT siRNA-transfected, mock-treated cells (B). (C) Alternatively, 24 hpt with the NT siRNA or the IFI6 siRNA, cells were either mock-transfected or transfected with poly(I:C) (1.5 μ g/mL). At 16 hpi, cells were infected with rVSV-GFP (MOI 0.1), and 24 hpi, supernatants from infected cells were titrated in Vero E6 cells by plaque assay (PFU/mL). Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in A, B, and C). Three different experiments were performed, with similar results.

Given that all previous experiments were performed in tumor-derived cells lines, additional experiments were performed in the non-tumor BEAS-2B cell line, a human bronchial epithelial cell line that is used as a cell system to study infectious diseases from the respiratory tract, for instance RSV [277] or IAV [278]. These cells are able to strongly response to infections and poly(I:C) treatments and produce high amounts of type I and III IFNs [277–279]. Thus, BEAS-2B cells were transfected with either an IFI6-siRNA or a NT siRNA and 24 hpt, cells were infected with SeV (MOI 3). At 24 hpi, mRNA levels of *IFI6*, *IFNL1*, and *IFIT2* were determined. The transfection of cells with the IFI6-specific siRNA led to a 20-fold reduction of *IFI6* mRNA levels, confirming an effective knock-down (Fig. R15A). After SeV-infection of BEAS-2B cells, *IFNL1* and *IFIT2* mRNA levels were highly induced (~6100- and ~110- fold, respectively) respect to mock-infected cells (Fig. R15B). Interestingly, the induction of *IFNL1* and *IFIT2* was 3- and 2-fold higher, respectively, when SeV-infected cells were previously transfected with the IFI6-specific siRNA compared to the control cells transfected with the NT siRNA (Fig. R15C). These results further prove that IFI6 is able to negatively modulate IFN-induced responses, in different cell lines, including non-tumorigenic-derived cells.

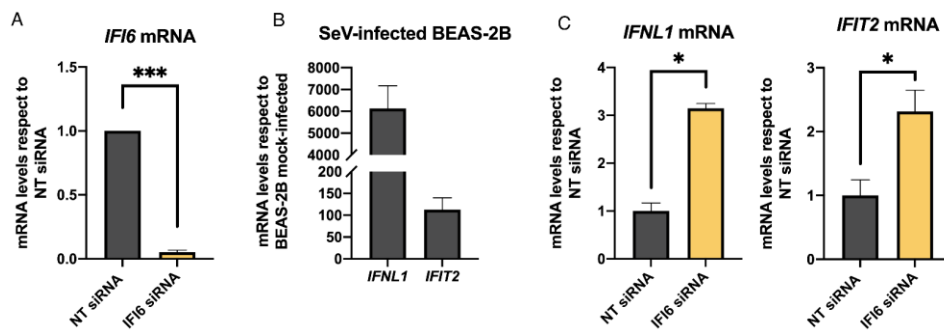


Figure R15. Effect of IFI6 on the modulation of innate immune responses induced in non-tumorigenic epithelial cells. (A–C) BEAS-2B cells were transfected with the IFI6-specific siRNA or the NT siRNA as a control, and 24 hpt, cells were infected with SeV (MOI 3). 24 hpi, total RNA was harvested, purified, and retrotranscribed to cDNA. mRNA levels of *IFI6* (A), *IFNL1*, *IFIT2* (B, C), and *GAPDH* (as housekeeping gene) (A, B, and C), were determined by qRT-PCR. mRNA levels are expressed as fold change (increases) in comparison to NT-siRNA transfected cells, used as controls (A, C). Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in A and C). Three different experiments were performed, with similar results.

All in all, by using different cell lines, we have been able to verify the following general points with respect to IFI27 and IFI6:

- Both genes are strongly induced by IFN- α and poly(I:C) treatments, and by IAV, SARS-CoV-2, and DENV2 infections (Fig. R1).
- Knock-down and KO of IFI27 or IFI6 lead to reduced viral replication of IAV, SARS-CoV-2, and DENV2 (Figs. R2–R4).
- Knock-down and KO of IFI27 or IFI6 lead to increased induction of IFN-response genes as well as proinflammatory cytokines, while the overexpression of IFI27 or IFI6 has the opposite effect (Figs. R5–R15).

4.2.3. IFI27 and IFI6 counteract the induction of IAV-induced innate immune responses *in vivo*

Building upon our previous findings *in vitro*, we tested whether these molecular effects are observed within a more physiological context. While *in vitro* data is highly useful to describe new mechanistic processes and to study the function of genes with loss or gain-of function approaches, the complexity of a living organism create new variables that may lead to inconsistencies between *in vitro* and *in vivo* data [280]. Thus, we sought to validate the previous results in an *in vivo* model, to be able to provide greater importance and translation to our observations. With this objective in mind, we decided to test the effect of human IFI27 or IFI6 protein overexpression in a murine model of IAV infections.

First, to carry out these experiments, we generated recombinant IAVs (rIAVs) encoding human IFI27 and IFI6 in their viral genomes as explained in materials and methods sections 3.5.3 and 3.6. As previously mentioned, IAV genome is formed by eight negative sense, single-stranded, RNA (-ssRNA) viral segments (vRNAs) [47] (Fig. 13). Specifically, we employed the IAV PR8 H1N1 strain, in which we engineered a modification in the 8th segment, named NS segment. To this end, we followed the protocol described by Nogales *et al.* [236]. They generated a replication-competent IAV PR8 expressing the mCherry fluorescent protein. The NS segment (Fig. 13) codifies for two proteins: NS1 and NEP. The viral protein NS1, had previously been shown to tolerate fusion to exogenous proteins such as for instance, GFP [281,282]. However, they decided to use mCherry as a reporter instead of GFP, due to red fluorescent proteins being preferred for *in vivo* studies [283]. After generation of the IAV PR8-mCherry, they assessed the growth properties of the virus in MDCK cells, and they described that albeit a low reduction in the total virus yield respect to the IAV PR8 WT, the kinetics were very similar [236]. NS1 is a multifunctional, non-structural protein of IAV that uses a plethora of mechanisms to counteract type I IFN responses during infections. They also confirmed that NS1 maintains its ability to antagonize IFN- β activation as NS1-WT, so mCherry does not alter the functionality of the protein, validating this method. Finally, they infected mice with IAV PR8-mCherry, confirming that this recombinant virus led to a lethal infection. Also, they detected a dose-dependent mCherry fluorescence within the lungs after infection with the IAV PR8-mCherry, and they demonstrated that the kinetics of PR8 replication in mice lungs are similar to that of PR8 WT virus infection [236].

As the viability of this model (introducing the coding sequence of a protein of interest in the NS segment, achieving its overexpression in all infected cells *in vivo*) had been demonstrated, we investigated the effect of human IFI27 and IFI6 overexpression during *in vivo* infections in mice with IAV PR8. To this end, rIAVs expressing either IFI27 or IFI6 tagged with HA (IAV-IFI27-HA or IAV-IFI6-HA) were generated, plus an IAV expressing mCherry as a negative control (IAV-mCherry) (Fig. R16A). As explained before, IFI27-HA, IFI6-HA, or mCherry ORFs were cloned in plasmids encoding an IAV NS split segment, so that this NS segment codifies for NS1, our protein of interest, and NEP. These proteins are translated individually thanks to being autoproteolytically processed, as the sequences are flanked by the 2A autoproteolytic sites from TAV (between the NS1 ORF and the IFI6/IFI27 ORFs) and from PTV (between the IFI6/IFI27 ORFs and the NEP ORF) (Fig. R16A). First, to demonstrate that the infectious viruses were able to properly produce the exogenous protein, MDCK cells were mock-infected or infected with either the IAV-mCherry control virus, or the IAV-IFI27-HA or IAV-IFI6-HA viruses (MOI 0.1). At 24 hpi, the expression of mCherry, IFI27-HA, and IFI6-HA was analyzed by detection of red fluorescence for mCherry, and by immunofluorescence against HA to detect IFI27 and IFI6,

respectively. Additionally, the expression of IAV NP protein was assessed by immunofluorescence, to show that infected cells (positive NP staining) also had a positive staining for either mCherry or HA (to detect IFI27-HA or IFI6-HA) (Fig. R16B).

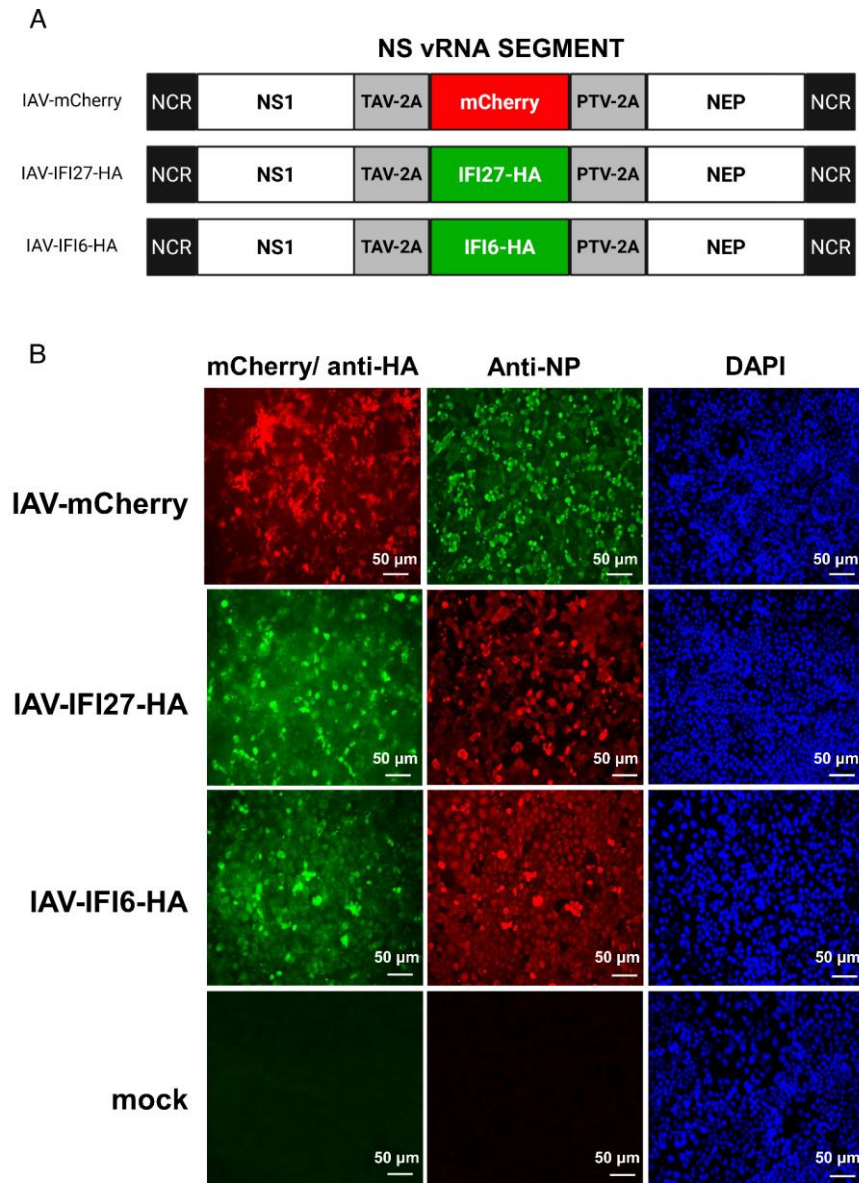


Figure R16. Generation of rIAV-PR8 expressing IFI27, IFI6 or mCherry proteins. (A) Schematic representation of modified IAV-PR8 NS segments generated to express an exogenous protein, either mCherry, as a control virus (top), IFI27-HA (middle), or IFI6-HA (bottom). The codified proteins in each NS segment are NS1 and NEP (white boxes) and the exogenous protein (mCherry in red and IFI27-HA or IFI6-HA in green). Black boxes flanking each segment represent non-coding regions (NCR) at the viral RNA 3' and 5' ends. Grey boxes represent the *Thosia asigna* virus 2A autoproteolytic cleavage site (TAV-2A) used for the proper individual expression of NS1 and mCherry/IFI27/IFI6, and the porcine teschovirus 2A autoproteolytic cleavage site (PTV-2A) used for the proper individual expression of mCherry/IFI27/IFI6 and NEP. **(B)** Confirmation of exogenous protein expression after viral infection. MDCK cells were either mock-infected or infected with the different recombinant viruses: IAV-mCherry, IAV-IFI27-HA, or IAV-IFI6-HA (MOI 0.1). At 24 hpi, infected cells were fixed, and permeabilized. First, mCherry expression was detected due to mCherry protein fluorescence (showed in red). Next, to detect IFI27-HA and IFI6-HA expression, and to analyze infection, cells were stained with anti-HA (showed in green) and anti-NP (to stain NP viral protein, showed in green for IAV-mCherry and in red for IAV-

IFI27/IFI6-HA). DAPI was used to stain nuclei, showed in blue. Representative images (20× magnification) are included. Scale bar, 50 μ m.

Once we confirmed that the recombinant viruses were infectious in MDCK cells and efficiently expressed the exogenous protein of interest (Fig. R16), we proceeded with *in vivo* infection experiments to investigate the potential effect of IFI27 or IFI6 overexpression in the lungs of mice infected with IAV. To this end, groups of C57BL/6 mice (n=4 per group) were infected intranasally with 2,000 FFU of the recombinant viruses (IAV-mCherry as a control, IAV-IFI27-HA, and IAV-IFI6-HA) (Fig. R17A). At 24 and 48 hpi, viral titers in mice lungs were determined (Fig. R17B). Conversely and correlating with the data in the figure R4C, at 24 hpi, viral titers were slightly increased in mice infected with the IFI27-expressing virus compared to mice infected with the control IAV-mCherry virus (Fig. R17B). However, at 48 hpi, we detected no differences in viral titers, indicating that in these conditions, IFI27 may only influence replication at early stages of the infection (Fig. R17B). For IFI6-expressing virus, we did not detect any significant differences in viral replication at 24 or 48 hpi compared to the IAV-mCherry control virus (Fig. R17B). So, even though *in vitro* both IFI27 and IFI6 had a similar effect, as the KO of these proteins led to a lower IAV replication (Figs. R4C and R4H, respectively), *in vivo*, IFI6 overexpression did not affect viral replication to a sufficient degree at the time points analyzed. Moreover, at 24 and 48 hpi, we assessed the mRNA levels of *Ifnl3*, *Ifit2* (an IFN and ISG, respectively), and *Ccl2* and *Tnf* (proinflammatory cytokines) in the lungs of infected mice, in order to study the possible effect of IFI27 or IFI6 overexpression on the induction of the innate immune responses after IAV infection (Fig. R17C). At 24 hpi, we detected little to no induction of *Ifit2*, *Ccl2*, or *Tnf* expression compared to mock-infected mice. Only *Ifnl3* showed a limited induction at this time point following infection with the IAV-mCherry control virus (2-fold), whereas in the mice infected with the IFI27 and IFI6-expressing viruses, *Ifnl3* mRNA levels were not upregulated compared to mock-infected mice, showing that IFI27 or IFI6 overexpression limited IFN induction (Fig. R17C). In contrast, at 48 hpi, the mRNA levels of *Ifnl3*, *Ifit2*, *Ccl2*, and *Tnf* were highly upregulated in the lungs of infected mice (Fig. R17C). Interestingly, at 48 hpi, the mRNA levels of *Ifnl3*, *Ifit2*, *Ccl2*, and *Tnf* were decreased after the infection with the IFI27 and IFI6-expressing viruses compared to the levels after the infection with the IAV-mCherry control virus (Fig. R17C). These results correlate with the previous data showing that IFI27 and IFI6 overexpression led to a lower induction of antiviral responses after SeV infections (Figs. R10C, R10D) and correlate with all the data obtained using the knock-down cells and the KO cells for IFI27 and IFI6. All in all, *in vivo* assays further corroborate that IFI27 and IFI6 are able to negatively modulate the induction of innate immune responses *in vitro* and *in vivo*. Furthermore, it validates the generation of rIAV expressing human proteins to study the function of these proteins *in vivo* within the context of a viral infection.

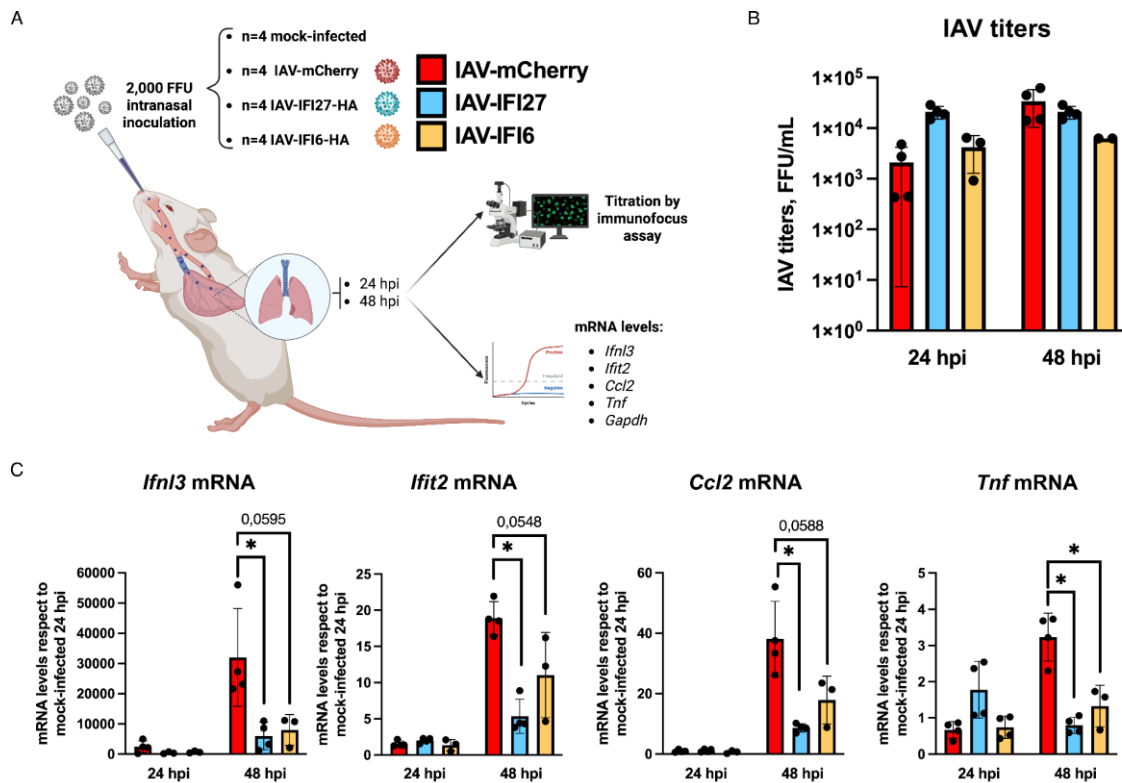


Figure R17. IFI27 or IFI6 expression negatively modulates the induction of innate immune responses after IAV infection in mice. (A) Experimental timeline. C57BL/6 mice (n=4 for each group) were either mock-infected or infected intranasally with 2,000 FFU of rIAV-PR8 (IAV-mCherry, IAV-IFI27-HA, or IAV-IFI6-HA). (B) On the one hand, mice were sacrificed at 24 and 48 hpi and the right lung lobules were extracted, homogenized, and employed to determine viral titers by immunofocus assay in MDCK cells, represented as FFU/mL. (C) On the other hand, at 24 and 48 hpi, left lung lobules of sacrificed mice were extracted and processed to extract total RNA, which was isolated and retrotranscribed to cDNA. mRNA levels of murine *Ifnl3*, *Ifit2*, *Ccl2*, *Tnf*, and *Gapdh* (to normalize the mRNA levels) were determined. mRNA levels were compared respect to mock-infected mice. Individual data points correspond to independent measures from different mice in each group. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or the exact p value in some cases (for comparisons using unpaired two-tailed Student's t test in B and C).

PART III: MOLECULAR MECHANISMS UNDERLYING THE MODULATION OF INNATE IMMUNE RESPONSES BY IFI27 AND IFI6

Previous sections show that both *IFI27* and *IFI6* are ISGs, that are able to negatively modulate the innate immune responses. Several ISGs, such as *ISG15*, *IFI44*, and *IFI44L*, among others, also have a similar effect on antiviral responses, and the molecular mechanisms of some of these ISGs have been already described [126,127,284]. Thus, our next objective was to elucidate the potential molecular mechanisms through which IFI27 and IFI6 mediate a negative regulation of antiviral responses. Both proteins were or will be studied individually in order to obtain clearer conclusions regarding the function and molecular mechanism of both.

4.3.1 Elucidating the molecular mechanisms of IFI27 and IFI6 on innate immunity modulation

4.3.1.1. IFI27 and IFI6 are RNA-binding proteins (RBPs)

One of the main inducers of innate immune responses after the infections with RNA viruses is the recognition of viral RNA by the PRRs, such as the cytosolic PRRs RIG-I and MDA5, which are able to distinguish between self- and viral-nucleic acids [80,285]. After RNA recognition, PRRs change their conformation and activate MAVS protein, which is a downstream member of the signaling pathway. After several steps, this pathway leads to the production of inflammatory cytokines and IFNs, and these IFNs will induce the expression of ISGs in an autocrine and paracrine manner [98,118]. In previous sections, we have described that IFI27 and IFI6 negatively modulate the induction of IFNs, ISGs, and proinflammatory cytokines both *in vitro* (Figs. R5-R15) and *in vivo* (Fig. R17).

To begin exploring the molecular mechanisms mediating IFI27 and IFI6 functions, we performed a bioinformatic prediction to test if these proteins could bind RNAs, which could explain whether IFI27 and IFI6 could interfere with viral RNA recognition by PRRs. To this end, we used RNABindRPlus [247], a predictor that combines machine learning and sequence homology-based methods to improve predictions of RNA-binding residues in proteins. This method assigns values to each amino acid from 0 (lowest probability) to 1 (highest probability), being 0.5 the minimum to consider that a residue could bind RNA. On the one hand, according to RNABindRPlus, the 122-amino acid length IFI27 isoform encodes 13 amino acids which are predicted to bind RNA, based on score predictions higher than 0.5, as follows: amino acid 60 (0.68), 61 (0.61), 62 (0.60), 63 (0.53), 64 (0.70), 65 (0.69), 68 (0.64), 69 (0.65), 82 (0.53), 83 (0.58), 84 (0.60), 85 (0.61), and 86 (0.62). In contrast, 90% of the remaining residues had predicted scores under 0.1, and none exceeded 0.41. On the other hand, according to RNABindRPlus, the 138-amino acid length IFI6 isoform has 11 amino acids which are predicted to bind RNA, based on score predictions higher than 0.5, as follows: amino acid 73 (0.65), 74 (0.57), 75 (0.57), 76 (0.52), 77 (0.69), 78 (0.68), 81 (0.64), 82 (0.68), 97 (0.64), 98 (0.60), 99 (0.53). Interestingly, 80% of the other residues had predicted scores lower than 0.1.

We decided to experimentally test these predictions by assessing the binding of IFI27 or IFI6 to poly(I:C). To this end, HEK-293T cells were transiently transfected with either the pCAGGS-IFI27-HA or the pCAGGS-IFI6-HA plasmids, pCAGGS plasmids expressing GFP (as a control of a protein which does not bind RNA) or FLAG-tagged PACT, a dsRBP, that was used as a positive control of RNA binding [140], or an empty plasmid, as a negative control of transfection (Fig. R18A). At 24 hpt, cellular lysates were obtained and incubated with agarose beads that had been previously conjugated to poly(I:C), as an analog of dsRNA, or to poly(C), as a negative

control (Fig. R18A). Interestingly, IFI27 (Fig. R18B) and IFI6 (Fig. R18C) bound to the poly(I:C)-conjugated agarose beads, but not to the control poly(C)-conjugated agarose beads (data not shown). As we could expect, GFP bound neither to poly(I:C)- nor poly(C)-conjugated beads, whereas PACT bound poly(I:C)- but not poly(C)-conjugated beads, validating the experiment results (Fig. R18B, R18C, and data not shown for poly(C)-control), and suggesting that both IFI27 and IFI6 bind poly(I:C).

To support this ability of IFI27 to bind to dsRNA by a different approach, HEK-293T cells were transfected with either the pCAGGS-IFI27-HA plasmid, or an empty pCAGGS plasmid as a control, and 24 hpt, cells were transfected with poly(I:C) conjugated or not conjugated (as a negative control of the assay) to biotin. At 24 hpt with poly(I:C), cellular extracts were harvested and incubated with streptavidin-conjugated beads (Fig. R18D). Biotin-streptavidin is one of the strongest non-covalent bonds known (association rate constant, $K_a \approx 10^{13} \text{ M}^{-1}$) which has led to its use for many different applications [286]. We could not detect the binding of IFI27 to streptavidin-conjugated agarose beads when cells had been transfected with non-biotinylated poly(I:C) (data not shown). However, IFI27 bound to streptavidin-conjugated agarose beads when cells had been transfected with biotinylated-poly(I:C), further showing that IFI27 binds to poly(I:C) (Fig. R18E), and most likely to dsRNAs. Additionally, as an alternative method to further confirm the interaction between IFI6 and poly(I:C), instead of using biotinylated-poly(I:C) such as we did for IFI27 (Fig. R18E), for this experiment, we used a recombinant IFI6 protein fused to GST and 6xHisidine (6xHis) tags, which was expressed in *E. coli* and purified using a Ni column, commercially available. This purified IFI6 protein was incubated with the poly(I:C)-conjugated agarose beads, or the poly(C)-conjugated agarose beads, as control. Interestingly, purified IFI6 only co-immunoprecipitated with the poly(I:C) beads, further confirming that IFI6 interacts with poly(I:C), and most likely with dsRNAs (Fig. R18F). This finding demonstrates that the interaction is not mediated by other cellular proteins, as IFI6 was purified in this experiment.

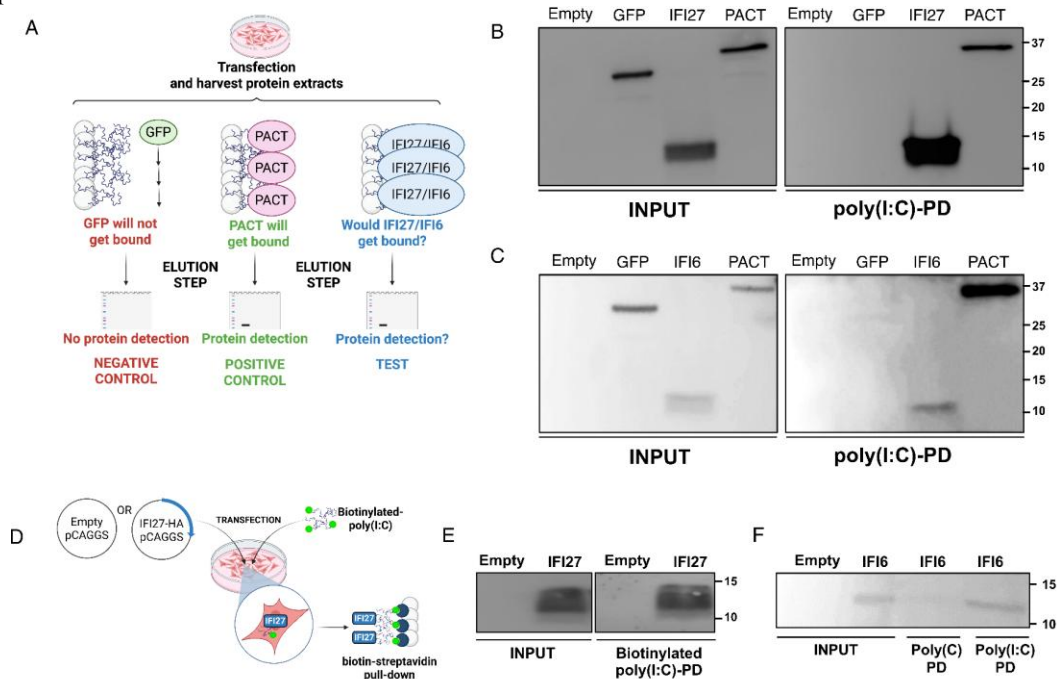


Figure R18. IFI27 and IFI6 bind to poly(I:C). (A) HEK-293T cells were transfected with the pCAGGS-GFP plasmid as a negative control of dsRNA binding; pCAGGS-PACT-FLAG plasmid as a positive control of dsRNA binding; pCAGGS-IFI27-HA plasmid (B) or pCAGGS-IFI6-HA (C); and an empty-pCAGGS

plasmid as a negative control. **(B and C)** At 24 hpt, cellular extracts were incubated with poly(I:C)-conjugated agarose beads or poly(C)-conjugated agarose beads (data not shown). A pull-down (PD) was performed and eluted proteins were analyzed by Western blot. Western blot was performed to detect GFP (using an anti-GFP antibody), PACT (using an anti-FLAG antibody), and IFI27 or IFI6 (using an anti-HA antibody), both in the cellular lysates (Input) and after the pull-down with poly(I:C)-agarose beads (Poly(I:C)-PD). Molecular weight markers are indicated on the right of the blot (in kDa). **(D and E)** As an alternative, HEK-293T cells were transiently transfected with an empty-pCAGGS plasmid or the pCAGGS-IFI27-HA plasmid, and 24 hpt, cells were transfected with either biotin-conjugated poly(I:C) or with unconjugated poly(I:C), as a control (data not shown). At 24 hpt, cellular extracts were harvested and pulled-down with streptavidin-conjugated agarose beads. **(E)** Eluates were analyzed by Western blot, using the specific antibody to detect IFI27 (anti-HA) both in the cellular lysates prior to the pull-down (Input), and in the eluates of the pull-down (Biotynilated Poly(I:C)-PD). **(F)** As an alternative approach for analyzing the binding of IFI6 to poly(I:C), a purified, recombinant IFI6 protein with N-terminal GST and C-terminal 6xHis tag was incubated with poly(I:C)-conjugated agarose beads or poly(C)-conjugated agarose beads, as control. A Western blot was performed to detect purified recombinant IFI6 both in the Input and after the pull-down with either poly(C)- or poly(I:C)-conjugated agarose beads by using the anti-GST antibody. Molecular weights are indicated on the right of the blot (in kDa). Three different experiments were performed, with similar results.

Once we confirmed that IFI27 and IFI6 are RBPs, we performed more detailed studies of targeted mutagenesis taking into account the amino acid residues that RNABindRPlus predicted to bind RNA. In order to validate the information of the specific residues that could bind RNA, we decided to generate two IFI27 variants, each one encoding only one point mutation in one of the specific residues that RNABindRPlus predicted as RNA-binding amino acids: S63L (modification of Serine to Leucine in the amino acid in position 63) and V82A (modification of Valine to Alanine in the amino acid in position 82). Once we generated these variants, we tested their ability to bind to poly(I:C). To this end, HEK-293T cells were transfected with the pCAGGS plasmids expressing IFI27-WT, IFI27-S63L, and IFI27-V82A variants fused to HA tags; with a pCAGGS plasmid expressing GFP, as a negative control; or with the plasmid expressing PACT fused to a FLAG tag (pCAGGS-PACT-FLAG), as a positive control of a dsRBP [140]. Cellular lysates were harvested and tested for proper protein expression (Fig. R19A, Input), and then, lysates were incubated with either poly(I:C)-conjugated agarose beads, or poly(C)-conjugated agarose beads (negative control, data not shown). As expected (Fig. R18B and data not shown), IFI27-WT only bound to poly(I:C)-conjugated agarose beads but not to the poly(C)-conjugated agarose beads, used as control, GFP bound neither poly(I:C)- nor poly(C)-conjugated beads, and PACT bound poly(I:C)- but not poly(C)-conjugated beads (Fig. R19B and data not shown), so the assay worked properly. Strikingly, the binding to poly(I:C) of variants S63L and V82A of IFI27 was heavily impaired or abolished, respectively (Fig. R19B, Poly(I:C)-PD). This result strongly indicates that these two residues are essential for RNA binding, as suggested by RNABindRPlus, and a mutation in just one of them, severely impair their ability to interact with poly(I:C). Therefore, these RNABindRPlus predictions were confirmed experimentally. In addition, these results indicate that the binding of IFI27-HA to poly(I:C) is not mediated through the HA tag.

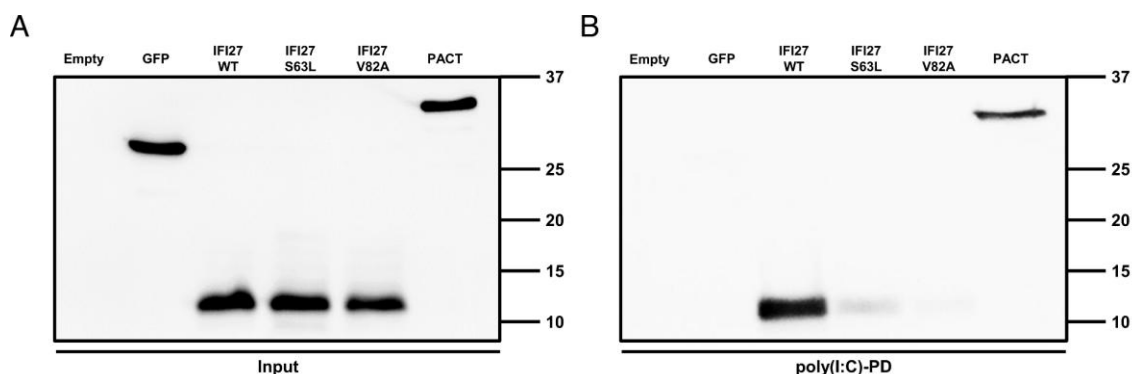


Figure R19. Binding of IFI27-WT and IFI27 variants S63L and V82A to poly(I:C). HEK-293T cells were transiently transfected with the pCAGGS-GFP plasmid as a negative control of dsRNA binding; pCAGGS-PACT-FLAG plasmid as a positive control of dsRNA binding; pCAGGS-IFI27-HA or one of the two IFI27 variants, pCAGGS-IFI27-S63L or pCAGGS-IFI27-V82A; or an empty pCAGGS plasmid, as a negative control. At 24 hpt, cellular extracts were harvested and the proper expression of the different codified proteins was analyzed by Western blot (Input, **A**). (**B**) Cellular extracts were subjected to a pull-down with poly(I:C)-conjugated agarose beads (Poly(I:C)-PD). (**A and B**) Inputs (**A**) and eluates of poly(I:C)-bound proteins (**B**) were analyzed by Western blot using the specific antibodies for GFP, FLAG (to detect PACT) or HA (to detect the different variants of IFI27). Molecular weights are indicated on the right of the blots (in kDa). Three different experiments were performed, with similar results.

4.3.1.2. IFI27 and IFI6 interact with RIG-I-like receptors and negatively modulate their activation

For the first time, we have described that IFI27 and IFI6 are RBPs, a common characteristic to many cellular factors that respond to viral infections by modulating the function of PRRs. RBPs are one of the major regulators of innate immune responses, as they are able to regulate every stage of the RNA lifecycle, including RNA splicing, mRNA synthesis, and mRNA maturation, which are essential steps in the life cycle of viruses [285]. Furthermore, emerging research highlights the crucial role of RBPs in the modulation of host antiviral responses by contributing to different processes such as the recognition of viral RNA, or by regulating the activation of PRRs such as the functional members of RLR family, RIG-I and MDA5 [285]. PRRs play the main role in the transmission of antiviral signals and subsequently, in the production of IFNs, ISGs and proinflammatory cytokines. RIG-I and MDA5 have been shown to recognize and respond to both poly(I:C) and to infections with SARS-CoV-2, IAV and SeV [83–85,88,91,92,96,97,274,287–289]. In our previous results, IFI27 and IFI6 have shown a negative effect on the induction of antiviral responses against all these stimuli that RIG-I and MDA5 are able to respond to. Knowing this, and that both proteins are RBPs, we hypothesized that IFI27 and IFI6 could be negative modulators of RIG-I and MDA5 activation and function. First, we assessed the ability of IFI27 and IFI6 to bind to RIG-I, and then, we also tested the ability of IFI27 to bind and modulate MDA5, the other functional RLR. As RIG-I and MDA5 structure and function are highly similar, studying both proteins could provide deeper insights into the possible modulation of RLRs family by IFI27 and IFI6.

4.3.1.2.1. IFI27 and IFI6 interact with RIG-I through RNA binding

To test the hypothesis that IFI27 and IFI6 could modulate RIG-I function, we started by studying a possible interaction between these proteins through their common ability to bind to RNAs. To this end, first, HEK-293T cells were transfected with different combinations of the pCAGGS-IFI27-HA plasmid or alternatively the pCAGGS-IFI6-HA plasmid, a pCAGGS plasmid expressing RIG-I tagged with FLAG (pCAGGS-RIG-I-FLAG) and an empty pCAGGS plasmid,

when needed, to keep the amount of plasmid constant in every sample (Fig. R20A). At 24 hpt, cells were transfected with poly(I:C) (Fig. R20B, R20D) or infected with SeV (Fig. R20C, R20E) as exogenous RNAs stimuli for an additional 24 hours. Subsequently, cell extracts were harvested, and the interaction between IFI27 or IFI6 and RIG-I was analyzed under poly(I:C) or SeV conditions, by performing a co-IP with an anti-FLAG antibody. Interestingly, after the co-IP, IFI27-HA or IFI6-HA were detected when RIG-I was overexpressed, and IFI27-HA or IFI6-HA were not detected when RIG-I was not overexpressed (Figs. R20B-E), suggesting a direct or indirect interaction between RIG-I and IFI27 and IFI6 proteins (Fig. R18) and RIG-I [287] bind to poly(I:C), we repeated the same co-IP, but now, cellular extracts were previously treated with ssRNases (RNase T1 and RNase A) and/or dsRNases (RNase III), in order to determine the possible importance of RNA in this interaction. While the amount of IFI27-HA (Figs. R20B, R20C), IFI6-HA (Figs. R20D, R20E), and RIG-I-FLAG (Figs. R20B-R20E) remained the same with or without the RNase treatment (Input), the amount of IFI27 or IFI6 that co-immunoprecipitated together with RIG-I was strongly decreased by the treatment with both ssRNases and dsRNases individually, or in combination (Figs. R20B-R20E), highlighting the importance of RNAs in the interaction between IFI27 and IFI6 proteins and RIG-I, both after poly(I:C) treatments and after SeV infection. Interestingly, these results strongly suggest that apart from synthetic dsRNA such as poly(I:C), IFI27 and IFI6 are also able to bind to SeV viral RNAs, in the context of viral infections.

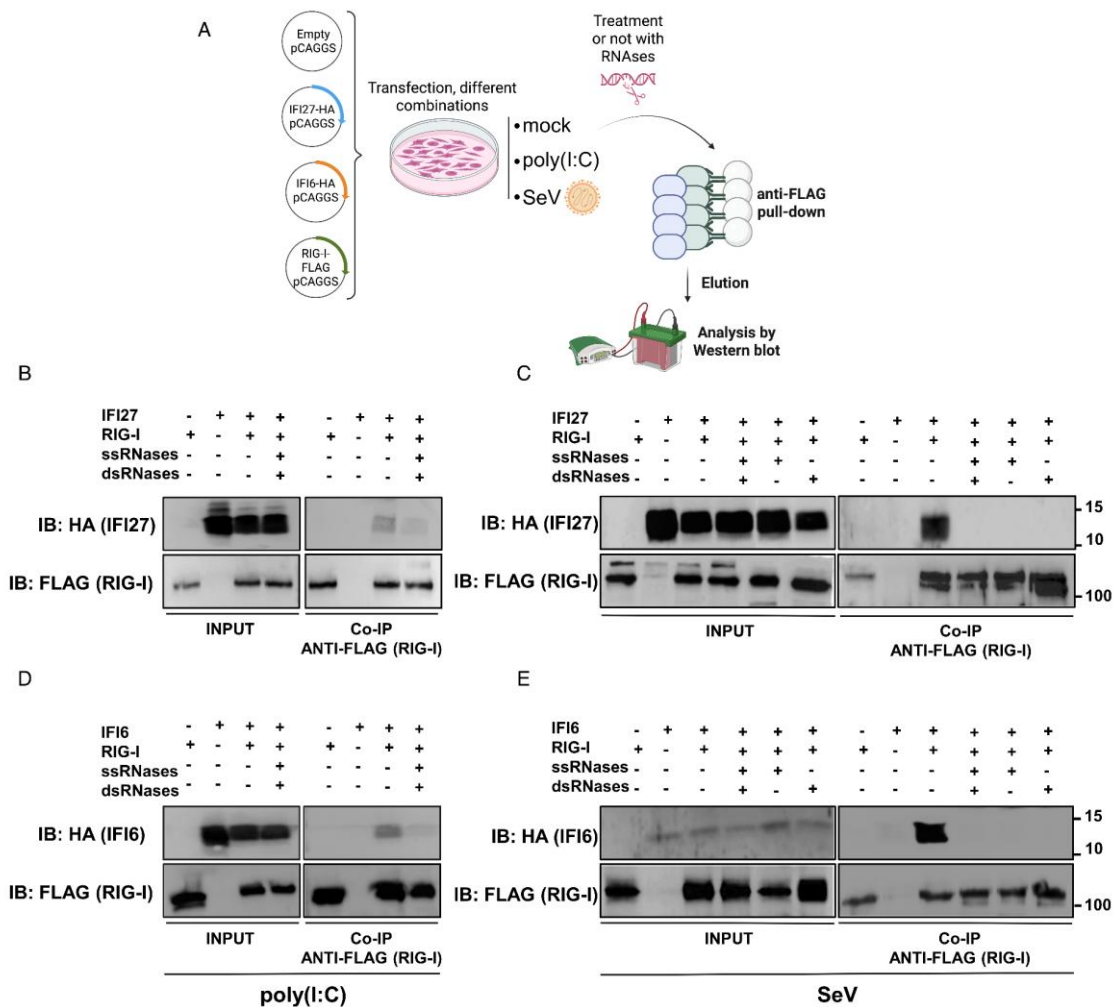


Figure R20. IFI27 and IFI6 interact with RIG-I through RNA binding. (A) Schema of the experimental procedure. (B-E) HEK-293T cells were transiently co-transfected with the pCAGGS plasmids encoding

IFI27-HA or IFI6-HA, and RIG-I-FLAG. To maintain a stable amount of DNA, we transfected cells also with an empty pCAGGS plasmid. 24 hpt, cells were either poly(I:C)-treated (3 µg/mL) (**B**, **D**), or SeV-infected (MOI 3) (**C**, **E**) for 24 hours. Next, lysates were treated or not with ssRNases and/or dsRNases and co-immunoprecipitated with anti-FLAG-conjugated agarose beads (**B-E**). The proper expression of the codified proteins in cellular lysates was confirmed by Western blot (**B-E**, Input). Co-IP of IFI27 or IFI6 with RIG-I under poly(I:C) conditions (**B**, **D**) and SeV-infection conditions (**C**, **E**) were analyzed by Western blot, using the specific antibodies for FLAG (to detect RIG-I), or HA (to detect IFI27 and IFI6) IB, immunoblot. Molecular weight markers are indicated on the right of the blots (in kDa). Two different experiments were performed, with similar results.

4.3.1.2.2. IFI27 and IFI6 partially co-localize with RIG-I in the cell cytoplasm

To support the interactions between IFI27 or IFI6 and RIG-I, we carried out intracellular colocalization studies by immunofluorescence and confocal microscopy. To this end, HEK-293T cells were transiently transfected with the pCAGGS-IFI27-HA plasmid (Figs. R21A, R21B) or pCAGGS-IFI6-HA plasmid (Figs. R21C, R21D), together with the pCAGGS-RIG-I-FLAG plasmid (Figs. R21A-R21D), or the empty control plasmid as a negative control of transfection. Next, cells were either mock- (Figs. R21A, R21C) or poly(I:C)- (Figs. R21B, R21D) transfected, and 24 hpt, the cells were fixed and permeabilized. IFI27-HA, IFI6-HA, or RIG-I-FLAG signals were not detected in non-transfected cells, as control (data not shown). To quantify colocalization, we calculated the “pearson coefficient”, a well-established measure of correlation, which applied to colocalization, consisting of estimating the degree of overlap between two fluorophores. If there is a total positive correlation between both fluorophores, the given value will be 1, and if there is a total negative correlation, the given value will be -1. If there is no correlation, the value will be near to 0 [243,244]. We observed a partial colocalization between RIG-I and IFI27 in the cell cytoplasm. In mock-conditions, the Pearson coefficient value was 0.677, while in poly(I:C)-transfected cells, this value was incremented to 0.828 (Fig. R21A, R21B, respectively). This reinforces the data obtained in the co-IP (Figs. R20B, R20C), which show how RNA favors the interaction between the two proteins. Similarly, IFI6 and RIG-I partially colocalized in distal regions of the cell cytoplasm. Under mock-conditions, we measured a Pearson coefficient of 0.504 (Fig. R21C), while this value was incremented to 0.609 after poly(I:C) treatment (Fig. R21D). Thus, poly(I:C) treatment resulted in a modest increase in the colocalization of IFI6 and RIG-I, consistent with previous findings indicating that the interaction between these two proteins is mediated by RNAs (Figs. R20D, R20E).

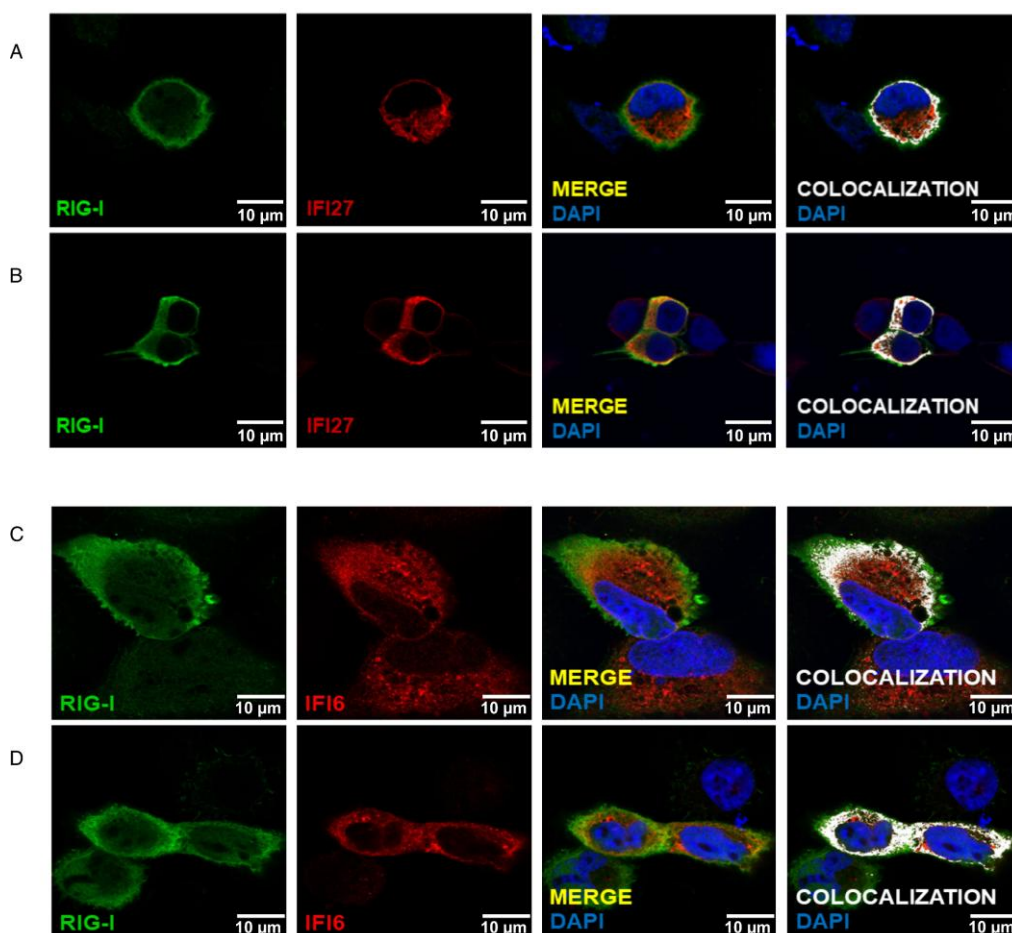


Figure R21. IFI27 and IFI6 partially colocalize with RIG-I in the cell cytoplasm. HEK-293T were transiently co-transfected with the pCAGGS plasmid encoding RIG-I-FLAG and IFI27-HA (**A, B**), or RIG-I-FLAG and IFI6-HA (**C, D**). At 24 hpt cells were either mock- (**A, C**) or poly(I:C)-treated (3 $\mu\text{g}/\text{mL}$) (**B, D**). 24 hpt, cells were fixed with formaldehyde and permeabilized. RIG-I-FLAG, IFI27-HA, and IFI6-HA were stained by incubation with the anti-FLAG (RIG-I, **A-D**) or anti-HA (IFI27 (**A, B**) and IFI6 (**C, D**)) antibodies, and the appropriate secondary antibodies conjugated to fluorophores (Alexa-488, green, for RIG-I-FLAG, and Alexa-594, red, for IFI27-HA and IFI6-HA). Nuclei were stained with DAPI. Areas of co-localization between both proteins appear in yellow in the third picture, and in white in the fourth. Scale bar, 10 μm . Three different experiments were performed, with similar results. For this figure, we show one representative image of the total of cells analyzed.

4.3.1.2.3. IFI27 and IFI6 negatively affect RIG-I activation

After the confirmation that due to their RNA binding capacity, both IFI27 and IFI6 are able to interact with RIG-I (Figs. R20-R21), we wanted to describe the possible functional significance of these interactions. We sought to determine if the negative effect that both proteins had on the induction of antiviral responses could be attributed to a negative modulation of RIG-I function. This would explain why IFI27 and IFI6 positively affect viral replication (section 4.1.2) and negatively affect innate immune responses both *in vitro* and *in vivo* (sections 4.2.1-4.2.3).

In order to test if the interactions of IFI27 and IFI6 with RIG-I lead to a negative effect on the induction of innate immune responses mediated by RIG-I, HEK-293T cells were transiently co-transfected with the RIG-I-FLAG coding plasmid or with an empty plasmid, in the presence or absence of either IFI27-HA or IFI6-HA coding plasmids (Fig. R22A, R22B, R22D, R22E). IFI27-HA (Fig. R22A), IFI6-HA (Fig. R22D), and RIG-I-FLAG (Fig. R22A, R22D) expression was

confirmed by Western blot by using anti-HA and anti-FLAG antibodies. At 24 hpt, cells were either mock- or SeV-infected for 24 hours, and then, total RNA was harvested and mRNA levels of *IFNL1* were determined, representative of the induction of innate immune responses (Fig. R22B, R22E). We observed that RIG-I overexpression, even in the absence of infection, strongly induced the expression of *IFNL1* (near to 20,000-fold) compared to mock-infected cells transfected with the empty plasmid (Fig. R22B, R22E), data which correlates with previously published studies [290]. *IFNL1* was further induced to near 80,000-fold when RIG-I was overexpressed and cells were infected with SeV (Fig. R22B, R22E). Notably, overexpression of IFI27 or IFI6 results in a strong reduction of mRNA levels of *IFNL1*, both in empty and RIG-I transfected cells infected with SeV. Thus, IFI27 and IFI6 present a similar effect on RIG-I activation and seem to be effective negative regulators of RIG-I-induced antiviral responses (Fig. R22B, R22E).

To corroborate these results, we took the opposite approach, by transfecting HEK-293T cells with either two different IFI27-siRNAs (Fig. R22C), or two different IFI6-siRNAs (Fig. R22F), or with the NT siRNA as a control, in order to analyze the effect of IFI27 or IFI6 knock-down instead of overexpression. At 24 hpt, cells were transfected again with the RIG-I-FLAG expressing plasmid or an empty plasmid as a control, and 24 hours later, cells were either mock- or SeV-infected. Next, at 24 hpi, total RNA was purified from the cells and mRNA levels of *IFNL1* were measured (Fig. R22C, R22F). Correlating with the overexpression results (Fig. R22B, R22E), IFI27 or IFI6 knock-down led to higher mRNA levels of *IFNL1* after RIG-I overexpression than in the control cells transfected with the NT siRNA, both in infected and uninfected cells (Fig. R22C, R22F). These findings further support that IFI27 and IFI6 negatively modulate RIG-I activity.

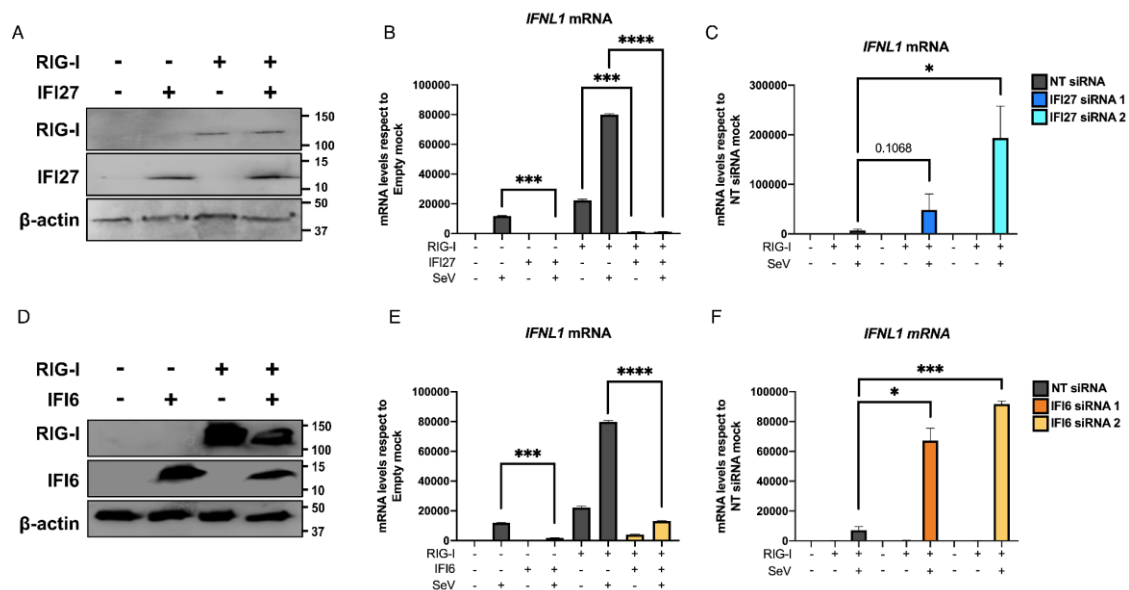


Figure R22. IFI27 and IFI6 negatively modulate RIG-I activation. HEK-293T cells were co-transfected with IFI27-HA (A, B) or IFI6-HA (D, E) together with a RIG-I-FLAG-expressing pCAGGS plasmid, or with the empty pCAGGS plasmid (A, B, D, E). First, proper protein expression of IFI27-HA and RIG-I-FLAG (A) or IFI6-HA and RIG-I-FLAG (D) was analyzed by Western blot by using the specific antibodies anti-FLAG (RIG-I-FLAG), anti-HA (IFI27-HA and IFI6-HA), and anti-β-actin, as a loading control. Molecular weight markers are indicated on the right of the blots (in kDa) (A, D). (C, F) As an alternative, HEK-293T cells were transfected with either two different IFI27-specific siRNAs (C) or two different IFI6-specific siRNAs (F) or with the NT siRNA as a negative control (C, F), and then, cells were transfected with a pCAGGS plasmid encoding RIG-I-FLAG or an empty-pCAGGS plasmid as control. (B, C, E, F) At

24 hpt, cells were either mock- or SeV-infected (MOI 3) for 24 hours. Next, total RNAs were harvested and the mRNA levels of *IFNL1* and *GAPDH* (as a housekeeping gene) were measured by qRT-PCR. mRNA levels are expressed as fold of change respect to mock-infected cells transfected with the empty plasmid (**B, E**) or to mock-infected cells transfected with the NT siRNA (**C, F**). Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or the exact p value (for comparisons using unpaired two-tailed Student's t test in **B, C, E, and F**). Three different experiments were performed, with similar results.

4.3.1.2.3.1 IFI27 negatively affects RIG-I ability to interact with viral RNAs

Given that the IFI27 V82A variant lost its RNA-binding ability (Fig. R19), we determined whether this loss impairs its capacity to negatively modulate the innate immune responses. To this end, HEK-293T cells were transiently transfected with pCAGGS plasmid expressing IFI27-WT, IFI27-V82A, or an empty pCAGGS plasmid as a negative control. At 24 hpt, cells were infected with SeV (MOI 3), and 24 hpi, total RNA and protein extracts were harvested from the cells. Protein extracts were used to confirm the proper and comparable expression of both IFI27-WT and IFI27-V82A in the cellular extracts by Western blot (Fig. R23A). Next, we measured the mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10*, in order to assess the induction of antiviral responses. As expected, IFI27-WT overexpression led to an 80-, 50- and 740-fold reduction of mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10*, respectively, compared to cells transfected with the empty plasmid (Fig. R23B). However, after IFI27-V82 overexpression, mRNA levels of *IFNL1* did not change significantly respect to the cells transfected with the empty plasmid (Fig. R23B), and the reduction of *IFIT2* and *CXCL10* induction after IFI27-V82A overexpression was much lower than in the cells overexpressing the IFI27-WT protein (4- and 5- fold only, compared to a 50- and 740-fold reduction for the IFI27-WT protein (Fig. R23B)). This result suggests that the loss of the ability of IFI27-V82A to bind RNA (Fig. R19), and presumably RIG-I, leads to a significant loss of the ability of IFI27 to negatively modulate the induction of innate immune responses.

A previous study of Yamada *et al.* [85] described that RIG-I protein is a RBP which recognizes and binds the 3'UTR region of SARS-CoV-2 RNA genome. As IFI27 is also a RBP (Figs. R18B, R18E), we hypothesized that IFI27 could compete or alter the ability of RIG-I to recognize and bind to viral RNAs, which would explain the effect that IFI27 has on negatively modulating RIG-I activity, and therefore, the antiviral responses. Thus, we assessed if IFI27 could alter the capacity of RIG-I to recognize and bind to SARS-CoV-2 genomic RNA. As we have observed that, due to its impaired RNA-binding (Fig. R19), the IFI27-V82A variant has a reduced capacity of negatively modulating antiviral responses compared to IFI27-WT (Fig. R23B), we also included V82A in this assay. To do this, we followed a previously described methodology by Gagliardi and Matarazzo [249]. First, HEK-293T-hACE2 cells were transfected with either the pCAGGS-RIG-I-FLAG plasmid together with the pCAGGS-IFI27-HA plasmid, the pCAGGS-RIG-I-FLAG plasmid together with the pCAGGS-IFI27-V82A-HA plasmid, or the pCAGGS-IFI27-HA plasmid alone. At 24 hpt, cells were infected with SARS-CoV-2 (MOI 1), and 24 hpi, cell lysates were harvested following the protocol described in materials and methods (Section 3.10.3). In short, once the cellular lysates were harvested, we performed a co-IP with anti-FLAG-conjugated agarose beads, in order to bind RIG-I-FLAG, as well as all the proteins and RNAs bound to RIG-I. After several washing steps, RNA from these RNPs was purified and retrotranscribed to cDNAs. The level of SARS-CoV-2 genomic RNAs was analyzed by measuring the level of SARS-CoV-2 RdRp RNA by qRT-PCR (Figs. R23C, R23D). As expected, in the control containing IFI27-HA alone, we did not detect RNA, since in the absence of RIG-I-FLAG in such

cell extracts, IFI27-HA will not bind to anti-FLAG antibodies. Surprisingly, when extracts contained both the RIG-I-FLAG and IFI27-HA proteins, the amount of RdRp cDNA we detected did not increase relative to the control sample, transfected only with the IFI27-HA plasmid. Remarkably, when the cell extracts contained the proteins RIG-I-FLAG and IFI27-V82A, we detected a near 8-fold increase in the amount of RdRp RNA compared to the sample transfected with the RIG-I-FLAG + IFI27-WT-coding plasmids (Fig. R23D). These results strongly suggest that IFI27-V82A, by not binding RNA, does not alter the ability of RIG-I to recognize and interact with SARS-CoV-2 RNA, whereas IFI27 WT, by binding RNA, could be competing with or altering the ability of RIG-I to recognize and bind SARS-CoV-2 RNA.

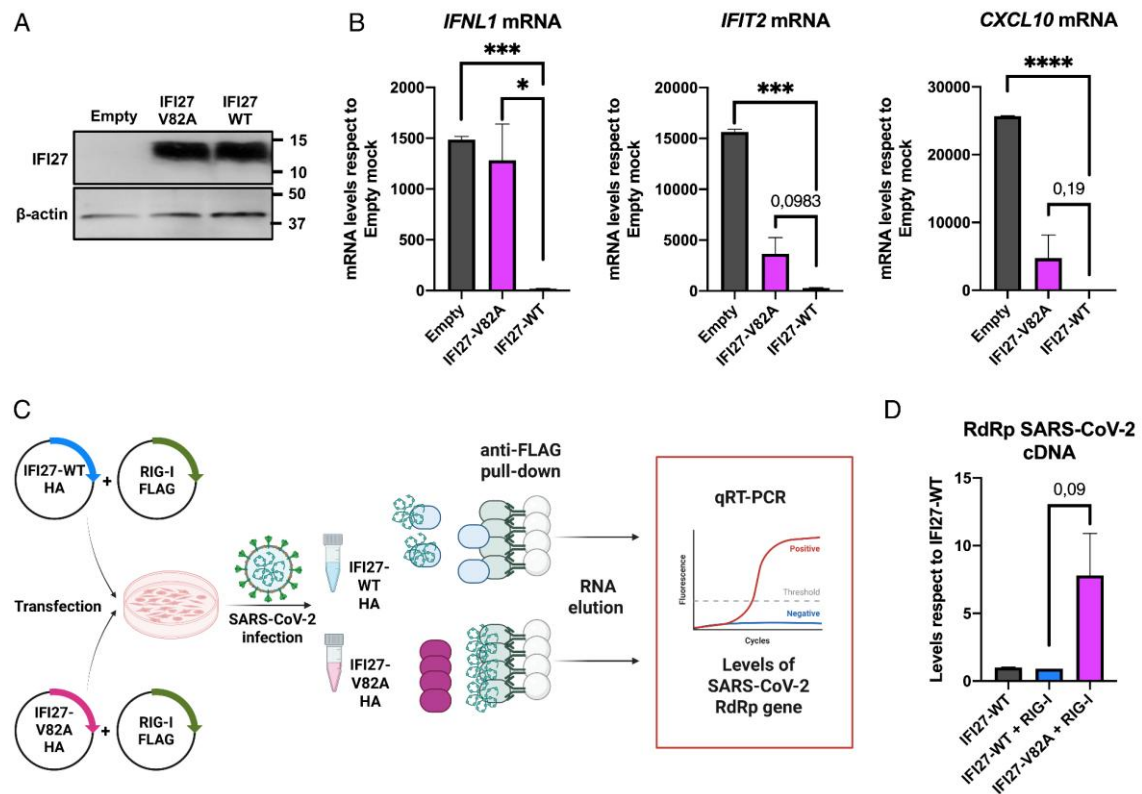


Figure R23. IFI27-V82 variant partially loses its ability to negatively modulate the innate immune responses. (A, B) HEK-293T cells were transfected with either the IFI27-WT-expressing pCAGGS plasmid, the IFI27-V82A-expressing pCAGGS plasmid, or an empty pCAGGS plasmid as negative control. 24 hpt, cells were infected with SeV (MOI 3) for 24 hours. Next, both (A) protein extracts and (B) total RNAs were harvested. (A) First, proper protein expression of IFI27-HA and IFI27-V82A-HA was analyzed by Western blot by using the specific antibodies anti-HA and anti-β-actin, as a loading control. Molecular weight markers are indicated on the right of the blot (in kDa). (B) mRNA levels of *IFNL1*, *IFIT2*, *CXCL10*, and *GAPDH* (housekeeping gene) were measured by qRT-PCR. (C) Experimental timeline schema. (C, D) HEK-293T-hACE2 cells were co-transfected with the pCAGGS plasmid expressing RIG-I-FLAG together with pCAGGS-IFI27-WT-HA or the pCAGGS-IFI27-V82A-HA. Alternatively, cells were transfected with pCAGGS-IFI27-WT-HA together with the empty pCAGGS plasmid as a negative control. At 24 hpt, cells were infected with SARS-CoV-2 (MOI 1), and 24 hpi, cellular extracts were harvested and used to perform a co-IP with anti-FLAG-conjugated agarose beads. Anti-FLAG-bound RNP complexes were eluted and RNAs from these complexes were isolated and purified. Total RNAs were retrotranscribed to cDNAs, and a qPCR was performed to detect the total amount of RNA of the RdRp gene of SARS-CoV-2, which was represented as fold of change respect to the cells transfected with the IFI27-WT-HA plasmid alone. Data is represented as the means and standard deviations of the different measures. *p < 0.05, **p < 0.01,

*** $p < 0.001$, **** $p < 0.0001$, or the exact p value (for comparisons using unpaired two-tailed Student's t test in **B** and **D**). Two different experiments were performed, with similar results.

4.3.1.2.3.2. Ubiquitination levels of RIG-I are reduced due to IFI6-mediated negative regulation

One of the hallmarks of RIG-I activation is the (K)63-linked polyubiquitination. As mentioned before, upon RNA binding to the C terminal domain of RIG-I, this protein undergoes a conformational change that exposes the CARD domains, which are the responsible to interact with MAVS and trigger the downstream signaling. In this process, K63-linked polyubiquitination of RIG-I is essential for its activation. K63-linked polyubiquitin chains are primarily added by different E3 ligases (Fig. R24A)[100,111]. Considering this information, we aimed to determine whether IFI6 could induce changes in RIG-I polyubiquitination levels, which would be indicative of alterations in its activation status. To this end, HEK-293T cells were transfected with an IFI6-specific siRNA, to knock-down IFI6 expression, or the NT siRNA as a control. Next, cells were transfected with a RIG-I-FLAG coding plasmid and infected with SeV to induce RIG-I activation. At 24 hpi, cellular extracts were harvested, and RIG-I-FLAG was immunoprecipitated using anti-FLAG-conjugated agarose beads. Subsequently, we measured protein levels of RIG-I (with an anti-FLAG antibody) and ubiquitinated RIG-I (with an anti-ubiquitin antibody) in the pull-down (Fig. R24B). Remarkably, while total RIG-I levels were similar, ubiquitinated RIG-I was significantly increased (6.8-fold) in IFI6-knocked-down cells compared to the control cells (Fig. R24B), which correlates with a higher activation status. This correlates with the previous results showing that IFI6 counteracts the activation of innate immune responses mediated by RIG-I (Fig. R22).

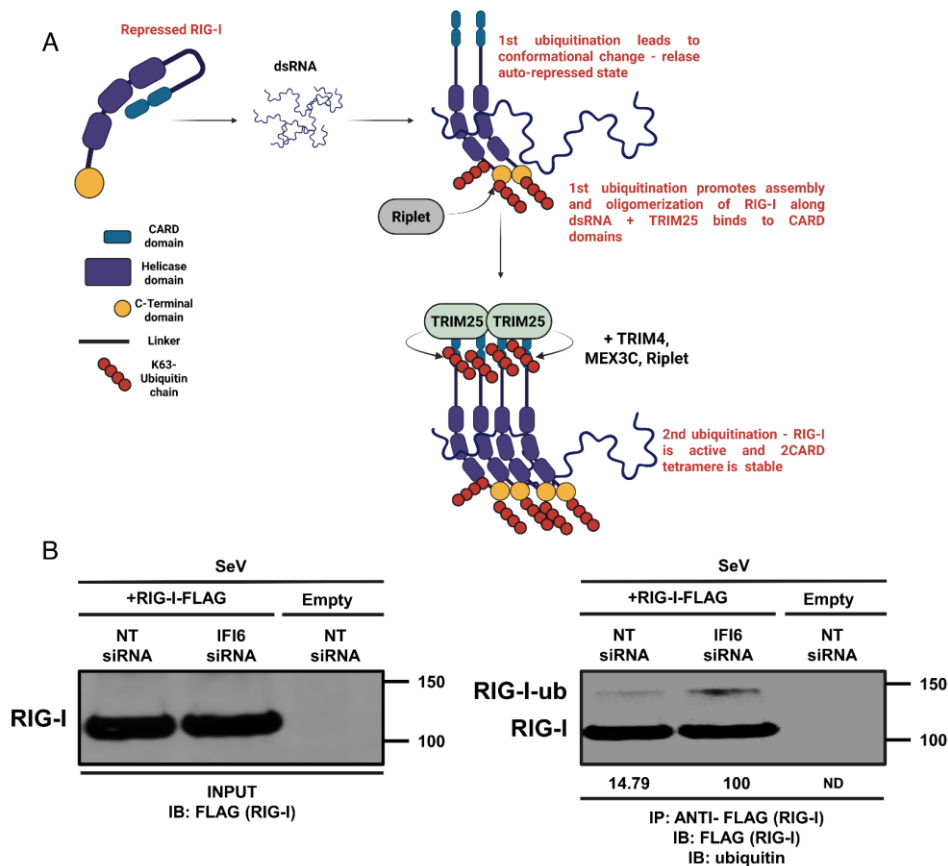


Figure R24. IFI6 negatively affects RIG-I ubiquitination. (A) Schema of the mechanism of activation of RIG-I by ubiquitination. In basal conditions, RIG-I is in a repressed state and closed conformation. When RIG-I recognizes and binds to dsRNAs, this binding changes its conformation, which is also favored by the poly-ubiquitination on the C-terminal domain and linker of RIG-I by Riplet, a ubiquitin E3-ligase. Next, RIG-I oligomerizes throughout the dsRNA molecule, and another ubiquitin E3-ligase, TRIM25, binds to CARD domains. TRIM25 poly-ubiquitinates CARD domains, together with other ubiquitin E3-ligases (Riplet, TRIM4, and Mex-3 RNA binding family member C (Mex3C)). This second round of ubiquitination fully activates RIG-I function, which forms a stable CARD tetramer. Schema adapted from [111]. **(B) IFI6 knock-down increments the levels of RIG-I ubiquitination.** HEK-293T cells were transfected with an IFI6-specific siRNA or a NT siRNA as a negative control. At 24 hpt, cells were transfected with either an empty pCAGGS plasmid or a RIG-I-FLAG coding plasmid and 24 hours later, the cells were infected with SeV (MOI 3) for an additional 24 hours. Then, cellular extracts were harvested and protein levels of total RIG-I were determined by Western blot (Input), using the anti-FLAG antibody. Next, cellular extracts were incubated with anti-FLAG-conjugated agarose beads to perform a co-IP. Anti-FLAG bound proteins were eluted, and protein levels of total RIG-I (using anti-FLAG antibody) and ubiquitinated-RIG-I (using anti-ubiquitin antibody) were determined by Western blot. The relative levels of ubiquitinated-RIG-I are shown in numbers below each line in the blot and were determined by densitometry using ImageJ software and relativized to the levels of total RIG-I. IB, immunoblot. Molecular weight markers are indicated on the right of the blots (in kDa). Two different experiments were performed with similar results.

4.3.1.2.4. IFI27 negatively regulates MDA5 activation

After describing that IFI27 and IFI6 are able to negatively modulate RIG-I function, our next step was to determine the possible effect that these proteins could have on MDA5. As previously mentioned, our objective was to learn how IFI27 and IFI6 could affect the RLR family, so both RIG-I and MDA5 were included. In this doctoral thesis, we exclusively focused on the potential modulation that IFI27 could exert on MDA5 function, but in future studies, IFI6 role in this regard will also be assessed. Knowing that IFI27 and IFI6 interact with RIG-I through RNA-binding and negatively modulate its function, and given the structural and functional similarities between RIG-I and MDA5 (Fig. 15), we hypothesized that IFI27 may also interact with and negatively modulate MDA5, in response to viral RNAs and/or poly(I:C).

4.3.1.2.4.1. IFI27 interacts with MDA5 through RNA binding

First, we analyzed IFI27 interaction with MDA5, now using IFI27-RIG-I interaction as a positive control. Again, we performed a co-IP by using anti-FLAG-conjugated agarose beads, which bind the FLAG tag of RIG-I and MDA5 (Fig. R25A). Interestingly, in poly(I:C)-treated cells, IFI27 co-immunoprecipitated together with MDA5, in a similar fashion as with RIG-I (Fig. R25B), as we could only detect IFI27-HA when RIG-I or MDA5 were overexpressed, suggesting a direct or indirect interaction of IFI27 with both proteins (Fig. R25B). Also, we tested this same procedure, but in mock-treated cells, to determine if endogenous RNA that could be generated within cells, can also mediate the interaction between IFI27 and RIG-I or MDA5. Surprisingly, we could detect a certain amount of IFI27 co-immunoprecipitated together with RIG-I or MDA5 (Fig. R25C), but to a much lesser extent than in the case of poly(I:C)-treated cells (Fig. R25B). As mentioned before, these proteins are RBPs, so in order to determine the importance of RNA on these interactions, we repeated the same procedure both in mock- and poly(I:C)-treated cells, but now, before the co-IP, the extracts were incubated with both the ssRNases and dsRNases. In both poly(I:C)-treated and mock-treated cells, while the amount of MDA5 and IFI27 in the protein extracts was maintained after the treatment with RNases (Input, Figs. R25B, R25C), the amount of IFI27 that co-immunoprecipitated together with MDA5 was highly decreased, almost to

undetectable levels (co-IP, Figs. R25B, R25C). These findings strongly suggest that IFI27 interaction with MDA5 is RNA-mediated, similarly as what we observe for IFI27-RIG-I interaction (Figs. R20B, R20C). Also, these results could denote that after plasmid transfection and protein overexpression in mock-treated cells, RIG-I and MDA5 could bind to cellular RNAs, as has been previously published [291]. However, endogenous levels of expression of RIG-I and MDA5 in non-infected cells, or non-poly(I:C) treated cells are close to zero, so the biological importance of these interactions in mock-conditions is limited.

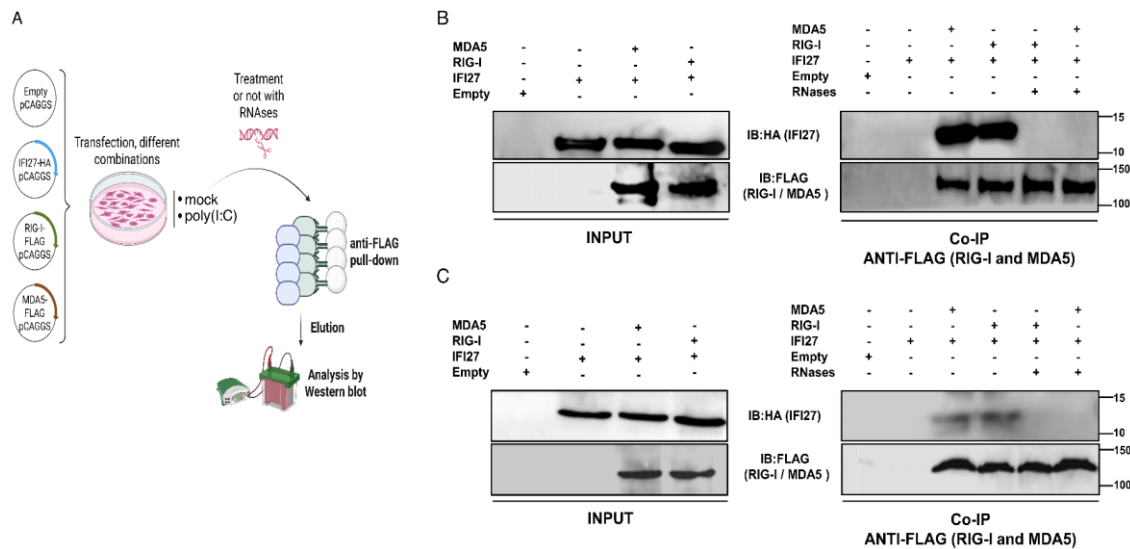


Figure R25. IFI27 interacts with RIG-I and MDA5 through RNA binding. (A) Schema of experimental timeline. (B, C) HEK-293T-hACE2 cells were transiently co-transfected with the pCAGGS plasmids encoding IFI27-HA and RIG-I-FLAG or MDA5-FLAG. To maintain a stable amount of DNA, we transfected cells also with an empty pCAGGS plasmid, as a negative control. At 24 hpt, cells were either poly(I:C) (3 μ g/mL)- (B) or mock-transfected (C) for 24 hours. Cellular lysates were harvested and the proper expression of the codified proteins was confirmed by Western blot, using the specific antibodies for FLAG (to detect RIG-I and MDA5) or for HA (to detect IFI27) (B and C, Input). Next, lysates were treated or not with ssRNases and dsRNases (denoted as RNases), and co-IPs with anti-FLAG-conjugated agarose beads were performed. Co-IPs of IFI27 with RIG-I and MDA5 under poly(I:C) conditions (B) and mock conditions (C) were analyzed by Western blot, using the specific antibodies for RIG-I and MDA5 (anti-FLAG) and IFI27 (anti-HA). IB, immunoblot. Molecular weight markers are indicated on the right of the blots (in kDa). Three different experiments were performed, with similar results.

Next, we wanted to test these interactions in the context of a real viral infection, as done with RIG-I and SeV infections (Fig. R20). To this end, we performed co-IPs of IFI27 and MDA5 after SARS-CoV-2 infection (Fig. R26A), as both MDA5 and RIG-I are able to sense and respond to SARS-CoV-2 infections [85,91], but multiple studies have established that, in SARS-CoV-2 infections, MDA5 is the primary and most important sensor for SARS-CoV-2 recognition in lung epithelial cells [91,92]. To this end, HEK-293T-hACE2 cells were transfected with the pCAGGS-IFI27-HA plasmid, pCAGGS-MDA5-FLAG plasmid, or the empty plasmid, in different combinations, and 24 hpt, cells were infected with SARS-CoV-2. At 24 hpi, cellular extracts were harvested. First, we confirmed SARS-CoV-2 infection by analyzing the protein expression levels of N protein of SARS-CoV-2 by Western blot (Fig. R26A). Once we confirmed that cells were properly infected, we performed a co-IP assay using anti-FLAG or anti-HA-conjugated agarose beads. Interestingly, MDA5 and IFI27 co-immunoprecipitated together when both proteins were overexpressed together using both anti-FLAG and anti-HA (Fig. R26B, R26C) agarose beads, suggesting that MDA5 and IFI27 directly or indirectly interact in SARS-CoV-2 infected cells.

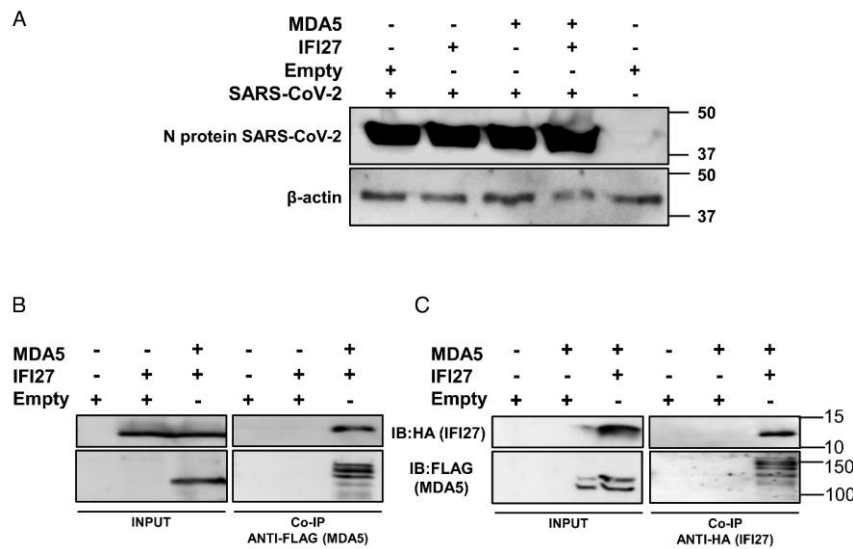


Figure R26. IFI27 co-immunoprecipitates with MDA5 after SARS-CoV-2 infection. (A-C) HEK-293T-hACE2 were transiently co-transfected with the pCAGGS plasmid encoding MDA5-FLAG and IFI27-HA, or with an empty pCAGGS plasmid, as a negative control. At 24 hpt, cells were either mock-infected (**A**) or infected with SARS-CoV-2 (MOI 0.5) for 24 hours (**A-C**). (**A**) Infection with SARS-CoV-2 was confirmed by Western blot by analyzing the protein levels of SARS-CoV-2 N-protein, using an anti-N antibody and anti-β-actin antibody, as a loading control. (**B, C**) Cellular extracts were used to perform co-IP experiments using anti-FLAG conjugated agarose beads to pull-down MDA5-FLAG (**B**) or anti-HA conjugated agarose beads to pull-down IFI27-HA (**C**). IFI27 and MDA5 were detected by Western blot in the cellular lysates (**B, C**, Input) and after the co-IPs (**B, C**, co-IP), using the specific antibodies to HA (to detect IFI27) and to FLAG (to detect MDA5). IB, immunoblot. Molecular weight markers (in kDa) are indicated on the right of the blots. Three different experiments were performed, with similar results.

To further analyze the interaction between IFI27 and MDA5, A549-hACE2 cells stably overexpressing IFI27-HA (A549-hACE2-IFI27-HA) and control (A549-hACE2-empty) cells, were generated. To this end, the IFI27-KO cells were transfected with a pCAGGS-IFI27-HA plasmid or an empty-pCAGGS plasmid (as control) that had been linearized and that codified an hygromycin-resistance gene. Transfected cells were selected in the presence of hygromycin, so that only cells that had integrated this plasmid into their genomes survived, presenting these cells a stable and long-lasting expression of IFI27-HA. These cells were either mock-treated or treated with poly(I:C) for 24 hours, and cellular extracts were collected. First, the proper expression of IFI27-HA was confirmed both by Western blot and immunofluorescence (Fig. R27B, Input, and Fig. R28B, respectively). Next, using these cells, we performed a co-IP of endogenous MDA5, using anti-MDA5 antibodies which were bound to protein A and coupled to sepharose beads. MDA5 and IFI27 co-immunoprecipitated together in poly(I:C)-transfected cells, but not in mock-transfected cells, as the levels of MDA5 in mock-transfected cells are very low, as expected [292](Fig. R27B). Thus, we confirmed that IFI27 interacts with endogenous MDA5.

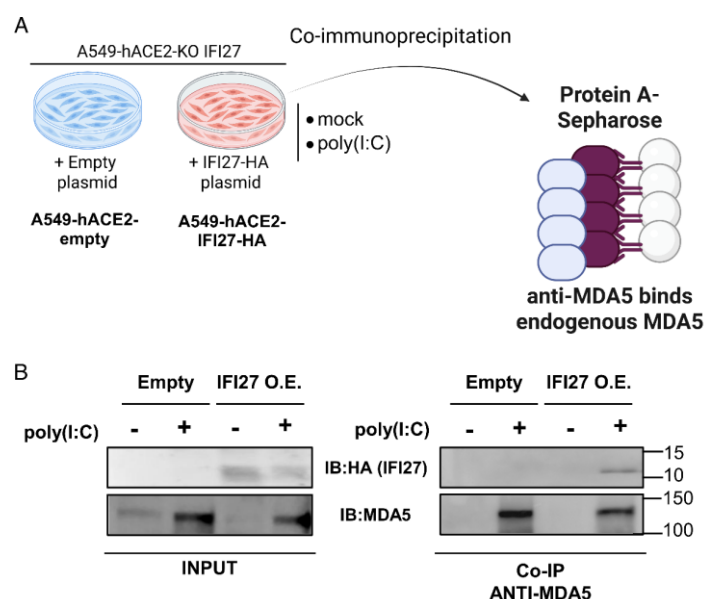


Figure R27. IFI27 binds MDA5 in A549-hACE2 cells stably overexpressing IFI27-HA. (A) Schema of experimental timeline. (B) A549-hACE2-IFI27-HA and A549-hACE2-empty cells were either mock- or poly(I:C)-treated (60 ng/mL), and 24 hpt, cell lysates were harvested. Proper protein expression of IFI27-HA (using an anti-HA antibody) and endogenous MDA5 (using an anti-MDA5 antibody) was measured by Western blot, using the appropriate antibodies (B, Input). A co-IP assay was performed by incubating cellular lysates with anti-MDA5 antibodies coupled to protein A-conjugated sepharose beads. MDA5 and IFI27 were detected by Western blot using the specific antibodies for HA (to detect IFI27) and for MDA5 (B, co-IP). IB, immunoblot. Molecular weight markers are indicated (in kDa) on the right of the blots. Three different experiments were performed, with similar results.

4.3.1.2.4.2. IFI27 partially colocalizes with MDA5 at the cell cytoplasm

As previously described for RIG-I, and to support the co-IP studies, we analyzed the potential intracellular colocalization between IFI27 and MDA5. Following this approach, HEK-293T-hACE2 cells were transiently transfected with the pCAGGS-IFI27-HA plasmid, pCAGGS-MDA5-FLAG plasmid, or the empty control plasmid as a negative control of transfection. Next, cells were either mock- or poly(I:C)-transfected, or infected with SARS-CoV-2, and 24 hours later, the cells were fixed and permeabilized. As a control, in non-transfected cells, IFI27-HA and MDA5-FLAG were not detected (data not shown). Again, Pearson coefficient was employed to analyze the degree of colocalization between fluorophores. Importantly, in the cell cytoplasm, we observed a partial co-localization between MDA5 and IFI27, which was more prominent in cells treated with poly(I:C) and infected with SARS-CoV-2 (Pearson coefficients of 0.75 and 0.84, respectively) compared to mock-treated cells (Pearson coefficient of 0.43) (Fig. R28A). Previously, we showed the co-IP interaction between IFI27 and endogenous MDA5 in the A549-hACE2 cells stably expressing IFI27-HA (A549-hACE2-IFI27-HA) (Fig. R27B). Therefore, we tested the possible colocalization between endogenous MDA5 and IFI27 in the A549-hACE2-IFI27-HA cells after mock conditions, poly(I:C)-treatment, and SARS-CoV-2 infection (Fig. R28C). First, we confirmed the proper detection of IFI27-HA expression in the A549-hACE2-IFI27-HA in comparison with the control cells (A549-hACE2-empty), where IFI27 expression was not detected (Fig. R28B). SARS-CoV-2 infection was confirmed by immunofluorescence using an anti-N protein antibody (Fig. R28C). Poly(I:C) treatment and SARS-CoV-2 infection, strongly induced MDA5 expression, which was undetectable in mock conditions (Fig. R28C).

Interestingly, MDA5 and IFI27 showed a partial colocalization in the cell cytoplasm after poly(I:C) treatment and SARS-CoV-2 infection (Fig. R28C). Thus, immunofluorescence and confocal microscopy strongly support the data obtained from the co-IPs showing that both proteins interact together (Figs. R26 and R27).

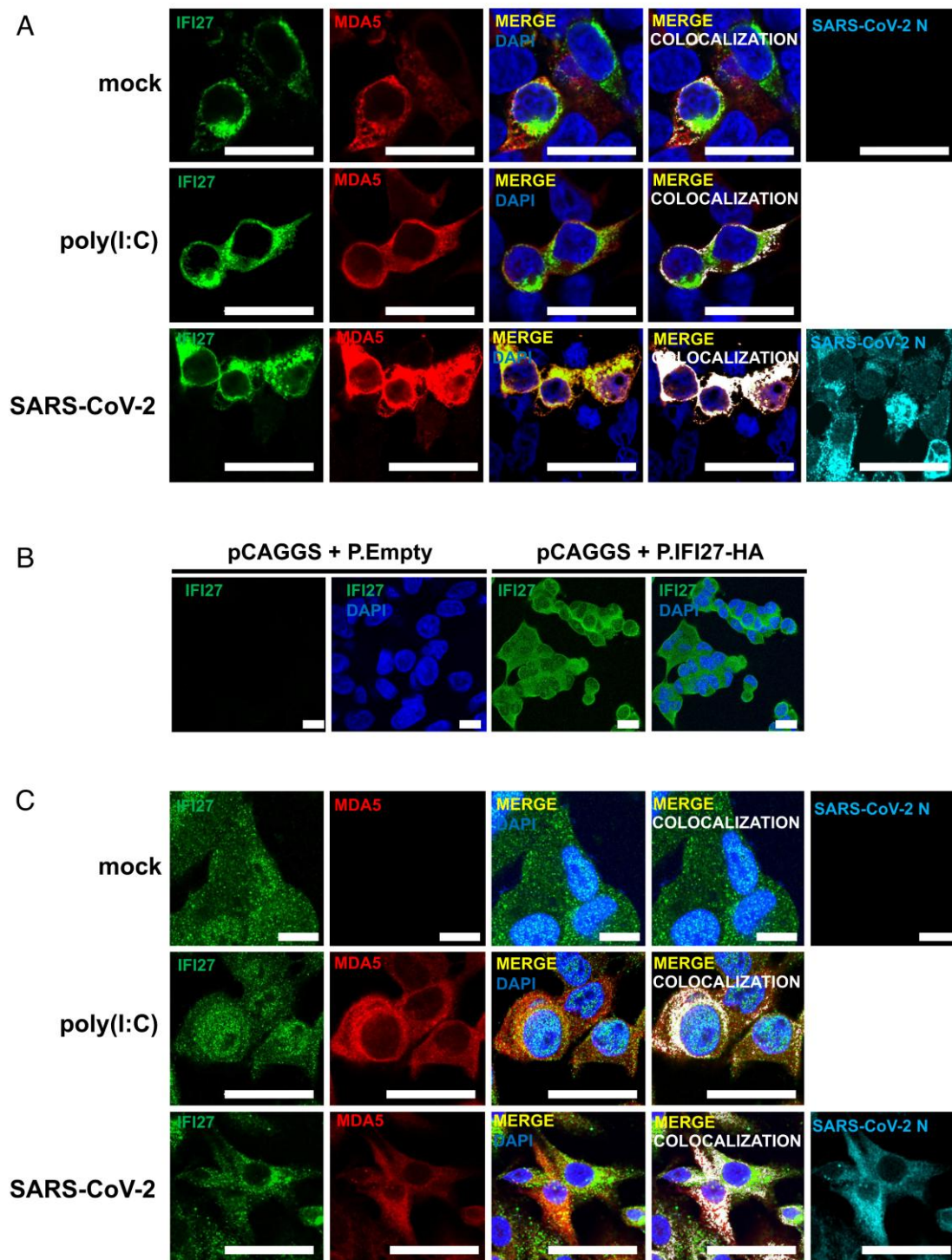


Figure R28. IFI27 partially colocalizes with MDA5 at the cell cytoplasm. (A) HEK-293T-hACE2 were transiently co-transfected with the pCAGGS plasmid encoding MDA5-FLAG and IFI27-HA. As a negative control, cells were transfected with an empty pCAGGS plasmid (data not shown). At 24 hpt, cells were either mock- or poly(I:C)-treated (60 ng/mL) or infected with SARS-CoV-2 (MOI 1). (B, C) Alternatively, A549-hACE2 KO IFI27 stably expressing IFI27-HA (A549-hACE2-IFI27-HA), or A549-hACE2-empty cells, were mock- or poly(I:C)-treated (60 ng/mL) or infected with SARS-CoV-2 (MOI 1). (A-C) At 24 hpt

or hpi, cells were fixed with formaldehyde and permeabilized. MDA5-FLAG, IFI27-HA, and SARS-CoV-2 N protein were stained by incubation with the anti-MDA5 (**A, C**), anti-HA (for IFI27 in **A-C**), the anti-SARS-CoV-2, N protein (in **A** and **C**), and the appropriate secondary antibodies conjugated to fluorophores (Alexa-488, green, for IFI27, and Alexa-594, red, for MDA5, and Alexa-647, light blue, for N-protein of SARS-CoV-2). Nuclei were stained with DAPI. Areas of co-localization between both proteins appear in yellow in the third picture, and in white in the fourth. Scale bar, 10 μ m. Three different experiments were performed, with similar results. For this figure, we show one representative image of the total of cells analyzed.

4.3.1.2.4.3. IFI27 negatively affects MDA5 oligomerization and activation, as both proteins compete for RNA binding

Once we had studied IFI27 effect on RIG-I function, we decided to test the same for MDA5, in order to have a broader view of the effect of IFI27 on RLR family of receptors. When MDA5 binds its target RNAs, it changes its conformation and goes through an oligomerization process. This oligomerization leads to its activation and transduction of antiviral signaling [80,105] (**Fig. R29A**). With this understanding, we decided to analyze the effect of IFI27 expression on the oligomerization of MDA5 by Western blot, using a high-affinity antibody for the detection of endogenous MDA5. To this end, the A549-hACE2-IFI27-HA and the control cells, transfected with the empty plasmid (A549-hACE2-empty), were either mock- or poly(I:C)-transfected. First, we could observe that in mock-treated cells, MDA5 oligomers (molecular weight near 250 kDa) were undetectable. However, after poly(I:C) treatment, MDA5 oligomers strongly increased, confirming that poly(I:C) stimulates MDA5 oligomerization (**Fig. R29B**), as previously described [105]. Whereas the expression of MDA5 monomers (molecular weight between 100 and 150 kDa) was similar in cells expressing IFI27-HA and the control cells transfected with the empty plasmid, oligomerized-MDA5 was significantly decreased in cells overexpressing IFI27 compared to the control cells transfected with the empty plasmid (**Fig. R29B**). This result shows that IFI27 negatively affects MDA5 oligomerization. Complementarily, following the same protocol and conditions, we analyzed mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10*. Supporting previous data, poly(I:C) strongly induced the mRNA levels of all these genes compared to mock-conditions. Interestingly, in IFI27-HA expressing cells, the mRNA levels for all these genes were significantly lower than in the empty control cells (**Fig. R29C**), correlating with the lower activation of MDA5 (**Fig. R29B**).

As previously mentioned, MDA5 is essential in the recognition of SARS-CoV-2 and induction of innate immune responses [91,92]. In line with this, we analyzed MDA5 activation after SARS-CoV-2 infection (**Fig. R29D**) and mRNA levels of *IFNL1* and *CXCL10* in the A549-hACE2-IFI27-HA cells and in control A549-hACE2-empty cells (**Fig. R29E**). For this purpose, cells were mock-transfected or transfected with either the pCAGGS-empty plasmid or the pCAGGS-MDA5-FLAG plasmid. Then, the cells were infected with SARS-CoV-2 for 24 hours. In correlation with poly(I:C) data (**Fig. R29B**), MDA5 oligomers were reduced in cells overexpressing IFI27 compared to the control cells (**Fig. R29D**). Also, transfection alone induced MDA5 activation compared to non-transfection conditions, suggesting that transfection activates MDA5 (**Fig. R29D**). mRNA levels of *IFNL1* and *CXCL10* were significantly lower in IFI27-HA expressing cells compared to empty control cells (**Fig. R29E**). Furthermore, viral titers were measured in supernatants of SARS-CoV-2-infected empty and IFI27-HA expressing cells. We observed that viral titers were higher in cells overexpressing IFI27-HA compared to control cells (**Fig. R29F**). All these data show that IFI27 acts as a negative regulator of MDA5 activation.

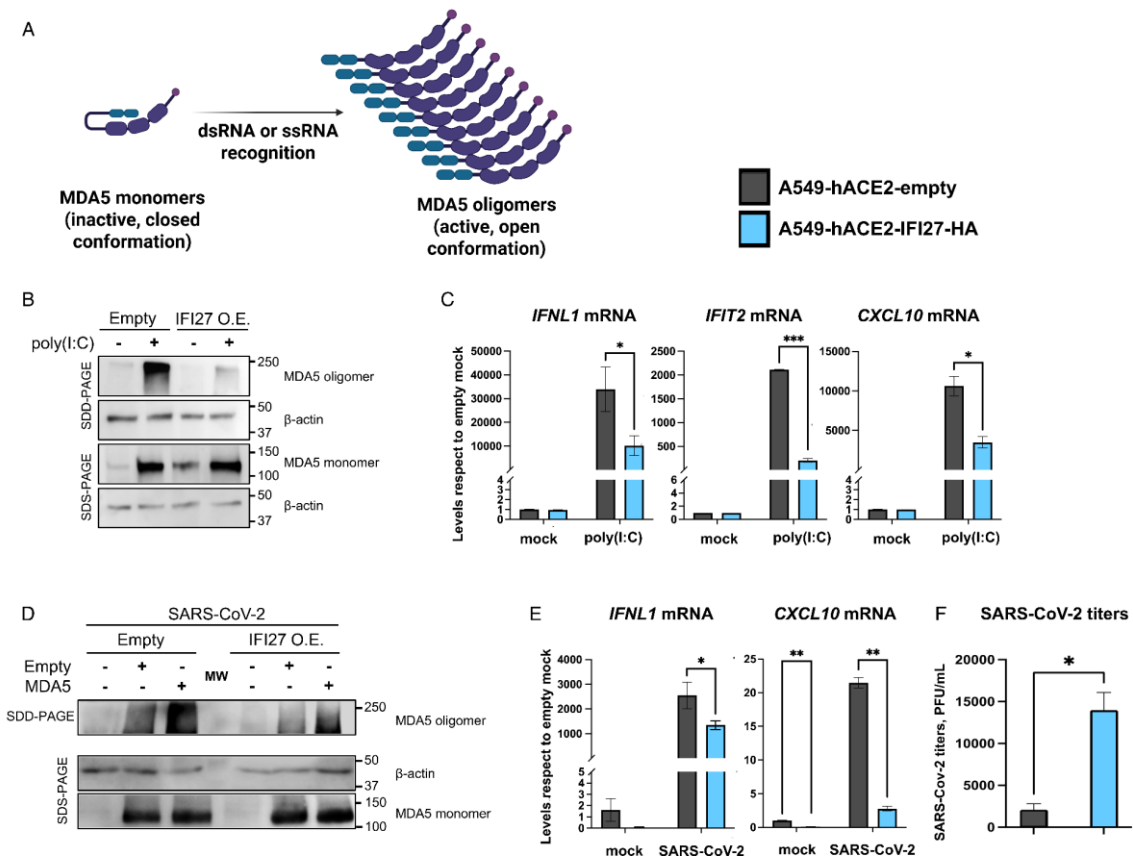


Figure R29. IFI27 negatively affects MDA5 oligomerization and function, hampering the induction of innate immune responses. **(A)** Graphical representation of MDA5 activation process. **(B, C)** A549-hACE2-IFI27-HA cells (IFI27 overexpression, O.E.) or control cells (empty), were treated with poly(I:C) (60 ng/mL) for 24 hours or left untreated. Protein extracts **(B)** or extracts to purify total RNAs **(C)** were harvested. **(D, E)** A549-hACE2-IFI27-HA (IFI27 O.E.) or control (empty) cells, were transfected with an MDA5-expressing pCAGGS plasmid or an empty pCAGGS plasmid as a control. At 24 hpt, cells were infected with SARS-CoV-2 (MOI 1), and proteins extracts **(D)** or extracts to purify total RNAs **(E)** were harvested. **(B, D)** Both SDD-PAGE (to detect MDA5 oligomers) and SDS-PAGE (to detect MDA5 monomers) Western blots were performed using anti-MDA5 (to detect both monomers and oligomers) and anti-β-actin antibodies. Molecular weights (in kDa) are on the right of the blots. **(C, E)** mRNA levels of *IFIT2* **(C)**, *IFNL1*, *CXCL10*, and *GAPDH* (to normalize mRNA levels) **(C, E)** were measured. **(F)** The A549-hACE2-IFI27-HA and A549-hACE2-empty cells were infected with SARS-CoV-2 at a MOI of 1. At 24 hpi, the viral titers in culture supernatants were analyzed by plaque assay (PFU/mL) in Vero E6 cells. Results are shown as fold of change respect to mock-infected, A549-hACE2-empty control cells. Data is represented as the means and standard deviations of three independent replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in **C, E, and F**). Two different experiments were performed, with similar results.

Next, we compared MDA5 activation in A549-hACE2 IFI27-KO cells respect to WT cells, to obtain complementary results with IFI27-KO cells. These cells were either mock-treated or treated with two different concentrations of poly(I:C). Subsequently, we assessed the generation of MDA5 oligomers (Fig. R30A) and the induction of *IFNL1*, *IFIT2*, and *CXCL10* (Fig. R30B). In IFI27-KO cells, the expression of MDA5 monomers, and the presence of oligomers were both increased (Fig. R30A), which correlated with a higher induction of the expression of *IFNL1*, *IFIT2*, and *CXCL10* in IFI27-KO cells respect to WT cells, both in mock- and poly(I:C)-transfected cells (Fig. R30B).

Similarly, as an alternative approach, we also assessed MDA5 activation in A549-hACE2 cells transfected either with an IFI27-specific siRNA, for knocking-down IFI27 expression, or a NT siRNA, as a control. At 24 hpt, cells were either mock-treated or treated with increasing concentrations of poly(I:C) (Fig. R30D), or SARS-CoV-2-infected (Fig. R30E). Firstly, after siRNA transfection and mock- or poly(I:C)-treatment, mRNA levels of *IFI27* were analyzed, confirming that IFI27-siRNA had properly achieved an IFI27 knock-down of expression by reducing *IFI27* mRNA levels 10-fold (Fig. R30C). In IFI27-siRNA transfected cells, the presence of MDA5 oligomers was increased respect to the control cells transfected with the NT siRNA, after poly(I:C) treatments (Fig. R30D) and SARS-CoV-2 infection (Fig. R30E). These results strongly support that IFI27 is a negative regulator of MDA5 activation and induction of innate immune responses after poly(I:C) treatment and SARS-CoV-2 infection.

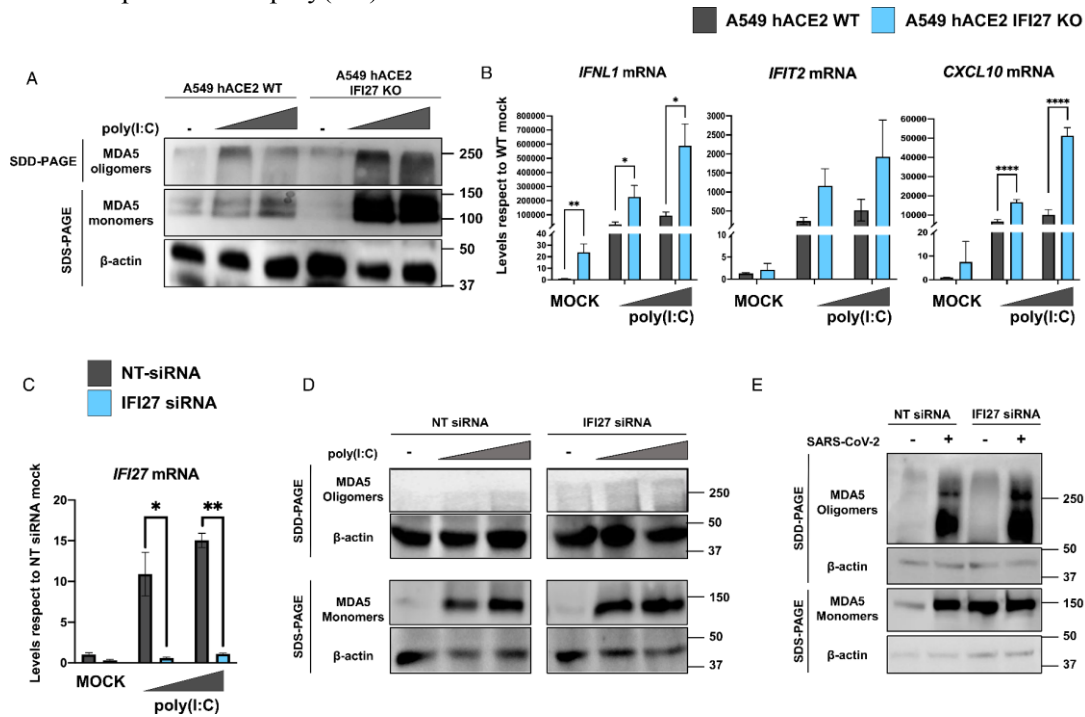


Figure R30. KO and knock-down of IFI27 leads to a higher MDA5 activation. (A, B) A549-hACE2-IFI27-KO cells or WT control cells, were mock-treated or treated with poly(I:C) at two different concentrations (5 and 12.5 ng/mL) for 24 hours. (A) Protein extracts were harvested. (B) Total RNAs were harvested and mRNA levels of *IFNL1*, *IFIT2*, *CXCL10*, and *GAPDH* (to normalize mRNA levels) were measured. Results are shown as fold-changes respect to mock-transfected A549-hACE2 WT cells. (C-E) A549-hACE2 WT cells were transfected twice, 24 hours apart, with either an IFI27 siRNA or a NT siRNA, as a negative control. At 24 hours after the second transfection, (C, D) cells were mock-transfected or transfected with poly(I:C) at two different concentrations (5 ng/mL and 12.5 ng/mL) for 24 hours or (E) cells were infected with SARS-CoV-2 (MOI 1) for 24 hours. (A, D, E) Protein extracts were harvested and both SDD-PAGE (to detect MDA5 oligomers) and SDS-PAGE (to detect MDA5 monomers) Western blots were performed using anti-MDA5 (to detect both monomers and oligomers) and anti- β -actin antibodies. Molecular weights (in kDa) are on the right of the blots. (C) 24 hours after the second transfection, total RNA was harvested, purified, and retrotranscribed, and the mRNA levels of *IFI27* and *GAPDH* (housekeeping gene) were measured by qRT-PCR. Data is represented as the means and standard deviations of three independent replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's *t* test in B and C). Two different experiments were performed, with similar results.

Previously, we described that IFI27 can alter the ability of RIG-I to bind to SARS-CoV-2 RNA (Fig. R23D). Building on this observation, we aimed to determine if IFI27 could have a similar

effect on the capacity of MDA5 to recognize and bind to RNA (Fig. R31A). To this end, we specifically tested the competition for poly(I:C) binding between MDA5 and IFI27, as both proteins bind to it [90] (Fig. R18). HEK-293T cells were transiently transfected with the pCAGGS plasmids encoding GFP (negative control of RNA binding), IFI27-HA, MDA5-FLAG, and both IFI27-HA plus MDA5-FLAG, or an empty plasmid as a negative control of transfection. At 24 hpt, protein extracts were harvested and the appropriate expression of the proteins of interest (GFP, IFI27-HA, and MDA5-FLAG) was confirmed by Western blot (Fig. R31B, Input). Next, these protein extracts were incubated with poly(I:C)-conjugated agarose beads, and eluates containing poly(I:C)-bound proteins were obtained. As expected, GFP did not interact with poly(I:C), and when IFI27-HA and MDA5-FLAG were overexpressed alone, these proteins interacted and immunoprecipitated with poly(I:C) (Fig. R31C). However, whereas the amount of MDA5 was similar when MDA5 was expressed alone or in combination with IFI27, in the protein extracts overexpressing both proteins, the amount of MDA5 bound to poly(I:C) was clearly decreased when IFI27 was also overexpressed (Fig. R31B), compared to when MDA5 was overexpressed alone (Fig. R31C). This means that MDA5 interaction with poly(I:C) in presence of IFI27 is compromised, strongly suggesting that IFI27 competes with MDA5 for poly(I:C) binding. This alteration in the RNA-binding properties of MDA5 could, at least in part, underlie the diminished activation of MDA5 due to IFI27 expression and the subsequent decrease in antiviral responses.

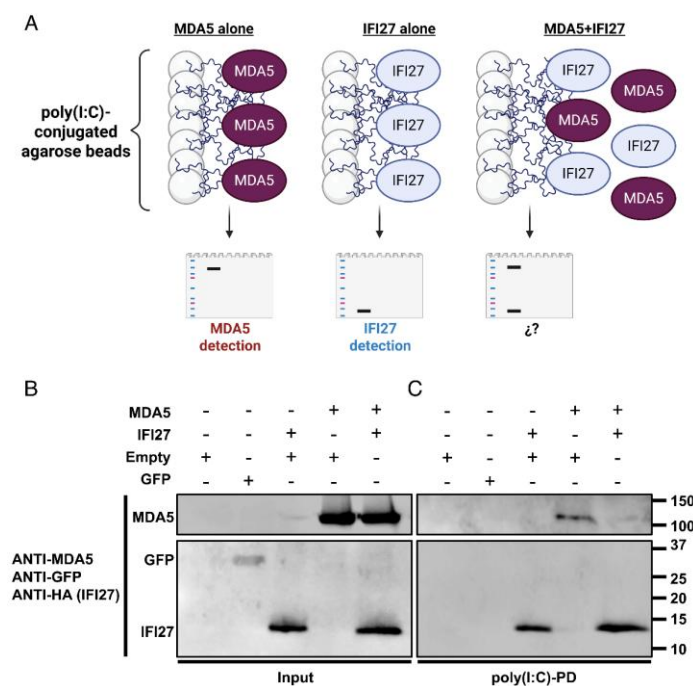


Figure R31. MDA5 and IFI27 compete for poly(I:C) binding. (A) Schematic representation of experimental concept to test the competition between MDA5 and IFI27 to poly(I:C) binding. (B) HEK-293T cells were transfected with the pCAGGS-GFP plasmid, as a negative control of RNA binding; pCAGGS-MDA5-FLAG plasmid alone, pCAGGS-IFI27-HA plasmid alone, or a combination of both pCAGGS-MDA5-FLAG and pCAGGS-IFI27-HA plasmids, to test poly(I:C) binding. At 24 hpt, cellular extracts were harvested and analyzed for proper protein expression (B, Input) by Western blot. (C) Next, a pull-down experiment was performed, by incubating cellular extracts with poly(I:C)-conjugated agarose beads. Poly(I:C)-bound proteins (Poly(I:C)-PD) were eluted and analyzed by Western blot. (B, C) For both Input and Poly(I:C)-PD samples, detection was achieved by using the specific antibodies for MDA5, anti-HA (to detect IFI27) and anti-GFP. Molecular weights are indicated on the right of the blots (in kDa). Two different experiments were performed, with similar results.

4.3.1.3. IFI27 affects protein translation by interacting with PACT and PKR

By different methods, we have shown that IFI27 is an RBP, and this function is highly conserved throughout different proteins that recognize viral PAMPs or act like modulators of innate immune responses. Thus, having demonstrated that IFI27 is an RBP that interacts with RIG-I and MDA5 and negatively modulates their activation, we wanted to search for new protein partners of IFI27, that could shed light about possible new molecular mechanisms and processes in which IFI27 could be implicated within the innate immune responses. As the study on IFI27 functions has been more extensive, as we have demonstrated its capacity to modulate both RIG-I and MDA5, in this section we will focus on analyzing new protein partners of IFI27. However, in future work, this analysis will be extended to IFI6 as well.

4.3.1.3.1. IFI27 interacts with PACT and PKR proteins

To start looking for additional possible functions of IFI27 within the innate immune responses, IFI27 protein was overexpressed by transfecting HEK-293T cells with the pCAGGS-IFI27-HA plasmid. Alternatively, cells were transfected with a pCAGGS-IFI44-HA plasmid, coding for IFI44 protein fused to an HA tag, or with an empty plasmid, as control. At 24 hpt, the cells were treated with poly(I:C), to promote the activation of the different players of the responses against RNA viruses. Next day, cells were lysed, and the protein extracts were incubated with anti-HA-conjugated agarose beads. By doing this, IFI27-HA and IFI44-HA bind to the anti-HA beads, as well as the IFI27- or IFI44-associated proteins. Next, proteins bound to anti-HA-conjugated agarose beads were eluted and analyzed by mass spectrometry (MS). The complete list for the IFI27-bound proteins showing a protein score higher than 40, eliminating the proteins that were also identified in the cells transfected with the empty plasmid (which were considered as non-specific), is shown in the [Table A3 \(Appendix\)](#). All proteins with a score higher than 40 were selected and analyzed by gene ontology, using NIH DAVID software (<https://davidbioinformatics.nih.gov/>). Selected proteins were classified according to their biological process ([Table A4](#), and [Fig. R32A](#)) and molecular function ([Table A5](#), and [Fig. R32B](#)), and we further studied those groups of gene ontology terms that showed an FDR-adjusted p-value lower than 0.05. With these standards, “RNA binding” category of [Fig. R32B](#) (including GO:0003723~RNA binding; GO:0003725~dsRNA binding; GO:0003729~mRNA binding; GO:0070883~pre-miRNA binding) was the main molecular function identified within the IFI27 interactome, representing a high proportion of all found proteins, with a total count of 62 proteins. GO: 0003723~RNA binding term showed the lower FDR-adjusted p-value, 2.27E-22 ([Table A5](#), and [Fig. R32B](#)). As previously described, IFI27 is an RBP ([Figs. R18, R19](#)), so it was really interesting and remarkable that more than half of IFI27 interactome is related to RNA binding processes. Also, as previously mentioned, RNA binding is closely related to RNA virus detection and response, as well as translational regulation [285,293]. Interestingly, the fifth molecular function with the lower FDR-adjusted p-value within IFI27 interactome was “structural constituent of ribosome (GO: 0003735) ([Fig. R32B](#)), which showed a fold enrichment of 13.7 and a FDR-adjusted p-value of 3.36E-07 ([Fig. R32B](#), [Table A5](#)), supporting the idea of a possible effect of IFI27 on modulation of translation [294]. In this context, it was remarkable to find that one of the most overrepresented biological processes within IFI27 interactome was “translation” which includes GO:0006412~translation, GO:0002181~cytoplasmic translation, and GO:0032543~mitochondrial translation, which showed a fold enrichment of 10.1, 16.03 and 15.68, respectively, and FDR-adjusted p-values of 4.1E-4, 2.85 E-3, and 2.85 E-3, respectively ([Table A4](#), [Fig. R32A](#)). Thus, all these findings suggested that IFI27 could be implicated in the regulation of translation processes. Considering this information, we decided to specifically select

some proteins within the interactome to perform further studies, and to do this selection, we searched for proteins that were included both in RNA-binding and translational processes. We were particularly interested in two proteins that belonged to both groups: PKR and PACT proteins (Fig. R33A). PKR's main function is to phosphorylate the Ser52 of the eukaryotic translation initiation factor 2 α (eIF2 α). Phosphorylated eIF2 α can no longer initiate new translation cycles, and this leads to a blocked translation initiation [76,138]. On the other hand, PACT is one of the best-known regulators of PKR function. PACT, in the presence of stress, is phosphorylated at Ser287 residue, and this was identified as a hallmark of downstream activation of PKR [130,142]. However, under certain conditions, PACT acts as a negative regulator of PKR activation. For instance, it has been described that during HIV-1 infections, PACT becomes a PKR inhibitor by a mechanism not completely understood that involves an interaction between ADAR1 and PACT [150,151]. Thus, the search for new proteins modulating this axis is fundamental to comprehend it better.

PKR and PACT proteins were not found in the IFI44-HA interactome (data not shown), suggesting that the binding was specific to IFI27 and not the HA tag. Furthermore, their scores among the IFI27 interactome were very high: 565 and 494 for PKR and PACT, respectively (Table A3, and Fig. R33A). To compare which of these two proteins is more abundant within IFI27 interactome, we analyzed the exponentially modified Protein Abundance Index (emPAI), which estimates the absolute amount of protein by quantifying the number of observed peptides of a protein divided by the number of observable peptides per protein [295]. PACT showed a higher emPAI value (1.92) than PKR (0.71), suggesting that PACT was more abundant in the IFI27 interactome than PKR (Fig. R33A). Other proteins known to modulate translation processes were found in the IFI27-interactome, such as protein phosphatase 1C (PPP1CC or PP1 γ), showing a protein score 279, and TARBP2 with a score of 373 (Table A3). These two proteins could affect host translation and innate immunity. As such, PPP1CC encodes the gamma isoform of the phosphatase catalytic subunit, which dephosphorylates eIF2 α [296], counteracting PKR function. Also, it is known that PPP1CC dephosphorylates the transcription factor IRF-3, thereby impairing RLRs mediated signaling [297]. Furthermore, TARBP2 is known to interact with PACT and to modulate PKR activity [141,145]. However, for the scope of this doctoral thesis, and due to the much higher score, we decided to focus on the possible effect of IFI27 on PKR and PACT functions.

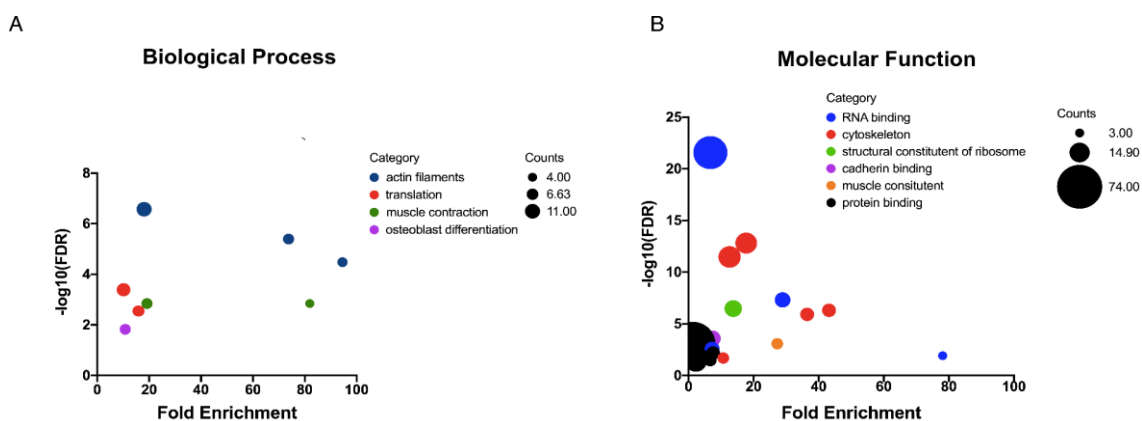


Figure R32. Bubble-plots of IFI27-interacting proteins categorized by biological processes and molecular functions. To generate this bubble-plot, we utilized mass-spectrometry data (Tables A4 and A5). All the proteins with a minimum score of 40 (Table A3) were selected and analyzed by gene ontology using the NIH DAVID software (<https://davidbioinformatics.nih.gov/>). Selected proteins were categorized according to their “Biological Process” (A) (Table A4) and “Molecular Functions” (B) (Table A5). FDR lower than 0.05 was selected to ensure that the rate of type I errors (false positives) is lower than 5% of the

findings. Subsequent analysis focused on proteins groups whose GO terms displayed an FDR-adjusted p-value lower than 0.05. **(A, B)** In the bubble-plots, the x-axis indicates the Fold Enrichment of the specific GO term, which indicates how much more (positive values) or less (negative values) a particular GO term is represented in our data set compared to a reference or background set. In the Y-axis, we represent the $-\log_{10}$ of the FDR-adjusted p-value for each GO term. The size of each bubble accounts for the number of proteins that have been classified within that category or GO term (an estimate of the bubble sizes is exemplified at the right of the graph, indicating the relation between the number of proteins and the bubble size).

Once MS data was explored, we analyzed the likely interaction of IFI27 with PACT. To this end, HEK-293T cells were transfected with the pCAGGS-IFI27-HA plasmid, together with the pCAGGS-PACT-FLAG plasmid, expressing the PACT protein fused to a FLAG tag. As a negative control, pCAGGS-PACT-FLAG was co-transfected with an empty pCAGGS plasmid. Next, 24 hpt, transfected cells were either mock- or poly(I:C)-transfected, and 24 hours later, cellular extracts were harvested. These extracts were employed to perform co-IPs with anti-HA-conjugated agarose beads, and then, proteins were eluted and analyzed by Western Blot (Fig. R33B). We could confirm that IFI27 and PACT co-immunoprecipitated together when cells had been treated previously with poly(I:C) (Fig. R33B, co-IP). In mock-treated cells, while the amount of PACT and IFI27 in the cellular extracts was similar (Fig. 33B, Input), the amount of PACT that co-immunoprecipitated together with IFI27 was clearly decreased compared to poly(I:C)-treated cells (Fig. R33B, co-IP), suggesting that the presence of exogenous dsRNA is pivotal for this interaction. Interestingly, when both mock- and poly(I:C)-treated extracts were incubated with a cocktail of RNases (ss and dsRNases) previously to the co-IP, the amount of PACT co-immunoprecipitating together with IFI27 was decreased to undetectable levels (Fig. R33B, co-IP). This further supports the idea that RNA presence is essential for the interaction between IFI27 and PACT, which could be explained by the ability of both IFI27 (Fig. R18) and PACT [140,142] to bind to dsRNA. Also, as in mock-treated cells, RNases have a small but clear effect too, suggesting that IFI27 and PACT interact to some degree in this condition, probably through endogenous RNAs (Fig. R33B, co-IP).

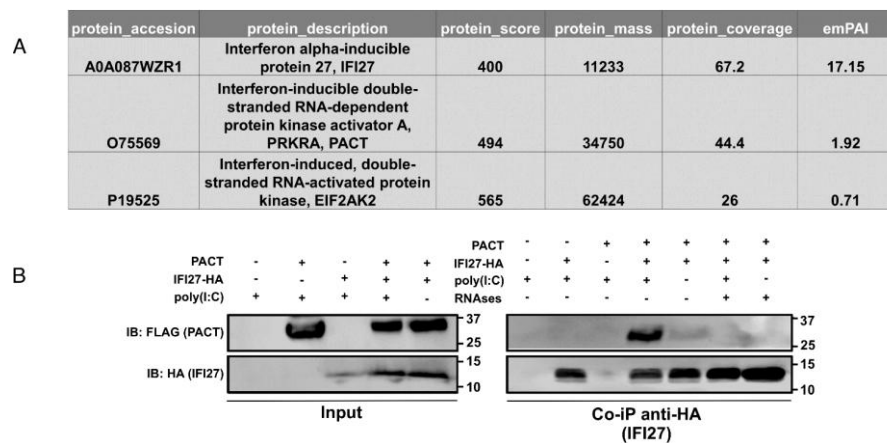


Figure R33. IFI27 interacts with PACT and PKR proteins through an interaction mediated by RNA. **(A)** HEK-293T cells were transfected with the pCAGGS-IFI27-HA plasmid and 24 hpt, cells were treated with poly(I:C) (3 μ g/mL). 24 hours after treatment with poly(I:C), cellular extracts were harvested and incubated with anti-HA-conjugated agarose beads. Extracts were pulled-down to retain IFI27-HA and its interacting proteins, which were then eluted and identified by MS. Protein accession number, description, score, mass, coverage, and emPAI index are indicated for IFI27, PACT and PKR proteins. **(B) Western blot confirmation of IFI27-PACT interaction.** HEK-293T cells were co-transfected with the pCAGGS-PACT-FLAG plasmid together with pCAGGS-IFI27-HA or empty pCAGGS plasmids. At 24 hpt, cells

were either mock- or poly(I:C)-transfected (3 μ g/mL) for 24 hours. Next, cellular extracts were incubated or not with a cocktail of RNases (both ss and dsRNases), and a co-IP using anti-HA-conjugated agarose beads was performed. Protein expression of PACT and IFI27 in the cellular extracts (Input) and after the co-IP was confirmed by Western blot using the specific antibodies anti-FLAG (for PACT, top panels), and anti-HA (for IFI27, bottom panels). IB, immunoblot. Molecular weights are indicated on the right of the panels (in kDa). B was performed three different times with similar results.

To further study PACT-IFI27 interaction, we analyzed the co-IP of both proteins after SARS-CoV-2 infection (Fig. R34), instead of poly(I:C) treatment (Fig. R33). In this case, instead of using anti-HA-conjugated agarose beads, we employed anti-FLAG-conjugated agarose beads, to bind PACT-FLAG. Thus, HEK-293T-hACE2 cells were transfected with pCAGGS-IFI27-HA and pCAGGS-PACT-FLAG plasmids, or pCAGGS-IFI27-HA and an empty pCAGGS plasmid, as a control. At 24 hpt, cells were infected with SARS-CoV-2 for an additional 24 hours. Next, cellular extracts were harvested and a co-IP using anti-FLAG-conjugated agarose beads was performed and subsequently analyzed by Western blot (Fig. R34). First, SARS-CoV-2 infection was confirmed by Western blot employing an anti-N protein antibody (Fig. R34A). Noteworthy, we could confirm that IFI27 and PACT interact after SARS-CoV-2 infection, further supporting the interaction between these two proteins (Fig. R34B).

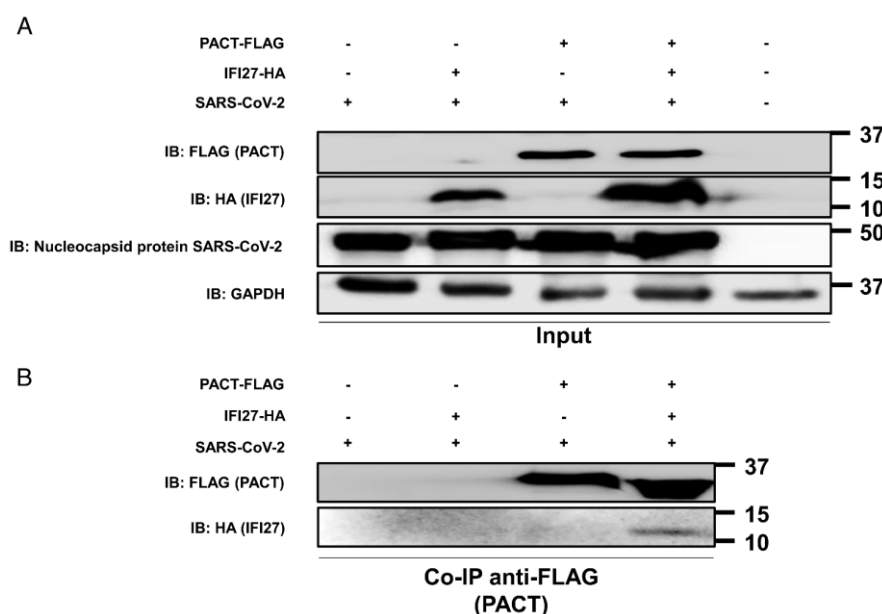


Figure R34. IFI27 co-immunoprecipitates with PACT after SARS-CoV-2 infection. (A, B) HEK-293T-hACE2 were transiently transfected with a pCAGGS-PACT-FLAG plasmid, a pCAGGS-IFI27-HA plasmid, a combination of both, or an empty pCAGGS plasmid as a negative control. 24 hpt, cells were infected with SARS-CoV-2 (MOI 0.5) for 24 hours. (A) Protein lysates were obtained and analyzed by Western blot to confirm PACT (anti-FLAG) and IFI27 (anti-HA) overexpression and SARS-CoV-2 infection (anti-Nucleocapsid protein), as well as GAPDH expression, as loading control (anti-GAPDH). (B) Protein extracts were incubated with anti-FLAG conjugated agarose beads to bind PACT-FLAG and its associated proteins. Bound proteins were eluted and analyzed by Western blot to detect PACT (anti-FLAG, top panel) and IFI27 (anti-HA, bottom panel). IB, immunoblot. Molecular weight markers are indicated on the right of the blots (in kDa). This experiment was performed twice, with similar results.

Given that we demonstrated that residues 63 and 82 are essential for IFI27's ability to bind RNAs (Fig. R19) and that results in Fig. R33 and R34 strongly suggest that IFI27 interacts with PACT through its ability to bind dsRNA, we performed co-IP assays of IFI27 and PACT using the two IFI27 mutated variants that are unable to bind to RNA, in order to determine whether the IFI27

binding to RNA is an essential requirement for its interaction with PACT. To test this, HEK-293T cells were transfected with the plasmids pCAGGS-IFI27-HA, pCAGGS-IFI27-S63L-HA, or pCAGGS-IFI27-V82A-HA alone or together with the pCAGGS-PACT-FLAG plasmid. At 24 hpt, cells were treated with poly(I:C) for 24 hours, and then, a co-IP using anti-HA-conjugated agarose beads was performed and analyzed by Western blot (Fig. R35). Again, as expected, PACT co-immunoprecipitated with IFI27-WT (Fig. R35). However, the S63L and V82A IFI27 variants, which are defective in RNA binding (Fig. R19) did not co-immunoprecipitate with PACT (Fig. R35), indicating that the interaction between IFI27 and PACT is mediated by the binding of both proteins to dsRNA. Furthermore, these co-IPs indicate that PACT does not interact with IFI27 via the HA tag, as these variants (which present a single point mutation in one residue, but also contain the intact HA tag) are unable to bind PACT.

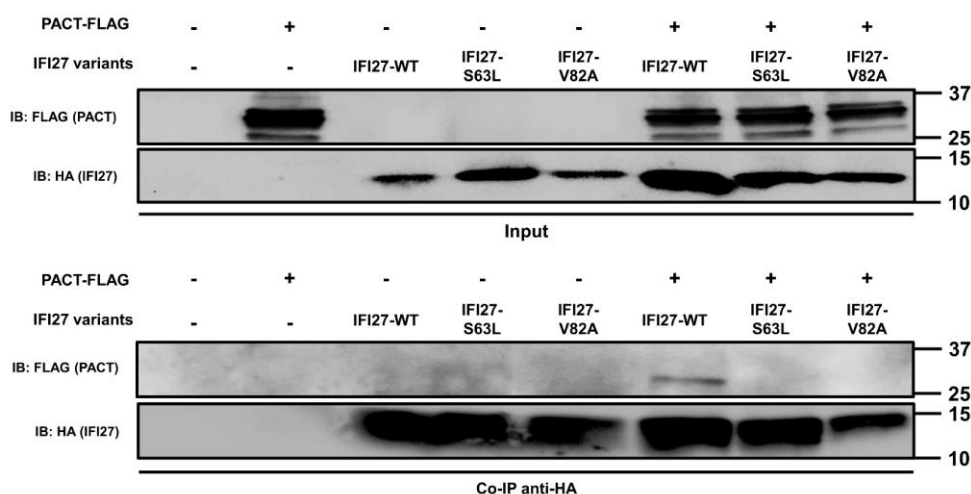


Figure R35. IFI27 variants S63L and V82A do not co-immunoprecipitate with PACT. HEK-293T cells were transiently transfected with the pCAGGS plasmid encoding PACT-FLAG together with the pCAGGS plasmid encoding for IFI27-WT or IFI27 variants, or with an empty pCAGGS plasmid as a negative control. At 24 hpt, cells were treated with poly(I:C) (3 µg/mL) for 24 hours. A co-IP with anti-HA-conjugated agarose beads was performed. PACT and IFI27 variants were detected both in the cellular lysates (Input) and after the co-IP (co-IP anti-HA) using the specific antibodies (anti-FLAG for PACT and anti-HA for the different variants of IFI27). IB, immunoblot. Molecular weight markers are indicated on the right of the blots (in kDa). These measures were performed in two independent experiments with similar results.

Secondly, we wanted to confirm the PKR-IFI27 interaction by co-IP and Western blot. To this end, HEK-293T cells were transfected with either an empty-pCAGGS plasmid, as a negative control, or the pCAGGS-IFI27-HA plasmid. Next day, cells were either mock- or poly(I:C)-transfected, and 16 hpt, cellular extracts were obtained. In this case, instead of using anti-HA beads, we used an anti-PKR antibody bound to a protein A-sepharose resin, in order to bind the endogenous PKR of cells. We could confirm that IFI27 co-immunoprecipitated with PKR in poly(I:C)-treated conditions (Fig. R36), whereas in mock conditions, we detected a weak interaction between both proteins, showing that endogenous RNAs could also participate, but to a lower extent, in PKR-IFI27 interactions (Fig. R36).

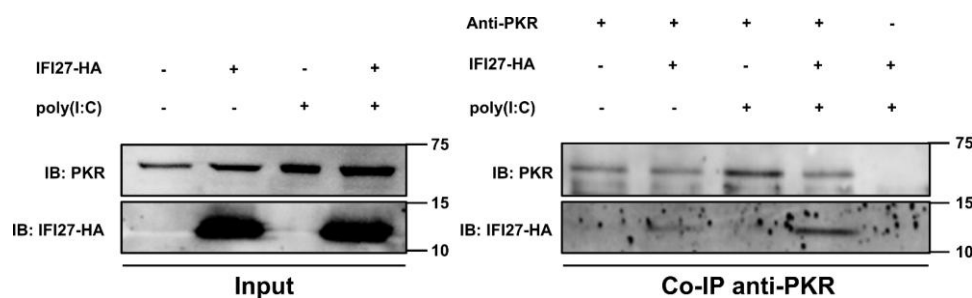


Figure R36. IFI27 and PKR co-immunoprecipitate together. HEK-293T cells were transfected with either the IFI27-HA encoding pCAGGS plasmid or with an empty-pCAGGS plasmid as a negative control. At 24 hpt, cells were either mock- or poly(I:C)-transfected (3 μ g/mL) for an additional 16 hours. Next, cell lysates were incubated overnight with the specific anti-PKR antibody at 4°C, as well as with protein A-sepharose resin, except for the last sample, which was incubated with the protein A-sepharose resin but without the anti-PKR antibody (negative control). Proteins in the cellular extracts (Input) and proteins bound to the resin were eluted and analyzed by Western blot using the specific antibodies (anti-PKR for endogenous PKR, top panels; anti-HA for IFI27, bottom panels). These measures were performed in three independent experiments with similar results.

4.3.1.3.2. IFI27 partially colocalizes with PACT and PKR in the cell cytoplasm

Once we had corroborated the interaction of IFI27 with PACT and PKR and to further support these experiments, we sought to analyze if these proteins colocalize within the cellular cytoplasm (Fig. R37). To this end, HEK-293T cells were transfected with pCAGGS-IFI27-HA plasmid alone or in combination with a pCAGGS-PACT-FLAG plasmid. IFI27-HA and PACT-FLAG were both transfected to detect these proteins using antibodies directed to their tags (anti-HA and anti-FLAG), as the antibodies against the endogenous protein did not provide a detectable staining (data not shown). However, for PKR, we detected the endogenous protein, since the anti-PKR antibody we used yielded a sufficiently strong signal, making it unnecessary to overexpress a tagged version of the protein. At 24 hpt, cells were mock- or poly(I:C)-transfected, as we wanted to compare the levels of colocalization in both conditions. Next, 24 hours later, cells were fixed and permeabilized, and an immunofluorescence to detect the intracellular localization of IFI27-HA, PACT-FLAG, and endogenous PKR was performed (Fig. R37A, R37B). We observed a partial colocalization in the cytoplasm between IFI27-HA and PACT-FLAG (Fig. R37A), and between IFI27-HA and PKR (Fig. R37B). To quantify the colocalization levels, we measured the area of the colocalization of both fluorophores and divided it with the total cell area (of at least $n=29$ cells for each condition). By doing this, we obtained a ratio of colocalization, which was then transformed to a percentage of colocalization within cells. Under mock conditions, PACT and PKR colocalized with IFI27 in an 8.27% and 8.38% of the surface area within the cells, respectively. Interestingly, these percentages of colocalization of PACT and PKR with IFI27 were significantly incremented when cells were previously treated with poly(I:C), showing a 13.6% and 13.7% of colocalization, respectively (Fig. R37C). These data further support the interactions observed by co-IP followed by mass-spectrometry or Western blotting, as well as the fact that poly(I:C) promotes the interaction of IFI27 with PACT and PKR (Fig. R32-R36).

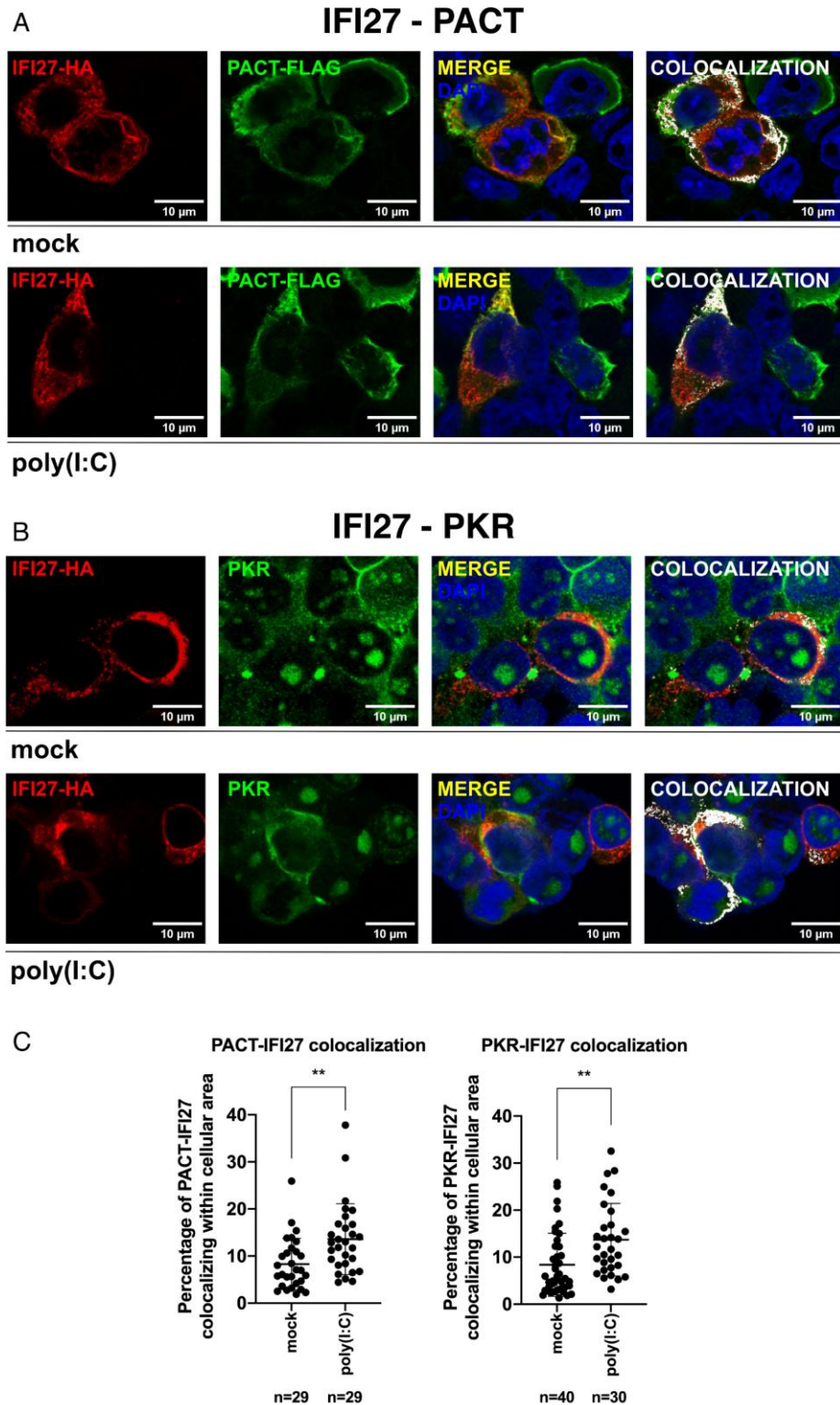


Figure R37. IFI27 partially colocalizes with PACT and PKR in the cell cytoplasm. HEK-293T cells were co-transfected with pCAGGS-IFI27-HA and pCAGGS-PACT-FLAG plasmids (A, for IFI27-PACT colocalization), or with pCAGGS-IFI27-HA only (B, for IFI27-PKR colocalization). At 24 hpt, cells were either mock- or poly(I:C)-transfected (3 μ g/mL) for 24 hours. Next, cells were fixed with formaldehyde and permeabilized, and an immunofluorescence to detect IFI27-HA (A, B), PACT-FLAG (A), or endogenous PKR (B) was performed, using the specific primary antibodies anti-HA, anti-FLAG, and anti-PKR, respectively, and the following secondary antibodies: anti-rabbit conjugated to Alexa Fluor 594 (to detect anti-HA, red fluorophore) or anti-mouse conjugated to Alexa Fluor 488 (to detect anti-FLAG or anti-PKR, green fluorophore). Nuclei were stained using DAPI. Cytoplasm colocalization is shown in yellow

(merge) in the third picture of each row, and in white (Colocalization) in the fourth picture. (C) For each cell, colocalization of IFI27 and PACT or IFI27 and PKR was measured and compared between mock- and poly(I:C)-conditions. To measure this, we divided the area where IFI27 (Alexa Fluor 594, red) colocalized with PACT or PKR (Alexa Fluor 488, green) between the total cell area. This ratio was transformed into percentage of colocalization and represented on the Y-axis. The number of cells analyzed for each condition is shown below the graphs. Each cell result for each condition is represented with dots. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in C). Scale bar: 10 μm . These measures were performed in two independent experiments, obtaining similar results.

4.3.1.3.3. IFI27 enhances PKR activation through a PACT-dependent mechanism

PKR protein is constitutively expressed within all vertebrates, but its expression is further induced by IFNs in the context of a pathogen infection, as PKR gene contains an ISRE element in its promoter [128,129]. Activated PKR leads to host shut-off or translation stalling [76,138], being this function of PKR also regulated by different protein partners, with PACT as one of the best studied.

4.3.1.3.3.1 IFI27 overexpression promotes PKR activation and consequently suppresses *de novo* protein synthesis

Previously, we observed that IFI27 protein is able to interact with both PACT and PKR, especially in presence of dsRNA (Figs. R33-R36). Therefore, we wanted to study the biological importance of these interactions, being one likely possibility the modulation of PKR function. To explore this, we transfected HEK-293T cells with the pCAGGS-IFI27-HA plasmid, or the empty pCAGGS plasmid as a negative control, plus a pRL plasmid expressing the Renilla Luciferase (RLuc, pRL-RLuc). RLuc luminescence assay allows us to quantify the total protein synthesis within cells, as the more RLuc that is translated, the more luminescence signal we will detect. That way, if PKR is more active, it will phosphorylate eIF2 α , translation rate will be reduced, and less luminescence will be detected (Fig. R38). 24 hpt, cells were either mock-infected or infected with SARS-CoV-2 (MOI 1) for 6 or 24 hours, or with rVSV-GFP (MOI 0.1) for 8 or 24 hours (Fig. R39A), two different, unrelated, RNA viruses. PKR is an important host factor that responds to the infection with these two viruses by inhibiting viral protein synthesis in order to stop viral replication [269,298]. Viral titers were measured in the cell supernatants by plaque assay, confirming that cells were properly infected with SARS-CoV-2 (24 hours) (Fig. R39B) or rVSV-GFP (8 and 24 hours) (Fig. R39C).

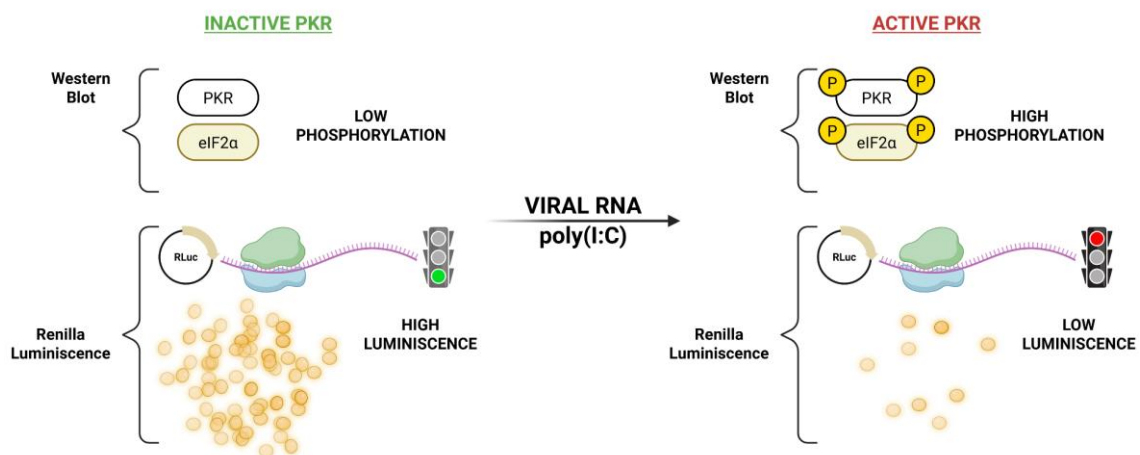


Figure R38. Schema of the experimental concept for analyzing PKR activation levels and the overall state of cellular translation by Western blot and RLuc luminescence. When PKR is in an inactive state, phosphorylation levels of both PKR and eIF2 α are low, which correlates with a higher translational rate in cells. Thus, this results in a higher translation of RLuc, and higher luminescence levels detected by a luminometer. However, when PKR is activated by the recognition of viral RNAs or poly(I:C), the phosphorylation levels of PKR and eIF2 α will increase substantially, and this will lead to a lower translational rate in cells. Therefore, less RLuc will be produced, and less luminescence will be detected by a luminometer.

Then, at 24 hpi, the levels of luminescence were analyzed with a luminometer. Interestingly, IFI27 overexpression, confirmed by Western blot using an anti-HA specific antibody (Fig. R39E, R39H), led to a reduction of RLuc luminescence compared to the empty plasmid-transfected cells in mock-infected (Fig. R39D and R39G), SARS-CoV-2-infected (Fig. 39D), and rVSV-GFP-infected (Fig. R39G) cells. A non-canonical PKR activation in mock-infected cells could explain this observation, as endogenous RNAs such as nuclear and mitochondrial [165,299] have been described to activate PKR functions.

To investigate if the reduction in RLuc levels could be due to an increase in PKR activity, the same protein extracts used to measure RLuc luminescence levels were employed to perform a Western blot to measure S52-phosphorylated eIF2 α (eIF2 α -P) and T446-phosphorylated PKR (PKR-P) levels, which were normalized to total eIF2 α and total PKR levels, respectively, as well as to the total levels of β -actin as a loading control (Fig. R39E, R39H). As expected, at 6 hours, SARS-CoV-2 infection increased eIF2 α -P levels by 2-fold compared to mock-infected cells, both in empty plasmid and IFI27-plasmid transfected cells (Fig. R39E, R39F). Interestingly, at 24 hpi, we observed that eIF2 α -P levels were increased 2-fold on empty plasmid-transfected cells, while in IFI27-plasmid transfected cells this increase was higher, up to 3.9-fold, compared to the control mock-infected cells transfected with the empty plasmid (Fig. R39E, R39F). In mock conditions, we observed similar levels of PKR-P in empty plasmid- and IFI27 plasmid-transfected cells at 6 h, but at 24 h, PKR-P levels were incremented by 1.8-fold in IFI27-overexpressing cells compared to the empty plasmid-transfected cells (Fig. R39E, R39F). After SARS-CoV-2 infection, at 6 hpi, we observed an increment in PKR-P levels of 7.4- and 9.6-fold in empty and IFI27 plasmid-transfected cells, respectively, compared to cells transfected with the empty plasmid and mock-infected (Fig. R39E, R39F). However, at 24 hpi with SARS-CoV-2, PKR-P levels were reduced by 1.2- and 1.7-fold in empty and IFI27 plasmid-transfected cells, respectively, compared to mock-infected cells at the same time point (Fig. R39E, R39F). This shows that this virus, at later infection time points, is able to efficiently counteract the activation of PKR, an effect which has extensively been described before. Various proteins of SARS-CoV-2, and other coronaviruses, have been shown to inhibit PKR mediated pathway. For example, the N proteins of SARS-CoV-2, as well as its closely related virus, the bat coronavirus RaTG13 (bat-CoV-RaTG13) [300], and other HCoV-229E [301], are able to inhibit PKR activation. Also, the nsp15 proteins of SARS-CoV-2 and MERS-CoV, have been described to act as potent inhibitors of dsRNA-induced innate immune pathways such as the PKR pathway [302].

Next, to have a broader view of PKR activation after the infection of different RNA viruses, we analyzed eIF2 α -P and PKR-P levels under rVSV-GFP infections. eIF2 α -P and PKR-P levels behaved similarly after IFI27 overexpression, as we observed a 1.5- and 2-fold increase at 24 hpi compared to empty plasmid-transfected cells. PKR-P levels, also showed a 2-fold increment at 8 hpi (Fig. R39H, R39I). This again demonstrates that IFI27 overexpression leads to higher levels of PKR phosphorylation, and consequently, increased PKR activation and increased eIF2 α phosphorylation, correlating with the results from RLuc luminescence measurements, where

IFI27 overexpression reduced luminescence, a reduction attributable to heightened PKR activity. Thus, the results obtained after SARS-CoV-2 and rVSV-GFP infections were comparable, showing that IFI27 mediated regulation of PKR activity could be broad to infections with different RNA viruses.

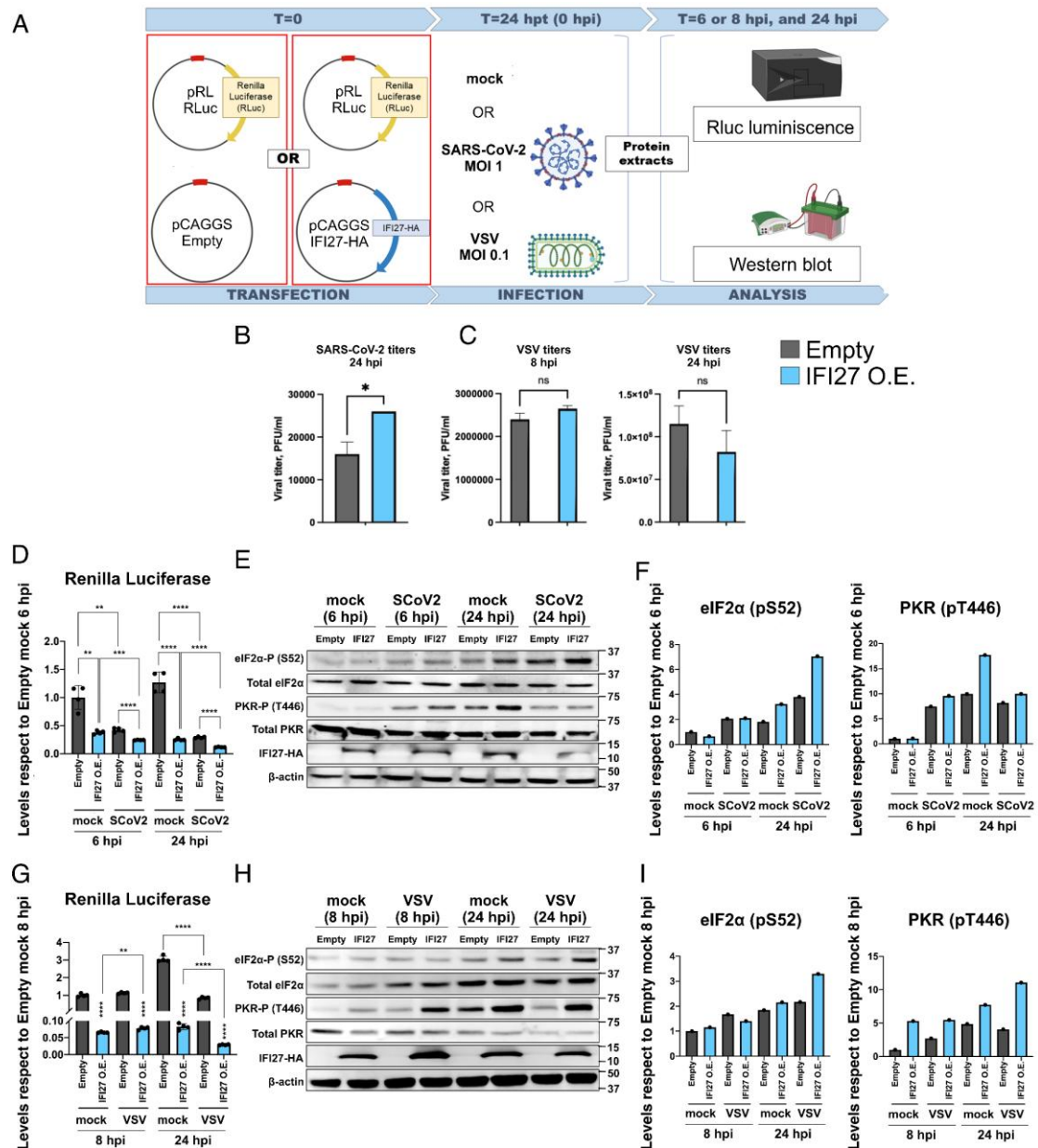


Figure R39. IFI27 overexpression enhances PKR activation and function. (A) Schema of experimental timeline. (B, D, E, F) HEK-293T-hACE2 or (C, G, H, I) HEK-293T cells were transiently transfected with a pCAGGS-IFI27-HA plasmid (IFI27 overexpression, O.E.) or an empty pCAGGS plasmid (Empty) as a negative control, together with the RLuc encoding pRL plasmid. At 24 hpt, cells were infected with SARS-CoV-2 (SCoV2) (MOI 1) (B, D, E, F) or rVSV-GFP (MOI 0.1) (C, G, H, I). (B, C) SARS-CoV-2 (SCoV2) (B) or rVSV-GFP (C) titers on the supernatants of transfected and infected cells were determined by plaque assay (PFU/mL) in confluent monolayers of Vero E6 cells, as previously described in materials and methods section 3.3.2, Fig. M6. Data is represented as PFU/mL. (D, G) At 6 and 24 hpi for SARS-CoV-2 infections (D) or 8 and 24 hpi for rVSV-GFP infections (G), protein extracts were harvested and the levels of RLuc luminescence were measured. Individual data points correspond to independent measures from different technical samples. Luminescence levels were represented as fold-change respect to mock-infected cells transfected with the empty plasmid at 6 hpi (D) or 8 hpi (G). (E, H) Protein extracts were also

employed to measure protein levels of eIF2 α -P (S52), total eIF2 α , PKR-P (T446), total PKR, IFI27-HA, and β -actin (loading control) by Western blot, using their respective specific antibodies. Molecular weights are indicated on the right of the blots (in kDa). Protein abundance of eIF2 α -P and PKR-P was measured by densitometry using ImageJ software (**F, I**). eIF2 α -P levels were normalized with total eIF2 α and β -actin (loading control); and PKR-P levels were normalized with total PKR and β -actin (loading control). The results of the quantifications are shown in bars on the right of the associated blot and represented as fold-changes respect to mock-infected, empty plasmid-transfected cells at the earliest time point. Data is represented as the means and standard deviations of the different measures. Ns (non-significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in **B, C, D, and G**). These measures were performed in two or more independent experiments with similar results.

4.3.1.3.3.2. KO and knock-down of IFI27 reduce PKR activation and increases *de novo* protein synthesis

To further dissect the modulation of PKR activity by IFI27, and since previous experiments involved the overexpression of IFI27 protein, we wanted to test this in a different experimental system. With this objective, we used both parental A549-hACE2 WT cells and the previously generated A549-hACE2-IFI27-KO cells, to assess the impact of IFI27 deficiency on PKR activation. First, WT and IFI27-KO cell lines were transfected with the pRL-RLuc plasmid, and 24 hours later, cells were either mock-transfected or transfected with increasing concentrations of poly(I:C) (**Fig. R40A**). As expected, the transfection with increasing concentrations of poly(I:C) resulted in a reduction of RLuc luminescence levels, consistent with poly(I:C)-mediated activation of PKR [303]. Remarkably, in the absence of IFI27 expression (IFI27-KO cell line), the levels of RLuc luminescence were 2.2- to 2.5-fold higher than in WT cells both in mock- and poly(I:C)-transfected cells (**Fig. R40B**). To confirm that the increment in RLuc luminescence was due to a reduction in PKR activation, the same cellular extracts were analyzed by Western blot to detect PKR-P, as well as total PKR and β -actin for normalization (**Fig. R40C**). As expected, PKR-P levels increased together with increasing concentrations of poly(I:C), and the levels of PKR-P were 1.5-fold higher in WT cells compared to IFI27-KO cells, both in mock and poly(I:C) conditions (**Fig. R40C, R40D**). Therefore, higher RLuc luminescence correlated with lower PKR-P levels, as it has been previously published [304]. These findings show that IFI27 absence leads to a significant reduction in PKR activity, which correlates with an increment in RLuc luminescence, as previously explained (**Fig. R38**).

Next, since IFI27-KO led to a clear phenotype (reduced PKR activation in response to poly(I:C)), and to further prove that this effect was caused by IFI27 absence and not to an unspecific effect, we sought to determine whether restoring IFI27 expression could restore higher levels of PKR pathway activation and rescue the phenotype. To achieve this, we used the previously described (**Fig. R27**) A549-hACE2-empty cells (IFI27 KO cells stably transfected with an empty plasmid, as a control) or the A549-hACE2-IFI27-HA cells, knocked-out for endogenous IFI27 expression, but stably overexpressing IFI27-HA (**Fig. R40E**). These cells were transfected with the RLuc expressing plasmid, and 24 hpt, cells were treated with poly(I:C) for 8 hours. IFI27 expression in IFI27-KO cells alone was able to restore PKR activation, showed by a significant reduction of RLuc luminescence levels compared to IFI27-KO cells transfected with the empty plasmid (**Fig. R40F**), indicating that the lower PKR activation in the IFI27-KO cells is due to the lack of IFI27 expression and not to something else.

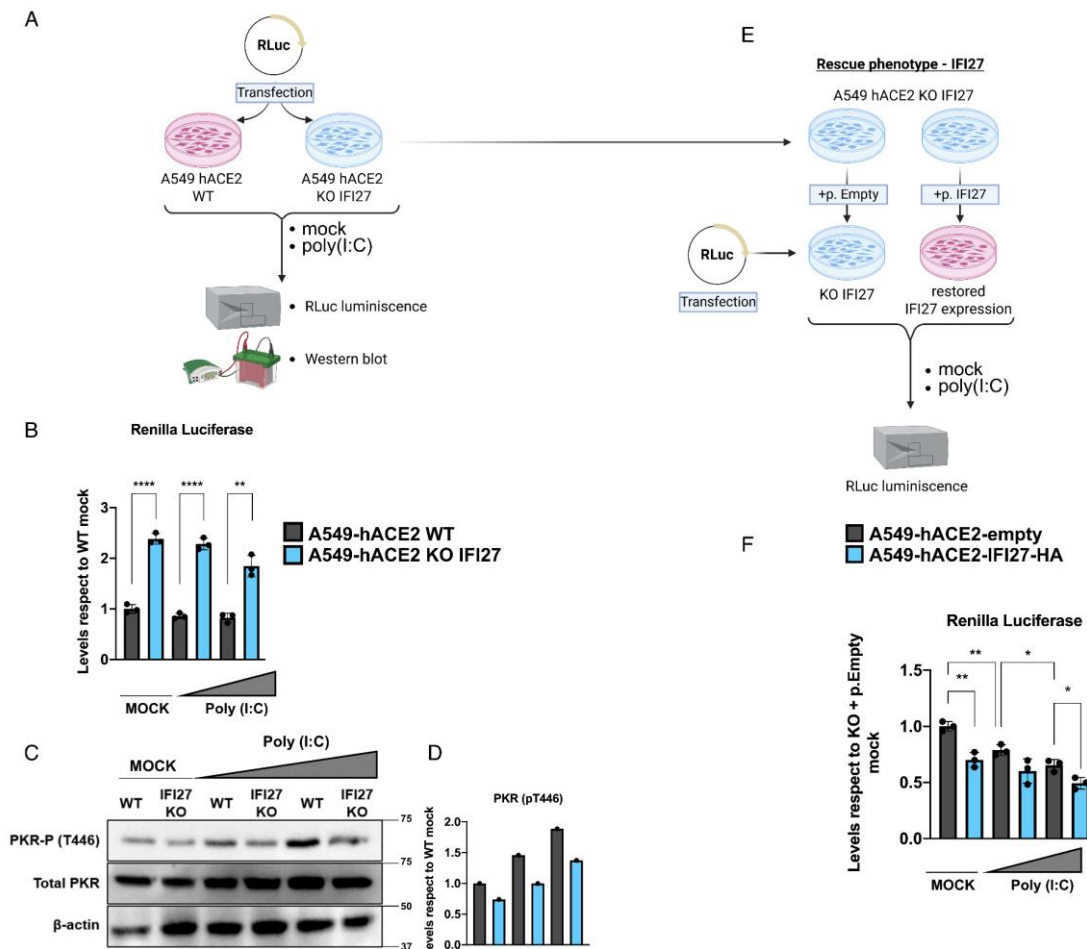


Figure R40. IFI27 KO leads to a phenotype of lower PKR activation, which is rescued by restoring IFI27 expression. **(A)** Schema of experimental timeline. **(A-D)** WT or IFI27-KO A549-hACE2 cells were transfected with the pRL plasmid expressing RLuc, and 24 hpt cells were either mock-transfected or transfected with increasing concentrations of poly(I:C) (50 and 250 ng/mL) for 7 hours. Next, protein lysates were obtained and the levels of RLuc luminescence were measured in a luminometer **(B)**. Values are represented as fold of change respect to mock-transfected WT cells. Individual data points correspond to independent measures from different technical samples. **(C)** Same cellular extracts were analyzed by Western blot in order to measure the protein levels of PKR-P (T446), total PKR, and β -actin (loading control) with the specific antibodies. **(D)** Protein amounts were measured by densitometry using ImageJ software; the levels of PKR-P (T446) were normalized by total PKR and by β -actin (loading control). Results of the quantification are shown as fold of change respect to the amount of normalized PKR-P in mock-treated WT cells. Molecular weights are indicated on the right of the panels (in kDa). **(E)** Schema of experimental timeline. **(E, F)** A549-hACE2 IFI27-KO cells stably transfected with a linearized pCAGGS plasmid expressing IFI27-HA (A549-hACE2-IFI27-HA), to restore IFI27 expression, or a linearized empty pCAGGS plasmid (A549-hACE2-empty), as a control, were transfected with a pRL plasmid encoding RLuc and 24 hpt, mock- or poly(I:C)-transfected, with increasing concentrations (50 and 250 ng/mL) for 8 hours. Next, protein lysates were extracted, and the level of RLuc luminescence was measured. **(F)** Luminescence levels are represented as fold of change respect to mock-transfected A549-hACE2-empty cells. Individual data points correspond to independent measures from different technical samples. Data is represented as the means and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in **B** and **F**). These measures were performed in two or more independent experiments with similar results.

Transfection procedures have been shown to activate IFN pathways as well as the PKR-dependent pathways [140,305]. To avoid this potential bias, in all experiments we use the same amounts of

lipofectamine reagents for every condition. Also, empty-plasmid transfections are included as controls to equal the amount of transfected DNA in every sample, and when necessary, cells were co-transfected with the empty plasmid. However, to strongly support all our previous findings, we studied PKR modulation by IFI27 in the absence of transfection. To achieve this, A549-hACE2 WT cells or A549-hACE2 IFI27-KO cells were infected with SARS-CoV-2, and 6 or 24 hpi, cellular extracts were harvested and used to detect the levels of eIF2 α -P and PKR-P by Western blot. In IFI27-KO cells, protein levels of eIF2 α -P were near 2-fold lower in IFI27-KO cells compared to WT cells, both at 6 and 24 hpi with SARS-CoV-2 (Fig. R41A, R41B). Similarly, PKR-P levels were near 2-fold lower in IFI27-KO cells compared to WT cells at 24 hpi with SARS-CoV-2 (Fig. R41A, R41B). Thus, even without transfection procedures, IFI27 functions as a positive modulator of PKR, as its KO leads to a clear reduction in PKR activation.

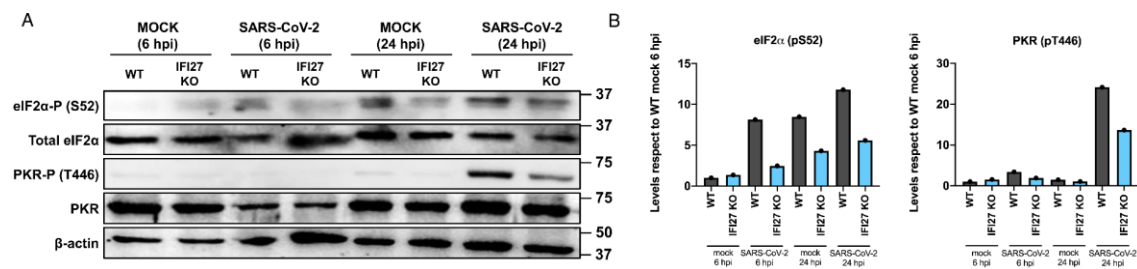


Figure R41. IFI27 KO leads to a lower PKR and eIF2 α phosphorylation levels after SARS-CoV-2 infection. (A, B) A549 hACE2 IFI27-KO or WT cells were either mock-infected or infected with SARS-CoV-2 (MOI 0.5). (A) At 6 and 24 hpi, cellular extracts were harvested and subjected to a Western blot analysis to measure the protein levels of eIF2 α -P (S52), total eIF2 α , PKR-P (T446), total PKR, and β -actin (as a loading control), using their respective antibodies. Molecular weights are indicated on the right of the panels (in kDa). (B) Protein levels were quantified by densitometry using the ImageJ software. eIF2 α -P and PKR-P levels were normalized to the amount of total eIF2 α and β -actin, and to the amount of total PKR and β -actin, respectively. Values representing the fold of change respect to mock-infected WT cells at 6 hpi. These measures were performed in two independent experiments with similar results.

In addition to employing the IFI27-KO cell lines we developed, as an alternative approach we used specific siRNAs targeting IFI27. After confirming the proper knock-down of IFI27 expression in mock and SARS-CoV-2-infected cells by qRT-PCR (Fig. R42A), RLuc expression (Fig. R42B) and phosphorylation levels of eIF2 α and PKR (Fig. R42C) were measured. In concordance with the KO results (Fig. R40, R41), IFI27 knock-down resulted in 2.2-fold higher RLuc luminescence (Fig. R42B) and a lower band intensity of both eIF2 α -P and PKR-P (Fig. R42C) compared to the control cells, indicating that IFI27 absence leads to a lower PKR activation. This IFI27 knock-down was also performed in the HeLa cell line following the same procedure, to rule out the possibility that the observed modulation of PKR function is cell-specific. Both in mock and poly(I:C) conditions, IFI27 knock-down resulted in a near 3-fold increment of RLuc luminescence, compared to the control cells (Fig. R42D), which correlated with a ~2-fold reduction in both eIF2 α -P and PKR-P levels in poly(I:C)-treated cells transfected with the IFI27-specific siRNA, compared to cells transfected with the NT siRNA (Fig. R42E). All in all, both in A549 and HeLa cells, IFI27 absence leads to a weaker PKR activation, validating these results in two different cell lines.

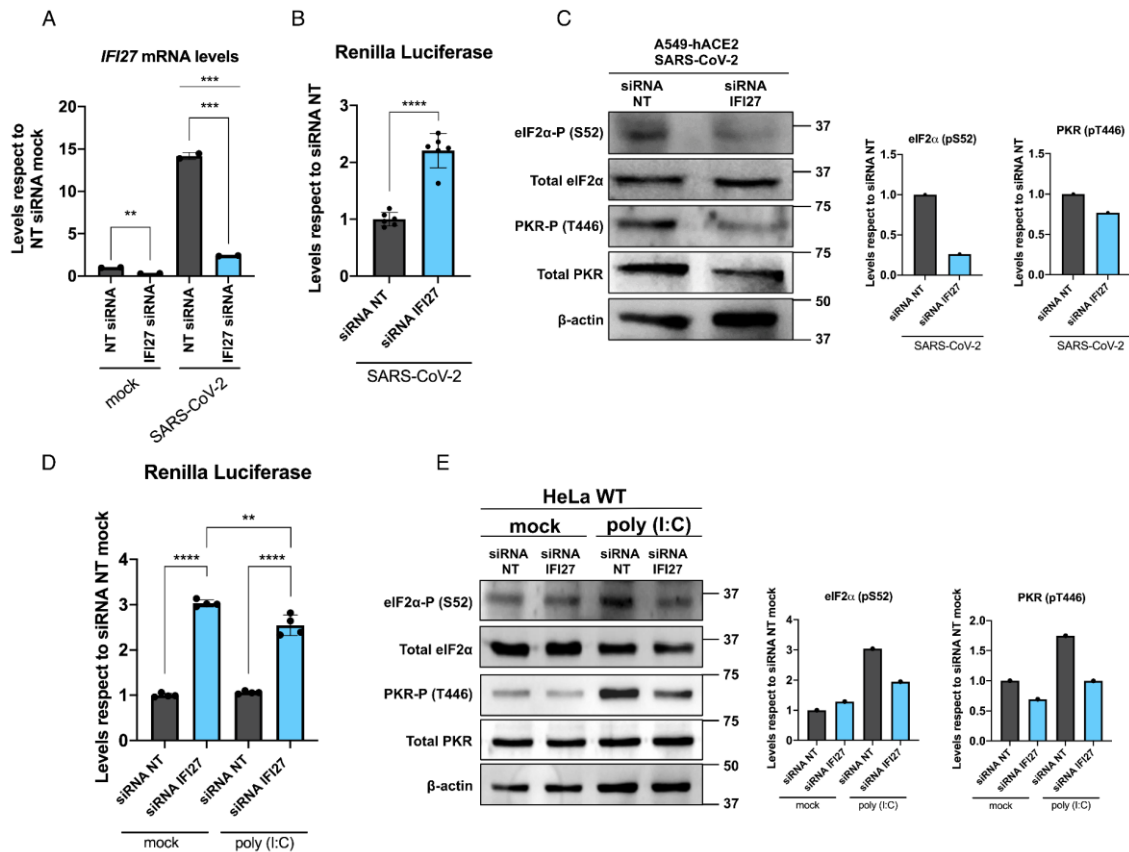


Figure R42. IFI27 knock-down hampers PKR activation both after SARS-CoV-2 infection and poly(I:C) treatment. A549-hACE2 (A-C) or HeLa (D-E) WT cells were transfected twice, with a 24-hour gap between each transfection, with an IFI27 siRNA, or alternatively, with the NT siRNA as a control. **(A)** At 24 hours after the second transfection, cells were either mock-infected or infected with SARS-CoV-2 (MOI 1) for 24 hours. Total RNA was extracted, and the mRNA levels of *IFI27* and *GAPDH* (as a housekeeping gene) were measured by qRT-PCR. mRNA levels are represented as fold of change respect to mock-treated cells transfected with the NT siRNA. **(B, C, D, E)** 24 hours after the second siRNA transfection, cells were transfected with a pRL plasmid encoding RLuc, and 6 hours later, cells were infected with SARS-CoV-2 (MOI 1) for 24 hours **(B, C)** or cells were either mock-treated or treated with poly(I:C) (2 µg/ml) for 16 hours **(D, E)**. Protein extracts were obtained by lysis. **(B, D)** RLuc luminescence levels were measured and represented as fold of change respect to control cells transfected with the NT siRNA. Individual data points correspond to independent measures from different technical samples. **(C, E)** Same protein extracts were employed to perform a Western blot to detect protein levels of eIF2α-P (S52), total eIF2α, PKR-P (T446), total PKR, and β-actin by Western blot employing their respective antibodies. Molecular weights are indicated on the right of the panels (in kDa). Western blots were quantified by densitometry using ImageJ software. The amount of eIF2α-P (S52) was normalized by total eIF2α and β-actin. The amount of PKR-P (T446) was normalized by total PKR and β-actin. Levels are shown as fold-changes respect to the control cells transfected with the NT siRNA **(C, E)**. Data is represented as the mean and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in **A, B, and D**). These measures were performed in two independent experiments with similar results.

4.3.1.3.3. PACT is essential for IFI27-mediated PKR activation

As we have previously mentioned, PACT is a dsRNA binding protein and regulator of PKR function [140]. Different studies describe that under stress conditions, PACT binds PKR, and shifts the equilibrium to the active form of PKR [130,132,248]. However, some studies show that PACT can also act as an inhibitor of PKR function [150–152].

We have demonstrated that IFI27 positively affects PKR function (Figs. R39-R42), and we detected both PKR and PACT in the IFI27 protein interactome (Fig. R33, Table A3). Thus, we wanted to test the importance of PACT in this interaction. To this end, Prof. Thomas Michiels from Université Catholique de Louvain, Belgium, kindly provided us with different HeLa cell lines KO for PACT, PKR, and the control WT cells [131]. With these cell lines, we can test the importance of PACT on the PKR activation mediated by IFI27 protein. First, KO of both PACT and PKR cell lines were confirmed by Western blot (Fig. R43C, R43D, respectively). Next, HeLa WT, PACT-KO, and PKR-KO were transiently co-transfected with either the pCAGGS-IFI27-HA plasmid or the pCAGGS empty plasmid, together with the pRL-RLuc plasmid. At 24 hpt, cells were either mock-transfected or transfected with poly(I:C) for 7 hours and RLuc luminescence was measured (Fig. R43B). When PKR activity is lower, translational activity is higher and RLuc luminescence is increased [304], so as expected, in PKR-KO cells, the levels of RLuc luminescence were highly increased compared to WT cells (Fig. R43B). Also, correlating with previous results, IFI27 overexpression in HeLa WT cells led to a 3-fold decrease on RLuc luminescence compared to empty control, both in mock- and poly(I:C)-transfected cells (Fig. R43B). In mock conditions, the overexpression of IFI27 in PACT- and PKR-KO cells resulted in only a slight reduction in RLuc luminescence relatively to cells transfected with the empty plasmid (Fig. R43B). However, remarkably, following poly(I:C) treatment, IFI27 overexpression did not reduce RLuc luminescence levels in either PACT- or PKR-KO cells (Fig. R43B). These results strongly suggest that the ability of IFI27 to reduce luminescence by PKR activation is impaired in the absence of PACT and PKR, meaning that the effect of IFI27 is both PACT- and PKR-dependent. As mentioned before, in the absence of exogenous stressors such as viral infections, PACT and ADAR1 suppress PKR activity, avoiding PKR activation in response to endogenous dsRNAs ligands, which could lead to deleterious effects [152]. So, it is noteworthy that in cells transfected with the empty plasmid in mock conditions, the RLuc luminescence levels are lower in the PACT-KO than in the WT cells (Fig. R43B). This observation could mean that in the absence of PACT, PKR is insufficiently suppressed and reacts against endogenous or plasmid-derived dsRNAs, leading to the enhanced PKR-mediated translation stalling that we observe in empty mock conditions in the PACT-KO cells.

To further confirm that the observations with RLuc luminescence can be attributed to PKR activity, same cellular extracts were used to perform Western blot analysis to detect the protein band intensities of eIF2 α -P and PKR-P. As expected [303], in the empty plasmid-transfected, HeLa WT cells, poly(I:C) treatment led to the proper activation of PKR, visualized by a 1.8- and 4.7-fold increase of band intensity of normalized eIF2 α -P and PKR-P, respectively (Fig. R43C). We observed that IFI27 overexpression in poly(I:C)-treated HeLa WT cells resulted in an increase of 2.1- and 2.4-fold of protein band intensity of eIF2 α -P and PKR-P, respectively, compared to the control cells (Fig. R43C). However, this effect was lost in HeLa PACT-KO cells, as IFI27 overexpression did not modify the band intensity of eIF2 α -P and PKR-P compared to the empty plasmid-transfected cells (Fig. R43C). These results are consistent with corresponding RLuc luminescence measurements (Fig. R43B), further supporting that IFI27-mediated modulation of PKR function, even under transfection conditions, critically depends on the expression of both PACT and PKR proteins.

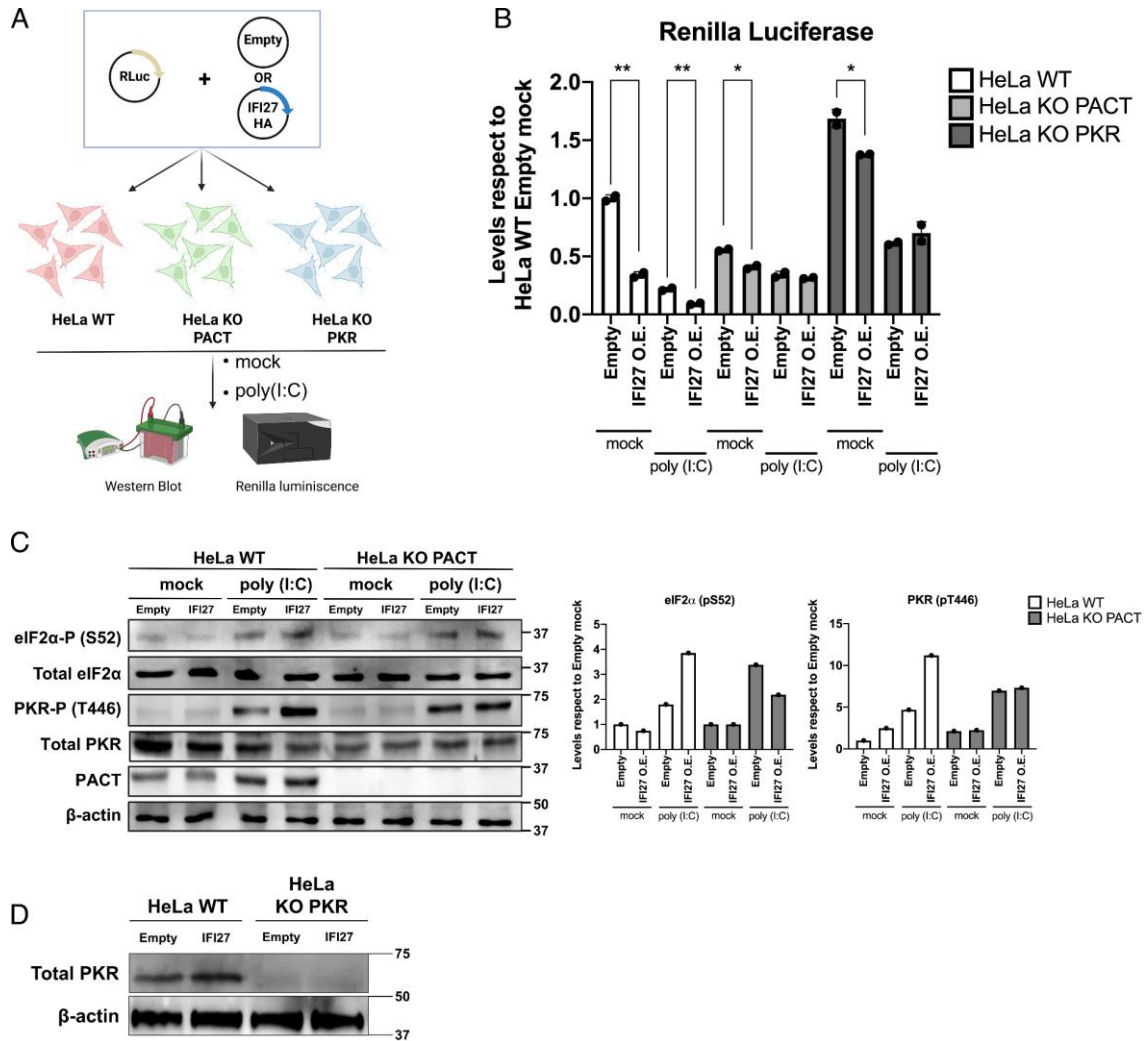


Figure R43. PACT deficiency impairs the activation of PKR by IFI27. (A) Schema of experimental timeline. **(B)** HeLa WT, PACT-KO, and PKR-KO cells were transiently transfected with the plasmids pCAGGS-IFI27-HA (IFI27 O. E.) or the pCAGGS-empty (Empty), as a negative control, together with the pRL-RLuc plasmid. At 24 hpt, cells were either mock-transfected or transfected with poly(I:C) (2 μ g/ml) for 7 hours. Next, cellular extracts were harvested and we measured RLuc luminescence levels, which were represented as fold of change respect to empty plasmid-transfected, mock-treated HeLa WT cells. Individual data points correspond to independent measures from different technical samples. **(C, D)** Same protein extracts from HeLa WT and HeLa PACT-KO **(C)** or HeLa WT and HeLa PKR-KO **(D)** cells were employed to perform a Western blot in order to measure protein levels of eIF2 α -P, total eIF2 α , PKR-P, total PKR, PACT, and β -actin (loading control) **(C)**, or total PKR and β -actin (loading control) **(D)**. Molecular weights are indicated on the right of the panels (in kDa). Western blots were quantified by densitometry using the ImageJ software. eIF2 α -P amount was normalized with total eIF2 α and β -actin. PKR-P was normalized with total PKR and β -actin. Protein levels from densitometry quantification are shown as fold of change respect to empty plasmid-transfected, mock-treated HeLa WT cells. Data is represented as the mean and standard deviations of the different measures. $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in **B**). These measures were performed in two independent experiments with similar results.

4.3.1.3.4. IFI27 overexpression potentiates stress granules (SGs) formation in a PKR and PACT-dependent manner

As mentioned in the introduction, SGs are membrane-less aggregates that are generated in the cell cytoplasm in response to a variety of cellular stressors, for instance, after viral infections. When a viral infection is detected, PKR is activated and translation is stopped following eIF2 α phosphorylation. Translation arrest leads to the dissociation of translation initiation complexes, and the different components of SGs are recruited and aggregated. Consequently, the generation of SGs is a reliable indicator of PKR activation in response to stimuli such as viral infections or poly(I:C) [156,158]. Therefore, the analysis of the assembly of these cytoplasmic structures provides an additional way to study how IFI27 modulates PKR function.

First, we confirmed the SGs formation in HeLa WT cells by transfecting these cells with poly(I:C), to induce a stressor stimulus. At 7 hours after poly(I:C) treatment, SG formation was detected by immunostaining of the SG's markers eIF3 η (also referred to as EIF3A) and G3BP1 [131,154,156,157,303], which appeared as discrete and brilliant dots. Also, these SG's markers (eIF3 η and G3BP1) colocalized with PKR foci (Fig. R44A), confirming that these three elements are present in poly(I:C)-induced SGs. As PKR is one of the main inducers of SGs generation [156], we studied whether IFI27 overexpression could promote the generation of SGs within the cell cytoplasm by enhancing PKR activation, and we also considered the potential modulatory role of PACT. To this end, both HeLa WT and HeLa PACT-KO cells were transfected with either the pCAGGS-IFI27-HA plasmid, or the empty pCAGGS plasmid as a control. At 24 hpt, cells were treated with poly(I:C) for 7 hours, and then, cells were fixed and permeabilized. SGs formation was studied by immunofluorescence, as we measured the percentage of cells containing SGs out of the total number of analyzed cells in each condition, considering the staining of both eIF3 η and G3BP1 proteins. In cells transfected with the empty plasmid, 25.8% of HeLa WT and 26.6% of PACT-KO cells contained SGs (Fig. R44B, R44C). Interestingly, after IFI27 overexpression, we observed a significative increment of the percentage of SGs-containing HeLa WT cells to 47.4%, a ~2-fold increase compared to the percentage observed in the control cells (Fig. R44B, R44C). Remarkably and consistent with previous results (Fig. R43), in PACT-KO cells overexpressing IFI27, the percentage of cells containing SGs was 32.1%, which did not represent a statistically significant increase compared to the 26.6% observed in the control PACT-KO cells transfected with the empty plasmid (Fig. R44B, R44C).

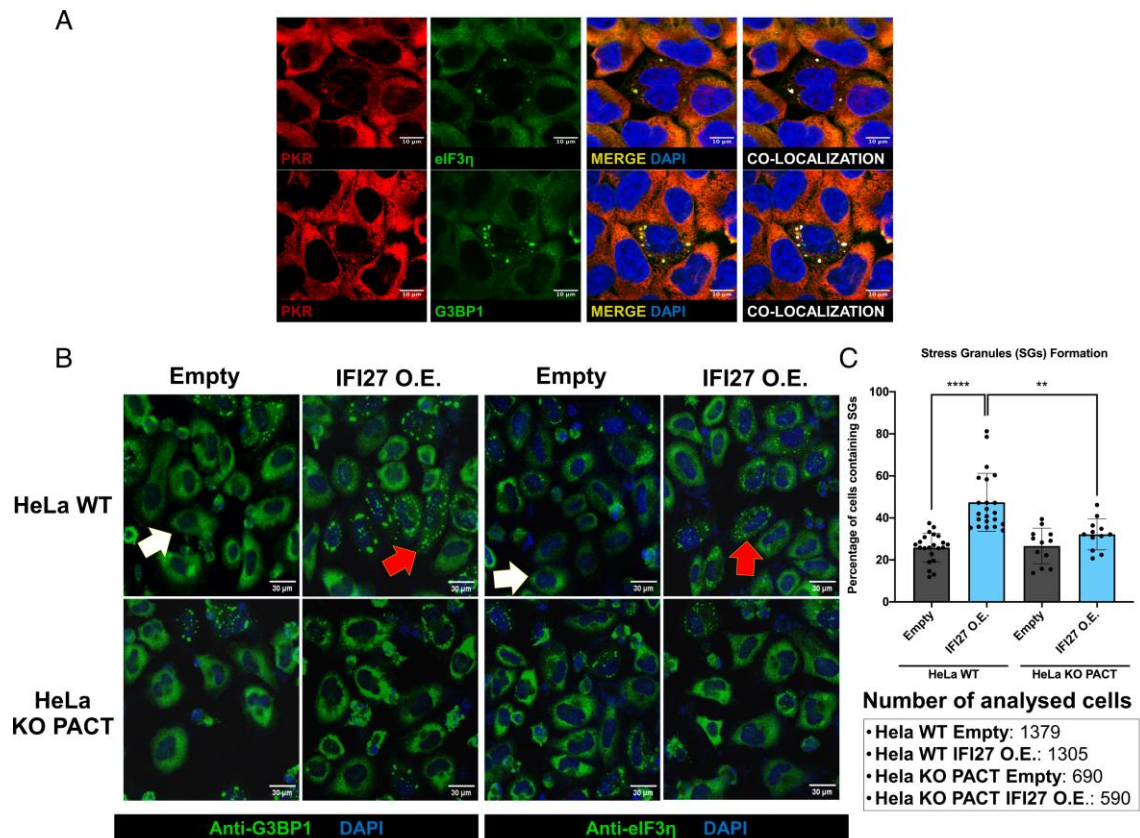


Figure R44. IFI27 overexpression promotes SGs generation in a PACT-dependent manner. (A) HeLa WT cells were treated for 7 hours with poly(I:C) (2 μ g/mL) and then, fixed with formaldehyde and permeabilized to perform an immunofluorescence. PKR and eIF3 η or PKR and G3BP1 were labelled with specific antibodies for each protein. PKR is shown in red, while eIF3 η and G3BP1 are shown in green. Colocalization between PKR and eIF3 η or PKR and G3BP1 appear in yellow in the third picture of each line (merge) and in white in the fourth (co-localization). Nuclei are stained with DAPI and shown in blue. Scale bar, 10 μ m. **(B, C)** HeLa WT and PACT-KO cells were transfected with either pCAGGS-IFI27-HA plasmid, or the empty pCAGGS plasmid as a control. At 24 hpt, cells were treated with poly(I:C) (2 μ g/mL) during 7 hours, and then, fixed with formaldehyde and permeabilized. An immunofluorescent was performed to detect the generation of SGs by staining of the SGs markers eIF3 η and G3BP1 with their respective antibodies (both shown in green). The white arrow **(B)** indicates a cell with no presence of SGs, while the red arrow **(B)** indicates a cell with positive presence SGs, shown by the staining of eIF3 η or G3BP1 in discrete dots. **(C)** The formation of SGs inside the cells was quantified. Each independent dot in the bars **(C)** indicates the percentage of SGs-containing cells within one specific field analyzed, considering both eIF3 η and G3BP1 staining. Each field contain several cells, so the total number of cells analyzed is indicated. Scale bar, 30 μ m. Data is represented as the mean and standard deviations of the different measures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in C). **B** and **C** are representative of three independent experiments.

Transfection efficiency in HeLa cells using Lipofectamine 3000 can reach levels up to a 70% [306]. To further dissect the effect of IFI27 on SGs generation, we wanted to measure the percentage of cells containing SGs only on cells that had been properly transfected, confirmed by staining for IFI27-HA expression. To this end, HeLa WT and HeLa PACT-KO cells were transfected and treated with poly(I:C) as before. The cells were fixed and permeabilized, and an immunostaining of IFI27-HA and G3BP1 (as a SG marker) was performed. In this case, within the total of cells with a positive staining for IFI27-HA (shown in red in Fig. R45A, and as a white bar in R45B) we measured the percentage of cells containing SGs (positive signal of G3BP1,

shown in green or merge in [R45A](#) or as a grey bar in [R45B](#)). By measuring this, we ensured that we considered IFI27 overexpression. IFI27 overexpression resulted in the assembly of detectable SGs in 44.1% of HeLa WT cells, while only 17% of HeLa PACT-KO cells overexpressing IFI27 were positive for SGs formation ([Fig. R45A, R45B](#)). This confirms previous results, supporting that IFI27 induces PKR activation and SGs formation, and this is dependent on the presence of PACT protein.

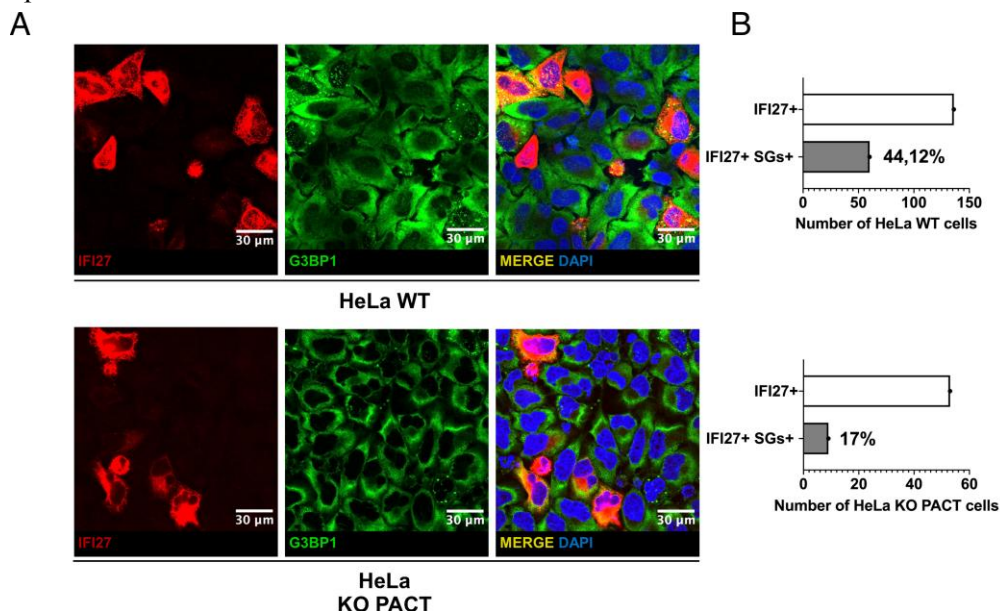


Figure R45. SGs generation in IFI27-overexpressing cells is reduced in the absence of PACT. **(A, B)** HeLa WT and HeLa PACT-KO cells were transfected with an IFI27-HA overexpressing plasmid and 24 hpt, treated with poly(I:C) (2 μ g/mL) for 7 hours. Next, cells were fixed with formaldehyde and permeabilized to perform an immunofluorescence. IFI27-HA (shown in red) and G3BP1 (shown in green) were labelled using their specific antibodies. The third picture shows colocalization of both fluorophores in yellow (merge). Nuclei are stained with DAPI and shown in blue. Scale bar, 30 μ m. **(B)** A quantification of the total IFI27-HA positive cells counted (white bar), and the number of SGs positive cells within the total IFI27-HA positive cells (grey bar) is represented for both HeLa WT and PACT-KO cells, and this quantification is shown as a percentage on the right of the grey bar. Also, the total number of counted cells is shown in the x-axis: 136 IFI27+ cells and 60 IFI27+SG+ cells for HeLa WT cells; 53 IFI27+ and 9 IFI27+ SG+ cells for HeLa KO PACT cells. **A** and **B** are representative of two independent experiments.

It has been previously shown that PKR overexpression is sufficient for its own activation, even in the absence of exogenous stressors [131]. Our next objective was to determine whether the presence of PACT is essential for IFI27-mediated regulation of PKR activity following PKR overexpression. To this end, we employed HeLa WT, PACT-KO, PKR-KO, and HeLa cells KO for both PACT and PKR (DKO) cells. Each of these cell lines were transfected with a RLuc expressing plasmid plus a PKR-myc overexpressing plasmid alone (PKR O.E.), or in combination with a IFI27-HA overexpressing plasmid (PKR+IFI27 O.E.). At 24 hpt, cells were either mock- or poly(I:C)-transfected for 7 hours, to further stimulate PKR activation. Next, cellular extracts were harvested and RLuc luminescence levels were measured ([Fig. R46A](#)). On the one hand, in mock conditions in HeLa WT cells, co-overexpression of PKR and IFI27, reduced RLuc luminescence levels to less than half of the luminescence levels observed with PKR overexpression alone ([Fig. R46B](#)). In mock-transfected PACT-KO, PKR-KO and DKO, IFI27 overexpression together with PKR only slightly reduced RLuc luminescence levels compared to PKR overexpression alone ([Fig. R46B](#)), and to a much lesser extent than in WT cells. On the other hand, when cells were treated with poly(I:C), it is very interesting to observe that only in

HeLa WT and HeLa PKR-KO cells, PKR+IFI27 overexpression led to a significant reduction of RLuc luminescence levels compared to PKR overexpression alone (Fig. R46B). These results showed that PKR overexpression in PKR-KO cells compensated for the absence of endogenous PKR, thus enabling IFI27 to further enhance PKR activation. Conversely, when PACT was absent (as in PACT-KO and DKO cells), the overexpression of IFI27 together with PKR did not result in a reduction of RLuc luminescence compared to PKR alone (Fig. R46B). These findings underscore the importance of PACT in IFI27-mediated modulation of PKR activation and support that PKR expression is essential for IFI27-mediated inhibition of translation. Moreover, we analyzed SG formation after the overexpression of PKR+IFI27 or PKR alone in HeLa WT or PACT-KO cells. In poly(I:C)-treated HeLa WT cells, the overexpression of PKR and IFI27 together led to the generation of SGs in a 34.4% of analyzed cells, which was significantly higher than the 21.5% observed in cells overexpressing PKR alone (Fig. R46C, R46D). In contrast, in PACT-KO cells, the percentage of SGs-containing cells was comparable for cells overexpressing both PKR and IFI27 and cells overexpressing PKR alone (Fig. R46C, R46D). These results correlate with our previous results (Fig. R43, R44, R45), showing, again, that PACT presence is pivotal for IFI27-mediated activation of PKR.

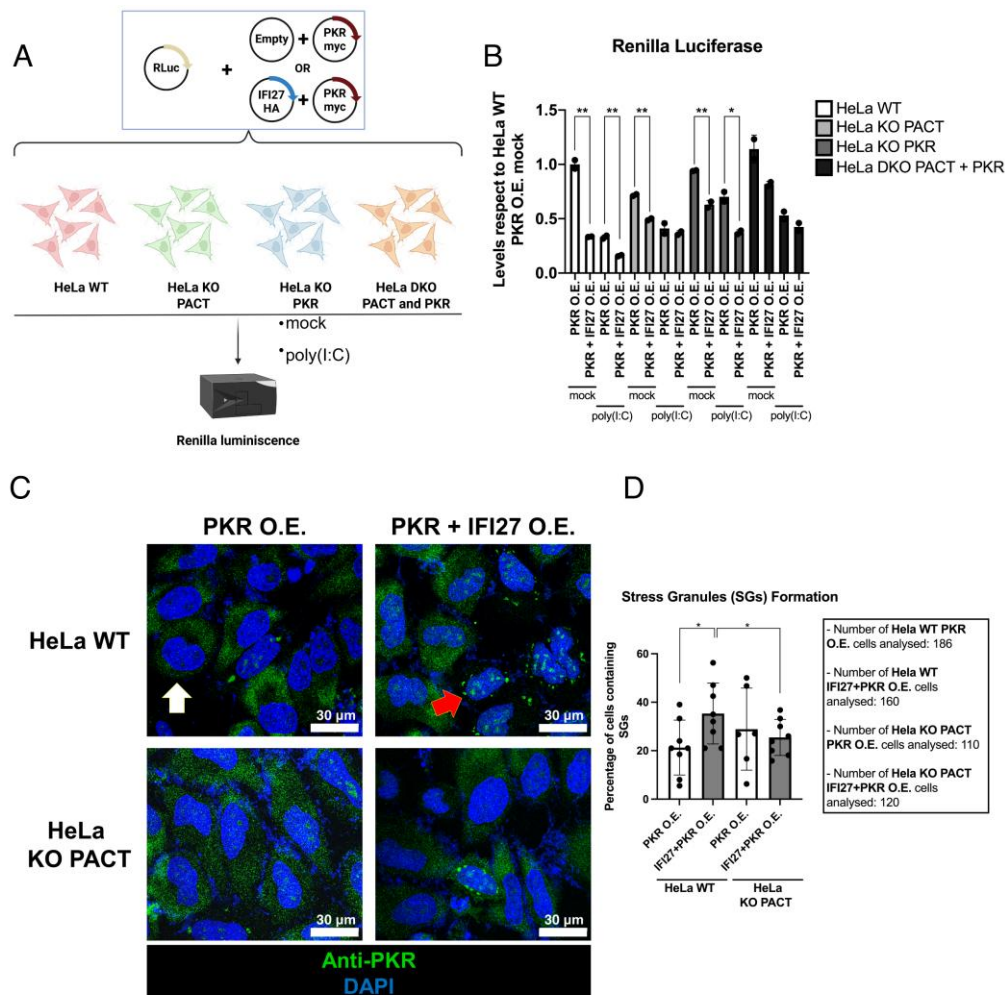


Figure R46. PKR overexpression promotes its own activation, which is further enhanced by IFI27 overexpression in a PACT-dependent manner. (A, B) HeLa WT, PACT-KO, PKR-KO, or double KO for PACT and PKR (DKO), were transiently transfected with a pCAGGS-PKR-myc plasmid (PKR overexpression, O.E.) alone or in combination with a pCAGGS-IFI27-HA plasmid (PKR + IFI27 O.E.). These plasmids were co-transfected with the pRL-RLuc plasmid. At 24 hpt, cells were either mock- or poly(I:C)-transfected (2 μ g/mL) for 7 hours, and then, protein extracts were harvested to measure RLuc

luminescence levels. RLuc luminescence levels are shown as fold-changes respect to the luminescence levels in control mock-transfected WT cells overexpressing PKR (**B**). Individual data points correspond to independent measures from different technical samples. Two independent experiments were performed with similar results. (**C and D**) Similarly, HeLa WT and HeLa PACT-KO cells were transfected with PKR-myc plasmid only (PKR O.E.) or in combination with the IFI27-HA plasmid (PKR+IFI27 O.E.). At 24 hpt, cells were treated with poly(I:C) (2 μ g/mL) for 7 hours, and then, fixed with formaldehyde and permeabilized. An immunofluorescence was performed for PKR (shown in green), and nuclei were stained with DAPI (shown in blue). The white arrow points to cells with a homogeneous distribution of PKR (no SGs), and the red arrow show cells with PKR-foci (SGs). (**D**) The percentage of SGs-containing cells within the total number of cells was quantified and represented. Each individual dot in (**D**) represents the percentage of SGs-containing cells within the total number of cells in one single image field, having each field several cells. Also, the total number of counted cells for each condition is indicated on the right of the bars. Scale bar, 30 μ m. Data is represented as the mean and standard deviations of the different measures. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (for comparisons using unpaired two-tailed Student's t test in **B** and **D**). These experiments were performed twice, with similar results.

4.3.1.3.5. PACT is a critical factor in IFI27-PKR interaction

MS data showed that both PACT and PKR interact with IFI27, and both proteins had very similar scores (494 and 565, respectively). However, emPAI value of PACT was significantly higher than that of PKR (1.92 and 0.71, respectively) (Fig. R33A and Table A3). Given that PACT is essential for the ability of IFI27 to modulate PKR function, we hypothesized that IFI27 interacts directly with PACT, and then, as PACT binds to PKR, this would facilitate the incorporation of IFI27 into the PKR-containing complex, thereby allowing IFI27 to modulate PKR function (Fig. R47A, schema of this hypothesis). According to this hypothesis, IFI27 and PKR would only interact if PACT is present. To test this hypothesis, we transfected both HeLa WT and PACT-KO cells with the IFI27-HA overexpressing plasmid, or the empty plasmid as a control. Following transfection, these cells were treated with poly(I:C) for 24 hours, and cellular extracts were harvested. Protein A sepharose beads conjugated with anti-PKR antibodies were used to perform co-IP assays, enabling the isolation of PKR and PKR-bound proteins. Interestingly, even though the expression of IFI27-HA was similar in both cell lines (Fig. R47B, Input), IFI27-HA only co-immunoprecipitated together with PKR in HeLa WT cells (Fig. R47B, co-IP), indicating that PACT mediates the interaction of IFI27 with PKR, supporting our hypothesis. These findings provide a molecular mechanism that explains the need for PACT in IFI27-mediated PKR activation.

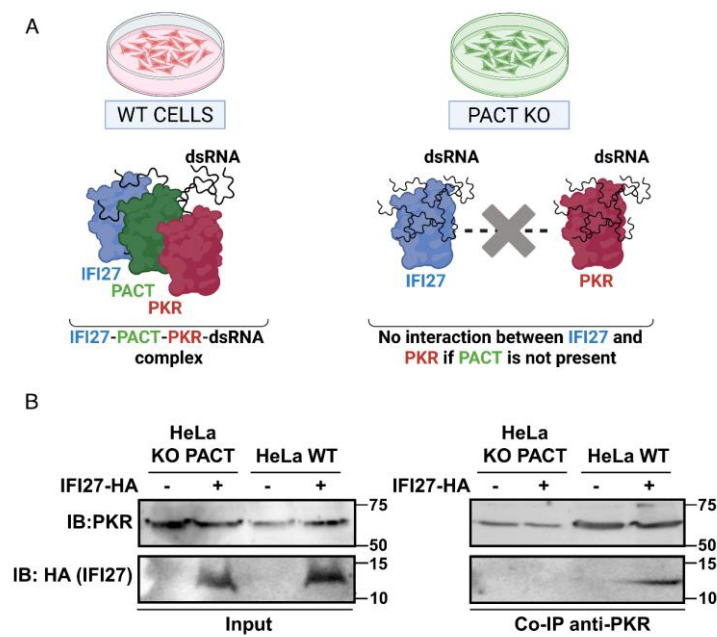


Figure R47. The interaction between IFI27 and PKR is facilitated by PACT. (A) Schematic representation of the hypothesis to test. IFI27, PACT, and PKR are dsRBPs that interact in a ternary complex. However, in the absence of PACT, IFI27 and PKR would be unable to interact. (B) To test this hypothesis, HeLa WT and HeLa PACT-KO cells were transiently transfected with a pCAGGS-IFI27-HA plasmid or an empty pCAGGS plasmid, and 24 hpt, cells were treated with poly(I:C) (3 μ g/mL) for 24 h. Cell lysates were harvested and a co-IP was performed by incubation overnight with anti-PKR- protein A-sepharose beads. Then, eluates were obtained and analyzed by Western blot using the specific antibodies for IFI27-HA (anti-HA, bottom panel) and endogenous PKR (anti-PKR, top panel), both in the cell lysates (Input) and after the co-IP (co-IP anti-PKR). Molecular weights are indicated on the right of the panels (in kDa). These co-IPs were performed twice, in independent experiments.

DISCUSSION

5. DISCUSSION

RNA viruses represent one of the major challenges for our health and economic systems, due to the high pandemic potential posed by these pathogens. Our immune system has evolved and adapted to counteract the infections by RNA viruses, and in this process, viral RNA recognition or sensing has become a pivotal mechanism to properly detect and attack these challenges. However, RNA viruses have also adapted, developing numerous strategies aimed at restricting and counteracting antiviral responses, turning this co-evolution into an arms race [307,308]. Thus, our best chance to be able to be on top of this situation, and improve prevention and treatment of viral infections, is to achieve a better knowledge of innate immune responses against RNA viruses. These responses are the first line of defense against the infection, and determine the fate of our reaction to it: limiting replication and returning to homeostasis, or losing the battle against viral replication, or even causing excessive responses that lead to immune-mediated damage [124,309]. Thus, the objective of this doctoral thesis is to search for new players and functions within the antiviral pathways, and specifically, focusing on two ISGs that had not been studied in depth before: *IFI27* and *IFI6*. This thesis describes the importance of these two ISGs on the infections with RNA viruses such as IAV, SARS-CoV-2, and DENV2, which constitute three of the greatest current challenges in global health [18,40,41,58]. The levels of induction of both genes after the infection with these RNA viruses and the effect that their function has on replication and viral titers have been analyzed. Furthermore, for the first time, the role and effects of *IFI27* and *IFI6* on the innate immune responses against IAV, SARS-CoV-2, and SeV have been studied, as well as the molecular mechanisms underlying the observed effects, in order to describe novel interactions and functions within the antiviral responses.

5.1. STUDY OF THE IMPORTANCE OF *IFI27* AND *IFI6* ON THE REPLICATION OF RNA VIRUSES: IAV, SARS-CoV-2, AND DENV2

The first objective was to confirm that *IFI27* and *IFI6* were induced to significant levels after different stimuli inducing innate immune responses, such as poly(I:C) or IFN treatment, as well as after IAV, SARS-CoV-2, and DENV2 infections. This first step was essential to confirm that *IFI27* and *IFI6* behave as ISGs in our cell systems, and validate subsequent studies aimed at analyzing their effect on RNA viral infections. Several studies had previously shown that *IFI27* expression is induced after the infections by RNA viruses such as the respiratory syncytial virus (RSV) in patients' blood, IAV in patients' blood or in infected cell-cultures, SARS-CoV-2 both in patients' blood and respiratory swabs [190,191,194,197,198,200,209,210], in peripheral blood mononuclear cells from DENV patients [206,207], as well as by type I IFN in most IFN-responsive cells [172–175,310]. In addition, another study demonstrated that the induction of *IFI27* by type I IFNs is compromised in cells lacking *IFNARI*, a gene encoding a component of the type I IFN receptor [311], further supporting the importance of type I IFNs for the induction of *IFI27* expression. Similarly, *IFI6* has also been shown to be an ISG [175], whose expression is induced after the infection with different RNA viruses such as after IAV infections of A549 cells [312], or in cell lines infected with different flaviviruses, as DENV2 [182]. Also, after analyzing mRNA expression profiles of upper airway samples from 234 patients with either COVID-19 or other viral or non-viral respiratory disease, *IFI6* mRNA was found to be significantly upregulated in COVID-19 patients [313]. Correlating with these previous studies, we confirmed that both the treatments with poly(I:C) and IFN, and the infections with IAV, SARS-CoV-2, and DENV2 for 24 and 48 hours in A549 cells, led to a significative and strong increment of *IFI27* and *IFI6* mRNA levels (Fig. R1). In the case of *IFI27*, this was also

demonstrated at protein levels (Fig. R1B, R1E, R1H) after poly(I:C) and IFN treatments, and after IAV and SARS-CoV-2 infections, showing a very good correlation with the mRNA values obtained for each treatment/infection and timepoint. We were not able to test the endogenous protein levels of IFI6, as no commercial antibodies yielded sufficiently strong signal. However, *IFI6* mRNA levels were also shown to be strongly induced after poly(I:C) and IFN treatments in HAP-1 cells, further supporting the role of *IFI6* as an ISG in a different cell line (Fig. R1C). The magnitude of the increment of mRNA or protein levels differed between infections, and in *IFI27* and *IFI6* cases. However, in every case, a sustained expression was observed at both time points, showing that *IFI27* and *IFI6* transcription is maintained throughout the infection process, at least until 48 hpi with three different RNA viruses. Thus, we could confirm that both *IFI27* and *IFI6* expression is strongly induced after different challenges that activate the antiviral responses, validating further studies aimed at modulating the level of expression of these two genes in order to assess the phenotypic effects derived from their absence or overexpression.

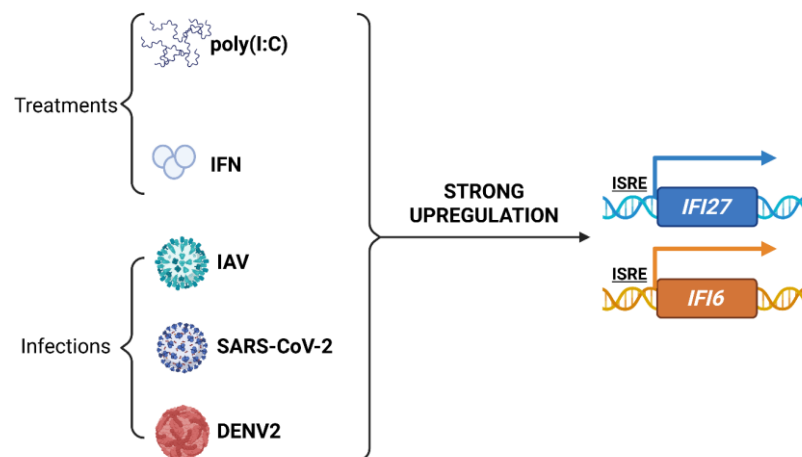


Figure D1. The expression of IFI27 and IFI6 is strongly induced after the treatments with poly(I:C) or IFN, and after the infections with IAV, SARS-CoV-2, and DENV2. These findings can be found in Figure R1.

Once we confirmed the induction of the expression of both IFI27 and IFI6 after the infection with different RNA viruses or the treatment with either poly(I:C) or IFN, the second step was to study the possible role of these two ISGs, to study if their induction could be associated with any positive or negative effect on the replication of RNA viruses. ISGs present a plethora of different antiviral functions and mechanisms directed towards the restriction of the replication of viruses. For instance, some described antiviral proteins, encoded by ISGs, against RNA virus infections are: Mx, a barrier for zoonotic transmission of avian influenza viruses that inhibits vRNPs import, transcription and replication [314]; Lymphocyte antigen 6 complex, locus E (LY6E), which has been demonstrated to protect against SARS-CoV-2 and other coronaviruses such as the mouse hepatitis virus (MHV) in the respiratory tract [315]; or IFN-induced transmembrane (IFITM) proteins, which has been described to restrict the entry of DENV as well as IAV H1N1 [314,316]. However, the picture is much more complex than this, as these antiviral factors, in certain conditions and specific infections, have shown proviral effects, with LY6E enhancing IAV uncoating [317] and DENV internalization [318], or IFITM proteins, which have been described to promote the infection by human coronavirus such as HCoV-OC43 [319]. Considering the enormous complexity of the function of ISGs and the IFN-induced responses, to have a broader view, we decided to test the effect of their loss-of function in the replication of IAV, SARS-CoV-

2, and DENV2. As ISG's functions can be very different in different types of infection and conditions, we tested this in three different, non-related RNA viruses, to get broader results.

Interestingly, knock-down of IFI27 led to a consistently lower level of viral titers of IAV, SARS-CoV-2, and DENV2 compared to a NT-siRNA control condition in lung epithelial cells (Figs. R2E, R2F, and R2G). Similarly, the absence of IFI27, by using IFI27-KO lung epithelial cells, had a negative effect on the replication and viral titers of these different, non-related, RNA viruses (Fig. R4C-R4F). However, these results were obtained in A549 cells, which are respiratory epithelial cells, not a normal physiological target for DENV infections *in vivo*. Due to this, to validate these findings in a more appropriate model for DENV infections, and using shRNA-coding lentiviruses, we tried to knock-down the expression of IFI27 in MoDCs. However, we did not achieve a proper reduction of the expression of IFI27 compared to the NT-shRNA control (data not shown). Nonetheless, in future studies we plan to refine this protocol to study IFI27 knock-down in MoDCs and DENV2 infections. Thus, IFI27's effect on DENV2 infection needs to be further explored to be able to strongly support our findings. Previous studies have shown that IFI27 behaves as one of the best biomarkers to discriminate between both IAV or SARS-CoV-2-infected patients and uninfected individuals, as well as it can be used as a prognosis tool, as its elevated levels are associated with worsened prognosis and severe disease [195,196,199]. For instance, SARS-CoV-2 patients who died due to development of ARDS showed an increased *IFI27* expression in M1-like macrophages in lung infected tissue, compared to tissue regions without infection [195]. Similarly, several studies have found *IFI27* to be upregulated in response to DENV infections, as previously mentioned [206,207]. In one specific study, a transcriptional meta-analysis of the interferome indicated that elevated levels of *IFI27*, together with *ISG15* and cytochrome B reductase 1 (*CYBRD1*), could be associated with the development of DSS and DHF, the severe forms of the disease [208]. Similarly, in other RNA virus infections, such as RSV, high *IFI27* levels have been associated with more severe cases, longer hospital stays and increased need for mechanical ventilation [177,210]. This is also observed after enterovirus 71 infection, where higher *IFI27* levels associated with an increased disease severity [320]. Furthermore, *IFI27* was described as the most differentially expressed gene (DEG) in HIV-1-infected patients, and its overexpression correlated with a worse response to the ART, thus behaving as a prognostic marker of severe disease and inflammation, and associated with worsened outcome [321,322]. All this data could mean that, as we have shown that IFI27 acts as a proviral factor for SARS-CoV-2, IAV, and DENV2 replication, greater IFI27 expression could be correlated with greater viral replication, and therefore, with a more severe disease after viral infections with these RNA viruses. Nonetheless, another study shows that IFI27 knock-down enhanced hepatitis B virus (HBV) replication [323], showing that, for certain infections, IFI27 behaves as an antiviral factor. HBV is a virus from the *Hepadnaviridae* family, that presents a dsDNA of 3.2 kb as a genome. Thus, it will be interesting to test in future studies, if the difference of presenting an RNA or DNA genome could be the explanation of the different effects of IFI27 protein.

Similar to IFI27, we have described that the knock-down and KO of the expression of IFI6 have a negative effect on the replication and viral titers of IAV and SARS-CoV-2 (Fig. R2H, R4H, R4I). Thus, in these two models of infection, IFI6 could either have a proviral role, or the ability to modify the antiviral responses leading to the observed effects on viral replication. *IFI6* expression has also been described to be upregulated in SARS-CoV-2 patients, both in nasopharyngeal and oropharyngeal samples, and this upregulation correlated with increased viral loads [192,193]. Same observations were performed for IAV infected patients, with *IFI6*

upregulation allowing to distinguish infected patients from healthy controls and *IFI6* being identified as one of the core transcriptions signatures of IAV infections [201,312,324]. However, its role in these infections and possible effect on replication had not been explored before.

In addition, we have shown that using a WT isolate of DENV2 virus (Thailand/16681/84 strain) in A549 cells, *IFI6* knock-down and KO led to a lower DENV2 replication and titers (Fig. R2I, R4J, R4K) in A549 cells, which are capable of inducing a strong type I and III IFN responses after the infection with different viruses as previously shown [254,325]. Furthermore, we also observed the same effect on human MoDCs derived from blood of healthy donors: DENV2 replication was significantly reduced after *IFI6* knock-down using three different *IFI6* shRNA-coding lentiviruses, in comparison to a NT-shRNA-coding lentivirus as a negative control (Fig. R3). Together with blood monocytes and tissue macrophages, DCs are one of the main cellular targets of DENV infection [326,327]. Therefore, this result is particularly interesting and relevant, as this is the first time that this effect of *IFI6* absence has been reported on DENV2 infections in a cellular target that is much closer to physiological reality *in vivo*. Nevertheless, more assays will be performed to further confirm these findings and better understand their significance. In contrast to our results, a CRISPR screening proposed that *IFI6* could be an ER-resident effector that block the replication of different viruses that generate replication organelles within the ER, for instance, flaviviruses like DENV2 [183]. However, this study used a mutated reporter version of DENV2 (DENV2-Fluc), presenting a mutation in NS4B protein, and Huh7.5 cells, which are highly permissive, as these cells are deficient in some antiviral signaling pathways [328], therefore, the effect of *IFI6* on antiviral responses could be underrepresented in these cells, and the effect of *IFI6* could vary depending on the characteristics of the used cell lines.

Given the identification of *IFI6* and *IFI27* as proviral factors, at least for IAV, SARS-CoV-2, and DENV, drugs inhibiting the functions of these proteins could be developed as antiviral treatments for these, and likely other viral infections. In this sense, the implementation of antiviral therapies targeting host factors or viral-host interactions could represent a better strategy to fight viral infections compared to drugs directed towards viral proteins, as the probability of generating resistant-viruses is negligible. These therapies may also present a broad spectrum against different viruses, being helpful to treat opportunistic pathogens that become virulent in unhealthy individuals and for emerging pathogens in case a new pandemic begins. The selection of drug-resistant viral strains has been a problem for antiviral development. For instance, in the case of IAV, 90% of the currently circulating IAV strains from the subtypes H1N1 and H3N2, which are the subtypes currently circulating in humans, are resistant to the viral M2 inhibitors amantadine and rimantadine, which are no longer recommended for monotherapy due to drug resistance. Furthermore, NA and PA-inhibitor resistant IAVs have been reported [329]. For SARS-CoV-2, remdesivir (a nucleoside analog) and nirmatrelvir (a viral 3CL protease inhibitor) are the only FDA-approved antivirals, authorized in October 2020 and May 2023, respectively, while molnupiravir (a ribonucleoside analog) is permitted solely for emergency usage. However, SARS-CoV-2 showed resistance against remdesivir in less than a year from its approval by the FDA to treat SARS-CoV-2 infections, and some resistance reports are available against 3CL protease inhibitors such as GRL-001 [330]. To date, therapeutic approaches against dengue infections are predominantly focused on symptom management and complication prevention. Although antiviral drugs and immunotherapies are under investigation, none have exhibited clinical success [331].

In conclusion, for this first part of the doctoral thesis, we have described that both *IFI27* and *IFI6* are strongly induced after the infection with different RNA viruses, and that the knock-down or

KO of IFI27 or IFI6 expression has a negative effect on the levels of replication of these same viruses. Thus, to have a better view of these findings, we decided to analyze whether IFI27 and IFI6 could, through yet unknown mechanisms, modulate the induction of antiviral responses after the infection with RNA viruses. This would improve our understanding of the status of antiviral response pathways, and help us to explain the observed effects with greater precision.

5.2. EFFECT OF IFI27 AND IFI6 ON ANTIVIRAL RESPONSES AFTER RNA VIRUS INFECTIONS

Some ISGs are not simply anti- or proviral factors, but important regulators of the induction of innate immune responses. In response to a viral infection, type I and III IFNs are produced, and these proteins bind their receptors in an autocrine and paracrine manner. Type I IFNs bind to their receptor IFNAR1-IFNAR2, while type III IFNs bind to the receptor IFNLR1-IL-10R2. Once IFNs bind their receptors, this initiates a signaling cascade through JAK/STAT pathway, leading to the generation of the ISGF3 complex, formed by phosphorylated STAT1 and STAT2 together with IRF-9. This complex translocates to the nucleus where it binds to ISRE sequences and induces the transcription of a plethora of ISGs [118]. Some of these ISGs act to amplify the signal to achieve a sufficiently strong response. Some examples of this are ISGs such as TRIM25 or RNF6 E3 ubiquitin ligases, which further activate RIG-I and the induction of more IFN and proinflammatory cytokines [122,123]. In contrast, some ISGs can negatively modulate the induction of the IFN pathways, by exerting a negative feedback mechanism, in order to return to homeostasis when needed, and to avoid an excessive response or inflammation, which can be deleterious to the host [124,309]. One previous report by Mick *et al.* [194] showed that in SARS-CoV-2 infections, the innate immune responses were suppressed compared to other viral pathogens, which was detected as lower enrichment of genes participating in innate immune responses, TLR signaling, proinflammatory cytokines, chemokines, ILs, etc. However, both *IFI27* and *IFI6*, were more upregulated than with the other viruses, even though general antiviral responses were attenuated. With this knowledge, together with our observations on the exerted effects on viral replication, we assessed whether both IFI27 and IFI6 could be negative modulators of innate immune responses. To achieve this, we followed loss-of function studies by using knocked-down and KO cells, or gain-of function studies by overexpression for either IFI27 or IFI6 and employing a wide range of stimuli to activate the antiviral responses: infections with IAV, SARS-CoV-2, or SeV, or treatments with poly(I:C). Remarkably, in A549 cells, IFI27 or IFI6 knock-down (Fig. R5) or KO (Fig. R6) led to a significantly higher induction of *IFNL1* (a type III IFN), *IFIT2* (an ISG), and *CXCL10* (a proinflammatory cytokine) than in negative control cells, after treatment with poly(I:C) (Figs. R5 and R6). Similarly, in IFI27 or IFI6 KO cells that were infected with IAV or SARS-CoV-2, the mRNA levels of *IFNL1*, *IFIT2*, and *CXCL10* were highly upregulated compared to the WT negative control A549 cells (Fig. R7 for IFI27 KO, Fig. R8 for IFI6 KO). This effect on antiviral responses, supports the previous observations of reductions of viral titers observed in IFI27 KO and IFI6 KO cells compared to WT cells, both after IAV (Fig. R4C for IFI27 KO and Fig. R4H or IFI6 KO) or SARS-CoV-2 infections (Fig. R4D for IFI27 KO and Fig. R4I or IFI6 KO), showing a strong correlation between the level of replication and the antiviral responses of the cells. To further analyze the antiviral state, A549 WT cells or A549 KO for either IFI27 or IFI6 were infected with rVSV-GFP, a virus that has been used only as tool because it is highly sensitive to the antiviral state induced by poly(I:C) and other IFN-inducible treatments, which highly impairs its replication [253]. In mock-treated cells prior to infection, rVSV-GFP viral titers were equal in WT and KO cells, however, interestingly, when cells were treated with poly(I:C) prior to infection, rVSV-GFP viral titers in both IFI27 and

IFI6 KO cells were strongly reduced compared to WT cells (Fig. R9), suggesting that in the absence of IFI27 or IFI6, the antiviral responses induced by poly(I:C) are much stronger or abundant than in the WT cells. Again, this correlates with the higher induction of *IFNL1*, *IFIT2*, and *CXCL10* in KO cells (Figs. R7 and R8) and in knocked-down cells (Fig. R10E, R10F). The opposite approach was also followed, showing that the overexpression of IFI27 or IFI6 correlated with a lower induction of *IFNL1* and *CXCL10* after SeV infections (Fig. R10C, R10D), in line with previously published results showing that the upregulation of IFI27 and IFI6 was associated with a lower innate immune activation [194]. All these results show that the negative effect of IFI27 and IFI6 on antiviral responses is broad, as similar results are obtained in response to poly(I:C) (Fig. R6, Fig. R9B, R9C), IAV (Fig. R7D, R8B), SARS-CoV-2 (Fig. R7E, R8C), and SeV (Fig. R10C-R10F), different, non-related RNA viruses and an analog of viral dsRNA.

Furthermore, IFI6 effect as a negative modulator of innate immune responses was also confirmed in HAP-1 cells and BEAS-2B cells (Figs. R13-R15). Furthermore, an RNA seq of HAP-1 WT vs IFI6 KO cells treated with poly(I:C) showed that the absence of IFI6 led to a strong upregulation of different biological processes related to the cellular response to viral infection, the induction of, and response to IFNs, and different processes associated with cell signaling in innate immune modulation, that point to IFI6 as an important negative modulator of IFN pathways and the production of proinflammatory cytokines (Figs. R11, R12, and Table A2). These results further support previous data showing the upregulation of *IFNL1*, *IFIT2*, and *CXCL10* in IFI6 KO cells compared to WT cells, and by performing a complete RNAseq, it expands the number of genes and processes analyzed, providing a wealth of valuable information to have a broader view of the effect that IFI6 could have on antiviral responses. In future studies, it would be really interesting to perform a similar assay also on IFI27 KO cells, as well as RNAseq studies on IFI6 and IFI27 KO cells after IAV and/or SARS-CoV-2 infections, to have extensive data to compare the innate immune responses against these two viruses, and against poly(I:C) treatment. All these results are remarkable, as they confirm the reproducibility of the obtained results in different cell lines for IFI6 loss-of-function: A549 (Figs. R6B, R8, R9C), HEK-293T (Fig. R10D, R10F), HAP-1 (Fig. R11-R14), and the non-tumorigenic human bronchial epithelial cell line, BEAS-2B (Fig. R15). All these findings correlated with previous works for other host factors acting as negative modulators of innate immune responses. For instance, we can highlight proteins such as ISG15, which sequesters RIG-I [125], or ISG56, which associates with stimulator of IFN genes (STING) protein and disrupts its interaction with MAVS or TBK1, key on the activation and translocation of IRF-3, and thus reducing the induction of type I IFNs and cellular antiviral responses [332]. Furthermore, the knock-down of IFI44 or IFI44L leads to a similar increase in the induction of *IFNL1* and *IFIT2*, and negatively affects the replication of either IAV, HCoV-229E, and the LCMV virus, or IAV, LCMV, and SeV, respectively [126,127]. The molecular mechanism involves the interaction of IFI44 and IFI44L with the cellular protein FKBP5, which in turn interacts with IKK α , IKK β , and IKK ϵ , leading to lower phosphorylation of IRF-3 and I κ B α , which results in a lower transcription of type I and III IFNs and different proinflammatory cytokines [126,127]. Likewise, studies by other groups, such as Das *et al.* [333], show that the ISG35 protein acts similarly to what we observe with IFI27 and IFI6, since silencing IFI35 leads to a reduction in VSV and SeV replication and an increase in *IFN- β* induction, while its overexpression has the opposite effect. In this work, the authors show that IFI35 interacts with RIG-I and both suppresses its dephosphorylation (which is required for activation) and induces proteasome-mediated degradation of RIG-I via K48-linked ubiquitination, resulting in a lower induction of IFNs and proinflammatory cytokines [333]. Thus, this type of effect has already been observed for different ISGs in recent years, highlighting the importance of negative feedback mechanisms in IFN

responses, probably to avoid an excessive response that could lead to immune-mediated damage by uncontrolled responses [124,309].

Next, in order to give greater relevance to our *in vitro* data, we decided to conduct *in vivo* studies in mice. Human *IFI27* and *IFI6* belong to the *FAM14* family [174], which is comprised of four different members, *ISG12a* (*IFI27*), *G1P3* (*IFI6*), and two other members (*ISG12b* and *ISG12c*) which are not IFN-inducible. However, this is different in mice, as in mice, there are homolog genes to human *IFI27* (mouse *Ifi27*), human *ISG12b* (mouse *ISG12b1*), and human *ISG12c* (mouse *ISG12b2*), clustered at the chromosome 12F1, but there is not a mouse homolog for human *IFI6* [175]. Thus, although future options could include using *IFI27*-KO mice (commercially available), this was not an option for *IFI6*, and therefore, we decided to overexpress the human *IFI6* and *IFI27* proteins using rIAVs to study *IFI6* and *IFI27* functions *in vivo*, in mice infected with these rIAVs. We generated three different viruses: IAV-mCherry, that codifies for the fluorescent mCherry protein, being useful both as a control and as a reporter; IAV-*IFI27*-HA, overexpressing the human *IFI27*; IAV-*IFI6*-HA, overexpressing the human *IFI6*. Remarkably, and supporting previous *in vitro* findings, at 48 hpi, the infection of mice with both IAV-*IFI27*-HA and IAV-*IFI6*-HA viruses led to a much lower induction of *Ifnl3*, *Ifit2*, *Ccl2*, and *Tnf* than the infection with the IAV-mCherry control virus, suggesting that *IFI27* and *IFI6* overexpression negatively modulate the induction of antiviral responses in lungs of infected mice (Fig. R17C). However, viral titers in lungs were not significantly modified in IAV-*IFI6*-HA-infected mouse, compared to the IAV-mCherry-infected mouse, with just a slight increment of IAV viral titers at 24 hpi in mice that were infected with the IAV-*IFI27*-HA virus compared to the mice infected with the mCherry virus, used as a control (Fig. R17B). This could mean that, *in vivo*, induced modulation of innate immune responses by *IFI27* or *IFI6* is not strong enough to affect viral titers, or that *IFI27*, *IFI6*, and mCherry are modulating viral replication by additional mechanisms that are still unknown, different from the modulation of IFN responses.

In this second part, we can conclude that *IFI27* and *IFI6* are able to impair host IFN and inflammatory responses. The absence of these two proteins led to a lower replication of several RNA viruses (IAV, SARS-CoV-2, and DENV2), which correlate with stronger IFN and inflammatory responses both *in vitro* and *in vivo*, while the overexpression shows the opposite effect. Consequently, to follow up our studies, our next objective was to explore the possible molecular mechanisms of *IFI27* and *IFI6* that could explain the observed effects as negative modulators of innate immune responses.

5.3. MOLECULAR MECHANISMS UNDERLYING THE MODULATION OF INNATE IMMUNE RESPONSES BY *IFI27* AND *IFI6*

5.3.1. *IFI27* and *IFI6* are RBPs

The innate immune responses against infections by RNA viruses such as IAV, SARS-CoV-2, or SeV, as well as against treatments with poly(I:C) have a common ground: these challenges are recognized by specialized receptors in our cells, such as the PRRs RIG-I and MDA5, which sense the presence of specific RNAs [80,334]. RNA binding and recognition is an essential functional asset that many players of innate immunity present, as the discerning between self and exogenous RNAs is a powerful tool to detect invading viral pathogens. RNA lifecycle is a highly dynamic process, with steps including splicing, mRNA synthesis and maturation, stability, etc. and all these steps are coordinated by the presence of a high number of RBPs, including the orchestration of host antiviral responses [285]. Here, we can differentiate canonical RBPs, which contain typical, well-defined RBDs such as RNA recognition motifs (RRMs) found in Non-POU domain-

containing octamer-binding protein (NONO), K homology (KH) domains found in Mex3B, zinc-finger domain (ZNF) found in TRIM56, DExD/H box domains found in RIG-I and MDA5, dsRBDs found in ADAR1 or PKR, and others [98,285].

Many of these RBPs, such as the aforementioned PRRs like RIG-I or MDA5, are essential to recognize and bind viral RNAs, and then initiate and orchestrate the antiviral response. However, many other RBPs are modulators of these PRRs, regulating the strength of their activation. For instance, RIG-I and MDA5 are targeted by TRIM25 [100], PACT [103,105], LGP2 [108,109], or different RNA helicases [81,110], among many others.

With this knowledge, we thought about the possibility that IFI27 and IFI6 could also be RBPs, modulating the PRR's levels of activation. Even though there is no knowledge of IFI27 and IFI6 presenting any typical canonical RRM, it is interesting to mention that non-canonical RBPs make up the majority, approximately three-quarters, of the total number of RBPs codified in our genomes [285]. These non-canonical RBPs have intrinsically disordered regions that lack the characteristics of typical RRMs, or RBDs that have not been described yet, but are demonstrated to bind to RNA sequences [285]. One clear example is TRIM25 protein [335] or OAS proteins [336]. Interestingly, we carried out a bioinformatic analysis using RNABindRPlus, showing that within IFI27 and IFI6 sequences, 13 and 15 aminoacids had a significative probability to bind to RNA, respectively. Next, we tested this experimentally, confirming that both IFI27 and IFI6 were able to interact and co-immunoprecipitate with poly(I:C), a dsRNA homolog (Fig. R18). Furthermore, by mutating two of the residues in IFI27 sequence that RNABindRPlus predicted to bind RNA (Ser63, mutated to Leu63 (S63L) and Val82, mutated to Ala82 (V82A)), we observed that IFI27 almost totally (S63L) or totally (V82A) lost its ability to bind to dsRNA (Fig. R19), validating the bioinformatic prediction. Also, these results confirmed that the IFI27 interactions with dsRNA are not mediated by the HA tag, as one single mutation in IFI27 abolished its interaction with dsRNA, even though HA tag remained unchanged.

5.3.2. IFI27 and IFI6 interact with RLRs and negatively modulate their function

Once we confirmed that IFI27 and IFI6 are RBPs, and considering previous results suggesting that these proteins act like negative regulators of innate immune responses induced by poly(I:C) or RNA viruses such as SARS-CoV-2, IAV, or SeV, we tested the possible modulation of RIG-I or MDA5 function by these proteins. As previously mentioned, RIG-I and MDA5 are one of the main inducers of antiviral responses against poly(I:C) treatments or against infections with SARS-CoV-2, IAV, and SeV [80,334]. Interestingly, we showed that both IFI27 and IFI6 proteins co-immunoprecipitated together with RIG-I after poly(I:C) treatment or after SeV infection, being these interactions dependent on RNA (Fig. R20B and R20C for IFI27-RIG-I, and Fig. R20D and R20E for IFI6-RIG-I). Additionally, these same results were also obtained for IFI27 and MDA5 interaction after poly(I:C) (Fig. R25B and R27B), after SARS-CoV-2 infections (Fig. R26B, R26C), and even in mock conditions (Fig. R25C), although to a lower extent, suggesting that IFI27 binds to both functional RLRs through its ability to interact with viral RNAs. In future studies, we will also test the possible interaction of IFI6 with MDA5, which was not assessed for the scope of this doctoral thesis. Thus, IFI27 and IFI6 are RBPs, and this ability to bind to RNA allow them to interact with RLRs.

The next step was to determine if these interactions lead to a modulation of RIG-I and/or MDA5 function in response to such stimuli. After viral RNA recognition, RIG-I and MDA5 change their conformation, and translocate to the mitochondria, where they interact with MAVS protein, activating its function, and subsequently leading to the activation of the transcription factors IRF-

3, IRF-7, and NF- κ B (Fig. I6). These transcription factors induce the transcription of IFNs and proinflammatory cytokines, which in turn activate further signaling pathways in neighbor cells activating the production of ISGs [98,118]. Thus, to study the possible modulation of RIG-I and MDA5 function, after different stimuli such as poly(I:C) (Fig. R29B, R29C, R30A, R30B, R30D) or infections such as SeV (Fig. R22, R23B, R24) or SARS-CoV-2 (Fig. R29D, R29E, R30E), we studied the induction of mRNA levels of IFNs (*IFNL1*), ISGs (*IFIT2*), and proinflammatory cytokines (*CXCL10*), and also, for MDA5 activation, the generation of MDA5 oligomers (active form of the protein).

On the one hand, we could observe that IFI27 or IFI6 overexpression, led to a strong reduction of the induction of *IFNL1* by RIG-I after SeV infection (Fig. R22B, R22E), while knock-down of IFI27 or IFI6, had the opposite effect (Fig. R22C, R22F). Also, we described that IFI27 is able to reduce the ability of RIG-I to bind to SARS-CoV-2 viral RNA, while the mutated version of IFI27 (IFI27 V82A) which does not bind to RNA (Fig. R19), did not present this effect (Fig. R23D), and had a reduced capacity to negatively modulate RIG-I-mediated induction of *IFNL1*, *IFIT2*, and *CXCL10* compared to the WT version of IFI27 (Fig. R23B). On the other hand, IFI6 knock-down led to an increase of ubiquitination of RIG-I (Fig. R24B), which is usually associated with RIG-I activation, as different E3 ubiquitin ligases such as Riplet, TRIM25, TRIM4 or Mex3C add K63-linked ubiquitin chains to stabilize and further activate RIG-I [111]. These findings provide possible molecular mechanisms of the observed results, suggesting that IFI27 and/or IFI6 bind to viral RNAs and subsequently, to RIG-I, and negatively affect the interaction between RIG-I and those RNAs, possibly leading to RIG-I dissociation and its inactivation, which correlates with a reduction on RIG-I ubiquitination in the presence of these proteins. On the other hand, IFI27 overexpression led to a reduction of the generation of MDA5 oligomers in response to poly(I:C) (Fig. R29B) or SARS-CoV-2 infection (Fig. R29D) and thus, a reduction on the induction of *IFNL1*, *IFIT2* and *CXCL10* (Fig. R29C, R29E, respectively). Conversely, IFI27 KO and knock-down led to an increase in MDA5 oligomers (Fig. R30A, R30D) and induction of *IFNL1*, *IFIT2*, and *CXCL10* after poly(I:C) treatment (Fig. R30B). Therefore, both results point to the same fact, which is that IFI27 is a negative regulator of MDA5 activation. To explore the possible molecular mechanisms that explain this finding, we tested MDA5-poly(I:C) binding in presence or absence of IFI27, showing that IFI27 overexpression led a strong decrease in the interaction between MDA5 and poly(I:C) (Fig. R31B).

With all these results for RIG-I and MDA5, we can conclude that IFI27 and/or IFI6 interact with viral derived RNAs and RIG-I and/or MDA5, and negatively affect the ability of the last to bind to those RNAs, leading to a reduction of their activation and subsequently, a reduction in the induction of innate immune responses against viral infections (Fig. D2). Furthermore, both IFI27 and IFI6 main subcellular location is believed to be the mitochondrial membrane [178,179,182], an organelle that functions as a critical platform for the transduction of antiviral signaling, since this is where the interaction between RIG-I and MDA5 with MAVS occurs, leading to the subsequent recruitment of TBK1 and IKK ϵ (Fig. I6) [337]. Attending to the colocalization experiments shown (Fig. R21, R28) where we observe a partial colocalization between IFI27 and IFI6 with RIG-I and/or MDA5, it is reasonable to think that these regulatory processes are concentrated in the mitochondrial membrane. However, they could occur also in other cellular locations such as peroxisomes, where MAVS can be also located [113], or at MAM [114], as IFI27 and IFI6 have also been described as proteins located at the ER [177,183]. Since the description of MAVS antiviral signaling from peroxisomes by Dixit *et al.* [113], several studies have described that MAVS activity in mitochondria and peroxisomes is slightly different, with

MAVS in mitochondria carrying out the “canonical” response by activating IRF-3, IRF-7 and NF- κ B in order to induce the transcription of IFNs and proinflammatory cytokines and establish an antiviral state in surrounding cells, while MAVS from peroxisomes follows a different pathway, inducing the generation of ISGs independently of the induction of IFN to provide a short-term protection [113,338]. Due to their well-described mitochondrial localization and that they behave as negative modulators of RLRs-dependent IFN responses induction, we would expect IFI27 and IFI6 to affect the here named “canonical” pathway, in order to reduce inflammation and antiviral responses and return to homeostasis, avoiding an excessive and deleterious sustained response. However, a much more insightful study of the subcellular localization of IFI27- and IFI6-mediated modulation of RLRs should be a priority in future studies to be able to properly affirm this and to dissect their effect the induction of IFNs, proinflammatory cytokines, and ISGs.

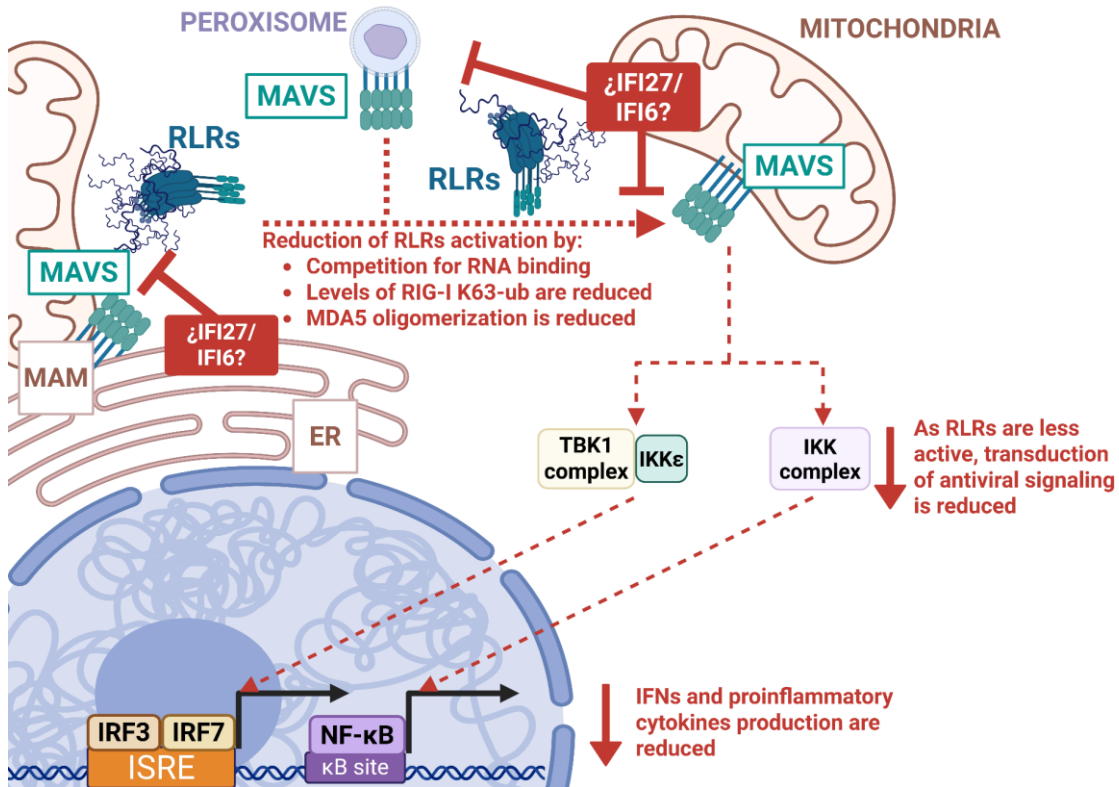


Figure D2. Graphical schema of IFI27 and IFI6 effect on the induction of innate immune responses.

Many ISGs or RBPs have been described as modulators of RIG-I and MDA5 activation and modulators of the transduction of the signal to MAVS, usually activators. Some examples are helicases such as: DDX60, which binds to both RIG-I and MDA5, promoting their activation, and type I IFN induction, and suppressing viral VSV and poliovirus replication [339]; DHX15, which binds to both the RNA PAMPs and to RIG-I CARD domains and promotes RIG-I function. DHX15 knock-down leads to an increased susceptibility to infection with RNA viruses of different families, such as *Paramyxoviridae*, *Rhabdoviridae*, and *Picornaviridae*, demonstrating that it is a regulatory factor in RNA virus infections in a broad manner [340]; DHX29, which is a co-sensor of pathogen nucleic acids that interacts with RIG-I and MAVS promoting their activation and downstream signaling [341]; DHX16, which is able to sense viruses such as IAV, ZIKV, and SARS-CoV-2, and interact with RIG-I in response to these stimuli, favoring IFN type I induction [342]; and DDX6, which co-immunoprecipitates with RIG-I, and binds to stimulatory RNAs, favoring their detection by RIG-I, as well as RIG-I activation, displaying antiviral activity

against enteroviruses [343,344]. All these examples clearly recall the results that we obtained for IFI27 and IFI6: RBPs that, after binding to viral RNA, are able to interact with RLRs and modulate their activation. However, the effect of IFI27 and IFI6 is opposite, as we see how interaction with RIG-I and MDA5 proteins leads to a decrease in their activation and therefore, a decrease in the induction of antiviral responses (Fig. D2). Interestingly, IFI27 and IFI6 are not the only examples of RLR partners that negatively modulate their activation. The aforementioned LGP2, the last member of RLR family, is also an RBP, but it lacks the functionality of RIG-I and MDA5 since it does not present CARD domains. However, recent studies show that LGP2 interacts with RNAs, acting as a negative regulator of RIG-I-mediated signaling [107].

Gain of function of RLRs have been associated with aberrant type I IFN production (IFN-I signature) and extensive organ damage due to an excessive response. For instance, mutations in *IFIH1* gene (coding for MDA5) have been reported in patients with autoimmune disorders such as Systemic Lupus Erythematosus or Aicardi Goutieres Syndrome, due to an hyperactivation of MDA5 [345]. This puts into perspective why mechanisms that negatively modulate RLRs signaling are needed, in order to fine-tune the antiviral and inflammatory responses, and terminate them to avoid sustained and excessive responses. In this sense, in the case of SARS-CoV-2 and IAV infections, the functions of IFI6 and IFI27 in negatively modulating the inflammatory responses might be beneficial for the host in order to induce a milder disease, since it has been described that exacerbated inflammatory responses correlate with disease severity after IAV [346,347] and SARS-CoV-2 [348] infections. Furthermore, many studies have suggested that systemic chronic inflammation caused by residual viral components may be one of the pathophysiologic mechanisms of long-term complications and sequelae after SARS-CoV-2 acute infections [349–351].

All in all, *IFI27* and *IFI6* could be members of a group of ISGs that are needed to stop the induction of antiviral responses, to avoid excessive inflammation and IFN induction, which can be deleterious to the host.

5.3.3. IFI27-protein interactome: describing novel protein partners, such as PACT, PKR, and others

Next, to further elucidate and understand IFI27 function on innate immune responses, we decided to perform a MS analysis to study IFI27-protein interactome. By studying the interactome, we could infer new functions or processes in which IFI27 could be implicated or even find new partners that could explain the observed previous results. This section was only performed for IFI27, to be able to conduct a focused, in-depth study of a single protein. However, likewise, one future objective is to analyze IFI6 protein interactome, to follow up this project.

In order to do this, we performed a Co-IP and MS study, obtaining a list of IFI27-bound proteins (Table A3). The main molecular function within IFI27-interactome was RNA binding (Table A5, Fig. R32B). This is remarkable, as in this thesis, we found that IFI27 is an RBP (Fig. R18, R19), so it is coherent that many interacting proteins are also RBPs, as they could be interacting through their shared ability to bind to RNA. Also, RNA binding is a common molecular function for many proteins that participate in innate immune responses, such as DDX60, DHX15, LGP2, examples given in this discussion. Thus, an interactome with an overrepresentation of RBPs could support the finding of IFI27 being a modulator of innate immune responses. Next, one of the most overrepresented processes was translation, as it showed an FDR of $3.36\text{E-}7$ for the molecular function “structural constituent of ribosome” (Table A5, Fig. R32B) and an FDR of $4.1\text{E-}4$ for “translation” in biological process, as well as FDRs of 0.0028 for both “cytoplasmic translation”

and mitochondrial translation” (Table A4, Fig. R32A), suggesting that IFI27 could be implicated somehow in the translational process in response to exogenous RNAs. Translational regulation is tightly associated with the recognition and response to viral RNA, as one of the best-known antiviral mechanisms is the inhibition of *de novo* protein synthesis, in order to restrict viruses from producing their own proteins to generate new viral particles. One of the main players in translation regulation is the PKR protein [293]. We selected these two characteristics, RNA binding and translation as starting point to search for interesting partners in the IFI27 interactome and remarkably found two proteins that are pivotal within the antiviral responses: PKR and PACT. Both proteins were found in IFI27-interactome with a high score (565 for PKR and 494 for PACT) (Fig. R33A). PKR protein is constitutively expressed in vertebrates, and in basal state, it remains inactive through autoinhibitory interactions. After the infection with an RNA virus, PKR expression is further induced, as it is an ISG that presents ISRE sequences on its promoter [128,129]. After an infection, PKR recognizes dsRNAs or RNA duplexes through two dsRBM, and this interaction leads to a conformational change that liberates its kinase domain from autorepression, leading to PKR dimerization and transphosphorylation in Thr residues, which constitute the hallmark of its activation [119,131]. Once activated, PKR molecules phosphorylate eIF2 α at the Ser52 residue, hampering new translation cycles, leading to the process known as host shut-off, in which translation can no longer occur [119]. This constitutes a pivotal antiviral mechanism, aimed at preventing viruses from producing their own viral proteins [128]. PKR is not only activated by the presence of dsRNAs, as its interaction with some protein regulators can induce PKR activation or further enhance its function, being PACT one of the best studied [132]. PACT is also a dsRBP, that in response to certain types of cellular stressors activates PKR, even in the absence of dsRNA, just through direct protein-protein interactions [130,140,144]. Therefore, the search for new proteins modulating this axis is fundamental to comprehend it better. In this regard, seeing that IFI27 interacts with both proteins with a high score, and taking into account all the previous results on its ability to modulate innate immune responses, we were very interested in analyzing the possibility of IFI27 being a modulator of PKR and PACT functions.

Nonetheless, even though we focused on the study of PKR and PACT, the detailed examination of IFI27-interactome provided interesting findings that will be worth-studying in the future, and that could support obtained results. First, other proteins known to modulate translation processes were found on IFI27-interactome, such as PPP1CC and TARBP2, with high scores (279 and 373, respectively) (Table A3). PPP1CC is a phosphatase that has been described to dephosphorylate eIF2 α , counteracting PKR function [296]. After the infection with HSV-1, PKR is activated and translation is halted, but HSV-1 has developed a strategy, in which its γ 134.5 protein interacts with PPP1CC and promotes eIF2 α dephosphorylation, restoring cellular translation [352]. Also, Opaluch *et al.* [353] showed that PPP1CC promotes NF- κ B mediated induction of proinflammatory cytokines. As we have described that IFI27 negatively modulates this process, these data could indicate that the interaction of IFI27 with PPP1CC could have a functional effect on the induction of innate immune responses. On the other hand, Gu *et al.* [297] demonstrated that PPP1CC attenuates RLR and TLR-mediated induction of type I IFNs in macrophages by dephosphorylating IRF-3. Hence, PPP1CC seems to be a really interesting target to study in future projects, since it has described roles both in the RLR pathway and a clear effect on the translational process after infection, both antiviral processes that we have studied in this doctoral thesis. On the other hand, TARBP2 is a known modulator of PKR function. Similar to PACT, TARBP2 has three dsRBDs, but conversely, it acts as a negative modulator of PKR activity as it promotes the generation of TARBP2-PKR heterodimers, instead of the PKR homodimers that

lead to PKR activation [141,145]. Furthermore, TARBP2 together with PACT, are known cofactors for DICER in the generation of micro RNAs (miRNAs) and siRNAs. Interestingly, “pre-miRNA binding” molecular function shows a ~78-fold enrichment in IFI27-interactome (Table A5), suggesting the potential participation of IFI27 in this process. dsRNAs are processed into siRNAs or miRNAs [354] that are loaded into the RNA-induced silenced complex (RISC, composed by the siRNA, DICER, TARBP2, PACT, and other proteins) [355], which is essential for RNA interference processes (RNAi), as this complex finds complementary sequences on RNAs and cleaves them [354]. This constitutes an antiviral mechanism that has been demonstrated to be of major importance in plants and invertebrates, as they lack an IFN system and rely on antiviral RNAi. However, in recent years, some authors claim that antiviral RNAi could still be important on cell-intrinsic restriction of viruses in mammals [356,357], although this is a matter of debate. For instance, Zhang *et al.* [357] found that upon activation of RNAi, extracellular vesicles in mice contained virus-derived siRNAs from Nodamura virus (NoV), Sindbis virus (SINV), and ZIKV.

Furthermore, other members of IFI27 interactome that caught our attention were RNA-binding protein Staufen homologs 1 (STAU1) and 2 (STAU2), with scores of 925 and 505 (Table A3). Interestingly, STAU1 has been described to modulate the induction of ISGs and proinflammatory cytokines such as *IFIT2*, *IFIT3*, *OASL*, and *CCL2* [358], while chicken STAU2 interacts with the NS1 protein of AIV H5N1, resulting in an increased replication of this virus [359]. In addition, a dsRNA-interactome study in PaKi cells (immortalized kidney cells from *Pteropus alecto* bats) infected with SINV, showed that STAU1, together with ADAR1, PACT, and DICER proteins were interacting with dsRNAs. By using STRING, a database that predicts protein-protein interactions (<https://string-db.org/>), Dai *et al.* [360] suggested that STAU1, STAU2, ADAR1, TARBP2, PACT, and DICER, are all interacting partners that modulate the RNAi pathway and could influence the binding affinity of RLRs to dsRNA. Additional studies support this theory [361], by arguing that the RNAi pathway and RLRs could intersect and affect each other. This would be a really interesting theory to explore in future studies, and a plausible additional explanation for the effect of IFI27 on RLRs activity, as we have shown that IFI27 negatively influences RIG-I- and MDA5-mediated induction of antiviral responses.

Other proteins found on the interactome that could support the activity of IFI27 as a negative modulator of IFN and proinflammatory cytokines induction (Table A3) are: (i) MIF, which is a proinflammatory cytokine which functions as a critical upstream mediator of host innate and adaptative immune responses [362]; (ii) the IL enhancer binding factors 3 (ILF3) and 2 (ILF2), which presented really high scores (1414 and 940, respectively) (Table A3). ILF3 has been described to be an essential negative regulator of type I IFN responses and DCs maturation, as its depletion in MoDCs led to an strong increase of innate immune responses [363]. Similarly, ILF2, usually described as an ILF3 partner, has been shown to inhibit NLRP3 inflammasome activation [364] and to suppress type I IFN production through the degradation of IRF-7 in Mallards [365]; (iii) the helicase DDX21, which presented a high score of 904, and competes with RIG-I for RNA binding, negatively modulating the induction of innate immune responses [366]. Interestingly, DDX21 KO led to an enhanced production of SeV-induced IFN- β production and ISGs expression, which strongly recalls our results for IFI27 KO cells. This strong correlation should be studied in future studies, as the similarities of their function could be due to a cooperative mechanism; (iv) the DDX3X helicase, which participates in translation initiation, and is exploited by many viruses such as HSV-1, human cytomegalovirus (HCMV), HCV, HIV-1, and SARS-CoV-2 to facilitate viral replication [367]. Also, Oshiumi *et al.* [368] described that DDX3X could

bind to RIG-I, MDA5, and MAVS, and modulate MAVS activity on transducing the antiviral signaling; and finally, (v) the Poly(rC)-binding protein 2 (PCBP2), with a score of 549, which is a known antagonist of cGAS-STING pathway [369], an essential antiviral response against DNA viruses, which recently, has been proposed as an important mechanism also against RNA viruses. RNA viruses as ZIKV, DENV, and chikungunya virus (CHIKV) present different factors that antagonize cGAS-STING pathway [370]. Thus, given that IFI27 interacts with PCBP2, it would be interesting to investigate the possible effect of IFI27 on cGAS-STING pathway.

Additionally, molecular function classification showed a great enrichment of components of actin cytoskeleton (“actin filament binding”, “actin binding”, “microfilament motor activity”, “cytoskeletal motor activity”, and “structural constituent of cytoskeleton”; all of these are categorized as “cytoskeleton” in Fig. R32B) (Table A5), and this was also observed for biological process classification (“actin filament organization”, “actin filament based movement”, and “vesicle transport along actin filament”; all of these are categorized as “Actin filaments” in Fig. R32A) (Table A4). Although in this doctoral thesis the possible function of IFI27 within actin cytoskeleton organization or activity has not been analyzed, actin cytoskeleton has been linked to the proper function and signaling of the innate immune responses, and particularly of RLRs. A recent study by Acharya *et al.* [371] suggests that RLR activation is a two-step process: first, the actin cytoskeleton remodeling prime RLRs, and then, immunostimulatory RNA completes the activation. Many viruses disrupt the correct function of actin cytoskeleton, leading to a relocation of PPP1R12C, a regulatory subunit of the PPP1CC protein. Then, PPP1CC dephosphorylates CARD domains of RIG-I and MDA5, which mediates RLRs priming and together with the RNA agonist, induces effective RLR initiation of antiviral signaling [371]. Therefore, it will be interesting to seek possible connections within these pathways in the future.

5.3.3.1. IFI27 affects protein translation by interacting with PACT and PKR

After analyzing MS data, next step we took was to confirm the interactions between IFI27 and PACT, and IFI27 and PKR by co-IP followed by Western blot. Similar to the results obtained for Co-IPs between IFI27 and RIG-I (Fig. R20B) or MDA5 (Fig. R25B), IFI27 co-immunoprecipitated together with PACT both after poly(I:C) treatment (Fig. R33B) and after SARS-CoV-2 infection (Fig. R34B), while in mock conditions, the interaction was very weak (Fig. R33B). Furthermore, correlating with previous data (Fig. R20B, R25B), if prior to co-IP, samples were treated with RNAses, we could observe that the interaction between IFI27 and PACT was lost (Fig. R33B). To support these results, we again performed co-IPs of IFI27 and PACT after treatment with poly(I:C), but in this case, in addition to IFI27 WT, we analyzed the possible binding to PACT of IFI27 variants that do not bind dsRNA (IFI27-S63L and IFI27-V82A) (Fig. R19 and Fig. R35). Interestingly, both IFI27-S63L and IFI27-V82A lost their ability to bind to PACT (Fig. R35), supporting an RNA-dependent interaction of IFI27 with PACT. Also, IFI27-PKR interaction was confirmed by Co-IP, showing that after poly(I:C) treatment, IFI27 and PKR strongly co-immunoprecipitated (Fig. R36). In mock conditions, even though the amount of IFI27 that co-immunoprecipitated together with PKR was strongly reduced, some interaction was detected (Fig. R36). This interaction in mock conditions is probably mediated by endogenous RNAs such as mitochondrial, non-coding, or dsRNAs formed by inverted Alu repeats, specifically during mitosis, as it has been previously reported that PKR can bind these endogenous RNAs [128,165,299]. Furthermore, and similar to RIG-I and MDA5 (Fig. R21 and R28, respectively), poly(I:C) treatment increased the level of colocalisation between IFI27 and PACT (Fig. R37A) and IFI27 and PKR (Fig. R37B), compared to mock conditions. Thus, the co-IPs and

colocalization experiments of IFI27 with RIG-I, MDA5, PACT, and PKR are very similar, showcasing the RNA binding nature of all these proteins [80,128,140] and (Fig. R18).

In this doctoral thesis, we found that IFI27 overexpression, either after VSV or SARS-CoV-2 infections (Fig. R39), or poly(I:C) treatment (Fig. R43B, R43C), and even in mock-infected cells, leads to an enhancement of PKR activation and function, thus promoting host-shut off and halting of *de novo* protein synthesis. Conversely and correlating with these results, in IFI27 KO cells, the level of activation of PKR was significantly lower than in WT cells, both after mock and poly(I:C) treatment (Fig. R40), and after SARS-CoV-2 infection (Fig. R41). Remarkably, the phenotype-rescue by overexpressing IFI27 in IFI27-KO cells, led to a recovery of PKR activation levels, which were even higher than in WT cells (Fig. R40). All these results clearly show that IFI27 is an important host-factor for PKR activity, and remarkably, this effect is observed after a broad variety of stimuli, showing that it could be a molecular mechanism common to many RNA viruses, and even in the absence of infection, probably due to the activation of PKR by the aforementioned endogenous RNAs such as mitochondrial, non-coding, or Alu repeats-dsRNAs [128,165,299]. A possibility that could have created an experimental bias is transfection processes, which can stimulate IFN pathways [140,305] and subsequently activate PKR. However, aside from the usual experimental controls, we always included the same amount of lipofectamine and DNA plasmids in transfection and co-transfection experiments. In addition, we showed that even on the absence of any transfection, both in mock- and SARS-CoV-2-infected cells, IFI27 KO led to a significant decrease on PKR and eIF2 α phosphorylation (Fig. R41), which is indicative of a lower PKR activity.

Next, we wanted to decipher PACT importance and analyze if this protein could be playing any role on IFI27-mediated modulation of PKR activity. As we previously mentioned, PACT effect on PKR activity is highly complex and dependent on the co-interaction with other players such as ADAR1 or TARBP2 [150,151]. Thus, IFI27 could be a novel modulator that participates on PACT-dependent modulation of PKR activity. To investigate this possibility, we employed both HeLa WT and KO for either PACT or PKR genes, and we described that PACT presence is essential for IFI27-mediated modulation of PKR activity (Fig. R43). Furthermore, PKR overexpression rescued the phenotype of KO PKR and DKO cells, but IFI27 overexpression only further enhanced PKR activity in WT and PKR KO cells, and not in PACT KO or DKO cells, showing that PACT is needed for the modulation of PKR activity by IFI27 (Fig. R46B). Interestingly, we found that PACT is necessary for the binding of IFI27 to PKR (Fig. R47), providing a mechanistic explanation of the need of PACT in this modulatory complex of PKR activity (Fig. D3).

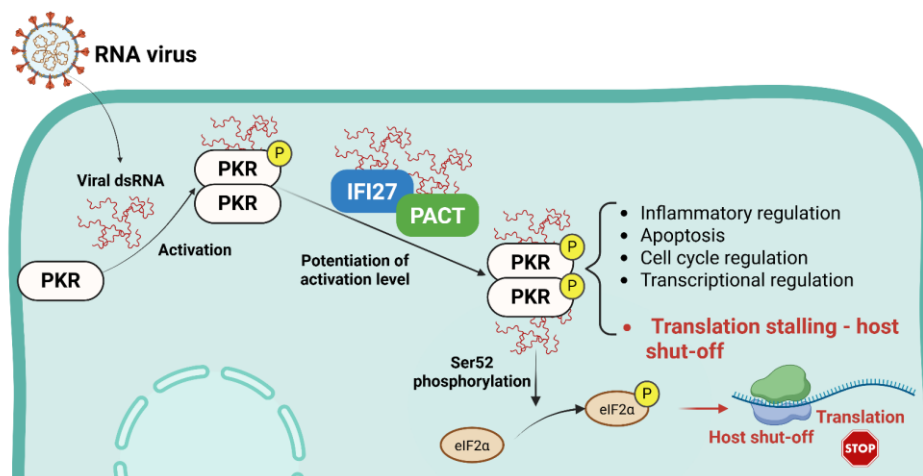


Figure D3. Schematic representation of IFI27-PACT-mediated modulation of PKR activity in response to RNA viruses.

5.3.4. Integration of IFI27-mediated modulation of RLRs and PKR: two arms of innate immune responses

RLRs and PKR are RBPs that detect and respond to the presence of exogenous viral dsRNAs or RNAs containing duplex regions. Both arms are activated in response to viral infections, but their function and objectives are different [372]. RLRs trigger the induction of IFNs and proinflammatory cytokines, and the objective is to initiate and promote the antiviral gene expression program in infected tissues, in order to prevent and ultimately stop viral spreading. On the other hand, the activation of PKR aims to stop translation in individual cells infected by viruses, strongly impairing the translation of viral proteins, but also affecting host translation and promoting apoptosis of infected cells [133,373]. Interestingly, the induction of apoptosis by PKR activation is also a shared function with IFI27, which has been described to be a pro-apoptotic factor by destabilizing mitochondrial membrane potential [174,176] and to favor apoptosis induced by TRAIL [186]. Thus, the positive modulation of PKR by IFI27 could provide a novel molecular mechanism to the pro-apoptotic potential of IFI27, which needs further investigation. In this doctoral thesis, we have shown that IFI27 negatively modulates RIG-I- and MDA5-mediated induction of IFNs and proinflammatory cytokines, and at the same time, IFI27 positively affects PKR activity, promoting translational shut-off. Thus, IFI27-induced PKR activation enhancement, could be contributing to reduce the production of IFNs and cytokines by reducing translation, providing an additional explanation on the possible molecular mechanism that could be leading to the observed effects of IFI27 on innate immune responses. Similar effects have been described before, as Garaigorta *et al.* [133] and Daito *et al.* [134] showed that HCV hamper IFN-induced antiviral mechanisms by inducing phosphorylation of PKR, which leads to a decreased translation of effector ISGs. Also, ZIKV promotes PKR activation and reduces translation efficiency of IFNs and ISGs [135]. In line with these studies, here we showed that IFI27 overexpression induced PKR activation in HEK-293T-hACE2 cells infected with SARS-CoV-2 (Fig. R39D, R39E), which was associated with an increase in viral titers (Fig. R39B) compared to the empty control. In previous data, we observed that IFI27 absence led to an increase on the induction of IFNs, ISGs and proinflammatory cytokines (Fig. R6A, R7D, R7E) and a reduction of SARS-CoV-2 viral titers (Fig. R4D). Integration of these different evidences led us to conclude that as IFI27 negatively modulate RLRs and activates PKR, so that the mRNA induction and translation of antiviral proteins is reduced, leading to an increased viral replication. Thus, the

coordination of RLR- and PKR-dependent responses requires precise regulation in order to achieve an efficient antiviral response, and in this process, the existence of novel players that modulate activation or inhibition of both pathways seems essential.

Even though we described that RIG-I and MDA5 co-immunoprecipitated with IFI27 after different stimuli (Fig. R20B, R20C, R25, R26, R27), we did not detect their presence in the MS study of IFI27-interactome (Table A3). Knowing that PACT is essential for the interaction between IFI27 and PKR, it is a plausible possibility that the interaction between IFI27 and RIG-I or MDA5, could also be mediated by some intermediate partner, as well as by RNA, as described (Fig. R18, R19). In this sense, PACT could be an option as an intermediary of IFI27 interaction with RLRs. Several studies have described that PACT directly binds RIG-I and MDA5 and contribute to their function on the induction of type I IFNs. Kok *et al.* [103] described that PACT could bind to the C-terminal of RIG-I and potentiate its activation in response to poly(I:C). Other studies showed that in response to viruses such as VSV, SeV, EMCV, and MHV, PACT is needed for a proper activation of RLRs [374]. Similarly, PACT and MDA5 have also been described to interact, leading to a potentiation of the oligomerization of MDA5 [105]. Furthermore, a recent study described that LGP2's ability to negatively modulate RIG-I function depends on its binding to PACT, as mutations in LGP2 residues required for its interaction with PACT, resulted in the loss of its regulatory effect on RLRs [375]. This last study clearly recalls our observations, as IFI27 binds PACT and negatively modulates both RIG-I and MDA5 function, but IFI27 variants that do not bind to PACT partially lose their ability to reduce the induction of *IFNL1*, *IFIT2*, and *CXCL10* (Fig. R23B). Most studies describe that PACT is an activator of RLRs and PKR. However, in specific conditions, PACT act as a inhibitor of PKR by interacting with TARBP2 [150] or ADAR1 [152]. Interestingly, PACT and ADAR1 cooperate to avoid an unneeded hyperactivation of PKR by self-dsRNAs in uninfected cells [152]. Similarly, IFI27 could interact with PACT and change it to an RLR inhibitor, and cooperatively, avoid an hyperactivation of RLRs that could lead to immune-mediated damage. Therefore, taking into account these results, we will analyze this possibility in future experiments by studying if IFI27 losses its ability to interact with RIG-I and MDA5 and modulate their function in KO cells for PACT. Other possible intermediate mediator of IFI27 interaction with RLRs could be DDX3X, which was found in IFI27-protein interactome (Table A3), and as previously mentioned, has been described to interact with RIG-I, MDA5, and MAVS [368]. An alternative explanation is that the level of expression of endogenous RIG-I and MDA5 in HEK-293T cells, which were the cells used for MS analysis, is very low, so not enough protein is detected in the MS analysis if the proteins are not overexpressed. Human Protein Atlas database provides data on relative abundance of a gene's RNA transcripts in a specific sample, such as cell lines. The values of normalised transcripts per million (nTPM) of *RIG-I* and *MDA5* in HEK-293T are 0.9 and 4, respectively, while the values of nTPM for *PACT* and *PKR* are 23.5 and 11.4, respectively (<https://www.proteinatlas.org/>). Thus, these data could support that the lower abundance of RIG-I and MDA5 is the reason why we did not detect these proteins in IFI27-protein interactome.

Cláudio *et al.* [376] explain that PKR pathway could be described as part of a microbial stress response that intersects and coordinates with the innate immune sensing to achieve a proper response. This intersection is exemplified by the generation of crucially important structures, the SGs, which function as signaling hubs that coordinate antiviral responses [161]. The generation of SGs is an important hallmark of PKR activation, as translational arrest induced by the phosphorylation of eIF2 α leads to the dissociation of translation initiation complexes and to the recruitment of different components of SGs, which aggregate in membrane-less cytoplasmic foci.

These structures drive translational recovery if the stressful conditions disappear, but they also function as hubs from which antiviral responses are coordinated [153–157]. A good indicator of the pivotal role of SGs is that many studies have found that crucial players of innate immune responses are recruited to SGs, such as PKR, RIG-I, MDA5, OAS/RNaseL, ADAR1, TRIM25, PACT, apoptosis regulators, viral components, regulators of host gene expression, and cytokine mRNAs [155,158], and that SGs function as important connectors of different antiviral pathways, including PKR-dependent and RLR-dependent antiviral responses. In this regard, Onomoto *et al.* [155] found that in response to IAV infection, the generation of antiviral SGs (avSGs) was induced in a PKR-dependent manner, and these structures were essential for a proper IFN- α induction and restriction of viral replication. Additionally, in response to vaccinia virus lacking the PKR antagonist E3L, PKR was found to be required for the proper activation of MAVS by MDA5, by a mechanism distinct to eIF2 α phosphorylation, and reciprocally, MDA5 potentiated PKR activation, as catalytic activity of PKR was impaired in MDA5-KO cells [160]. Thus, PKR- and RLR-dependent pathways are coordinated and intricate processes that integrate to achieve a proper antiviral response. Very interestingly, in this doctoral thesis, we have described that IFI27 overexpression increases the generation of SGs in response to poly(I:C) (Fig. R44B, R44C, R45, R46C and R46D) due to its ability to activate PKR. Importantly, this effect was not observed in HeLa PACT-KO cells, again showing how IFI27-mediated modulation of PKR activity is dependent on the presence of PACT. We could confirm the presence of PKR on these SGs, as described before [158], by studying the colocalization with the SGs markers G3BP1 and eIF3 η (Fig. R44A). However, IFI27 did not show a clear and specific co-localization with SGs (Fig. R45), meaning that IFI27 could be acting upstream, inducing PKR activation which then promotes SGs generation. Thus, indirectly, IFI27 is an important modulator of SGs formation, which are essential to coordinate and modulate PKR and RLRs activity (Fig. D4). As previously mentioned, we did not explore the potential involvement of IFI6 on PKR modulation, but in future studies, due to the similarities between IFI27 and IFI6, and that both proteins act as negative modulators of RIG-I function, it will be interesting to study the possible interaction between IFI6, PACT, and PKR.

A clear illustration of the importance of PKR and RLRs in the antiviral response is the high number of virus-encoded antagonists, proteins that allow viruses to counteract or bypass the effect of these two pathways. Some examples of RIG-I and MDA5 antagonists are: (i) the V proteins of several paramyxoviruses, which block MDA5 activity [377]; (ii) the VP35 of Ebola virus (EboV), which blocks PACT-mediated RIG-I activation [378]; (iii) the NS1 protein of IAV, which specifically inhibits the activity of RIG-I and its stimulation by PACT [379]; (iv) the 2A protease from enteroviruses, which cleaves both MDA5 and MAVS [380,381]; or (v) even ORF7b protein of SARS-CoV-2, which inhibits RIG-I-mediated MAVS activation [382]. On the other hand, we can provide many examples of PKR inhibitors, such as (i) Vaccinia virus K3L and E3L proteins, which inhibit eIF2 α phosphorylation [383]; (ii) the IAV NS1 protein, which directly binds PKR and inhibits its function [384]; (iii) the HSV-1 γ 34.5 or Us11 proteins, which prevent accumulation of phosphorylated eIF2 α [385]; or (iv) SARS-CoV-2 N protein, which sequesters dsRNA through domain N2b and prevents PKR activation [301].

Some viruses have developed strategies to counteract some host factors of the innate immunity while still being sensitive to others. For instance, for a proper induction of IFN- β after SeV-infections, RIG-I and MAVS functions are essential [386], while SeV C protein is a well-described PKR inhibitor, as it limits the generation of dsRNAs within infected cells [86]. On the other hand, IAV NS1 protein is a well-known inhibitor of RIG-I and PKR activity [234,384], but

interestingly, the absence of PKR boosted IFN levels in response to the infection with IAV lacking NS1 [160], as inhibition of translation also reduces the production of host proteins, showing the high complexity of these responses and the need for a proper regulation. Therefore, upon infection with viruses that lack PKR inhibitors and are more sensitive to its function, IFI27 could be a target to potentiate this pathway and efficiently restrict the replication of these viruses, while upon infection with viruses that are able to efficiently counteract PKR function, IFI27 could be used as a target to switch the balance and achieve a higher activation of PKR-dependent pathway. Conversely, for viruses that are able to efficiently counteract RIG-I and/or MDA5 function, IFI27 and IFI6 could be seen as “proviral” factors, further favoring the inhibition of these pathways. Thus, IFI27 and IFI6 effect on innate immune responses is broad after infection with different RNA viruses, but the outcome of their function on viral replication and disease could be different depending on the specific virus and its characteristics.

As conclusion, in this doctoral thesis we described that the expression of the IFN-induced proteins IFI27 and IFI6 is strongly induced by the infection with different RNA viruses such as IAV, SARS-CoV-2, and DENV, and for the first time, we showed that the absence of IFI27 and IFI6 leads to a lower replication of these three viruses. We found that this effect on viral replication could be explained by a negative modulation on the induction of innate immune responses exerted by both IFI27 and IFI6, as the absence of these two proteins resulted in stronger antiviral responses after infection with IAV, SARS-CoV-2, SeV, or treatment with poly(I:C). On the one hand, the study of the possible molecular mechanisms behind these observations demonstrated that IFI27 and IFI6 are RBPs that interact with both poly(I:C) and viral RNAs, and negatively modulate RIG-I function, and IFI27 also negatively modulates MDA5 function, which results in a lower production of IFNs, proinflammatory cytokines, and ISGs. On the other hand, we demonstrated that IFI27 interacts with PACT and PKR proteins, which results in an enhancement of PKR activity and the generation of dsRNA-induced SGs. Furthermore, we observed that this IFI27-mediated induction of PKR activity was dependent on the presence of PACT. All in all, these evidences suggest that IFI27 and IFI6 are pivotal modulators of innate immune responses. IFI27 could be a pivotal modulator, that shifts antiviral responses to “pro-PKR-dependent pathways” to promote the inhibition of host and viral protein synthesis, likely delaying viral replication, inducing apoptosis of infected cells, and reducing the expression of IFNs and proinflammatory cytokines (Fig. D4). This could be representative of a process that is induced to avoid sustained and deleterious inflammatory responses and restore homeostasis by eliminating infected cells. Therefore, this work sheds new light on the complexity of antiviral responses, describes for the first time the role of IFI27 and IFI6 in these responses, and raises new questions to be answered that could help us develop new therapies or preventive treatments focused on adjusting this innate immunity signaling pathways to the needs of each infection process, to obtain a more efficient and controlled response.

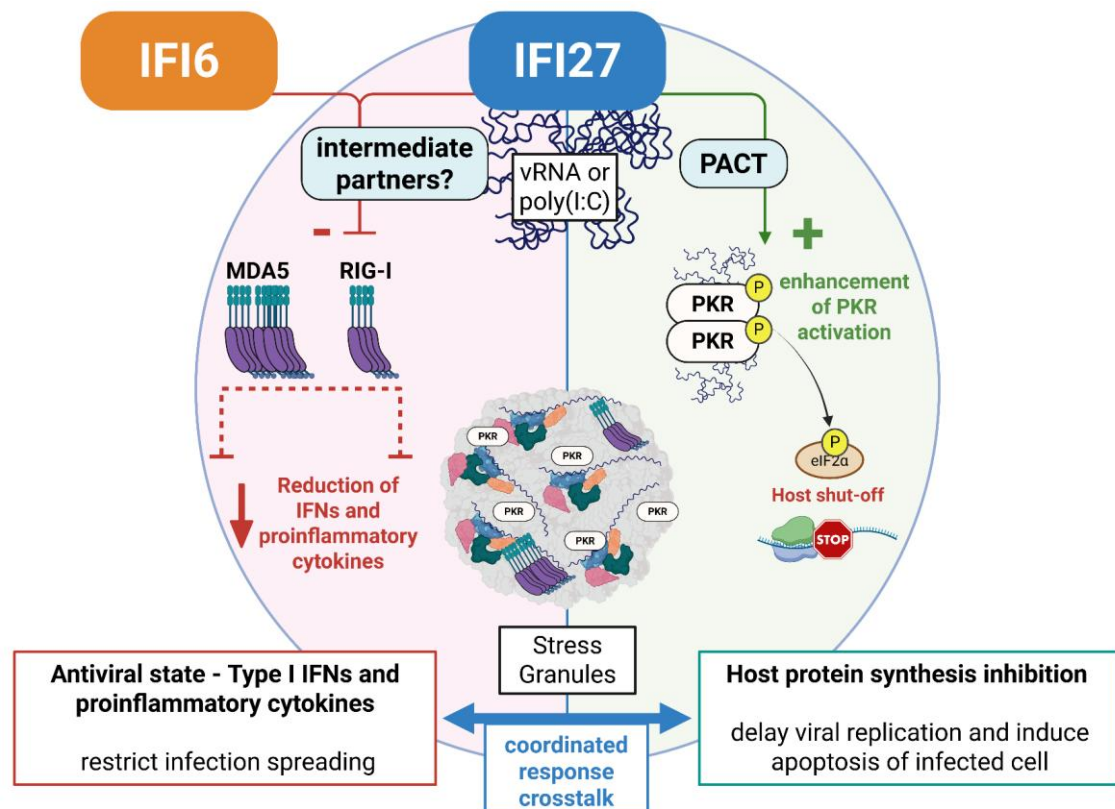


Figure D4. Graphical schema of the integration of the different IFI27 and IFI6 findings of this doctoral thesis.

CONCLUSIONS

6. CONCLUSIONS

1. The expression of IFN alpha-inducible proteins IFI27 and IFI6 is strongly induced in tissue-culture cells after the treatments with the dsRNA analog poly(I:C), and IFN- α ; and after the infection with either IAV, SARS-CoV-2, or DENV2.
2. IFI27 and IFI6 knock-down or KO in A549 cells led to lower viral titers of IAV, SARS-CoV-2, and DENV2 than in the control cells. Similarly, DENV2 infection in MoDCs after knocking-down IFI6, led to markedly lower levels of DENV2 total RNA than in the control cells. Thus, the absence of either IFI27 or IFI6 expression negatively affects viral replication of three, non-related RNA viruses.
3. After poly(I:C) treatment, or infections with different RNA viruses, such as IAV, SARS-CoV-2, and SeV, knock-down and KO of IFI27 and IFI6 led to a higher induction of mRNA levels of innate immune genes, such as *IFNL1*, *IFIT2*, and *CXCL10*, than in the control cells, correlating with previous data showing lower viral replication in the knocked-down and KO cells. Additionally, treatment of IFI27 and IFI6 KO cells with poly(I:C) led to a higher induction of antiviral responses than in WT cells, which correlated with lower viral titers after rVSV-GFP infection. Conversely, the overexpression of IFI27 or IFI6 proteins associated with lower levels of *IFNL1* and *CXCL10* mRNAs than in the control cells. These data indicate that IFI6 and IFI27 counteract the innate immune responses after the infections with the mentioned RNA viruses and after poly(I:C) treatment.
4. RNAseq study of poly(I:C)-treated, IFI6 KO HAP-1 cells, compared to poly(I:C)-treated WT HAP-1 cells, showed at least a 2-fold upregulation of genes involved different biological processes, such as: regulation of viral replication, responses to viral infection, IFN responses, regulation of viral entry, cell migration and chemotaxis, metabolic modulation and signaling, and regulation of immune system process, indicating that IFI6 negatively regulates the innate immune responses after poly(I:C) treatment.
5. Infection of mice with recombinant IAVs overexpressing either mCherry, IFI27, or IFI6 proteins, showed that mRNA levels of *Ifnl3*, *Ifit2*, *Ccl2*, and *Tnf* in mice lungs were significantly decreased when IFI27 or IFI6 proteins were overexpressed in comparison to mice infected with the control virus overexpressing the mCherry protein. These results indicate that IFI6 and IFI27 negatively modulate the innate immune responses after IAV infections *in vivo*.
6. IFI27 and IFI6 are dsRNA binding proteins. Through its RNA binding capacity, IFI27 interacts with RIG-I and MDA5, and IFI6 interacts with RIG-I. In presence of RNAses, these interactions are severely impaired, showing that either dsRNAs, and/or ssRNAs are required for the interaction between IFI27 or IFI6 and these RLRs.
7. IFI27 and IFI6 counteract the induction of innate immune responses mediated by RLRs. IFI27 competes with RIG-I and MDA5 for RNA binding, so that these PRRs have a reduced capacity to recognize viral RNAs, and the induction of innate immune responses is diminished. Likewise, IFI6 affects RIG-I-ubiquitination, which could be a consequence of reduced activation of RIG-I.

8. IFI27-protein interactome was studied by MS, observing a significant overrepresentation of RNA binding proteins and proteins participating in mRNA translation. We specifically studied two IFI27-interacting proteins that were included in both groups and had high scores: PKR and PACT. The interaction of IFI27 with these two proteins was verified by co-IP followed by Western blot analysis and was shown to be dependent on the presence of RNA. Furthermore, we demonstrated that the interaction of IFI27 with PKR is mediated by PACT.
9. IFI27 enhances PKR activation in response to poly(I:C), SARS-CoV-2, and VSV infections, and after PKR overexpression in a PACT-dependent manner, given that in PACT KO cells, overexpression of IFI27 failed to enhance PKR activity.
10. IFI27 promotes the generation of dsRNA-induced SGs in WT cells but not in KO-PACT cells, further showing the importance of PACT on IFI27-mediated modulation of PKR function and subsequent SG formation.

BIBLIOGRAPHY

7. BIBLIOGRAPHY

- [1] Domingo E, Sheldon J, Perales C. Viral quasispecies evolution. *Microbiol Mol Biol Rev* 2012;76:159–216. <https://doi.org/10.1128/MMBR.05023-11>.
- [2] Payne S. Family Coronaviridae. *Viruses* 2017;149–58. <https://doi.org/10.1016/B978-0-12-803109-4.00017-9>.
- [3] Woo PCY, de Groot RJ, Haagmans B, Lau SKP, Neuman BW, Perlman S, et al. ICTV Virus Taxonomy Profile: Coronaviridae 2023. *J Gen Virol* 2023;104:001843. <https://doi.org/10.1099/jgv.0.001843>.
- [4] Pringle CR. Virus taxonomy 1996 - a bulletin from the Xth International Congress of Virology in Jerusalem. *Arch Virol* 1996;141:2251–6. <https://doi.org/10.1007/BF01718231>.
- [5] de Vries AAF, Horzinek MC, Rottier PJM, de Groot RJ. The Genome Organization of the Nidovirales: Similarities and Differences between Arteri-, Toro-, and Coronaviruses. *Semin Virol* 1997;8:33–47. <https://doi.org/10.1006/smv.1997.0104>.
- [6] Cui J, Li F, Shi Z-L. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol* 2019;17:181–92. <https://doi.org/10.1038/s41579-018-0118-9>.
- [7] Tang G, Liu Z, Chen D. Human coronaviruses: Origin, host and receptor. *J Clin Virol* 2022;155:105246. <https://doi.org/10.1016/j.jcv.2022.105246>.
- [8] Current ICTV Taxonomy Release | ICTV n.d. <https://ictv.global/taxonomy> (accessed September 4, 2025).
- [9] Li Q, Shah T, Wang B, Qu L, Wang R, Hou Y, et al. Cross-species transmission, evolution and zoonotic potential of coronaviruses. *Front Cell Infect Microbiol* 2023;12:1081370. <https://doi.org/10.3389/fcimb.2022.1081370>.
- [10] Wille M, Holmes EC. Wild birds as reservoirs for diverse and abundant gamma- and deltacoronaviruses. *FEMS Microbiol Rev* 2020;44:631–44. <https://doi.org/10.1093/femsre/fuaa026>.
- [11] Kesheh MM, Hosseini P, Soltani S, Zandi M. An overview on the seven pathogenic human coronaviruses. *Rev Med Virol* 2022;32:e2282. <https://doi.org/10.1002/rmv.2282>.
- [12] Liu DX, Liang JQ, Fung TS. Human Coronavirus-229E, -OC43, -NL63, and -HKU1 (*Coronaviridae*). In: Bamford DH, Zuckerman M, editors. *Encyclopedia of Virology* (Fourth Edition), Oxford: Academic Press; 2021, Volume 2, p. 428–40. <https://doi.org/10.1016/B978-0-12-809633-8.21501-X>.
- [13] Arden KE, Nissen MD, Sloots TP, Mackay IM. New human coronavirus, HCoV-NL63, associated with severe lower respiratory tract disease in Australia. *J Med Virol* 2005;75:455–62. <https://doi.org/10.1002/jmv.20288>.
- [14] Bastien N, Anderson K, Hart L, Caesele PV, Brandt K, Milley D, et al. Human Coronavirus NL63 Infection in Canada. *J Infect Dis* 2005;191:503–6. <https://doi.org/10.1086/426869>.
- [15] Woo PCY, Lau SKP, Chu C, Chan K, Tsoi H, Huang Y, et al. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol* 2005;79:884–95. <https://doi.org/10.1128/JVI.79.2.884-895.2005>.
- [16] Pene F, Merlat A, Vabret A, Rozenberg F, Buzyn A, Dreyfus F, et al. Coronavirus 229E-related pneumonia in immunocompromised patients. *Clin Infect Dis* 2003;37:929–32. <https://doi.org/10.1086/377612>.
- [17] MERS-CoV Dashboard. WHO Data n.d. [https://www.who.int/news-room/fact-sheets/detail/middle-east-respiratory-syndrome-coronavirus-\(mers-cov\)](https://www.who.int/news-room/fact-sheets/detail/middle-east-respiratory-syndrome-coronavirus-(mers-cov)) (accessed September 4, 2025).
- [18] COVID-19 cases | WHO COVID-19 dashboard. WHO Data n.d. <https://data.who.int/dashboards/covid19/cases> (accessed September 4, 2025).
- [19] Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020;395:565–74. [https://doi.org/10.1016/S0140-6736\(20\)30251-8](https://doi.org/10.1016/S0140-6736(20)30251-8).

- [20] Chung C, Irudayaraj P, Lallow E, Xu Z, Park YK, Kudchodkar SB, et al. An overview of SARS-CoV-2 viral proteins with relevance to improved diagnostic and therapeutic platforms. *Front Virol* 2024;4. <https://doi.org/10.3389/fviro.2024.1399993>.
- [21] Wang M-Y, Zhao R, Gao L-J, Gao X-F, Wang D-P, Cao J-M. SARS-CoV-2: Structure, Biology, and Structure-Based Therapeutics Development. *Front Cell Infect Microbiol* 2020;10. <https://doi.org/10.3389/fcimb.2020.587269>.
- [22] Yang H, Rao Z. Structural biology of SARS-CoV-2 and implications for therapeutic development. *Nat Rev Microbiol* 2021;19:685–700. <https://doi.org/10.1038/s41579-021-00630-8>.
- [23] Lamers MM, Haagmans BL. SARS-CoV-2 pathogenesis. *Nat Rev Microbiol* 2022;20:270–84. <https://doi.org/10.1038/s41579-022-00713-0>.
- [24] Dharra R, Kumar Sharma A, Datta S. Emerging aspects of cytokine storm in COVID-19: the role of proinflammatory cytokines and therapeutic prospects. *Cytokine* 2023;156287. <https://doi.org/10.1016/j.cyto.2023.156287>.
- [25] Del Valle DM, Kim-Schulze S, Huang H-H, Beckmann ND, Nirenberg S, Wang B, et al. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat Med* 2020;26:1636–43. <https://doi.org/10.1038/s41591-020-1051-9>.
- [26] Ghaffarpour S, Ghazanfari T, Ardestani SK, Naghizadeh MM, Vaez Mahdavi MR, Salehi M, et al. Cytokine profiles dynamics in COVID-19 patients: a longitudinal analysis of disease severity and outcomes. *Sci Rep* 2025;15:14209. <https://doi.org/10.1038/s41598-025-98505-y>.
- [27] Relich RF, Loeffelholz MJ. Taxonomic Changes for Human Viruses, 2020 to 2022. *J Clin Microbiol* 2022;61:e00337-22. <https://doi.org/10.1128/jcm.00337-22>.
- [28] Javanian M, Barary M, Ghebrehewet S, Koppolu V, Vasigala V, Ebrahimpour S. A brief review of influenza virus infection. *J Med Virol* 2021;93:4638–46. <https://doi.org/10.1002/jmv.26990>.
- [29] Guo X, Zhou Y, Yan H, An Q, Liang C, Liu L, et al. Molecular Markers and Mechanisms of Influenza A Virus Cross-Species Transmission and New Host Adaptation. *Viruses* 2024;16:883. <https://doi.org/10.3390/v16060883>.
- [30] Carter T, Iqbal M. The Influenza A Virus Replication Cycle: A Comprehensive Review. *Viruses* 2024;16:316. <https://doi.org/10.3390/v16020316>.
- [31] Influenza (seasonal) n.d. [https://www.who.int/news-room/fact-sheets/detail/influenza-\(seasonal\)](https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)) (accessed September 10, 2025).
- [32] Ashraf MA, Raza MA, Amjad MN, ud Din G, Yue L, Shen B, et al. A comprehensive review of influenza B virus, its biological and clinical aspects. *Front Microbiol* 2024;15:1467029. <https://doi.org/10.3389/fmicb.2024.1467029>.
- [33] Jackson D, Elderfield RA, Barclay WS. Molecular studies of influenza B virus in the reverse genetics era. *J Gen Virol* 2011;92:1–17. <https://doi.org/10.1099/vir.0.026187-0>.
- [34] Njouom R, Monamele GC, Ermetat B, Tchatchouang S, Moyo-Tetang S, McCauley JW, et al. Detection of Influenza C Virus Infection among Hospitalized Patients, Cameroon. *Emerg Infect Dis* 2019;25:607–9. <https://doi.org/10.3201/eid2503.181213>.
- [35] Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, et al. Isolation of a Novel Swine Influenza Virus from Oklahoma in 2011 Which Is Distantly Related to Human Influenza C Viruses. *PLoS Pathog* 2013;9:e1003176. <https://doi.org/10.1371/journal.ppat.1003176>.
- [36] Yu J, Wen Z, Hu W, Chen M, Zhang Y, Liu S, et al. Influenza D virus infection in China, 2022–2023. *Emerg Microbes & Infect* 2024;13:2343907. <https://doi.org/10.1080/22221751.2024.2343907>.
- [37] Snoeck CJ, Oliva J, Pauly M, Losch S, Wildschutz F, Muller CP, et al. Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012–2016. *Emerg Infect Dis* 2018;24:1388–9. <https://doi.org/10.3201/eid2407.171937>.
- [38] Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, et al. New world bats harbor diverse influenza A viruses. *PLoS Pathog* 2013;9:e1003657. <https://doi.org/10.1371/journal.ppat.1003657>.

- [39] Webster RG, Govorkova EA. Continuing challenges in influenza. *Ann N Y Acad Sci* 2014;1323:115–39. <https://doi.org/10.1111/nyas.12462>.
- [40] Dunning J, Thwaites RS, Openshaw PJM. Seasonal and pandemic influenza: 100 years of progress, still much to learn. *Mucosal Immunol* 2020;13:566–73. <https://doi.org/10.1038/s41385-020-0287-5>.
- [41] Harrington WN, Kackos CM, Webby RJ. The evolution and future of influenza pandemic preparedness. *Exp Mol Med* 2021;53:737–49. <https://doi.org/10.1038/s12276-021-00603-0>.
- [42] Reid AH, Fanning TG, Hultin JV, Taubenberger JK. Origin and evolution of the 1918 “Spanish” influenza virus hemagglutinin gene. *Proc Natl Acad Sci U S A* 1999;96:1651–6. <https://doi.org/10.1073/pnas.96.4.1651>.
- [43] Peacock TP, Moncla L, Dudas G, VanInsberghe D, Sukhova K, Lloyd-Smith JO, et al. The global H5N1 influenza panzootic in mammals. *Nature* 2025;637:304–13. <https://doi.org/10.1038/s41586-024-08054-z>.
- [44] Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2024, 12 December 2024 n.d. [https://www.who.int/publications/m/item/cumulative-number-of-confirmed-human-cases-for-avian-influenza-a\(h5n1\)-reported-to-who--2003-2024--20-december-2024](https://www.who.int/publications/m/item/cumulative-number-of-confirmed-human-cases-for-avian-influenza-a(h5n1)-reported-to-who--2003-2024--20-december-2024) (accessed September 10, 2025).
- [45] Capelastegui F, Goldhill DH. H5N1 2.3.4.4b: a review of mammalian adaptations and risk of pandemic emergence. *J Gen Virol* 2025;106:002109. <https://doi.org/10.1099/jgv.0.002109>.
- [46] Gamblin SJ, Skehel JJ. Influenza Hemagglutinin and Neuraminidase Membrane Glycoproteins. *J Biol Chem* 2010;285:28403–9. <https://doi.org/10.1074/jbc.R110.129809>.
- [47] Nogales A, DeDiego ML, Martínez-Sobrido L. Live attenuated influenza A virus vaccines with modified NS1 proteins for veterinary use. *Front Cell Infect Microbiol* 2022;12:954811. <https://doi.org/10.3389/fcimb.2022.954811>.
- [48] Chiem K, López-García D, Ortego J, Martinez-Sobrido L, DeDiego ML, Nogales A. Identification of Amino Acid Residues Required for Inhibition of Host Gene Expression by Influenza Virus A/Viet Nam/1203/2004 H5N1 PA-X. *J Virol* 2022;96:e00408-21. <https://doi.org/10.1128/JVI.00408-21>.
- [49] Muramoto Y, Noda T, Kawakami E, Akkina R, Kawaoka Y. Identification of Novel Influenza A Virus Proteins Translated from PA mRNA. *J Virol* 2013;87:2455–62. <https://doi.org/10.1128/jvi.02656-12>.
- [50] Chauhan RP, Gordon ML. An overview of influenza A virus genes, protein functions, and replication cycle highlighting important updates. *Virus Genes* 2022;58:255–69. <https://doi.org/10.1007/s11262-022-01904-w>.
- [51] Márquez-Bandala AH, Gutierrez-Xicotencatl L, Esquivel-Guadarrama F. Pathogenesis Induced by Influenza Virus Infection: Role of the Early Events of the Infection and the Innate Immune Response. *Viruses* 2025;17:694. <https://doi.org/10.3390/v17050694>.
- [52] An W, Lakhina S, Leong J, Rawat K, Husain M. Host Innate Antiviral Response to Influenza A Virus Infection: From Viral Sensing to Antagonism and Escape. *Pathogens* 2024;13:561. <https://doi.org/10.3390/pathogens13070561>.
- [53] Gu Y, Zuo X, Zhang S, Ouyang Z, Jiang S, Wang F, et al. The Mechanism behind Influenza Virus Cytokine Storm. *Viruses* 2021;13:1362. <https://doi.org/10.3390/v13071362>.
- [54] Short KR, Kroeze EJBV, Fouchier RAM, Kuiken T. Pathogenesis of influenza-induced acute respiratory distress syndrome. *Lancet Infect Dis* 2014;14:57–69. [https://doi.org/10.1016/S1473-3099\(13\)70286-X](https://doi.org/10.1016/S1473-3099(13)70286-X).
- [55] de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 2006;12:1203-1207. <https://doi.org/10.1038/nm1477>.

- [56] de Almeida PR, Weber MN, Sonne L, Spilki FR. Aedes-borne orthoflavivirus infections in neotropical primates – Ecology, susceptibility, and pathogenesis. *Exp Biol Med* (Maywood) 2024;248:2030–8. <https://doi.org/10.1177/15353702231220659>.
- [57] Vasilakis N, Shell EJ, Fokam EB, Mason PW, Hanley KA, Estes DM, et al. Potential of ancestral sylvatic dengue-2 viruses to re-emerge. *Virology* 2007;358:402–12. <https://doi.org/10.1016/j.virol.2006.08.049>.
- [58] Dengue n.d. <https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue> (accessed September 10, 2025).
- [59] Pourzangiabadi M, Najafi H, Fallah A, Goudarzi A, Pouladi I. Dengue virus: Etiology, epidemiology, pathobiology, and developments in diagnosis and control – A comprehensive review. *Infect Genet Evol* 2025;127:105710. <https://doi.org/10.1016/j.meegid.2024.105710>.
- [60] Thai KTD, Henn MR, Zody MC, Tricou V, Nguyet NM, Charlebois P, et al. High-Resolution Analysis of Intrahost Genetic Diversity in Dengue Virus Serotype 1 Infection Identifies Mixed Infections. *J Virol* 2012;86:835–43. <https://doi.org/10.1128/jvi.05985-11>.
- [61] Obi JO, Gutiérrez-Barbosa H, Chua JV, Deredge DJ. Current Trends and Limitations in Dengue Antiviral Research. *Trop Med Infect Dis* 2021;6:180. <https://doi.org/10.3390/tropicalmed6040180>.
- [62] Lee MF, Long CM, Poh CL. Current status of the development of dengue vaccines. *Vaccine: X* 2025;22:100604. <https://doi.org/10.1016/j.jvacx.2024.100604>.
- [63] Katzelnick LC, Gresh L, Halloran ME, Mercado JC, Kuan G, Gordon A, et al. Antibody-dependent enhancement of severe dengue disease in humans. *Science* 2017;358:929–32. <https://doi.org/10.1126/science.aan6836>.
- [64] Perera R, Kuhn RJ. Structural Proteomics of Dengue Virus. *Curr Opin Microbiol* 2008;11:369–77. <https://doi.org/10.1016/j.mib.2008.06.004>.
- [65] Nasar S, Rashid N, Iftikhar S. Dengue proteins with their role in pathogenesis, and strategies for developing an effective anti-dengue treatment: A review. *J Med Virol* 2020;92:941–55. <https://doi.org/10.1002/jmv.25646>.
- [66] Bhatt P, Sabeena SP, Varma M, Arunkumar G. Current Understanding of the Pathogenesis of Dengue Virus Infection. *Curr Microbiol* 2021;78:17–32. <https://doi.org/10.1007/s00284-020-02284-w>.
- [67] Chaturvedi UC, Agarwal R, Elbishbishi EA, Mustafa AS. Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. *FEMS Immunol Med Microbiol* 2000;28:183–8. <https://doi.org/10.1111/j.1574-695X.2000.tb01474.x>.
- [68] Malavige GN, Jeewandara C, Ogg GS. Dysfunctional Innate Immune Responses and Severe Dengue. *Front Cell Infect Microbiol* 2020;10. <https://doi.org/10.3389/fcimb.2020.590004>.
- [69] Dayarathna S, Jeewandara C, Gomes L, Somathilaka G, Jayathilaka D, Vimalachandran V, et al. Similarities and differences between the ‘cytokine storms’ in acute dengue and COVID-19. *Sci Rep* 2020;10:19839. <https://doi.org/10.1038/s41598-020-76836-2>.
- [70] Xie Y, Yu Y, Zhao L, Ning P, Luo Q, Zhang Y, et al. Specific Cytokine Profiles Predict the Severity of Influenza A Pneumonia: A Prospectively Multicenter Pilot Study. *Biomed Res Int* 2021;2021:9533044. <https://doi.org/10.1155/2021/9533044>.
- [71] Bradley-Stewart A, Jolly L, Adamson W, Gunson R, Frew-Gillespie C, Templeton K, et al. Cytokine responses in patients with mild or severe influenza A(H1N1)pdm09. *J Clin Virol* 2013;58:100–7. <https://doi.org/10.1016/j.jcv.2013.05.011>.
- [72] Beachboard DC, Horner SM. Innate immune evasion strategies of DNA and RNA viruses. *Curr Opin Microbiol* 2016;32:113–9. <https://doi.org/10.1016/j.mib.2016.05.015>.
- [73] Jensen S, Thomsen AR. Sensing of RNA Viruses: a Review of Innate Immune Receptors Involved in Recognizing RNA Virus Invasion. *J Virol* 2012;86:2900–10. <https://doi.org/10.1128/jvi.05738-11>.
- [74] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783–801. <https://doi.org/10.1016/j.cell.2006.02.015>.

- [75] Said EA, Tremblay N, Al-Balushi MS, Al-Jabri AA, Lamarre D. Viruses Seen by Our Cells: The Role of Viral RNA Sensors. *J Immunol Res* 2018;2018:9480497. <https://doi.org/10.1155/2018/9480497>.
- [76] Peisley A, Hur S. Multi-level regulation of cellular recognition of viral dsRNA. *Cell Mol Life Sci* 2012;70:1949–63. <https://doi.org/10.1007/s00018-012-1149-4>.
- [77] Kawai T, Ikegawa M, Ori D, Akira S. Decoding Toll-like receptors: Recent insights and perspectives in innate immunity. *Immunity* 2024;57:649–73. <https://doi.org/10.1016/j.immuni.2024.03.004>.
- [78] Nie L, Cai S-Y, Shao J-Z, Chen J. Toll-Like Receptors, Associated Biological Roles, and Signaling Networks in Non-Mammals. *Front Immunol* 2018;9:1523. <https://doi.org/10.3389/fimmu.2018.01523>.
- [79] Feng S, Wierzbowski MC, Hrovat-Schaa K, Dumortier A, Zhang Y, Zyulina M, et al. Mechanisms of NLRP3 activation and inhibition elucidated by functional analysis of disease-associated variants. *Nat Immunol* 2025;26:511–23. <https://doi.org/10.1038/s41590-025-02088-9>.
- [80] Rehwinkel J, Gack MU. RIG-I-like receptors: their regulation and roles in RNA sensing. *Nat Rev Immunol* 2020;20:537–51. <https://doi.org/10.1038/s41577-020-0288-3>.
- [81] Yoneyama M, Kato H, Fujita T. Physiological functions of RIG-I-like receptors. *Immunity* 2024;57:731–51. <https://doi.org/10.1016/j.immuni.2024.03.003>.
- [82] Zhu Z, Zhang X, Wang G, Zheng H. The Laboratory of Genetics and Physiology 2: Emerging Insights into the Controversial Functions of This RIG-I-Like Receptor. *Biomed Res Int* 2014;2014:960190. <https://doi.org/10.1155/2014/960190>.
- [83] Zheng J, Shi W, Yang Z, Chen J, Qi A, Yang Y, et al. Chapter One - RIG-I-like receptors: Molecular mechanism of activation and signaling. In: Alt FW, Murphy KM, editors. *Advances in Immunology*, vol. 158, Academic Press; 2023, p. 1–74. <https://doi.org/10.1016/bs.ai.2023.03.001>.
- [84] Wang Y, Ludwig J, Schuberth C, Goldeck M, Schlee M, Li H, et al. Structural and functional insights into 5'-ppp RNA pattern recognition by the innate immune receptor RIG-I. *Nat Struct Mol Biol* 2010;17:781–7. <https://doi.org/10.1038/nsmb.1863>.
- [85] Yamada T, Sato S, Sotoyama Y, Orba Y, Sawa H, Yamauchi H, et al. RIG-I triggers a signaling-abortive anti-SARS-CoV-2 defense in human lung cells. *Nat Immunol* 2021;22:820–8. <https://doi.org/10.1038/s41590-021-00942-0>.
- [86] Takeuchi K, Komatsu T, Kitagawa Y, Sada K, Gotoh B. Sendai virus C protein plays a role in restricting PKR activation by limiting the generation of intracellular double-stranded RNA. *J Virol* 2008;82:10102–10. <https://doi.org/10.1128/JVI.00599-08>.
- [87] Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-Stranded RNA Is Produced by Positive-Strand RNA Viruses and DNA Viruses but Not in Detectable Amounts by Negative-Strand RNA Viruses. *J Virol* 2006;80:5059–64. <https://doi.org/10.1128/JVI.80.10.5059-5064.2006>.
- [88] Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, Weber F, et al. RIG-I-Mediated Antiviral Responses to Single-Stranded RNA Bearing 5'-Phosphates. *Science* 2006;314:997–1001. <https://doi.org/10.1126/science.1132998>.
- [89] Runge S, Sparrer KMJ, Lässig C, Hembach K, Baum A, García-Sastre A, et al. In Vivo Ligands of MDA5 and RIG-I in Measles Virus-Infected Cells. *PLoS Pathog* 2014;10:e1004081. <https://doi.org/10.1371/journal.ppat.1004081>.
- [90] Wu B, Peisley A, Richards C, Yao H, Zeng X, Lin C, et al. Structural Basis for dsRNA Recognition, Filament Formation, and Antiviral Signal Activation by MDA5. *Cell* 2013;152:276–89. <https://doi.org/10.1016/j.cell.2012.11.048>.
- [91] Yin X, Riva L, Pu Y, Martin-Sancho L, Kanamune J, Yamamoto Y, et al. MDA5 Governs the Innate Immune Response to SARS-CoV-2 in Lung Epithelial Cells. *Cell Rep* 2021;34:108628. <https://doi.org/10.1016/j.celrep.2020.108628>.
- [92] Sampaio NG, Chauveau L, Hertzog J, Bridgeman A, Fowler G, Moonen JP, et al. The RNA sensor MDA5 detects SARS-CoV-2 infection. *Sci Rep* 2021;11:13638. <https://doi.org/10.1038/s41598-021-92940-3>.

- [93] Li Y, Yu P, Qu C, Li P, Li Y, Ma Z, et al. MDA5 against enteric viruses through induction of interferon-like response partially via the JAK-STAT cascade. *Antiviral Res* 2020;176:104743. <https://doi.org/10.1016/j.antiviral.2020.104743>.
- [94] Ye S, Liang Y, Chang Y, Lai B, Zhong J. Dengue Virus Replicative-Form dsRNA Is Recognized by Both RIG-I and MDA5 to Activate Innate Immunity. *J Med Virol* 2025;97:e70194. <https://doi.org/10.1002/jmv.70194>.
- [95] Feng Q, Hato SV, Langereis MA, Zoll J, Virgen-Slane R, Peisley A, et al. MDA5 detects the double-stranded RNA replicative form in picornavirus-infected cells. *Cell Rep* 2012;2:1187–96. <https://doi.org/10.1016/j.celrep.2012.10.005>.
- [96] Benitez AA, Panis M, Xue J, Varble A, Shim JV, Frick AL, et al. In vivo RNAi screening identifies MDA5 as a significant contributor to the cellular defense against influenza A virus. *Cell Rep* 2015;11:1714–26. <https://doi.org/10.1016/j.celrep.2015.05.032>.
- [97] Gitlin L, Benoit L, Song C, Cella M, Gilfillan S, Holtzman MJ, et al. Melanoma Differentiation-Associated Gene 5 (MDA5) Is Involved in the Innate Immune Response to Paramyxoviridae Infection In Vivo. *PLoS Pathog* 2010;6:e1000734. <https://doi.org/10.1371/journal.ppat.1000734>.
- [98] Brisse M, Ly H. Comparative Structure and Function Analysis of the RIG-I-Like Receptors: RIG-I and MDA5. *Front Immunol* 2019;10:1586. <https://doi.org/10.3389/fimmu.2019.01586>.
- [99] Kuniyoshi K, Takeuchi O, Pandey S, Satoh T, Iwasaki H, Akira S, et al. Pivotal role of RNA-binding E3 ubiquitin ligase MEX3C in RIG-I-mediated antiviral innate immunity. *Proc Natl Acad Sci U S A* 2014;111:5646–51. <https://doi.org/10.1073/pnas.1401674111>.
- [100] Gack MU, Shin YC, Joo C-H, Urano T, Liang C, Sun L, et al. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 2007;446:916–20. <https://doi.org/10.1038/nature05732>.
- [101] Wang W, Götte B, Guo R, Pyle AM. The E3 ligase Riplet promotes RIG-I signaling independent of RIG-I oligomerization. *Nat Commun* 2023;14:7308. <https://doi.org/10.1038/s41467-023-42982-0>.
- [102] Parchure A, Cesarec M, Braut A, Kolman R, Ivanišević V, Čunko M, et al. ADAR1 p150 prevents HSV-1 from triggering PKR/eIF2 α -mediated translational arrest and is required for efficient viral replication. *PLoS Pathog* 2025;21:e1012452. <https://doi.org/10.1371/journal.ppat.1012452>.
- [103] Kok K-H, Lui P-Y, Ng M-HJ, Siu K-L, Au SWN, Jin D-Y. The Double-Stranded RNA-Binding Protein PACT Functions as a Cellular Activator of RIG-I to Facilitate Innate Antiviral Response. *Cell Host Microbe* 2011;9:299–309. <https://doi.org/10.1016/j.chom.2011.03.007>.
- [104] Yu M, Levine SJ. Toll-like Receptor 3, RIG-I-like Receptors and the NLRP3 Inflammasome: Key Modulators of Innate Immune Responses to Double-stranded RNA Viruses. *Cytokine Growth Factor Rev* 2011;22:63–72. <https://doi.org/10.1016/j.cytogfr.2011.02.001>.
- [105] Lui P-Y, Wong L-YR, Ho T-H, Au SWN, Chan C-P, Kok K-H, et al. PACT Facilitates RNA-Induced Activation of MDA5 by Promoting MDA5 Oligomerization. *J Immunol* 2017;199:1846–55. <https://doi.org/10.4049/jimmunol.1601493>.
- [106] Lian H, Zang R, Wei J, Ye W, Hu M-M, Chen Y-D, et al. The Zinc-Finger Protein ZCCHC3 Binds RNA and Facilitates Viral RNA Sensing and Activation of the RIG-I-like Receptors. *Immunity* 2018;49:438–448.e5. <https://doi.org/10.1016/j.immuni.2018.08.014>.
- [107] Quicke KM, Kim KY, Horvath CM, Suthar MS. RNA Helicase LGP2 Negatively Regulates RIG-I Signaling by Preventing TRIM25-Mediated Caspase Activation and Recruitment Domain Ubiquitination. *J Interferon Cytokine Res* 2019;39:669–83. <https://doi.org/10.1089/jir.2019.0059>.
- [108] Rothenfusser S, Goutagny N, DiPerna G, Gong M, Monks BG, Schoenemeyer A, et al. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by

- retinoic acid-inducible gene-I. *J Immunol* 2005;175:5260–8.
<https://doi.org/10.4049/jimmunol.175.8.5260>.
- [109] Bruns AM, Leser GP, Lamb RA, Horvath CM. The innate immune sensor LGP2 activates antiviral signaling by regulating MDA5-RNA interaction and filament assembly. *Mol Cell* 2014;55:771–81. <https://doi.org/10.1016/j.molcel.2014.07.003>.
- [110] Onomoto K, Onoguchi K, Yoneyama M. Regulation of RIG-I-like receptor-mediated signaling: interaction between host and viral factors. *Cell Mol Immunol* 2021;18:539–55. <https://doi.org/10.1038/s41423-020-00602-7>.
- [111] Okamoto M, Kouwaki T, Fukushima Y, Oshiumi H. Regulation of RIG-I Activation by K63-Linked Polyubiquitination. *Front Immunol* 2018;8:1942. <https://doi.org/10.3389/fimmu.2017.01942>.
- [112] Jacobs JL, Coyne CB. Mechanisms of MAVS Regulation at the Mitochondrial Membrane. *J Mol Biol* 2013;425:5009–19. <https://doi.org/10.1016/j.jmb.2013.10.007>.
- [113] Dixit E, Boulant S, Zhang Y, Lee ASY, Odendall C, Shum B, et al. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* 2010;141:668–81. <https://doi.org/10.1016/j.cell.2010.04.018>.
- [114] Horner SM, Liu HM, Park HS, Briley J, Gale M. Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. *Proc Natl Acad Sci U S A* 2011;108:14590–5. <https://doi.org/10.1073/pnas.1110133108>.
- [115] Ren Z, Ding T, Zuo Z, Xu Z, Deng J, Wei Z. Regulation of MAVS Expression and Signaling Function in the Antiviral Innate Immune Response. *Front Immunol* 2020;11:1030. <https://doi.org/10.3389/fimmu.2020.01030>.
- [116] Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and Essential Roles of Transcription Factors IRF-3 and IRF-7 in Response to Viruses for *IFN- α/β* Gene Induction. *Immunity* 2000;13:539–48. [https://doi.org/10.1016/S1074-7613\(00\)00053-4](https://doi.org/10.1016/S1074-7613(00)00053-4).
- [117] Hinz M, Scheidereit C. The I κ B kinase complex in NF- κ B regulation and beyond. *EMBO Rep* 2014;15:46–61. <https://doi.org/10.1002/embr.201337983>.
- [118] Schneider WM, Chevillotte MD, Rice CM. Interferon-Stimulated Genes: A Complex Web of Host Defenses. *Annu Rev Immunol* 2014;32:513–45. <https://doi.org/10.1146/annurev-immunol-032713-120231>.
- [119] Back SH. Roles of the Translation Initiation Factor eIF2 α Phosphorylation in Cell Structure and Function. *Cell Struct Funct* 2020;45:65–76. <https://doi.org/10.1247/csf.20013>.
- [120] Drappier M, Michiels T. Inhibition of the OAS/RNase L pathway by viruses. *Curr Opin Virol* 2015;15:19–26. <https://doi.org/10.1016/j.coviro.2015.07.002>.
- [121] Verhelst J, Hulpiau P, Saelens X. Mx Proteins: Antiviral Gatekeepers That Restrain the Uninvited. *Microbiol Mol Biol Rev* 2013;77:551–66. <https://doi.org/10.1128/mmbr.00024-13>.
- [122] Yang E, Huang S, Jami-Alahmadi Y, McInerney GM, Wohlschlegel JA, Li MMH. Elucidation of TRIM25 ubiquitination targets involved in diverse cellular and antiviral processes. *PLoS Pathog* 2022;18:e1010743. <https://doi.org/10.1371/journal.ppat.1010743>.
- [123] Sun W, Hua X, Gu Y, Xu Q, Zhu S, Lv T, et al. E3 ubiquitin ligase RNF6 promotes antiviral immune responses through enhancing the expression of interferon stimulated genes in myeloid cells. *Clin Immunol* 2022;242:109099. <https://doi.org/10.1016/j.clim.2022.109099>.
- [124] Richards KH, Macdonald A. Putting the brakes on the anti-viral response: negative regulators of type I interferon (IFN) production. *Microbes Infect* 2011;13:291–302. <https://doi.org/10.1016/j.micinf.2010.12.007>.
- [125] Kim M-J, Hwang S-Y, Imaizumi T, Yoo J-Y. Negative Feedback Regulation of RIG-I-Mediated Antiviral Signaling by Interferon-Induced ISG15 Conjugation. *J Virol* 2008;82:1474–83. <https://doi.org/10.1128/jvi.01650-07>.

- [126] DeDiego ML, Martinez-Sobrido L, Topham DJ. Novel Functions of IFI44L as a Feedback Regulator of Host Antiviral Responses. *J Virol* 2019;93:10.1128/jvi.01159-19. <https://doi.org/10.1128/jvi.01159-19>.
- [127] DeDiego ML, Nogales A, Martinez-Sobrido L, Topham DJ. Interferon-Induced Protein 44 Interacts with Cellular FK506-Binding Protein 5, Negatively Regulates Host Antiviral Responses, and Supports Virus Replication. *mBio* 2019;10:10.1128/mbio.01839-19. <https://doi.org/10.1128/mbio.01839-19>.
- [128] Gal-Ben-Ari S, Barrera I, Ehrlich M, Rosenblum K. PKR: A Kinase to Remember. *Front Mol Neurosci* 2018;11:480. <https://doi.org/10.3389/fnmol.2018.00480>.
- [129] Cesaro T, Michiels T. Inhibition of PKR by Viruses. *Front Microbiol* 2021;12:757238. <https://doi.org/10.3389/fmicb.2021.757238>.
- [130] Bou-Nader C, Gordon JM, Henderson FE, Zhang J. The search for a PKR code—differential regulation of protein kinase R activity by diverse RNA and protein regulators. *RNA* 2019;25:539–56. <https://doi.org/10.1261/rna.070169.118>.
- [131] Cesaro T, Hayashi Y, Borghese F, Vertommen D, Wavreil F, Michiels T. PKR activity modulation by phosphomimetic mutations of serine residues located three aminoacids upstream of double-stranded RNA binding motifs. *Sci Rep* 2021;11:9188. <https://doi.org/10.1038/s41598-021-88610-z>.
- [132] Chukwurah E, Farabaugh KT, Guan B-J, Ramakrishnan P, Hatzoglou M. A tale of two proteins: PACT and PKR and their roles in inflammation. *FEBS J* 2021;288:6365–91. <https://doi.org/10.1111/febs.15691>.
- [133] Garaigorta U, Chisari FV. Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. *Cell Host Microbe* 2009;6:513–22. <https://doi.org/10.1016/j.chom.2009.11.004>.
- [134] Daito T, Watashi K, Sluder A, Ohashi H, Nakajima S, Borroto-Esoda K, et al. Cyclophilin inhibitors reduce phosphorylation of RNA-dependent protein kinase to restore expression of IFN-stimulated genes in HCV-infected cells. *Gastroenterology* 2014;147:463–72. <https://doi.org/10.1053/j.gastro.2014.04.035>.
- [135] Pallarés HM, González López Ledesma MM, Oviedo-Rouco S, Castellano LA, Costa Navarro GS, Fernández-Alvarez AJ, et al. Zika virus non-coding RNAs antagonize antiviral responses by PKR-mediated translational arrest. *Nucleic Acids Res* 2024;52:11128–47. <https://doi.org/10.1093/nar/gkae507>.
- [136] Li H, Liu S, Feng Q, Deng R, Wang J, Wang X, et al. Regulation of PKR-dependent RNA translation inhibition by TRIM21 upon virus infection or other stress. *PLoS Pathog* 2023;19:e1011443. <https://doi.org/10.1371/journal.ppat.1011443>.
- [137] Dauber B, Wolff T. Activation of the Antiviral Kinase PKR and Viral Countermeasures. *Viruses* 2009;1:523–44. <https://doi.org/10.3390/v1030523>.
- [138] Walsh D, Mohr I. Viral subversion of the host protein synthesis machinery. *Nat Rev Microbiol* 2011;9:860–75. <https://doi.org/10.1038/nrmicro2655>.
- [139] Sadler AJ, Williams BRG. Structure and Function of the Protein Kinase R. In: Pitha PM, editor. *Interferon: The 50th Anniversary*, Berlin, Heidelberg: Springer; 2007, p. 253–92. https://doi.org/10.1007/978-3-540-71329-6_13.
- [140] Patel RC, Sen GC. PACT, a protein activator of the interferon-induced protein kinase, PKR. *EMBO J* 1998;17:4379–90. <https://doi.org/10.1093/emboj/17.15.4379>.
- [141] Ukhueduan B, Chukwurah E, Patel RC. Regulation of PKR activation and apoptosis during oxidative stress by TRBP phosphorylation. *Int J Biochem Cell Biol* 2021;137:106030. <https://doi.org/10.1016/j.biocel.2021.106030>.
- [142] Heyam A, Lagos D, Plevin M. Dissecting the roles of TRBP and PACT in double-stranded RNA recognition and processing of noncoding RNAs. *Wiley Interdiscip Rev RNA* 2015;6:271–89. <https://doi.org/10.1002/wrna.1272>.
- [143] Dabo S, Maillard P, Collados Rodriguez M, Hansen MD, Mazouz S, Bigot D-J, et al. Inhibition of the inflammatory response to stress by targeting interaction between PKR and its cellular activator PACT. *Sci Rep* 2017;7:16129. <https://doi.org/10.1038/s41598-017-16089-8>.

- [144] Ahmad S, Zou T, Hwang J, Zhao L, Wang X, Davydenko A, et al. PACT prevents aberrant activation of PKR by endogenous dsRNA without sequestration. *Nat Commun* 2025;16:3325. <https://doi.org/10.1038/s41467-025-58433-x>.
- [145] Park H, Davies MV, Langland JO, Chang HW, Nam YS, Tartaglia J, et al. TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR. *Proc Natl Acad Sci U S A* 1994;91:4713–7. <https://doi.org/10.1073/pnas.91.11.4713>.
- [146] Peters GA, Li S, Sen GC. Phosphorylation of Specific Serine Residues in the PKR Activation Domain of PACT Is Essential for Its Ability to Mediate Apoptosis. *J Biol Chem* 2006;281:35129–36. <https://doi.org/10.1074/jbc.M607714200>.
- [147] Singh M, Castillo D, Patel CV, Patel RC. Stress-induced phosphorylation of PACT reduces its interaction with TRBP and leads to PKR activation. *Biochemistry* 2011;50:4550–60. <https://doi.org/10.1021/bi200104h>.
- [148] Peters GA, Khoo D, Mohr I, Sen GC. Inhibition of PACT-mediated activation of PKR by the herpes simplex virus type 1 Us11 protein. *J Virol* 2002;76:11054–64. <https://doi.org/10.1128/jvi.76.21.11054-11064.2002>.
- [149] Tseng Y-Y, Liao G-R, Sen GC, Lin F-Y, Hsu W-L. Regulation of PACT-Mediated Protein Kinase Activation by the OV20.0 Protein of Orf Virus. *J Virol* 2015;89:11619–29. <https://doi.org/10.1128/JVI.01739-15>.
- [150] Clerzius G, Shaw E, Daher A, Burugu S, G  linas J-F, Ear T, et al. The PKR activator, PACT, becomes a PKR inhibitor during HIV-1 replication. *Retrovirology* 2013;10:96. <https://doi.org/10.1186/1742-4690-10-96>.
- [151] Burugu S, Daher A, Meurs EF, Gatignol A. HIV-1 translation and its regulation by cellular factors PKR and PACT. *Virus Res* 2014;193:65–77. <https://doi.org/10.1016/j.virusres.2014.07.014>.
- [152] Manjunath L, Santiago G, Ortega P, Sanchez A, Oh S, Garcia A, et al. Cooperative role of PACT and ADAR1 in preventing aberrant PKR activation by self-derived double-stranded RNA. *Nat Commun* 2025;16:3246. <https://doi.org/10.1038/s41467-025-58412-2>.
- [153] Reineke LC, Kedersha N, Langereis MA, van Kuppeveld FJM, Lloyd RE. Stress granules regulate double-stranded RNA-dependent protein kinase activation through a complex containing G3BP1 and Caprin1. *mBio* 2015;6:e02486. <https://doi.org/10.1128/mBio.02486-14>.
- [154] Gao P, Liu Y, Wang H, Chai Y, Weng W, Zhang Y, et al. Viral evasion of PKR restriction by reprogramming cellular stress granules. *Proc Natl Acad Sci U S A* 2022;119:e2201169119. <https://doi.org/10.1073/pnas.2201169119>.
- [155] Onomoto K, Jogi M, Yoo J-S, Narita R, Morimoto S, Takemura A, et al. Critical Role of an Antiviral Stress Granule Containing RIG-I and PKR in Viral Detection and Innate Immunity. *PLoS One* 2012;7:e43031. <https://doi.org/10.1371/journal.pone.0043031>.
- [156] McCormick C, Khapersky DA. Translation inhibition and stress granules in the antiviral immune response. *Nat Rev Immunol* 2017;17:647–60. <https://doi.org/10.1038/nri.2017.63>.
- [157] Marcelo A, Koppenol R, de Almeida LP, Matos CA, N  brega C. Stress granules, RNA-binding proteins and polyglutamine diseases: too much aggregation? *Cell Death Dis* 2021;12:592. <https://doi.org/10.1038/s41419-021-03873-8>.
- [158] Eiermann N, Haneke K, Sun Z, Stoecklin G, Ruggieri A. Dance with the Devil: Stress Granules and Signaling in Antiviral Responses. *Viruses* 2020;12:984. <https://doi.org/10.3390/v12090984>.
- [159] Matsumiya T, Shiba Y, Ding J, Kawaguchi S, Seya K, Imaizumi T. The double-stranded RNA-dependent protein kinase PKR negatively regulates the protein expression of IFN-   induced by RIG-I signaling. *FASEB J* 2023;37:e22780. <https://doi.org/10.1096/fj.202201520RR>.
- [160] Pham AM, Santa Maria FG, Lahiri T, Friedman E, Mari   JJ, Levy DE. PKR Transduces MDA5-Dependent Signals for Type I IFN Induction. *PLoS Pathog* 2016;12:e1005489. <https://doi.org/10.1371/journal.ppat.1005489>.

- [161] Zhang P, Li Y, Xia J, He J, Pu J, Xie J, et al. IPS-1 plays an essential role in dsRNA-induced stress granule formation by interacting with PKR and promoting its activation. *J Cell Sci* 2014;127:2471–82. <https://doi.org/10.1242/jcs.139626>.
- [162] Hu S, Sun H, Yin L, Li J, Mei S, Xu F, et al. PKR-dependent cytosolic cGAS foci are necessary for intracellular DNA sensing. *Sci Signal* 2019;12:eaav7934. <https://doi.org/10.1126/scisignal.aav7934>.
- [163] Kumar A, Haque J, Lacoste J, Hiscott J, Williams BR. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. *Proc Natl Acad Sci U S A* 1994;91:6288–92. <https://doi.org/10.1073/pnas.91.14.6288>.
- [164] García MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, et al. Impact of Protein Kinase PKR in Cell Biology: from Antiviral to Antiproliferative Action. *Microbiol Mol Biol Rev* 2006;70:1032–60. <https://doi.org/10.1128/MMBR.00027-06>.
- [165] Kim Y, Lee JH, Park J-E, Cho J, Yi H, Kim VN. PKR is activated by cellular dsRNAs during mitosis and acts as a mitotic regulator. *Genes Dev* 2014;28:1310–22. <https://doi.org/10.1101/gad.242644.114>.
- [166] Xu C, Gamil AAA, Munang'andu HM, Evensen Ø. Apoptosis Induction by dsRNA-Dependent Protein Kinase R (PKR) in EPC Cells via Caspase 8 and 9 Pathways. *Viruses* 2018;10:526. <https://doi.org/10.3390/v10100526>.
- [167] Zhao R, Li X. Multi-Faceted Roles of Stress Granules in Viral Infection. *Microorganisms* 2025;13:1434. <https://doi.org/10.3390/microorganisms13071434>.
- [168] Paget M, Cadena C, Ahmad S, Wang H-T, Jordan TX, Kim E, et al. Stress granules are shock absorbers that prevent excessive innate immune responses to dsRNA. *Mol Cell* 2023;83:1180–1196.e8. <https://doi.org/10.1016/j.molcel.2023.03.010>.
- [169] Yang E, Li MMH. All About the RNA: Interferon-Stimulated Genes That Interfere With Viral RNA Processes. *Front Immunol* 2020;11. <https://doi.org/10.3389/fimmu.2020.605024>.
- [170] Friedman RL, Manly SP, McMahon M, Kerr IM, Stark GR. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. *Cell* 1984;38:745–55. [https://doi.org/10.1016/0092-8674\(84\)90270-8](https://doi.org/10.1016/0092-8674(84)90270-8).
- [171] Kelly JM, Gilbert CS, Stark GR, Kerr IM. Differential regulation of interferon-induced mRNAs and c-myc mRNA by α - and γ -interferons. *Eur J Biochem* 1985;153:367–71. <https://doi.org/10.1111/j.1432-1033.1985.tb09312.x>.
- [172] Kelly JM, Porter AC, Chernajovsky Y, Gilbert CS, Stark GR, Kerr IM. Characterization of a human gene inducible by alpha- and beta-interferons and its expression in mouse cells. *EMBO J* 1986;5:1601–6. <https://doi.org/10.1002/j.1460-2075.1986.tb04402.x>.
- [173] Rasmussen UB, Wolf C, Mattei MG, Chenard MP, Bellocq JP, Chambon P, et al. Identification of a new interferon-alpha-inducible gene (p27) on human chromosome 14q32 and its expression in breast carcinoma. *Cancer Res* 1993;53:4096–101.
- [174] Cheriya V, Leaman DW, Borden EC. Emerging Roles of FAM14 Family Members (G1P3/ISG 6–16 and ISG12/IFI27) in Innate Immunity and Cancer. *J Interferon Cytokine Res* 2011;31:173–81. <https://doi.org/10.1089/jir.2010.0105>.
- [175] Parker N, Porter AC. Identification of a novel gene family that includes the interferon-inducible human genes 6–16 and ISG12. *BMC Genomics* 2004;5:8. <https://doi.org/10.1186/1471-2164-5-8>.
- [176] Rosebeck S, Leaman DW. Mitochondrial localization and pro-apoptotic effects of the interferon-inducible protein ISG12a. *Apoptosis* 2008;13:562–72. <https://doi.org/10.1007/s10495-008-0190-0>.
- [177] Shojaei M, McLean AS. Interferon-stimulated gene IFI27 as a multifaceted candidate target in precision medicine. *Trends Immunol* 2025;46:219–28. <https://doi.org/10.1016/j.it.2025.01.008>.
- [178] Cui X, Liu H, Shi T, Zhao Q, Li F, Lv W, et al. IFI27 Integrates Succinate and Fatty Acid Oxidation to Promote Adipocyte Thermogenic Adaption. *Adv Sci (Weinh)* 2023;10:2301855. <https://doi.org/10.1002/advs.202301855>.

- [179] Jin W, Jin W, Pan D. Ifi27 is indispensable for mitochondrial function and browning in adipocytes. *Biochem Biophys Res Commun* 2018;501:273–9. <https://doi.org/10.1016/j.bbrc.2018.04.234>.
- [180] UniProt IFI27_HUMAN. UniProt n.d. <https://www.uniprot.org/uniprotkb/P40305/entry> (accessed September 11, 2025).
- [181] Martensen PM, Sogaard TMM, Gjermansen IM, Buttenschøn HN, Rossing AB, Bonnevie-Nielsen V, et al. The interferon alpha induced protein ISG12 is localized to the nuclear membrane. *Eur J Biochem* 2001;268:5947–54. <https://doi.org/10.1046/j.0014-2956.2001.02545.x>.
- [182] Qi Y, Li Y, Zhang Y, Zhang L, Wang Z, Zhang X, et al. IFI6 Inhibits Apoptosis via Mitochondrial-Dependent Pathway in Dengue Virus 2 Infected Vascular Endothelial Cells. *PLoS One* 2015;10:e0132743. <https://doi.org/10.1371/journal.pone.0132743>.
- [183] Richardson RB, Ohlson MB, Eitson JL, Kumar A, McDougal MB, Boys IN, et al. A CRISPR screen identifies IFI6 as an ER-resident interferon effector that blocks flavivirus replication. *Nat Microbiol* 2018;3:1214–23. <https://doi.org/10.1038/s41564-018-0244-1>.
- [184] Dukhovny A, Lamkiewicz K, Chen Q, Fricke M, Jabrane-Ferrat N, Marz M, et al. A CRISPR Activation Screen Identifies Genes That Protect against Zika Virus Infection. *J Virol* 2019;93:e00211-19. <https://doi.org/10.1128/JVI.00211-19>.
- [185] Sajid M, Ullah H, Yan K, He M, Feng J, Shereen MA, et al. The Functional and Antiviral Activity of Interferon Alpha-Inducible IFI6 Against Hepatitis B Virus Replication and Gene Expression. *Front Immunol* 2021;12:634937. <https://doi.org/10.3389/fimmu.2021.634937>.
- [186] Liu N, Wu Z, Chen A, Chai D, Li L, Zhang L, et al. ISG12a and its interaction partner NR4A1 are involved in TRAIL-induced apoptosis in hepatoma cells. *J Cell Mol Med* 2019;23:3520–9. <https://doi.org/10.1111/jcmm.14251>.
- [187] Cheriya V, Glaser KB, Waring JF, Baz R, Hussein MA, Borden EC. G1P3, an IFN-induced survival factor, antagonizes TRAIL-induced apoptosis in human myeloma cells. *J Clin Invest* 2007;117:3107–17. <https://doi.org/10.1172/JCI31122>.
- [188] Gytz H, Hansen MF, Skovbjerg S, Kristensen ACM, Hørlyck S, Jensen MB, et al. Apoptotic properties of the type 1 interferon induced family of human mitochondrial membrane ISG12 proteins. *Biol Cell* 2017;109:94–112. <https://doi.org/10.1111/boc.201600034>.
- [189] Cheriya V, Kaur J, Davenport A, Khalel A, Chowdhury N, Gaddipati L. G1P3 (IFI6), a mitochondrial localised antiapoptotic protein, promotes metastatic potential of breast cancer cells through mtROS. *Br J Cancer* 2018;119:52–64. <https://doi.org/10.1038/s41416-018-0137-3>.
- [190] Gupta RK, Rosenheim J, Bell LC, Chandran A, Guerra-Assuncao JA, Pollara G, et al. Blood transcriptional biomarkers of acute viral infection for detection of pre-symptomatic SARS-CoV-2 infection: a nested, case-control diagnostic accuracy study. *Lancet Microbe* 2021;2:e508–17. [https://doi.org/10.1016/S2666-5247\(21\)00146-4](https://doi.org/10.1016/S2666-5247(21)00146-4).
- [191] Huang L, Shi Y, Gong B, Jiang L, Zhang Z, Liu X, et al. Dynamic blood single-cell immune responses in patients with COVID-19. *Sig Transduct Target Ther* 2021;6:110. <https://doi.org/10.1038/s41392-021-00526-2>.
- [192] Gajate-Arenas M, García-Pérez O, Chao-Pellicer J, Domínguez-De-Barros A, Dorta-Guerra R, Lorenzo-Morales J, et al. Differential expression of antiviral and immune-related genes in individuals with COVID-19 asymptomatic or with mild symptoms. *Front Cell Infect Microbiol* 2023;13. <https://doi.org/10.3389/fcimb.2023.1173213>.
- [193] Rajagopala SV, Strickland BA, Pakala SB, Kimura KS, Shilts MH, Rosas-Salazar C, et al. Mucosal Gene Expression in Response to SARS-CoV-2 Is Associated with Viral Load. *J Virol* 2023;97:e01478-22. <https://doi.org/10.1128/jvi.01478-22>.
- [194] Mick E, Kamm J, Pisco AO, Ratnasiri K, Babik JM, Castañeda G, et al. Upper airway gene expression reveals suppressed immune responses to SARS-CoV-2 compared with other respiratory viruses. *Nat Commun* 2020;11:5854. <https://doi.org/10.1038/s41467-020-19587-y>.

- [195] Tan CW, Chen J, Liu N, Bhuva DD, Blick T, Monkman J, et al. In situ single-cell profiling sheds light on IFI27 localisation during SARS-CoV-2 infection. *eBioMedicine* 2024;101. <https://doi.org/10.1016/j.ebiom.2024.105016>.
- [196] Shojaei M, Shamshirian A, Monkman J, Grice L, Tran M, Tan CW, et al. IFI27 transcription is an early predictor for COVID-19 outcomes, a multi-cohort observational study. *Front Immunol* 2023;13. <https://doi.org/10.3389/fimmu.2022.1060438>.
- [197] Zhai Y, Franco LM, Atmar RL, Quarles JM, Arden N, Bucacas KL, et al. Host Transcriptional Response to Influenza and Other Acute Respiratory Viral Infections – A Prospective Cohort Study. *PLoS Pathog* 2015;11:e1004869. <https://doi.org/10.1371/journal.ppat.1004869>.
- [198] Ravi N, Chang SE, Franco LM, Nagamani SCS, Khatri P, Utz PJ, et al. A GMR-based assay for quantification of the human response to influenza. *Biosens Bioelectron* 2022;205:114086. <https://doi.org/10.1016/j.bios.2022.114086>.
- [199] Schughart K, Smith AM, Tsalik EL, Threlkeld SC, Sellers S, Fischer WA, et al. Host response to influenza infections in human blood: association of influenza severity with host genetics and transcriptomic response. *Front Immunol* 2024;15:1385362. <https://doi.org/10.3389/fimmu.2024.1385362>.
- [200] Tang BM, Shojaei M, Parnell GP, Huang S, Nalos M, Teoh S, et al. A novel immune biomarker IFI27 discriminates between influenza and bacteria in patients with suspected respiratory infection. *Eur Respir J* 2017;49:1602098. <https://doi.org/10.1183/13993003.02098-2016>.
- [201] Zhang Y, Zong L, Zheng Y, Zhang Y, Li N, Li Y, et al. A single-cell atlas of the peripheral immune response in patients with influenza A virus infection. *iScience* 2023;26:108507. <https://doi.org/10.1016/j.isci.2023.108507>.
- [202] Schoggins JW, Dorner M, Feulner M, Imanaka N, Murphy MY, Ploss A, et al. Dengue reporter viruses reveal viral dynamics in interferon receptor-deficient mice and sensitivity to interferon effectors in vitro. *Proc Natl Acad Sci U S A* 2012;109:14610–5. <https://doi.org/10.1073/pnas.1212379109>.
- [203] Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 2011;472:481–5. <https://doi.org/10.1038/nature09907>.
- [204] Neufeldt CJ, Cortese M, Acosta EG, Bartenschlager R. Rewiring cellular networks by members of the Flaviviridae family. *Nat Rev Microbiol* 2018;16:125–42. <https://doi.org/10.1038/nrmicro.2017.170>.
- [205] Ci Y, Shi L. Compartmentalized replication organelle of flavivirus at the ER and the factors involved. *Cell Mol Life Sci* 2021;78:4939–54. <https://doi.org/10.1007/s00018-021-03834-6>.
- [206] Suppiah J, Md Sani SS, Hassan SS, Nadzar NIF, Ibrahim N ‘Izzah, Thayan R, et al. Unraveling potential gene biomarkers for dengue infection through RNA sequencing. *Virus Genes* 2025;61:26–37. <https://doi.org/10.1007/s11262-024-02114-2>.
- [207] Jiang C, He C, Kan J, Guan H, Zhou T, Yang Y. Integrative bulk and single-cell transcriptome profiling analysis reveals IFI27 as a novel interferon-stimulated gene in dengue. *J Med Virol* 2023;95:e28706. <https://doi.org/10.1002/jmv.28706>.
- [208] Usuda JN, Praça DR, Fonseca DLM, Marques AHC, Filgueiras IS, Chaves VGB, et al. Interferome signature dynamics during the anti-dengue immune response: a systems biology characterization. *Front Immunol* 2023;14. <https://doi.org/10.3389/fimmu.2023.1243516>.
- [209] Fjaerli H-O, Bukholm G, Krog A, Skjaeret C, Holden M, Nakstad B. Whole blood gene expression in infants with respiratory syncytial virus bronchiolitis. *BMC Infect Dis* 2006;6:175. <https://doi.org/10.1186/1471-2334-6-175>.
- [210] Gao J, Zhu X, Wu M, Jiang L, Wang F, He S. IFI27 may predict and evaluate the severity of respiratory syncytial virus infection in preterm infants. *Hereditas* 2021;158:3. <https://doi.org/10.1186/s41065-020-00167-5>.

- [211] Huang H, Lv J, Huang Y, Mo Z, Xu H, Huang Y, et al. IFI27 is a potential therapeutic target for HIV infection. *Ann Med* 2022;54:314–25. <https://doi.org/10.1080/07853890.2021.1995624>.
- [212] Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, et al. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med* 2011;17:589–95. <https://doi.org/10.1038/nm.2341>.
- [213] Meyer K, Kwon Y-C, Liu S, Hagedorn CH, Ray RB, Ray R. Interferon- α inducible protein 6 impairs EGFR activation by CD81 and inhibits hepatitis C virus infection. *Sci Rep* 2015;5:9012. <https://doi.org/10.1038/srep09012>.
- [214] Rawle DJ, Le TT, Dumenil T, Yan K, Tang B, Nguyen W, et al. ACE2-lentiviral transduction enables mouse SARS-CoV-2 infection and mapping of receptor interactions. *PLoS Pathog* 2021;17:e1009723. <https://doi.org/10.1371/journal.ppat.1009723>.
- [215] Shang J, Wan Y, Luo C, Ye G, Geng Q, Auerbach A, et al. Cell entry mechanisms of SARS-CoV-2. *Proc Natl Acad Sci U S A* 2020;117:11727–34. <https://doi.org/10.1073/pnas.2003138117>.
- [216] Chang C-W, Parsi KM, Somasundaran M, Vanderleeden E, Liu P, Cruz J, et al. A Newly Engineered A549 Cell Line Expressing ACE2 and TMPRSS2 Is Highly Permissive to SARS-CoV-2, Including the Delta and Omicron Variants. *Viruses* 2022;14:1369. <https://doi.org/10.3390/v14071369>.
- [217] Shukla N, Roelle SM, Suzart VG, Bruchez AM, Matreyek KA. Mutants of human ACE2 differentially promote SARS-CoV and SARS-CoV-2 spike mediated infection. *PLoS Pathog* 2021;17:e1009715. <https://doi.org/10.1371/journal.ppat.1009715>.
- [218] Gödecke N, Hauser H, Wirth D. Stable Expression by Lentiviral Transduction of Cells. *Methods Mol Biol* 2018;1850:43–55. https://doi.org/10.1007/978-1-4939-8730-6_4.
- [219] Blesch A. Lentiviral and MLV based retroviral vectors for ex vivo and in vivo gene transfer. *Methods* 2004;33:164–72. <https://doi.org/10.1016/j.ymeth.2003.11.005>.
- [220] Cronin J, Zhang X-Y, Reiser J. Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther* 2005;5:387–98. <https://doi.org/10.2174/1566523054546224>.
- [221] Duvergé A, Negroni M. Pseudotyping Lentiviral Vectors: When the Clothes Make the Virus. *Viruses* 2020;12:1311. <https://doi.org/10.3390/v12111311>.
- [222] Kimple ME, Brill AL, Pasker RL. Overview of Affinity Tags for Protein Purification. *Curr Protoc Protein Sci* 2013;73:Unit-9.9. <https://doi.org/10.1002/0471140864.ps0909s73>.
- [223] Rodriguez-Madoz JR, Bernal-Rubio D, Kaminski D, Boyd K, Fernandez-Sesma A. Dengue Virus Inhibits the Production of Type I Interferon in Primary Human Dendritic Cells. *J Virol* 2010;84:4845–50. <https://doi.org/10.1128/jvi.02514-09>.
- [224] Aguirre S, Maestre AM, Pagni S, Patel JR, Savage T, Gutman D, et al. DENV Inhibits Type I IFN Production in Infected Cells by Cleaving Human STING. *PLoS Pathog* 2012;8:e1002934. <https://doi.org/10.1371/journal.ppat.1002934>.
- [225] Schickli JH, Flandorfer A, Nakaya T, Martinez-Sobrido L, García-Sastre A, Palese P. Plasmid-only rescue of influenza A virus vaccine candidates. *Philos Trans R Soc Lond B Biol Sci* 2001;356:1965–73. <https://doi.org/10.1098/rstb.2001.0979>.
- [226] Baker SF, Guo H, Albrecht RA, García-Sastre A, Topham DJ, Martínez-Sobrido L. Protection against Lethal Influenza with a Viral Mimic. *J Virol* 2013;87:8591–605. <https://doi.org/10.1128/JVI.01081-13>.
- [227] Wang L, Guzman M, Muñoz-Santos D, Honrubia JM, Ripoll-Gomez J, Delgado R, et al. Cell type dependent stability and virulence of a recombinant SARS-CoV-2, and engineering of a propagation deficient RNA replicon to analyze virus RNA synthesis. *Front Cell Infect Microbiol* 2023;13. <https://doi.org/10.3389/fcimb.2023.1268227>.
- [228] Hamlin RE, Rahman A, Pak TR, Maringer K, Mena I, Bernal-Rubio D, et al. High-dimensional CyTOF analysis of dengue virus-infected human DCs reveals distinct viral signatures. *JCI Insight* 2017;2:e92424. <https://doi.org/10.1172/jci.insight.92424>.

- [229] Kochs G, García-Sastre A, Martínez-Sobrido L. Multiple Anti-Interferon Actions of the Influenza A Virus NS1 Protein. *J Virol* 2007;81:7011–21. <https://doi.org/10.1128/jvi.02581-06>.
- [230] Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S, et al. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* 2003;4:263–75. [https://doi.org/10.1016/s1535-6108\(03\)00241-1](https://doi.org/10.1016/s1535-6108(03)00241-1).
- [231] Green MR, Sambrook J. *Molecular cloning: a laboratory manual*. 4th ed. Cold Spring Harbor: Cold Spring Harbor laboratory press; 2012.
- [232] Hitoshi N, Ken-ichi Y, Jun-ichi M. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991;108:193–9. [https://doi.org/10.1016/0378-1119\(91\)90434-D](https://doi.org/10.1016/0378-1119(91)90434-D).
- [233] pCAGGS vector map and sequence n.d. <https://www.novoprolabs.com/vector/Vgm3tqm> (accessed October 15, 2025).
- [234] Mibayashi M, Martínez-Sobrido L, Loo Y-M, Cárdenas WB, Gale M, García-Sastre A. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J Virol* 2007;81:514–24. <https://doi.org/10.1128/JVI.01265-06>.
- [235] pRL Renilla Luciferase Control Reporter Vectors n.d. <https://www.promega.es/products/luciferase-assays/genetic-reporter-vectors-and-cell-lines/prl-renilla-luciferase-control-reporter-vectors/> (accessed October 15, 2025).
- [236] Nogales A, Baker SF, Martínez-Sobrido L. Replication-competent influenza A viruses expressing a red fluorescent protein. *Virology* 2015;476:206–16. <https://doi.org/10.1016/j.virol.2014.12.006>.
- [237] Chua MA, Schmid S, Perez JT, Langlois RA, tenOever BR. Influenza A virus utilizes suboptimal splicing to coordinate the timing of infection. *Cell Rep* 2013;3:23–9. <https://doi.org/10.1016/j.celrep.2012.12.010>.
- [238] Witkowski W, Vermeire J, Landi A, Naessens E, Vanderstraeten H, Nauwynck H, et al. Vpx-Independent Lentiviral Transduction and shRNA-Mediated Protein Knock-Down in Monocyte-Derived Dendritic Cells. *PLoS One* 2015;10:e0133651. <https://doi.org/10.1371/journal.pone.0133651>.
- [239] Smith N, Vidalain P-O, Nisole S, Herbeuval J-P. An efficient method for gene silencing in human primary plasmacytoid dendritic cells: silencing of the TLR7/IRF-7 pathway as a proof of concept. *Sci Rep* 2016;6:29891. <https://doi.org/10.1038/srep29891>.
- [240] Telesnitsky A, Goff SP. Reverse Transcriptase and the Generation of Retroviral DNA. In: Coffin JM, Hughes SH, Varmus HE, editors. *Retroviruses*, Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1997.
- [241] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* 2001;25:402–8. <https://doi.org/10.1006/meth.2001.1262>.
- [242] González-Cabaleiro L, Fernández-Lodeiro C, Vázquez-Iglesias L, Soriano-Maldonado P, van Raaij MJ, Bodelón G, et al. Pushing the Limits of Lateral Flow Immunoassay by Digital SERS for the Ultralow Detection of SARS-CoV-2 Virus. *Small Sci* 2024;4:2400259. <https://doi.org/10.1002/ssmc.202400259>.
- [243] Pike JA, Styles IB, Rappoport JZ, Heath JK. Quantifying receptor trafficking and colocalization with confocal microscopy. *Methods* 2017;115:42–54. <https://doi.org/10.1016/j.ymeth.2017.01.005>.
- [244] Adler J, Parmryd I. Quantifying colocalization by correlation: The Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry A* 2010;77A:733–42. <https://doi.org/10.1002/cyto.a.20896>.
- [245] RNABindRPlus: Honavar (PSU) & Dobbs (ISU) Laboratories n.d. (accessed October 15, 2025).
- [246] Walia RR, El-Manzalawy Y, Honavar VG, Dobbs D. Sequence-Based Prediction of RNA-Binding Residues in Proteins. *Methods Mol Biol* 2017;1484:205–35. https://doi.org/10.1007/978-1-4939-6406-2_15.

- [247] Walia RR, Xue LC, Wilkins K, El-Manzalawy Y, Dobbs D, Honavar V. RNABindRPlus: A Predictor that Combines Machine Learning and Sequence Homology-Based Methods to Improve the Reliability of Predicted RNA-Binding Residues in Proteins. *PLoS One* 2014;9:e97725. <https://doi.org/10.1371/journal.pone.0097725>.
- [248] Peters GA, Hartmann R, Qin J, Sen GC. Modular structure of PACT: distinct domains for binding and activating PKR. *Mol Cell Biol* 2001;21:1908–20. <https://doi.org/10.1128/MCB.21.6.1908-1920.2001>.
- [249] Gagliardi M, Matarazzo MR. RIP: RNA Immunoprecipitation. *Methods Mol Biol* 2016;1480:73–86. https://doi.org/10.1007/978-1-4939-6380-5_7.
- [250] Navajas R, Ramos-Fernandez A, Herraiz I, Galindo A, Bartha JL, Corrales F, et al. Quantitative proteomic analysis of serum-purified exosomes identifies putative pre-eclampsia-associated biomarkers. *Clin Proteomics* 2022;19:5. <https://doi.org/10.1186/s12014-022-09342-4>.
- [251] Felgenhauer U, Schoen A, Gad HH, Hartmann R, Schaubmar AR, Failing K, et al. Inhibition of SARS-CoV-2 by type I and type III interferons. *J Biol Chem* 2020;295:13958–64. <https://doi.org/10.1074/jbc.AC120.013788>.
- [252] Horisberger MA, de Staritzky K. A Recombinant Human Interferon- α B/D Hybrid with a Broad Host-range. *J Gen Virol* 1987;68:945–8. <https://doi.org/10.1099/0022-1317-68-3-945>.
- [253] DeDiego ML, Nogales A, Lambert-Emo K, Martinez-Sobrido L, Topham DJ. NS1 Protein Mutation I64T Affects Interferon Responses and Virulence of Circulating H3N2 Human Influenza A Viruses. *J Virol* 2016;90:9693–711. <https://doi.org/10.1128/JVI.01039-16>.
- [254] Chiu H-C, Hannemann H, Heesom KJ, Matthews DA, Davidson AD. High-Throughput Quantitative Proteomic Analysis of Dengue Virus Type 2 Infected A549 Cells. *PLoS One* 2014;9:e93305. <https://doi.org/10.1371/journal.pone.0093305>.
- [255] Cloherty APM, Rader AG, Patel KS, Eidsen T-JTHD, van Piggelen S, Schreurs RRCE, et al. Dengue virus exploits autophagy vesicles and secretory pathways to promote transmission by human dendritic cells. *Front Immunol* 2024;15. <https://doi.org/10.3389/fimmu.2024.1260439>.
- [256] Schmid MA, Diamond MS, Harris E. Dendritic Cells in Dengue Virus Infection: Targets of Virus Replication and Mediators of Immunity. *Front Immunol* 2014;5:647. <https://doi.org/10.3389/fimmu.2014.00647>.
- [257] Bartlett DW, Davis ME. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res* 2006;34:322–33. <https://doi.org/10.1093/nar/gkj439>.
- [258] Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen T, et al. Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. *Science* 2014;343:84–7. <https://doi.org/10.1126/science.1247005>.
- [259] Sullivan OM, Nesbitt DJ, Schaack GA, Feltman EM, Nipper T, Kongsomros S, et al. IFIT3 RNA-binding activity promotes influenza A virus infection and translation efficiency. *J Virol* 2025;99:e00286-25. <https://doi.org/10.1128/jvi.00286-25>.
- [260] Ahmed MM, Okesanya OJ, Ukoaka BM, Ibrahim AM, Lucero-Prisno DE. Vesicular Stomatitis Virus: Insights into Pathogenesis, Immune Evasion, and Technological Innovations in Oncolytic and Vaccine Development. *Viruses* 2024;16:1933. <https://doi.org/10.3390/v16121933>.
- [261] Rehman S, Chavan PM, Chaturvedi A, Nayak D. Negative-sense RNA virus nucleocapsid as a versatile platform for gene delivery, vaccine development, and antiviral screening. *Sci Rep* 2025;15:38367. <https://doi.org/10.1038/s41598-025-22282-x>.
- [262] Nikolic J, Belot L, Raux H, Legrand P, Gaudin Y, A. Albertini A. Structural basis for the recognition of LDL-receptor family members by VSV glycoprotein. *Nat Commun* 2018;9:1029. <https://doi.org/10.1038/s41467-018-03432-4>.

- [263] Blanchard L, Tarbouriech N, Blackledge M, Timmins P, Burmeister WP, Ruigrok RWH, et al. Structure and dynamics of the nucleocapsid-binding domain of the Sendai virus phosphoprotein in solution. *Virology* 2004;319:201–11. <https://doi.org/10.1016/j.virol.2003.10.029>.
- [264] Gómez Á, Reina R. Recombinant Sendai Virus Vectors as Novel Vaccine Candidates Against Animal Viruses. *Viruses* 2025;17:737. <https://doi.org/10.3390/v17050737>.
- [265] Syedbasha M, Egli A. Interferon Lambda: Modulating Immunity in Infectious Diseases. *Front Immunol* 2017;8:119. <https://doi.org/10.3389/fimmu.2017.00119>.
- [266] Fensterl V, Sen GC. Interferon-induced Ifit proteins: their role in viral pathogenesis. *J Virol* 2015;89:2462–8. <https://doi.org/10.1128/JVI.02744-14>.
- [267] Elemam NM, Talaat IM, Maghazachi AA. CXCL10 Chemokine: A Critical Player in RNA and DNA Viral Infections. *Viruses* 2022;14:2445. <https://doi.org/10.3390/v14112445>.
- [268] Wang J, Nikrad MP, Phang T, Gao B, Alford T, Ito Y, et al. Innate Immune Response to Influenza A Virus in Differentiated Human Alveolar Type II Cells. *Am J Respir Cell Mol Biol* 2011;45:582–91. <https://doi.org/10.1165/rcmb.2010-0108OC>.
- [269] Li Y, Renner DM, Comar CE, Whelan JN, Reyes HM, Cardenas-Diaz FL, et al. SARS-CoV-2 induces double-stranded RNA-mediated innate immune responses in respiratory epithelial-derived cells and cardiomyocytes. *Proc Natl Acad Sci U S A* 2021;118:e2022643118. <https://doi.org/10.1073/pnas.2022643118>.
- [270] D’agostino PM, Amenta JJ, Reiss CS. IFN- β -Induced Alteration of VSV Protein Phosphorylation in Neuronal Cells. *Viral Immunol* 2009;22:353–69. <https://doi.org/10.1089/vim.2009.0057>.
- [271] Bedsaul JR, Zaritsky LA, Zoon KC. Type I Interferon-Mediated Induction of Antiviral Genes and Proteins Fails to Protect Cells from the Cytopathic Effects of Sendai Virus Infection. *J Interferon Cytokine Res* 2016;36:652–65. <https://doi.org/10.1089/jir.2016.0051>.
- [272] Goodbourn S, Randall RE. The Regulation of Type I Interferon Production by Paramyxoviruses. *J Interferon Cytokine Res* 2009;29:539–47. <https://doi.org/10.1089/jir.2009.0071>.
- [273] Strähle L, Marq J-B, Brini A, Hausmann S, Kolakofsky D, Garcin D. Activation of the Beta Interferon Promoter by Unnatural Sendai Virus Infection Requires RIG-I and Is Inhibited by Viral C Proteins. *J Virol* 2007;81:12227–37. <https://doi.org/10.1128/jvi.01300-07>.
- [274] Yount JS, Gitlin L, Moran TM, López CB. MDA5 Participates in the Detection of Paramyxovirus Infection and Is Essential for the Early Activation of Dendritic Cells in Response to Sendai Virus Defective Interfering Particles1. *J Immunol* 2008;180:4910–8. <https://doi.org/10.4049/jimmunol.180.7.4910>.
- [275] Llargués-Sistac G, Bonjoch L, Castellvi-Bel S. HAP1, a new revolutionary cell model for gene editing using CRISPR-Cas9. *Front Cell Dev Biol* 2023;11:1111488. <https://doi.org/10.3389/fcell.2023.1111488>.
- [276] Han X, Na T, Wu T, Yuan B-Z. Human lung epithelial BEAS-2B cells exhibit characteristics of mesenchymal stem cells. *PLoS One* 2020;15:e0227174. <https://doi.org/10.1371/journal.pone.0227174>.
- [277] Hillyer P, Shepard R, Uehling M, Krenz M, Sheikh F, Thayer KR, et al. Differential Responses by Human Respiratory Epithelial Cell Lines to Respiratory Syncytial Virus Reflect Distinct Patterns of Infection Control. *J Virol* 2018;92:10.1128/jvi.02202-17. <https://doi.org/10.1128/jvi.02202-17>.
- [278] Seng L-G, Daly J, Chang K-C, Kuchipudi SV. High basal expression of interferon-stimulated genes in human bronchial epithelial (BEAS-2B) cells contributes to influenza A virus resistance. *PLoS One* 2014;9:e109023. <https://doi.org/10.1371/journal.pone.0109023>.
- [279] Mathur SK, Fichtinger PS, Kelly JT, Lee W-M, Gern JE, Jarjour NN. Interaction between allergy and innate immunity: model for eosinophil regulation of epithelial cell

- interferon expression. *Ann Allergy Asthma Immunol* 2013;111:25–31. <https://doi.org/10.1016/j.anai.2013.05.010>.
- [280] Liu Y, Jing R, Wen Z, Li M. Narrowing the Gap Between In Vitro and In Vivo Genetic Profiles by Deconvoluting Toxicogenomic Data In Silico. *Front Pharmacol* 2020;10. <https://doi.org/10.3389/fphar.2019.01489>.
- [281] Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, García-Sastre A. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. *Proc Natl Acad Sci U S A* 2010;107:11531–6. <https://doi.org/10.1073/pnas.0914994107>.
- [282] Perez JT, García-Sastre A, Manicassamy B. Insertion of a GFP reporter gene in influenza virus. *Curr Protoc Microbiol* 2013;Chapter 15:15G.4.1–15G.4.16. <https://doi.org/10.1002/9780471729259.mc15g04s29>.
- [283] Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Methods* 2005;2:905–9. <https://doi.org/10.1038/nmeth819>.
- [284] Zhang X, Bogunovic D, Payelle-Brogard B, Francois-Newton V, Speer SD, Yuan C, et al. Human intracellular ISG15 prevents interferon- α/β over-amplification and auto-inflammation. *Nature* 2015;517:89–93. <https://doi.org/10.1038/nature13801>.
- [285] Li J, Yu J, Shen A, Lai S, Liu Z, He T-S. The RNA-binding proteins regulate innate antiviral immune signaling by modulating pattern recognition receptors. *Virol J* 2024;21:225. <https://doi.org/10.1186/s12985-024-02503-x>.
- [286] DeChancie J, Houk KN. The origins of femtomolar protein-ligand binding: hydrogen-bond cooperativity and desolvation energetics in the biotin-(strept)avidin binding site. *J Am Chem Soc* 2007;129:5419–29. <https://doi.org/10.1021/ja066950n>.
- [287] Daulethaev N, Cammisano M, Herscovitch K, Lands LC. Stimulation of the RIG-I/MAVS Pathway by Polyinosinic:Polycytidylic Acid Upregulates IFN- β in Airway Epithelial Cells with Minimal Costimulation of IL-8. *J Immunol* 2015;195:2829–41. <https://doi.org/10.4049/jimmunol.1400840>.
- [288] Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006;441:101–5. <https://doi.org/10.1038/nature04734>.
- [289] Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, et al. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell* 2010;140:397–408. <https://doi.org/10.1016/j.cell.2010.01.020>.
- [290] Österlund PI, Pietilä TE, Veckman V, Kotenko SV, Julkunen I. IFN Regulatory Factor Family Members Differentially Regulate the Expression of Type III IFN (IFN- λ) Genes1. *J Immunol* 2007;179:3434–42. <https://doi.org/10.4049/jimmunol.179.6.3434>.
- [291] Straub S, Sampaio NG. Activation of cytosolic RNA sensors by endogenous ligands: roles in disease pathogenesis. *Front Immunol* 2023;14. <https://doi.org/10.3389/fimmu.2023.1092790>.
- [292] Yang K, Wang J, Xiang AP, Zhan X, Wang Y, Wu M, et al. Functional RIG-I-like receptors control the survival of mesenchymal stem cells. *Cell Death Dis* 2013;4:e967. <https://doi.org/10.1038/cddis.2013.504>.
- [293] Hur S. Double-Stranded RNA Sensors and Modulators in Innate Immunity. *Annu Rev Immunol* 2019;37:349–75. <https://doi.org/10.1146/annurev-immunol-042718-041356>.
- [294] Green R, Noller HF. Ribosomes and translation. *Annu Rev Biochem* 1997;66:679–716. <https://doi.org/10.1146/annurev.biochem.66.1.679>.
- [295] Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol Cell Proteomics* 2005;4:1265–72. <https://doi.org/10.1074/mcp.M500061-MCP200>.
- [296] Rojas M, Vasconcelos G, Dever TE. An eIF2 α -binding motif in protein phosphatase 1 subunit GADD34 and its viral orthologs is required to promote dephosphorylation of eIF2 α . *Proc Natl Acad Sci U S A* 2015;112:E3466–3475. <https://doi.org/10.1073/pnas.1501557112>.

- [297] Gu M, Zhang T, Lin W, Liu Z, Lai R, Xia D, et al. Protein phosphatase PP1 negatively regulates the Toll-like receptor- and RIG-I-like receptor-triggered production of type I interferon by inhibiting IRF3 phosphorylation at serines 396 and 385 in macrophage. *Cell Signal* 2014;26:2930–9. <https://doi.org/10.1016/j.cellsig.2014.09.007>.
- [298] Stojdl DF, Abraham N, Knowles S, Marius R, Brasey A, Lichty BD, et al. The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. *J Virol* 2000;74:9580–5. <https://doi.org/10.1128/jvi.74.20.9580-9585.2000>.
- [299] Kim Y, Park J, Kim S, Kim M, Kang M-G, Kwak C, et al. PKR Senses Nuclear and Mitochondrial Signals by Interacting with Endogenous Double-Stranded RNAs. *Mol Cell* 2018;71:1051-1063.e6. <https://doi.org/10.1016/j.molcel.2018.07.029>.
- [300] LeBlanc K, Lynch J, Layne C, Vendramelli R, Sloan A, Tailor N, et al. The Nucleocapsid Proteins of SARS-CoV-2 and Its Close Relative Bat Coronavirus RaTG13 Are Capable of Inhibiting PKR- and RNase L-Mediated Antiviral Pathways. *Microbiol Spectr* 2023;11:e0099423. <https://doi.org/10.1128/spectrum.00994-23>.
- [301] Aloise C, Schipper JG, van Vliet A, Oymans J, Donselaar T, Hurdiss DL, et al. SARS-CoV-2 nucleocapsid protein inhibits the PKR-mediated integrated stress response through RNA-binding domain N2b. *PLoS Pathog* 2023;19:e1011582. <https://doi.org/10.1371/journal.ppat.1011582>.
- [302] Otter CJ, Bracci N, Parenti NA, Ye C, Asthana A, Blomqvist EK, et al. SARS-CoV-2 nsp15 endoribonuclease antagonizes dsRNA-induced antiviral signaling. *Proc Natl Acad Sci U S A* 2024;121:e2320194121. <https://doi.org/10.1073/pnas.2320194121>.
- [303] Borghese F, Sorgeloos F, Cesaro T, Michiels T. The Leader Protein of Theiler's Virus Prevents the Activation of PKR. *J Virol* 2019;93:e01010-19. <https://doi.org/10.1128/JVI.01010-19>.
- [304] Zhang K, Wang S, Chen T, Tu Z, Huang X, Zang G, et al. ADAR1p110 promotes Enterovirus D68 replication through its deaminase domain and inhibition of PKR pathway. *Virol J* 2022;19:222. <https://doi.org/10.1186/s12985-022-01952-6>.
- [305] Gantke T, Boussouf S, Janzen J, Morrice NA, Howell S, Mühlberger E, et al. Ebola virus VP35 induces high-level production of recombinant TPL-2–ABIN-2–NF- κ B1 p105 complex in co-transfected HEK-293 cells. *Biochem J* 2013;452:359–65. <https://doi.org/10.1042/BJ20121873>.
- [306] Pakdel F, Hosseini SM, Soleimani N, Farhadi A. Small T Oncoprotein of Merkel Cell Polyomavirus Attenuates Cisplatin-Induced Apoptosis and Enhances E1, E6/E7, MMP-1, and Ki-67 Expression in HeLa Cervical Cancer Cells. *Adv Pharm Bull* 2025;15:194–205. <https://doi.org/10.34172/apb.43882>.
- [307] Tenthorey JL, Emerman M, Malik HS. Evolutionary Landscapes of Host-Virus Arms Races. *Annu Rev Immunol* 2022;40:271–94. <https://doi.org/10.1146/annurev-immunol-072621-084422>.
- [308] Markiewicz L, Drazkowska K, Sikorski PJ. Tricks and threats of RNA viruses – towards understanding the fate of viral RNA. *RNA Biol* 2021;18:669–87. <https://doi.org/10.1080/15476286.2021.1875680>.
- [309] Komuro A, Bamming D, Horvath CM. Negative regulation of cytoplasmic RNA-mediated antiviral signaling. *Cytokine* 2008;43:350–8. <https://doi.org/10.1016/j.cyto.2008.07.011>.
- [310] Liu C, Zhu H, Subramanian GM, Moore PA, Xu Y, Nelson DR. Anti-hepatitis C virus activity of albinterferon alfa-2b in cell culture. *Hepatol Res* 2007;37:941–7. <https://doi.org/10.1111/j.1872-034X.2007.00142.x>.
- [311] Crowl JT, Stetson DB. SUMO2 and SUMO3 redundantly prevent a noncanonical type I interferon response. *Proc Natl Acad Sci U S A* 2018;115:6798–803. <https://doi.org/10.1073/pnas.1802114115>.
- [312] Zhou A, Dong X, Liu M, Tang B. Comprehensive Transcriptomic Analysis Identifies Novel Antiviral Factors Against Influenza A Virus Infection. *Front Immunol* 2021;12. <https://doi.org/10.3389/fimmu.2021.632798>.

- [313] Qi M, Liu B, Li S, Ni Z, Li F. Construction and Investigation of Competing Endogenous RNA Networks and Candidate Genes Involved in SARS-CoV-2 Infection. *Int J Gen Med* 2021;14:6647–59. <https://doi.org/10.2147/IJGM.S335162>.
- [314] Husain M. Influenza Virus Host Restriction Factors: The ISGs and Non-ISGs. *Pathogens* 2024;13:127. <https://doi.org/10.3390/pathogens13020127>.
- [315] Mar KB, Wells AI, Caballero Van Dyke MC, Lopez AH, Eitson JL, Fan W, et al. LY6E is a pan-coronavirus restriction factor in the respiratory tract. *Nat Microbiol* 2023;8:1587–99. <https://doi.org/10.1038/s41564-023-01431-w>.
- [316] Brass AL, Huang I-C, Benita Y, John SP, Krishnan MN, Feeley EM, et al. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 2009;139:1243–54. <https://doi.org/10.1016/j.cell.2009.12.017>.
- [317] Mar KB, Rinkenberger NR, Boys IN, Eitson JL, McDougal MB, Richardson RB, et al. LY6E mediates an evolutionarily conserved enhancement of virus infection by targeting a late entry step. *Nat Commun* 2018;9:3603. <https://doi.org/10.1038/s41467-018-06000-y>.
- [318] Hackett BA, Cherry S. Flavivirus internalization is regulated by a size-dependent endocytic pathway. *Proc Natl Acad Sci U S A* 2018;115:4246–51. <https://doi.org/10.1073/pnas.1720032115>.
- [319] Zhao X, Guo F, Liu F, Cuconati A, Chang J, Block TM, et al. Interferon induction of IFITM proteins promotes infection by human coronavirus OC43. *Proc Natl Acad Sci U S A* 2014;111:6756–61. <https://doi.org/10.1073/pnas.1320856111>.
- [320] Min Z, Ye Z, Gang L, Boyu D, Xueyan X. IFI27 as a potential indicator for severe Enterovirus 71-infected hand foot and mouth disease. *Virus Res* 2020;289:198149. <https://doi.org/10.1016/j.virusres.2020.198149>.
- [321] Palm AA, Veerla S, Lindman J, Isberg P-E, Johansson E, Biague A, et al. Interferon Alpha-Inducible Protein 27 Expression Is Linked to Disease Severity in Chronic Infection of Both HIV-1 and HIV-2. *Front Virol* 2022;2. <https://doi.org/10.3389/fviro.2022.929053>.
- [322] Liu X, Lin L, Lu L, Li X, Han Y, Qiu Z, et al. Comparative Transcriptional Analysis Identified Characteristic Genes and Patterns in HIV-Infected Immunological Non-Responders. *Front Immunol* 2022;13:807890. <https://doi.org/10.3389/fimmu.2022.807890>.
- [323] Ullah H, Sajid M, Yan K, Feng J, He M, Shereen MA, et al. Antiviral Activity of Interferon Alpha-Inducible Protein 27 Against Hepatitis B Virus Gene Expression and Replication. *Front Microbiol* 2021;12:656353. <https://doi.org/10.3389/fmicb.2021.656353>.
- [324] Sohail A, Waqas FH, Braubach P, Czichon L, Samir M, Iqbal A, et al. Differential transcriptomic host responses in the early phase of viral and bacterial infections in human lung tissue explants ex vivo. *Respir Res* 2024;25:369. <https://doi.org/10.1186/s12931-024-02988-8>.
- [325] Jiao P, Fan W, Cao Y, Zhang H, Tian L, Sun L, et al. Robust induction of interferon and interferon-stimulated gene expression by influenza B/Yamagata lineage virus infection of A549 cells. *PLoS One* 2020;15:e0231039. <https://doi.org/10.1371/journal.pone.0231039>.
- [326] Schmid MA, Diamond MS, Harris E. Dendritic Cells in Dengue Virus Infection: Targets of Virus Replication and Mediators of Immunity. *Front Immunol* 2014;5:647. <https://doi.org/10.3389/fimmu.2014.00647>.
- [327] Fernandes-Santos C, de Azeredo EL. Innate Immune Response to Dengue Virus: Toll-like Receptors and Antiviral Response. *Viruses* 2022;14:992. <https://doi.org/10.3390/v14050992>.
- [328] Sumpter R, Loo Y-M, Foy E, Li K, Yoneyama M, Fujita T, et al. Regulating Intracellular Antiviral Defense and Permissiveness to Hepatitis C Virus RNA Replication through a Cellular RNA Helicase, RIG-I. *J Virol* 2005;79:2689–99. <https://doi.org/10.1128/jvi.79.5.2689-2699.2005>.

- [329] Sesifredo I, Batalha ÍL, Amorim MJ. Fast and flu-rious: How to prevent and treat emerging influenza A viruses. *PLoS Pathog* 2025;21:e1013135. <https://doi.org/10.1371/journal.ppat.1013135>.
- [330] Dinata R, Baindara P, Mandal SM. Evolution of Antiviral Drug Resistance in SARS-CoV-2. *Viruses* 2025;17:722. <https://doi.org/10.3390/v17050722>.
- [331] Farsiu N, Zandi K, Rukerd MRZ, Charostad J, Bashash D, Ghafari S, et al. Challenges in the Prevention and Treatment of Dengue Fever: An Emerging Issue. *Am J Trop Med Hyg* 2025;tpmd250049. <https://doi.org/10.4269/ajtmh.25-0049>.
- [332] Li Y, Li C, Xue P, Zhong B, Mao A-P, Ran Y, et al. ISG56 is a negative-feedback regulator of virus-triggered signaling and cellular antiviral response. *Proc Natl Acad Sci U S A* 2009;106:7945–50. <https://doi.org/10.1073/pnas.0900818106>.
- [333] Das A, Dinh PX, Panda D, Pattnaik AK. Interferon-Inducible Protein IFI35 Negatively Regulates RIG-I Antiviral Signaling and Supports Vesicular Stomatitis Virus Replication. *J Virol* 2014;88:3103–13. <https://doi.org/10.1128/JVI.03202-13>.
- [334] Li D, Wu M. Pattern recognition receptors in health and diseases. *Sig Transduct Target Ther* 2021;6:291. <https://doi.org/10.1038/s41392-021-00687-0>.
- [335] Álvarez L, Haubrich K, Iselin L, Gillioz L, Ruscica V, Lapouge K, et al. The molecular dissection of TRIM25's RNA-binding mechanism provides key insights into its antiviral activity. *Nat Commun* 2024;15:8485. <https://doi.org/10.1038/s41467-024-52918-x>.
- [336] Schwartz SL, Conn GL. RNA regulation of the antiviral protein 2'-5'-oligoadenylate synthetase. *Wiley Interdiscip Rev RNA* 2019;10:e1534. <https://doi.org/10.1002/wrna.1534>.
- [337] Ren Z, Ding T, Zuo Z, Xu Z, Deng J, Wei Z. Regulation of MAVS Expression and Signaling Function in the Antiviral Innate Immune Response. *Front Immunol* 2020;11:1030. <https://doi.org/10.3389/fimmu.2020.01030>.
- [338] Ferreira AR, Marques M, Ramos B, Kagan JC, Ribeiro D. Emerging roles of peroxisomes in viral infections. *Trends in Cell Biology* 2022;32:124–39. <https://doi.org/10.1016/j.tcb.2021.09.010>.
- [339] Miyashita M, Oshiumi H, Matsumoto M, Seya T. DDX60, a DEXD/H box helicase, is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling. *Mol Cell Biol* 2011;31:3802–19. <https://doi.org/10.1128/MCB.01368-10>.
- [340] Pattabhi S, Knoll ML, Gale M, Loo Y-M. DHX15 Is a Coreceptor for RLR Signaling That Promotes Antiviral Defense Against RNA Virus Infection. *J Interferon Cytokine Res* 2019;39:331–46. <https://doi.org/10.1089/jir.2018.0163>.
- [341] Sugimoto N, Mitoma H, Kim T, Hanabuchi S, Liu Y-J. Helicase proteins DHX29 and RIG-I cosense cytosolic nucleic acids in the human airway system. *Proc Natl Acad Sci U S A* 2014;111:7747–52. <https://doi.org/10.1073/pnas.1400139111>.
- [342] Hage A, Bharaj P, van Tol S, Giraldo MI, Gonzalez-Orozco M, Valerdi KM, et al. The RNA helicase DHX16 recognizes specific viral RNA to trigger RIG-I-dependent innate antiviral immunity. *Cell Rep* 2022;38:110434. <https://doi.org/10.1016/j.celrep.2022.110434>.
- [343] Zhang R, Cheng M, Liu B, Yuan M, Chen D, Wang Y, et al. DEAD-Box Helicase DDX6 Facilitated RIG-I-Mediated Type-I Interferon Response to EV71 Infection. *Front Cell Infect Microbiol* 2021;11:725392. <https://doi.org/10.3389/fcimb.2021.725392>.
- [344] Núñez RD, Budt M, Saenger S, Paki K, Arnold U, Sadewasser A, et al. The RNA Helicase DDX6 Associates with RIG-I to Augment Induction of Antiviral Signaling. *Int J Mol Sci* 2018;19:1877. <https://doi.org/10.3390/ijms19071877>.
- [345] Song J, Li M, Li C, Liu K, Zhu Y, Zhang H. Friend or foe: RIG- I like receptors and diseases. *Autoimmun Rev* 2022;21:103161. <https://doi.org/10.1016/j.autrev.2022.103161>.
- [346] Ma W, Belisle SE, Mosier D, Li X, Stigger-Rosser E, Liu Q, et al. 2009 pandemic H1N1 influenza virus causes disease and upregulation of genes related to inflammatory and immune responses, cell death, and lipid metabolism in pigs. *J Virol* 2011;85:11626–37. <https://doi.org/10.1128/JVI.05705-11>.

- [347] Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* 2009;460:1021–5. <https://doi.org/10.1038/nature08260>.
- [348] Silva MJA, Ribeiro LR, Gouveia MIM, Marcelino BDR, Santos CSD, Lima KVB, et al. Hyperinflammatory Response in COVID-19: A Systematic Review. *Viruses* 2023;15:553. <https://doi.org/10.3390/v15020553>.
- [349] Sariol A, Perlman S. Lung inflammation drives Long Covid. *Science* 2025;387:1039–40. <https://doi.org/10.1126/science.adw0091>.
- [350] Bachiller S, Vitallé J, Camprubi-Ferrer L, García M, Gallego I, López-García M, et al. SARS-CoV-2 post-acute sequelae linked to inflammation via extracellular vesicles. *Front Immunol* 2025;16:1501666. <https://doi.org/10.3389/fimmu.2025.1501666>.
- [351] Yin K, Peluso MJ, Luo X, Thomas R, Shin M-G, Neidleman J, et al. Long COVID manifests with T cell dysregulation, inflammation and an uncoordinated adaptive immune response to SARS-CoV-2. *Nat Immunol* 2024;25:218–25. <https://doi.org/10.1038/s41590-023-01724-6>.
- [352] He B, Gross M, Roizman B. The γ 134.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1 α to dephosphorylate the α subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci U S A* 1997;94:843–8. <https://doi.org/10.1073/pnas.94.3.843>.
- [353] Opaluch AM, Schneider M, Chiang C, Nguyen QT, Maestre AM, Mulder LCF, et al. Positive regulation of TRAF6-dependent innate immune responses by protein phosphatase PP1- γ . *PLoS One* 2014;9:e89284. <https://doi.org/10.1371/journal.pone.0089284>.
- [354] Maillard PV, van der Veen AG, Poirier EZ, Reis e Sousa C. Slicing and dicing viruses: antiviral RNA interference in mammals. *EMBO J* 2019;38:e100941. <https://doi.org/10.15252/embj.2018100941>.
- [355] Zhang R, Jing Y, Zhang H, Niu Y, Liu C, Wang J, et al. Comprehensive Evolutionary Analysis of the Major RNA-Induced Silencing Complex Members. *Sci Rep* 2018;8:14189. <https://doi.org/10.1038/s41598-018-32635-4>.
- [356] Li Y, Lu J, Han Y, Fan X, Ding S-W. RNA interference functions as an antiviral immunity mechanism in mammals. *Science* 2013;342:231–4. <https://doi.org/10.1126/science.1241911>.
- [357] Zhang Y, Dai Y, Wang J, Xu Y, Li Z, Lu J, et al. Mouse circulating extracellular vesicles contain virus-derived siRNAs active in antiviral immunity. *EMBO J* 2022;41:e109902. <https://doi.org/10.15252/embj.2021109902>.
- [358] Zhong Y, Hu Z, Wu J, Dai F, Lee F, Xu Y. STAU1 selectively regulates the expression of inflammatory and immune response genes and alternative splicing of the nerve growth factor receptor signaling pathway. *Oncol Rep* 2020;44:1863–74. <https://doi.org/10.3892/or.2020.7769>.
- [359] Wang Q, Zhang Q, Zheng M, Wen J, Li Q, Zhao G. Viral–Host Interactome Analysis Reveals Chicken STAU2 Interacts With Non-structural Protein 1 and Promotes the Replication of H5N1 Avian Influenza Virus. *Front Immunol* 2021;12:590679. <https://doi.org/10.3389/fimmu.2021.590679>.
- [360] Dai Y, Wang B, Wang J, Wei X, Liu X, Che X, et al. Increased viral tolerance mediates by antiviral RNA interference in bat cells. *Cell Reports* 2024;43:114581. <https://doi.org/10.1016/j.celrep.2024.114581>.
- [361] MacKay CR, Wang JP, Kurt-Jones EA. Dicer’s role as an antiviral: still an enigma. *Current Opinion in Immunology* 2014;26:49–55. <https://doi.org/10.1016/j.coi.2013.10.015>.
- [362] Sumaiya K, Langford D, Natarajaseenivasan K, Shanmughapriya S. Macrophage migration inhibitory factor (MIF): A multifaceted cytokine regulated by genetic and physiological strategies. *Pharmacol Ther* 2022;233:108024. <https://doi.org/10.1016/j.pharmthera.2021.108024>.

- [363] Nazitto R, Amon LM, Mast FD, Aitchison JD, Aderem A, Johnson JS, et al. ILF3 is a negative transcriptional regulator of innate immune responses and myeloid dendritic cell maturation. *J Immunol* 2021;206:2949–65. <https://doi.org/10.4049/jimmunol.2001235>.
- [364] Jin J, Li A, Wang W, Wu J. Interleukin-enhanced binding factor 2 interacts with NLRP3 to inhibit the NLRP3 inflammasome activation. *Biochem Biophys Res Commun* 2018;500:398–404. <https://doi.org/10.1016/j.bbrc.2018.04.087>.
- [365] Pang AN, Chen SN, Tian B, Liu LH, Zhang S, Song JW, et al. Mallard ILF2 negatively regulates type I IFN production through autophagy-lysosome-dependent degradation of IRF7. *J Immunol* 2025;214:2371–84. <https://doi.org/10.1093/jimmun/vkaf118>.
- [366] Li J, Fang P, Zhou Y, Wang D, Fang L, Xiao S. DEAD-box RNA helicase 21 negatively regulates cytosolic RNA-mediated innate immune signaling. *Front Immunol* 2022;13:956794. <https://doi.org/10.3389/fimmu.2022.956794>.
- [367] Ma S, Mao Q, Weng S, Teng M, Luo J, Zhang K. DDX3X and virus interactions: functional diversity and antiviral strategies. *Front Microbiol* 2025;16. <https://doi.org/10.3389/fmicb.2025.1630068>.
- [368] Oshiumi H, Sakai K, Matsumoto M, Seya T. DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN- β -inducing potential. *Eur J Immunol* 2010;40:940–8. <https://doi.org/10.1002/eji.200940203>.
- [369] Gu H, Yang J, Zhang J, Song Y, Zhang Y, Xu P, et al. PCBP2 maintains antiviral signaling homeostasis by regulating cGAS enzymatic activity via antagonizing its condensation. *Nat Commun* 2022;13:1564. <https://doi.org/10.1038/s41467-022-29266-9>.
- [370] Webb LG, Fernandez-Sesma A. RNA viruses and the cGAS-STING pathway: reframing our understanding of innate immune sensing. *Curr Opin Virol* 2022;53:101206. <https://doi.org/10.1016/j.coviro.2022.101206>.
- [371] Acharya D, Reis R, Volcic M, Liu G, Wang MK, Chia BS, et al. Actin cytoskeleton remodeling primes RIG-I-like receptor activation. *Cell* 2022;185:3588–3602.e21. <https://doi.org/10.1016/j.cell.2022.08.011>.
- [372] Ahmad S, Mu X, Hur S. The Role of RNA Editing in the Immune Response. *Methods Mol Biol* 2021;2181:287–307. https://doi.org/10.1007/978-1-0716-0787-9_17.
- [373] Dalet A, Gatti E, Pierre P. Integration of PKR-dependent translation inhibition with innate immunity is required for a coordinated anti-viral response. *FEBS Lett* 2015;589:1539–45. <https://doi.org/10.1016/j.febslet.2015.05.006>.
- [374] Sharma N, Kessler P, Sen GC. Cell-type-specific need of Ddx3 and PACT for interferon induction by RNA viruses. *J Virol* 2023;97:e0130423. <https://doi.org/10.1128/jvi.01304-23>.
- [375] Sanchez David RY, Combredet C, Najburg V, Millot GA, Beauclair G, Schwikowski B, et al. LGP2 binds to PACT to regulate RIG-I- and MDA5-mediated antiviral responses. *Sci Signal* 2019;12:eaar3993. <https://doi.org/10.1126/scisignal.aar3993>.
- [376] Cláudio N, Dalet A, Gatti E, Pierre P. Mapping the crossroads of immune activation and cellular stress response pathways. *EMBO J* 2013;32:1214–24. <https://doi.org/10.1038/emboj.2013.80>.
- [377] Rodriguez KR, Horvath CM. Amino Acid Requirements for MDA5 and LGP2 Recognition by Paramyxovirus V Proteins: a Single Arginine Distinguishes MDA5 from RIG-I. *J Virol* 2013;87:2974–8. <https://doi.org/10.1128/jvi.02843-12>.
- [378] Luthra P, Ramanan P, Mire CE, Weisend C, Tsuda Y, Yen B, et al. Mutual antagonism between the Ebola virus VP35 protein and the RIG-I activator PACT determines infection outcome. *Cell Host Microbe* 2013;14:74–84. <https://doi.org/10.1016/j.chom.2013.06.010>.
- [379] Tawaratsumida K, Phan V, Hrincius ER, High AA, Webby R, Redecke V, et al. Quantitative proteomic analysis of the influenza A virus nonstructural proteins NS1 and NS2 during natural cell infection identifies PACT as an NS1 target protein and antiviral host factor. *J Virol* 2014;88:9038–48. <https://doi.org/10.1128/JVI.00830-14>.
- [380] Wang B, Xi X, Lei X, Zhang X, Cui S, Wang J, et al. Enterovirus 71 Protease 2Apro Targets MAVS to Inhibit Anti-Viral Type I Interferon Responses. *PLoS Pathog* 2013;9:e1003231. <https://doi.org/10.1371/journal.ppat.1003231>.

- [381] Feng Q, Langereis MA, Lork M, Nguyen M, Hato SV, Lanke K, et al. Enterovirus 2Apro targets MDA5 and MAVS in infected cells. *J Virol* 2014;88:3369–78. <https://doi.org/10.1128/JVI.02712-13>.
- [382] Xiao X, Fu Y, You W, Huang C, Zeng F, Gu X, et al. Inhibition of the RLR signaling pathway by SARS-CoV-2 ORF7b is mediated by MAVS and abrogated by ORF7b-homologous interfering peptide. *J Virol* 2024;98:e01573-23. <https://doi.org/10.1128/jvi.01573-23>.
- [383] Davies MV, Chang HW, Jacobs BL, Kaufman RJ. The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. *J Virol* 1993;67:1688–92. <https://doi.org/10.1128/jvi.67.3.1688-1692.1993>.
- [384] Schierhorn KL, Jolmes F, Bernalow J, Saenger S, Peteranderl C, Dzieciolowski J, et al. Influenza A Virus Virulence Depends on Two Amino Acids in the N-Terminal Domain of Its NS1 Protein To Facilitate Inhibition of the RNA-Dependent Protein Kinase PKR. *J Virol* 2017;91:e00198-17. <https://doi.org/10.1128/JVI.00198-17>.
- [385] Poppers J, Mulvey M, Khoo D, Mohr I. Inhibition of PKR Activation by the Proline-Rich RNA Binding Domain of the Herpes Simplex Virus Type 1 Us11 Protein. *J Virol* 2000;74:11215–21. <https://doi.org/10.1128/jvi.74.23.11215-11221.2000>.
- [386] Loo Y-M, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, et al. Distinct RIG-I and MDA5 Signaling by RNA Viruses in Innate Immunity. *J Virol* 2008;82:335–45. <https://doi.org/10.1128/JVI.01080-07>.

APPENDIX

8. APPENDIX

8.1. APPENDIX TABLES

Table A1. RNAseq study comparing poly(I:C)-treated HAP-1 WT versus mock-treated HAP-1 WT cells. This table shows upregulated genes (at least 2-fold) in HAP-1 WT, poly(I:C)-treated cells compared to HAP-1 WT mock-treated cells. Upregulated genes were classified by gene ontology (GO) according to their biological process. *Homo sapiens REFLIST* column represents the total number of listed genes within that GO term biological process in *H. sapiens* genome; *Genes upregulated in the RNAseq experiment* column represents the number of upregulated genes within that GO term category; knowing that the total number of upregulated genes in the whole RNAseq dataset is 28, *Number of expected genes considering the number of upregulated genes in the RNAseq experiment* column represent the expected number of upregulated genes of each GO term; *Fold Enrichment in the uploaded upregulated genes* column represents the enrichment of upregulated genes (3rd column) respect to the expected number of upregulated genes of that GO term category (4th column); False Discovery Rate-adjusted p-value for each GO term category data.

Gene ontology (GO) terms	<i>Homo sapiens</i> REFLIST: 20589 genes	Genes upregulated in the RNAseq experiment: 28 genes	Number of expected genes considering the number of upregulated genes in the RNAseq experiment (28)	Fold Enrichment in the uploaded upregulated genes	FDR adjusted p-value
negative regulation of viral genome replication (GO:0045071)	57	8	0.07	> 100	1.48E-11
positive regulation of interferon-beta production (GO:0032728)	41	6	0.05	> 100	2.05E-08
interleukin-27-mediated signaling pathway (GO:0070106)	7	4	0.01	> 100	4.44E-07
antiviral innate immune response (GO:0140374)	22	4	0.03	> 100	1.62E-05
regulation of defense response to virus (GO:0050688)	76	5	0.1	50.17	2.79E-05
regulation of ribonuclease activity (GO:0060700)	9	3	0.01	> 100	1.76E-04
cytoplasmic pattern recognition receptor signaling pathway in response to virus (GO:0039528)	11	3	0.01	> 100	2.70E-04
positive regulation of RIG-I signaling pathway (GO:1900246)	11	3	0.01	> 100	2.76E-04
cellular response to interferon-alpha (GO:0035457)	12	3	0.02	> 100	3.29E-04
cellular response to exogenous dsRNA (GO:0071360)	19	3	0.02	> 100	9.71E-04
response to bacterium (GO:0009617)	754	8	0.99	8.09	1.18E-03

positive regulation of interferon-alpha production (GO:0032727)	25	3	0.03	91.51	1.74E-03
positive regulation of tumor necrosis factor production (GO:0032760)	101	4	0.13	30.2	2.50E-03
positive regulation of MDA-5 signaling pathway (GO:1900245)	4	2	0.01	> 100	5.53E-03
type I interferon signaling pathway (GO:0060337)	42	3	0.06	54.47	6.05E-03
regulation of type III interferon production (GO:0034344)	5	2	0.01	> 100	7.52E-03
ISG15-protein conjugation (GO:0032020)	6	2	0.01	> 100	9.75E-03
regulation by virus of viral protein levels in host cell (GO:0046719)	8	2	0.01	> 100	1.43E-02
negative regulation of cytokine-mediated signaling pathway (GO:0001960)	75	3	0.1	30.5	2.66E-02
negative regulation of innate immune response (GO:0045824)	76	3	0.1	30.1	2.73E-02

Table A2. RNAseq study comparing HAP-1 WT and HAP-1 IFI6 KO cells, both treated with poly(I:C). This table shows upregulated genes (at least 2-fold) in HAP-1, IFI6 KO, poly(I:C)-treated cells compared to control, HAP-1 WT cells treated with poly(I:C). Upregulated genes were classified by gene ontology (GO) according to their biological process. *Homo sapiens REFLIST* column represents the total number of listed genes within that GO term biological process in *H. sapiens* genome; *Genes upregulated in the RNAseq experiment* column represents the number of upregulated genes within that GO term category; knowing that the total number of upregulated genes in the whole RNAseq dataset is 1204, *Number of expected genes considering the number of upregulated genes in the RNAseq experiment* column represent the expected number of upregulated genes of each GO term; *Fold Enrichment in the uploaded upregulated genes* column represents the enrichment of upregulated genes (3rd column) respect to the expected number of upregulated genes of that GO term category (4th column); False Discovery Rate-adjusted p-value for each GO term category data.

Gene ontology (GO) terms	<i>Homo sapiens</i> REFLIST: 20589 genes	Genes upregulated in the RNAseq experiment: 1204 genes	Number of expected genes considering the number of upregulated genes in the RNAseq experiment (1204)	Fold Enrichment in the uploaded upregulated genes	FDR adjusted p-value
negative regulation of viral process (GO:0048525)	95	31	5.56	5.58	6.38E-10
negative regulation of viral genome replication (GO:0045071)	57	23	3.33	6.9	1.18E-08
defense response to virus (GO:0051607)	253	47	14.79	3.18	3.08E-08
response to virus (GO:0009615)	357	54	20.88	2.59	9.63E-07
regulation of viral genome replication (GO:0045069)	87	23	5.09	4.52	5.07E-06
angiogenesis (GO:0001525)	327	49	19.12	2.56	5.78E-06
positive regulation of cell migration (GO:0030335)	532	64	31.11	2.06	4.98E-05
regulation of epithelial cell proliferation (GO:0050678)	369	47	21.58	2.18	3.79E-04
negative regulation of cell migration (GO:0030336)	288	39	16.84	2.32	6.79E-04
cellular response to fibroblast growth factor stimulus (GO:0044344)	85	18	4.97	3.62	1.18E-03
regulation of response to external stimulus (GO:0032101)	973	93	56.9	1.63	1.20E-03
regulation of MAP kinase activity (GO:0043405)	184	28	10.76	2.6	1.92E-03
regulation of animal organ morphogenesis (GO:2000027)	129	22	7.54	2.92	2.39E-03
response to interferon-alpha (GO:0035455)	23	9	1.34	6.69	3.08E-03

collagen fibril organization (GO:0030199)	60	14	3.51	3.99	3.44E-03
response to type I interferon (GO:0034340)	52	13	3.04	4.28	3.46E-03
regulation of peptidyl-tyrosine phosphorylation (GO:0050730)	261	34	15.26	2.23	3.62E-03
negative regulation of fibroblast growth factor receptor signaling pathway (GO:0040037)	18	8	1.05	7.6	3.88E-03
regulation of vesicle-mediated transport (GO:0060627)	552	58	32.28	1.8	4.44E-03
response to hormone (GO:0009725)	767	74	44.85	1.65	5.16E-03
response to hypoxia (GO:0001666)	277	35	16.2	2.16	5.20E-03
regulation of axon extension involved in axon guidance (GO:0048841)	33	10	1.93	5.18	5.90E-03
cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	267	34	15.61	2.18	6.28E-03
peptidyl-lysine oxidation (GO:0018057)	5	5	0.29	17.1	6.39E-03
canonical Wnt signaling pathway (GO:0060070)	102	18	5.96	3.02	6.47E-03
positive regulation of interferon-beta production (GO:0032728)	41	11	2.4	4.59	6.48E-03
regulation of Wnt signaling pathway (GO:0030111)	332	39	19.41	2.01	7.43E-03
regulation of transmembrane receptor protein serine/threonine kinase signaling pathway (GO:0090092)	275	34	16.08	2.11	7.77E-03
gastrulation with mouth forming second (GO:0001702)	28	9	1.64	5.5	8.30E-03
negative regulation of cell-substrate adhesion (GO:0010812)	60	13	3.51	3.71	9.37E-03
regulation of blood coagulation (GO:0030193)	70	14	4.09	3.42	1.09E-02
positive regulation of cell death (GO:0010942)	583	58	34.09	1.7	1.16E-02
response to interferon-gamma (GO:0034341)	130	20	7.6	2.63	1.25E-02
BMP signaling pathway (GO:0030509)	91	16	5.32	3.01	1.39E-02
positive regulation of ossification (GO:0045778)	55	12	3.22	3.73	1.46E-02

interleukin-27-mediated signaling pathway (GO:0070106)	7	5	0.41	12.21	1.51E-02
negative chemotaxis (GO:0050919)	47	11	2.75	4	1.51E-02
positive regulation of axon extension (GO:0045773)	39	10	2.28	4.38	1.52E-02
regulation of angiogenesis (GO:0045765)	288	34	16.84	2.02	1.55E-02
glomerulus development (GO:0032835)	56	12	3.27	3.66	1.60E-02
response to retinoic acid (GO:0032526)	113	18	6.61	2.72	1.62E-02
regulation of synapse assembly (GO:0051963)	103	17	6.02	2.82	1.63E-02
regulation of viral entry into host cell (GO:0046596)	48	11	2.81	3.92	1.69E-02
positive regulation of neural precursor cell proliferation (GO:2000179)	57	12	3.33	3.6	1.79E-02
regulation of phosphatidylinositol 3-kinase activity (GO:0043551)	57	12	3.33	3.6	1.79E-02
metanephros development (GO:0001656)	85	15	4.97	3.02	1.87E-02
regulation of Notch signaling pathway (GO:0008593)	95	16	5.56	2.88	1.91E-02
response to interferon-beta (GO:0035456)	33	9	1.93	4.66	1.92E-02
regulation of apoptotic process (GO:0042981)	1468	120	85.85	1.4	1.92E-02
cell-substrate junction organization (GO:0150115)	41	10	2.4	4.17	1.97E-02
regulation of ERK1 and ERK2 cascade (GO:0070372)	303	35	17.72	1.98	1.97E-02
negative regulation of MAP kinase activity (GO:0043407)	58	12	3.39	3.54	1.98E-02
regulation of epithelial to mesenchymal transition (GO:0010717)	97	16	5.67	2.82	2.22E-02
negative regulation of vascular permeability (GO:0043116)	20	7	1.17	5.99	2.39E-02
negative regulation of axon extension involved in axon guidance (GO:0048843)	27	8	1.58	5.07	2.42E-02
negative regulation of wound healing (GO:0061045)	69	13	4.03	3.22	2.45E-02
negative regulation of endothelial cell proliferation (GO:0001937)	43	10	2.51	3.98	2.58E-02

negative regulation of viral life cycle (GO:1903901)	28	8	1.64	4.89	2.87E-02
cellular response to type I interferon (GO:0071357)	44	10	2.57	3.89	2.95E-02
regulation of ribonuclease activity (GO:0060700)	9	5	0.53	9.5	2.99E-02
regulation of immune system process (GO:0002682)	1520	122	88.89	1.37	3.19E-02
endocardial cushion morphogenesis (GO:0003203)	37	9	2.16	4.16	3.43E-02
regulation of response to biotic stimulus (GO:0002831)	364	39	21.29	1.83	3.56E-02
regulation of tumor necrosis factor production (GO:0032680)	163	22	9.53	2.31	3.72E-02
aorta morphogenesis (GO:0035909)	30	8	1.75	4.56	3.90E-02
neutrophil homeostasis (GO:0001780)	16	6	0.94	6.41	4.00E-02
heart valve development (GO:0003170)	65	12	3.8	3.16	4.11E-02
hair follicle morphogenesis (GO:0031069)	31	8	1.81	4.41	4.49E-02
negative regulation of viral entry into host cell (GO:0046597)	24	7	1.4	4.99	4.94E-02

Table A3. IFI27-HA-bound proteins. HEK-293T cells were transfected with a pCAGGS plasmid encoding for IFI27 fused to HA tag (pCAGGS-IFI27-HA) or a pCAGGS plasmid encoding for IFI44 fused to a HA tag (pCAGGS-IFI44-HA), as control. 24 hours later, cells were treated with poly(I:C) at 3 µg/mL. At 24h after poly(I:C) transfection, protein extracts were obtained by lysis and were incubated with HA-bound agarose beads to retain IFI27-HA and all its associated proteins, which were then identified by MS (90 proteins). Protein accession number in UniProt (<https://www.uniprot.org/>) (prot_acc), description (prot_desc), prot_score, prot_mass, prot_matches_sig, prot_sequences_sig, prot_coverage, and prot_PI (isoelectric point) are indicated. These terms were previously explained in (https://www.matrixscience.com/help/csv_headers.html).

prot_acc	prot_desc	prot_score	Prot mass	Prot matches_sig	Prot sequences_sig	Prot cover	prot_pi
Q7Z406	Myosin-14 OS=Homo sapiens GN=MYH14 PE=1 SV=2	1554	228514	37	29	16	5.52
O95793	Double-stranded RNA-binding protein Stauf homolog 1 OS=Homo sapiens GN=STAU1 PE=1 SV=2	925	63373	20	17	36.7	9.46
O94832	Unconventional myosin-Id OS=Homo sapiens GN=MYO1D PE=1 SV=2	889	116772	19	19	21.6	9.44
F5H6E2	Unconventional myosin-Ic OS=Homo sapiens GN=MYO1C PE=1 SV=1	881	119564	17	17	21.7	9.46
A0A0A0MRM8	Unconventional myosin-VI OS=Homo sapiens GN=MYO6 PE=1 SV=1	842	146028	15	15	17.5	8.78
E9PDF6	Unconventional myosin-Ib OS=Homo sapiens GN=MYO1B PE=1 SV=1	835	129228	18	17	18.5	9.36
G8JLA2	Myosin light polypeptide 6 OS=Homo sapiens	651	17216	18	10	75.7	4.46

	GN=MYL6 PE=1 SV=1						
A0A1X7SBS1	Heterogeneous nuclear ribonucleoprotein U OS=Homo sapiens GN=HNRNPU PE=1 SV=1	623	82296	12	12	20.7	5.64
P11142	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1	606	71038	12	11	23.5	5.37
P19525	Interferon-induced, double-stranded RNA-activated protein kinase OS=Homo sapiens GN=EIF2AK2 PE=1 SV=2	565	62424	11	11	26	8.58
Q15366	Poly(rC)-binding protein 2 OS=Homo sapiens GN=PCBP2 PE=1 SV=1	553	38878	13	9	45.2	6.33
P68032	Actin, alpha cardiac muscle 1 OS=Homo sapiens GN=ACTC1 PE=1 SV=1	544	42268	58	11	28.6	5.23
A0A087WWU8	Tropomyosin alpha-3 chain OS=Homo sapiens GN=TPM3 PE=1 SV=1	511	26588	10	10	39.6	4.75
P52907	F-actin-capping protein subunit alpha-1 OS=Homo sapiens GN=CAPZA1 PE=1 SV=3	511	33040	9	9	45.1	5.45
Q5T6W2	Heterogeneous nuclear ribonucleoprotein K (Fragment) OS=Homo sapiens	508	41965	11	11	35.9	5.43

	GN=HNRNPK PE=1 SV=1						
A0A0A0MTC4	Double-stranded RNA-binding protein Staufen homolog 2 OS=Homo sapiens GN=STAU2 PE=1 SV=1	505	43343	8	8	31.9	9.44
P14649	Myosin light chain 6B OS=Homo sapiens GN=MYL6B PE=1 SV=1	495	22842	13	10	48.1	5.56
O75569	Interferon- inducible double- stranded RNA- dependent protein kinase activator A OS=Homo sapiens GN=PRKRA PE=1 SV=1	494	34750	10	10	44.4	8.69
O77727	Keratin, type I cytoskeletal 15 (Contact-Cont) OS=Ovis aries GN=KRT15 PE=2 SV=1	490	48740	32	10	15.9	4.73
Q9UHB6	LIM domain and actin-binding protein 1 OS=Homo sapiens GN=LIMA1 PE=1 SV=1	480	85541	10	10	20.2	6.41
Q9NYL9	Tropomodulin-3 OS=Homo sapiens GN=TMOD3 PE=1 SV=1	427	39708	8	8	29.3	5.08
A0A087WZR1	Interferon alpha- inducible protein 27, mitochondrial OS=Homo sapiens GN=IFI27 PE=1 SV=1	400	11233	15	5	67.2	11.07
P32969	60S ribosomal protein L9 OS=Homo sapiens GN=RPL9 PE=1 SV=1	387	21942	7	7	53.6	9.96

Q15633	RISC-loading complex subunit TARBP2 OS=Homo sapiens GN=TARBP2 PE=1 SV=3	373	39521	6	6	22.4	6.11
H0YK48	Tropomyosin alpha-1 chain OS=Homo sapiens GN=TPM1 PE=1 SV=1	372	28609	7	7	26.2	4.74
Q16643	Drebrin OS=Homo sapiens GN=DBN1 PE=1 SV=4	369	71753	7	7	16	4.41
B1AK87	Capping protein (Actin filament) muscle Z-line, beta, isoform CRA_a OS=Homo sapiens GN=CAPZB PE=1 SV=1	363	29507	7	7	29.2	6.45
P67936	Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3	346	28596	6	6	21.8	4.67
H0Y6E7	RNA-binding motif protein, X chromosome (Fragment) OS=Homo sapiens GN=RBMX PE=1 SV=2	342	31837	7	6	21.6	9.84
B7Z645	Heterogeneous nuclear ribonucleoprotein Q OS=Homo sapiens GN=SYNCRIP PE=1 SV=1	338	52152	6	6	14	9.19
F8VR82	Serine/threonine-protein phosphatase OS=Homo sapiens GN=PPP1CC PE=1 SV=1	279	31610	5	5	25.2	5.57
H0YKX5	Tropomyosin alpha-1 chain	277	16401	6	6	31.7	4.7

	(Fragment) OS=Homo sapiens GN=TPM1 PE=1 SV=1						
A0A087WXM6	60S ribosomal protein L17 (Fragment) OS=Homo sapiens GN=RPL17 PE=3 SV=1	271	19757	7	6	49.1	10.05
H0YKS4	Annexin (Fragment) OS=Homo sapiens GN=ANXA2 PE=1 SV=1	266	19610	5	5	38.6	5.68
I3L3P7	40S ribosomal protein S15a OS=Homo sapiens GN=RPS15A PE=1 SV=1	266	11562	6	6	53	10.18
D6RJ07	Zinc finger protein 346 OS=Homo sapiens GN=ZNF346 PE=1 SV=1	264	35129	4	4	24.5	9.34
A0A0C4DGB6	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=1	261	70791	6	6	10.3	5.99
I3L1L3	Myb-binding protein 1A (Fragment) OS=Homo sapiens GN=MYBBP1A PE=1 SV=1	260	140937	5	5	7.5	9.34
M0R1A7	60S ribosomal protein L18a OS=Homo sapiens GN=RPL18A PE=1 SV=1	259	17604	7	6	40.1	10.89
Q96SI9	Spermatid perinuclear RNA- binding protein OS=Homo sapiens GN=STRBP PE=1 SV=1	227	74158	6	6	7.7	8.91
E9PFP8	Poly(rC)-binding protein 3 OS=Homo sapiens	222	38393	4	4	14.1	8.22

	GN=PCBP3 PE=1 SV=1						
P62280	40S ribosomal protein S11 OS=Homo sapiens GN=RPS11 PE=1 SV=3	193	18557	4	4	32.3	10.31
A0A087WVQ9	Elongation factor 1-alpha 1 OS=Homo sapiens GN=EEF1A1 PE=1 SV=1	191	48129	4	4	12.9	9.12
Q6WCQ1	Myosin phosphatase Rho- interacting protein OS=Homo sapiens GN=MPRIIP PE=1 SV=3	184	117105	4	4	6.2	5.89
O15145	Actin-related protein 2/3 complex subunit 3 OS=Homo sapiens GN=ARPC3 PE=1 SV=3	173	20717	4	4	23.6	8.78
H0YB22	40S ribosomal protein S14 (Fragment) OS=Homo sapiens GN=RPS14 PE=1 SV=1	157	13165	3	3	35	9.91
F8WCF6	Actin-related protein 2/3 complex subunit 4 OS=Homo sapiens GN=ARPC4- TTLL3 PE=3 SV=1	151	21137	4	4	21	8.89
A0A087WY00	Unconventional myosin-Va OS=Homo sapiens GN=MYO5A PE=1 SV=1	145	213447	3	3	1.8	8.82
A0A0U1RR32	Histone H2A OS=Homo sapiens GN=HIST1H3D PE=3 SV=1	144	18470	4	3	20.7	11.52
P62241	40S ribosomal protein S8 OS=Homo sapiens	139	24420	3	3	19.7	10.32

	GN=RPS8 PE=1 SV=2						
P40939	Trifunctional enzyme subunit alpha, mitochondrial OS=Homo sapiens GN=HADHA PE=1 SV=2	132	83545	3	3	7.2	9.16
Q86SG5	Protein S100-A7A OS=Homo sapiens GN=S100A7A PE=1 SV=3	124	11390	4	2	22.8	6.89
H3BTH6	F-box only protein 22 (Fragment) OS=Homo sapiens GN=FBXO22 PE=1 SV=1	121	26852	3	3	15.8	6.66
A0A0D9SET8	F-actin-capping protein subunit alpha-2 OS=Homo sapiens GN=CAPZA2 PE=1 SV=1	108	20390	1	1	16.1	6.07
O75475	PC4 and SFRS1- interacting protein OS=Homo sapiens GN=PSIP1 PE=1 SV=1	106	60159	2	2	8.1	9.15
J3KQE5	GTP-binding nuclear protein Ran (Fragment) OS=Homo sapiens GN=RAN PE=1 SV=1	101	26983	2	2	9	9.62
A0A0D9SF53	ATP-dependent RNA helicase DDX3X OS=Homo sapiens GN=DDX3X PE=1 SV=1	87	81978	2	2	3.1	8.29
M0QZ52	Calmodulin OS=Homo sapiens GN=CALM3 PE=1 SV=1	87	9348	1	1	36.1	3.89
A2A2V2	RNA-binding protein 34 (Fragment) OS=Homo sapiens	84	45982	2	2	6.4	10.09

	GN=RBM34 PE=1 SV=1						
C9JI87	Voltage-dependent anion-selective channel protein 1 (Fragment) OS=Homo sapiens GN=VDAC1 PE=1 SV=6	80	20308	2	2	13.1	6.74
O43143	Pre-mRNA- splicing factor ATP-dependent RNA helicase DHX15 OS=Homo sapiens GN=DHX15 PE=1 SV=2	77	91519	1	1	1.5	7.12
Q9ULJ8	Neurabin-1 OS=Homo sapiens GN=PPP1R9A PE=1 SV=2	77	123497	2	2	1.6	5.01
X6R631	39S ribosomal protein L55, mitochondrial OS=Homo sapiens GN=MRPL55 PE=1 SV=2	77	6845	1	1	29.1	9.3
P27824	Calnexin OS=Homo sapiens GN=CANX PE=1 SV=2	75	67894	3	3	6.8	4.47
Q5SSJ5	Heterochromatin protein 1-binding protein 3 OS=Homo sapiens GN=HP1BP3 PE=1 SV=1	73	61399	3	3	5.8	9.69
Q9Y2Q9	28S ribosomal protein S28, mitochondrial OS=Homo sapiens GN=MRPS28 PE=1 SV=1	72	20968	1	1	10.2	9.21
F5GXS2	Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2	69	59757	2	2	4.2	4.82
V9GYA7	28S ribosomal protein S29,	68	23681	1	1	7.1	7.52

	mitochondrial (Fragment) OS=Homo sapiens GN=DAP3 PE=1 SV=1						
A0A087X035	Interferon alpha- inducible protein 27, mitochondrial (Fragment) OS=Homo sapiens GN=IFI27 PE=4 SV=1	63	12189	1	1	10	6.74
A0A1B0GTT5	Vimentin (Fragment) OS=Homo sapiens GN=VIM PE=1 SV=1	62	16415	1	1	6.7	10.26
A8MUF7	Hemoglobin subunit epsilon (Fragment) OS=Homo sapiens GN=HBE1 PE=1 SV=1	56	9464	1	1	11.5	9.1
P56182	Ribosomal RNA processing protein 1 homolog A OS=Homo sapiens GN=RRP1 PE=1 SV=1	56	52990	1	1	2.8	9.39
M0QX71	Glutamate-rich WD repeat- containing protein 1 (Fragment) OS=Homo sapiens GN=GRWD1 PE=1 SV=1	53	25599	2	2	9.2	4.76
P07900	Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5	50	84929	2	2	3.3	4.94
A0A0A0MT26	Sodium/potassium- transporting ATPase subunit alpha-3 OS=Homo sapiens GN=ATP1A3 PE=1 SV=1	49	134795	1	1	1.2	5.76
P82663	28S ribosomal protein S25,	49	20287	1	1	6.4	8.99

	mitochondrial OS=Homo sapiens GN=MRPS25 PE=1 SV=1						
B4DUR8	T-complex protein 1 subunit gamma OS=Homo sapiens GN=CCT3 PE=1 SV=1	48	56053	1	1	3.8	5.47
P82673	28S ribosomal protein S35, mitochondrial OS=Homo sapiens GN=MRPS35 PE=1 SV=1	48	37051	1	1	3.1	8.4
Q92665	28S ribosomal protein S31, mitochondrial OS=Homo sapiens GN=MRPS31 PE=1 SV=3	48	45382	1	1	5.8	9.32
A0A0A0MRA5	Heterogeneous nuclear ribonucleoprotein U-like protein 1 OS=Homo sapiens GN=HNRNPUL1 PE=1 SV=1	47	86346	1	1	1.8	8.94
P60059	Protein transport protein Sec61 subunit gamma OS=Homo sapiens GN=SEC61G PE=1 SV=1	47	7782	1	1	17.6	10.01
O14896	Interferon regulatory factor 6 OS=Homo sapiens GN=IRF6 PE=1 SV=1	46	53555	1	1	4.1	5.18
Q9BRT6	Protein LLP homolog OS=Homo sapiens GN=LLPH PE=1 SV=1	44	15307	1	1	9.3	10.38
E7ESE2	Prohibitin (Fragment) OS=Homo sapiens GN=PHB PE=1 SV=2	43	22431	1	1	4.5	5.97

Q9P0K7	Ankycorbin OS=Homo sapiens GN=RAI14 PE=1 SV=2	43	110479	1	1	2.3	5.87
H0YCK3	Double-stranded RNA-specific adenosine deaminase (Fragment) OS=Homo sapiens GN=ADAR PE=1 SV=1	42	133530	1	1	1	8.71
P60468	Protein transport protein Sec61 subunit beta OS=Homo sapiens GN=SEC61B PE=1 SV=2	42	10014	1	1	15.6	11.57
I3L3B0	Complement component 1 Q subcomponent- binding protein, mitochondrial OS=Homo sapiens GN=C1QBP PE=1 SV=1	41	20168	1	1	7.3	4.1
U3KQF2	ADP-ribosylation factor 4 OS=Homo sapiens GN=ARF4 PE=1 SV=1	41	8072	1	1	11.3	4.81
Q96BK5	PIN2/TERF1- interacting telomerase inhibitor 1 OS=Homo sapiens GN=PINX1 PE=1 SV=2	40	37150	1	1	4.9	9.61

Table A4. Biological process organization of mass spectrometry data. All proteins found on IFI27 interactome with a score higher than 40 (Table A3) were analysed by gene ontology (GO), using NIH DAVID software (<https://davidbioinformatics.nih.gov/>). Proteins were classified by biological process, and we selected all those groups of proteins whose function showed an FDR-adjusted p-value lower than 0.05. GO term of biological process, count of proteins found within that biological process, pvalue, fold enrichment of the specific GO term in IFI27 interactome, FDR-adjusted p-value, and the specific genes within each group are indicated.

GOTERM_Biological process	Count	p-value	Fold Enrichment	FDR-adjusted p-value	Genes
GO:0007015~actin filament organization	11	4.49E-10	18.02329114	2.69E-07	<i>TMOD3, TPM3, TPM1, MYO1C, MYO1D, MYO6, ACTC1, DBN1, TPM4, PPP1R9A, MYO1B</i>
GO:0030048~actin filament-based movement	6	1.36E-08	73.73164557	4.06E-06	<i>MYO1C, MYO1D, MYO6, ACTC1, MYH14, MYO1B</i>
GO:0030050~vesicle transport along actin filament	5	1.68E-07	94.52775073	3.34E-05	<i>ACTN4 hCG_1996371, MYO1C, MYO1D, MYO6, MYO1B</i>
GO:0006412~translation	9	2.74E-06	10.10022542	4.10E-04	<i>EIF2K2, RPL9, EEF1A1, RPL17, RPS8, RPL18A, MRPL55, RPS11, RPS15A</i>
GO:0030049~muscle filament sliding	4	1.34E-05	81.92405063	0.001443513	<i>TPM3, TPM1, MYL6, MYL6B</i>
GO:0006936~muscle contraction	6	1.45E-05	19.15107677	0.001443513	<i>TMOD3, TPM3, TPM1, MYL6, MYL6B, TPM4</i>
GO:0002181~cytoplasmic translation	6	3.44E-05	16.0286186	0.002852071	<i>RPL9, RPL17, RPS8, RPL18A, RPS11, RPS15A</i>
GO:0032543~mitochondrial translation	6	3.82E-05	15.68758416	0.002852071	<i>MRPS31, MRPS35, MRPS25, MRPL55, MRPS28, DAP3</i>

Table A5. Molecular functions organization of mass spectrometry data. All proteins found on IFI27 interactome with a score higher than 40 (Table A3) were analysed by gene ontology (GO), using NIH DAVID software (<https://davidbioinformatics.nih.gov/>). Proteins were classified by molecular functions, and we selected all those groups of proteins whose function showed an FDR-adjusted p-value lower than 0.05. GO term of molecular function, count of proteins found within that molecular function, pvalue, fold enrichment of the specific GO term in IFI27 interactome, FDR-adjusted p-value, and the specific genes within each group are indicated.

GOTERM_Molecular function	Count	p-value	Fold Enrichment	FDR-adjusted p-value	Genes
GO:0003723~RNA binding	42	1.06E-24	6.679052208	2.72E-22	<i>STRBP, LLPH, TARBP2, SYNCRIP, HSP90AA1, RPL9, VIM, PRKRA, RPS11, MRPS28, STAU1, EIF2AK2, ANXA2, MYBBP1A, EEF1A1, RPL17, RPL18A, PPP1CC, ZNF346, RPS15A, CANX, HNRNPU, PCBP2, RBMX, RRP1, ACTN4, DHX15, DDX3X, HNRNPUL1, RAN, GRWD1, PSIP1, HNRNPK, DAP3, HSPA8, PCBP3, CCT3, RBM34, RPS8, MRPS31, MRPS35, SEC61B</i>
GO:0051015~actin filament binding	17	1.21E-15	17.69842818	1.55E-13	<i>ACTN4, ARPC3, MYO1D, CAPZB, MPRIP, LIM1, PPP1R9A, MYH14, TPM3, TPM1, MYO1C, CAPZA1, MYO6, DBN1, TPM4, ARPC4, MYO1B</i>
GO:0003779~actin binding	18	3.98E-14	12.58623953	3.41E-12	<i>TMOD3, ACTN4, ARPC3, MYO1D, CAPZB, MYH14, TPM3, TPM1, RAI14, MYO1C, CAPZA1, MYO6, HNRNPU, DBN1, TPM1, TPM4, ARPC4, MYO1B</i>
GO:0003725~double-stranded RNA binding	9	7.56E-10	28.87938523	4.86E-08	<i>EIF2K2, STRBP, DHX15, TARBP2, VIM, PRKRA, ZNF346, HNRNPU, STAU1</i>
GO:0003735~structural constituent of ribosome	11	6.53E-09	13.70576025	3.36E-07	<i>RPL9, RPL17, RPS8, RPL18A, MRPS31, MRPS35, MRPS25, MRPL55, RPS11, RPS15A, DAP3</i>

GO:0000146~microfilament motor activity	7	1.15E-08	43.15019255	4.95E-07	<i>MYL6, MYO1C, MYO1D, MYO6, ACTC1, MYH14, MYO1B</i>
GO:0003774~cytoskeletal motor activity	7	3.33E-08	36.43794038	1.22E-06	<i>MYL6, MYO1C, MYL6B, MYO1D, MYO6, MYH14, MYO1B</i>
GO:0045296~cadherin binding	10	8.25E-06	7.297317833	2.65E-04	<i>DDX3X, HSPA8, CAPZA1, RAN, CAPZB, HNRNPK, DBN1, MPRIP, LIM1, MYO1B</i>
GO:0008307~structural constituent of muscle	5	3.20E-05	27.23766307	8.47E-04	<i>TPM3, TPM1, MYL6, MYL6B, TPM4</i>
GO:0005515~protein binding	74	3.30E-05	1.268035756	8.47E-04	<i>TMOD3, SYNCRIP, hCG_401112, HSP90AA1, ALB, IFI27, RPS11, MPRIP, LIM1, STAU1, PPP1R9A, EIF2AK2, ANXA2, S100A7A, EEF1A1, RPL17, ZNF346, SEC61G, CANX, RPS15A, RBMX, ATP1A3, ARPC4-TTLL3, DDX3X, CALM3, hCG_20313, MYL6B, RAN, MYO1D, CAPZB, ACTC1, PHB, DAP3, HSPA8, RAI14, CCT3, RPS8, MRPS35, DBN1, TPM4, SEC61B, MYO1B, STRBP, LLPH, TARBP2, MYL6, RPL9, VIM, ARPC3, HP1BP3, PRKRA, VDAC1, TPM3, TPM1, MYO1C, RPL18A, MYO6, PPP1CC, HNRNPU, C1QBP, hCG_2040042, PCBP2, RRP1, ACTN4, DHX15, FBXO22, HNRNPUL1, HADHA, GRWD1, PSIP1, PINX1, HNRNPK, ARF4, PCBP3, CAPZA1, MRPS31, IRF6</i>
GO:0003729~mRNA binding	8	1.14E-04	7.20750469	0.002658723	<i>DDX3X, HSP90AA1, PCBP3, HNRNPK, C1QBP, hCG_2040042, RBMX, PCBP2, STAU1</i>
GO:0019904~protein domain specific binding	7	3.19E-04	7.521593198	0.00682722	<i>VIM, MYO1D, RAN, MRPS31, PPP1CC, HNRNPK, RBMX</i>

GO:0070883~pre-miRNA binding	3	6.20E-04	78.08130081	0.012262679	<i>TARBP2, PRKRA, RAN</i>
GO:0019899~enzyme binding	8	0.001013374	5.010564758	0.018602659	<i>HSPA8, TARBP2, HNRNPUL1, PRKRA, ZNF346, PHB, PCBP2, ARPC4-TTLL3</i>
GO:0005200~structural constituent of cytoskeleton	5	0.001207131	10.64745011	0.020682179	<i>TPM3, TPM1, VIM, ARPC3, ARPC4-TTLL3</i>
GO:0005516~calmodulin binding	6	0.002028567	6.629544409	0.03258385	<i>MYO1C, EEF1A1, MYO1D, MYO6, MYH14, MYO1B</i>
GO:0042802~identical protein binding	17	0.00275398	2.240937727	0.041633705	<i>TARBP2, HSP90AA1, VIM, ALB, PRKRA, IFI27, HNRNPK, VDACL1, EIF2K2, TPM3, ANXA2, TPM1, S100A7A, MYO6, HNRNPU, TPM4, RBMX</i>

8.2. RESEARCH ARTICLES IN WHICH I HAVE PARTICIPATED DURING THIS PHD THESIS

Those articles in which results presented in this doctoral thesis are published, are highlighted in bold.

Following the list of articles, the first page of each one is attached (Journal Citation Report (JCR) impact factor).

1. DeDiego ML, López-Fernández-Sobrino R, Pedragosa J, López-García D, Nogales A, Durbán J, Cardona F, Llucià-Carol L, Rivero V, Vazquez-Utrilla P, Palomo Sanchez-Grande L, Cobo M, Lloret L, Márquez-Kisinousky L, Ruiz-Jaén F, Lozano F, Sibila O, Faner R, Castro P, Domingo C, Robles V, Bedini JL, Rico V, Agüero D, Soriano A, Martín Nalda A, Parra Martínez A, Colobran R, Soler-Palacín P, Serra-Llovich A, Dietl B, Arranz ML, Dalmau D, Signes-Costa J, Gil-Carbonell J, Todolí J, Martínez J, Rojo S, Fiz-López A, Arribas E, Cal-Sabater P, Bernardo D, Vogel M, Wiemann S, Abolhassani H, Pan-Hammarström Q, Pujol A, COVID Human Genetic Effort, Su HC, Lee D, Zhang SY, Casanova JL, Fernández-Cadenas I, Pérez-Tu J, and Planas AM. OAS1 and OAS3 genetic variants enhance inflammatory responses to SARS-CoV. *iScience* 2025. doi: <https://doi.org/10.1016/j.isci.2025.113966>. Impact factor: 4.1. Q1 journal.
2. **López-García D, Rivero V, Villamayor L, DeDiego ML. IFN alpha inducible protein 27 (IFI27) acts as a positive regulator of PACT-dependent PKR activation after RNA virus infections. *PLoS Pathog* 2025;21(6):e1013246. doi: 10.1371/journal.ppat.1013246. Impact factor: 4.9. Q1 journal.**
3. Ibargüen-Gonzalez L, Heller S, López-García D, Dietenberger H, Barth T, Gallego P, Fernandez-Cadenas I, Alzate-Piñol S, Crespi C, Mena-Guerrero J, Cisneros-Barroso E, Ugalde A, Bretones G, Steenblock C, Kleger A, DeDiego ML*, Barcelo C*. Host factor PLAC8 is required for pancreas infection by SARS-CoV-2. *Commun Med (Lond)*. 2025;5(1):34. doi: 10.1038/s43856-025-00745-6. *Co-corresponding authors. Impact factor 6.3. Q1 journal.
4. DeDiego ML*, Portilla Y, Daviu N, López-García D, Villamayor L, Vázquez-Utrilla P, Mulens-Arias V, Pérez-Yagüe S, Nogales A, Ovejero JG, Gallo-Cordova A, Enjuanes L, Veintemillas-Verdaguer S, Morales MP, Barber DF*. Biocompatible Iron Oxide Nanoparticles Display Antiviral Activity Against Two Different Respiratory Viruses in Mice. *Int J Nanomedicine* 2024;19:13763-13788. doi: 10.2147/IJN.S475323. *co-corresponding authors. Impact factor 6.5. Q1 journal.
5. Rivero V, Carrión-Cruz J, López-García D, DeDiego ML*. The IFN-induced protein IFI27 binds MDA5 and counteracts its activation after SARS-CoV-2 infection. *Front Cell Infect Microbiol* 2024;14:1470924. doi:10.3389/fcimb.2024.1470924. *corresponding author. Impact factor 4.8. Q1 journal.
6. DeDiego ML, Portilla Y, López-García D, Daviu N, Vázquez-Utrilla P, Gallo-Cordova A, Morales MP, Barber DF*. Chapter 13: Antiviral Activity of Iron Oxide and Iron Oxyhydroxide Nanoparticles with a Focus on Respiratory Influenza and Coronavirus Infections. López-Ortega A, Gómez Roca A, editors. *Magnetic Nanoparticles. Nanoscience & Nanotechnology Series*. The Royal Society of Chemistry 2024. <https://doi.org/10.1039/9781837672967-00330>. *corresponding author. *Magnetic Nanoparticles: Materials Engineering, Properties and Applications Book*.
7. Villamayor L, López-García D*, Rivero V*, Martínez-Sobrido L, Nogales A, DeDiego ML*. The IFN-stimulated gene IFI27 counteracts innate immune responses after viral infections by interfering with RIG-I signaling. *Front Microbiol*

- 2023;14:1176177. doi: 10.3389/fmicb.2023.1176177. *last and corresponding author. ‡These authors have contributed equally to this work. Impact factor 4.5. Q1 journal.
8. Villamayor L, Rivero V[‡], López-García D^{*}, Topham DJ, Martínez-Sobrido L, Nogales A, DeDiego ML*. Interferon alpha inducible protein 6 is a negative regulator of innate immune responses by modulating RIG-I activation. *Front Immunol* 2023 Jan 30;14:1105309. doi: 10.3389/fimmu.2023.1105309. *last and corresponding author. ‡These authors have contributed equally to this work. Impact factor 5.9. Q1 journal.
 9. DeDiego ML*, Portilla Y, Daviu N, López-García D, Villamayor L, Mulens-Arias V, Ovejero JG, Gallo-Cordova Á, Veintemillas-Verdaguer S, Morales MP*, Barber DF*. Iron oxide and iron oxyhydroxide nanoparticles impair SARS-CoV-2 infection of cultured cells. *J Nanobiotechnology* 2022;20(1):352. doi: 10.1186/s12951-022-01542-2. *Co-Corresponding authors. Impact factor 12.6. D1 journal (Author: 4/11)
 10. Chiem K, López-García D, Ortego J, Martínez-Sobrido L, DeDiego ML*, Nogales A*. Identification of Amino Acid Residues Required for Inhibition of Host Gene Expression by Influenza Virus A/Viet Nam/1203/2004 H5N1 PA-X. *J Virol* 2022;96(5):e0040821. doi: <https://doi.org/10.1128/JVI.00408-21>. *Co-Corresponding authors. Impact factor 3.8. Q2 journal (Author: 2/6)
 11. Saiz ML*, DeDiego ML*, López-García D, Corte-Iglesias V, Baragaño-Raneros A, Astola I, Asensi V, López-Larrea C, Suárez-Alvarez B. Epigenetic targeting of the ACE2 and NRP1 viral receptors limits SARS-CoV-2 infectivity. *Clin Epigenetics* 2021;13(1):187. doi: 10.1186/s13148-021-01168-5. *co-first authors. Impact factor 4.4. Q1 journal (Author: 3/9)

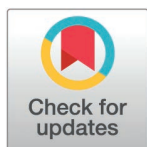
RESEARCH ARTICLE

IFN alpha inducible protein 27 (IFI27) acts as a positive regulator of PACT-dependent PKR activation after RNA virus infections

Darío López-García, Vanessa Rivero, Laura Villamayor, Marta L. DeDiego^{ID}*

Department of Molecular and Cell Biology, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CNB-CSIC), Madrid, Spain

* marta.lopez@cnb.csic.es



OPEN ACCESS

Citation: López-García D, Rivero V, Villamayor L, DeDiego ML (2025) IFN alpha inducible protein 27 (IFI27) acts as a positive regulator of PACT-dependent PKR activation after RNA virus infections. PLoS Pathog 21(6): e1013246. <https://doi.org/10.1371/journal.ppat.1013246>

Editor: Peter Sarnow, Stanford University, UNITED STATES OF AMERICA

Received: November 28, 2024

Accepted: May 28, 2025

Published: June 16, 2025

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.ppat.1013246>

Copyright: © 2025 López-García et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium,

Abstract

Protein kinase R (PKR) expression is induced by interferons. This protein is activated by double-stranded (ds) RNAs or RNAs containing duplex regions, produced after different stimuli, such as after viral infections, leading to the phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), and subsequently inhibiting cellular and viral protein translation. This function may lead to different effects such as to impairing the replication of RNA viruses by inhibiting viral protein translation, and to modulating the innate immune responses after viral infections by affecting the translation of effector proteins. In this work, we identify, for the first time, an interaction of IFN alpha inducible protein 27 (IFI27) with PKR-activating protein (PACT or PRKRA) and with PKR, showing that the interaction of IFI27 with PACT is likely mediated by dsRNAs or RNAs containing duplex regions, and that the interaction of IFI27 with PKR is PACT-dependent. Interestingly, using IFI27 knocked-down, knocked-out and overexpressing tumour-derived, established cells, we show that these interactions trigger a potentiation of the activity of PKR and, therefore, a decrease in protein translation. Moreover, we find that IFI27 increases PKR function in cells infected with different RNA viruses such as Severe Acute Respiratory virus 2 (SARS-CoV-2), and Vesicular Stomatitis virus (VSV), and in cells transfected with the dsRNA analog poly(I:C), suggesting a broad effect of IFI27 on PKR activation. Moreover, we show that IFI27 expression increases the formation of stress granules (SGs) at the cell cytoplasm, correlating with the increased PKR activation mediated by IFI27, as it has been shown that the translational arrest induced by activated PKR leads to the formation of SGs. Mechanistically, we describe that this ability of IFI27 to activate PKR is dependent on its interaction with PACT. Further understanding of the regulation of PKR activity will allow us to develop new antiviral drugs to modulate this signalling axis, which is crucial in RNA virus infections.



OPEN ACCESS

EDITED BY

Sonia Navas-Martin,
Drexel University, United States

REVIEWED BY

Ulfert Rand,
German Collection of Microorganisms and
Cell Cultures GmbH (DSMZ), Germany
Irene Ramos,
Icahn School of Medicine at Mount Sinai,
United States

*CORRESPONDENCE

Marta L. DeDiego

✉ marta.lopez@cnb.csic.es

RECEIVED 26 July 2024

ACCEPTED 06 September 2024

PUBLISHED 04 October 2024

CITATION

Rivero V, Carrión-Cruz J, López-García D and DeDiego ML (2024) The IFN-induced protein IFI27 binds MDA5 and counteracts its activation after SARS-CoV-2 infection. *Front. Cell. Infect. Microbiol.* 14:1470924. doi: 10.3389/fcimb.2024.1470924

COPYRIGHT

© 2024 Rivero, Carrión-Cruz, López-García and DeDiego. This is an open-access article distributed under the terms of the [Creative Commons Attribution License \(CC BY\)](#). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

The IFN-induced protein IFI27 binds MDA5 and counteracts its activation after SARS-CoV-2 infection

Vanessa Rivero, Julia Carrión-Cruz, Darío López-García and Marta L. DeDiego*

Department of Molecular and Cell Biology, Centro Nacional de Biotecnología (CNB-CSIC), Campus Universidad Autónoma de Madrid, Madrid, Spain

Innate immune responses are induced after viral infections, being these responses essential to establish an antiviral response in the host. The RIG-I-like receptors (RLRs), RIG-I and MDA5 are pivotal for virus detection by recognizing viral RNAs in the cytoplasm of infected cells, initiating these responses. However, since excessive responses can have a negative effect on the host, regulatory feedback mechanisms are needed. In this work, we describe that IFN alpha-inducible protein 27 (IFI27) co-immunoprecipitates with melanoma differentiation-associated protein 5 (MDA5), being this interaction likely mediated by RNAs. In addition, by using IFI27 overexpression, knock-out, and knock-down cells, we show that IFI27 inhibits MDA5 oligomerization and activation, counteracting the innate immune responses induced after SARS-CoV-2 infections or after polyinosinic-polycytidylic acid (poly(I:C)) transfection. Furthermore, our data indicate that IFI27 competes with MDA5 for poly(I:C) binding, providing a likely explanation for the effect of IFI27 in inhibiting MDA5 activation. This new function of IFI27 could be used to design target-driven compounds to treat diseases associated with an exacerbated induction of innate immune responses, such as those induced by SARS-CoV-2.

KEYWORDS

IFI27, MDA5, innate immune responses, interferon, inflammation, SARS-CoV-2, innate immunity, RIG-I-like receptors

Introduction

The host innate immune system detects viral infections, relying on pattern recognition receptors (PRRs), which sense pathogen-associated molecular patterns (PAMPs), such as viral RNAs. The retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), RIG-I and melanoma differentiation-associated protein 5 (MDA5) are pivotal for virus detection by recognizing viral RNAs in the cytoplasm of infected cells. Severe Acute Respiratory



OPEN ACCESS

EDITED BY

Jose Angel Regla Nava,
University of Guadalajara, Mexico

REVIEWED BY

Jia Xue,
China Agricultural University, China
Shuvojit Banerjee,
IDT Biologika GmbH, Germany

*CORRESPONDENCE

Marta L. DeDiego
✉ marta.lopez@cnb.csic.es

*PRESENT ADDRESS

Laura Villamayor,
Instituto de Investigaciones Biomédicas
"Alberto Sols" (IIBm-CSIC-UAM),
Madrid, Spain

[†]These authors have contributed equally to this work

RECEIVED 28 February 2023

ACCEPTED 13 April 2023

PUBLISHED 28 April 2023

CITATION

Villamayor L, López-García D, Rivero V,
Martínez-Sobrido L, Nogales A and
DeDiego ML (2023) The IFN-stimulated gene
IFI27 counteracts innate immune responses
after viral infections by interfering with RIG-I
signaling.
Front. Microbiol. 14:1176177.
doi: 10.3389/fmicb.2023.1176177

COPYRIGHT

© 2023 Villamayor, López-García, Rivero,
Martínez-Sobrido, Nogales and DeDiego. This
is an open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](#). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic practice.
No use, distribution or reproduction is
permitted which does not comply with these
terms.

The IFN-stimulated gene IFI27 counteracts innate immune responses after viral infections by interfering with RIG-I signaling

Laura Villamayor^{1†}, Darío López-García^{1†}, Vanessa Rivero^{1†},
Luis Martínez-Sobrido², Aitor Nogales³ and Marta L. DeDiego^{1*}

¹Department of Molecular and Cell Biology, Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain, ²Texas Biomedical Research Institute, San Antonio, TX, United States, ³Center for Animal Health Research, CISA-INIA-CSIC, Madrid, Spain

The recognition of viral nucleic acids by host pattern recognition receptors (PRRs) is critical for initiating innate immune responses against viral infections. These innate immune responses are mediated by the induction of interferons (IFNs), IFN-stimulated genes (ISGs) and pro-inflammatory cytokines. However, regulatory mechanisms are critical to avoid excessive or long-lasting innate immune responses that may cause detrimental hyperinflammation. Here, we identified a novel regulatory function of the ISG, IFN alpha inducible protein 27 (IFI27) in counteracting the innate immune responses triggered by cytoplasmic RNA recognition and binding. Our model systems included three unrelated viral infections caused by Influenza A virus (IAV), Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2), and Sendai virus (SeV), and transfection with an analog of double-stranded (ds) RNA. Furthermore, we found that IFI27 has a positive effect on IAV and SARS-CoV-2 replication, most likely due to its ability to counteract host-induced antiviral responses, including *in vivo*. We also show that IFI27 interacts with nucleic acids and PRR retinoic acid-inducible gene I (RIG-I), being the interaction of IFI27 with RIG-I most likely mediated through RNA binding. Interestingly, our results indicate that interaction of IFI27 with RIG-I impairs RIG-I activation, providing a molecular mechanism for the effect of IFI27 on modulating innate immune responses. Our study identifies a molecular mechanism that may explain the effect of IFI27 in counterbalancing innate immune responses to RNA viral infections and preventing excessive innate immune responses. Therefore, this study will have important implications in drug design to control viral infections and viral-induced pathology.

KEYWORDS

IFI27, innate immune responses, interferon, inflammation, influenza, SARS-CoV-2, virus-host interactions

Introduction

Some pathogen molecules, such as lipopolysaccharides, glycoproteins, proteoglycans and nuclear acid motifs, such as double-stranded RNA (ds) RNA and 5'-phosphate single stranded RNA (ssRNA) motifs, are known as pathogen-associated molecular patterns (PAMPs), and are essential components to initiate an innate immune response upon recognition by pattern recognition receptors (PRRs) (Wilkins and Gale, 2010; Jensen and Thomsen, 2012). There are



OPEN ACCESS

EDITED BY

Pei-Hui Wang,
Shandong University, China

REVIEWED BY

Lizhong Liu,
University of Texas Southwestern Medical
Center, United States
Jinxu Hou,
Immunology, China

*CORRESPONDENCE

Marta L. DeDiego
✉ marta.lopez@cnb.csic.es

†PRESENT ADDRESS

Laura Villamayor,
Instituto de Investigaciones Biomédicas
“Alberto Sols” (IIBm-CSIC-UAM), Arturo
Duperier 4, Madrid, Spain

†These authors have contributed equally to
this work

SPECIALTY SECTION

This article was submitted to
Viral Immunology,
a section of the journal
Frontiers in Immunology

RECEIVED 22 November 2022

ACCEPTED 05 January 2023

PUBLISHED 30 January 2023

CITATION

Villamayor L, Rivero V, López-García D,
Topham DJ, Martínez-Sobrido L, Nogales A
and DeDiego ML (2023) Interferon alpha
inducible protein 6 is a negative regulator
of innate immune responses by modulating
RIG-I activation.
Front. Immunol. 14:1105309.
doi: 10.3389/fimmu.2023.1105309

COPYRIGHT

© 2023 Villamayor, Rivero, López-García,
Topham, Martínez-Sobrido, Nogales and
DeDiego. This is an open-access article
distributed under the terms of the [Creative
Commons Attribution License \(CC BY\)](#). The
use, distribution or reproduction in other
forums is permitted, provided the original
author(s) and the copyright owner(s) are
credited and that the original publication in
this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Interferon alpha inducible protein 6 is a negative regulator of innate immune responses by modulating RIG-I activation

Laura Villamayor^{1†}, Vanessa Rivero^{1†}, Darío López-García^{1†},
David J. Topham², Luis Martínez-Sobrido³, Aitor Nogales⁴
and Marta L. DeDiego^{1*}

¹Department of Molecular and Cell Biology, Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain, ²David H. Smith Center for Vaccine Biology and Immunology, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, United States, ³Disease Intervention and Prevention and Population Health Programs, Texas Biomedical Research Institute, San Antonio, TX, United States, ⁴Center for Animal Health Research, CISA-INIA-CSIC, Valdeolmos, Madrid, Spain

Interferons (IFNs), IFN-stimulated genes (ISGs), and inflammatory cytokines mediate innate immune responses, and are essential to establish an antiviral response. Within the innate immune responses, retinoic acid-inducible gene I (RIG-I) is a key sensor of virus infections, mediating the transcriptional induction of IFNs and inflammatory proteins. Nevertheless, since excessive responses could be detrimental to the host, these responses need to be tightly regulated. In this work, we describe, for the first time, how knocking-down or knocking-out the expression of IFN alpha-inducible protein 6 (IFI6) increases IFN, ISG, and pro-inflammatory cytokine expression after the infections with Influenza A Virus (IAV), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), and Sendai Virus (SeV), or poly(I:C) transfection. We also show how overexpression of IFI6 produces the opposite effect, *in vitro* and *in vivo*, indicating that IFI6 negatively modulates the induction of innate immune responses. Knocking-out or knocking-down the expression of IFI6 diminishes the production of infectious IAV and SARS-CoV-2, most likely because of its effect on antiviral responses. Importantly, we report a novel interaction of IFI6 with RIG-I, most likely mediated through binding to RNA, that affects RIG-I activation, providing a molecular mechanism for the effect of IFI6 on negatively regulating innate immunity. Remarkably, these new functions of IFI6 could be targeted to treat diseases associated with an exacerbated induction of innate immune responses and to combat viral infections, such as IAV and SARS-CoV-2.

KEYWORDS

IFI6, interferon, innate immunity, RIG-I, RNA binding, inflammation, influenza virus, coronavirus



OAS1 and OAS3 genetic variants enhance inflammatory responses to SARS-CoV-2

Marta L. DeDiego, Raúl López-Fernández-Sobrino, Jordi Pedragosa, Darío López-García, Aitor Nogales, Jordi Durbán, Fernando Cardona, Laia Llucià-Carol, Vanessa Rivero, Paula Vazquez-Utrilla, Laura Palomo Sanchez-Grande, Miriam Cobo, Lara Lloret, Leonardo Márquez-Kisinousky, Francisca Ruiz-Jaén, Francisco Lozano, Oriol Sibila, Rosa Faner, Pedro Castro, Carlos Domingo, Verónica Robles, Josep L. Bedini, Verónica Rico, Daiana Aguero, Alex Soriano, Andrea Martín Nalda, Alba Parra Martínez, Roger Colobran, Pere Soler-Palacín, Alexandre Serra-Llovich, Beatriz Dietl, Ma Jesús Arranz, David Dalmau, Jaime Signes-Costa, Joan Gil-Carbonell, José Todolí, Jacobo Martínez, Silvia Rojo, Aida Fiz-López, Elisa Arribas, Paloma Cal-Sabater, David Bernado, Marina Vogel, Stefan Wiemann, Hassan Abolhassani, Qiang Pan-Hammarström, Aurora Pujol, COVID Human Genetic Effort, Helen C. Su, Danyel Lee, Shen-Ying Zhang, Jean-Laurent Casanova, Israel Fernández-Cadenas, Jordi Pérez-Tur, Anna M. Planas

PII: S2589-0042(25)02227-8

DOI: <https://doi.org/10.1016/j.isci.2025.113966>

Reference: ISCI 113966

To appear in: *iScience*

Received Date: 22 July 2024

Revised Date: 15 August 2025

Accepted Date: 4 November 2025

Please cite this article as: DeDiego, M.L., López-Fernández-Sobrino, R., Pedragosa, J., López-García, D., Nogales, A., Durbán, J., Cardona, F., Llucià-Carol, L., Rivero, V., Vazquez-Utrilla, P., Sanchez-Grande, L.P., Cobo, M., Lloret, L., Márquez-Kisinousky, L., Ruiz-Jaén, F., Lozano, F., Sibila, O., Faner, R., Castro, P., Domingo, C., Robles, V., Bedini, J.L., Rico, V., Aguero, D., Soriano, A., Nalda, A.M., Martínez, A.P., Colobran, R., Soler-Palacín, P., Serra-Llovich, A., Dietl, B., Arranz, M.J., Dalmau, D., Signes-Costa, J., Gil-Carbonell, J., Todolí, J., Martínez, J., Rojo, S., Fiz-López, A., Arribas, E., Cal-Sabater, P., Bernado, D., Vogel, M., Wiemann, S., Abolhassani, H., Pan-Hammarström, Q., Pujol, A., COVID Human Genetic Effort, Su, H.C., Lee, D., Zhang, S.-Y., Casanova, J.-L., Fernández-Cadenas, I., Pérez-Tur, J., Planas, A.M., OAS1 and OAS3 genetic variants enhance inflammatory responses to SARS-CoV-2, *iScience* (2025), doi: <https://doi.org/10.1016/j.isci.2025.113966>.

<https://doi.org/10.1038/s43856-025-00745-6>

Host factor PLAC8 is required for pancreas infection by SARS-CoV-2

Check for updates

Lesly Ibargüen-González^{1,14}, Sandra Heller^{2,14}, Darío López-García³, Hanna Dietenberger⁴, Thomas FE Barth⁴, Patricia Gallego⁵, Israel Fernández-Cadenas⁶, Sayoa Alzate-Piñol⁶, Catalina Crespi^{5,7}, Julieth A. Mena-Guerrero¹, Eugenia Cisneros-Barroso^{7,8}, Alejandro P. Ugalde⁹, Gabriel Bretones⁹, Charlotte Steenblock¹⁰, Alexander Kleger^{2,11}, Marta L. DeDiego³ ✉ & Carles Barceló^{1,12,13} ✉

Abstract

Background Although COVID-19 initially caused great concern about respiratory symptoms, mounting evidence shows that also the pancreas is productively infected by SARS-CoV-2. However, the severity of pancreatic SARS-CoV-2 infection and its pathophysiology is still under debate. Here, we investigate the consequences of SARS-CoV-2 pancreatic infection and the role of the host factor Placenta-associated protein (PLAC8).

Methods We analyze plasma levels of pancreatic enzymes and inflammatory markers in a retrospective cohort study of 120 COVID-19 patients distributed in 3 severity-stratified groups. We study the expression of SARS-CoV-2 and PLAC8 in the pancreas of deceased COVID-19 patients as well as in non-infected donors. We perform pseudovirus infection experiments in PLAC8 knock-out PDAC and human beta cell-derived cell lines and validate results with SARS-CoV-2 virus.

Results We find that analysis of circulating pancreatic enzymes aid the stratification of patients according to COVID-19 severity and predicts outcomes. Interestingly, we find an association between PLAC8 expression and SARS-CoV-2 infection in postmortem analysis of COVID-19 patients both in the pancreas and in other bonafide SARS-CoV-2 target tissues. Functional experiments demonstrate the requirement of PLAC8 in SARS-CoV-2 pancreatic productive infection by pseudovirus and full SARS-CoV-2 infectious virus inoculum from Wuhan-1 and BA.1 strains. Finally, we observe an overlap between PLAC8 and SARS-CoV-2 immunoreactivities in the pancreas of deceased patients.

Conclusions Our data indicate the human pancreas as a SARS-CoV-2 target with plausible signs of injury and demonstrate that the host factor PLAC8 is required for SARS-CoV-2 pancreatic infection, thus defining new target opportunities for COVID-19-associated pancreatic pathogenesis.

Plain language summary

SARS-CoV-2 virus can be found in the pancreas following infection. We investigated whether the pancreas is damaged by the infection and whether there are changes of the pancreatic enzymes that the pancreas produces and circulates around the rest of the body. A particular protein called the placenta-associated protein 8 (PLAC8) expression was found in people who died following SARS-CoV-2 infection. We show this protein is required for SARS-CoV-2 pancreatic infection and viral replication. PLAC8 could be further investigated as a target for drugs to treat COVID-19 that could reduce the impact on the pancreas.

COVID-19, the respiratory disease caused by SARS-CoV-2, has been associated with a wide range of symptoms and complications, affecting not only the lung but also other organs in the body including the gastrointestinal system^{1,2}. Among the less studied, but potentially occurring with severe complications, is the infection of the pancreas, a gland responsible for producing hormones like insulin and digestive enzymes. While the exact mechanism of how SARS-CoV-2 affects the pancreas is not yet fully understood, several studies reported pancreatic inflammation, injury, and

dysfunction in some COVID-19 patients^{3–8}. Taken together, this raises concerns about the potential long-term impact on pancreatic function and the overall metabolic health of those affected.

Placenta-associated 8 (PLAC8) is a highly conserved protein that is expressed in various tissues like the placenta as well as the respiratory system or the gastrointestinal tract⁹. PLAC8 has been implicated in the regulation of multiple cellular processes like autophagy or cell motility¹⁰. Previous studies have suggested that PLAC8 also plays a prominent role in pancreatic

A full list of affiliations appears at the end of the paper. ✉ e-mail: marta.lopez@cnb.csic.es; carles.barcelo@gmail.com; CBarcelo@umanresa.cat

Biocompatible Iron Oxide Nanoparticles Display Antiviral Activity Against Two Different Respiratory Viruses in Mice

Marta L DeDiego^{1,*}, Yadileiny Portilla^{2,*}, Neus Daviu², Darío López-García¹, Laura Villamayor¹, Paula Vázquez-Utrilla¹, Vladimir Mulens-Arias², Sonia Pérez-Yagüe², Aitor Nogales³, Jesús G Ovejero⁴, Alvaro Gallo-Cordova⁴, Luis Enjuanes¹, Sabino Veintemillas-Verdaguer⁴, M Puerto Morales⁴, Domingo F Barber²

¹Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain; ²Department of Immunology, Oncology and Nanobiomedicine Initiative, Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain; ³Center for Animal Health Research, CISA-INIA-CSIC, Madrid, Spain; ⁴Department of Nanoscience and Nanotechnology, Instituto de Ciencia de Materiales de Madrid (ICMM-CSIC), Madrid, Spain

*These authors contributed equally to this work

Correspondence: Domingo F Barber; Marta L DeDiego, Email dfbarber@cnb.csic.es; marta.lopez@cnb.csic.es

Background: Severe Acute Respiratory syndrome coronavirus 2 (SARS-CoV-2) and Influenza A viruses (IAVs) are among the most important causes of viral respiratory tract infections, causing similar symptoms. IAV and SARS-CoV-2 infections can provoke mild symptoms like fever, cough, sore throat, loss of taste or smell, or they may cause more severe consequences leading to pneumonia, acute respiratory distress syndrome or even death. While treatments for IAV and SARS-CoV-2 infection are available, IAV antivirals often target viral proteins facilitating the emergence of drug-resistant viral variants. Hence, universal treatments against coronaviruses and IAVs are hard to obtain due to genus differences (in the case of coronavirus) or subtypes (in the case of IAV), highlighting the need for novel antiviral therapies. Interestingly, iron oxide nanoparticles (IONPs) with a 10 nm core size and coated with the biocompatible dimercaptosuccinic acid (DMSA: DMSA-IONP-10) display antiviral activity against SARS-CoV-2 in vitro.

Methods: We analyzed the antiviral activity of DMSA-IONP-10 against SARS-CoV-2 infection in vivo, and against IAV infection in vitro and in vivo.

Results: DMSA-IONP-10 treatment of mice after SARS-CoV-2 infection impaired virus replication in the lungs and led to a mildly reduced pro-inflammatory cytokine induction after infection, indicating that these IONPs can serve as COVID-19 therapeutic agents. These IONPs also had a prophylactic and therapeutic effect against IAV in tissue cultured cells at non-cytotoxic doses, and a therapeutic effect in IAV-infected-mice, inhibiting viral replication and slightly dampening the inflammatory response after viral infection. As an exacerbated inflammatory response to IAVs and SARS-CoV-2 is detrimental to the host, weakening this response in mice through IONP treatment may reduce disease severity. Interestingly, our data suggest that IONP treatment affects oxidative stress and iron metabolism in cells, which may influence IAV production.

Conclusion: This study highlights the antiviral activity of DMSA-IONP-10 against important human respiratory viruses.

Keywords: iron oxide nanoparticles, SARS-CoV-2, IAV, viral infection, oxidative stress, iron metabolism

Introduction

Severe Acute Respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to the *Coronaviridae* family, and it is an enveloped virus with a positive-sense, single-stranded RNA (ssRNA) genome of around 30 kb that can infect humans and other mammals. SARS-CoV-2 infection has spread globally, ultimately provoking the coronavirus pandemic that caused over 776 million confirmed cases up to October 2024 and over 7 million deaths globally, although these numbers are most likely underestimated.¹ Influenza viruses (IVs) belong to the *Orthomyxoviridae* family and they are also

CHAPTER 13

Antiviral Activity of Iron Oxide and Iron Oxyhydroxide Nanoparticles with a Focus on Respiratory Influenza and Coronavirus Infections

MARTA L. DEDIEGO^{*a}, YADILEINY PORTILLA^{b,†},
DARÍO LÓPEZ-GARCÍA^{a,†}, NEUS DAVIU^{b,†},
PAULA VÁZQUEZ-UTRILLA^{a,†}, ÁLVARO GALLO-CORDOVA^{c,†},
M. PUERTO MORALES^{*c} AND DOMINGO F. BARBER^{*b}

^aDepartment of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, 28049 Madrid, Spain; ^bDepartment of Immunology and Oncology & Nanobiomedicine Initiative, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, 28049 Madrid, Spain; ^cDepartment of Energy, Environment and Health, Instituto de Ciencia de Materiales de Madrid (ICMM-CSIC), Sor Juana Inés de la Cruz 3, 28049 Madrid, Spain

^{*}E-mail: marta.lopez@cnb.csic.es, puerto@icmm.csic.es, dfbarber@cnb.csic.es

[†]These authors contributed equally to this work.

Nanoscience & Nanotechnology Series No. 63
Magnetic Nanoparticles: Materials Engineering, Properties and Applications
Edited by Alberto López-Ortega and Alejandro Gómez Roca
© The Royal Society of Chemistry 2024
Published by the Royal Society of Chemistry, www.rsc.org

RESEARCH

Open Access



Iron oxide and iron oxyhydroxide nanoparticles impair SARS-CoV-2 infection of cultured cells

Marta L. DeDiego^{1*†}, Yadileiny Portilla^{2†}, Neus Daviu², Darío López-García¹, Laura Villamayor^{1,4}, Vladimir Mulens-Arias^{2,5}, Jesús G. Ovejero^{3,6}, Álvaro Gallo-Cordova³, Sabino Veintemillas-Verdaguer³, M. Puerto Morales^{3*} and Domingo F. Barber^{2*}

Abstract

Background: Coronaviruses usually cause mild respiratory disease in humans but as seen recently, some human coronaviruses can cause more severe diseases, such as the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the global spread of which has resulted in the ongoing coronavirus pandemic.

Results: In this study we analyzed the potential of using iron oxide nanoparticles (IONPs) coated with biocompatible molecules like dimercaptosuccinic acid (DMSA), 3-aminopropyl triethoxysilane (APS) or carboxydextran (FeraSpinTM R), as well as iron oxyhydroxide nanoparticles (IOHNPs) coated with sucrose (Venofer[®]), or iron salts (ferric ammonium citrate -FAC), to treat and/or prevent SARS-CoV-2 infection. At non-cytotoxic doses, IONPs and IOHNPs impaired virus replication and transcription, and the production of infectious viruses in vitro, either when the cells were treated prior to or after infection, although with different efficiencies. Moreover, our data suggest that SARS-CoV-2 infection affects the expression of genes involved in cellular iron metabolism. Furthermore, the treatment of cells with IONPs and IOHNPs affects oxidative stress and iron metabolism to different extents, likely influencing virus replication and production. Interestingly, some of the nanoparticles used in this work have already been approved for their use in humans as anti-anemic treatments, such as the IOHNP Venofer[®], and as contrast agents for magnetic resonance imaging in small animals like mice, such as the FeraSpinTM R IONP.

Conclusions: Therefore, our results suggest that IONPs and IOHNPs may be repurposed to be used as prophylactic or therapeutic treatments in order to combat SARS-CoV-2 infection.

[†]Marta L. DeDiego and Yadileiny Portilla contributed equally to this work

*Correspondence: marta.lopez@cnb.csic.es; puerto@icmm.csic.es; dfbarber@cnb.csic.es

¹ Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, 28049 Madrid, Spain

² Department of Immunology, Oncology and Nanobiomedicine Initiative, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, 28049 Madrid, Spain

³ Department of Energy, Environment and Health, Instituto de Ciencia de Materiales de Madrid (ICMM-CSIC), Sor Juana Inés de la Cruz 3, 28049 Madrid, Spain

Full list of author information is available at the end of the article



© The Author(s) 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.



Identification of Amino Acid Residues Required for Inhibition of Host Gene Expression by Influenza Virus A/Viet Nam/1203/2004 H5N1 PA-X

Kevin Chiem,^a Darío López-García,^b Javier Ortego,^c Luis Martínez-Sobrido,^a Marta L. DeDiego,^b Aitor Nogales^c

^aDepartment of Disease Intervention and Prevention, Texas Biomedical Research Institute, San Antonio, Texas, USA

^bDepartment of Molecular and Cell Biology, Centro Nacional de Biotecnología (CNB-CSIC), Campus Universidad Autónoma de Madrid, Madrid, Spain

^cCenter for Animal Health Research, INIA-CISA, Valdeolmos, Madrid, Spain

ABSTRACT PA-X is a nonstructural protein of influenza A virus (IAV), which is encoded by the polymerase acidic (PA) N-terminal region that contains a C-terminal +1 frameshifted sequence. IAV PA-X protein modulates virus-induced host innate immune responses and viral pathogenicity via suppression of host gene expression or cellular shutoff, through cellular mRNA cleavage. Highly pathogenic avian influenza viruses (HPAIV) of the H5N1 subtype naturally infect different avian species, they have an enormous economic impact in the poultry farming, and they also have zoonotic and pandemic potential, representing a risk to human public health. In the present study, we describe a novel bacterium-based approach to identify amino acid residues in the PA-X protein of the HPAIV A/Viet Nam/1203/2004 H5N1 that are important for its ability to inhibit host protein expression or cellular shutoff activity. Identified PA-X mutants displayed a reduced shutoff activity compared to that of the wild-type A/Viet Nam/1203/2004 H5N1 PA-X protein. Notably, this new bacterium-based screening allowed us to identify amino acid residues widely distributed over the entire N-terminal region of PA-X. Furthermore, we found that some of the residues affecting A/Viet Nam/1203/2004 H5N1 PA-X host shutoff activity also affect PA polymerase activity in a mini-genome assay. This information could be used for the rational design of new and more effective compounds with antiviral activity against IAV. Moreover, our results demonstrate the feasibility of using this bacterium-based approach to identify amino acid residues important for the activity of viral proteins to inhibit host gene expression.

IMPORTANCE Highly pathogenic avian influenza viruses continue to pose a huge threat to global animal and human health. Despite of the limited genome size of Influenza A virus (IAV), the virus encodes eight main viral structural proteins and multiple accessory nonstructural proteins, depending on the IAV type, subtype, or strain. One of the IAV accessory proteins, PA-X, is encoded by the polymerase acidic (PA) protein and is involved in pathogenicity through the modulation of IAV-induced host inflammatory and innate immune responses. However, the molecular mechanism(s) of IAV PA-X regulation of the host immune response is not well understood. Here, we used, for the first time, a bacterium-based approach for the identification of amino acids important for the ability of IAV PA-X to induce host shutoff activity and describe novel residues relevant for its ability to inhibit host gene expression, and their contribution in PA polymerase activity.

KEYWORDS baloxavir, H5N1, influenza A virus, minigenome, PA-X protein, shutoff, avian viruses

Influenza are enveloped viruses belonging to the *Orthomyxoviridae* family and are the causative agents of flu (1–3). There are four recognized influenza types: A, B, C, and D (1–6). Influenza A viruses (IAV) are divided into subtypes based on the antigenic

Editor Colin R. Parrish, Cornell University

Copyright © 2022 American Society for Microbiology. All Rights Reserved.

Address correspondence to Marta L. DeDiego, marta.lopez@cnb.csic.es, or Aitor Nogales, nogales.aitor@inia.es.

Received 8 March 2021

Accepted 31 March 2021

Accepted manuscript posted online 14 April 2021

Published 9 March 2022

RESEARCH

Open Access



Epigenetic targeting of the ACE2 and NRP1 viral receptors limits SARS-CoV-2 infectivity

Maria Laura Saiz^{1†}, Marta L. De Diego^{2†}, Darío López-García², Viviana Corte-Iglesias¹, Aroa Baragaño Raneros¹, Ivan Astola^{3,4}, Victor Asensi⁵, Carlos López-Larrea^{1,6*} and Beatriz Suarez-Alvarez¹ 

Abstract

Background: SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) and neuropilin-1 (NRP1) receptors for entry into cells, and the serine protease TMPRSS2 for S protein priming. Inhibition of protease activity or the engagement with ACE2 and NRP1 receptors has been shown to be an effective strategy for blocking infectivity and viral spreading. Valproic acid (VPA; 2-propylpentanoic acid) is an epigenetic drug approved for clinical use. It produces potent antiviral and anti-inflammatory effects through its function as a histone deacetylase (HDAC) inhibitor. Here, we propose VPA as a potential candidate to tackle COVID-19, in which rapid viral spread and replication, and hyperinflammation are crucial elements.

Results: We used diverse cell lines (HK-2, Huh-7, HUVEC, Caco-2, and BEAS-2B) to analyze the effect of VPA and other HDAC inhibitors on the expression of the ACE-2 and NRP-1 receptors and their ability to inhibit infectivity, viral production, and the inflammatory response. Treatment with VPA significantly reduced expression of the ACE2 and NRP1 host proteins in all cell lines through a mechanism mediated by its HDAC inhibitory activity. The effect is maintained after SARS-CoV-2 infection. Consequently, the treatment of cells with VPA before infection impairs production of SARS-CoV-2 infectious viruses, but not that of other ACE2- and NRP1-independent viruses (VSV and HCoV-229E). Moreover, the addition of VPA 1 h post-infection with SARS-CoV-2 reduces the production of infectious viruses in a dose-dependent manner without significantly modifying the genomic and subgenomic messenger RNAs (gRNA and sg mRNAs) or protein levels of N protein. The production of inflammatory cytokines (TNF- α and IL-6) induced by TNF- α and SARS-CoV-2 infection is diminished in the presence of VPA.

Conclusions: Our data showed that VPA blocks three essential processes determining the severity of COVID-19. It downregulates the expression of ACE2 and NRP1, reducing the infectivity of SARS-CoV-2; it decreases viral yields, probably because it affects virus budding or virions stability; and it dampens the triggered inflammatory response. Thus, administering VPA could be considered a safe treatment for COVID-19 patients until vaccines have been rolled out across the world.

Keywords: SARS-CoV-2, ACE2, HDAC, Epigenetic, NRP1, Valproic acid

Background

SARS-CoV-2 is the causal agent of the coronavirus-induced disease 2019 (COVID-19) pandemic, which had cost more than 3.4 million lives worldwide by the end of May 2021 [1]. The scenario has drastically changed since the start of the pandemic. Although several vaccines have been approved for use in humans [2–6], there are many concerns regarding their worldwide accessibility,

*Correspondence: inmuno@hca.es

[†]Maria Laura Saiz and Marta L. De Diego have contributed equally to this work

¹ Translational Immunology Laboratory, Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), Oviedo, Spain

Full list of author information is available at the end of the article



© The Author(s) 2021. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.