Université de Montréal

Spliceosome SNRNP200 Promotes Viral RNA Sensing and IRF3 Activation of Antiviral Response

par Nicolas Tremblay

Département de Médecine
Faculté de Médecine

Thèse présentée
en vue de l'obtention du grade de Philosophiae doctor
en Biologie moléculaire option Biologie des systèmes

Novembre, 2017

Résumé

Le système immunitaire innée est la première ligne de défense de l'organisme contre une multitude d'agents pathogènes tel que les bactéries, les virus, les parasites et les champignons. Afin d'identifier de nouveau régulateur de l'immunité antivirale innée, nous avons complété le premier criblage pangénomique par ARN interférent (RNAi) s'intéressant à la réponse transcriptionnelle de l'interféron-β (IFNB1) suite à une infection par le virus Sendai (SeV). De façon surprenante, une analyse d'enrichissement génomique (GESA) nous a permis d'identifier 114 gènes régulateurs dont plusieurs facteurs du splicéosome. Par eux, nous avons priorisé la caractérisation de SRNNP200, une protéine clé de la machinerie d'épissage des introns et une hélicase de la famille Ski2, sur la base de similitudes entre sa structure et celle d'autres hélicases antivirales tel que RIG-I et MDA5. Dans cette thèse, nous montrons, pour la première fois, un rôle distinct, pour SNRNP200, de sa fonction canonique dans l'épissage des pré-ARNs. En effet, le silençage de l'expression de SNRNP200 dans des lignées de cellules humaines primaires entraîne une réduction de l'immunité antivirale et une augmentation de la susceptibilité à une infection virale. Plus spécifiquement, nous montrons que SNRNP200 est un régulateur positif de l'activation de IRF3 via une interaction protéine-protéine avec la sérine/thréonine-kinase TBK1. Additionnement, nous avons montré que, lors d'une infection, SNRNP200 est capable de lier l'ARN viral cytoplasmique et qu'il relocalise, du noyau au cytoplasme, avec TBK1 dans des structures périnucléaires distinctes et spécifiques. En lien avec la clinique, nous avons observé une réponse antivirale réduite dans les cellules mononucléées du sang périphérique (PBMC) de patients atteints de rétinite pigmentaire de type 33 (RP33) causée par des mutations dans le gène SNRNP200. De plus, nous avons démontré qu'un mutant de SNRNP200 associé à RP33 n'était plus en mesure de lier l'ARN viral cytoplasmique ou de rétablir l'immunité antivirale de cellules ciblée par un RNAi lors d'une expérience de sauvetage. Ainsi, cette thèse présente les premiers travaux portant sur la fonction immunomodulatrice de SNRNP200 et de son rôle comme senseur d'ARN viral et de protéines adaptatrice de TBK1 et d'IRF3. **Mots-clés** : Criblage pangénomique, Immunité innée, Virus de Sendai, Interféron de type I, RIG-I, Voie de signalisation RLR, SNRNP200, IRF3, TBK1, Senseur d'ARN viral, Protéine adaptatrice, Rétinite pigmentaire .

Abstract

The innate immune system is the first line of defense against invading pathogens of many kind such as bacteria, viruses, parasites and fungi. Its role is straightforward: it acts within minutes of a pathogenic engagement to control and restrict the microscopic invasion using non-specific mechanisms while the host mounts an induced, and specific, innate and adaptive response. To identify new regulators of antiviral innate immunity, we have completed the first genome-wide gene RNAi screen assessing the transcriptional response at the interferon-β (IFNB1) promoter following Sendai virus (SeV) infection. Interestingly, a Gene Set Enrichment Analysis (GSEA) of the 114 gene hits revealed that many of these proteins were spliceosome-associated. Among them, we further prioritized the characterization of SNRNP200, a core and unique spliceosomal member of the Ski2-like RNA helicase family based on its structural similarities to other antiviral RNA helicase like RIG-I and MDA5. In this thesis, we provide evidence for a role of the spliceosomal SNRNP200 that is clearly distinguishable of the one in pre-mRNA splicing. Indeed, the depletion of SNRNP200 in human cells resulted in a reduced antiviral response and increased susceptibility to viral infection. We specifically showed that SNRNP200 positively regulates activation of the key antiviral transcriptional factor IRF3 via a protein-protein interaction with the serine/threonine-protein kinase TBK1. Additionally, we showed that upon infection, SNRNP200 binds viral RNA and relocalizes into TBK1-containing cytoplasmic structures to promote innate signaling. Of clinical relevance, we observed a significantly hindered antiviral response of PBMCs from patients carrying a dominant SNRNP200 mutation associated to the retina pigmentosa type 33 (RP33), an inherited degenerative eye disease. We showed that expression of the RP33-associated mutant has lost the ability to bind RNA and to rescue antiviral response in SNRNP200 silenced cells. Thus, this thesis provides new insights into an immunoregulatory role of spliceosome SNRNP200 acting as an RNA sensor and adaptor of TBK1 to promote IRF3 signaling in antiviral response.
Keywords : RNAi screen, Innate Antiviral Immunity, Sendai Virus, Type I Interferon, RIG-I, RLR Pathway Signalling, SNRNP200, IRF3, TBK1, Viral RNA Sensor, Adaptor Protein, Retinitis Pigmentosa

Table of content

List of tables

List of figures

List of supplemental figures

List of acronyms

cGAS: Cyclic GMP-AMP synthase, cGAMP synthase

DDX58: DExD/H-Box Helicase 58

dsRNA: Double-Stranded RNA

CBP: CREB Binding Protein

ER: Endoplasmic Reticulum

FOXO1: Forkhead Box O1

IFIH1: Interferon Induced With Helicase C Domain 1, a.k.a. MDA5

IKBKE: Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Epsilon, a.k.a. IKKE

JUN: Jun Proto-Oncogene, AP-1 Transcription Factor Subunit, a.k.a. AP-1

LGP2: Probable ATP-Dependent RNA Helicase DHX58

IL-1B: Interleukin 1B

IL-6: Interleukin 6

IRF3: Interferon Regulatory Factor 3

IRF7: Interferon Regulatory Factor 7

IFIT1: InterFeron-Induced protein with Tetratricopeptide repeats 1, a.k.a. ISG56

IFNB1: Interferon Beta 1

ISG56: Interferon Stimulated Gene 56 KDa Protein

MAVS: Mitochondrial Antiviral Signaling Protein, a.k.a. IPS-1, VISA and CARDIF

MDA5: Melanoma Differentiation-Associated Protein 5, a.k.a. IFIH1

miR-155: Micro-RNA 155

mRNA: Messenger RNA

NEMO: Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Gamma

NF-κB: Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells

NLRP4: Forkhead Box O1

OPTN: Optineurin

P300: Histone Acetyltransferase P300

Poly (dA:dT): Poly(deoxyadenylic-deoxythymidylic) acid sodium salt

Poly (I:C): Poly(inosinic:cytidylic) acid sodium salt

Poly (dG:dC): Poly(deoxyguanylic-deoxycytidylic) acid sodium salt

RAUL: Ubiquitin Protein Ligase E3C

RBCK1: Forkhead Box O1

RIG-I: Retinoic Acid-Inducible Gene 1 Protein, a.k.a. DDX58

RNA: RiboNucleic Acid

ssRNA: Single-stranded RNA

SIGLEC1: Sialic Acid Binding Ig Like Lectin 1

SOCS3: Ubiquitin Protein Ligase E3C

STING: Stimulator Of Interferon Genes Protein

TBK1: TANK-Binding Kinase 1

TANK: TRAF Family Member Associated NFKB Activator, a.k.a. TRAF2

TBK1: TANK Binding Kinase 1, a.k.a. NAK

TNF: Tumor Necrosis Factor-Alpha

TRAF2/3/5: TNF Receptor Associated Factor 2/3/5

TRIM: Tripartite Motif Containing

List of abbreviations

5’ppp: 5’ triphosphate

AdV: Adenovirus

APCs: Antigen presenting cells

Asp: Aspartic acid

CARD: Caspase activation and recruitment domains

cKO: Conditional Knock-out

CSFV: Classical Swine Fever Virus

CVB3: Coxsackievirus B3

DAAs: Direct Acting Antivirals

DC: Dendritic Cells

EBV: Epstein-Barr Virus

ESI-LC-MS/MS: Electrospray Ionization LC-MS/MS

FLUA: Influenza Virus A

GBV-B: GB virus B

HAV/HBV/HCV/HDV: Hepatitis A/B/C/D virus

HIV-1: Human Immunodeficiency Virus type 1

HPV: Human Papillomavirus

HRV1a: Human Rhinovirus 1a

IFNs: Interferons

KD: Knock-Down

KO: Knock-Out

KFSV: Kaposi's Sarcoma Virus

LC/MS-MS: Liquid-chromatography Tandem Mass Spectrometry

LNA: Locked Nucleic Acid

LPS: Bacterial Lipopolysaccharide

MAC: Membrane-Attack Complex

MBL: Mannose-Binding Lectin

MDMs: Monocyte-Derived Macrophages

miRNA: Micro RNA

MHC: Major Histocompatibility Complex

MOI: Multiplicity Of Infection

NK: Natural Killer Cells

NLRs: NOD-like receptors

NT: Non-Target

NP: Nucleocapsid Protein

PAMPs: Pathogen-Associated Molecular Patterns

PBMCs: Peripheral Blood Mononuclear Cells

PEG-IFN: Pegylated Interferon

PRRs: Pathogen-Recognition Receptors

PTM: Post-transcriptional Modification

RLRs: Retinoic acid-inducible gene-I-Like Receptors or RIG-I-like receptors

RSV: Respiratory Syncytial Virus

RP33: Retinitis Pigmentosa 33

SARS-CoV: SARS coronavirus

SCR: Scrambled

SEV: Sendai Virus

shRNA: Short Hairpin RNA

Ser: Serine

Thr: Threonine

Tyr: Tyrosine

TLRs: Toll-like receptors

UPR: Unfolded Protein Response

VACV: Vaccinia Virus

VSV: Vesicular Stomatitis Virus

WNV: West Nile Virus

WT: Wild-Type

"Never discourage anyone...who continually makes progress, no matter how slow."

-Plato

Foreword

The body of this thesis comes from various research and review articles that I co-authored during my Ph.D. To give credit where credit is due, the foreword of the thesis acknowledges all authors and their respective contributions that have contributed to the text presented herein.

Introduction – Part 1.2. and 1.5.

The text is adapted from a review article that I co-authored: "Viruses Seen by our Cells: The Role of Viral RNA Sensors" by Elias Said*, Nicolas Tremblay*, Mohammed Al-Balushi, Ali A. Al-Jabri and Daniel Lamarre. Contributions: I have designed the annotated outline of the review with ES and DL. I have written the content related to the RLR-pathway, RLR and TLR usage for the development of therapeutics (antiviral therapies and vaccines) and the conclusions and perspectives. These sections were edited and reviewed by ES and DL. I have edited and reviewed the content related to the TLR-pathway that was written by ES.

Background information

The two figures used in preliminary data where taken from: "Genome-wide RNAi Screen Reveals a New Role of a WNT/CTNNB1 Signaling Pathway as Negative Regulator of Virus-induced Innate Immune Responses" by Martin Baril , Salwa Es-Saad , Laurent Chatel-Chaix, Karin Fink, Tram Pham, Valérie-Ann Raymond, Karine Audette, Anne-Sophie Guenier, Jean Duchaine, Marc Servant, Marc Bilodeau, Éric Cohen, Nathalie Grandvaux, Daniel Lamarre. This work was done before the start of my Ph.D. and the two figures were generated by Martin Baril. They are included in my thesis to provide context and relevance to my research hypothesis.

Results

The main research article presented in this thesis is taken from "Spliceosome SNRNP200 Promotes Viral RNA Sensing and IRF3 Activation of Antiviral Response" by Nicolas Tremblay*, Martin Baril*, Laurent Chatel-Chaix, Salwa Es-Saad, Alex Young Park, Robert K. Koenekoop, Daniel Lamarre. For this article, I have:

- I have used western blots, Elisa, RT-QPCR, viral plaque assays and rescue experiments to show that SNRNP200 is required to initiate a type I interferon response upon infection with several RNA viruses (Figure 1, 2).
- I have analyzed the data from microarray experiments to identify genes that are essential for the early (IRF3-dependent) and late (IFN-α/β dependent) antiviral response and affected by the depletion of SNRNP200 (Figure 3).
- I have mapped the functional domains that are required for SNRNP200 antiviral activity and mapped the interaction with the TBK1 protein kinase using cloning and directed mutagenesis in combination with western blots, co-immunoprecipitation, Elisa, reporter assays and confocal microscopy (Figure 4, 6, 7).
- I have used DNA/RNA-coupled to streptavidin beads to assess the role of SNRNP200 in viral nucleotide sensing using co-immunoprecipitation, western blot and RT-QPCR (Figure 5).
- I have used western blots, RT-QPCR and ELISA to confirm the role of SNRNP200 in innate antiviral immunity in primary cells (macrophages) and PBMCs of RP33 patients that are carrying mutations in SNRNP200 (Figure 9).
- I have made significant intellectual contributions to the proposed model that recapitulates the role of SNRNP200 in innate antiviral immunity (Figure 10.)
- I have also performed and analyzed the experiments presented in figure, S1A, S2, S6C, S7, S8, S9, S10, S11, S12 and S13.

The reminder of the experiments was performed by Martin Baril.

Annex A

The article attached in Annex A is taken from " Development of Panviral Therapeutics Requires a Better Understanding of Pathogen-induced Immune Response." by Salwa Es-Saad*, Nicolas Tremblay, Martin Baril and Daniel Lamarre. For this review article, I have have drafted the outline of the review article and written the first part of the review on the role of RNA sensors and sentinels in innate antiviral immunity.

I have reviewed and edited the content on TLR-based therapies that was written by SES and MB. Lastly, I have updated the review in 2017 and added two sections to cover new materials.

Annex C

The article attached in Annex B is taken from "Importin β1 targeting by hepatitis C virus NS3/4A protein restricts IRF3 and NF-κB signaling of IFNB1 antiviral response." by Bridget Gagné, Nicolas Tremblay, Alex Young Park, Martin Baril and Daniel Lamarre.

For this article, I have created a clear and concise graphical abstract that shows that IMPβ1 mediates the nuclear translocation of IRF3, essential to produce IFN-β, upon viral infection. In HCV infection, IMPβ1 is cleaved by HCV-NS3/4a protease as a novel mechanism of immune evasion. To elucidate the contribution of this mechanism to the RLR-pathway, I have generated a MAVS knock out (KO) cell line using CRISPR-Cas9 genome editing, along with various NS34/a cleavage-resistant MAVS and IMPβ1 clones. I then conducted various experiments (reporter assays, western blots and confocal microscopy) and showed that NS3/4A-mediated cleavage of importin β1 (IMPβ1) and interferon-β (IFNB1) inhibition are completely restored by expression of NS3/4A cleavage-resistant IMPβ1 variant (IMPβ1CR) and treatment with BILN 2061 NS3/4a protease inhibitor (Figure 8 of the paper). In addition, I have written and formatted the manuscript for publication and drafted Table 1 and Table 2 that summarize the gene enrichment analysis and the functional classification of the genes that affects the nuclear trafficking of IRF3 and p65, respectively.

1.1. Introduction

Antiviral immunity is the epitome of an evolutionary conserved system that has been shaped by host-pathogen interactions. On one side, the viruses are trying to hijack host machinery to his own benefit to fulfil its lifecycle, while deploying active and passive countermeasures to alleviate the ability of the host to prevent his enslavement. On the other side, the host must be able to balance its early innate immune response to a level that will restrict viral entry, replication and egress, but that will not seal its fate towards apoptotic death. While our understanding of the battle tactics used by both sides has increased over the last 15 years to this date, we still need to understand the finer molecular mechanics that govern the doctrine used by both armies.

To contribute to the advancement of knowledge in that field, I joined Dr. Lamarre's Lab following the completion of the first genome-wide RNA interference (RNAi) screen that identified over 100 novel regulators of type I interferon (IFN) response against RNA viruses. With that in mind, my thesis work has been focused on the characterization of a highly enriched family of spliceosomal proteins that showed great potential as positive regulators of an interferon-beta (IFN-β) driven antiviral response. Of interest, SNRNP200, an RNA helicase that shares many similarities with classical RNA sensors and sentinels, was revealed to be a bona fide sensor of viral RNA and an adapter-like platform that was shown to be essential for TBK1-mediated IRF3 activation.

This thesis focuses, for the first part, on the validation of the phenotypic observations that led us to prioritize SNRNP200 following the genome-wide screen and, for the second part, on the functional studies designed to assess SNRNP200 role in type I IFNresponse to viral pathogens.

As an introduction to this thesis, we will begin with a review of the literature to highlight key concepts of antiviral immunity from the recognition of viral nucleic acids from pathogen recognition receptors (PRRs) and to the expression of IFN stimulated genes (ISGs).

1.1.1. Plan to introduce the innate antiviral immune system

In the next section of the introduction, we will try to understand how an organism react to a pathogenic engagement. First, we will begin with a summary of the innate, and early induced, immune response. Then, we will move to an RLR-centric view of innate immunity that will be indispensable to understand the work presented in this thesis.

1.1.2. The innate immune system

The innate immune system is the first line of defense against invading pathogens of many kind such as bacteria, viruses, parasites and fungi. Its role is straightforward: it acts within minutes of a pathogenic engagement to control and restrict the microscopic invasion using non-specific mechanisms while the host mounts an induced, and specific, innate and adaptive response.

1.1.3. Specific innate response comes from many inducible protein-based systems

The transition from innate immunity (non-specific) to an induced early innate response (specific) is mediated by a complex network of anti-pathogenic soluble factors (interferons, cytokines and chemokines) that are primarily produced by specialized immune cells. A hallmark of this transition is the recognition, ingestion and destruction of pathogens that are killed by phagocytosis. Indeed, macrophages, granulocytes (neutrophils, eosinophils, basophils) and immature dendritic cells (DCs) patrol human tissues in a search for micro-organisms. Upon recognition of a pathogen via surface receptors, that are specific to some microbial components (ex. mannose, complement-coated, dectin-1), the phagocytic cells will internalize the pathogen and destroy it by means of acidification, production of reactive oxygen/nitrogen species, antimicrobial peptides and enzymes. This engagement by immune cells will, in turn, lead to the production of cytokines and chemokines that will induce a pro-inflammatory state that aim to recruit additional immune cells to the site of infection, but also to hinder the spreading of the infection and eventually promote the healing of the injury.

While some of the pro-inflammatory response mediated by phagocytic cells is broadly unspecific (ex. secretion of prostaglandins, leukotrienes, platelet-activating factor, TNF-α), most of the secreted cytokines and chemokines are specifically tailored towards a certain class of pathogens. This specification arises from the engagement of a specific set of PRRs that recognize a precise set of PAMPs derived from bacteria, viruses and parasites. The main pathogen recognition pathways include the Toll-like receptors (TLR), the NOD-like receptors (NLR), the RIG-I-like receptors (RLR) and the cGAS receptor (cGAS-STING) (Figure 1). In addition, the mammalian innate defense system relies on other inducible antiviral proteins and viral restriction factors such as RNA-dependent Protein Kinase (PKR), ribonuclease L (RNase L), myxovirus-resistance protein (Mx) GTPase, Oligo-adenylate Synthetase (OAS), tripartite motif (TRIM), APOBEC3G, TRIM5α to name a few. In the next section, we will provide a broad overview of the TLR and cGAS-STING pathway. Then we will look at the role of some inducible antiviral proteins before moving on to an RLR-centric view of innate antiviral immunity that is required to position the work presented in this thesis.

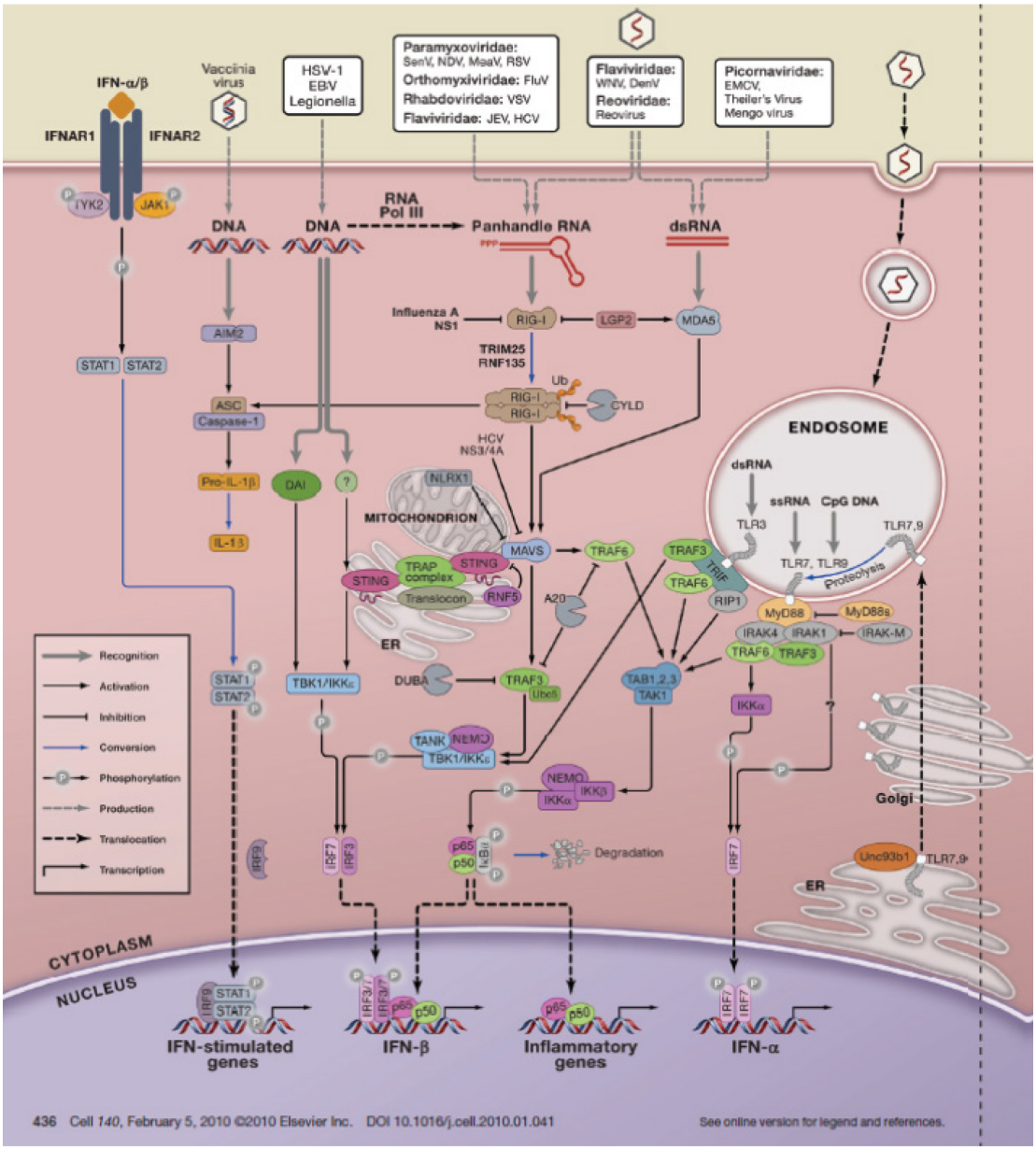


Figure 1. Innate signaling pathways triggered by viral nucleic acids. A hallmark of antiviral innate immune responses is the production of type I interferons and inflammatory cytokines. Recent research has unveiled multiple signaling pathways that detect viral infection, with several pathways detecting the presence of viral nucleic acids. Used with permission. [1]

1.2. The TLR pathway

The TLR pathways relies on a family of 13 receptors that are widely expressed on immune and non-immune cells (as reviewed in [2]). They are made of cell-surface receptors (TLR1/2/4/5/6/10) and intracellular receptors (TLR3/7/8/9/11/12/13) that are anchored to the different cellular compartments via a transmembrane domain and a cytoplasmic TIR domain that is necessary for downstream signaling (Figure 2).

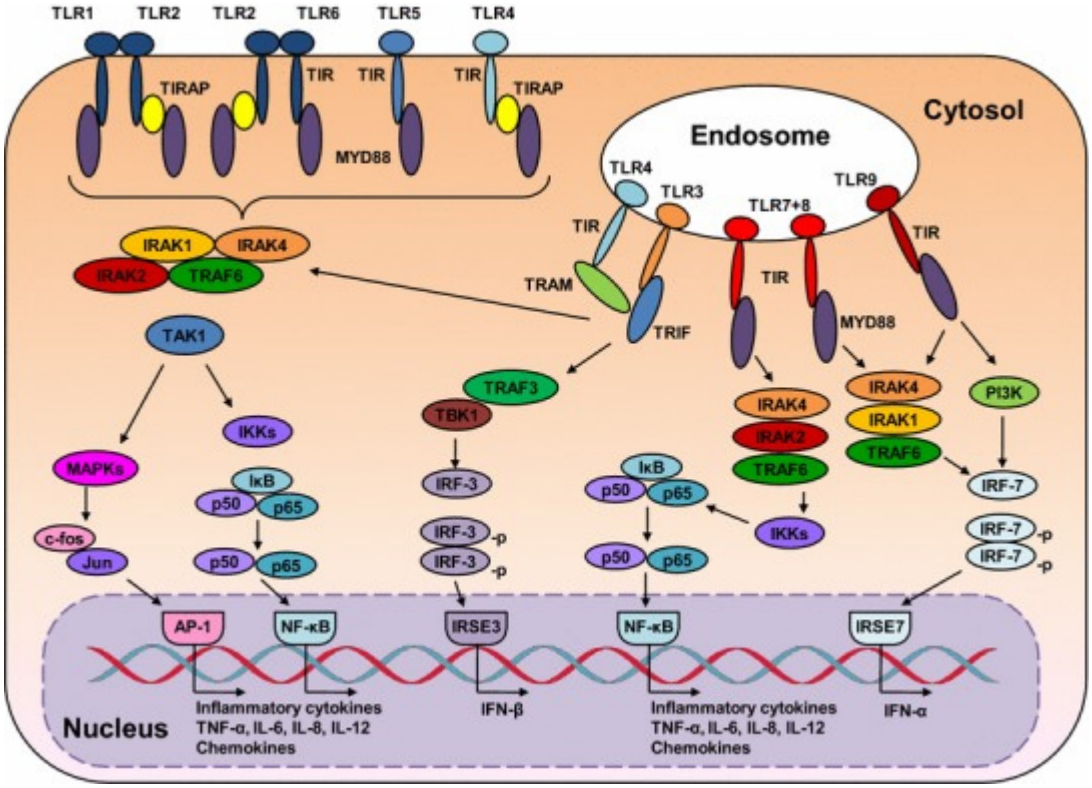


Figure 2. TLR signaling. Upon the activation of TLRs by their respective ligands, the adaptor molecules MYD88, TIRAP, TRIF, and TRAM are recruited and further activate the kinases TAK1, MAPKs, TRAF3, TBK1, and IKKs, resulting in nuclear translocation of transcriptions factors AP-1, NF-κB, IRF3, or IRF7, and subsequent transcription of IFNs and pro-inflammatory cytokines. Used with permission. CC BY 4.0. [3]

TLRs have a significant role in recognizing molecular patterns associated with different pathogens. The majority of the TLRs are found on the plasma membrane, while TLRs3, 7, 8 and 9 are present in the endosomal compartment [4]. Whereas those expressed on the cell surface predominantly recognize molecules of the microbial membrane e.g. proteins, lipids and lipoproteins, endosomal TLRs detect viral, bacterial or self-nucleic acids. In this section, we will focus on TLRs 3, 7 and 8 for their role in detecting extracellular RNA and viral particles [4].

1.2.1. TLR3 expression and ligands

TLR3 is expressed in the endosomes of immune cells i.e. monocytes, macrophages, dendritic cells (DC) (other than plasmacytoid DC), natural killers (NK) cells, T and B lymphocytes, mast cells, eosinophils and basophils. Non-immune cells, such as epithelial and endothelial cells, keratinocytes, fibroblasts, hepatocytes, astrocytes and microglia also express TLR3 [5, 6]. TLR3 recognizes dsRNA, the synthetic polyinosinic-polycytidylic acid (poly I:C) and polyadenylic–polyuridylic acid (poly A:U) [5, 6]. Moreover, TLR3 may be triggered by single –stranded RNA (ssRNA) with stable stem structures as described based on poliovirus RNA sequences [7]. However, further studies may be required to elucidate the exact mechanisms of such triggering.

TLR3 plays a significant role in the modulation of RNA and DNA virus-mediated innate immune responses. TLR3 senses dsRNA viruses such as members of the Reoviridae family including the rotavirus by sensing their genomic RNA; this recognition leads to the induction of inflammatory cytokines and type-I IFNs [5, 8]. Moreover, TLR3 recognizes intermediate RNAs that are produced during the replication of other viruses such as the herpes simplex virus-1 (HSV-1), respiratory syncytial virus (RSV), West Nile virus (WNV), coxsackievirus B3 (CVB3), poliovirus and influenza A virus (FLUA). The viral dsRNAs can reach the TLR3 in the endosomes upon phagocytosis of the dying infected cells or by direct uptake from the medium by antigen presenting cells [5, 8]. The possibility of the presence of intermediate viral ssRNAs with stable stem structures as a reason for the detection of these viruses by TLR3, as observed in the case of the poliovirus, remains to be investigated [7].

1.2.2. TLR3 structure and signaling pathways

TLR3 has a C-terminal cytoplasmic toll-interleukin 1 receptor (TIR) domain used for signaling, an N-terminal extracellular domain (ECD) and a single transmembrane alpha helix. The ECD has 23 leucine-rich repeats (LRRs); it is responsible for the binding of dsRNA. The dimerization of ECDs initiates the signaling [5, 9]. The TIR domain containing adaptor protein inducing IFN-β (TRIF) is then recruited and undergoes slight conformational changes [10] to form a signaling complex together with TNF receptor-associated factor 6 (TRAF6), TRAF3, TBK1 , IKKε and IKK (Figure 2). This leads to the activation of IRF3/IRF7 and NF-κB, which results in the production of type I IFNs and inflammatory cytokines respectively [5, 9].

To control the levels of inflammation induced by the triggering of TLR3, its signaling pathway is regulated by different molecules. Some act as positive regulators; such as serine/threonine kinase receptor associated protein (STRAP) that interacts with TBK1 and IRF3 [11]; munc18-1-interacting protein 3 (Mint3) that stimulates the K63-linked polyubiquitination of TRAF3 [12]; Src-associated substrate in mitosis of 68 kDa (Sam68) that may balance NF-κB p65 and c-Rel activation [13] and finally S100A9 that acts during the early stages of TLR3 activation by easing the maturation of TLR3-containing early endosomes into late endosomes [14]. Other molecules act as negative regulators; such as Rho proteins that decrease the production of pro-inflammatory cytokines upon TLR3 triggering [15]; SUMO-specific protease 6 (SENP6) that inhibits the NF-κB-mediated expression of the pro-inflammatory genes [16] and miR-155 that controls TLR3 signaling by repressing molecules such as TAB2, IKK-ε and RIP [17]. Interestingly, some oncogenic herpes viruses such as Kaposi's-sarcoma-associated herpes virus (KSHV) and Epstein-Barr virus (EBV) induce cellular miR-155 expression or encode functional orthologous of miR-155, which might constitute a strategy to escape the immune responses induced upon TLR3 triggering [17]. In addition, several proteins in the TLR3 pathway are targeted by different PTMs, which also participate in the regulation of responses initiated by TLR3 triggering [18].

1.2.3. TLR3 and the pathogenesis of viral infections

TLR3 has an important impact on the pathogenesis and the outcome of several RNA virus infections. In fact, the level of expression of TLR3 is associated with the severity and outcome of HCV infection [19]. Moreover, single nucleotide polymorphisms (SNPs) in the *TLR3* gene are associated with HCV-mediated liver disease progression and the development of hepatic fibrosis [20]. As mentioned above, TLR3 also plays an important role in establishing immune responses against HSV-1. Different studies showed that mutations in the *TLR3* gene are associated with the predisposition to HSV-1 encephalitis (HSE) in children [21-24] and adults [25, 26]. These mutations in TLR3 were shown to result in a lack of response to poly I:C and HSV-1 as observed in fibroblasts and induced pluripotent stem cells (iPSCs)-differentiated neural stem cells (NSCs), neurons, astrocytes and oligodendrocytes [21, 22]. This impairment was characterized by the absence of production of IFN-β and IFN-λ in these cells [21, 22]. The association of mutations in TLR3 gene with varicella-zoster virus encephalitis was also shown [25]. Other studies have shown that TLR3 may influence the pathogenesis of RSV, CB3 and enterovirus 71 (EV71), severe fever with thrombocytopenia syndrome (SFTS) and HBV infections [27-31]. This highlights the important role played by TLR3 in the innate immune responses to viruses, although the exact mechanisms of recognition and how it is involved often remain elusive.

1.2.4. Targeting TLR3 in anti-viral therapies and vaccines

The potential use of TLR3 ligands in anti-viral therapies and vaccines is suggested by different studies. For example, recently TLR3 ligands were shown to be efficient in reversing the latency of the human immunodeficiency virus (HIV) by the reactivation of HIV transcription in microglial cells [32]. Another study reported TLR3 ligands as candidates for anti-HIV immunotherapeutic strategies because these ligands increased the ability of HIV-infected DC to activate HIV-specific cytotoxic T lymphocytes [33]. TLR3 ligands were also shown to be potent adjuvants for vaccine preparations targeting influenza virus, HIV and HSV-2 [34-36]. Interestingly, poly I:C derivatives (known as Ampligen) are potential adjuvants tested in vaccine preparations targeting influenza virus, HIV and HPV [34].

1.2.5. TLRs7 and 8 expression and ligands

TLRs7 and 8 are expressed in the endosomes of a wide variety of cells including immune cells such as monocytes, macrophages, DC and NK cells [37]. The expression of TLR7 is also reported in T and B-cells [37, 38]. TLR8 is also expressed in mast cells and regulatory T-cells [39, 40]. The expression of TLRs 7 and 8 is not restricted to immune cells, as they are also expressed in endothelial and epithelial cells, astrocytes, microglia, hepatocytes as well as tumor cells [41-43]. TLRs7 and 8 share a lot of similarities, and recent findings suggest a potential compensatory role played by TLR8 in the absence of TLR7 [44]. TLRs7 and 8 recognize guanosine and uridine (GU)-rich or U-rich ssRNA sequences [45, 46]. However, we have shown that the presence of GU-rich sequences in ssRNA might not be sufficient, although necessary, to stimulate these TLRs [47]. In this study, several GU-rich sequences in HCV genome were described; however, not all these sequences were able to trigger TLRs7 and 8. In fact, the capacity of these sequences to trigger TLRs7 and 8 was not influenced by their length or the number of GU repeats that they contain [47]. Interestingly, some cellular defense mechanisms that target vRNA may influence its detection by TLRs7 and 8. In fact, the detection of phagocytosed vRNA by TLRs7 and 8 is facilitated by the adenosine-to-inosine (A-to-I) editing, which is an important arm of the antiviral response [48]. Furthermore, 2'-O-methylation within an RNA sequence shapes differential activation of TLRs7 and 8 [49, 50]. This modification leads to the triggering of TLR8 but not TLR7 by an RNA that was initially able to trigger both TLRs. The hypothesis that this might be due to a stronger binding by TLR7 than TLR8 will require further investigation. This change in the triggering leads to different secretion of pro-inflammatory cytokines as it impairs IFN-α production but not IL-6 [50].

Because of the capacity to sense ssRNA, TLRs7 and 8 have an important role in detecting RNA viruses and inducing anti-viral immune responses. They can be triggered by viral GU and U-rich ssRNA sequences, such as those in highly conserved untranslated terminal regions (UTR) of viral genomes that have a crucial role in viral protein translation and RNA replication [51]. The implication of TLR7 or TLR8 in detecting RNA viruses is different depending on the virus and the cell in which these TLRs are expressed. Viruses such as yellow fever virus (YFV), rhinoviruses and HIV can be detected by both TLR7 and

TLR8 [45, 52, 53]. However, the expression of TLRs 7 and 8 in a cell does not always guarantee their triggering by an RNA virus, even though the latter has RNA sequences that can be detected by these TLRs. This was shown in the case of HCV genome, which has sequences that stimulate both TLRs7 and 8 [47]. Nevertheless, the complete HCV particles do not induce responses through these TLRs in myeloid and plasmacytoid DC subsets and monocytes, whereas such stimulation takes place in macrophages without stimulating anti-viral responses [47]. Differences in the ability of cells to detect an RNA virus via TLRs7 and 8 were also described for Zika virus (ZIKV) infection, as no TLR7 activation was detected in primary human fibroblasts [54], while genes implicated in TLRs7 and 8 pathways were found to be upregulated in the human neural progenitor cells (hNPCs) infected with this virus [55]. Moreover, some vRNAs are recognized by TLR7 but not by TLR8. This may suggest the presence of differences in the conditions that lead to the detection of ssRNA sequences by TLR7 and TLR8. For example, the measles virus (MV), Ebola virus (EV), dengue virus (DV), human T-lymphotropic virus type 1 (HTLV-I) and poliovirus are able to trigger TLR7 only, while the role of TLR8 in such recognition remains unclear [5, 56]. Nevertheless, SNPs in TLR7 and TLR8 genes were associated with immune responses to MV suggesting a role for both TLRs during MV infection [57].

1.2.6. TLRs7 and 8 structures and signaling pathways

TLRs 7 and 8 are single-pass transmembrane receptors composed of a pathogen-recognition LRR-containing ectodomain and a TIR domain [58]. TLRs7 and 8 have 26 LRRs motifs in their extracellular domain, which contain multiple insertions such as the Z-loop or undefined region situated between LRR14 and 15 [59]. Both TLRs are proteolytically cleaved in the endosomes at the level of the Z-loop by arginine endopeptidase and cathepsins, and the cleaved fragments are linked together [60]. This is essential for the dimerization and activation of these TLRs [61]. TLRs7 and 8 dimers have a binding site for small chemical stimuli or degradation products of ssRNA, and a second binding site that recognizes ssRNA oligonucleotides. Both these sites are required for ssRNA-induced activation [62, 63]. The TIR domains multimerize following the interaction of TLRs7 and 8 with their agonists, which is important for the recruitment of myeloid differentiation primary response gene 88 (MyD88) [64]. MyD88 forms a complex

with interleukin-1 receptor-associated kinases (IRAKs) molecules. The pathway will eventually lead to the activation of transcription factors including IRF7 and NF-κB, which will cause the production of type I IFNs and inflammatory cytokines respectively (Figure 2) [64].

A number of molecules regulate TLRs7 and 8 signaling pathways and control the immune responses that are triggered upon stimulation of these TLRs. Some of these molecules are positive regulators such as UNC93B1, which physically associates with TLRs7 and 8 and delivers them to endolysosomes [65]; hepatocyte growth factor regulated the tyrosine kinase substrate (HRS) that is required for a proper TLR7 trafficking to endolysosomal networks [66]; CCAAT/enhancer-binding protein beta (C/EBPδ) that enhances the transcription of TLR8 [67]; triggering receptor expressed on myeloid cells like 4 (TREML4) that enhances TLR7 signaling [68] and pyruvate dehydrogenase kinase isozyme 2 (PDK2) that physically interacts with TRAF6 [66]. Spleen tyrosine kinase (Syk) was also shown as a positive regulator of the TLR7 pathway in the plasmacytoid DC (pDC) subsets. However, Syk may also negatively regulate the TLR7 pathway upon the stimulation of the regulatory immunoreceptors CD303 and CD85g in pDC, which suggests the presence of a dual role for Syk in the regulation of the TLR7 pathway [69]. Other molecules are also considered as negative regulators for TLR7 pathway such as tripartite motif 35 (TRIM35) that stimulates the K48-linked ubiquitination of IRF7 [70] and SENP6 described above in the TLR3 section [16]. More studies are required to identify molecules that negatively regulate TLR8 signaling. Furthermore, different proteins implicated in the TLRs7/8 pathway are subject to PTMs, which has a direct impact on the regulation of TLRs7 and 8-induced responses [18].

1.2.7. TLRs7 and 8 and the pathogenesis of viral infections

TLRs7 and 8 influence the pathogenesis and the outcome of several RNA virus infections such as HCV. In fact, the spontaneous resolution of the HCV infection has been shown to be associated with a sustained hyper-responsiveness of pDCs and mDCs to TLR7/8 stimulation [71], and the clearance and progression of the HCV infection is modulated by variations in the TLR7 and TLR8 genes [72]. Moreover, the potential capacity of the vRNA of different influenza strains to stimulate TLRs7 and 8 was found to be correlated to the

virulence of the strains [73]. In addition, SNPs in the TLR7 and TLR8 genes were associated with the CD4 T cell count during an HIV infection [74] as well as the levels of type I IFN and pro-inflammatory cytokines and the progression to hepatocellular carcinoma during an HCV infection [75, 76]. Also, the low copy numbers of TLR7 gene is associated with the establishment of chronic HBV infection [77].

The triggering of TLRs7 and 8 by viruses is not always an advantage for the immune system. HIV infection provides several examples for this phenomenon. In fact, TLR7 stimulation by the HIV ssRNA in CD4 T cells induces the anergy of these cells [78]. HIV requires the stimulation of NFκB upon the triggering of TLR8 to replicate in DCs [79]. In addition, HIV takes advantage of the cellular protein Snapin that inhibits its detection by TLR8 in DCs to trans-infect other cells [80]. In fact, inhibiting Snapin expression leads to an increased localization of HIV-1 within the early endosomes that contain TLR8, the establishment of a pro-inflammatory response and the inhibition of CD4 T cells trans-infection [80].

1.2.8. Targeting TLRs7 and 8 in anti-viral therapies and vaccines

TLRs7 and 8 ligands are potential candidates for anti-viral therapeutic and vaccine strategies. Hence, the capacity of TLRs7 and 8 ligands to inhibit HIV replication and to activate HIV reservoir is being investigated [81, 82]. Moreover, TLRs 7 and 8 ligands were proposed to be used as adjuvants in FLU vaccine preparations [83]. Furthermore, TL7 agonist Imiquimod (R837 or trade name Aldara) and TLRs7/8 dual agonist Resiquimod (R-848) are topical treatments for HPV-induced warts [34]. Although Imiquimod systemic administration may be highly toxic, Resiquimod showed promising results as adjuvant in an anti-HSV trial [34].

1.3. The cGAS-STING pathway

A relatively new player in the realm of innate immunity is the cGAS-STING pathway implicated in the detection of cytoplasmic DNA. In viral infections, this pathway relies on the recognition of pathogenic cytoplasmic DNA via the soluble receptor cGAS that signal viral engagement via the ER-bound STING adaptor protein (Figure 3).

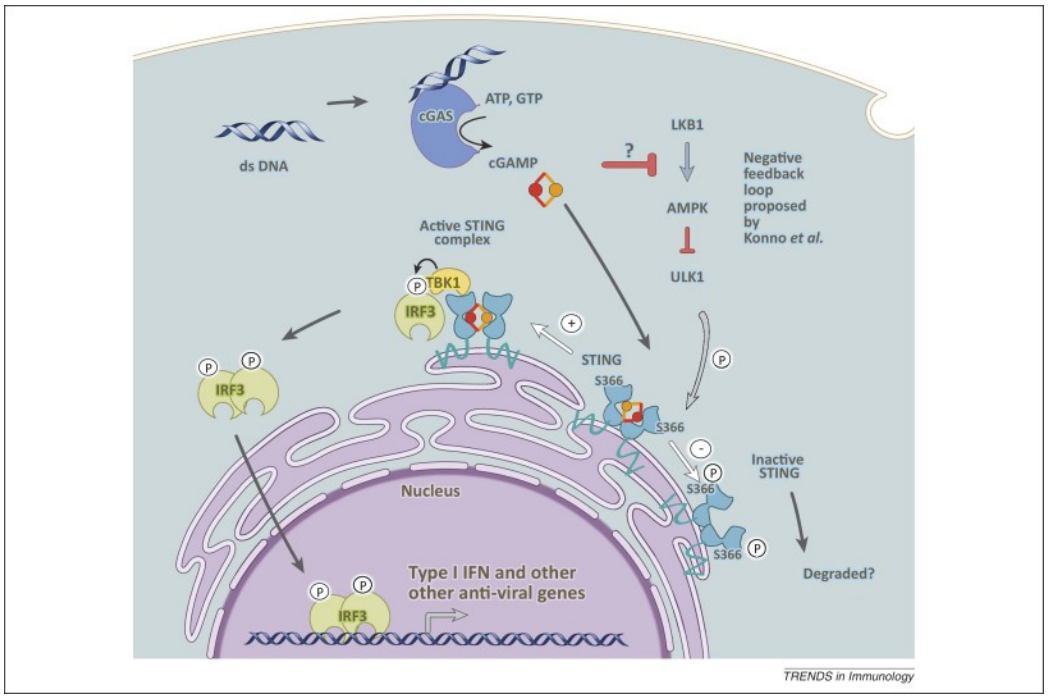


Figure 3. cGAS-STING signalling. Detection of cytosolic viral DNA activates a downstream signaling molecule called STimulator of INterferon Genes (STING) via the cytoplasmic sensor cGAS. Used with permission. [84]

A particularity of this pathway is that, upon binding to viral DNA, cGAS catalyzes the synthesis of a cyclic GMP-AMP (cGAMP), from ATP and GTP, that is used to indirectly

activate STING. This feature is unique to the cGAS-STING pathway since the activation of an immune signaling pathway, such as the TLR and the RLR, is normally mediated via direct protein-protein interactions and not via a chemical secondary messenger. Once activated, this pathway leads to the production of IRF3-dependent antiviral genes that culminate with the production of type I IFNs and TNF-α, IL-1B, IL6 pro-inflammatory cytokines. All-in-all, we can clearly see that many mechanisms are needed to coordinate an early immune response that will rapidly counter an infection. From an unspecific innate response (barriers, complement system, phagocytosis, inflammation), the host can transition towards an induced innate response that is tailored-made for a precise type of pathogenic entity by leveraging upon a complex set of innate immune pathways.

1.4. Interferon-induced Antiviral Proteins

Interferon-induced antiviral proteins are important to directly restrict viral replication, initiate translational arrest or apoptosis and to regulate the stress response that is initiated upon viral infection. In this section, we will examine the role of a selected subset of proteins to give the reader an idea of their contribution to innate antiviral immunity.

1.4.1. APOBEC3G

APOBEC3G (CEM15) is a cytidine deaminase that acts as a restriction factor for human immunodeficiency virus type 1 (HIV-1) and related lentiviruses. APOBEC3G is an interferon stimulated gene that is upregulated upon viral infection. In HIV-1 infected cells, APOBEC3G is incorporated into viral particles such as the Gag nucleocapsid [85-87]. Once incorporated into the viral progeny, APOBEC3G can directly interfere with viral replication by triggering G-A hypermutation via the deamination of nascent retroviral DNA. This results in an editing of the viral genome that introduces various errors like stop codons and non-synonymous mutations coding for unfunctional viral proteins [88-90]. More recently, it was seen that APOBEC3G can directly inhibit viral replication by preventing the elongation of HIV-1 cDNA via steric hindrance of the viral reverse transcriptase [91].

Besides HIV-1, APOBEC3G was also seen to have an antiviral function for various retrovirus viruses such as Equine Infectious Anemia Virus (EIAV), Murine Leukemia Virus (MuLV), Human foamy virus, and Human Hepatitis B Virus (HBV) [92]. Interestingly, it was recently reported that APOBEC3G can also inhibit the replication of a positive-strand RNA virus, HCV, and positive-strand RNA viruses like measles, mumps, and respiratory syncytial virus (RSV) [93, 94].

Additional studies will be required to pinpoint the APOBEC3G mechanism of action against RNA viruses. Overall, APOBEC3G is a prototypic ISG that acts by interfering with viral replication at various level.

1.4.2. TRIM family proteins

Tripartite Motif (TRIM) proteins are important regulators and effectors of the RLR-mediated antiviral response (for full review [95]). This family of protein acts at various levels of antiviral defenses: 1) they promote optimal activation of RIG-I and MDA5, 2) they facilitate the activation of the MAVS adaptor proteins and downstream effector proteins, and 3) they directly antagonize viral RNA replication and viral proteins to allow an optimal antiviral response.

First, let us examine how TRIMs can promote the optimal activation of the RLR-pathway (Figure 4). Upstream of the RLR-signaling pathway, TRIM25 and TRIM4 facilitate the K63-linked polyubiquitination of RIG-I CARDS that is required for its association with the adaptor protein MAVS [96-98]. Additionally, TRIM38 has been shown to SUMOylate RIG-I and MDA5 in order to prevent their K48-linked ubiquitination and targeting of the proteasome [99, 100]. Midstream, TRIM25 facilitates the release of TBK1 and NEMO from MAVS to allow for the efficient activation of antiviral transcription factors such as IRF3. In addition, TRIM31 facilitates MAVS K63-linked ubiquitination, which is required for the initiation of MAVS oligomerization and downstream antiviral signaling [101]. Downstream, many TRIMs are important for the activation of TBK1, NEMO, IRF3, and the feedback inhibition of IFN-β. TRIM14 mediates the K63-linked ubiquitination of NEMO that allows for its recruitment of TBK1-IKBKE to the activation complex of IRF3/7 and the recruitment of IKBKA-IKBKB to the activation complex of p65-p50 (NF-κB)

[102]. Additionally, TRIM14 and TRIM11 have been shown to be important for the optimal activity of TBK1, which is the main effector protein of IRF3 phosphorylation, and they undergo activation with both positive and negative regulatory function, respectively [102-104]. Interestingly, it was recently found that TRIM68 negatively regulate IFN-β production via the degradation of TRK-fused gene (TFG) [104]. All-in-all, TRIMs are important regulators of the RLR-pathway that enforce an optimal antiviral response by facilitating the activation and repression of key signaling events.

Second, let us consider the role of TRIMs as host factors that hinder the viral life cycle of many pathogens. TRIM5α is another example of a well-characterized HIV-1 restriction factor (see APOBEC3G). Primarily, TRIM5α antagonizes HIV-1 by inhibiting capsid processing by binding to the HIV-1 SPRY domain, thus, disrupting its normal conformation and inducing premature viral uncoating. Second, TRIM5α was shown to disrupt HIV-1 reverse transcription by inhibiting the nuclear translocation of reverse transcription products or by targeting reverse transcription products for proteasomal degradation. This ability to antagonize viral pathogenesis via direct host-virus interaction is also shared by other TRIM proteins, TRIM25, 22, 32, and 56, which were shown to inhibit the replication of FLUA, HCV, and Dengue Virus (DENV) [105-107]. In addition to direct antiviral capabilities, TRIM5α can also potentiate the production of antiviral type I IFNs in the presence of HIV-1 capsid proteins. Indeed, TRIM5α was recently shown to facilitate the phosphorylation of TAK1, via K63-linked ubiquitination, which is an important regulator of AP-1 and p65-p50 (NF-κB)[108]. Collectively, the role of TRIM5α as an antiviral protein is clearly seen from its role in HIV-1 infection. However, further studies will be required to explore its emerging role in the regulation of type I interferon.

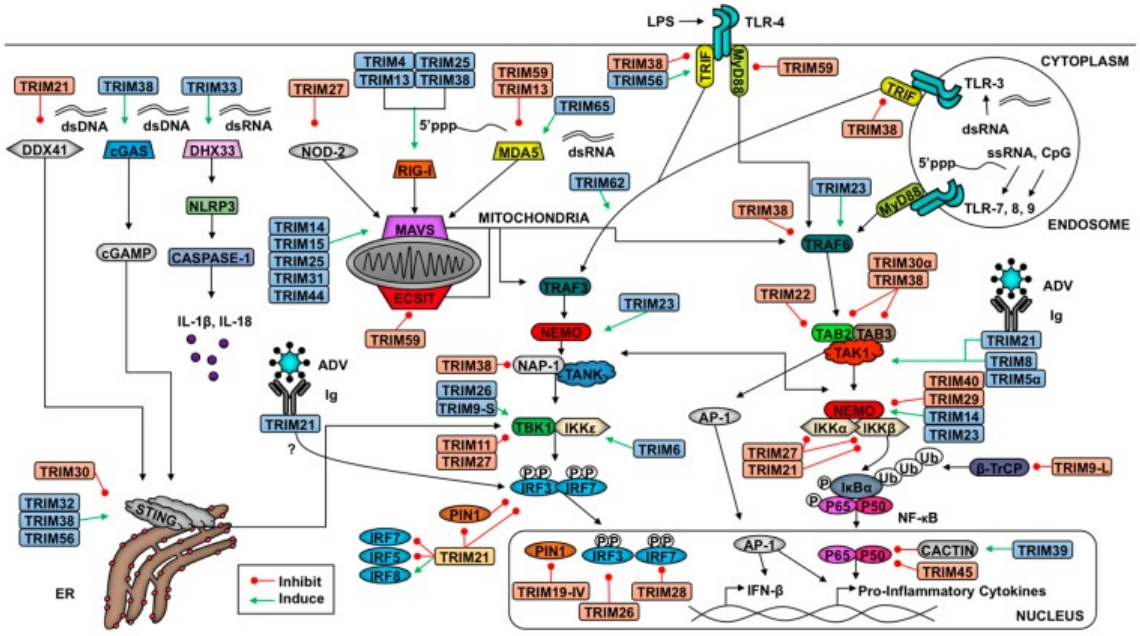


Figure 4. Regulation of pattern recognition receptor (PRR) signaling by tripartite motifs (TRIMs). TRIMs play an integral role in the positive and negative regulation of antiviral pathways. TRIMs can act as pathogen PRRs, as is the case for TRIM21 in the recognition of non-enveloped viruses bound by immunoglobulin (Ig). Additionally, these TRIMs can regulate the activation of other PRRs that recognize viral pathogen-associated molecular patterns (PAMPs) in the cytosol (DDX41 (DEAD-box helicase 41), cyclic GMP-AMP synthase (cGAS), DEAH-box helicase 33 (DHX33), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated protein (MDA5)) and at membrane surfaces (toll-like receptors, TLRs). Downstream of the initial pattern recognition, TRIMs also influence the recruitment and interaction of adaptor molecules (stimulator of IFN genes (STING), mitochondrial antiviral signaling protein (MAVS), TGF-β-activated kinase 1(TAK1)/MAP3K7-binding protein (TAB) 2, Myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), NF-κB essential modulator (NEMO), nucleosome assembly protein (NAP-1), and tumor necrosis factor (TNF) receptor-associated factors (TRAF) family member-associated NF-κB activator (TANK)) and enzymes (TRAF3, TRAF6, TAK1, inhibitor of NF-κB (IκB) kinase (IKK) α,β,ε, TANK binding kinase 1 (TBK1)) to signaling complexes in order to activate transcription factors. This includes IFN regulatory factor (IRF)3 and IRF7, important in type-I interferon (IFN) signaling, and NF-κB, important in expression of pro-inflammatory genes, which regulate the expression of antiviral effectors. Type-I IFN production is critical for an effective antiviral response. Used with permission. CC BY 4.0. [95]

1.4.3. Mx family proteins

Mx family proteins are made of a group of highly conserved antiviral proteins shared by all vertebrates. In humans, two isoforms, MX1 (MxA) and MX2 (MxB), contribute to intrinsic antiviral defenses. The expression of Mx proteins is under the exclusive control of type I and type III IFNs [109]. Mx proteins are expressed in many tissues, and their sub-cellular localization is sparse, ranging from the cytoplasm to the nucleus and the nucleopore. They are made of N-terminal GTPase domain, a middle domain (MD), and a C-terminal GTPase effector domain (GED). The N-terminal domain confers the biological activity of Mx proteins, while the MD and GED domains are required for oligomerization and host-virus interactions[110]. In humans, MX1 has been shown to prevent the FLUA vRNP transport into the nucleus, to inhibit the FUA RNA translation, to prevent the transcription of VSV RNA and to prevent the release of FLUA vRNPs into the cytoplasm [111, 112]. In contrast, MX2 is a restriction factor of HIV-1 by inhibiting capsid-dependent nuclear import of subviral complexes[113, 114].

This shows that the Mx family proteins have an extremely broad antiviral activity with the potential to hinder infections from Orthomyxoviridae, Rhabdoviridae, Paramyxoviridae, and other viral lineage. While there is no common antiviral mechanism against various viral lineage, the antiviral activity of Mx proteins seems to rely on an intact GTPase domain and the ability to oligomerize on viral targets, such as vRNP or large order viral proteins, to disrupt the viral life cycle at many steps: entry, replication, and assembly. As such, the Mx family proteins are a good example of IFN-inducible antiviral factors.

1.4.4. OAS & RNase L

Oligo-adenylate synthetase (OAS) and RNase L are two functionally-related IFN inducible antiviral proteins. They were discovered very early in the innate immunity field and are the first example of antiviral effector proteins. The human OAS consists of three isoforms (OAS1, OAS2, OAS3), which encode for antiviral proteins that can recognize dsRNA [115]. Upon binding, OASs become catalytically active and can convert ATP into 2-5A: a short oligoadenylates linked by 2′,5′-phosphodiester bond. The 2-5A can, in turn, be recognized by the latent endoribonuclease RNase L.

Upon binding with 2-5A, RNase L can dimerize and exert its antiviral activity. RNase L acts on two fronts: it can directly hinder the virus life cycle (viral genome degradation or vRNA degradation), and it can promote an optimal IFN response, reduce cellular translation, and promote apoptosis (Figure 5) [115]. Indeed, RNase L was seen to produce small RNA cleavage products from self-RNA that can initiate IFN production, effectively producing immune-stimulatory molecules from self-RNA rather than viral RNA [116]. Additionally, RNase L was recently reported to be involved in the arrest of the protein synthesis, in response to dsRNA, without any degradation of translation machinery [117]. Overall, it can be concluded that OAS/RNase L is an important intrinsic antiviral protein that exerts hinderance on the viral life cycle by using both host and viral targets.

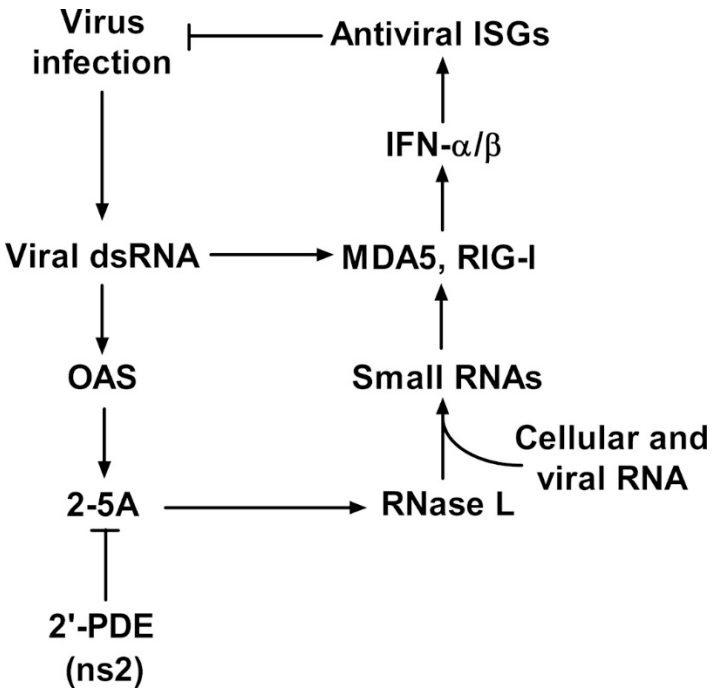


Figure 5. The Interferon Induced OAS-RNase L Pathway. Following infection, viral RNA is detected by pattern recognition receptors RIG-I and MDA5, resulting in the induction of IFN-α/β, which in turn induces ISGs, including OAS. OAS is activated by dsRNA to produce 2–5A, which activates RNase L. RNase L degrades cellular and viral RNA producing more RNA that is recognized by MDA5 and RIG-I, resulting in enhanced IFN induction. 2'-PDE cleaves 2–5A and inhibits the activation of RNase L. MHV ns2, like the cellular enzyme 2'-PDE, is a 2',5'-phosphodiesterase. OAS, 2'–5'-oligoadenylate synthetase; 2–5A, 2', 5' oligoadenylate; 2'-PDE, 2'-phosphodiesterase. [118]

1.4.5. PKR

PKR is a vRNA sensor that is ubiquitously expressed in many different cell types. Upon the binding of dsRNA, PRK can dimerize, undergo autophosphorylation, and be activated to exert its antiviral activity [118, 119]. Then, active PKR can bind to its cellular target, eukaryotic translation initiation factor 2 (eIF2α) [120, 121]. Binding of PRK to eIF2α leads to its phosphorylation, which allows for its interaction with eIF2B and subsequent functional sequestration. Since eIF2B is critical for protein synthesis initiation, its sequestration by eIF2α leads to an inhibition of translation initiation and, as a side-effect, to the hindrance of the viral life cycle that relies on host-machinery for replication and assembly.

Beyond a classical role in intrinsic antiviral immunity, PRK is also an important regulator of TLR/RLR-signaling and is involved in a wide variety of cellular processes from the regulation of stress response, cell growth, and apoptosis (for a full review see [122]). Overall, PKR is another good example of an intrinsic cellular factor that significantly contributes to innate antiviral immunity.

1.4.6. Beyond sensors and IFNs: the emerging important of stress granules

In recent years, the relationship between virus-induced translational arrest and stress granules has become topical in the field of antiviral immune responses. In short, there is much evidence that shows that stress granules are key players of an optimal antiviral response: they seem to act as organelles of vRNA sensing and immune signaling. Indeed, upon viral infection, pathogenic dsRNA and virus-induced endo-reticulum stress are sensed by PKR, and PKR-like endoplasmic reticulum kinase (PERK), respectively. These two pathways lead to the phosphorylation of eIF2α, the accumulation of stalled 48S mRNPs pre-initiation complex, and the initiation of cell-wide translational arrest. It is the accumulation of 48S mRNPs that will eventually result in the formation of stress granules and their aggregation into large order complex.

These stress granules have been classically defined as closed organelles that are characterized by the presence of aggregating T-cell restricted intracellular antigen (TIA1), TIA1-related protein (TIAR), and RAS GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) in the observed granules. Indirectly, stress granules can be considered as an important mechanism of antiviral immunity as translational arrest will restrict viral gene expression that relies on the host machinery to complete its life-cycle.

Interestingly, the role of stress granules might be larger than previously thought. First, it was recently seen that stress granules seem to potentiate virus recognition and innate signaling via the recruitment of viral RNA sensors (RIG-I and MDA5) and signaling molecules (TRIM25) from the cytoplasm into the stress-induced organelle. While the exact mechanisms that govern this recruitment and signaling remains to be elucidated, it can be suggested that the recruitment of RIG-I, MDA5, and TRIM25, in a closed environment that is rich in immune-stimulatory molecules such as vRNA, can be leveraged as an effective signaling platform to put the cell in an optimal antiviral state.

Second, stress granules have been shown to contain various viral proteins and host-factors that are required for optimal viral replication, assembly, and release. Thus, in combination with the translational arrest that disrupts viral gene expression, stress granules can co-opt viral proteins from the cytoplasm or the nucleus where they are normally used to support replication, immune evasion, or the hijacking of host factors. Lastly, it is worth mentioning that many antiviral effectors (ISG) are recruited to virus-induced stress granules. For example, PKR, OAS\RNase L, and APOBEC3G have been observed in virus-induced stress granules [123-126]. While their contribution to antiviral immunity have not been fully researched in the context of virus-induced stress granule, their recruitment suggests that like vRNA sensors and signaling molecules, the cell is actively shuffling antiviral proteins from the cytoplasm to closed organelles where they will be in contact with an increased amount of viral RNA or proteins. Overall, it can be noted that stress granules appear to be a critical component of the first line of defense against viral pathogens. Further research will be required to understand the kinetics of their initiation and destruction and to pinpoint their contribution and relationship with other known antiviral pathways.

1.5. Pathogen Recognition Receptors of the RLR-pathway

If a living organism wants to engage, control and eliminate a pathogenic entity, it must first be able to detect it. At first, this simple, yet elegant paradigm might seem easy enough to crack experimentally, but, in retrospective, it has been a central research question for more than 50 years, as of now.

From the pioneering studies to identify interferon (IFN)-inducing compounds, to the discovery of toll-like receptors (TLR), RIG-I-like receptors (RLR) and the cGAS-STING pathway, the quest to understand how pattern recognition receptors (PRR) recognized pathogen associated molecular patterns (PAMP) has shed light on a complex network of signaling pathways that are spatially compartmentalized, mostly pathogen specific and highly/tightly regulated.

In the first part of this review, we will focus on the significant contribution of cellular RNA helicases to antiviral defenses. To do so, we will use current research to answer three questions:

1. What are the determinants of viral RNA (vRNA) that are sensed by RNA helicases?
2. How can an RNA sensor distinguish cellular RNA from pathogenic or vRNA?
3. What are the emerging role of RNA sensors in cancer and autoimmune disease?

1.5.1. The three musketeers and squires of antiviral immunity

In the wake of the discovery of TLRs, it was historically postulated that antiviral immunity was mediated via TLR3 because this membrane-anchored receptor was essential to trigger the production of Type I IFNs and the activation of IFN stimulated genes (ISGs) when challenged with extracellular double-stranded RNA (dsRNA) poly(I:C), as a viral surrogate [127].

However, further investigation revealed that mouse TLR3-/- dendritic cells (BMDC) can produce high levels of IFNα when stimulated with intracellular dsRNA suggesting the existence of another type of RNA sensor, beside the TLRs, that would survey the cytoplasmic space for pathogenic nucleic acids [128].

From that point, the race was on, the hypothesis was solid: scientists wanted to identify the sensor(s) that had eluded them for almost two decades.

1.5.2. The classic RNA Helicases of antiviral innate immunity

It would not be long before scientific breakthroughs, such as the completion of the human genome project, synergize to identify RNA helicases as key players of antiviral innate immunity.

As soon as 2004, RIG-I, a DExD/H box RNA helicase, was shown to initiate antiviral signaling following intracellular dsRNA stimulation providing the first evidence of a novel class of cytoplasmic sensors of RNA virus replicating genomes [129]. Directed mutagenesis experiments revealed that RIG-I's N-terminal CARD domain was essential to antiviral responses following stimulation, while C-terminal regulatory domain (CTD) ablation would lead to a constitutive RIG-I activation hinting at a regulatory role of both moieties. Based on those functional structural insights and sequence homology analysis, MDA5 and CARD-less LGP2 were identified as putative vRNA sensors (Figure 6). Of importance, these RNA helicases have a similar ATPase/helicase domain that will prove to be essential for their function as vRNA sensor. Interestingly, the same structural rationale was used to identify MAVS, which contains a CARD domain, as the signaling adaptor between RIG-I and IRF3/NF-κB linking the mitochondria to innate immunity and completing the framework of what is now referred to as the RLR (or RLR/MAVS) signaling pathway. The next steps were to understand the regulatory role of both CARD domains and CTD, to decipher how RIG-I initiates antiviral signaling via the adaptor MAVS protein and to contrast the role and function of RIG-I, MDA5 and LGP2. For the latest, the answer would address a central paradigm of antiviral immunity: how does a cytoplasmic RNA helicase discriminates between sensing pathogenic and cellular RNA that is evidently abundant within this organelle.

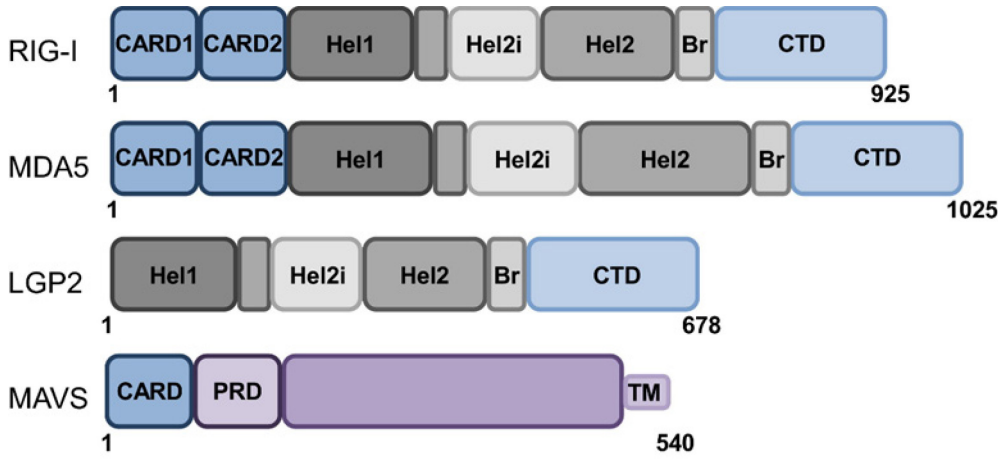


Figure 6. Schematic representation of RLR and MAVS domain structures. RIG-I and MDA5, but not LGP2, possess tandem caspase activation and recruitment domains (CARDs), a signaling module allowing for MAVS binding and IFN-α/β induction. In addition, all three RLR members have a helicase core consisting of two helicase domains (Hel1 and Hel2), a helicase insertion domain within Hel2 (Hel2i) with ATPase activity, a bridging domain (Br), and a C-terminal domain (CTD). Both the helicase and the CTD have RNA binding abilities. MAVS is comprised of a single CARD, a proline-rich domain (PRD), and a transmembrane (TM) domain that anchors it to mitochondria, peroxisomes, and MAM. Used with permission. [130]

1.5.3. RIG-I and MDA5 RNA helicases

As we have seen before, RIG-I and MDA5 are RNA helicases that survey the cytoplasm in search of PAMPs. But do they have similar or different pathogenic RNA preference? Initial studies in mouse embryonic fibroblast deficient for MDA5 (MDA5 -/-) could initiate an antiviral response when challenged with intracellular RNA molecules containing a triphosphate moiety at the 5'region (5'ppp) while RIG-I -/- could not [131]. Moreover, when the 5' region is capped, or the 5'ppp is treated with calf intestinal alkaline phosphatase to remove the phosphates, stimulations are not observed [132]. These were the first evidence that RIG-I can recognize uncapped 5' and phosphorylated single-stranded RNA genomes while MDA5 could not. Subsequent studies showed that RIG-I preferably recognizes short dsRNA molecules, while MDA5 is activated by long dsRNA [133-135].

More recently, Influenza U/A-rich 3' regions of viral RNA segments were also shown to activate RIG-I in a 5'ppp independent matter via an unknown mechanism [136].

This recognition might be mediated by RIG-I's helicase domain instead of the paradigmal CTD. Additional studies, such as the crystal structures of the full RIG-I/MDA5 proteins bound to vRNA, would be required to understand the fine molecular mechanisms that put together vRNA and sensor structural properties into one unifying and comprehensive theory. This correlates exceptionally well with the type of viruses that are recognized by RIG-I, such as Sendai virus (SeV), Vesicular stomatitis (VSV), Influenza A (FLUA) and hepatitis C virus (HCV), and by MDA5, such as Encephalomyocarditis virus (EMCV), Norovirus or murine hepatitis virus (MHV) [136-138]. Altogether, these data support the concept that cytoplasmic RNA helicases are sensors of non-self RNA (uncapped and 5'phosphorylated RNA) and that they work together to ensure an optimal coverage of the full spectrum of viral nucleic acids, including replication intermediates or copy-back defective interfering (DI) genomes.

Now, how these crucial events lead to the initiation of the RLR/MAVS antiviral signaling pathway. Under homeostatic conditions, RIG-I and MDA5 are kept in a close conformation (signal off) by the CTD. Upon contact with vRNA molecules, it is proposed that an ATP dependent translocation along the nucleotide strand lead to the high-affinity binding with the CTD to expose the CARD domains and to promote the formation of stable RIG-I dimers (Figure 7) [139-141]. Indeed, the ATP dependent translocation was recently showed to contribute to the self vs non-self RNA recognition as ATPase/translocase activity removes RIG-I from abundant self-RNA while locking it into the 5'ppp following translocation and binding to the viral determinant, reducing background signaling and increasing sensitivity of vRNA detection [139, 141]. Following the liaison, the exposed CARD domains are activated by the phosphatases PP1α/PP1γ and are ubiquitinated by TRIM25/Riplet to allow the conformational changes required for the CARD domains to interact with the MAVS adaptor (Figure 8) [96, 142]. The interactions of RIG-I and MAVS through their CARD domains contribute to the establishment of MAVS prion-like aggregates that become the immune platform for the phosphorylation of IRF3/NF-κB via IKBKE and TBK1 protein

kinases, leading to their nuclear translocation and production of type I IFN with subsequent expression of ISGs [143-148]

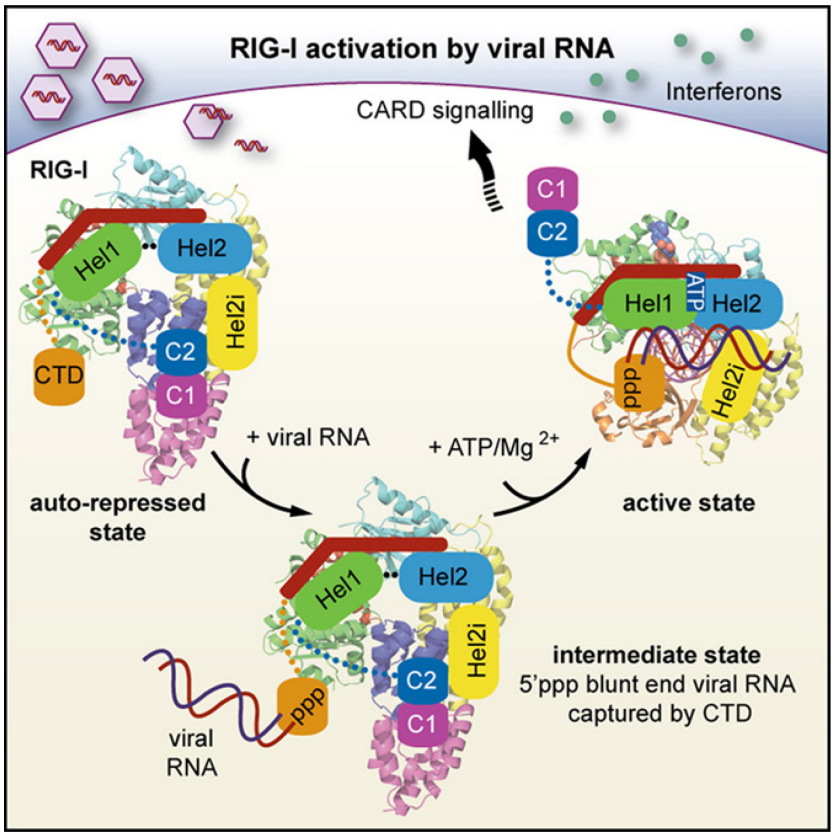


Figure 7. RIG-I is a key innate immune pattern-recognition receptor that triggers interferon expression upon detection of intracellular 5′triphosphate double-stranded RNA (5′ppp-dsRNA) of viral origin. RIG-I comprises N-terminal caspase activation and recruitment domains (CARDs), a DECH helicase, and a C-terminal domain (CTD). Inactive (auto-repressed) RIG-I has an open conformation with the CARDs sequestered by a helical domain inserted between the two helicase moieties. ATP and dsRNA binding induce a major rearrangement to a closed conformation in which the helicase and CTD bind the blunt end 5′ppp-dsRNA with perfect complementarity but incompatibly with continued CARD binding. After initial binding of 5′ppp-dsRNA to the flexibly linked CTD, co-operative tight binding of ATP and RNA to the helicase domain liberates the CARDs for downstream signaling. Used with permission. [149]

1.5.4. LGP2 and sentinel RNA helicases

LGP2 is an RNA helicase, homologous in structure to RIG-I and MDA5, but that lacks the CARD domains that are required to initiate antiviral signaling via the MAVS adaptor protein. Thus, LGP2 is not able to propagate the signal to produce IFN, and must have a role that is different from RIG-I and MDA5 in the RLR pathway. Initially, LGP2 was proposed as a negative feedback regulator of the RLR pathway that would act by sequestering vRNA from RIG-I [150] or by displacing IKBKE from MAVS in order to terminate IRF3-dependent antiviral signaling [151]. Moreover, later studies showed that LGP2 and RIG-I CTD, commonly referred as repressor domain (RD), are analogous and provided in vitro evidence that LGP2 CTD can interact with RIG-I to abolish its ability to initiate antiviral signaling [152, 153]. As an aside, the latest is reminiscent of the novel negative regulator of innate immunity KHSRP that associates with the CTD of RIG-I to maintain the receptor in an inactive state and attenuate its sensing of vRNA [133]. Upon viral infection, KHSRP competes with PAMP for the RNA recognition site located within RIG-I's CTD. This competition between KHSRP and vRNA is thought to be essential to maintain a proper activation threshold of RIG-I signalling and prevent unnecessary or disproportionate activation of the RLR pathway.

Despite some controversies about its function in antiviral signaling, LGP2 is emerging as a sentinel sensor that cooperates with RIG-I and MDA5 to enhance their recognition of vRNA substrate and initiate type IFN response against some viruses such as ECMV and HCV [154-156]. According to this model, LGP2 can leverage upon its ATP-dependent/RNA helicase activity to assist and increase interactions of a larger subset of nucleic acids-derived PAMPs with RIG-I/MDA5, and to finally potentiate the antiviral signaling. Additionally, it was recently shown that LGP2 inhibits a DICER-mediated processing of vRNA [138]. In contrast to the elaborated, protein-based system, found in mammals, plants and invertebrates rely on their RNA interference (RNAi) machinery to degrade vRNA and subvert viral replication [139]. This recent report provides evidence that LGP2 antagonizes the degradation of vRNA by DICER to keep the cytosolic PAMP intact and allow their detection by RNA sensors. Further studies should provide key insights about the relationship between the antiviral RNAi system, LGP2 and the RLR

pathway in mammalian cells. Interestingly, LGP2 sentinel function seems to be shared by many other DExD/H box RNA helicases such as DDX3, DHX9, DHX29, and DDX41, which bind directly to nucleic acids and interact with either RIG-I or MAVS, activating the pathway (see review [141, 157]).

Interestingly, LGP2 sentinel function seem to be shared by many other DExD/H box RNA helicases such as DDX3, DHX9, DHX29, DDX41 and DDX60 that have been extensively reviewed elsewhere [158, 159]. For the review, we will concentrate on DDX60, a Ski2 RNA helicase, to show prototypical characteristics of sentinel sensors. As an aside, Ski2-like RNA/DNA helicases are made of a group of seven helicases (Ski2, Mtr4, Brr2, Slh1, Hfm1/Mer3, Hel308 and DDX60) that are structurally similar and phylogenetically close (for a full review, see [160]. They were initially identified in the S. cerevisiae and annotated together because of their large size ranging from 120 to 220 kilodaltons (kDa). They all share an architectural DExH-box core made of two RecA domains, a winged helix domain and a ratchet domain that support an ATP-dependent unwinding and translocation activity along a nucleotide strand. Based on these structural insights, the Ski2-like helicases are thought to be processive helicases that can alter/modify the RNA/DNA template to which they are associated. As such, Ski2-like RNA helicases have been associated with many RNA degradation, processing and splicing pathways.

As an example, Ski2, a yeast ortholog of human DDX60, is a key player, and classical cofactor of the Ski complex of the cytoplasmic RNA exosome which mediates the 3′-to-5′ processing or degradation of many RNA molecules and contributes to cell proliferation and differentiation, telomerase RNA quality control and antiviral immunity [161]. Interestingly, Ski2 was associated with antiviral defenses almost twenty years before the discovery of the RNA exosome! Indeed, Ski2 or "Super-killer 2", was shown to restrict the expression of a toxin encoded by the M2 protein of the dsRNA L-A-HN yeast virus [162, 163]. More recently, another Ski complex cofactor, the human SKIV2L, was also shown to participate in the selective, exosome-mediated, degradation of the HBV RNA [164]. So, is DDX60 playing a role in innate immunity via a similar mechanism involving the RNA exosome?

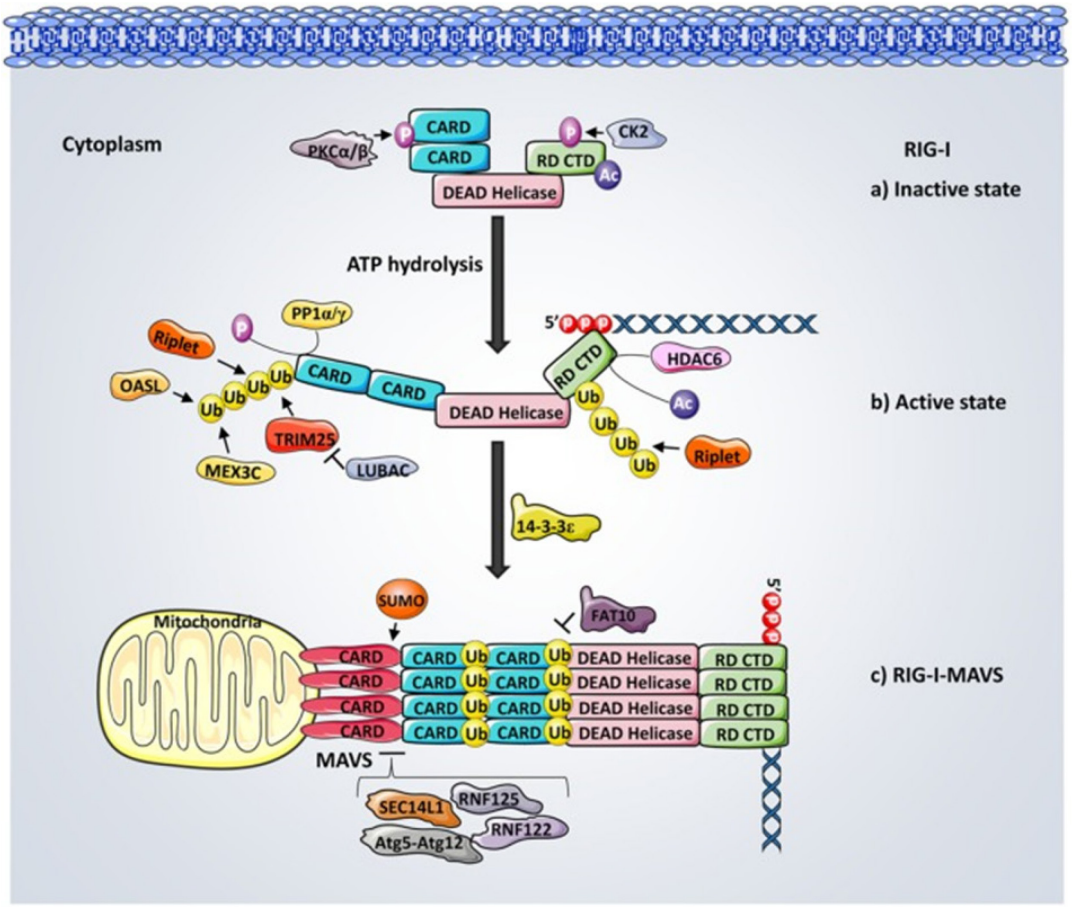


Figure 8. Regulation of retinoic acid-inducible gene-I (RIG-I) activation. (a) In resting cells, RIG-I is kept inactivated through the phosphorylation of caspase activation and recruitment domains (CARDs) and C-terminal domain (CTD) mediated by casein kinase II and protein kinase C-α/β, respectively. (b) Following the binding of 5′ triphosphate (5′ppp) RNA and ATP hydrolysis, RIG-I is dephosphorylated by phosphoprotein phosphatase 1-α/γ and results in a conformational change that opens CARDs. HDAC6-mediated deacetylation of RIG-I CTD is critical for RIG-I and 5′pppRNA binding. The Lys63-linked ubiquitination of RIG-I mediated by TRIM25, Riplet, oligoadenylate synthetases-like protein, and MEX3C at both CARDs and CTD further activate RIG-I and facilitate its tetramerization. (c) Interactions between RIG-I–TRIM25 complex and 14-3-3ϵ promote RIG-I translocation to mitochondrial mitochondrial antiviral signaling protein (MAVS) for downstream signaling, leading to interferon production. Interactions between TRIM25, RIG-I, and MAVS are further negatively regulated by the Lys48-linked ubiquitination, which is meditated by LUBAC, RNF125, and RNF122. SEC14L1 and Atg5–Atg12 both inhibit the signaling by interrupting RIG-I–MAVS interactions, whereas SUMOylation promotes RIG-I–MAVS binding. Used with permission. CC BY 4.0. [165]

Upon viral infection, DDX60 acts as an ISG that helps the cell suppress viral replication by increasing vRNA and RIG-I/MDA5 interactions to enhance antiviral signaling and IFN production [166]. Additionally, DDX60 is able to promote RNA exosome-mediated degradation of HCV RNA, which as a first line of defense reduces cell stress from viral replication, but that in turn, produces degraded vRNA agonists likely to be recognized by RIG-I/MDA5 and other sentinels, and in a feed-forward mechanism enhance IFN signaling [167]. While additional studies are required to access the role of DDX60 against many viruses and across different cell lines, the first insight into its mechanism of action highlights two important features of sentinel RNA helicases: 1) they are able to directly bridge vRNA with RIG-I/MDA5 to potentiate antiviral signaling and 2) they are able to leverage upon their intrinsic cellular function to edit cytoplasmic vRNA (edit transcripts or remove accessory proteins) and ultimately turn them into immunostimulatory RNA molecules that are preferentially recognized by RIG-I/MDA5. Thus, overall, it can be reasonably generalized that DDX60 main function in antiviral immunity is amendable to its association with the RNA exosome. In this perspective, it is reasonable to propose that antiviral RNA helicases are involved in the larger picture of RNA-responsiveness where they balance the need for innate defenses against pathogens and actively restrict involuntary RLR pathway activation.

1.5.5. A role for RNA helicases beyond innate antiviral immunity

Up to this point, we have positioned the key players and mechanisms of antiviral innate immunity protecting the host from RNA viruses. We have shown that RNA Helicases are essential nucleic acid sensors that survey the cytoplasmic space for threats and, upon engagement, elicit type I IFN to restrict and abrogate viral replication. To be successful, RNA sensors and sentinels must discriminate between pathogenic and self-RNA. Otherwise, what seems to be an efficient and tightly regulated system for sensing pathogen-derived nucleic acids can rapidly become deleterious for the host cell. As an example, naturally occurring mutations of RIG-I and MDA5 have been associated with Type I interferonopathies in which RLR-receptors are constitutively active and lead to autoimmune diseases such as Aicardi-Goutieres syndrome (AGS) and Singleton-Merten syndrome (SMS) [168-171].

Similarly, SKIV2L, another Ski2 RNA Helicase evolutionally related to SNRNP200, mediates RNA exosome degradation of endogenous ligands blocking RLR activation, while recapitulate RIG-I's associated autoimmune disorders in SKIV2L-deficient patients [172]. This shows the importance of proper ligand recognition, conferred by the ATPase/Helicase domain of RLRs and sentinels, without which autoimmune disorders are likely to occur. Future studies will surely identify additional layers of control that limit the activation of sentinels and RIG-I/MDA5 by endogenous ligands. Lastly, there are growing evidences that RIG-I, and most likely other sentinels, are involved in cancer biology (as fully reviewed in [77]). As an example, RIG-I has been associated with therapy resistance and progression in breast cancer and in colorectal carcinoma due to improper activation by endogenous RNA or cellular cofactor snU1/U2 [173, 174]. In opposition, constitutive activation of RIG-I in hepatocellular carcinoma has been shown to induce IFN-mediated tumor suppression [175] .

Notwithstanding its exact contribution to cancer etiology, an interesting observation regarding RLR-signaling is the subject of intense research towards the development of anti-cancer medicine. Indeed, there has been an emerging literature that shows that cytoplasmic RNA helicases, such as RIG-I and MDA5, are implicated in the regulation of cell death by apoptosis and autophagy in a capacity that goes beyond the induction of IFN and pro-inflammatory cytokines. Indeed, the RLR pathway has been shown to regulate the activity of many caspases that are required to induce apoptosis [176-180]. Additionally, Chattopadhyay et al. recently published a thought-provoking series of reports that shows that, upon activation by the RLR and TLR pathway, IRF3 can mediate two complementary antiviral responses by promoting the canonical nuclear transcription of ISGs or by mediating the cytoplasmic RIG-I-like receptor-induced IRF3 mediated pathway of apoptosis (RIPA) (Figure 9) [181-186]. Briefly, these reports collectively show that upon activation, IRF3 can bind the pro-apoptotic protein Bax to translocate to the mitochondria and trigger cellular apoptosis via the release of cytochrome C.

Thus, IRF3 is implicated in two distinct signaling pathways that complement each other to provide an optimal antiviral response. Thus, a finer molecular understanding of the RIPA pathway holds the promise to chemically manipulate apoptosis in cancer and thus using the RLR-pathway to reverse non-viral pathogenesis. Current proof-of-concept studies include the usage of RLR and TLR agonist to induce tumor cell apoptosis [187].

While the exact role of RIG-I/MDA5 and sentinels in cancer remains to be elucidated, it will be important to fully characterize the impact of RNA helicase sensors on cancer initiation, progression and resistance to therapy as it paves the way for the development of a novel class of therapeutic agents that could modify the course of the disease and improve cancer treatment as adjunct therapy.

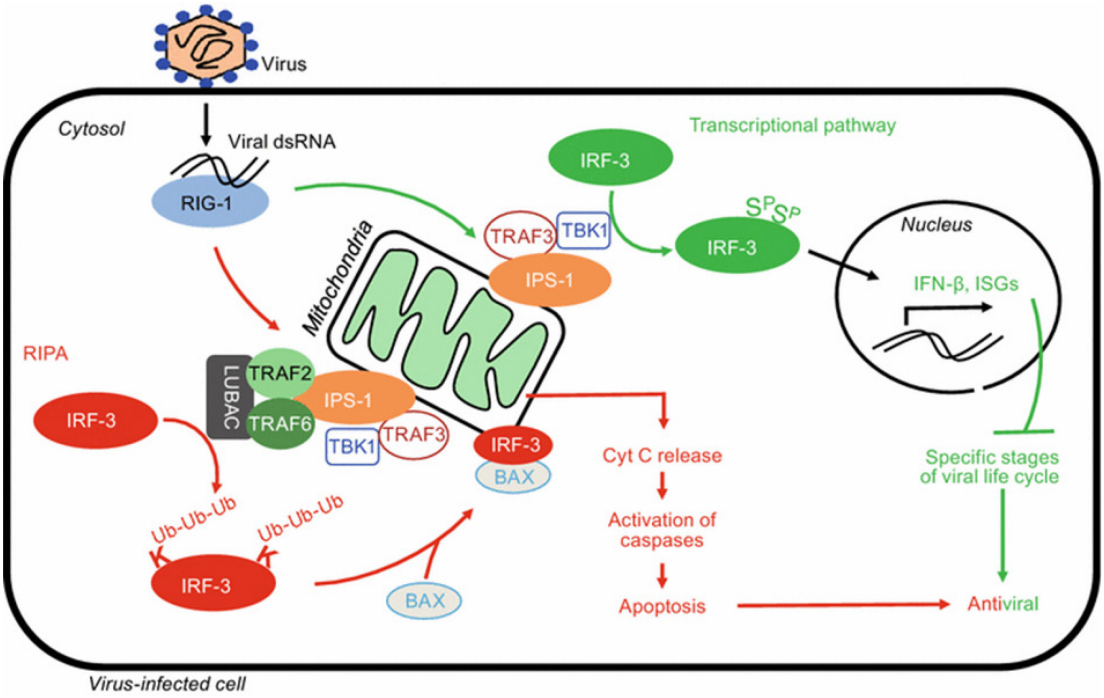


Figure 9. Dual functions of IRF3 in antiviral defense. Virus infection is recognized by the cytoplasmic sensor RIG-I, which binds to viral double-stranded RNA and triggers two signaling branches via mitochondrial adaptor IPS1. In the transcriptional pathway, IRF3 is translocated to the nucleus to induce antiviral genes, such as the interferon-beta (IFN-β) and interferon stimulated genes (ISGs). In contrast, in the RIPA branch, IRF3 is activated by LUBAC-mediated linear ubiquitination, which triggers its interaction with BAX to cause mitochondrial activation and apoptotic cell death. Both pathways contribute to the overall antiviral responses of the host. Used with permission. CC BY 4.0 [188]

1.6. Adaptors and effectors of the RLR-MAVS-IRF3 signaling

In Section 1.5., we have carefully reviewed the role of RNA sensors and sentinels in viral RNA detection. However, a detection system would not be very efficient without an efficient network of adaptor and effector proteins that relay a danger signal throughout the RLR pathway, raising the alarm against an invading pathogen and taking the appropriate steps to put a cell in an antiviral state. Interestingly, the same structural rationale was used to identify MAVS, which contain a CARD domain, as the signaling adaptor between RIG-I and IRF3/NF-κB. This links the mitochondria to the innate immunity and completes the framework of what is now referred to as the RLR (or RLR/MAVS) signaling pathway. In this section, we will focus on the role of the MAVS adaptor protein, the TBK1/IKBKE protein kinase and the transcription factor IRF3 to antiviral defenses. To do so, we will use current research to answer three questions:

1. How does the antiviral signal from the PRR travel to downstream effectors via the MAVS adaptor protein?
2. What are the roles of TBK1/IKBKE and IRF3 and how are they regulated?
3. What are the classical IRF3-dependent interferon stimulated genes?

1.6.1. Mitochondria: Command Centers of Antiviral Immunity?

In parallel with the discovery of RIG-I, MDA5 and LGP2, many groups tried to elucidate how an antiviral signal could travel from a pathogen recognition pattern (PRR) to downstream transcription factors and effector proteins such as IRF3/7 and type I IFNs. Consequently, many reports were published, almost at the same time, regarding the identification and characterization of MAVS, also called CARDIF and IPS-1 and VISA. Two reports comprehensively showed that MAVS is necessary for viral sensing and signal propagation [144, 146]. First, MAVS RNAi-mediated knockdown abrogates the antiviral signaling of Sendai and VSV infected HEK293, while its overexpression prior to infection potentiates antiviral response. This is measured by a decrease or an increase of RIG-I/IRF3/NF-κB dependent effector proteins such as IFN-β, RANTES and IFNα4/6 respectively. Second, using epistasis experiments, the authors could show that MAVS

signaling occurs downstream of RIG-I and upstream of TBK1/IKBKE—independent of TRIF, a TLR3 adaptor protein, as partial rescue of MAVS-depleted cells can only be achieved with the overexpression of MAVS itself or TBK1/IKBKE. Consequently, the gain of function, achieved via MAVS overexpression, is lost in TBK1$^{-/-}$-IKBKE$^{-/-}$ MEF but can be restored upon supplementation with TBK1 or IKBKE. Third, the protein biochemistry showed that MAVS C-terminal CARD and N-terminal mitochondrial signal-anchor (TM) domains are indispensable for their interaction with RIG-I and activation respectively. Activated MAVS, interestingly, was shown to be detergent resistant (1% Triton X-100), suggesting a spatial reorganization that is mediated by viral infection and most likely necessary for its signaling activity. Last, [145] showed that MAVS is specifically targeted by HCV NS3/4A that cleaves its transmembrane domain at the residue C508, resulting in a truncated protein lacking a mitochondrial anchor domain. As it turns out, the cleavage of MAVS by HCV NS3/4A disrupts its localization and impairs its ability to relay antiviral signaling [189]. Thus, this host-pathogen interaction results in a loss of antiviral response that cannot be rescued by overexpression of RIG-I or TBK1/IKBKE highlighting MAVS central role as the command center of the RLR pathway.

All in all, these initial observations conclusively showed that MAVS serves as an adaptor of antiviral signaling that links PRR (such as RIG-I) to downstream effector proteins (such as TBK1/IKBKE/IRF3 and, ultimately, type I IFN). The type I IFNs then trigger the activation of STAT1, STAT2 and IRF9, a transcription factor complex known as IFN-stimulated gene factor 3 (ISGF3) to induce many IFN-stimulated genes (ISGs). At that point in time, the next step was clear—more research was needed to understand the mechanism behind MAVS relocation and activation upon viral infection, and their contribution (if any) to the regulation of key transcription factors and effector proteins of innate antiviral activity.

1.6.2. MAVS, the "Mad Cow" of Innate Antiviral Immunity

To investigate the biochemical regulation behind RIG-I, MAVS and IRF3 activation, the research team of Dr. Zhijian Chen designed a cell-free system that would recapitulate a viral infection and allow for the easy *in vitro* monitoring of post-translational modifications (PTM) and signaling events. In short, whole cell homogenates from uninfected or infected cells are fractionated by centrifugation (nuclear, mitochondrial, cytosolic or supernatant fractions) and incubated with different components of the RLR pathway such as tagged-RIG-I, tagged-IRF3, viral or mimetic RNA, ATP and Ubiquitination proteins (E1, Ubc5, TRIM 25) to recreate an *ex cellulo* infection. For example, this method was used to identify and isolate the unanchored K63-polyubiquitin (polyUb) chains that are required to enable activation of RIG-I or IRF3 upon viral infection [96, 190]. In this section, we will discuss and analyze the results yielded from this system to understand the particularities of MAVS activation upon viral infection.

1.6.3. MAVS forms functional prion-like aggregates

The search for a mechanism that would recapitulate how MAVS gets activated upon viral infection would take the researchers down a very unexpected road. Indeed, Hou et al. (2011) [191] would uncover, using the previously discussed cell-free system, that following its initial association with RIG-I, MAVS gets converted into self-perpetuating aggregates. These fibrous aggregates are detergent and proteasome resistant and can convert inactive and unbound MAVS to an active conformation via a mechanism reminiscent of prions (Figure 10). To shift from an inactive to an active state, one must note that the first MAVS protein needs to interact with four active RIG-I (open conformation, ubiquitinated CARD), which is most likely to limit self-activation but achieve an initial hetero-pentameric conformation that allows for the recruitment of essential functional partners [192]. Once activated, MAVS prion-like aggregates can recruit TRAF2/3/5 that, in association with NEMO, becomes a ubiquitin-dependent signaling complex having the ability to prime other inactive MAVS, recruit TBK1 and IKBKE to propagate antiviral signaling downstream of the RLR pathway towards transcription factors such as IRF3/ NF-κB [193, 194].

Up to this point, we have discussed the role of MAVS from a very "IRF3-dependent / type I IFN" centric point of view. However, recent evidence shows that it might play a larger role in innate antiviral immunity. In fact, recent reports show that MAVS signaling occurs not only from the mitochondria but also from the peroxisomes [195, 196].

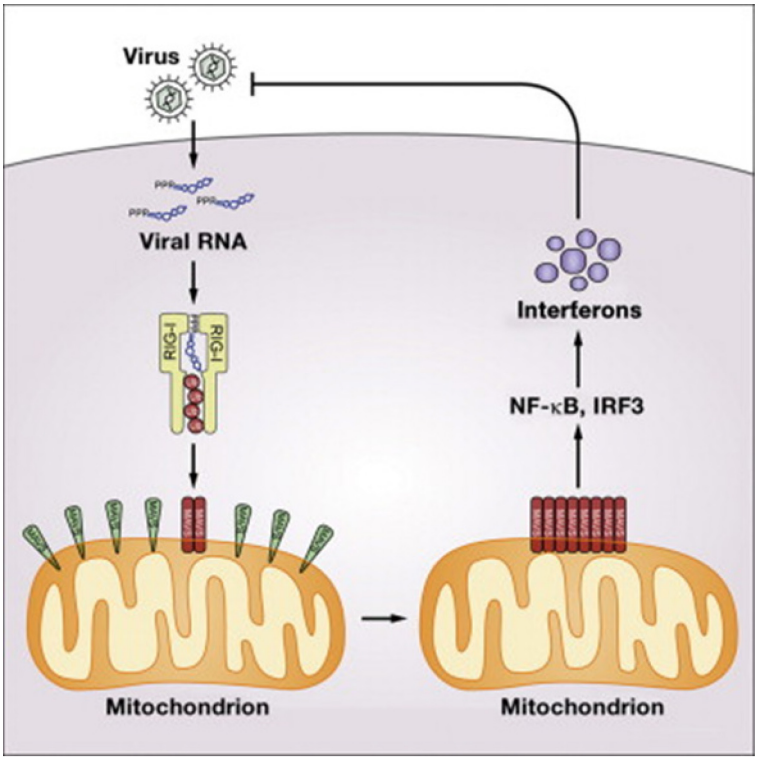


Figure 10. MAVS protein forms self-propagating fibrils (prions). In response to viral infection, RIG-I-like RNA helicases bind to viral RNA and activate the mitochondrial protein MAVS, which in turn activates the transcription factors IRF3 and NF-κB to induce type I interferons. RIG-I binds to unanchored lysine-63 (K63) polyubiquitin chains and that this binding is important for MAVS activation; however, the mechanism underlying MAVS activation is not understood. In this figure, a viral infection induces the formation of very large MAVS aggregates, which potently activate IRF3 in the cytosol. Indeed, a fraction of recombinant MAVS protein forms fibrils that are capable of activating IRF3. Remarkably, the MAVS fibrils behave like prions and effectively convert endogenous MAVS into functional aggregates. Additionally, in the presence of K63 ubiquitin chains, RIG-I catalyzes the conversion of MAVS on the mitochondrial membrane to prion-like aggregates. This suggest that a prion-like conformational switch of MAVS activates and propagates the antiviral signaling cascade. Used with permission. [191]

According to this model, upon engagement from RNA sensors, antiviral signaling is first transduced from MAVS-pex and then from MAVS-mito following a canonical pathway. The major difference being that MAVS-pex is resists promoting an IRF1-dependent, type III interferon response that provides short-term protection from viral pathogenesis while the cell establishes an IRF3-dependent, type I interferon response to induce a sustained antiviral state. Furthermore, it was also shown that MAVS-pex is the target of viral evasion strategies that are related to the viral arsenal tailored for MAVS-mito. Surely, HCV NS3/4A protease can relocate to and cleave both MAVS isoforms to counter the downstream antiviral response [197, 198]. Additionally, results from Bender et al. (2015) suggest that MAVS-pex also acts as a failsafe mechanism that, in MAVS-mito KO cells, can induce both type III and type I IFN in response to a viral challenge, but not vice versa. Thus, it is valid to ask ourselves about the role of type III IFN in the establishment of innate antiviral immunity (as fully reviewed in[199]).

Initially, type III interferons were thought to be an alternative group of three cytokines (referred as interferon-λ or, individually, IL28a (λ2), IL28b (λ3) and IL29 (λ1)) that acts in parallel to type I interferons to provide immunity from the viral infection [200, 201]. While this remains true today, additional research has shown subtle differences that suggest that the key role of type III interferons in antiviral immunity is most likely to dampen the pro-inflammatory properties that are associated with a type I IFN response [202-206]. Thus, by deploying two sets of interferons, the cell aims to strike a balance between viral restriction and a sequel-free survival. All-in-all, we have clearly established in this section that MAVS acts as the interface between PRRs and the TBK1/IRF3 dependent activation of effector proteins. Thus, the next step to better understand how a cell engages a virus is to further our understanding of the interplay between TBK1/IKBKE and IRF3, as it represents the last step in our signaling journey towards the induction of an antiviral state.

1.6.4. The Interplay Between TBK1/IKBKE and IRF3

The initial link between IRF3 and the RLR pathway cannot be untangled from the parallel characterization of TBK1 and IKBKE. Indeed, a lot has been learned about the function of these three proteins by studying their relationship using genomic, proteomic and molecular biology tools.

In this section, we will highlight the importance of TBK1 and IRF3 to initiate a type I interferon antiviral response following the recognition of viral nucleotides by PRRs and signal transduction by the adaptor protein MAVS. Following its initial description as a member of the IFN-β enhanceosome, IRF3 was subsequently characterized as a transcription factor that conferred an antiviral specialization to TLR3 [207, 208]. Certainly, upon stimulation with poly (I:C), Doyle et al. showed that IRF3 mediates early transcriptome of an NF-κB-independent subset of ISGs such as RANTES, IP10, ISG15, IFIT1/2/3, IRF7/IRF9, MX1 and PKR, which is essential for the induction and maintenance of a type I interferon antiviral response. The initiation of this gene program was subsequently showed to require IRF3's phosphorylation by TBK1 and/or IKBKE, followed by its nuclear translocation where it binds to an Interferon Response Element (ISRE) to mediate the transcription of antiviral genes via a mechanism like NF-κB and AP-1 [209-211]. Indeed, TBK1 knockout (KO) or knockdown (KD) MEF shows major defects in the antiviral response with hindered IRF3-dependent gene expression when challenged with Sendai virus or poly (I:C) that cannot be rescued by the expression of a kinase dead (K38A) TBK1 in contrast to WT TBK1. The link between IRF3 and the RLR pathway would then become apparent when Seth et al. (2005) identified MAVS as the adaptor protein that serves as a signaling relay between PRRs (such as RIG-I) and effector proteins (such as IRF3).

In summary, the transcription of interferon stimulated genes requires the activation of IRF3 downstream of the MAVS adaptor protein. This activation is mainly mediated by two protein kinases—TBK1 and IKBKE—that mediate IRF3 phosphorylation and the subsequent dimerization and nuclear import. Once in the nucleus, IRF3 can bind to specific Interferon-sensitive Response Elements (ISRE) to induce the transcription of ISGs. In the next sections, we will have a closer look at the finer molecular mechanisms at play at each of these major steps of the signaling pathway. From that point, multiple questions would need answers such as the following: What is the mechanism behind IRF3 activation? How are TBK1 and IKBKE regulated? Do all the antiviral pathways converge on IRF3? What is the result of IRF3 activation in terms of ISGs and interferon production? In the next sections, we will provide our answers to these questions using a broad conceptual framework that sets the tone for the next chapter of this thesis.

1.6.5. Key Points of TBK1/IKBKE and IRF3 Activation

The summary described in the last section might seem simple, but many signaling events must occur concurrently to allow for the activation of IRF3 following the engagement of a pathogen by viral RNA sensors. Imagine an orchestra playing your favorite opus—every musician must follow the partition to allow their instruments to shine at the right moment, following the tempo and the tune intended in the partition. The first step towards the activation of downstream effectors is the recruitment of TBK1 and IKBKE to the adaptor protein MAVS. Upon viral recognition, RIG-I will reorganize into active oligomers that will convert MAVS into prion-like structures. This will allow for the recruitment of TRAF2/5/6, three E3 ubiquitin ligases, at the MAVS adaptor protein [143, 190, 192, 193]. Once assembled, this RIG-I/MAVS/TRAFs complex will be ready to recruit TBK1 and IKBKE and mediate its activation via the transfer of K63 polyubiquitination chain [193, 212]. Once activated, TBK1 and IKBKE will then licentiate the signaling complex for IRF3 recruitment and activation by phosphorylation of the adaptor protein MAVS at a consensus motif, pLxIS, that is also found at the c-terminus of other innate immune adaptor proteins such as TRIF and STING [213]. MAVS is then able to bind to positively charge IRF3 and, in tandem with TBK1 and IKBKE, phosphorylate this transcription factor to induce its dimerization and nuclear translocation (Figure 11)

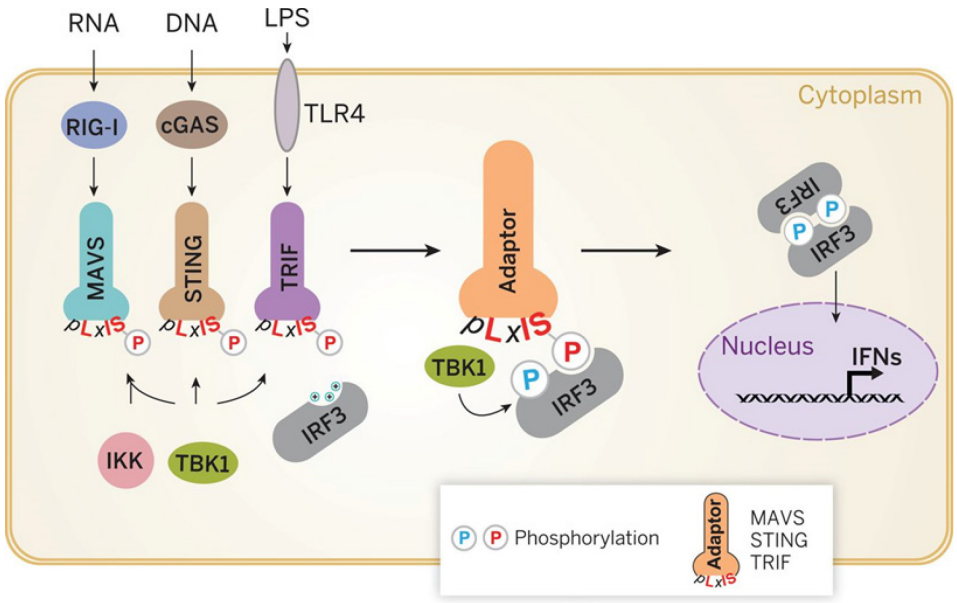


Figure 11. Phosphorylation of innate immune adaptor proteins licenses IRF3 activation. MAVS, STING, and TRIF—which are activated by viral RNA, cytosolic DNA, and bacterial lipopolysaccharide (LPS), respectively—activate the kinases IKK and TBK1. These kinases then phosphorylate the adaptor proteins, which in turn recruit IRF3, thereby licensing IRF3 for phosphorylation (P) by TBK1. Phosphorylated IRF3 dissociates from the adaptor proteins, dimerizes, and then enters the nucleus to induce IFNs. Used with permission. [213]

The phosphorylation of IRF3 is tightly regulated and mostly occurs within its C-terminal serine-rich region (SRR). Module 1 sites or 2S sites are located on Ser385/Ser386, while Module 2 sites or 5ST sites are located on Ser396/ Ser398/Ser402/Thr404/Ser405/ [214-217]. According to the structure-based canonical model, the first phosphorylation events take place at the 2S sites that relieve the IRF3 auto-inhibition conformational blockade of its C-terminal repressor domain (RD) and subsequently exposes Ser396/Ser398 of the 5ST sites to allow for their phosphorylation [218, 219]. Reciprocally, the dual phosphorylation-dependent switch model shows that phosphorylation at 5ST occurs prior to the phosphorylation at the 2S sites and suggests that phosphorylation of 5ST residues enable transition from an inactive to an active conformation; also, once in an active state, the 2S sites are available for phosphorylation and activation [220].

Once phosphorylated and activated, IRF3 is ready to dimerize and translocate to the nucleus, where it sequentially binds to CBP/p300 and ISRE to allow for the transcription of ISGs [214, 221-223]. So, one might wonder, what phosphorylation event is the key for IRF3 activation?

To answer this question, let us examine evidence from some functional studies. First, mutagenesis assays have shown that if IRF3's Ser385 and Ser386 are substituted for a neutral amine like Alanine, it completely blocks IRF3 activation—most likely due to the loss of phosphorylatable residue mediating the relief of IRF3 auto-inhibition [214]. On the other hand, mutations of the same amino acids with other polar residues like Asparagine also abrogate IRF3 activation by changing the recognition sequence required for its interaction with the licentiated MAVS complex [215]. Second, mutation of the 5ST sites either hinders or constitutively activates IRF3 following neutral (alanine) or phosphomimic (aspartic acid) substitution respectively [215, 221, 224, 225]. It was later shown that the minimal phosphorylation event that is required to fully activate IRF3 occurs at Ser396, and that a mutant bearing a Asp396 constitutively dimerizes, translocates and associates with CBP/p300 and ISRE [226]. Third, a recent study by a pioneer of IRF3 biology suggested that other phosphorylatable residues, such as Ser339, might also potentiate IRF3 activity or act as a back-up mechanism for activation if Ser385/386/396 are inefficiently activated due to the deployment of a viral evasion strategy [227]. All in all, it can be concluded that from a conservative point of view, IRF3 activation heavily relies on the efficient phosphorylation of Ser385/386 and Ser396 by TBK1/IKBKE for efficient antiviral activity. Lastly, while neither of the two proposed models of IRF3 activation have led to a consensus in the field, they highlight a very sophisticated system that relies on many redundant phosphorylation sites, which most likely enables a context specific (type of virus infection, kinetics of response) antiviral response. The next step in understanding the activation of downstream effectors is gaining a comprehensive understanding of the positive and negative regulation mechanisms that promote or alleviate IRF3 and TBK1/IKBKE antiviral activity.

1.6.5.1. Positive and Negative Regulation of IRF3 Activation

As one might imagine, RLR signaling must be tightly regulated to promote a robust antiviral response that can be balanced throughout the infection and terminated upon the clearance of the pathogen. Additionally, many mechanisms must be at play simultaneously to avoid the viral hijacking and evasion of key signaling proteins. In this section, we will analyze the regulatory mechanisms pertaining to IRF3 and TBK1/IKBKE activation and come to understand that they mostly rely on protein-protein interaction, which are governed by viral-induced post-transcriptional modification (PTM). Notably, many other conventional and non-conventional PTMs (phosphorylation, ubiquitination, methylation, acetylation, SUMOylation, ADP-ribosylation & glutamylation) are involved in the regulation and activation of innate antiviral signaling (as review in [228, 229]) However, we chose to focus on the mechanisms that are important to understand the work presented in this thesis. To facilitate our understanding, we will treat TBK1/IKBKE activation concurrently, as they are almost inseparable—if TBK1 activation is prevented, it will ultimately hinder IRF3 stimulation and vice versa. A summary is provided in Table 1.

1.6.5.2. Positive Regulation – Facilitation of the Recruitment at Adaptor Protein

To induce a rapid and robust antiviral response, a cell must be able to bring together RIG-I, MAVS and NEMO-TRAF2/5/6 into a signalosome that is geared towards the recruitment of TBK1 and IRF3. While this complex alone can do the job in a cell-free context, many accessory proteins seems to potentiate this recruitment in cellulo. For example, TRIM26 can serve as a bridge between TBK1 and NEMO to optimize an RNA virus-induced MAVS complex [230]. Another TRIM protein, TRIM32, can potentiate MAVS recruitment of TBK1 by contributing K63 polyubiquitination chains at residue K20/150/224/236, which promotes the interaction and activation of TBK1 [231]. To complement, DDX3 can act as an effector protein of TBK1 by mediating its Ser172 (NAK) phosphorylation, which is classically the hallmark of its activation, and thus it potentiates IRF3 activation [232]. All things considered, we could identify 14 proteins that potentiate IRF3 activation by promoting their recruitment at the adaptor protein mainly via ubiquitination or phosphorylation events (Table I).

1.6.5.3. Positive Regulation – K63 Poly-Ubiquitination

According to the classical model, K63 poly-ubiquitination chains are used by the MAVS adaptor protein to recruit TBK1, allowing for its activation and the subsequent phosphorylation of IRF3[193, 233, 234]. However, recent reports have shown the role of one other protein that can contribute to this mechanism by itself. As a matter of fact, RNF128 is upregulated upon both RNA and DNA virus infection and contributes, in a feed-forward fashion, to the K63 ubiquitination of TBK1. This facilitates its subsequent activation, which in turn potentiates IRF3 activation [235]. While this kind of mechanism was only reported once, it provides a interesting perspective on the possible regulatory mechanism of IRF3 activation.

1.6.5.4. Positive regulation – Misc.

Other positive regulation mechanisms of IRF3 activation have also been described. In one report, AGO2, an Argonaut family protein involved in the processing of RNA and microRNA, was shown to compete with IRF3 for CBP/p300 binding [236]. Upon viral infection, AGO2 is exported out of the nucleus to reduce the competition with IRF3, and thus facilitate its association with CBP/p300, consequently potentiating its binding to ISRE. In a completely unrelated mechanism, the short isoform of TRIM9 was characterized as a bridge between GSK3β and TBK1 to mediate its phosphorylation and facilitate the subsequent activation of IRF3 [237]. Interestingly, TRIM9 also seems to skew, via an unknown mechanism, the antiviral signaling towards a discrete IRF3-dependent gene programming—evidenced by a dose-dependent increase of type I interferons but not of NF-κB-mediated pro-inflammatory cytokines. Overall, these two mechanisms highlight the fact that the regulation of IRF3 activation goes beyond what happens at the adaptor protein.

1.6.5.5. Negative Regulation – K48 Poly-Ubiquitination

A major mechanism of negative regulation of IRF3 activation is the targeting of TBK1 for degradation via a K48 poly-ubiquitination mechanism. Overall, OPTN, SIGLEC1 , FOXO1, NLRP4/UPS38, RBCK1 and RAUL govern TBK1 degradation, acting as a negative regulator of antiviral signalling [140, 238-245]. However, as of now, the order of

magnitude and the kinetic of each individual mechanism remains unclear and additional research will be required to characterize if these negative regulators act as a constitutive or induced negative feedback loop across cell lines. As an example, SOCS3, another protein that targets TBK1 for K48 polyubiquitination degradation targets only its activated state (pSer172) suggesting that SOCS3 is involved in a feedback mechanism that is there to prevent or balance immune signalling [246]. Notwithstanding the limitations of these studies, it can be agreed that K48-mediated degradation of TBK1 represents a major negative regulation mechanism.

1.6.5.6. Negative Regulation - Competitive Phosphorylation

A novel negative regulation mechanism involving competitive phosphorylation was recently reported, which provides novel ideas behind the regulatory aspect of IRF3 activation. Indeed, INKIT (C7ORF41) was shown to be induced upon viral infection (SEV, HSV-1 and VSV) and compete with IRF3 for phosphorylation by TBK1 and IKBKE, resulting in a dose-dependent decrease of type I interferon response [247]. Consequently, the silencing or deletion of INKIT resulted in a potentiated antiviral response. This is an elegant and unique example of a viral evasion mechanism that aims to actively hinder antiviral signaling.

1.6.5.7. Negative Regulation – Inactivation via Phosphorylation

There is another type of regulatory mechanism that closely follows the main activation mechanism but has the opposite effect. In fact, three recent reports have shown that phosphorylation of TBK1 (Tyr354/394) and IRF3 (Thr75/Thr253; Ser97) by LCK/HCK/FGR, MST1 and PTEN respectively results in the inactivation of TBK1 or a prevention of IRF3 dimerization and subsequent nuclear import [248-250]. This is an interesting perspective into IRF3 activation, as phosphorylation is generally thought of as a positive post-transcriptional modification that mediates the progression of antiviral signaling.

1.6.5.8. Negative Regulation – Formation of an Inhibitory Complex

A competitive kind of negative regulation of IRF3 activation comes from the formation of inhibitory complexes that sequester TBK1 and/or IRF3 and limit its availability for antiviral signaling. For example, upon viral infection, ERRα and TRIM11 associates with TBK1, preventing its association with IRF3 and resulting in a hindered antiviral response [103, 251]. This is reminiscent of the constitutive interaction between YAP and IRF3/TBK1 that prevents unnecessary activation and is relieved by the IKBKE mediated phosphorylation of YAP upon viral infection [252]. While the kinetic and magnitude of these complex formation remain to be elucidated, it nonetheless shows that the spatial sequestration, into larger protein complexes, of both IRF3 and TBK1 seems to play a role in the negative regulation of their activation in resting cells. It also plays a role upon viral infection in what might be a viral evasion mechanism or simply a negative feedback loop aimed at balancing immune activation.

1.6.5.9. Negative regulation – Misc.

Ultimately, many other negative regulation mechanisms seem to negatively mediate IRF3 activation (Table 3). As a selected example, a recent report showed that FAF1, a protein containing many ubiquitin-related domain, can disrupt IRF3 nuclear translocation by reducing its interaction with IPO5 and Importin-β3—two proteins implicated in IRF3-dimer nuclear import [253]. This results in a dose-response abrogation of type I interferon response to poly (I:C) or RSV. This negative regulation mechanism of IRF3 activation is referred to in a recent report, which I co-authored, showing that many importin-β (IMPβ) receptors, importin-α adaptors and nucleocytoplasmic trafficking accessory proteins are required for the efficient nuclear import of IRF3 into the nucleus following an SeV infection; also putting into perspective that many of them are the direct target of viral evasion mechanisms [254]. For instance, we showed that HCV NS3/4A triggers the cleavage of IMPβ1 and inhibits the nuclear transport of IRF3 into the nucleus to disrupt the interferon production. While this research question remains open-ended and complex to address, it still shows that the nuclear import machinery most likely constitutes a major hub of negative, but potentially positive, regulators of IRF3 activation.

Overall, it can be concluded that the regulation of IRF3 activation is governed by many distinct mechanisms that, for the most part, rely on the post-translational modifications (PTM) of TBK1/IKBKE and IRF3. While our review has focused mainly on phosphorylation and ubiquitination, it cannot be ignored that many other PTM, such as methylation, acetylation, SUMOylation, ADP-ribosylation and glutamylation, oversee the activation of IRF3 and the overall RLR-pathway (as reviewed in [228, 229, 255]. At this time, one last question remains to be examined: what is the endpoint of IRF3 activation?

Table I. Positive and negative regulators of TBK1, IKBKE and IRF3.

Signaling protein	Target	Functions	Ref.
Positive regulation – Facilitate the recruitment at adaptor protein			
TRIM26	TBK1	• Serves as a bridge between TBK1 & NEMO that is required for optimal recruitment of TBK1 to the virus-induced MAVS signalosome	[230]
RIOK3	TBK1 IRF3	• Act as a downstream adaptor protein of TBK1 and IRF3 to facilitate MAVS & STING activation of type I interferons	[256]
FYN and SRPK1	IRF3	• Promotes an interferon-independent transcription of IFN-λ1 and CXCL10 that, in turn, increase IRF3 phosphorylation and contribute to an enhanced antiviral response	[257]
TRIM32	TBK1	• Indirectly promotes the recruitment of TBK1 at the adaptor protein MAVS & STING by increasing its ubiquitination upon viral infection	[231]
WDR5	IRF3	• WDR5 is recruited to the adaptor protein MAVS where it promotes IRF3 activation via an unknown mechanism	[258]
TOM70	TBK1 IRF3	• TOM70 acts as a bridge between the adaptor protein MAVS, TBK1 and IRF3 and, as such, is required for optimal antiviral signaling	[259]

SINTBAD, NAP1 & TANK	TBK1 IKBKE	• SINTBAD, NAP1 & TANK promotes the recruitment of TBK1 and IKBKE to MAVS adaptor protein via a direct interaction with TBK1/IBKBE binding domain (TBD)	[260-262]
NEMO	TBK1	• NEMO recruits K69, K154 and K372 ubiquitinated TBK1 to the adaptor protein MAVS where it can be fully activated and phosphorylate IRF3	[263]
DDX3	TBK1	• DDX3 is a critical effector of TBK1 phosphorylation and is, as such, required for IRF3 activation	[232]
TBK1/IKBKE	IRF3	• Original research papers that shows that IKBKE and TBK1 are the protein kinases responsible for IRF3 canonical phosphorylation and activation	[209, 264, 265]
Positive regulation – K63 poly-ubiquitination			
RNF128	TBK1	• RNF128 directly interacts with to catalyze the K63 polyubiquitination of TBK1, which leads to TBK1 activation	[235]
Positive regulation – Misc.			
AGO2	IRF3	• AGO2, which competes with IRF3 for CBP/p300 binding, is targeted to nuclear export upon viral infection to facilitate IRF3 signaling	[236]
TRIM9; short isoform	TBK1 IKBKE	• TRIM9 is K63 ubiquitinated, upon viral infection, and subsequently act as a bridge between GSK3β, a protein kinase, and TBK1 to promote its activation	[237]
Negative regulation – K48 poly-ubiquitination			
OPTN	TBK1	• OPTN recruits TBK1 and TRAF6 into an inhibitory complex aimed towards IRF3; Also, it bridges TBK1 with CYLD to promote its k48 polyubiquitination degradation. However, OPTN is required for optimal IRF3 activation suggesting that it plays a dual role, as a positive and negative regulator of innate antiviral immunity.	[239-241]

SIGLEC1	TBK1	• Siglec1 associates with DAP12 and SHP2 to recruit TRIM27 that induces TBK1 degradation via K48 ubiquitination of Lys25	[238]
FOXO1	IRF3	• Promotes IRF3 K48 polyubiquitination and degradation	[242]
NLRP4/UPS38	TBK1	• UPS38 via NLRP4 targets TBK1 for degradation by recruiting DTX4 and TRIP, an E3 ubiquitin kinase, to TBK1 leading to its K48 polyubiquitination at Lys670	[140, 243]
RBCK1	IRF3	• Promotes IRF3 K48 polyubiquitination and degradation	[245]
RAUL	IRF3	• Promotes IRF3 K48 polyubiquitination and degradation	[244]
SOCS3	TBK1	• SOCS3 promotes k48 polyubiquitination of TBK1 at Lys341/344 to induce its degradation. Interestingly, it seems to be involved in a feedback mechanism as it only targets activated TBK1 (pSer172)	[246]
Negative regulation - Competitive phosphorylation			
INKIT	IRF3	• INKIT is competitively phosphorylated by TBK1/IKBK1, and thus, decrease the magnitude of IRF3 activation	[247]
Negative regulation – phosphorylation for inactivation			
Lck/Hck/Fgr (SFKs)	TBK1	• Lck/Hck/Fgr directly phosphorylate TBK1 at Tyr354/394, to prevent TBK1 dimerization and activation.	[248]
MST1	IRF3	• Mst1 interacts and directly phosphorylates with IRF3 at Thr75/Thr253 to disrupt its dimerization and occupancy on chromatin	[249]
PTEN	IRF3	• PTEN directly phosphorylate IRF3 at Ser97 to prevent its dimerization and subsequent nuclear import	[250]
Negative regulation – Formation of an inhibitory complex			

ERRα	TBK1 IRF3	• Prevents the formation of functional TBK1-IRF3 complex	[251]
YAP	IFR3 TBK1	• YAP constitutively binds IRF3 and TBK1 to prevent antiviral activation; upon infection, YAP-mediated inhibition is relieved by IKBKE (IRF3) and Lats1/2 (TBK1)	[252]
TRIM11	TBK1	• Directly interacts with TBK1 and TBK1 enhancer complex proteins (NAP1, SINTBAD & TANK) to hinder TBK1 ability to phosphorylate IRF3	[103]
SIKE	TBK1	• SIKE binds TBK1 with high affinity to block its activation upon viral infection	[266]

Negative regulation – Misc.

FBXO17	IRF3	• FBXO17 recruits PP2A, a protein phosphatase, to IRF3 to induce its dephosphorization and, thus, promote its deactivation	[267]
DDX25	IRF3	• Promotes IRF3 cytoplasm localization via IκBα	[268]
PPM1a	TBK1 IKBKE	• PPM1a disrupts the recruitment of TBK1/IKBKE to the adaptor protein MAVS by promoting their dephosphorylation	[269]
RFN11	TBK1 IKBKE	• RNF11 attenuates K63 polyubiquitination of TBK1 by blocking TBK1-TRAF3 interaction, and thus, hinders TBK1 activation	[270]
TRIM59	IRF3	• TRIM59 inhibits IRF3 phosphorylation via an unknow mechanism	[271]

1.6.6. Upstream Divergence, but Downstream Convergence of Antiviral Immunity

Up to this point, we have gone over all the major signalling events of the RLR pathway. In this section, let us consider two aspects of antiviral immunity First, we need to understand that despite the diversity of upstream sensor and adaptor proteins, only a few downstream effectors are in play to modulate the antiviral gene expression. Second, and last, we need to be able to position the key ISGs and to understand their role in viral restriction and clearance. As we have seen in Figure 1, there are many antiviral signalling pathways that can be differentiated from one another based on their recognition of specific pathogen-associated molecular patterns (PAMP) or their spatial distribution inside the cell. In short, the RIG-I/MAVS pathway is specialized in the recognition and elimination of cytosolic RNA viruses, the TLR3/TRIF and TLR7/MyD88 pathways are geared toward the detection of endosomal RNA viruses, while the cGAS/STING pathway is useful to engage cytoplasmic DNA viruses. However, despite that upstream divergence, it can be observed that all the antiviral pathways converge on a limited set of downstream transcription factors, namely IRF3/7 and NF-κB (p65/p50). Once activated, theses transcription factors will mediate the first wave of antiviral defences by initiating the transcription of type I interferons (interferon α/β) and pro-inflammatory cytokines. Then, in an autocrine and paracrine fashion, these interferons will pledge to stimulate the second wave of antiviral defences through the cytoplasmic receptors and downstream effectors of the interferon amplification loop (IFNAR1/2-JAK-STAT1/2-IRF9 pathway). As such, the cell antiviral programming will be the same: notwithstanding the origin of the initial stimuli or the signalling pathway used to turn on the antiviral defences, it will beard the molecular signature of a type I antiviral response. So, what are the genes that are associated with a type I antiviral response?

1.7. Snapshot about Interferon Stimulated Genes

1.7.1. IFITs: Classical Marker of type I interferon antiviral immunity

A major family of ISGs are the ISG56/IFIT1 family of genes that are made of four distinct proteins namely ISG56/IFIT1, ISG54/IFIT2, ISG60/IFIT3, and ISG58/IFIT5 (as fully reviewed in [272].

Upon viral infection, IFITs are induced, at first, independently of interferons and, subsequently, reinforced via the JAK/STAT mediated interferon amplification loop [273, 274]. Interestingly, ISG54/56 ISRE are preferentially recognized by IRF3, and as such, they are commonly used as surrogate markers of IRF3 activation and transcription of antiviral genes [181, 275]. IFITs are direct acting antiviral proteins that mainly act by surveying the cytoplasm and binding to viral nucleic acids or host and pathogen cellular factors to block viral replication and translation [276-278]. As an example, IFIT1 can recognize and bind to viral 5'ppp-RNA, with a nano-molar affinity, making it an extremely efficient and broadly active antiviral protein. However, some viruses, like the West Nile virus, are able to mask their molecular signature by adding a 2'-O methylation to the 5' cap of their viral RNA to efficiently evade detection by the IFIT defense system [279]. Lastly, IFITs play a role in the feedback inhibition of the IFN-β induction by disrupting the interaction between MAVS or STING and TBK1 and, thus, dampening IRF3 activation to prevent over activation of antiviral programming [280]. Thus, for all these reasons, it can be agreed that IFITs, and especially ISG54/56, are excellent prototypical ISGs that can be used as a marker of type I interferon antiviral immunity.

1.7.2. Beyond the Nomenclature: Key Function of Interferon Stimulated Genes

As one can imagine, a viral infection can induce major cellular transcriptional variation as the cell transition from a resting to an antiviral state. As experimentally demonstrated using systems biology approaches and high-throughput methods, ISGs are identified using functional and biological prioritization to start from a list containing thousands of candidates genes and ending up with a handful of well-characterized antiviral proteins

[281]. As summarized by [282], the main functions of ISGs are to restrict viral replication, balance viral recognition by PRRs, balance downstream activation of transcription factors, such as IRF3, and balance the induction of the interferon amplification loop (Figure 12). However, this regulation comes at a price, because ISGs can also be co-opted and hijacked by viral pathogens to promote and support their lifecycle. Thus, while antiviral response to a specific viral pathogen might elicit the transcription of specific ISGs, it can be accepted that the major effector proteins will overlap and be representative of a type I interferon signature.

Figure 12. Diverse roles for ISGs in the IFN antiviral pathway. Incoming viruses are sensed by pattern reecognition receptors (PRR), leading to activation of interferon regulatory factors (IRFs) and transcriptional induction of IFNs. Antiviral IFNs signal through the JAK/STAT pathway to induce ISG production. ISGs can also be directly induced by some IRFs in an IFN-independent pathway (thin blue arrow). Some ISGs function to block virus replication (thick red bars), while others have the ability to promote or enhance replication of certain viruses (green arrow). A subset of ISGs are themselves components of the IFN pathway or promote its signaling (red dotted arrows). IFN also induces several negative regulators which can target PRR, IRFs, or JAK/STAT to dampen the response (thin red bars). Used with permission. [282]

To characterize the unique molecular signature of RIG-I-dependent antiviral responses, Goulet et al. (2013) used a system analysis of the genes induced early (6 hrs.) and late (24 hrs.) following the activation of the RLR pathway using 5'pppRNA as a viral surrogate [283]. In short, they showed that, early upon stimulation, 5'pppRNA induced the transcription of a diverse set of genes related to IRF, NFKB and STAT signalling, type I and III interferons as well as pro-inflammatory cytokines. Overall, the early activation of the RLR pathway showed a signature compatible with the establishment of an antiviral state, with a representative member of the pathway being upregulated, such as IRF1/3/7/9, STAT1/2, DDX58, DDX60, TRIM25, MAVS, OAS1/2/3/L, IFIT1/2/3 and IFNB1/IL28A/IL28B/IL29.

The late response was mostly characterized by a signature compatible with a sustained activation of the RLR pathway and functionally characterized by the induction of PRR signalling, ubiquitin signalling, apoptosis and STAT/NFKB signalling. While the two sets of data overlap, the late time point showed a dramatic upregulation of key players of the interferon amplification loop, such as STAT1/2, as well as a signature of cellular apoptosis and stress, compatible with a transition from early RIG-I-mediated signalling to an antiviral state that is now governed by interferons and ISGs. All-in-all, this shows that many of the key players of the antiviral immunity that we have described in the previous sections are ISGs themselves and that, as such, antiviral defences (ISGs) can be described as a two-prong system that is constantly balanced for efficient antiviral signalling and effective viral restriction.

1.8. Conclusion

The induction of innate antiviral defenses requires an efficient network of secondary messengers that are able to relay the signal of viral engagement from the activated PRRs all the way down to the transcription factors responsible for the initiation of a cellular antiviral state. In this chapter, we have described and analyzed various aspects of the innate antiviral immunity. For the RLR-pathway, we have seen that this pathway relies on key signalling events, such as the efficient formation of MAVS prion-like structure, the recruitment of MAVS-complex accessory proteins such as TRAF2/3/5 and NEMO, the

licensing of MAVS by TBK1 for IRF3 activation, the proper dimerization of IRF3, nuclear import and association of IRF3 with CBP/p300 and the robust induction of ISGs.

We have highlighted that throughout the pathway, signalling events are mediated by many post-translational modifications such as phosphorylation and ubiquitination. Lastly, we have seen that interferon stimulated genes are diverse in functions and origins, but all aim to support antiviral signaling, viral restriction, translational arrest and apoptosis. However, some limitations in our understanding of the regulation of the pathway still exists and additional research will be required to fully understand the finer molecular mechanisms at play during an antiviral immune response.

Further studies using a system-based approach, similar to the one used to identify KHSRP, together with the understanding of the nature of ligands and inhibitors of PRRs should provide additional knowledge to identify novel approaches for treatments and vaccine preparations directed against RNA viruses and beyond, in autoimmune diseases and cancers [34, 284]. Moreover, the potential ability of RNA viruses to interfere with the mechanisms regulating the signaling of these PRRs in order to escape detection necessitates more investigations. Additionally, with the description of a myriad of novel host factors involved in RLR signaling, one might wonder which components (RNA sensors, sentinels, positive and negative regulators) are required for the minimum or the optimal antiviral response, and what are the differences in this hierarchy according to cell type or pathogen. Also, there is a coordination between TLRs and RLRs, as seen in some autoimmune diseases and viral infections [285-288]. The mechanisms that control this cooperation in detecting RNA viruses, and the consequences of such collaboration deserve to be investigated in more depth. Lastly, PRR-targeting therapies have gained great momentum in the field of cancer immunotherapy. Recent reports have shown that RIG-I activation can induce tumor cell death directly via the production of IFN, or indirectly via the activation of cytotoxic CD8 T cells and NK cells, and via DC-mediated antigen cross presentation of tumors associated antigens to CD8 T cells [68, 224]. In addition, the modulation of TLR3 and 7 can be leveraged as anticancer therapies since their signaling can increase cytotoxic T-cell activity and directly induce cancer cell death via apoptosis,

pyroptosis, and autophagy. Thus, the recent advances in our understanding of innate antiviral immunity have clearly given a new momentum towards the development of therapeutic agents targeting PRR for infectious diseases and cancers. These strategies are in the pre-clinical or early clinical phase such that it is still unknown if these PPR-targeting agents will translate into effective, safe and tolerable anti-cancer therapeutics. ***A complete review of this perspective is provided in Annex A of this thesis.***

2.Background information on my main research project

2.1. Preliminary results

To identify new regulators of antiviral innate immunity, Dr. Lamarre's research unit completed the first genome-wide gene RNAi screen assessing the transcriptional response at the interferon-β (IFNB1) promoter following Sendai virus (SeV) infection [289]. To do so, the lab used the MISSION TRC shRNA lentiviral library (Sigma-Aldrich, MI, USA) made of about 75,000 individual lentivirus clones across 15,000 genes. Using a validated high throughput screening assay, HEK 293T cells expressing the IFNB1 promoter coupled to a luciferase reporter gene were transduced using a pool of three individual shRNA and, after 72 hours, challenged with SeV virus, a specific RIG-I agonist. If the silencing resulted in a "hit" (25% change in IFNB1 activity), each individual gene was retested using five individual shRNA and, after 72 hours, challenged with SeV virus or transfected with polyinosinic:polycytidylic acid (polyI:C), MAVS- or IRF3(5D)-expressing plasmids. The transfection of these constructs, in addition to viral infection, was designed as a secondary screen to assign a putative role of the candidate regulator in the RLR pathway based on the activation/inhibition pattern. Overall, this study yielded 114 gene hits (correlation between the primary/secondary and qPCR tertiary screen) with 59 gene hits additionally validated for knock-down specificity (Figure 13).

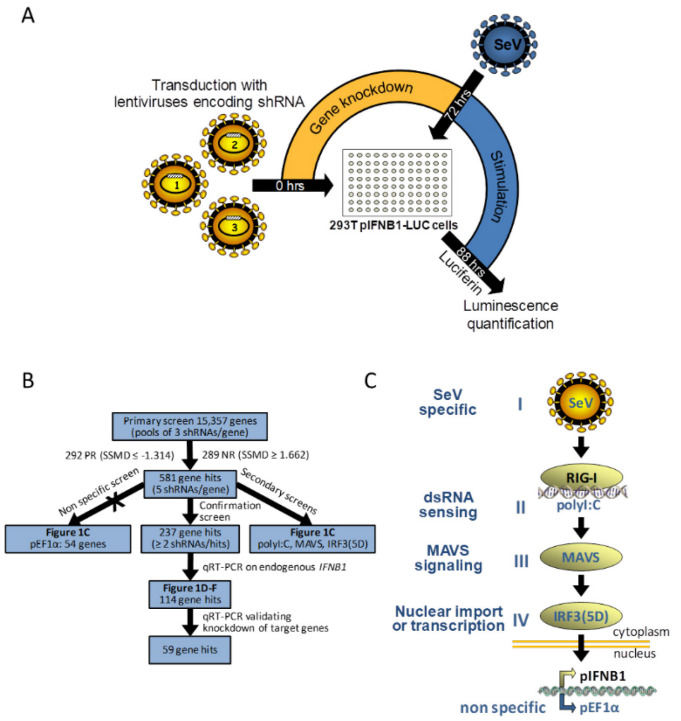


Figure 13. Genome-wide gene silencing study of virus-induced innate immune responses and bioinformatics analyses. (A) Schematic representation of the primary genome-wide screen and secondary screens. HEK 293T cells stably expressing the luciferase gene under the control of the IFNB1 promoter were transduced with arrayed lentiviruses combining three shRNAs per gene (primary screen) or with five individual shRNA-expressing lentiviruses for each gene hit (secondary screens) in a 96-well format. After 72 hours, cells were challenged with SeV virus (primary and confirmation screens) or transfected with polyI:C, MAVS or IRF3(5D) (secondary screens) for 16 hours before measuring IFNB1 promoter-driven luciferase activity. (B) Decision tree of primary screen and summary data of gene hits obtained in secondary and validation screens. Selected gene hits (114) that were confirmed and validated with endogenous IFNB1 screens by qRT-PCR induced a modulation of more than 25 % of the IFNB1 promoter activity with at least two independent shRNAs following SeV infection. Prioritized gene hits (59) for which knockdown of the target gene was greater than 40% with two independent shRNAs are also identified. (C) Schematic representation of confirmation and secondary assays for epistasis analysis of gene hits acting on the signaling cascade leading to IFNB1 production. SeV infection (primary and confirmation screens), polyI:C (dsRNA mimetic), MAVS or IRF3(5D) transfection (secondary screens) were used to activate innate immune response. A non-specific assay was used to discard gene hits affecting nonimmune-related transcription by measuring transcriptional activity of EF1α constitutive promoter.

Interestingly, a Gene Set Enrichment Analysis (GSEA) of the 114 gene hits revealed that many of these proteins were spliceosome-associated. In fact, with a fold enrichment of 8.5 ($p = 3.9e-5$), it was the most significant functional group identified among the gene set. Based on these results, our group decided to prioritize the characterization of these spliceosomal factors. Preliminary studies to assess the immune function of these proteins (SNRNP200, SF3A1, NHP2L1 and PHF5A; SFRS1 not shown) confirmed that they were most likely to play the role of a positive regulator of antiviral signalling as their silencing in SeV infected cells completely abrogated IFIT1 induction (Figure 14). From there, we further prioritized the characterization of SNRNP200, a core and unique spliceosomal member of the Ski2-like RNA helicase family.

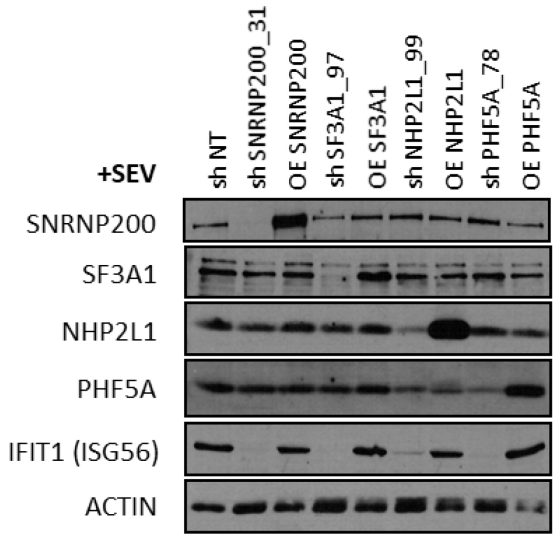


Figure 14. Specific spliceosome proteins are required for virus-induced IFIT1 induction. Immunoblot analysis of HEK 293T cells infected with SeV for 16 hours following SNRNP200, SF3A1, NHP2L1 and PHF5A 72 hours KD with corresponding shRNAs or overexpression (OE) by transient transfection for 16 hours. A non-target shRNA (NT) is used as a control.

2.2. SNRNP200: Function and structure

SNRNP200 (Brr2 or U5) is an essential member of the spliceosome complex, along with seven other RNA helicases, which is responsible for removing introns from the pre-mRNA and giving rise to coding mRNA (Figure 15) [290-294]. In the stepwise model of spliceosome activation, SNRNP200 is responsible for the ATP-dependent unwinding of U4/U6 snRNAs (ref). Additionally, it serves as the scaffolding protein that joins the pre-spliceosome A complex to the U4/U6.U5 tri.snRNP. It is the only member of the spliceosome that is required at every step of the splicing process from the activation of the pre-spliceosome to the dissociation of U2/U6 and U4/U6, which results in the disassembly of the spliceosome and the release of the spliced RNA.

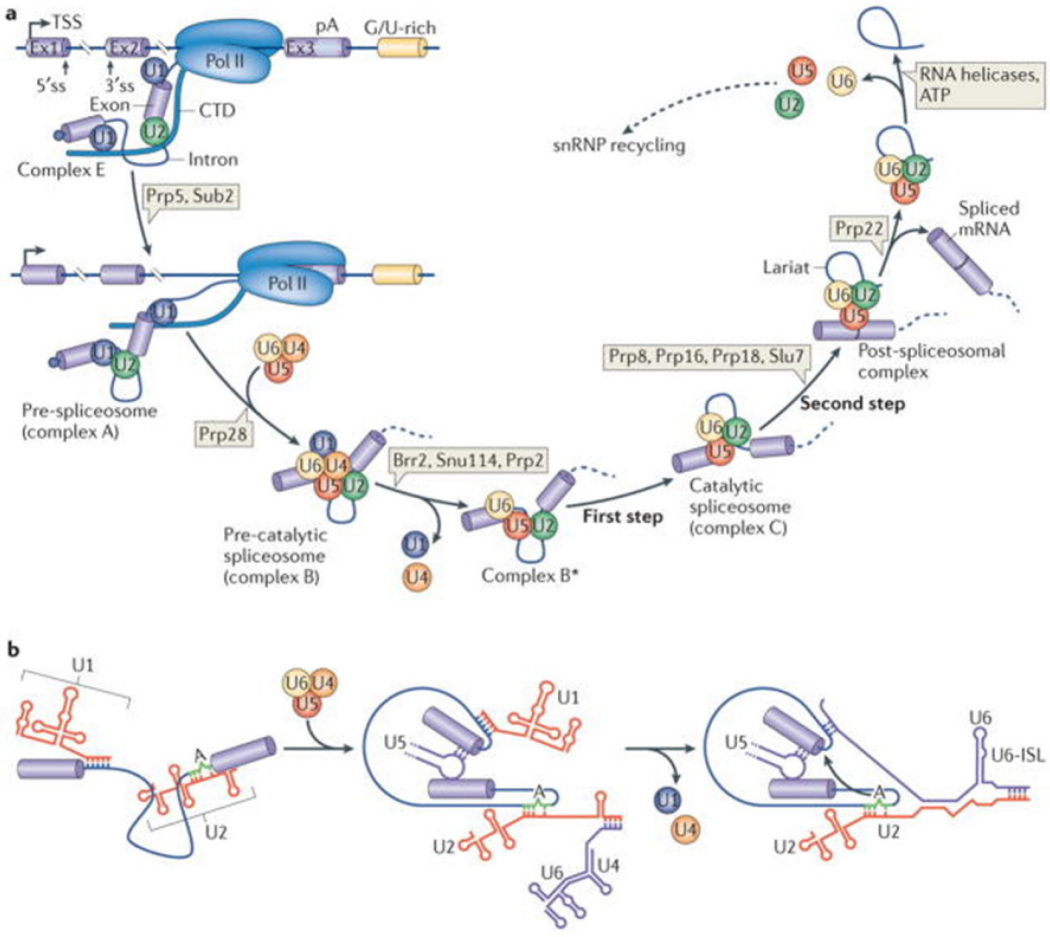


Figure 15. Step-wise assembly of the spliceosome and catalytic steps of splicing. Spliceosome assembly takes place at sites of transcription. (a) The U1 and U2 snRNPs assemble onto the pre-mRNA in a co-transcriptional manner through recognition of the 5′ and 3′ splice sites, which is mediated by the C-terminal domain (CTD) of pol II. The U1 and U2 snRNPs interact with each other to form the pre-spliceosome (complex A). This process is dependent on DExD/H helicases Prp5 and Sub2. In a subsequent reaction catalysed by Prp28, the preassembled tri-snRNP U4/U6•U5 is recruited to form complex B. The resulting complex B undergoes a series of rearrangements to form a catalytically active complex B (complex B*), which requires multiple RNA helicases (Brr2, Snu114 and Prp2) and results in the release of U4 and U1 snRNPs. Complex B* then carries out the first catalytic step of splicing, generating complex C, which contains the free exon 1 and the intron-exon 2 lariat intermediate. Complex C undergoes additional rearrangements and then carries out the second catalytic step, resulting in a post-spliceosomal complex that contains the lariat intron and spliced exons. Finally, the U2, U5 and U6 snRNPs are released from the mRNP particle and recycled for additional rounds of splicing. Release of the spliced product from the spliceosome is catalysed by the DExD/H helicase Prp22109, 110. (b) During splicing, RNA-RNA interactions are rearranged in a stepwise manner to create the catalytic center of the spliceosome. Initially, U1 and U2 snRNA pair with the 5′ss and the branch point sequence within complex A (left, the branch point adenosine is indicated). Subsequently, complex A associates with the U4/U6•U5 tri-snRNP, leading to new base pairs between U2 and U6 snRNA and between U5 snRNA and exonic sequences near the 5′ss (middle). The U4 snRNA is disassociated from U6 to expose the 5′ end of U6, which then base pairs with the 5′ss to displace U1 snRNA (right). In the end, an extensive network of base pairing interactions is formed between U6 and U2, juxtaposing the 5′ss and branch point adenosine for the first catalytic step of splicing. The central region of U6 snRNA forms an intramolecular stem-loop (the U6-ISL) that is key for splicing catalysis. Used with permission. [295]

Finally, the U2, U5 and U6 snRNPs are released from the mRNP particle and recycled for additional rounds of splicing. Release of the spliced product from the spliceosome is catalysed by the DExD/H helicase Prp22109, 110. (b) During splicing, RNA-RNA interactions are rearranged in a stepwise manner to create the catalytic center of the spliceosome. Initially, U1 and U2 snRNA pair with the 5′ss and the branch point sequence within complex A (left, the branch point adenosine is indicated). Subsequently, complex A associates with the U4/U6•U5 tri-snRNP, leading to new base pairs between U2 and U6 snRNA and between U5 snRNA and exonic sequences near the 5′ss (middle). The U4 snRNA is disassociated from U6 to expose the 5′ end of U6, which then base pairs with the 5′ss to displace U1 snRNA (right). In the end, an extensive network of base pairing

interactions is formed between U6 and U2, juxtaposing the 5′ss and branch point adenosine for the first catalytic step of splicing. The central region of U6 snRNA forms an intramolecular stem-loop (the U6-ISL) that is key for splicing catalysis. Used with permission. [295]

Structurally, SNRNP200 differs from other spliceosomal helicases and is classified in the Ski2-like subfamily, which is a small family of superfamily 2 helicases (founder member: yeast Ski2) involved in a variety of RNA processing and degradation events. It is the largest helicase known with a molecular weight of about 250 kDA. From the sequence analysis and resolution of its crystal structure, it has been determined that SNRNP200 is made of an N-terminal and C-terminal cassette composed of a duplicated RecA-like domain (ATP-dependent DEAD/H-box RNA helicase) and a Sec63 homology domain. Both Sec63 homology domains are made of a helical bundle (HB) and immunoglobulin-like (IG) sub-domains, which are separated by a helix loop helix (HLH) motif that contains the RNA binding tunnel of the N-terminal cassette (Figure 16).

Accordingly, it is thought that SNRNP200 N-terminal cassette is required for nucleotide hydrolysis and RNA unwinding and is tough enough to be the catalytic unit of SNRNP200, while the C-terminal cassette has been characterized as a protein-protein interaction domain. Interestingly, Sec63 homology domains have no precise biological function in vertebrates but were shown to be related to endoplasmic reticulum (ER)-associated shuttling in the yeast. Lastly, it is worth to mention that SNRNP200 yeast homologs, SNR Ski2 and Ski2-like helicase 1 (Slh1), have been reported as RNA helicases implicated in antiviral defense and were shown to block translation of RNA lacking a 3' poly(A) structure [296, 297].

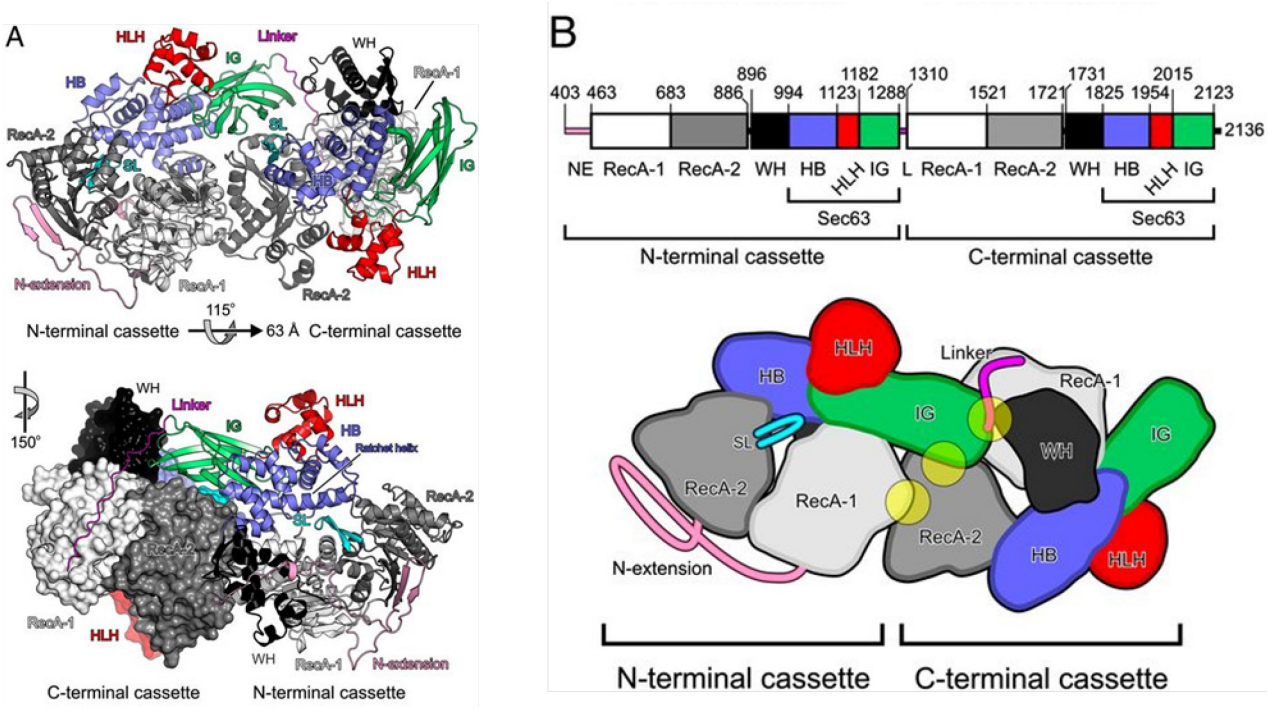


Figure 16. Overall structure of hSNRNP200HR. (A Upper) Ribbon plot of hBrr2HR. N-terminal extension, pink; RecA-1, light gray; RecA-2, dark gray; WH, black; HB, blue; HLH, red; IG, green; linker, magenta; separator loop (SL), cyan. Symbols below the image indicate the relationship between the cassettes within hBrr2HR. (Lower) Combined ribbon (N-terminal cassette) and surface (C-terminal cassette) plot showing the intercassette linker. Plot was rotated 150° counterclockwise as indicated. (B) Schematic representations of SNRNP200. (Upper) Domain borders. (Lower) A 2D scheme of Brr2HR. Intercassette contacts between the N-terminal IG domain and the C-terminal RecA-2 and WH domains and between the N-terminal RecA-1 and the C-terminal RecA-2 domains are indicated by semitransparent yellow circles. Used with permission. [298]

2.3. Role of SNRNP200 in the etiology of retinitis pigmentosa

Interestingly, SNRP200 has been associated with retinitis pigmentosa, which is an inherited eye disease characterized by a progressive degeneration of the retinal photoreceptors (rods and cones) that affects between 1:3000 and 1:8000 individuals worldwide [OMIM: 26800]. Clinical presentation is variable, but RP is generally diagnosed based on the observation of an abnormal fundus, alteration of a- and b-waves in an electroretinogram, and a reduced visual field [299]. RP can be syndromic (Usher syndrome and Bardet-Biedl syndrome) or non-syndromic.

The mode of inheritance of the non-syndromic form of RP can be autosomal dominant (adRP), autosomal recessive (arRP), or X-linked (xlRP). The genetics of RP is highly heterogeneous with an association or linkage to 58 genes spanning more than 40 distinct loci [300]. Among them, eight genes encode for six snRNP proteins (PRPF3/4/6/8/31 and SNRNP200) and two splicing factors (RP9 and DHX38) [301]. This enrichment for genes that are associated with pre-mRNA splicing suggests that the etiology of RP can be summarized as an impairment in the formation of the U4/U6.U5 tri-snRNP complex that is required for proper gene splicing. This is most likely detrimental for the retina that requires about seven times more RNA splicing than other tissues. About 1.3% of the RP cases are amenable to mutations found in SNRNP200 that are in the first RecA domain with the exception of the S1087L, which is located in the N-terminal Sec63 domain. Thus, it can be concluded that mutations occurring in the first RecA domain might cause RP due to a decrease in affinity with SNRNP00 regulatory proteins, such as PRP8, which leads to a hyperactive and error-prone RNA splicing. However, more studies will be required to understand the role and impact of the S1087L mutation in RP etiology.

2.4. Biological prioritization, research hypothesis and specific aims

Our prioritization of SNRNP200 was based on the following criteria:

- SNRNP200 bears structural similarities to other antiviral RNA helicases, such as RIG-I, as it is made of an N-terminal and a C-terminal cassette, both bearing a DEAD/DEAH box helicase and ATPase domain that are typically associated with the recognition of non-self RNA by RNA sensors and sentinels.
- SNRNP200 is a key player of intron splicing and could regulate antiviral gene expression. Other spliceosome proteins such as SF3A1 (alternative splicing of My88D) or SFSR1 (alternative splicing of IRF3) have been shown to control gene expression during viral infection. [302, 303].
- SNRNP200 is associated with retinitis pigmentosa, a degenerative eye disease, that was considered by some ophthalmologists, based on clinical observations, to be reminiscent of autoimmune disease [304, 305].
- A posteriori, SNRNP200 was identified as a functional interactor of FLUB NS1 protein suggesting that it might be involved in viral evasion or hijacking mechanism. [306]

Accordingly, the hypothesis of my research project was that SNRNP200 is required for RLR signalling, leading to the production of IFN-β, upon RNA virus infection.

The first aim was to validate the phenotype that is observed in SNRNP200-depleted cells by correlating the magnitude of IFN-β decrease with an increase in viral susceptibility using traditional virology and molecular biology tools (Western blot; CO-IP, Elisa, Viral plaque assays, Rescue experiments). The second aim was to characterize the mechanism of action of SNRNP200 by elucidating its role in the RLR pathway using functional assays to identify if SNRNP200 is required for viral RNA sensing, adaptor protein signal transduction, activation of IRFs or the regulation of interferon stimulated gene expression. In the next section, we will present the results of our investigation and show that we were able to show that SNRNP200 is a novel sensor of viral RNA that also acts as an adapter-like platform that is essential for TBK1-mediated IRF3 activation.

3. Spliceosome SNRNP200 amino-terminal Sec63 domain promotes viral RNA sensing and IRF3 activation in antiviral response.

Tremblay,N*. ,Baril,M*. , Chatel-Chaix, L., EsSaad, S., Park, A.Y., Koenekoop, R.K., Lamarre D. (2016) Spliceosome SNRNP200 Promotes Viral RNA Sensing and IRF3 Activation of Antiviral Response. PLoSPathog12(7): e1005772.doi:10.1371/journal. ppat.1005772

3.1.1. Acknowledgements

We thank R. Lührmann for the SNRNP200 (hBrr2) plasmid; C. Rice and T. Wakita for J6/JFH (p7-Rluc2a) and Huh7.5 cells; T.-S. Tep and P. Ancuta for preparation of MDM; S. Tran, N. Bédard and N. Shoukry for PBMCs; M. Servant and N. Grandvaux for reagents. We also thank A.-S. Guenier, K. Audette and J. Duchaine of the IRIC's screening facility; S. Drouin and R. Lambert from the IRIC's Genomics Core facility for microarray analysis and qRT-PCR; G. Chouinard from the CRCHUM's Cell Imaging Core Facility for confocal microscopy. We thank Prof. S. Bhattacharya and Dr. C. Chakarova for their help in patient identification, assessment and genotyping. We thank the patients for their enthusiastic participation.

3.1.2. Abstract

Spliceosomal SNRNP200 is a Ski2-like RNA helicase that is associated with retinitis pigmentosa 33 (RP33). Here we found that SNRNP200 promotes viral RNA sensing and IRF3 activation through the ability of its Sec63-1 domain to interact with TBK1. We show that SNRNP200 relocalizes into TBK1-containing cytoplasmic structures upon infection,

in contrast to the RP33-associated S1087L mutant, which is also unable to rescue antiviral response of SNRNP200 knockdown cells. This functional rescue correlates with the Sec63-1-mediated binding of viral RNA. The hindered IFN-β production in knockdown cells was further confirmed in peripheral blood cells of RP33 patients bearing missense mutation in SNRNP200. This work identifies a novel immunoregulatory role of the spliceosomal SNRNP200 helicase as an RNA sensor and TBK1 adaptor in activation of IRF3-mediated antiviral innate response.

3.3.3. Author Summary

The innate immune system is the first line of defense against pathogens and relies on the recognition of molecular structures specific to pathogens by sensor receptors. These receptors activate a signaling cascade and induce a protective cellular response. In this study, we provide evidence for a role of the spliceosomal SNRNP200 that is clearly distinguishable of the one in pre-mRNA splicing. The depletion of SNRNP200 in human cells resulted in a reduced antiviral response and increased susceptibility to viral infection. We showed that SNRNP200 positively regulates activation of the key transcriptional factor IRF3 via interaction with TANK kinase 1 (TBK1). Upon infection, SNRNP200 binds viral RNA and relocalizes into TBK1-containing cytoplasmic structures to promote innate signaling. Of clinical relevance, we observed a significantly hindered antiviral response of PBMCs from patients carrying a dominant SNRNP200 mutation associated to the retina pigmentosa type 33 (RP33), an inherited degenerative eye disease. We showed that expression of the RP33-associated mutant has lost the ability to bind RNA and to rescue antiviral response in SNRNP200 silenced cells. Our study provides new insights into an immunoregulatory role of spliceosome SNRNP200 acting as an RNA sensor and adaptor of TBK1 to promote IRF3 signaling in antiviral response.

3.2. Introduction

3.2.1. The RLR Pathway

The innate immune system is the first line of defense against pathogens and relies on the recognition of pathogen-associated molecular patterns (PAMPs) by specific pattern recognition receptors (PRRs). Upon viral infection, intracellular foreign nucleic acids are detected by specific DExD-box RNA helicases of the RIG-I-like receptors (RLRs) family: retinoic acid-inducible gene I (RIG-I also known as DDX58), melanoma differentiation–associated gene-5 (MDA5, also known as IFIH1) and laboratory of genetics and physiology 2 (LGP2, also known as DHX58)[307]. In response to the sensing of viral RNA, these RLRs associate with the mitochondrial antiviral signaling (MAVS) adaptor (also named IPS-1, Cardif and VISA)[144-146, 148] to induce its multimerization[191, 308] and to activate multiple kinases including the IκB kinase complex (IKK), the TNF receptor-associated factor (TRAF)-associated NF-κB activator (TANK) binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase ε (IKBKE). Upon signal transduction, activation of transcriptional factors activator protein 1 (AP-1, ATF-2/c-jun), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) and interferon regulatory factor 3 (IRF3) and IRF7, induces expression of pro-inflammatory and antiviral cytokines and chemokines. Type I IFNs then trigger the activation of STAT1, STAT2 and IRF9, a transcription factor complex known as IFN-stimulated gene factor 3 (ISGF3) to induce a large number of IFN-stimulated genes (ISGs).

3.2.2. Major Role of RNA Helicases in Innate Antiviral Immunity

In a recent genome-wide RNAi screen assessing virus-induced *IFNB1* transcription [289], we identified spliceosomal factors, including RNA helicase SNRNP200, that positively modulate the RLR-mediated antiviral pathway. Few studies have described a contribution of spliceosomal factors in immune response to pathogen invasion, and mainly described a role in alternative mRNA splicing of innate immunity genes such as DDX58, MyD88 and IRF3[302, 303, 309]. Interestingly, many DExD/H-box RNA helicases, beside DDX58 and MDA5, were recently identified as components of viral nucleic acids sensors and/or mediators of antiviral innate immunity including the DHX15 helicase involved in late-stage RNA splicing and spliceosome

disassembly [310, 311]. DHX15 and DHX9 helicases were showed to interact with MAVS following dsRNA recognition and to activate NF-κB and IRF3 in myeloid dendritic cells (mDC)[310, 312]. An RNA helicase complex composed of DDX1, DDX21 and DHX36 was also shown to induce type I IFN though a TRIF-dependent signaling in mDC[313]. Two other helicases, DDX60 and DDX3, were reported to bind to DDX58/MDA5 and to enhance their ability to recognize dsRNA and induce type I IFN production[166, 314]. DDX3 is acting as an adaptor protein of TBK1 and IKBKE that synergistically enhance IFNB1 promoter induction [232, 315]. Finally, DDX41 helicase is a DNA sensor that activates type I IFN via a STING-TBK1 complex [316].

In the present study, we found that the silencing of SNRNP200, a core and unique spliceosomal member of the Ski2-like RNA helicase family, leads to a strong decrease of antiviral innate response by positively regulating IRF3 signaling upon Sendai virus (SeV) infection. In SNRNP200 knockdown (KD) cells, expression of the retinitis pigmentosa 33 (RP33)-associated mutant S1087L is unable to rescue *IFNB1* transcription and innate response, in contrast to expression of the wild-type (WT) protein. The functional rescue phenotype correlates with the ability of SNRNP200 to bind surrogate polyinosinic-polycytidylic acid (poly I:C) or hepatitis C virus (HCV) RNA, allowing a subcellular fraction of the protein to re-localize into punctuate TBK1-containing cytosolic structures in infected cells. We further demonstrated a physical interaction between SNRNP200 and TBK1 and mapped this interaction to the N-terminal Sec63 domain (Sec63-1). Finally, we demonstrated a significantly hindered innate immune response to virus infection in human monocyte-derived macrophages (MDM) upon SNRNP200 silencing, as well as in peripheral blood cells of RP33 patients bearing pathogenic missense mutation in SNRNP200. Overall this study uncovers a novel immunoregulatory role of the spliceosome SNRNP200 helicase as both a viral RNA sensor and a TBK1 adaptor promoting IRF3-dependent antiviral innate immune response.

3.3. Results

3.3.1. Identification of spliceosome proteins required for SeV-induced IFNB1 transcription

A genome-wide gene silencing screen assessing the transcriptional response at the *IFNB1* promoter following SeV infection was previously performed by our group to identify novel regulators of innate immunity [289]. We identified six genes encoding spliceosome components that reduced *IFNB1* transcription upon gene silencing (Figure 17A), and for which two hit genes (SF3A1 and SRSF1) were shown to regulate innate immune response by alternative splicing of Myd88 and IRF3 respectively [302, 303]. To further explore RNA helicases that are central players in splicing and often function in proofreading events in pre-mRNA splicing [317], we performed a RNAi mini-screen targeting most spliceosomal RNA helicases assigned to the DEAD-box, DEAH-box or Ski2-like helicase subfamilies using five independent lentivirus expressing short hairpin RNA (shRNA) (Figure S1A). SNRNP200 was the only RNA helicase within the Ski2-like family that confirmed a significant reduction of IFNB1 promoter-driven reporter activity. The KD and specificity of the various shRNA were demonstrated with the decreased SNRNP200 mRNA and protein levels, as determined by qRT-PCR and western blot analysis, and with the lack of silencing of other spliceosome gene hits (Figure S1B-S1C).

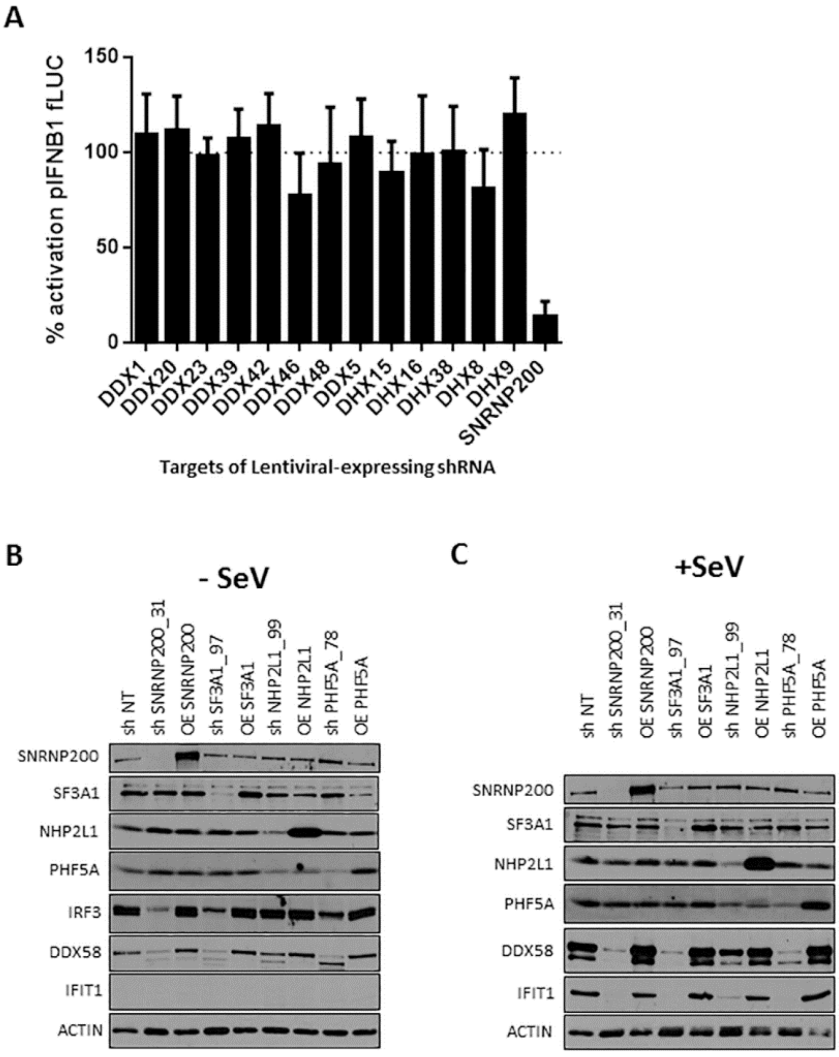


Figure S1. Screening of a subgroup of spliceosome members identified SNRNP200 as the only helicase required for the antiviral response of SeV infection

(A) HEK 293T pIFNB1-Luc cells are transduced with lentivirus-expressing shRNA targeting a subset of RNA helicases implicated in splicing for three days and stimulated with SeV for 16 hours. (B) HEK 293T are transduced with lentivirus-expressing shRNA targeting for 3 days or transfected for 48 hours with SNRNP200, SFRS1, SNRNP35, SF3A1, PHF5A and NHP2L1 expression plasmids. Protein KD and overexpression (OE) efficiencies of the various spliceosome proteins as well as IRF3, DDX58, IFIT1 and ACTIN protein levels are resolved by immunobloting of cell lysates and compared to shNT control cells. (C) HEK 293T are treated as indicated in (B) and infected with SeV for 16 hours.

SNRNP200 overexpression was not able to increase the representative interferon induced protein with Tetratricopeptide repeat 1 (*IFIT1)* gene expression in the absence of viral infection or its induction upon SeV infection. The depletion of SNRNP200 protein by gene silencing significanlty reduced IFN-β levels in a kinetic study of SeV infection comparable to the those obtained in RIG-I (DDX58) KD cells (Figure 17B), and completely inhibited IFIT1 protein induction (Figure S1C). To investigate such contribution to antiviral response, we monitored virus susceptibility of SNRNP200 KD cells by the production of infectious particles and of viral protein levels in parallel to IFIT1 induction in a time-course experiment (Figure 17C-17D). In control HEK 293T cells transduced with non-target sequence (NT) shRNA-expressing lentiviral particles, SeV nucleocapsid protein (NP) is weakly detected at 24 hours post-infection, a time point where IFIT1 is induced, while never detected at eight hours (Figure 17C).

In contrast, SeV protein is readily detectable at 8 hours and increased at 24 hours post-infection, a time point where IFIT1 is not induced in SNRNP200 KD cells. Importantly, we observed up to a 2-log increase in virus titers from 8 to 48 hours post-infection in SNRNP200 KD cells when compared to the control cells (Figure 17D). We confirmed an increase of influenza A virus (FLUA) and hepatitis C virus (HCV) replication upon depletion of SNRNP200 in HEK 293T and Huh7 respectively that correlates with the reduced early IFNB1 induction of SNRNP200 KD cells (Figure S2).

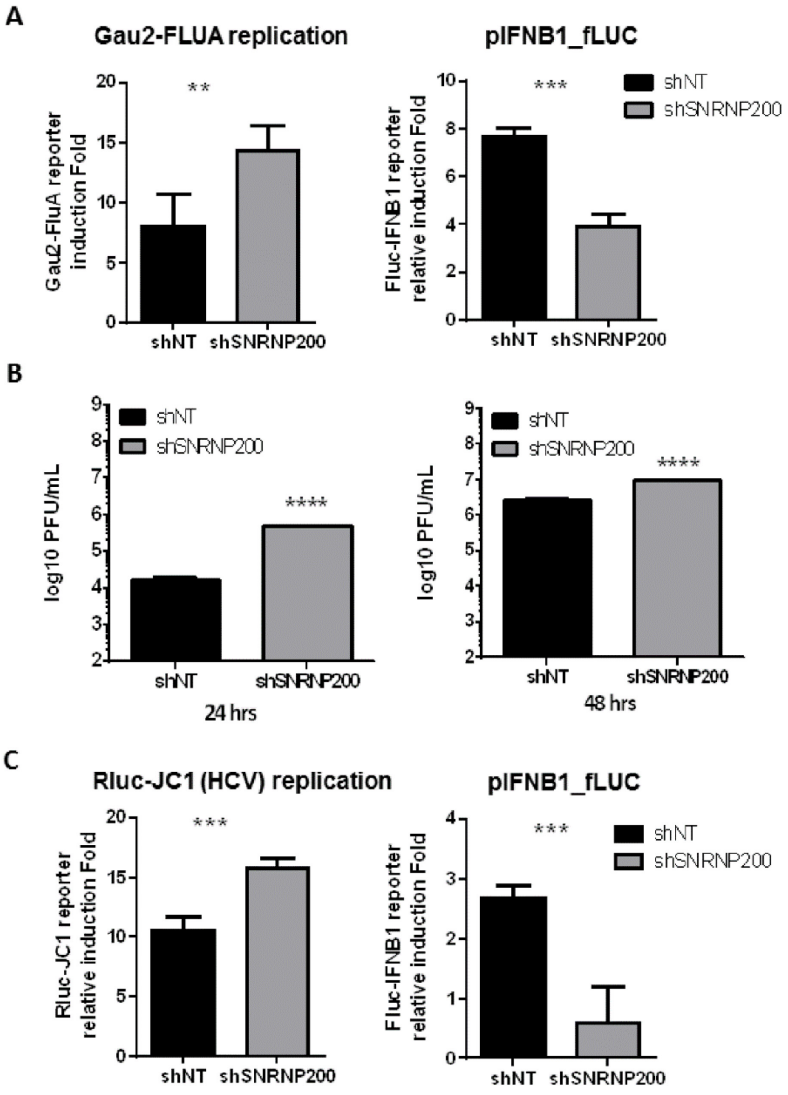


Figure S2. SNRNP200 KD enhances viral replication and restricts antiviral response.

(A) FLUA-Gaussia activity and IFNB1 promoter-driven luciferase activity of HEK 293T cells infected with FLUA for 24 hours and transduced with shNT or shSNRNP200 for three days. (B) HEK 293T cells are infected with FLUA for 24 and 48 hours and viral titers are determined by harvesting supernatants and subsequently infecting MDCK.2 cells using virus plaque assays. (C) HCV J6/JC1(2a)-Renilla luciferase activity and IFNB1 promoter-driven firefly luciferase activity of Huh7 cells transduced with shNT or shSNRNP200 for 4 days and infected with HCV for the three last days. P values <0.01 (**) or <0.001 (***) or <0.0001 (****) are indicated.

In epistasis analysis, we showed that IFNB1 activation was weakly affected with expression of constitutively active IRF3(5D) [224] while completely blocked with SeV infection or MAVS expression upon the stringent silencing of SNRNP200 in cells transduced at a high multiplicity of infection (MOI of 20) of shRNA-mediated lentiviral particles (Figure 17E). Comparable results were obtained in A549 cells (Figure S3A-B). The expression of IRF3(5D), in contrast to IRF3, can rescue the induction of IFIT1 protein upon SeV infection (Figure 17F), suggesting a role of SNRNP200 in IRF3 activation required for IFNB1 production. We next investigated if the KD of SNRNP200 affects NF-κB-dependent transcription using a reporter assay (p2xNF-κB_fLUC) in HEK 293T cells. We found that SNRNP200 KD cells display no attenuation of poly I:C-, MAVS-, TBK1- and p65-mediated activation of NF-κB transcription, in contrast to the significant inhibition of SeV-, poly I:C-, TBK1- and IFN-mediated activation of ISG56 promoter activity (Figure S4A-B). We confirmed that SNRNP200 silencing does not affect NF-κB-dependent transcription in SeV-infected A549 cells by quantification of NF-κB-dependent *TNF*, *NFKBIA* and *TNFAIP3* genes using qRT-PCR (Figure S3C). Interestingly, SNRNP200 KD cells neither affect TRIF nor cGAS/STING pathways, in contrast to the RLR pathway, which all converge to the TBK1-mediated phosphorylation of their respective adaptor (TRIF, STING and MAVS) to recruit IRF3, and license IRF3 for phosphorylation to activate IFN production [213]. These data suggest that SNRNP200 may function at the MAVS adaptor and TBK1-mediated IRF3 licensing step upon RNA virus infection. Altogether, these observations led us to explore a specific regulatory role of SNRNP200, a core component of U4/U6-U5 snRNP[318], in the downstream activation of IRF3 to drive IFNB1 production and elicit an optimal antiviral response.

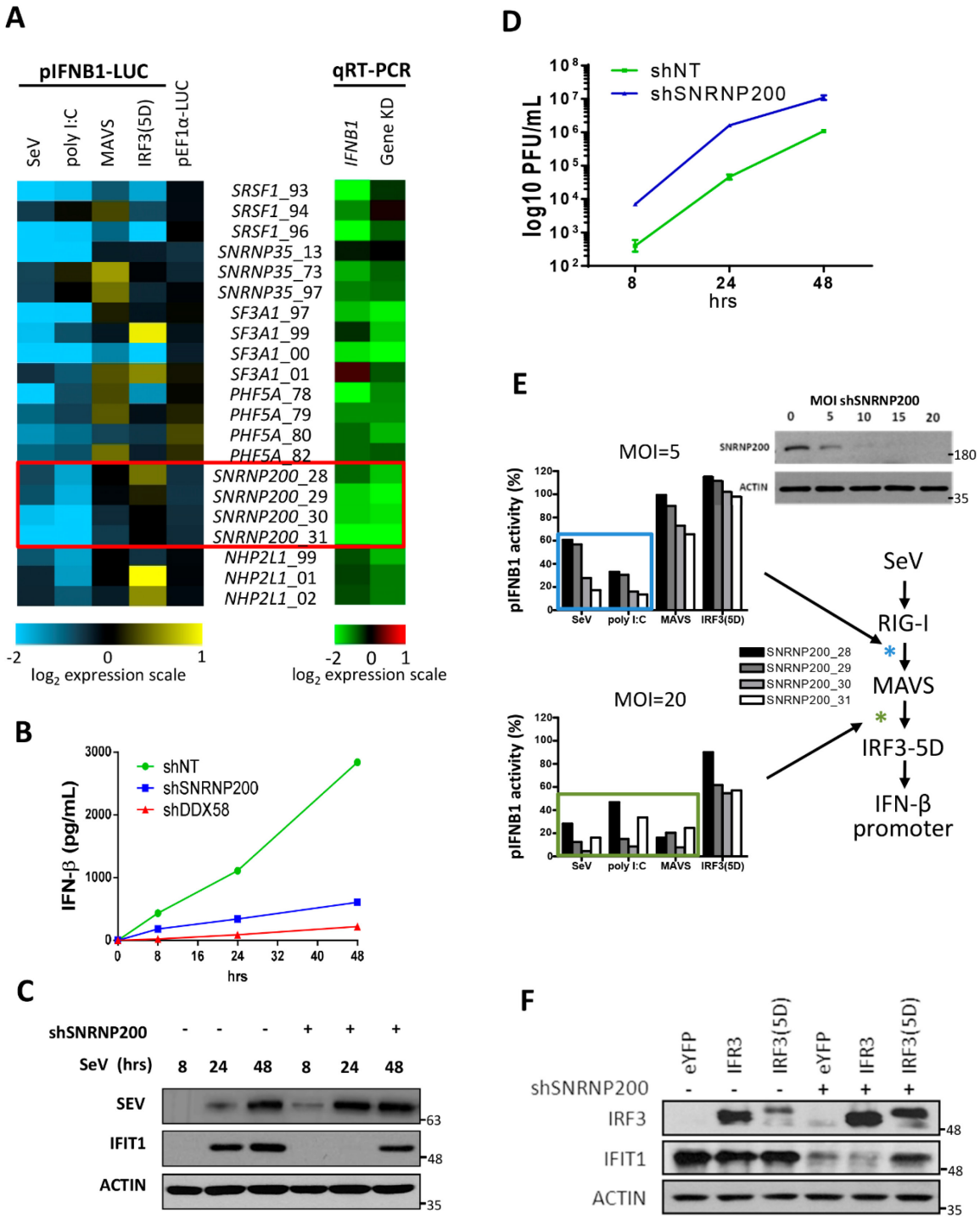


Figure 17. SNRNP200 spliceosome protein is required for virus-induced IFNB1 production to control viral replication.

(A) HEK 293T cells stably expressing an IFNB1promoter-driven luciferase gene (HEK 293T pIFNB1-Luc) are transduced with different lentiviral-expressing shRNA targeting SNRNP200, SFRS1, SNRNP35, SF3A1, PHF5A and NHP2L1 genes. Left panel - Heat map (log2 scale) indicating the modulation of IFNB1 promoter activity following silencing of spliceosome genes and infection with SeV or transfection of poly I:C, MAVS or IRF3(5D) expression plasmids for 16 hours. Right panel - qRT-PCR validation data of the endogenous IFNB1 mRNA levels and target gene KD efficiency of cells transduced with shRNA.

(B) HEK 293T are transduced with lentiviral-expressing shRNA control (shNT) or targeting SNRNP200 (shSNRNP200) or DDX58 (shDDX58) for three days and infected cells with SeV for 8, 24 or 48 hours. Supernatants are harvested and IFN-β secretion levels are measured by ELISA.

(C) Immunoblot analysis of HEK 293T cells infected with SeV for 8, 24 or 48 hours following treatment with shNT or shSNRNP200 for three days. SeV, IFIT1 and actin proteins are resolved by immunobloting at the indicated time.

(D) Infectivity titers of SeV particles produced as indicated in (C) are determined by harvesting supernatants at the indicated time and infecting VERO cells in virus plaque assays.

(E) HEK 293T pIFNB1-Luc cells are transduced with four different shSNRNP200 at a multiplicity of infection (MOI) of 5 and 20 for three days. Relative IFNB1 promoter activity are reported as percentage of the control shRNA NT after infection with SeV or transfection of poly I:C, MAVS or IRF3(5D) expression plasmids for 16 hours (left). Simplified schematic of RLR signaling pathway leading to IFN-β promoter induction (right). Deduced points of action of SNRNP200 are marked with asterisks (blue and green for MOI=5 and 20, respectively). Knockdown efficiencies at the various MOI are determined by immunobloting analysis of SNRNP200 protein levels.

(F) Immunoblot analysis of HEK 293T cells transduced with shNT or shSNRNP200 for three days and subjected to SeV infection for 16 hours. Plasmids encoding eYFP, IRF3 and IRF3(5D) are transfected for 48 hours. Following cell harvesting, IRF3 and IFIT1 protein levels are resolved by immunobloting analysis of cell lysates.

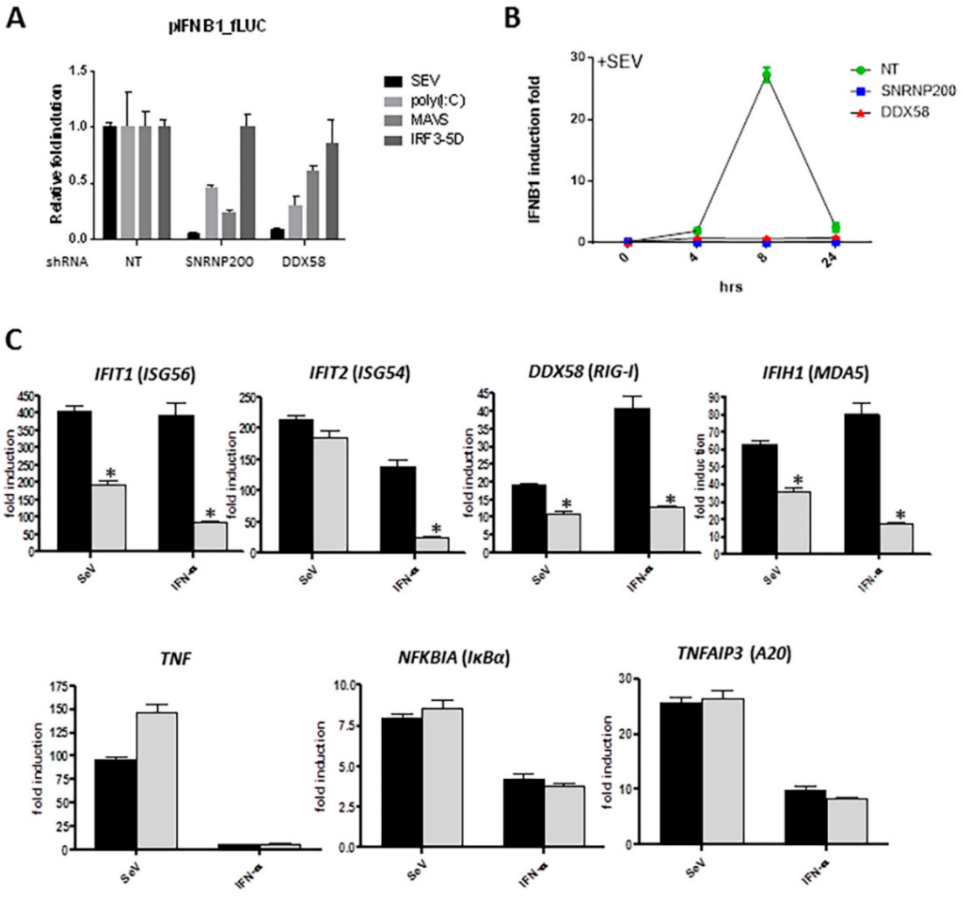


Figure S3. Silencing of SNRNP200 in A549 cells specifically inhibits activation of the RLR-dependent IFNB1 production and IFN-α signaling pathways, but does not affect activation of the canonical NF-κB pathway.

(A) A549 cells treated with lentiviral-expressing shRNA targeting SNRNP200 or DDX58 at a multiplicity of infection (MOI) of 10 for three days. Relative IFN-β promoter activity are reported as percentage of the control shNT following infection with SeV for 8 hours or transfection of poly I:C, MAVS or IRF3(5D) for 16 hours. Inhibition profile of sh*SNRNP200* maps its site of action between MAVS and IRF3(5D) of the RLR signaling pathway. (B) Time course SeV infection (4, 8, 24 hours) in cells treated as indicated in (A). (C) qRT-PCR quantification of *IFIT1*, *IFIT2, DDX58*, *IFIH1*, *TNF*, *NFKBIA* and *TNFAIP3* mRNA fold induction in A549 cells transduced with lentiviral-expressing shNT (black bars) or shSNRNP200 (grey bars) for four days and treated with SeV or IFN-α for four hours. mRNA RQ were normalized versus *GAPDH* and *HPRT1* mRNA. *P* values <0.05 (*) are indicated.

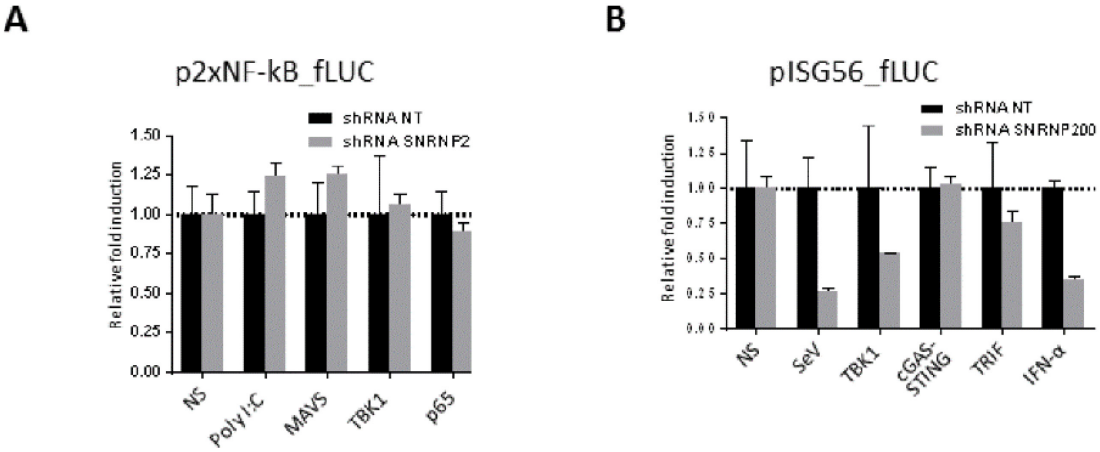


Figure S4. SNRNP200 KD specifically inhibits activation of the RLR-dependent pathway, but does not affect activation of the canonical NF-κB pathway.

(A) Relative NF-kB promoter-driven luciferase activity reported as percentage of the control shNT after transfection of HEK 293T cells with poly (I:C)/RIG-I, MAVS, TBK1 and p65 for 16 hours. (B) Relative ISG56 promoter-driven luciferase activity reported as percentage of the control shNT after SeV infection, transfection with TBK1, cGAS-STING and TRIF for 16 hours or IFN-α treatment.

3.3.2. SNRNP200 specifically regulates IRF3 signaling upon RNA virus infection

To understand how SNRNP200 contributes to IRF3-mediated IFNB1 production upon viral infection, we evaluated the effect of SNRNP200 silencing on the expression of established members of the RLR pathway by western blot analysis (Figures 18A and S5A). We first observed a decreased expression of IRF3 protein in SNRNP200 KD cells that correlates with the blockade of SeV-mediated induction of IFIT1, DDX58 and IFIH1 proteins. The decreased IRF3 protein levels in infected cells were further confirmed at the mRNA level by qRT-PCR that paralleled the reduced mRNA levels of SNRNP200 and effector genes (*IFNB1, IFIT1, DDX58* and *IFIH1)* (Figure S5B). While IRF3 protein levels were shown to be sufficient for activation of the cGAS/STING pathway in KD cells (Figure S4B), the phosphorylation of IRF3 at serine 386 was completely abrogated following SeV infection, suggesting a specific contribution of SNRNP200 at the IRF3 activation step to promote IFNB1 production.

We further observed a weak decrease of the basal levels of DDX58 protein in SNRNP200 KD cells and mRNA levels comparable to control shNT transduced cells (Figure S5A-B), thereby suggesting that SNRNP200 may enhance protein stability to promote the RLR-mediated antiviral signaling. In contrast, expression of MAVS, TBK1, IKBKE, RELA (p65) and TRAF3 proteins that contribute to signal propagation of *IFNB1* induction remained unchanged in all conditions, as well as the expression of housekeeping genes ACTIN, TUBULIN and GAPDH (Figure S5A).

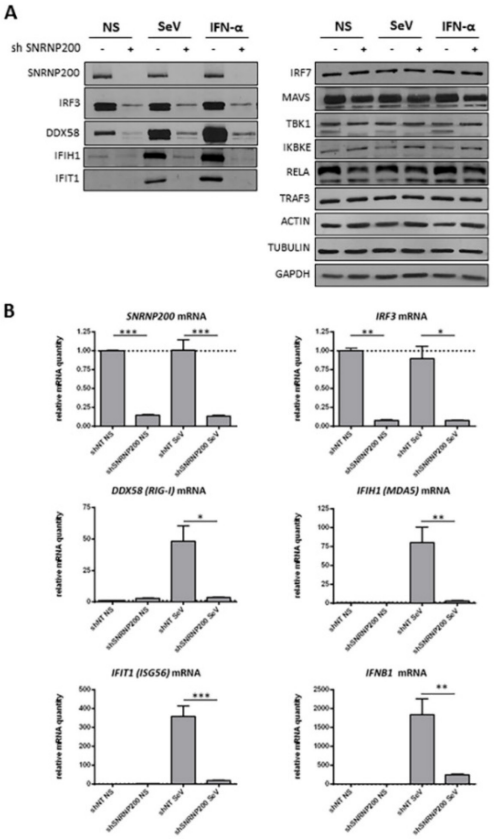


Figure S5. SNRNP200 KD restricts SeV- and IFN-α-mediated induction of antiviral response and affects IRF3 expression

HEK 293T cells are transduced with shSNRNP200 for three days and then either unstimulated (NS), infected with SeV or stimulated with IFN-α for 16 hours. Cells are harvested and selected proteins including known members of the RLR signaling pathway (SNRNP200, IRF3, DDX58, IFIH1, IFIT1, IRF7, MAVS, TBK1, IKBKE, RELA, TRAF3, ACTIN, TUBULIN, GAPDH) are resolved by immunobloting of cell lysates and compared to shNT cells. (B) HEK 293T cells are treated as indicated in (A) and relative gene expression was measured by qRTPCR for *SNRNP200, DDX58, IRF3, IFIH1, IFIT1 and IFNB1* and compared to control shNT cells. Average mRNA RQ normalized versus *ACTIN* and *HPRT1* mRNA. P values <0.05 (*), <0.01(**) and <0.001 (***) are indicated.

To better evaluate the outcome of reduced DDX58 and IRF3 protein levels for IFNB1 production, we attempted to restore antiviral response by ectopic protein expression in SNRNP200 KD cells. Surprisingly, overexpression of DDX58 and IRF3 alone or in combination were not able to restore SeV-mediated IFIT1 induction (Figures 18B and S6A-6B).

Furthermore, ectopic expression of DDX58 or IRF3 could neither restore IFNB1 promoter reporter activity nor IFN-β production upon SeV infection, in contrast to the almost complete rescue of IRF3-5D expression in SNRNP200 KD cells (Figure 18D-18E). We also investigated the phophorylation of IRF3 S386 (IRF3-p386) as a key step of IRF3 activation and determine the quantitative ratios of IRF3-p386 and IRF3 levels under endogenous or overexpressed DDX58, IRF3, IRF3-5D and cGAS/STING proteins (Figure 18B). We showed a significant reduction in IRF3-p386 /IRF3 ratios (ratios of 0.1-0.2) of SNRNP200 KD cells when compared to control shNT-treated infected cells (ratios of 0.6-0.8) that is unaltered by DDX58 or IRF3 overexpression, firmly establishing the requirement of SNRNP200 for downstream IRF3 activation independently of its level of expression. Furthermore, IRF3-p386 /IRF3 ratios (0.8) of overexpressed IRF3-5D in SNRNP200 KD cells are comparable to those of control shNT-treated cells (0.6 - 1.3), and correlate the almost complete restoration of SeV-mediated IFNB1 production. In addition, we investigated the activation of the cGAS/STING cytosolic DNA sensing pathway by overexpression of both proteins in SNRNP200 KD cells. We showed that SNRNP200 is dispensable for cGAS/STING-mediated IFIT1 induction, IFN-β production and IFNB1 promoter activity (Figure 18B-18D-18E), solidely establishing a specific role of SNRNP200 in the RLR-mediated IRF3 signaling pathway upon RNA virus infection. The higher IRF3-p386/IRF3 ratios (4.5) upon cGAS/STING expression in SNRNP200 KD cells versus control cells (2.3) largely reflect a significant increase of IRF3 activation leading to IFIT1 induction (Figure 17B), suggesting that SNRNP200 potentially competes with STING adaptor at the TBK1-mediated IRF3 phosphorylation step. Although IRF3 expression slightly increased IFN-β secretion and IFNB1 promoter activity of combined activation of cGAS/STING pathway and SeV infection in SNRNP200 KD cells (Figure 18D-18E), similar IFNB1 induction is observed in uninfected cells (Figure 18C) demonstrating that IRF3 protein levels in SNRNP200 KD cells has barely any functional consequence on the cytosolic DNA sensing pathway.

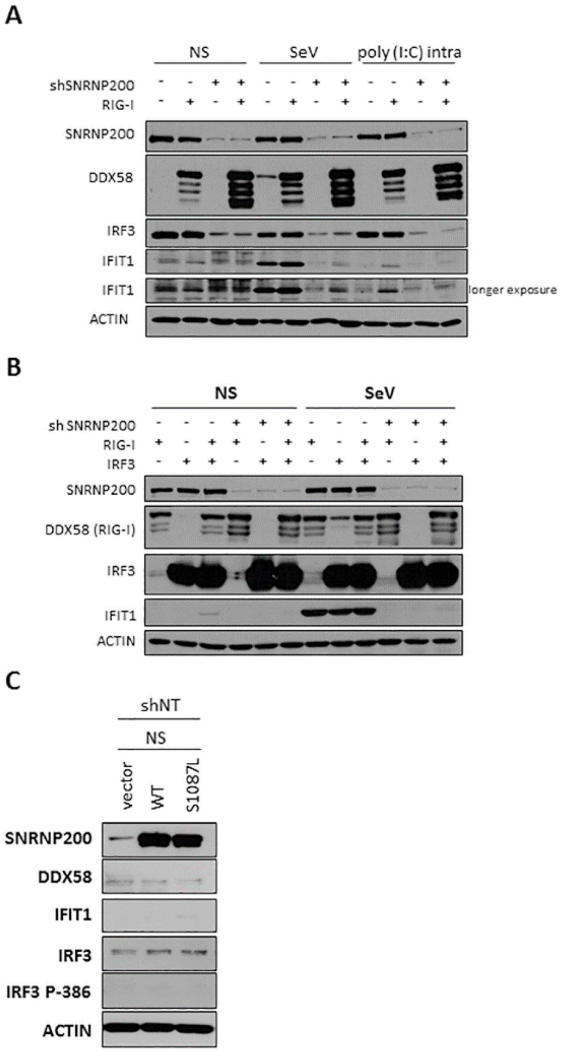


Figure S6. Ectopic expression of IRF3 and DDX58 or both does not rescue antiviral response of SNRNP200 KD cells.

(A) HEK 293T cells are transduced with shSNRNP200 for three days and transfected with DDX58 expression plasmid for the last 48 hours. Subsequently, cells are either untreated (NS), infected with SeV or stimulated with intracellular poly (I:C) for 16 hours. Cells are harvested and selected proteins (SNRNP200, DDX58, IRF3, IFIT1 and ACTIN) are resolved by immunobloting of cell lysates and compared to control shNT cells. (B) HEK 293T cells are transduced with shSNRNP200 for three days and transfected with DDX58 or IRF3 expression plasmids alone or in combination for the last 48 hours. Selected proteins are resolved as indicated in (A). (C) As a control experiment, unstimulated HEK 293T cells are transduced with shNT and transfected with SNRNP200 WT or S1087L variant expression plasmids for 48 hours. Cells are harvested and SNRNP200, DDX58, IFIT1, IRF3 and IRF3pS386 expression are resolved by immunobloting of cell lysates and compared to cells transfected with an empty expression plasmid (vector).

Finally, we investigated *IRF3* mRNA splice junctions to explain the reduced mRNA and protein levels of IRF3. We did not identified splicing variants, ruling out an alternative splicing regulation of IRF3 and point at a reduction in the efficiency of pre-mRNAs splicing to explain the observed phenotype in SNRNP200 KD cells (Figure S7). Interestingly, we further observed an inhibition of IFIT1, DDX58 and IFIH1 induction in IFN-α-treated SNRNP200 KD cells with similar levels of IFNα/β receptor alpha chain (IFNAR1), STAT1 and phosphorylation at tyrosine 701 (STAT1pY701), revealing the involvement of SNRNP200 at a later stage of IFNα/β signaling (Figure 18A). We confirmed that silencing SNRNP200 impedes IFN-α signaling as demonstrated by the reduced induction of IFIT1, DDX58 and IFIH1 genes in A549 cells (Figure S3C).

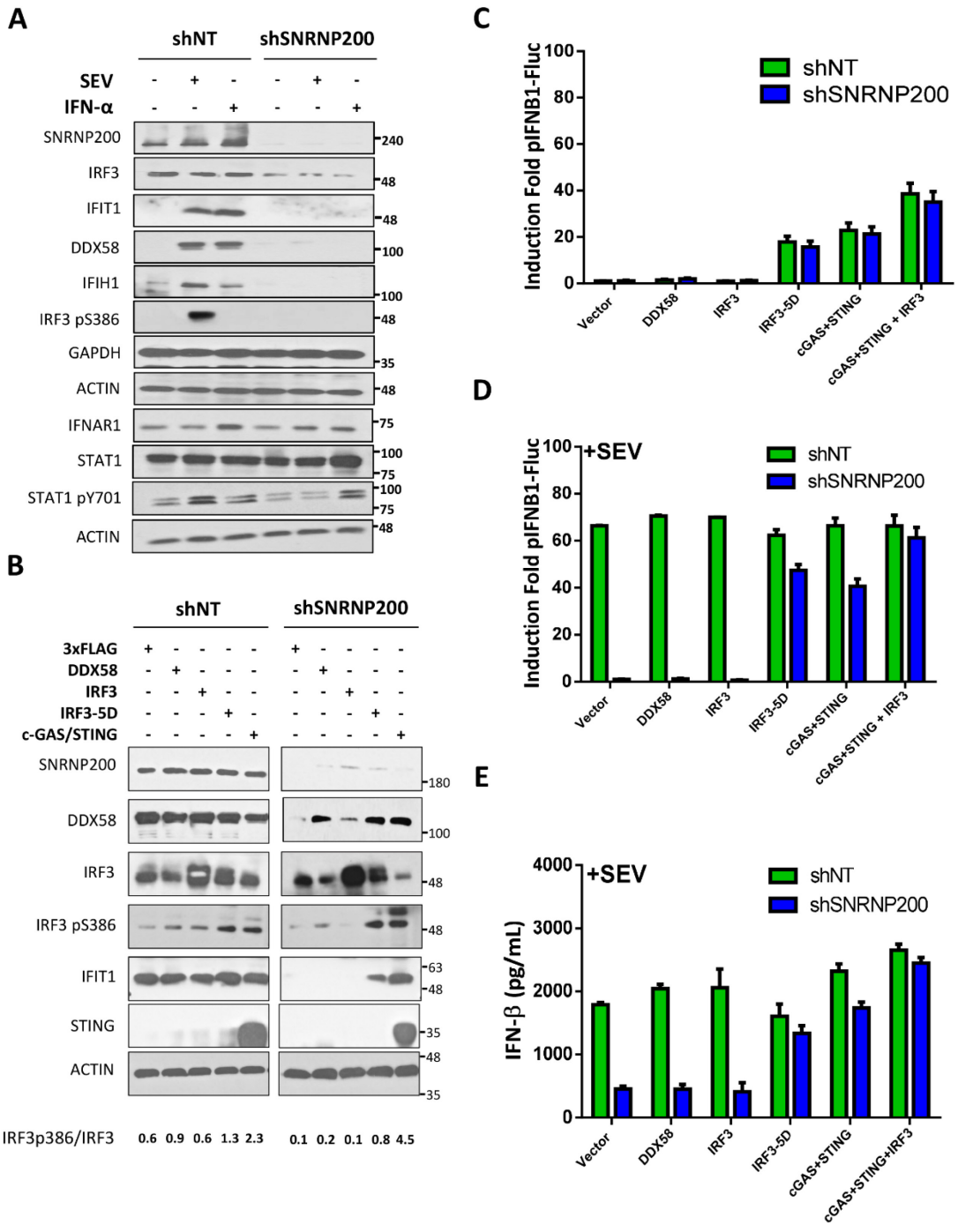


Figure 18. SNRNP200 KD restricts SeV- and type I IFN-mediated induction of antiviral response.

(A) HEK 293T cells are transduced with shNT or shSNRNP200 and infected with SeV or stimulated with IFN-α for 24 hours. Selected genes are resolved by immunobloting and compared to shNT control cells.

(B) HEK 293T are transduced with shNT or shSNRNP200 for 3 days and transfected with DDX58, IRF3, IRF3(5D) and cGAS-STING expression plasmids for the last 48 hours, and subsequently infected with SeV for 24 hours. Selected genes are resolved by immunobloting and compared with cells transduced with shNT.

(C) HEK 293T pIFNB1-Luc cells are transduced with shNT or shSNRNP200 for 3 days and transfected with DDX58, IRF3, IRF3(5D) and cGAS-STING expression plasmids for the last 48 hours. Luciferase levels are resolved and compared to shNT control cells.

(D) HEK 293T pIFNB1-Luc cells are transduced with shNT or shSNRNP200 for 3 days and transfected with DDX58, IRF3, IRF3(5D) and cGAS-STING expression plasmids for the last 48 hours, and subsequently infected with SeV for 24 hrs. Luciferase levels are resolved and compared to shNT cells.

(E) HEK 293T cells are treated as indicated in D. At 24 hrs post-infection, supernatants are harvested and IFN-β secretion levels are measured by ELISA.

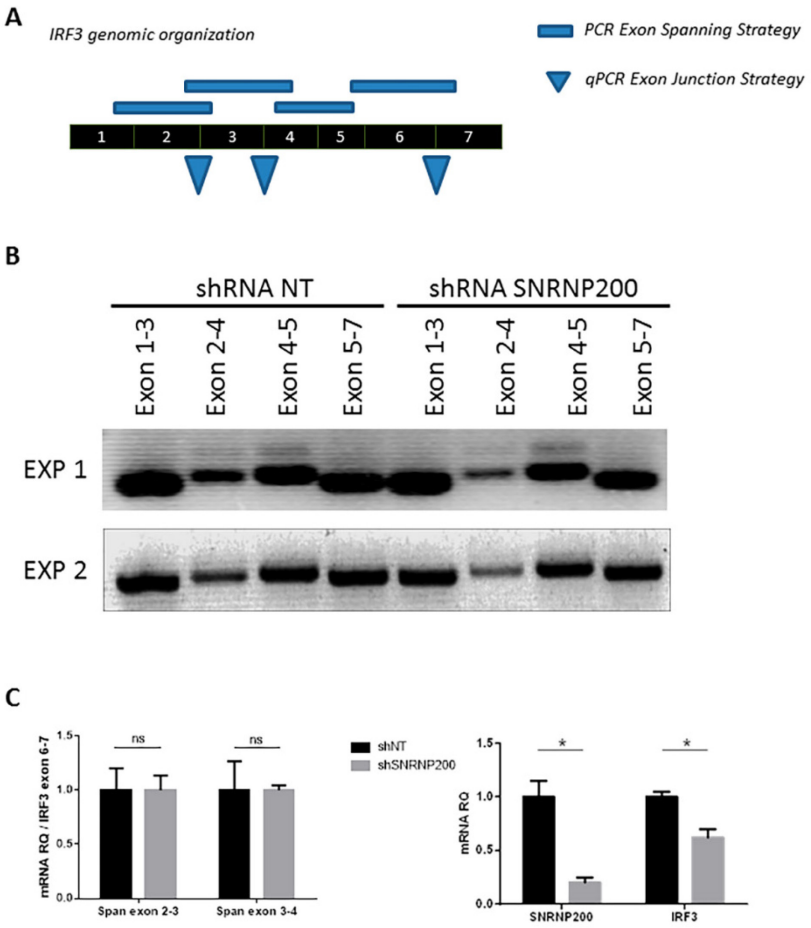
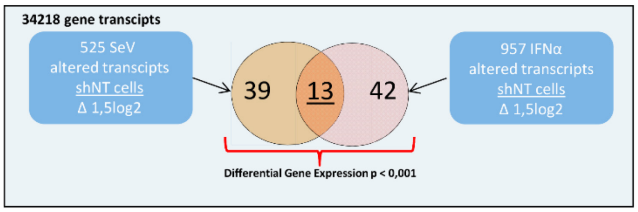


Figure S7. SNRNP200 KD does not induce *IRF3* mRNA alternative splicing.

(A) Schematic representation of *IRF3* genomic organization and theoretical PCR products for the PCR exon spanning or junction strategies. Exons 1-7 are represented by black boxes and primers used for the PCR analysis are represented by arrows. (B) DNA electrophoresis of PCR products described in (A) after mRNA extraction and subjected to RT of HEK 293T cells transduced with shNT or shSNRNP200 for four days. Two independent experiments are presented. (C) qRT-PCR of IRF3 exon junctions described in (A) for exon 2-3 and exon 3-4 (left) and treated as indicated in (B). qRT-PCR of SNRNP200 and IRF3 following SNRNP200 KD (right). *P* values <0.05 (*) are indicated.

To comprehensively understand the effect of SNRNP200 silencing on IFN production and signaling pathways, we performed transcriptional profiling studies of non-stimulated (NS), SeV-infected and IFN-α-treated SNRNP200 KD cells versus control shNT HEK 293T cells to assess differential gene expression (Figures 19 and S8). We first established the effect of SNRNP200 silencing on basal gene expression in NS cells and found overall 3,047 altered transcripts (cutoff of 1,5 log2 fold induction) of genes that are mainly enriched for immune system function and cell cycle regulation (Figure S8A). The analysis of SeV-infected gene expression profile revealed that SNRNP200 KD modulated a total of 1,333 genes and significantly affects the expression of 148 SeV-dependent genes when compared to control shNTcells. Similarly, analysis of IFN-α-treated gene expression profile showed that SNRNP200 KD significantly affects 399 IFN-α-dependent genes compared to control shNTcells out of a total of 2,177 IFN-α modulated genes (Figure S8B-S8C). To assess the biological relevance of these overlaps (148 for SeV and 399 for IFN-α), we then ranked and compared the differential gene expression between SNRNP200 KD cells and control shNT cells. This enabled the identification of 41 SeV-dependent genes, 18 IFN-α-dependent genes and 26 shared genes that decrease by more than 1,5 log2 expression fold in SNRNP200 KD cells (Figure 19). The resulting gene network shows that many of these genes are highly connected to IRF3 (25 edges) and IFNB1 (11 edges). Finally, a gene ontology (GO) enrichment analysis of the SNRNP200 gene network confirmed that it mapped to innate immunity gene function such as response to virus and type I interferon signaling pathway (Figure 19E). The molecular signature strengthens our observation that SNRNP200 silencing hinders IRF3-dependent gene induction that leads to a general atony of the RLR signaling pathway. Altogether, our results suggest that SNRNP200 specifically regulates IRF3 activation upon RNA virus infection to promote IFNB1 induction and IFN effector response, and as such, plays a key role in the early control of a viral infection.

A



B

Common	200_NS	200_SEV	NT_SEV	200_IFNa	NT_IFNa
IFIT1	0,07	2,81	7,04	0,77	7,07
IFIT3	-1,13	1,82	5,53	0,46	6,25
IFIH1	-0,70	0,54	5,49	-0,14	4,16
C17orf50	1,95	0,00	3,88	0,00	3,31
IFI6	0,04	1,02	3,80	0,33	4,57
IRF9	1,10	1,04	3,64	2,48	4,53
IFITM1	0,38	0,74	3,64	1,79	5,93
DDX60	-1,40	-0,48	3,63	-0,40	4,73
BST2	0,00	-1,15	2,85	-0,59	4,26
OAS3	0,18	1,07	2,70	-1,69	4,18
IFITM4P	0,78	0,75	2,53	1,19	4,38
PARP9	-1,72	-1,17	2,20	-0,81	3,84
SP110	-1,49	-0,48	2,19	0,45	3,92

C

SeV	200_NS	200_SEV	NT_SEV	200_IFNa	NT_IFNa
AKNA	0,00	5,90	6,36	0,00	0,00
OR5M3	0,00	0,00	5,34	0,00	0,00
IFIT2	0,90	1,67	4,48	1,36	0,89
KRT12	0,00	2,32	4,32	0,00	1,36
OR8K5	0,00	0,00	4,15	0,00	0,00
SIX6	0,00	0,00	4,13	0,00	0,00
HYDIN	1,48	2,09	4,06	-0,78	1,71
OR51G1	0,00	0,00	3,96	0,00	0,00
SVOP	1,39	1,63	3,67	1,66	2,02
LRRTM2	1,17	1,15	3,59	1,42	0,19
S100A8	0,00	0,77	3,58	4,26	0,00
HERC5	0,00	0,00	3,53	0,00	0,00
IL29	0,00	0,00	3,52	3,92	0,00
EYS	-0,10	1,50	3,46	0,00	1,53
TM4SF20	1,51	1,65	3,44	2,69	1,55

D

IFNa	200_NS	200_SEV	NT_SEV	200_IFNa	NT_IFNa
ACVR1C	-0,25	-0,25	0,54	4,31	6,54
C1QA	-0,21	0,00	2,56	0,00	6,26
C4orf7	-0,27	0,60	0,00	5,40	5,53
CD84	0,00	0,82	3,00	0,00	5,16
CIITA	0,70	1,51	0,99	2,69	4,68
DPCR1	2,50	-1,29	1,55	2,59	4,10
DTX3L	1,24	1,65	3,04	2,11	4,03
EIF2AK2	0,00	0,00	0,00	0,00	4,01
EPSTI1	1,12	1,41	0,12	2,11	3,84
HIF3A	-0,87	0,05	2,14	0,21	3,80
IFI44L	1,04	3,16	2,78	0,00	3,76
IFIT5	0,29	-0,54	0,29	2,17	3,74
IKZF1	0,00	0,00	1,32	-1,37	3,70
KRTAP13-1	0,00	0,00	0,00	0,00	3,70
KRTAP4-11	1,50	0,99	0,14	2,68	3,53

E

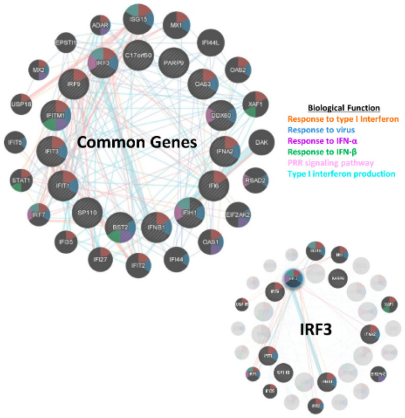


Figure 19. Transcriptional profiles of SNRNP200 KD cells reveal altered expression of genes induced by SeV infection and IFN-α treatment.

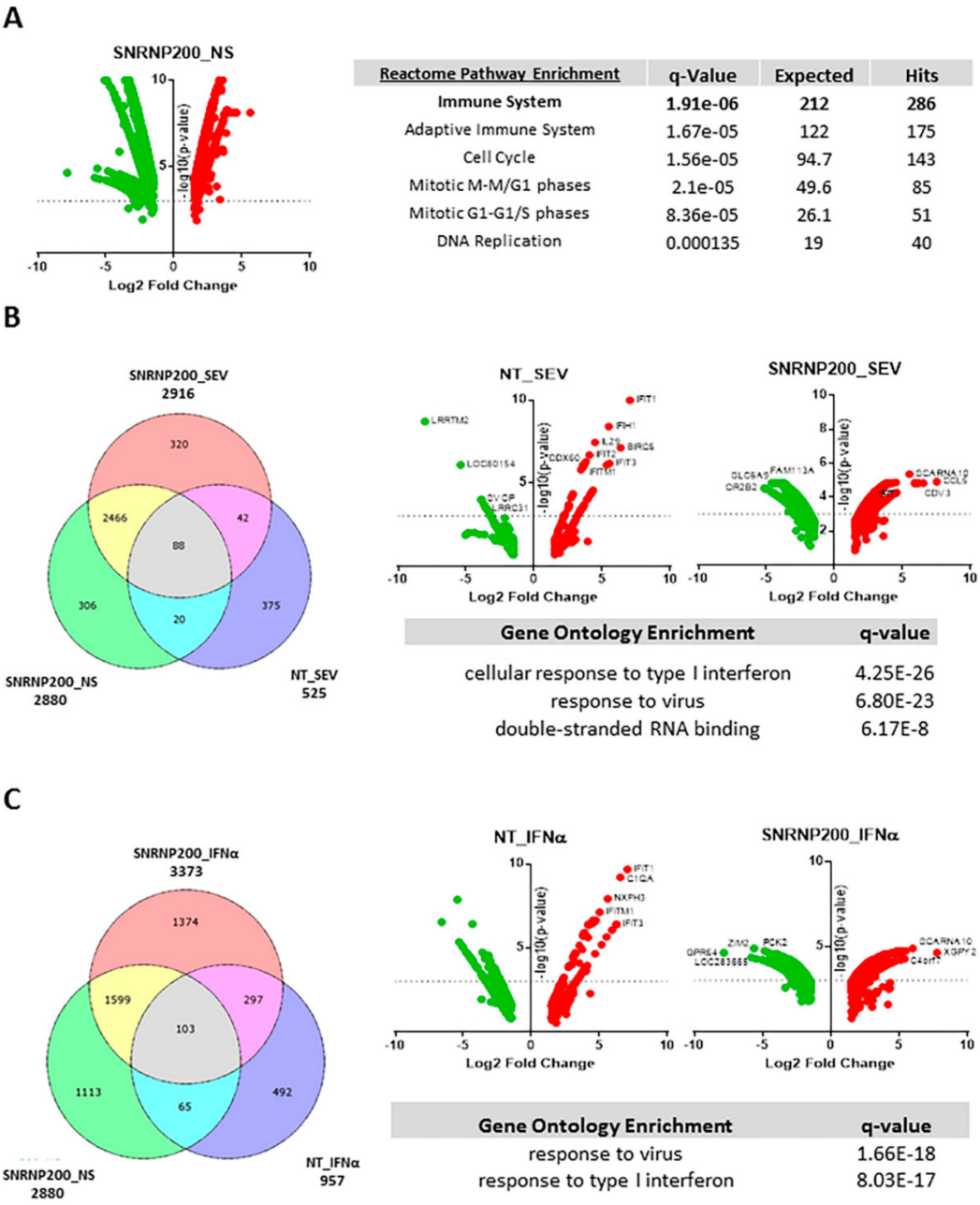
A) Summary diagram of the transcriptional analysis of shNT control cells to illustrate that out of the 525 SeV altered transcripts and 957 IFN-α altered transcripts, 52 and 55 genes have a differential gene expression ($p \geq 0.001$) upon SeV infection or IFN-α stimulation, respectively.

(B) Differential gene expression of the 13 transcripts affected by SeV infection and IFN-α stimulation. Expression Fold Change are shown for shSNRNP200 cells (200_NS), shSNRNP200 cells + SeV (200 SEV) and shSNRNP200 cells + IFN-α (200_IFNa) and for shNT control cells (NT_SEV, NT_IFNa). Numerical values are log2 fold change.

(C) Differential gene expression of the 39 transcripts affected by SeV infection. Expression Fold Change are shown for shSNRNP200 cells (200_NS), shSNRNP200 cells + SeV (200 SEV) and shSNRNP200 cells + IFN-α (200_IFNa) and for shNT control cells (NT_SEV, NT_IFNa). Numerical values are log2 fold change. Top 15 genes are displayed. Complete list and gene network are available in supporting information (S1 Fig and S1 Table).

(D) Differential gene expression of the 42 transcripts affected by IFN-α stimulation. Expression Fold Change are shown for shSNRNP200 cells (200_NS), shSNRNP200 cells + SeV (200 SEV) and shSNRNP200 cells + IFN-α (200_IFNa) and for shNT control cells (NT_SEV, NT_IFNa). Numerical values are log2 fold change. Top 15 genes are displayed. Complete list and gene network are available in supporting information.

(E) Interaction network of the 13 common genes shown in (a) and affected by SNRNP200 silencing. The colors inside the dot represent their biological processes (see legend on left). Lines represent physical interactions (protein-protein interactions), pathways (blue), and co-localization (purple) attributes. Shaded nodes represent input data; Black nodes represent most likely first-degree interactor. Higher magnification and input genes of this network can be found in supporting documentation. The network on the right corner represents the connections to IRF3.



Reactome Pathway Enrichment	q-Value	Expected	Hits
Immune System	**1.91e-06**	**212**	**286**
Adaptive Immune System	1.67e-05	122	175
Cell Cycle	1.56e-05	94.7	143
Mitotic M-M/G1 phases	2.1e-05	49.6	85
Mitotic G1-G1/S phases	8.36e-05	26.1	51
DNA Replication	0.000135	19	40

Gene Ontology Enrichment	q-value
cellular response to type I interferon	4.25E-26
response to virus	6.80E-23
double-stranded RNA binding	6.17E-8

Gene Ontology Enrichment	q-value
response to virus	1.66E-18
response to type I interferon	8.03E-17

Figure S8. SNRNP200 silencing leads to an impaired induction of innate immunity genes.

HEK 293T cells are transduced with shNT or shSNRNP200 for three days and either unstimulated (NS) infected with SeV or treated with IFN-α for 16 hours. Relative gene expression was measured by microarray and compared to control shNT cells. (A) Left - Volcano plot showing the effect of SNRNP200 silencing on gene expression level of untreated cells (SNRNP200_NS). Only genes > 1,5 log2 fold induction change are displayed. Right - Reactome Pathway Enrichment of up- or down-regulated genes upon SNRNP200 silencing. (B) Left - Venn diagram of the number of

altered genes (> 1,5 log2 fold) of shSNRNP200 unstimulated cells (SNRNP200_NS) or infected with SeV (SNRNP200_SEV) and compared to control shNT cells infected with SeV (NT_SEV).

Right - Volcano plot of the gene expression in shNT cells and shSNRNP200 following SeV infection. Table shows the gene ontology enrichment of the gene list used. (C) Left – Venn diagram of the number of altered genes (> 1,5 log2 fold) of shSNRNP200 unstimulated cells (SNRNP200_NS) or treated with IFN-α (SNRNP200_IFN-α) and compared to control shNT cells treated with IFN-α (NT_IFN-α). Right - Volcano plot of the gene expression in shNT cells and shSNRNP200 following IFN-α stimulation. Table shows the gene ontology enrichment of the gene list used.

3.3.3. Sec63-1 domain of SNRNP200 is required for virus-mediated *IFNB1* production

To examine how SNRNP200 directly contributes to IRF3-mediated IFNB1 activation upon SeV infection, we first tested a series of recombinant SNRNP200 truncation mutants for their ability to rescue IFNB1 reporter activity and ISG expression in SNRNP200 KD cells (Figures 20 and S9). We showed that expression of all truncated proteins are unable to induce antiviral response in SNRNP200 KD cells (beside weak IFNB1 activation by D1-D3 construct).

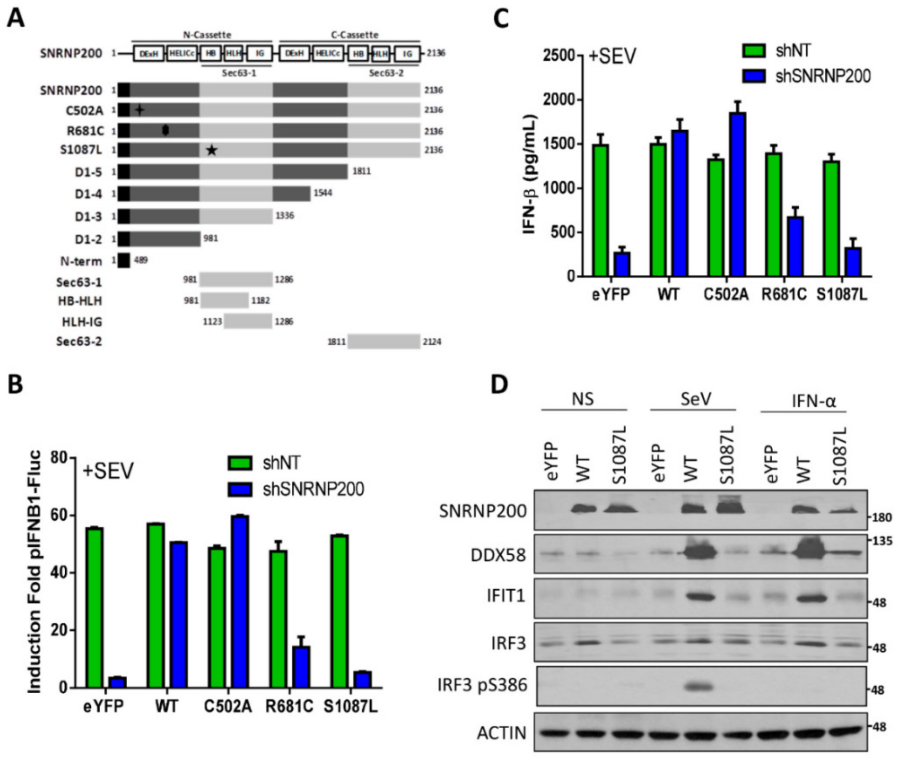


Figure 20. SNRNP200, but not Sec63-containing S1087L mutant, rescues SeV- and IFN-α-mediated induction of antiviral response in SNRNP200 KD cells.

(A) Schematic representation of SNRNP200 protein, C-terminal truncation and clinically relevant mutants.

(B) HEK 293T pIFNB1-Luc cells are transduced with shSNRNP200 and transfected with RNAi-resistant SNRNP200 WT and variants expression plasmids bearing the indicated mutation or eYFP as a control. Following 24 hours of SeV infection, total luciferase levels are measured and compared with control shNT cells.

(C) HEK 293T are treated as indicated in B. IFN-β secretion levels are measured by ELISA and compared with shNT cells.

(D) HEK 293T cells are transduced with shSNRNP200 and transfected with RNAi-resistant SNRNP200 WT and variants expression plasmids bearing the indicated mutation or eYFP as a control. At 24 hours, cells are harvested and DDX58, IFIT1, IRF3 and IRF3pS386 levels are resolved by immunobloting analysis of cell lysates.

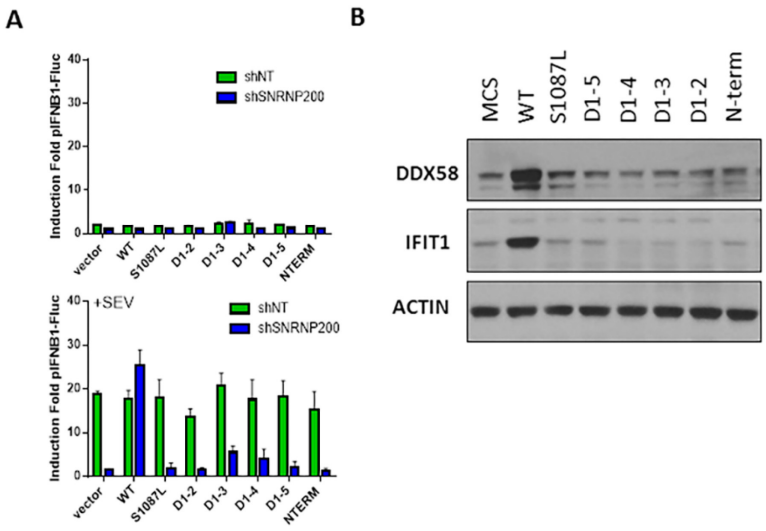


Figure S9. The full-length protein sequence of SNRNP200 is required to rescue SeV-mediated induction of antiviral response in SNRNP200 KD cells.

(A) HEK 293T pIFNB1-Luc cells are transduced with shSNRNP200 for three days and transfected with RNAi resistant expression plasmids for SNRNP200 WT, S1087L or C-terminal truncated mutants for the last 48 hours. Subsequently, cells are untreated (left panel) or infected with SeV for 16 hours (right panel). IFNB1 promoter-driven luciferase activities are measured and compared with control shNT cells. (B) HEK 293T cells are treated as indicated in (A). Cells are harvested and DDX58, IFIT1 and ACTIN proteins are resolved by immunobloting of cell lysates.

Indeed, its revealed that deletion of the C-terminal Sec63 domain (Sec63-2) solely, which was reported in yeast (Sec63-2 deleted Brr2 protein) to reduce ATPase/helicase activity and splicing [319], completely abolished activation of IFNB1 promoter-driven reporter activity and induction of IFIT1 and DDX58 upon SeV infection. To further explore a dual regulatory role in splicing and RNA-mediated antiviral response, we then took advantage of described SNRNP200 mutations associated to the retinal disorder retinitis pigmentosa 33 (RP33)[298, 320, 321]. Particularly, we investigated the RP33-associated SNRNP200 S1087L and R681C variants located respectively within the Sec63-1 homology domain and the N-terminal RecA-like ATPase/helicase domains (Figure 20A). We first demonstrated that the ectopic expression of RNAi-resistant SNRNP200 WT completely rescues SeV-mediated IFN-β secretion and IFNB1-driven reporter activity in KD cells, further validating the specificity and minimal off-target effects of shSNRNP200 to explain the immunoregulatory phenotype (Figure 20). Surprisingly, expression of SNRNP200 S1087L mutant has completely lost the ability to rescue inducible IFNB1 activation (Figure 20B-20C). Similar results were obtained with sole rescue of endogenous *IFNB1* mRNA levels by SNRNP200 WT using qRT-PCR (Figure S10).

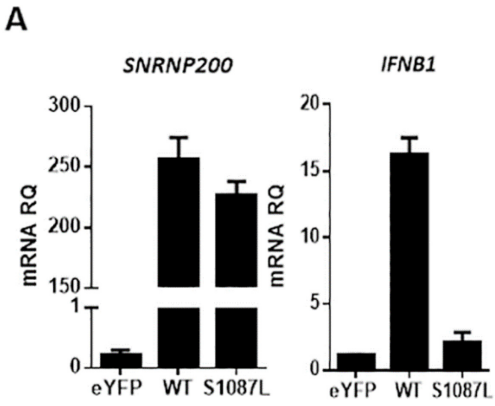


Figure S10. Ectopic expression of SNRNP200, but not Sec63-containing S1087L mutant, rescues SeV-mediated induction of *IFNB1* mRNA in SNRNP200 KD cells.

qRT-PCR quantification of *SNRNP200* and *IFNB1* mRNA levels of HEK 293T cells transduced with shSNRNP200 for three days and transfected with eYFP or RNAi resistant SNRNP200 WT or SNRNP200 S1087L expression plasmids for 48 hours and subjected to SeV infection for 16 hours.

Concordantly, SNRNP200 WT but not S1087L mutant restores IRF3 protein levels and, more importantly, IRF3 386 phosphorylation upon SeV infection as well as inducible levels of DDX58 and IFIT1 proteins (Figure 20D). SNRNP200 WT but not S1087L mutant also restores IFN-α-dependent IFIT1 and DDX58 induction. We further showed that expression of R681C variant barely rescues IFNB1 promoter-driven reporter activity and IFN-β secretion (Figure 20B-20C). More interestingly, when investigating a mutant within the ATP binding motif, we found that overexpression of the SNRNP200 C502A variant elicits an IFNB1 response that is independent of viral infection (Figure S11), as recently reported for the natural gain-of-function DDX58 and IFIH1 ATPase-deficient variants [322].

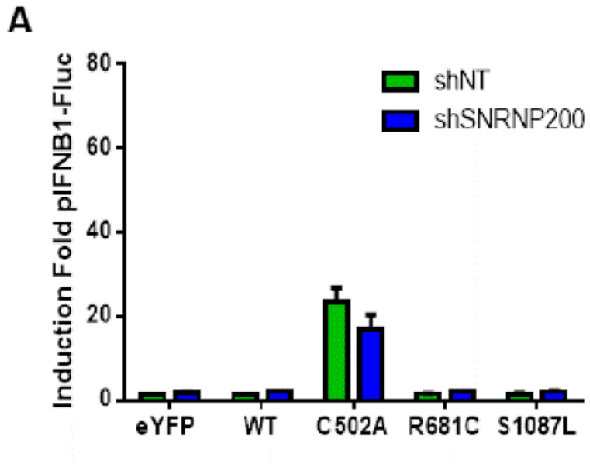


Figure S11. SNRNP200 C502A variant elicits an IFNB1 response independently of viral infection.

HEK 293T pIFNB1-Luc cells are transduced with shSNRNP200 for three days and transfected with RNAi resistant SNRNP200 WT or variants expression plasmids bearing the indicated mutation for 48 hours. IFNB1 promoter-driven luciferase activities are measured and compared with control shNT cells.

The constitutive induction of IFNB1 with expression of SNRNP200 C502A is further enhanced upon SeV infection to levels similar than with WT enzyme (Figure 20B-20C), thereby suggesting the requirement of a functional SNRNP200 ATPase in conferring specificity to viral RNA and preventing signaling through recognition of self-RNA. Altogether, the data firmly establish a critical role of the Sec63-1 domain and the functional requirement of the N-terminal ATPase/helicase domain to promote the virus-mediated innate immune response.

3.3.4. Sec63-1 domain of SNRNP200 is a major determinant of viral RNA recognition

DExD/H-box helicases such as RIG-I are engaged in antiviral innate immunity by detecting viral nucleic acids and preventing recognition of self-RNA through ATP hydrolysis. As the Sec63-1 containing S1087L mutation was reported to diminish binding to RNA duplex and to reduce RNA-stimulated ATPase/helicase activity without any discernible effect on the folding of SNRNP200 (27), we hypothesized that this natural loss-of-function mutation abolishes the recognition of viral RNA for IFNB1 induction. To determine if S1087L variant impaired the binding of immunostimulatory RNA in SeV-infected cells, we measured the *in vitro* ability of exogenously expressed SNRNP200 variant to bind biotinylated polyinosinic-polycytidylic acid (poly (I:C)) by RNA pull-down and western blot analysis of bead-bound fractions (Figure 21). We first showed that FLAG-SNRNP200 WT binds poly (I:C) used as virus surrogate double-stranded RNA (dsRNA) molecules solely from SeV-infected cell extracts (Figure 21A). Furthermore, we observed a complete loss of poly (I:C) binding by the FLAG-SNRNP200 S1087L variant. More interestingly, we showed that the FLAG-Sec63-1 domain, but not FLAG-Sec63-2, was sufficient to bind poly (I:C) (Figure 21B). These observations were confirmed by the RNA pull-down of SNRNP200 and its Sec63-1 domain with biotinylated HCV RNA genome, but not of SNRNP200 S1087L variant and Sec63-2 domain (Figure 21C). To provide insights into the binding of SNRNP200 to immunostimulatory RNA molecules, we next investigated the ability of the synthetic 5'-triphosphate (5'ppp) and double-stranded stretch of RNA using the full-length HCV genome produced by in vitro transcription with T7 polymerase, and known as a potent inducer of innate response (Figure 21D). We showed a comparable binding of the FLAG-SNRNP200 WT to the untreated and to the calf-intestine alkaline phosphatase (CIAP)-treated blunt-ended HCV RNA, suggesting that the 5'ppp moieties is not essential for the recognition of viral dsRNA by SNRNP200. However, FLAG-SNRNP200 do not bind dsDNA molecules as reflected by the lack of pull-down with biotinylated polydeoxyadenylic acid-polythymidylic acid (poly (dA:dT)) and polydeoxyguanylic acid-polydeoxycytidylic acid (poly (dG:dC)) homopolymer molecules, in contrast to the pull down of control FLAG-cGAS (Figure 21D)[323].

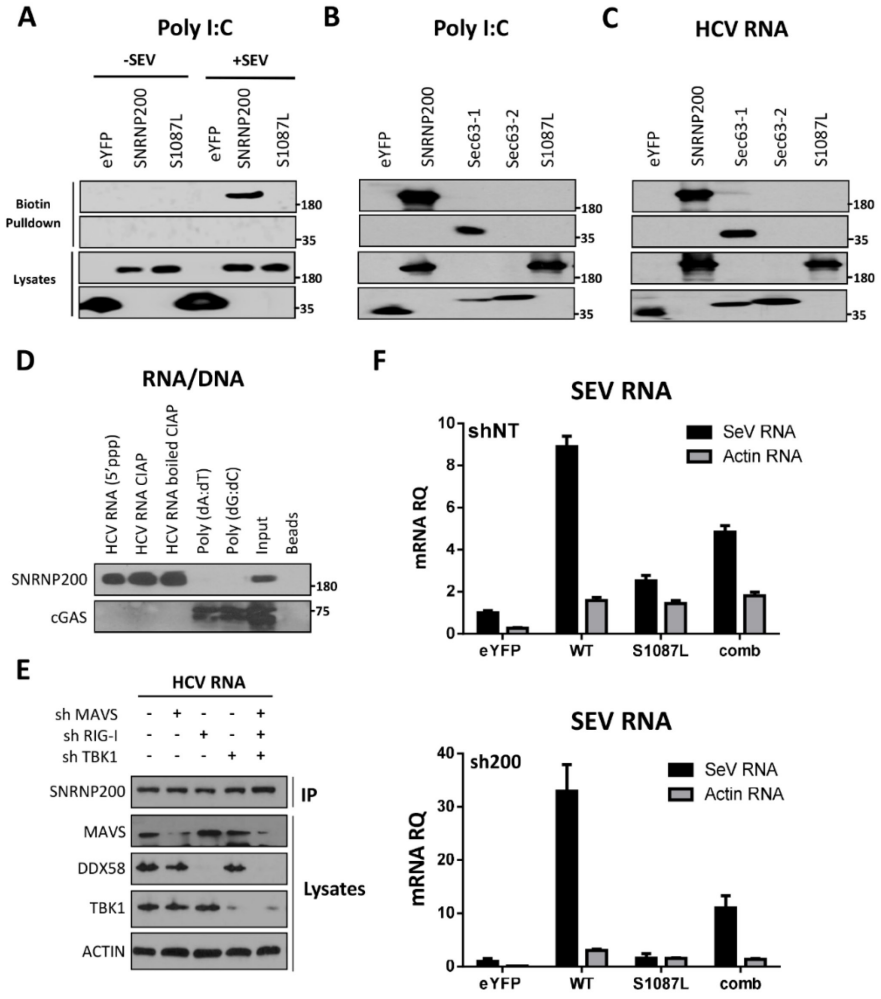


Figure 21. SNRNP200, but not S1087L mutant, binds viral RNA *in vitro*.

(A) HEK 293T cells are transfected with FLAG-eYFP (control), FLAG-SNRNP200 or FLAG-SNRNP200 S1087L mutant expression plasmids for 48 hours and infected with SeV for 16 hours. RNA pull-down assays are performed with cell lysates using biotinylated poly (I:C). Cell lysates and bead-bound complexes are analyzed by Western blotting and compared to uninfected control cells.

(B) HEK 293T cells are transfected with FLAG-Sec63-1 and FLAG-Sec63-2. RNA pull-down assays are performed and analyzed as indicated in (A).

(C) HEK 293T are treated as indicated in (B) and RNA pull-down assays are performed on cell lysates using biotinylated HCV RNA and analyzed as indicated in (A).

(D) HEK 293T cells are transfected with FLAG-SNRNP200 (top panel) or FLAG-cGAS (bottom) expression plasmids for 48 hours. RNA pull-down assays are performed with cell lysates using biotinylated HCV RNA (5'ppp) that is either left untreated or treated with CIAP or heat inactivated (h/i) CIAP (control), and with biotinylated poly (dA:dT) and poly (dG:dC) DNA molecules. Pull-down complexes are resolved by immunobloting and compared to protein input and uncoated beads as negative control.

(E) Pull-down assays with biotinylated HCV RNA and transfected FLAG-SNRNP200 are performed with lysates of cells transduced with shRNA targeting MAVS, RIG-I or TBK1, either alone or in combination.

(F) HEK 293T cells are transduced with shNT or shSNRNP200 and transfected with FLAG-eYFP, RNAi-resistant FLAG-SNRNP200 WT or S1087L variant expression plasmids and both (comb) for 48 hours. At 16 hours post-infection with SeV, cell lysates are subjected to an anti-FLAG immunoprecipitation, and RNA molecules are extracted from the immune complexes and analyzed by qRT-PCR. SeV and actin RNA levels are determined and normalized to RNA levels of cell lysates.

To assess the requirement of DDX58, MAVS or TBK1 in a protein complex with SNRNP200 for HCV RNA binding, we silenced expression of proteins individually or together and performed RNA pull-down assays to detect SNRNP200 (Figure 21E). We showed that SNRNP200 equally binds HCV RNA ruling out a contribution of these proteins for its ability to recognize viral RNA. Finally, we immunoprecipitated FLAG-tagged SNRNP200 WT and S1087L variant upon SeV infection of SNRNP200 KD and control shNT cells, and analyzed the co-purified RNA molecules by qRT-PCR detection (Figures 21F). We first found increased amounts of actin mRNA for both immunoprecipitated proteins when compared to eYFP control protein and normalized to RNA levels in whole cell lysates. We then observed a significant enrichment of SeV RNA upon immunoprecipitation of SNRNP200 WT, which is more important in KD cells than in shNT control cells expressing endogeneous SNRNP200 protein, demonstrating a direct binding to viral genome. The amount of SeV RNA recovered with the WT was almost 10- to 20-fold higher than with the S1087L variant in KD cells (and 3-fold in shNT cells) reflecting an altered RNA binding ability of the mutant. Despite the weak binding of SeV RNA by S1087L, such interaction is not productive for IFNB1 induction as revealed by its loss-of-function and is possibly due to its N-terminal RecA domains. Altogether, our data suggest that the Sec63-1 domain of SNRNP200 acts as a major determinant of viral RNA recognition to activate antiviral innate immune response.

3.3.5. Sec63-1 domain of SNRNP200 interacts with TBK1

To better define a specific immunoregulatory role of SNRNP200, we screened for binding partners of the antiviral signaling pathways by expression of the FLAG-tagged protein. This successfully detected a constitutive interaction between SNRNP200 and ubiquitously expressed kinase TBK1, which was also detected with expression of SNRNP200 S1087L mutant (Figure 22A). DDX58, MDA5, MAVS, IKBKE or IRF3 proteins were not detected in immunoprecipitated FLAG-SNRNP200 samples (data not shown). Various SNRNP200-truncated mutants (see Figure 20A) were expressed to map the TBK1 binding domain (Figure 21B). Mutagenesis analysis showed that the N-terminal Sec63 domain (Sec63-1) of SNRNP200 is required and sufficient for TBK1 interaction (Figure 22C), reminiscent of our observation for RNA binding (Figure 21B-21C). Both Sec63 homology domains of SNRNP200 contain a helical bundle (HB) and immunoglobulin like (IG) sub-domains, separated by a helix loop helix (HLH) motif, which were expressed separately in Sec63-1 to more precisely map the interaction with TBK1. We demonstrated that the integrity of the Sec63-1 domain is required for optimal TBK1 interaction, but a weak detection is observed with the HLH-IG sub-domain suggesting its contribution in binding TBK1 (Figure 22C). We also confirmed that the C-terminal Sec63-2 domain is not able to bind TBK1, which corroborates binding of the N-terminal truncated D1-3, D1-4 and D1-5 mutants (Figure 22B-22C). In reciprocal experiments, immunoprecipitation of FLAG-tagged TBK1 confirmed the interaction with ectopically expressed SNRNP200 from uninfected and SeV-infected cells (Figure 22D). In addition, we showed that the kinase-dead mutant of TBK1 (K38A) is still able to interact with SNRNP200, demonstrating that this interaction is not dependent on TBK1 activity (Figure 22D). Finally, the interaction was confirmed in A549 cells by co-immunoprecipitation of endogenous SNRNP200 and TBK1 proteins (Figure S12).

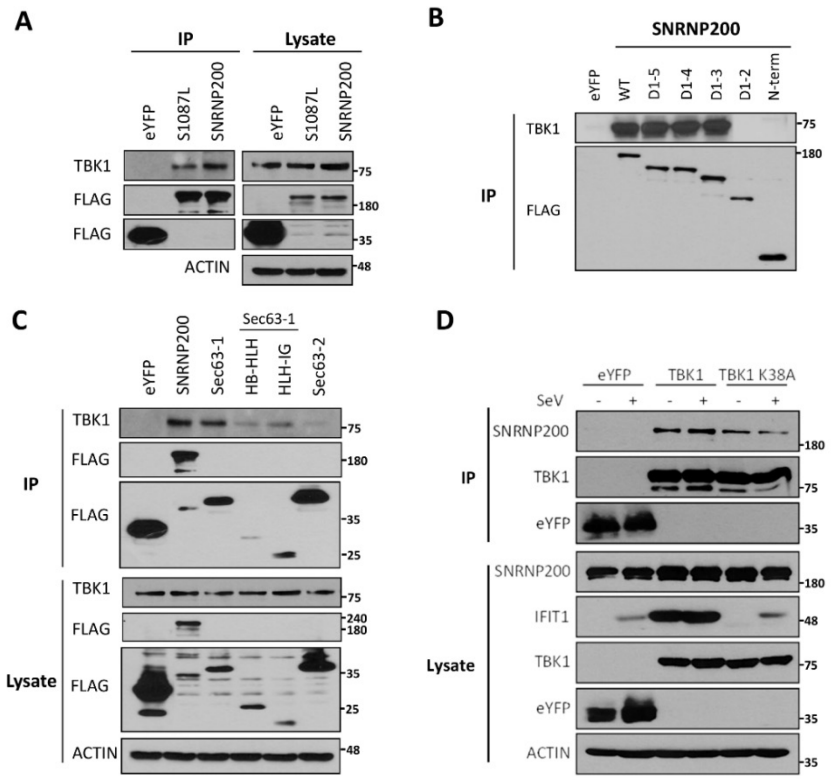


Figure 22. SNRNP200 Sec63-1 domain interacts with TBK1.

(A) HEK 293T cells are transfected with FLAG-eYFP (control), FLAG-SNRNP200 or FLAG-SNRNP200 S1087L mutant expressing plasmids for 48 hours. Cell lysates are prepared following 16 hours of SeV infection and subjected to immunoprecipitation with anti-FLAG antibodies. Cell lysates and immune complexes are resolved by immunobloting analysis using anti-FLAG and anti-TBK1 antibodies.

(B) Immunoprecipitation of FLAG-SNRNP200 C-terminal deletion mutants are performed and analyzed as indicated in (A).

C) Immunoprecipitation of FLAG-SNRNP200 Sec63-1, HB-HLH or HLH-IG subdomains of Sec63-1 and Sec63-2 are performed and analyzed as indicated in (A).

(D) Reciprocal immunoprecipitation of FLAG-eYFP (control), FLAG-TBK1 or FLAG-TBK1 K38A mutant following ectopic expression of SNRNP200 are performed as indicated in (A) and analyzed as indicated in (A). Cell lysates and immune complexes are resolved by immunobloting analysis using anti-FLAG and anti-SNRNP200 antibodies.

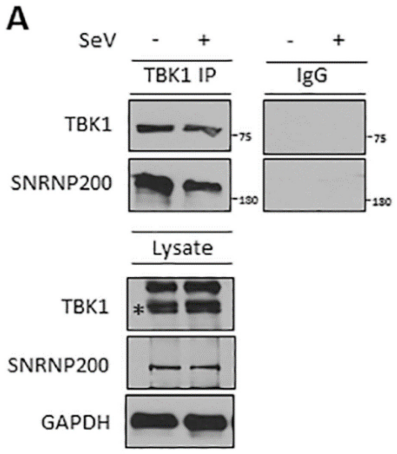


Figure S12. Constitutive interaction of TBK1 and SNRNP200 endogenous proteins in A549 cells.

(A) A549 cells are untreated or infected with SeV for 16 hours. Cell lysates are subjected to immunoprecipitation using anti-TBK1 or control IgG antibodies followed by incubation with protein G sepharose beads. TBK1 and SNRNP200 are resolved by immunoblotting of immune complexes (up) and cell lysates (down). Results are compared to untreated cells. TBK1 protein is indicated with an asterix.

To further assess the interaction of SNRNP200 and TBK1, we investigated their intracellular localization by confocal fluorescence microscope images of HEK 293T and HeLa cells in response to SeV infection (Figures 23 and S13). We observed that FLAG-SNRNP200 (HEK 293T cells) and endogenous SNRNP200 (Hela cells) are localized to nucleus and cytoplasm with a diffuse staining prior to stimulation. Interestingly, we observed a subcellular fraction of SNRNP200 that relocalizes with TBK1 into perinuclear cytoplasmic speckles upon viral infection (Figures 23A and S13B). SNRNP200 and TBK1 colocalization can easily be observed in the 3D-stack and lateral view of infected cells (Figures 23B and 23C). However, the staining of FLAG-SNRNP200 S1087L mutant does not show relocalization of the protein nor colocalization with TBK1 into cytoplasmic speckles upon infection (Figures 23A and S13A), which correlates with the lack of RNA binding (Figure 21). Altogether, our data suggest that viral RNA recognition by the Sec63-1 domain is responsible of the cytoplasmic relocalization of SNRNP200 to perinuclear cytoplasmic speckles, which possibly functions as an adaptor via its interaction with TBK1 to promote IRF3 phosphorylation and antiviral innate response.

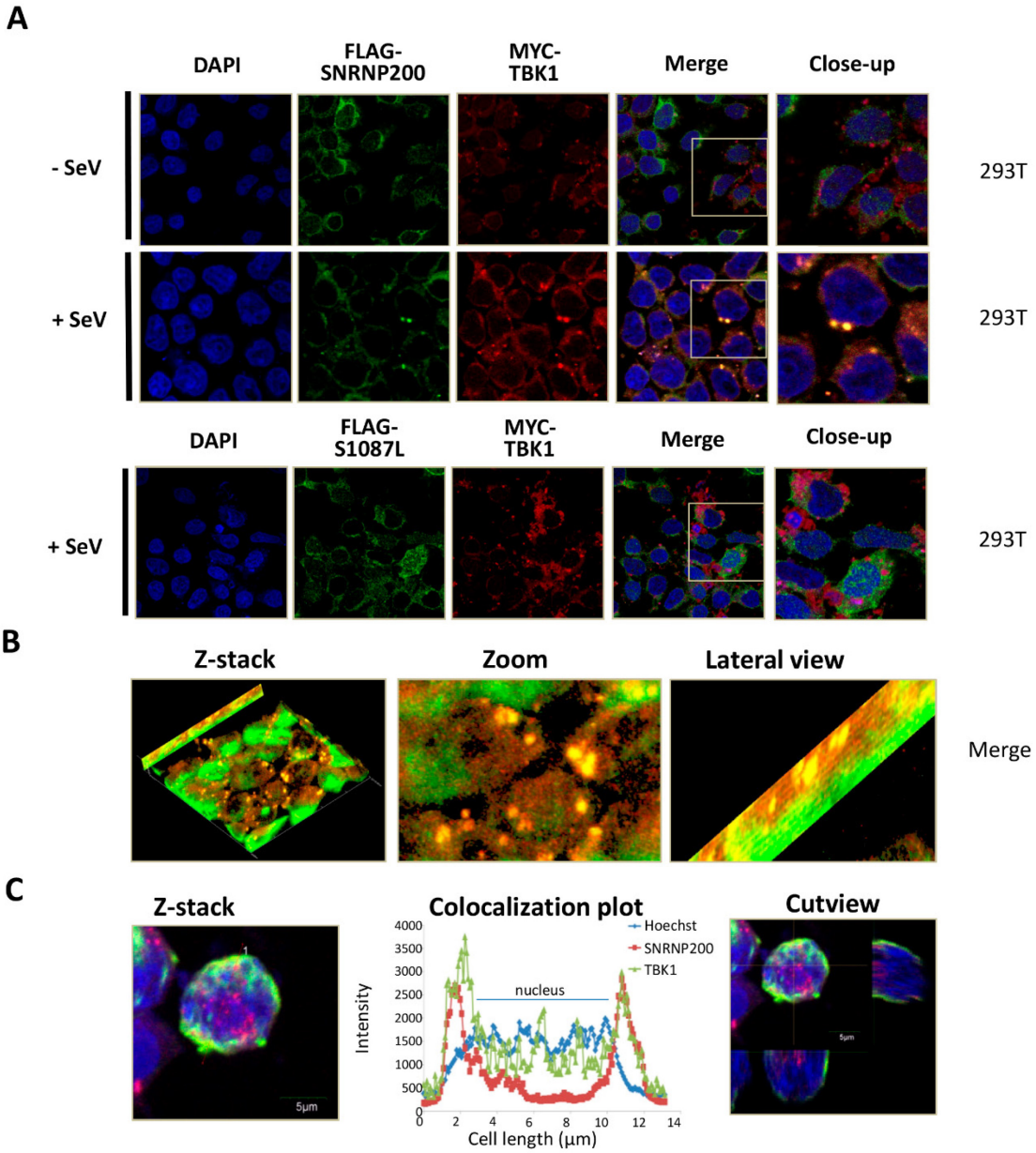


Figure 23. Re-localization of SNRNP200 in perinuclear cytoplasmic speckles and colocalization with TBK1 in response to SeV infection.

(A) Confocal analysis of HEK 293T co-transfected with FLAG-SNRNP200 or FLAG-SNRNP200-S1087L and MYC-TBK1 using Hoechst, anti-FLAG and anti-MYC antibodies without virus infection or following a 16-hour infection with SeV. Imaging was done using a 63x/1.40 Oil DIC objective. Intensity analysis showed that 19/19 cells have cytoplasmic colocalization between SNRNP200 and TBK1 in SeV-infected cells.

(B) Z-stack and lateral view of SNRNP200 and TBK1 in SeV-infected HEK 293T cells treated as indicated in (A).

(C) Z-stacks reconstitution of a complete cell with colocalization plot and cut view, showing an exclusive cytoplasmic colocalization of SNRNP200 and TBK1.

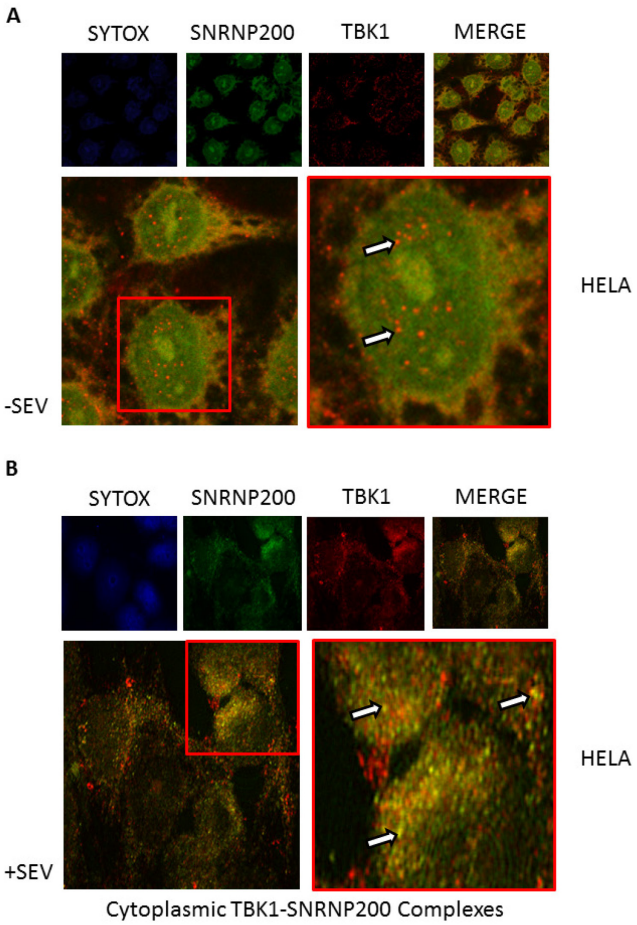


Figure S13. Relocalization of SNRNP200 into cytoplasmic speckles and co-staining with TBK1 is dependent on SeV infection in Hela cells.

(A) Hela cells are stained with anti-TBK1 and anti-SNRNP200 antibodies and analyzed by confocal microscopy. Nuclei are stained with Sytox Green. A merge for both protein is shown (Merge G/R) and at higher magnification (down panel). SNRNP200 staining is green and TBK1 is red. White arrows indicate TBK1 stained as red dots. Imaging was done using a 63x/1.40 Oil DIC objective.

(B) Hela cells are infected with SeV for 16 hours and analyzed as indicated in (B). White arrows indicate SNRNP200-TBK1 complex stained as yellow dots. Imaging was done using a 63x/1.40 Oil DIC objective.

3.3.6. SNRNP200 regulates innate immune response of SeV-infected human MDM

The regulation of antiviral response by SNRNP200 was further investigated in immune cells using primary cultures of purified human monocyte-derived macrophages (MDM). Interestingly, SeV infection leads to an increase in the immunodetection of SNRNP200 protein without affecting *SNRNP200* mRNA level (Figure 24A), as observed in SeV-infected and IFN-α-treated HEK 293T cells (Figure S14) and is possibly due to its relocalization into perinuclear speckles. More importantly, we showed that depletion of SNRNP200 in MDM decreases induction of IFIH1 and IFIT1 protein, and completely blocks IRF3 Ser386 phosphorylation within 3-hour post-infection (Figure 24B). Kinetic studies of IFN-β production further showed that secretion is completely abrogated from 3 hours post-infection (Figure 24C). Parallel decreased of *IFNB1* mRNA levels is observed at 1 hour post-infection of MDM, in contrast to comparable TNFα mRNA levels, which correlated the reduced levels of *SNRNP200* mRNA (Figure 24D, 24E and 24F). Interestingly, the duration of SNRNP200 gene silencing was not sufficient to affect steady-state levels of IRF3 protein as no reduction was observed with MDM. In addition, SNRNP200 KD also increased SeV protein levels as observed in HEK 293T cells (Figures 17C). These results confirm a regulatory role of SNRNP200 in IRF3-mediated antiviral response of human macrophages.

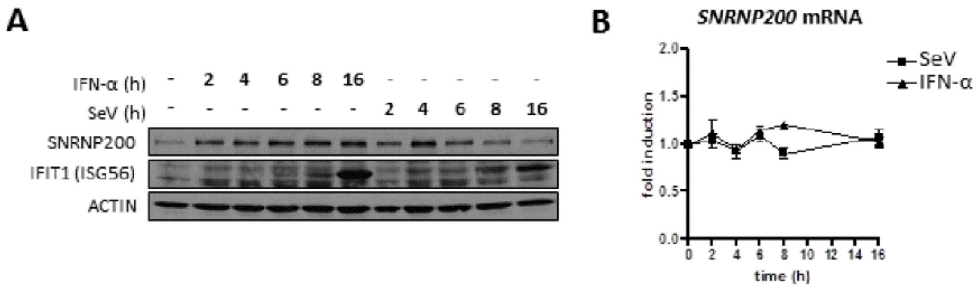


Figure S14. SNRNP200 protein accumulation in HEK 293T following SeV infection or IFN-α treatment does not result from an increase in mRNA levels.

(A) Immunoblot analysis of HEK 293T cells infected with SeV or treated with IFN-α for the indicated times. (B) qRT-PCR quantification of *SNRNP200* mRNA levels of HEK 293T cells

treated as indicated in (A).

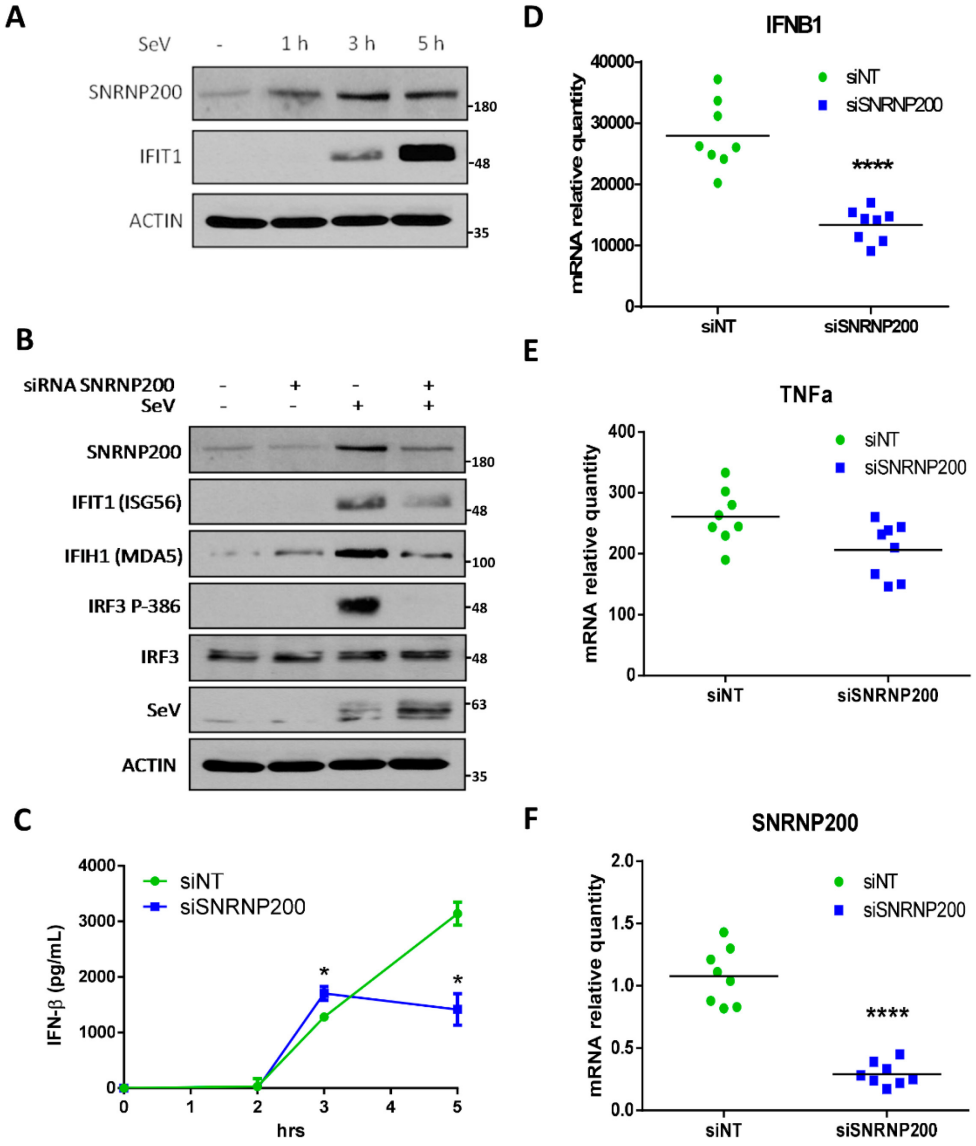


Figure 24. SNRNP200 KD restricts SeV-mediated antiviral response of human MDM.

(A) MDM are infected with SeV for 1, 3 or 5 hours. SNRNP200 and IFIT1 protein levels are resolved by immunobloting of cell lysates.

(B) MDM are transfected with a pool of siRNA targeting SNRNP200 for 48 hours and infected with SeV. At 3 hours post-infection, cells are harvested and selected proteins (SNRNP200, IFIT1, IFIH1, IRF3, IFR3-386, SeV and actin) are resolved by immunobloting of cells lysates and compared to control cells treated with scrambled siRNA.

(C) MDM are transfected with a pool of siRNA targeting SNRNP200 for 48 hours and infected with SeV for 1, 3 or 5 hours. Supernatants are harvested and IFN-β secretion levels are measured by ELISA and compared to control cells treated with scrambled siRNA (siNT).

(D-F) MDM are transfected with a pool of siRNA targeting SNRNP200 (siSNRNP200) or scrambled siRNA (siNT) and infected with SeV for 1 hour. Cells are harvested and relative gene expression of IFNB1 (D), TNFα (E) and SNRNP200 (F) are measured by qRT-PCR and compared with scrambled control cells. mRNA RQ are normalized versus *ACTIN* and *HPRT1* mRNA. P values <0.0001 (****) are indicated. Data are pooled results from two experiments of two biological replicates

3.3.7. Impaired antiviral response of PBMCs from RP33 patients

Retinitis Pigmentosa (RP) is an inherited degenerative eye disease that causes severe vision impairment and blindness due to mutations in several core spliceosomal proteins. Peripheral blood cells (PBMCs) of RP33 patients genotyped for the particular monoallelic mutation in SNRNP200: p.S1087L- c.3260C>T in the Sec63-1 domain and with p.R681C c.2122G>A in the N-terminal helicase domain were characterized for their innate immune response to viral infection (see Table S1 for donor information). Interestingly, all RP33 patients showed a complete block in IFN-β production from 3-hour post-infection with a significant two-fold reduction in IFN-β secretion at 7 hours (Figure 25A and 25B). The decreased IFN-β was corroborated by a reduction in virus-induced IRF3-dependent *IFNB1* and *IFIT1* mRNA levels while NF-κB-dependent *TNF* mRNA levels were not significantly affected (Figure 25C, 25D and 25E). The *IRF3* mRNA levels determined by qRT-PCR showed no significant difference between healthy donors (HD) and RP 33 patients (Figure 25F). Finally, a cytokine 41-plex assay performed on supernatants of infected PBMCs from HD and RP33 patients showed significant changes in IFN-α2 and similar cytokine/chemokine levels for RANTES, IL6, CXCL10 and IL1B (Figure S15). The data support a role of SNRNP200 in regulating IRF3-mediated antiviral response of PBMCs from RP33 patients, without altering the NF-κB-dependent inflammatory pathway.

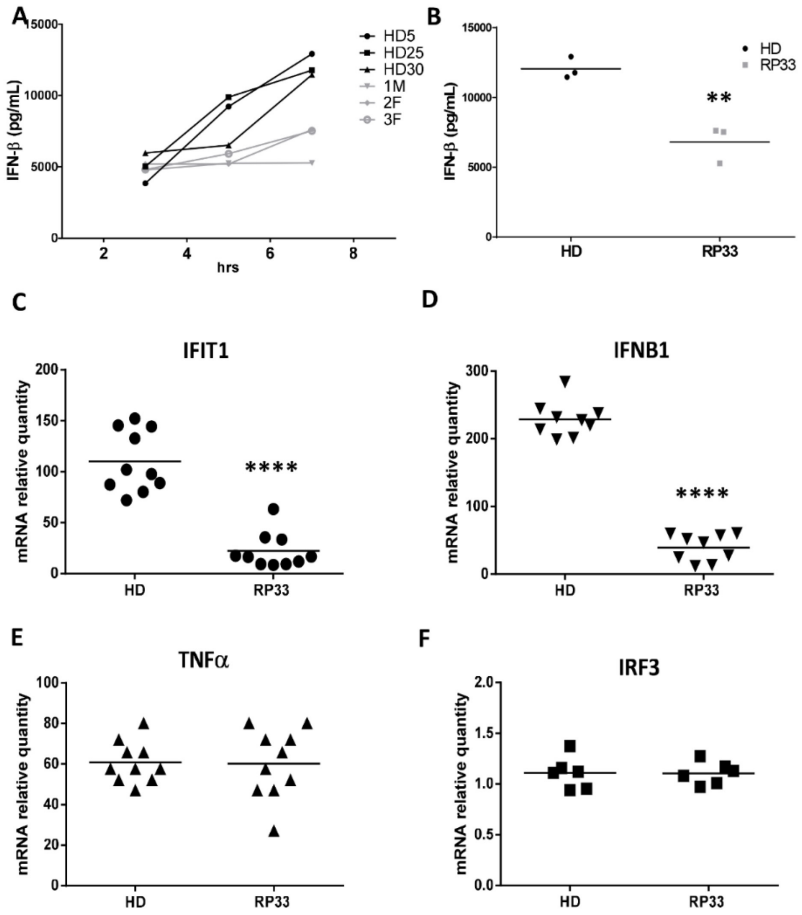


Figure 25. PBMCs of RP33 patients bearing monoallelic point mutation in SNRNP200 show hindered antiviral innate immune response.

(A) PBMCs of RP33 patients are infected for 3, 5 and 7 hours with SeV. Supernatants are harvested and IFN-β secretion levels are measured by ELISA and compared to PBMCs of three healthy donors (HD).

(B) Alternative representation of the 7-hours SeV infection of individual RP33 patients and HD as in (A), where the horizontal bar represents the mean of each group. P value <0.01(**) is indicated.

(C-F) PBMCs of RP33 patients are infected for 1 hour with SeV. Cells are harvested and relative gene expression of IFIT1(C), IFNB1 (D), TNFα (E) and IRF3 (F) are measured by qRT-PCR and compared with PBMCs of HD. mRNA RQ are normalized versus *ACTIN* and *HPRT1* mRNA. P values <0.0001 (****) are indicated.

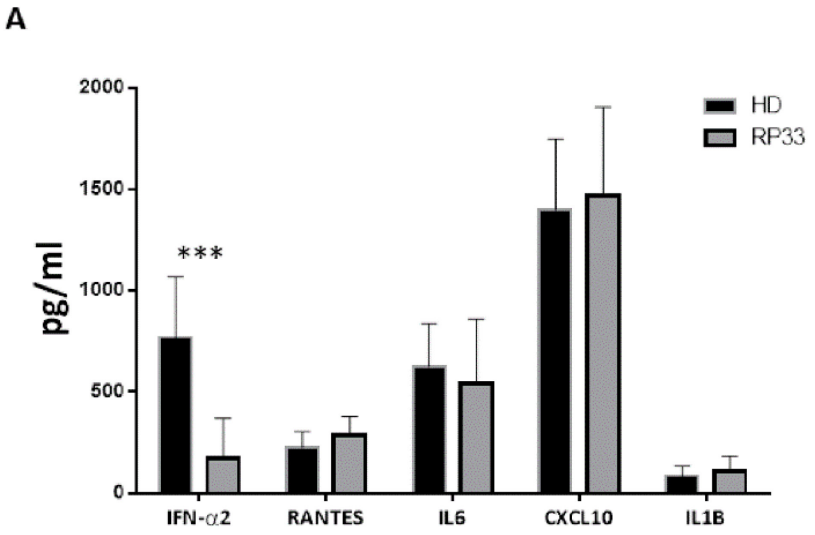


Figure S15. PBMCs from RP33 patients bearing monoallelic point mutation in SNRNP200 show hindered IFN-α2 secretion.

PBMCs of RP33 patients (RP33) or healthy donors (HD) are infected with SeV for 16 hours. Supernatants are then harvested and cytokine levels are measured by multiplex-ELISA. In total 42 cytokines are analyzed and representative results for IFN-α2, RANTES, IL6, CXCL10 and IL1B are shown.

Table SI. Description of the RP33 patients (Age, Sex, Ethnicity and Retinitis Pigmentosa associated polymorphism) who volunteered PBMCs used in the experiments presented in Fig 24. Mean ages of patients and healthy donors were matched (43.3 vs. 43.0).

Patient	Age	Sex	Ethnicity	ADRP-Mutation
1	21	M	Caucasian	p.S1087L
2	50	F	Caucasian	p.S1087L
3	59	F	Black	p.R681C

3.4. Discussion

SNRNP200 RNA helicase is ubiquitously expressed in cells and is a core component of the spliceosome. Its plays a key role in unwinding U4/U6 small nuclear RNA (snRNA) to form a highly structured RNA interaction network among the U2, U6, and U5 snRNAs and the pre-mRNA required for activation of the spliceosome [324, 325]. Despite such critical function for pre-RNA splicing, there are no data to our knowledge that suggests a role of SNRNP200 in host defense. Furthermore, few studies have described a contribution of spliceosomal proteins in immune innate immunity. Interestingly, two spliceosomal proteins (SRSF1 and SF3A1) were identified in our genome-wide gene silencing screen that have been previously reported in the generation of alternative splice variants of important innate immune regulators. Depletion of SRSF1 in human A549 lung cancer cells was shown to reduce IFN-β by expression of alternative IRF3 spliced variants [303], while SF3A1 silencing leads to decreased induction of pro-inflammatory cytokines by promoting alternative splice form of MyD88 [302]. In this study, we now provide evidence for a novel role of the spliceosomal SNRNP200 RNA helicase in the regulation of IRF3-mediated antiviral response upon RNA virus infection of human cells: 1. SNRNP200 KD cells infected with SeV and FLUA show higher virus titers and viral proteins (Figures 17 and S2), suggesting that SNRNP200 is involved in host defense mechanisms; 2. SNRNP200 KD cells reduce virus-mediated IFN-β production (Figures 17B, 18E, 24C and 25C); 3. Epistasis studies point out to a role for SNRNP200 at the step of IRF3 activation upon antiviral response (Figures 18E, 18F, 18B, 18D, 18E); 4. SNRNP200 regulates solely the RLR pathway and neither affects TRIF nor cGAS/STING pathways to activate IFN production (Figure 20B, 20D, 20E and S4); 5. SNRNP200 promotes IRF3 activation and requires a competent Sec63-1 domain and functional ATPase/helicase activity (Figure 20); 6. SNRNP200 Sec63-1 domain binds immunostimulatory RNA molecules (Figure 21); 7. SNRNP200 interacts with endogenous TBK1 through its Sec63-1 domain (Figure 22); 8. PBMCs of RP33 patients (who have one allele carrying the dominant S1087L or R681C mutation) show a reduction of IFN-β secretion when challenged with SeV (Figure 25).

Thus, we believed that SNRNP200, the only Ski2-like RNA helicase involved in pre-mRNA splicing, regulates IRF3-dependent IFNB1 production upon RNA virus infection by recognition of viral RNA that promotes phosphorylation of IRF3, possibly as an adaptor protein through a constitutive interaction with TBK1. Our results uncover unique molecular mechanisms of how SNRNP200 regulates antiviral response.

The major mechanism by which SNRNP200 functions is as a spliceosomal helicase that unwinds the U4/U6 di-snRNAs providing key remodeling activity for spliceosome catalytic activation, and thus regulates expression of a large and disparate group of genes associated to cell cycle [326] . Indeed, the gene profiling studies of HEK 293T cells revealed a large group of differentially expressed genes upon SNRNP200 depletion that are associated to immune system and cell cycle using a reactome pathway enrichment analysis. Nevertheless, among the total SeV- and IFN-induced genes of control shNT cells, the silencing of SNRNP200 KD affects a significant number of SeV- and/or IFN-inducible genes by more than 1,5 log2 fold induction (see Venn diagrams of Figure S8), for which analysis of the SNRNP200 gene network confirmed that it mapped to innate immunity gene function such as response to virus and type I IFN signaling pathway (Figure 19E). It also revealed that these altered genes (41+29 for SeV and 18+29 for IFN with 29 in common) are highly connected to IRF3 and IFNB1 with a molecular signature supporting that SNRNP200 silencing hinders IRF3-dependent gene induction. One possible mechanism is that SNRNP200 affects the pathway at a transcriptional level as first revealed with the observation that SNRNP200 alters expression of the key transcriptional factor IRF3, which is essential for *IFNB1* transcription. The decrease in IRF3 mRNA and protein levels correlated with a reduced SNRNP200 mRNA and protein levels, as well as with the reduced expression of effector genes upon infection of SNRNP200 KD cells (Figures S5). Our experiments did not identify splicing variants to explain the reduced IRF3 protein levels (Figure S8), ruling out alternative splicing regulation of *IRF3* mRNA in SNRNP200 depleted cells. While a reduction of IRF3 and of DDX58 protein expression (Figure S5A) is observed that may contribute to the phenotype, much evidence would suggest that this is not the main mechanism responsible for the reduced IFNB1 production in SNRNP200 KD cells.

First, ectopic expression of IRF3 and/or DDX58 in SNRNP200 KD cells failed to restore virus-induced IFNB1 production and IFIT1 expression, while expression of constitutively active IRF3(5D) fully rescued antiviral response (Figures 17F, 18B, 18D, 18E and S6). Second, we showed that activation of the cGAS/STING pathway involved in the recognition of cytosolic DNA is not affected by SNRNP200 KD as a result of the reduced IRF3 protein levels (Figure 18C), and weakly restrict IFNB1 production solely upon combination of cGAS/STING activation and SeV infection (Figure 18-18E, see cGAS+STING versus cGAS+STING+IRF3). Indeed, the full activation of the cGAS/STING/TBK1/IRF3 pathway in SNRNP200 KD cells further supports a specific role of SNRNP200 in the activation of the RLR/MAVS/TBK1/IRF3 pathway upon RNA virus infection. Finally, SNRNP200-depleted MDM completely block IRF3 Ser386 phosphorylation, in the presence of IRF3 protein levels similar to control cells, resulting in the abolishment of IFN-β secretion after 3 hours post-infection (Figure 24C). On the other hand, gene profiling data clearly illustrate that SNRNP200 regulates the expression of a large group of immune-related genes. Indeed, we observed that SNRNP200 KD also decreased type I IFN signaling pathway downstream to STAT1 phosphorylation, and through a molecular mechanism that requires further investigation. Thus, we cannot exclude that the perturbation of pre-mRNA processing leading to impaired expression of multiple genes possibly contributes via an indirect role of SNRNP200 to the reduced antiviral response of KD cells. Nonetheless, the abrogated phosphorylation of IRF3 (Figures 18A, 18B for IRF3p386/IRF3 ratios and 24B) provides the first mechanistic insight to explain the phenotype of SNRNP200 depleted cells. Building upon these results, we considered a direct role of SNRNP200 in IRF3 signaling. In order to pinpoint a regulatory function of SNRNP200 in IRF3 phosphorylation, we evaluated its ability to interact with known members of the RLR signaling pathway and identified the specific interaction with TBK1. We mapped precisely the TBK1 binding site to the Sec63-1 domain (Figure 22C) and corroborated a colocalization of SNRNP200 and TBK1 in cytoplasmic perinuclear speckles that is triggered by SeV infection (Figures 23 and S12). This points out to the involvement of a cytoplasmic SNRNP200-TBK1 protein complex modulating IRF3 phosphorylation, which is required to induce its dimerization and nuclear translocation for *IFNB1* transcription and production of ISGs [220], a mechanism reminiscent of the one described for DDX3 helicase [315].

We then sought to test our hypothesis that SNRNP200 directly operates as a sensor of viral RNA using RNA pull-down experiments. We were able to demonstrate that SNRNP200, and more specifically its Sec63-1 domain, binds both viral surrogate poly (I:C) and HCV RNA genome (Figure 21B-21C). The RNA-binding ability of SNRNP200 mainly involves recognition of dsRNA as seen with poly:IC, while the presence of the 5'triphosphate moiety as well as the presence of DDX58, MAVS and TBK1 proteins are not required for binding dsRNA molecules (Figure 21D, 21E). As expected, SNRNP200 RNA helicase does not bind dsDNA (Figure 21D), which correlates with the observation that SNRNP200 KD does not affect ISG56 promoter activation by the cGAS/STING pathway (Figure S4B). Surprisingly, the binding of SNRNP200 to dsRNA was only observed following SeV infection, in contrast to DDX58 that binds poly (I:C) in the absence of viral infection (data not shown). While we cannot explain this observation especially in the context that SNRNP200 directly binds SeV RNA (Figure 21F), we further demonstrated that expression of the naturally occurring SNRNP200 S1087L variant located in the Sec63-1 domain, which is associated to RP33 [OMIM:610359], is unable to bind dsRNA and SeV RNA (Figure 21A, 21F), does not relocalize with TBK1 upon SeV infection (Figure 23A) and is not able to restore the antiviral response in SNRNP200 KD cells (Figure 20D). Thus, a pre-activation of SNRNP200 upon recognition of viral RNA allows its relocalization to perinuclear speckles with TBK1. Finally, the study of ATP hydrolysis-deficient SNRNP200 variants that affect antiviral response, and the discovery of a mutant (C502A) that elicits an IFNB1 response independent of viral infection and fully rescues IFN-β in SNRNP200 KD cells (Figure 20C and S10), further supports the SNRNP200 ATPase function in conferring specificity to viral RNA and preventing signaling through recognition of self-RNA, as recently reported for the natural gain-of-function DDX58 and IFIH1 ATPase-deficient variants [322]. Altogether, the data demonstrate a direct regulatory role of SNRNP200 via its Sec63-1 domain and ATPase/helicase function for the recognition of viral RNA and relocalization into perinuclear cytoplasmic speckles with TBK1 to promote IRF3 activation and antiviral response.

In order to ascertain the regulatory role of SNRNP200 in immune cells, we carried out depletion experiments in purified MDM isolated from PBMCs of healthy donors. As observed in the cell lines, SNRNP200 KD MDM led to a hindered IFN-β production by blocking IRF3 phosphorylation but without altering IRF3 expression (Figure 24B, 24C).

This is accompanied by an increased viral susceptibility illustrating a relevant role of SNRNP200 for the antiviral response of human macrophages. We then exploited the loss-of-function mutations in the human *SNRNP200* gene that causes autosomal-dominant RP33. RP is a rare inherited disease of retinal dystrophies with an incidence of one in 3,000-4,000, of which 1.6% bear mutations in the *SNRNP200* gene [327]. The investigated S1087L is a disease-associated mutation with complete penetrance in RP33-linked family [328-330]. With access to peripheral blood cells of three RP33 patients who have one allele carrying the dominant S1087L or R681C mutation, we were able to confirm a decreased IRF3-dependent antiviral response when challenged with SeV by the specific reduction of IFN-β and IFN-α2 cytokine secretion while not affecting other tested cytokines (Figures 25 and S14). Thus, we presented further evidence with human cells of patients with RP33 disease that SNRNP200 positively regulates antiviral response independently from its primary core function in pre-mRNA splicing.

The recent resolution of SNRNP200 structure (aa 395-2129 hBrr2) provides the spatial relation between duplicated N-terminal and C-terminal cassettes both containing RecA1-RecA2 DEAD-box helicase domain and Sec63 homology domain [298]. Both cassettes are required for optimal helicase activity and splicing function, but only the N-terminal cassette was reported to be catalytically active [298]. The 3D structure of Sec63-1 homology region is constituted of a seven-helix bundle (HB), a helix–loop–helix (HLH), and an Ig-like (IG) domain and resemble the structure of isolated C-terminal Sec63 units of yeast and human enzyme [319, 331]. The serine 1087 is located to a long scaffolding helix (referred as ratchet-helix) within the HB domain that with RecA domains form respectively the top and bottom of a central tunnel for RNA, which is believed to constitute a strand separation device. The testing of a leucine at position 1087 exhibited decreased RNA binding and reduced ATPase and helicase activities compared with the WT variant [298]. The corresponding yeast homolog N1104L mutation was detrimental to U4/U6 unwinding and splicing [319, 331]. This is believed to decrease spliceosome activation and to explain its linkage to RP33 [319]. In our study, the S1087L mutant completely abolishes recognition of viral RNA molecules and is unable to relocalize into TBK1-containing cytoplasmic speckles, which could explain the hindered antiviral response.

Further proteomic studies should provide a more comprehensive picture of this mechanism with identification of interaction partners that trigger the cytoplasmic relocalization of a SNRNP200-TBK1 complex upon infection. Structurally, SNRNP200 also differs decisively from other spliceosomal helicases as it belongs to the Ski2-like subfamily, which is a small family of superfamily 2 helicases (founder member: yeast Ski2) involved in a variety of RNA processing and degradation events [160]. Beside SNRNP200 that exhibits ATP-dependent unwinding activity of U4/U6 RNA duplexes during pre-mRNA splicing [224, 332, 333], others such as SKIV2L and DDX60 promote exosome-mediated RNA decay [166, 172]. Long ago, the yeast Ski2 and Ski2-like helicase 1 (Slh1) have been reported as RNA helicases implicated in antiviral defense and were shown to block translation of RNA lacking a 3' poly(A) structure [296, 297]. Recently, a role of the Ski2-like RNA helicase SKIV2L was described in the elimination of incompletely spliced RNA transcripts upon stress response, which upon inhibition triggers a sterile RNA-activated antiviral innate response [172]. Indeed, SKIV2L-deficient patients exhibit a constitutive type I IFN signature in their peripheral blood resulting in a human auto-immune disorder. Further studies will be required to assess a potential role of SNRNP200 in the recognition of host RNA molecules upon stress response and in their elimination by the RNA exosome.

Finally, to our knowledge, there has been no association between the RP33 pathology and immune disorders. Our findings in PBMC of patients bearing monoallelic point mutation in SNRNP200 establish a deregulation of innate immunity, which may affect cell viability to different retina insults as these cells and neural cells are usually non-proliferative and long-lived. Although RP33 is a rare event, it may be clinically relevant to elucidate the mechanism of disease onset at a molecular level in relation to a deregulation of the innate response and control of cell viability. Indeed, optineurin (OPTN), a critical regulator of antiviral signaling [240], in which mutation E50K promoting interaction with TBK1 was associated to familial primary open-angle glaucoma [334]. The dysfunction of OPTN and TBK1 in retinal cells was proposed to play a significant role in glaucomatous and other retinal diseases by affecting an autophagy process and survival [335, 336].

In summary, we demonstrated that upon RNA virus infection, spliceosome SNRNP200 helicase in complex with TBK1 via its Sec63-1 domain binds dsRNA, relocalizes into TBK1-containing perinuclear structures and positively regulates IRF3 phosphorylation to promote antiviral response. The regulatory role of SNRNP200 was confirmed in MDM and in PBMCs of RP33 patients by an impaired production of IFN-β upon viral infection. Our data uncover a crucial immunoregulatory role of the Sec63-competent SNRNP200 helicase acting as an RNA sensor and adaptor for TBK1 to promote IRF3-mediated antiviral innate immune response. Altogether this illustrates a novel function of SNRNP200 clearly distinguishable of the one in spliceosome activation and pre-mRNA splicing (Figure 26). Exploiting the actions of human encoded regulators of antiviral response during infection by developing immunomodulatory molecules presents an alternative strategy to treat a broad range of viral infection or to limit virus-induced inflammation.

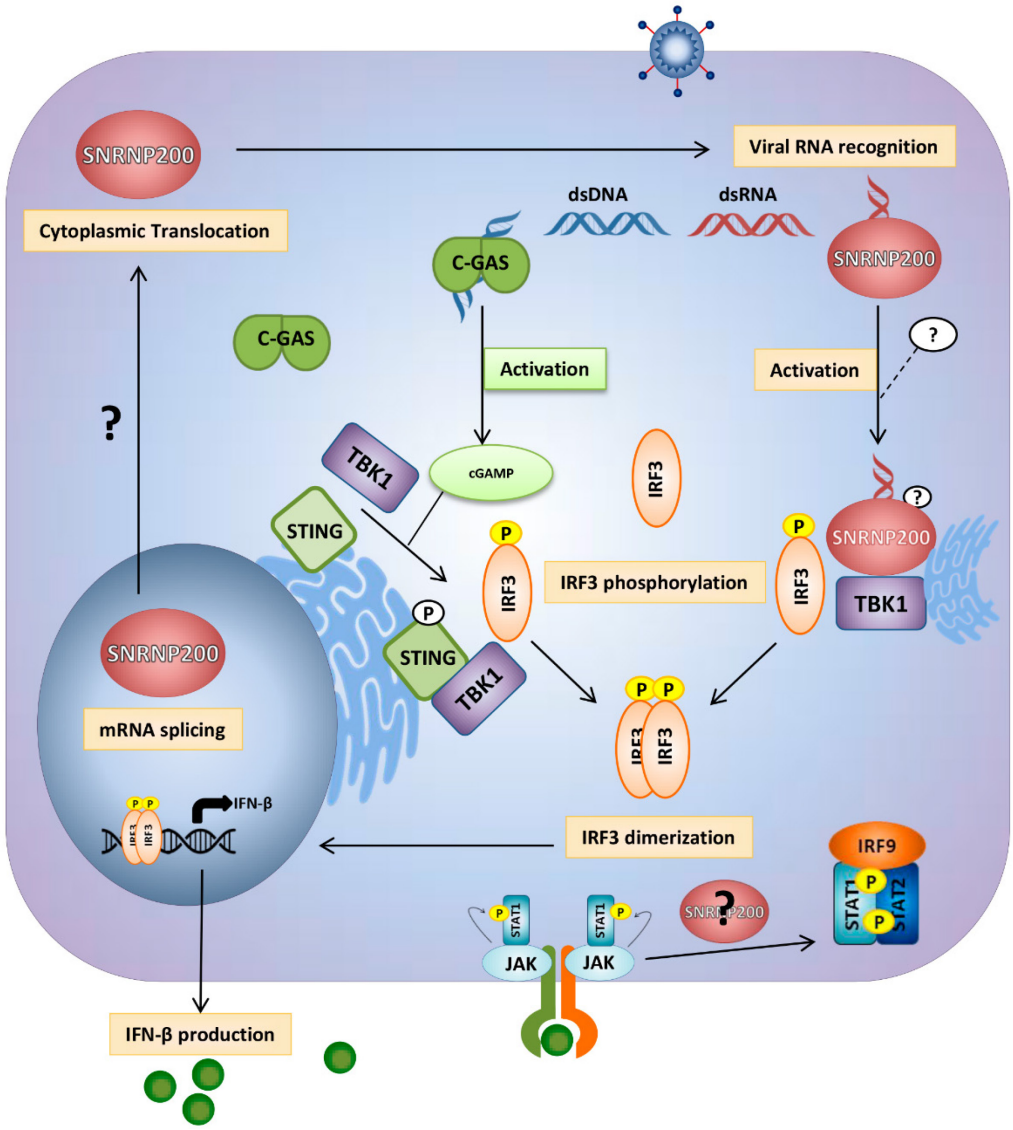


Figure 26. SNRNP200 regulates the antiviral response. Proposed model: Upon viral infection, SNRNP200 relocates to an undefined cytoplasmic structure where it is able to directly sense viral RNA via its Sec63-1 domain. This activation by viral nucleic acids, through an undefined mechanism, results in a virus-induced association of SNRNP200 with TBK1 into a larger order perinuclear structure. The mobilization of SNRNP200 with TBK1, downstream of DDX58/MAVS signaling, promotes IRF3 phosphorylation and IRF3's subsequent translocation to the nucleus. This nuclear translocation allows the transactivation of the IFN-β promoter and thus the production of type I IFNs and ultimately of ISGs. This model demonstrates that SNRNP200 is dispensable to cGAS/STING cytosolic DNA sensing but required for RLR/MAVS/TBK1/IRF3 signaling, by a novel mechanism, to engage antiviral immunity against RNA viruses.

3.5. Perspectives and Future Work

In this thesis, we have characterized the function of SNRNP200 in innate antiviral immunity. Using a variety of molecular biology and genomic tools, we showed that SNRNP200 is an essential component of the RLR-pathway that can act as a bona fide viral RNA sensor and adaptor of the TBK1 protein kinase required for IRF3 activation. In short, we have proposed a model in which SNRNP200 is moved to the cytoplasm of an infected cell, where it can act as a RNA viral sensor and then potentiate IRF3 phosphorylation by recruiting TBK1 to perinuclear speckles. However, the mechanism behind some key points of this model remain elusive and thus, are open for discussion, speculation and future work. The objective of this last section is to highlight some key points that will inform further investigations on the mechanisms by which SNRNP200 regulates innate antiviral immunity.

3.5.1. Further investigation regarding the in vivo relevance of SNRNP200 in the RLR pathway

An essential downstream study is required to confirm the immunoregulatory role of SNRNP200 *in vivo*. Certainly, while we provided a comprehensive assessment of the function of SNRNP200 in the regulation of the RLR pathway during a viral infection, our data only covers three different cell lines (293T, A549, Huh7) and two peripheral human primary cell subsets (PBMC, MDM). Hence, additional studies are required to confirm the role at the whole tissue and organism level.

To that end, it would be interesting to complete a series of studies across various human primary cell lines and animal models. First, we could generate a library of induced pluripotent stem cells (iPSCs) from skin biopsies of healthy individuals and patients with SNRNP200-related diseases such as RP33. Then, we would generate a sub-library of different primary cell types by inducing the iPSCs differentiation into pulmonary epithelial cells, hepatocytes or bone marrow cells. We would then have a series of "WT" and "SNRNP200-defective" (ex. S1087L) cell lines that we can use to assess the function of SNRNP200 in viral restriction and immune regulation in a similar fashion as that of the work presented in this thesis. Moreover, we would generate KO of terminally differentiated cells, using CRISPR-Cas9 technology, to assess the impact of a

complete loss-of-function. Together, these validation studies in primary cell lines would support a major role of SNRNP200 in the regulation of innate antiviral immunity.

Second, we would carry out similar experiments, in vivo, using a conditional KO (cKO) mouse model generated using a sgRNAs-loxP/LSL-Cas9 system [337]. Briefly, this novel strategy has many advantages over a traditional cKO system; it allows for a fast and efficient genomic editing of one or multiple genes using a one-step transgenic construction and it is a *trans* system that requires the deletion of only one allele to remove the gene of interest eliminating the need for laborious breeding and genotyping. Notably, we would need to use cKO of SNRNP200 since SNRNP200-deficient mice have been shown to exhibit embryonic lethality by the International Knockout Mouse Consortium. The cKO could be directly carried out in the mice strain already engineered for tissue specific tamoxifen-induced Cre-recombinase expression or directly in wild type mice for usage with bioengineered viruses encoding Cre-recombinase such as the influenza virus [338]. Using these animals, we would conduct survival studies, viral load assays and evaluate the antiviral responses in blood in specific tissues by measuring the production of type I and III IFNs and pro-inflammatory cytokines kinetically during the infection. Overall, this would provide us with the required information to assess the biological role of SNRNP200 in the regulation of antiviral innate immunity in an established animal model.

3.5.2. Further investigation regarding the role of the SNRP200 and TBK1 interaction

An interesting observation from our work is that both WT SNRNP200 and S1087L SNRNP200 can interact with TBK1, as shown by co-immunoprecipitation, but only WT SNRNP200 has the capacity to functionally rescue the production of virus-induced IFN-β. So, what is the biological significance of this interaction to our model? An interesting way to dissect this question would be to conduct directed mutagenesis experiments on both SNRNP200 and TBK1, and to identify a site that can be mutated to disrupt the protein-protein interaction but not the normal protein function. Thus, a refinement of our strategy would most likely identify the key residues of the SEC63-1 domain that allows SNRNP200 and TBK1 to interact.

Thereafter, we would need to measure the correlates of innate immune function during infection (ex. viral load, IFNB1 expression, IRF3 phosphorylation, pattern of expression using confocal microscopy) in SNRNP200 or TBK1 deleted cells that were rescued with a construct bearing the disruptive mutation. Then, we would have additional information to refine our model and account for the constitutive interaction between SNRNP200 and TBK1 that does not correlate with the immune function of WT SNRNP200 and S1087L SNRNP200. Last, we could integrate these results to our in vivo studies using a CRISPR-Cas9 knock-in strategy in our iPSCs libraries or mouse models.

3.5.3. Further investigation regarding the viral-induced cytoplasmic translocation of SNRNP200

In eukaryotes, RNA splicing is the mechanism by which introns are removed from pre-mRNA to give rise to protein-coding mRNA. This process relies on eight essential RNA helicases that govern the conformational changes required for a fully functional spliceosomal machinery [339]. Of interest for this thesis, SNRNP200 is the major component of the U5 small nuclear RNA proteins (snRNPs) subunit and thus, it is essential to this fundamental cellular process, in which it mainly serves as a scaffolding protein for ATP-dependent RNA unwinding [333, 340, 341]. As one might expect, the removal of introns, or splicing, occurs in the nucleus where the pre-mRNA is processed into a mature transcript before it is exported to the cytoplasm where it can be translated into a protein (as reviewed in [342]). Thus, SNRNP200 is considered, based on its biological/molecular function and annotation, to be a resident of the nucleus with limited needs to translocate to the cytoplasm of a cell where no canonical splicing occurs. However, in our study, we showed, using confocal microscopy, that SNRNP200 can be observed in the cytoplasm of infected cells. Significantly, upon viral infection, it seems to relocate to peri-nuclear speckles where it can be seen along with TBK1 suggesting that SNRNP200 antiviral function is most likely regulated by a spatiotemporal compartmentalization.

Two hypotheses, directly related to our work, might explain a need to SNRNP200 cytoplasmic translocation: SNRN200 needs to relocate to the cellular compartment where viral nucleic acids (role of vRNA sensor) and TBK1 (role of adaptor protein) can be found.

This movement into a non-traditional organelle might be reminiscent of the cellular function of other Ski2-like helicase that are part of the RNA exosome system. In fact, the human MTR4 protein is part of the TRAMP complex that is required for nuclear RNA surveillance of improperly processed mRNAs, rRNAs, snRNAs, snoRNAs, and tRNAs [343-346]. Upon viral infection, MTR4 and ZCCHC7, that are both exosome cofactors of the TRAMP complex, are translocated from the nucleus and repurposed into a smaller scale TRAMP-like complex that specifically recognizes and induces degradation of viral mRNA [347]. However, it has not yet been elucidated that the finer molecular mechanisms are this antiviral mechanism. Notwithstanding, it can be postulated that SNRNP200 could be translocated from the nucleus to the cytoplasm in a similar fashion. Thus, to fully understand SNRNP200 role in innate antiviral activity, it will be imperative to understand how, why and where it moves from the nucleus, to the cytoplasm and to perinuclear speckles, upon viral infection.

In addition, innate immunity relies heavily on spatial compartmentalization to regulate signal transduction using a combination of sorting-signaling adaptor pairs that allow the reliable detection of an activated receptor and facilitates the recruitment of activated receptors to various organelles [348, 349]. For instance, in the RLR pathway, active RIG-I (activated receptor) can be recruited to MAVS (sorting-signaling adaptor) located at the mitochondria, the peroxisomes and the mitochondria-associated membrane (Figure 27). Each combination of activated receptor/sorting-signaling adaptor (RIG-I/MAVS-mito, RIG-I/MAVS-pex and RIG-I/MAVS-mam) will result in distinct downstream events. Certainly, MAVS-mito favors a classical type I antiviral response, MAVS-pex favors the transcription of an early type III interferon antiviral response, while MAVS-mam acts as an immune synapse that seem to balance downstream signaling by allowing a direct interaction between MAVS-mito and MAVS-pex [195, 350, 351]. All in all, the aim of this compartmentalization of innate antiviral immunity is to allow the timely activation of the proper pathways and effector responses based on some upstream stimuli and provide alternative routes of signaling in the event of (as a generalization) viral subversion.

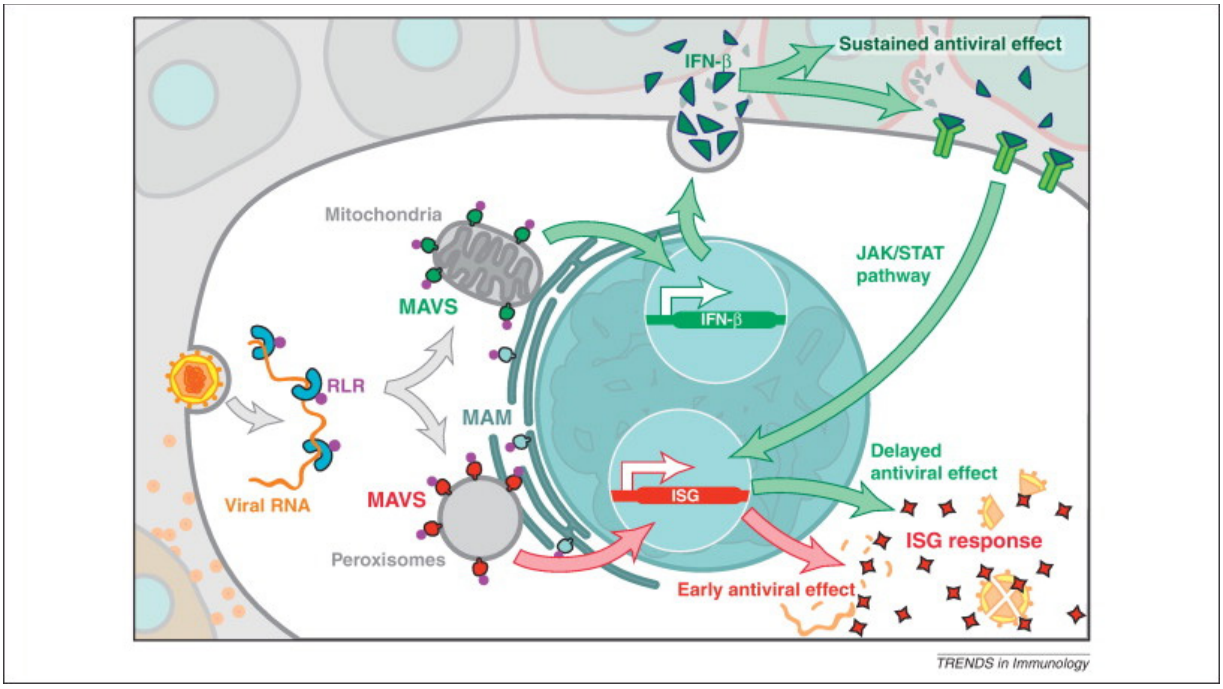


Figure 27. Retinoic acid-inducible gene 1 protein (RIG-I)-like receptor (RLR)-mediated detection of viral RNAs leads to receptor transport to the sorting–signaling adaptor hybrid mitochondrial antiviral-signaling protein (MAVS). MAVS is located on mitochondria, peroxisomes and the mitochondria-associated membrane (MAM). The docking of these organelles at the MAM creates an innate immune synapse that maximizes antiviral innate immunity. Used with permission [349].

Thus, the objective of mapping the route that SNRNP200 takes upon viral infection is three-fold, as it can provide information about where it signals from, what it signals to and how it mediates the antiviral signal.

To comeback to our initial observation, we just described that upon viral infection SNRNP200 relocates to TBK1-containing perinuclear speckles. Strikingly, perinuclear punctate structures, a hallmark feature of an activated cGAS-STING pathway, govern innate antiviral immunity against DNA viruses and converge towards TBK1-IRF3 signaling [352]. In fact, activation of the STING adaptor protein requires a licensing phosphorylation by TBK1 at Ser366, which allows subsequent recruitment and activation of IRF3 [213]. Interestingly, according to Liu et al., the pSer366-STING can be observed in "perinuclear punctate structures only in DNA-stimulated cells". This relocation of an active STING is mediated by EXOC2, a member of the exocyst complex, that facilitates vesicular trafficking [353]. Interestingly, TBK1 trafficking is

also associated with the EXOC2 via Ralb, a GTPase member of the Ras family of protein, that is essential for TBK1 activation upon SeV infection and also associated with tumor progression of TBK1-related cancers [354, 355]. Thus, since STING and SNRNP200 have been described to relocate with TBK1 in perinuclear speckles upon viral infection or mimetic stimulation, it can be reasonably postulated that both proteins might share a common activated receptor/sorting-signaling adaptor mechanism that is governed by perinuclear compartmentalization.

With that in mind, we propose that further investigations regarding the SNRNP200 movement due to viral infections focus on the following:

1. It is imperative to assess what post-translational modifications govern the functional re-localization of SNRNP200 to the cytoplasm and perinuclear speckles. Priority should be given to phosphorylation, but other PTM, such as ubiquitination, SUMOylation or acetylation should also be reasonably assessed.
2. The characterization of the SNRNP200-interactome, using total, nuclear and cytoplasmic sub-pools in resting and infected cells would allow for the identification of important functional partners that are most likely to assist in SNRNP200 infection-induced re-localization to both the cytoplasm and the perinuclear speckles.
3. The confirmation of these findings using conventional molecular biology along with the previously described cell-free model of RLR-pathway would culminate in solid findings pertaining to our model.
4. To assess if SNRNP200-STING colocalize to similar or different perinuclear vesicles upon viral infection using confocal microscopy

While challenging, a tandem mass spectrometry co-IP strategy could be implemented. This will allow for the PTM/Interactome characterization of not only the WT SNRNP200 but also of other interesting mutants (as FLAG-tagged protein), such as the S1087L, which does not bind to vRNA or relocate to perinuclear speckles but interacts with TBK1. It must be noted that these experiments should bear in mind that SNRNP200 does not have a pLxIS motif despite its constitutive interaction with TBK1 and that it is most likely to activate IRF3 via a mechanism that is different from other antiviral adaptor proteins, such as STING, MAVS and TRIF. It is also most likely analogous to DDX3 activation of IRF3 via IKBKE [356]. On the whole, we are

confident that a research plan, aligned with these four priorities, would open the discussion and provide the necessary information to determine whether SNRNP200, as a vRNA sensor and adaptor protein, is distinct from one another or intertwined in a unique mechanism.

3.5.4. Further investigation regarding SNRNP200 preference towards viral RNA

One of the most intriguing findings of my thesis is the observation that SNRNP200 can bind, via its SEC63-1 domain, poly (I:C) and full-length HCV RNA, independent of the presence of 5'ppp moieties and without the need to associate with DDX58 or MAVS. Additionally, in our experimental setup, only SNRNP200 purified from a SeV-infected whole cell lysate can bind to dsRNA, which suggests that it needs to be "activated" in order to exert its vRNA sensor capabilities. Thus, we have two open questions:

1) What are the determinants of vRNA that allow for recognition by SNRNP200?

2) Is the binding of vRNA by SNRNP200 a direct or an indirect phenomenon?

Regarding the first question, it has been demonstrated that vRNA sensors and sentinels display a preference towards a certain kind of RNA ligands. As a generalization, RIG-I favors short, 5'ppp, blunt-ended vRNA, while MDA5 prefers long stretches of vRNA (as reviewed in the introduction).

This allows for the recognition of a complete portfolio of PAMPs that are generated during viral replication and efficient immune surveillance by PRR. Thus, it can be agreed upon that a lot can be learned in terms of the specific function of an RNA sensor in innate immunity from its preference for a certain RNA ligand. Consequently, we propose to apply tagged protein affinity purification coupled to next-generation sequencing of SNRNP200-associated RNA-molecules from a broad sampling of representative RNA viruses. This method has been very effective to contrast classic RNA sensors, such as RIG-I, MDA5 and LGP2 [357, 358]. This will enable the comparison between SNRNP200 and a known vRNA sensor, which should provide the required information to determine whether SNRNP200 favors a classic pattern of vRNA, such as 5'ppp or whether it favors the recognition of non-traditional viral signatures, such as 3'UTR or an AU-rich region.

Regarding the second question, it would be extremely interesting to see if SNRNP200 binds vRNA in tandem with other host factors or if it acts alone. A limitation of our current study is that we can only correlate the presence of SNRNP200 and poly(I:C)/vRNA in a pulled-down sample without the required data to rule out the implication of other proteins apart from RIG-I, MDA5 and TBK1. As an example, DDX1, DDX21 and DHX36 act as a vRNA complex wherein DDX1 binds to vRNA and DDX21/DHX36 associates with TRIF to induce type I IFN (Zhang & Kim et al., 2011). However, their experiments demonstrated that proteins bound to poly (I:C) were resolved by co-immunoprecipitation against DDX1, DDX21, DHX36 and TRIF. Thus it can be reasonably assumed that, if the complex had not been identification using an LC-MS/MS screen or the more recent approach of electrospray ionization tandem mass spectrometry (ESI-MS/MS), it would have been more fastidious to directly resolve the existence of this tandem vRNA sensor where two proteins contribute to the interaction with an adaptor protein and one protein acts as a direct vRNA sensor. Consequently, it would be a priority to resolve the interactome of poly (I:C) and vRNA-bounded SNRNP200, as it would provide additional information regarding the mechanism behind the capabilities of SNRNP200 to act as a vRNA sensor.

3.5.5. Exploring the role of SNRNP200 in ER-stress response

As mentioned in the introduction, RNA sensors, such as RIG-I and MDA5, use an ATP dependent translocation along the nucleotide strand to recognize and bind with high affinity to PAMPs (Cui et al., 2012; Lässig et al., 2016; Rawling, Fitzgerald, & Pyle, 2015). Indeed, this ATP-dependent translocation is a key mechanism by which RIG-I, MDA and other sentinels can recognize self RNA vs non-self (pathogenic) RNA. As the helicase moves along the RNA stand, its ATPase/translocase activity removes it from cytoplasmic-abundant self-RNA while locking it into the 5'ppp or other non-conventional RNA motif following translocation (Lässig et al., 2016; Rawling et al., 2015). In our study, we showed that an ATP hydrolysis-deficient SNRNP200 mutant (C502A) can elicit an IFN-β response independent of viral infection. This observation supports the SNRNP200 ATPase function with regard to conferring specificity to viral RNA and preventing signaling through recognition of self-RNA, as recently reported for the natural gain-of-function DDX58 and IFIH1 ATPase-deficient variants (Lassig et al., 2015).

As our last perspective, it would be interesting to evaluate the contribution of SNRNP200 to the prevention of viral-induced unfolded protein response (UPR). Emerging literature certainly shows that the production of type I interferon, mediated by antiviral innate immunity, can be synergistically upregulated by UPR stress (as reviewed by [359]). Briefly, the UPR stress response is designed to balance protein processing at the ER with survival and thus, ensure that if an external factor, such as a viral infection that calls for the processing of viral proteins at the ER, increases the ER load, the feedback mechanism will kick in to transitionally decrease the ER processing and load and maintain homeostasis [360]. UPR stress is induced by three different pathways in mammalian cells, which induces the dissociation of folding chaperone BiP from the ER membrane and thus, prompts the ER to slow down the active processing of protein owing to an increased presence of stationary folded protein (Figure 28). Thus, upon viral infection, one might expect that a UPR pathway acts as a bystander mechanism that antagonizes the viral life cycle by limiting the cell's ability to process viral particles while the innate antiviral immunity clears the infection. Yet, viruses can also highjack UPR pathways to promote their replication and assembly by co-opting UPR cofactors, reducing apoptosis, promoting survival or subverting innate immunity [361-365].

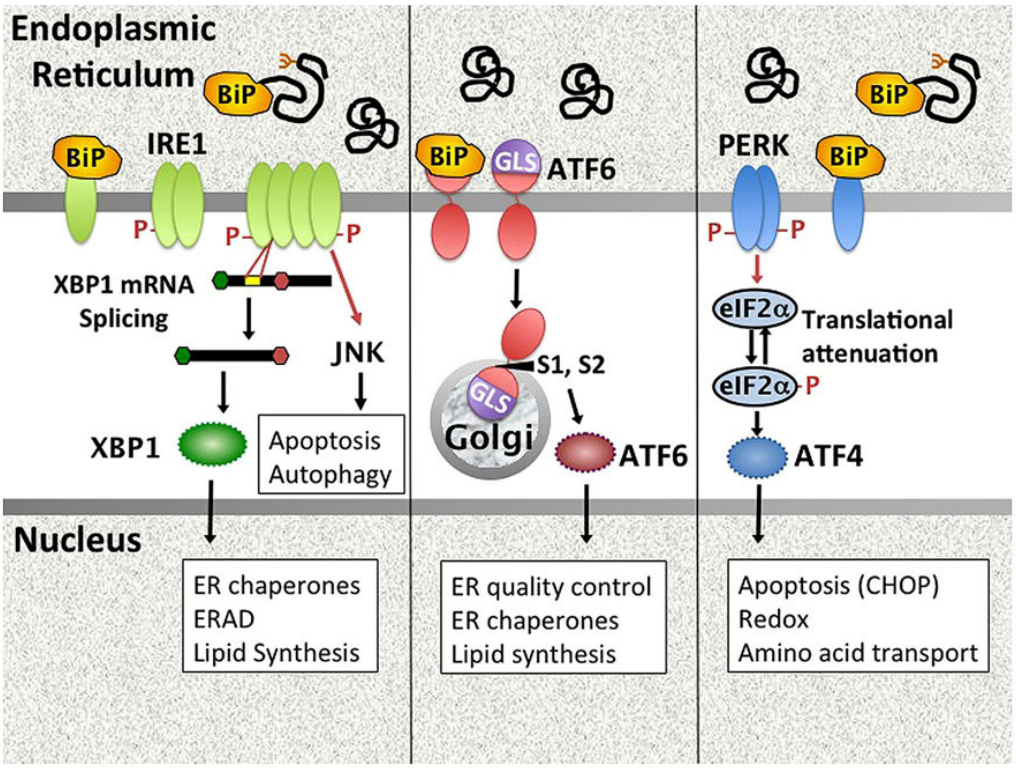


Figure 28. Mammalian UPR pathways. The UPR encompasses signaling pathways triggered by the activation of ER stress transducers IRE1, ATF6, and PERK. In unstressed cells, these molecules associate with the folding chaperone BiP. Upon accumulation of unfolded proteins in the ER, PERK, and IRE1 release BiP and oligomerize. IRE1 is both a kinase that phosphorylates targets such as JNK, and an endonuclease that splices 26bp from the XBP1 mRNA, removing a premature stop codon. Dissociation of ATF6 from BiP uncovers a Golgi localization signal. ATF6 traffics to the Golgi, where site-specific proteases (S1, S2) cleave it to an active transcription factor. PERK phosphorylates eIF2α, resulting in global translational attenuation apart from select open reading frames (e.g., ATF4). UPR gene targets (e.g., CHOP) and UPR regulated cellular processes are in boxes. ERAD = ER associated degradation. GLS = Golgi localization signal. Used under permission. CC BY 4.0 [359]

The mechanism by which viruses can highjack the UPR stress responses is either indirect or direct. As an example of an indirect mechanism, Influenza A and Hepatitis C viruses can compete with the host protein for post-translationtraal modifications, such as glycosylation, by interfering with many cellular processes that take place at the ER [366, 367]. In addition, these

two viruses along with others have also been shown to increase the calcium permeability of the ER membranes, which leads to a major influx of cytoplasmic calcium.

This results in an optimal configuration of cellular organelles (mitochondrial disruption, reduced vesicular pH, viroplasm formation) to promote viral particle assembly and release [368]. In terms of direct mechanisms, many viruses, such as Influenza and Hepatitis C, have been shown to manipulate the PERK-eIF2α-ATF4 kinase, ATF6 and IRE1-XBP1 pathways to inhibit the host protein translation involved in innate immunity and UPR stress induced apoptosis and thus, support an optimal viral replication [369]. As a consequence, it is evident that viruses have evolved to manipulate intrinsic cellular responses, such as UPR stress responses, that are normally present in order to maintain homeostasis to the advantage of their life cycle.

However, a more complex picture has emerged lately and revealed a novel paradigm regarding UPR stress. Recent studies have shown that some components of the UPR stress response can synergize the potential of an innate antiviral response. As an example, the IRE1-XBP1 UPR pathway can synergize, IRF3-dependent, type I interferon response upon viral infection through a XBP1-dependent enhancer element located upstream of the IRF3-CPB/p300 IFNB1 promoter [370-372]. Thus, while the UPR stress response is co-opted for the maintenance of the viral lifecycle, it seems that it is also co-opted by antiviral pathways to augment the immune response and act as co-stimulatory danger signals (Figure 28).

Thus, a fine balance seems to exist between the positive or deleterious effect of the activation/hijacking UPR pathways during infection. However, the mechanisms that govern this synergism between UPR and antiviral immunity remain evasive. Interestingly, SNRNP200 might play a role in sensing the abundant immuno-stimulatory host-RNAs upon the induction of UPR stress, which are normally recognized by specialized RNA sensors that direct them to the RNA-exosome for degradation. Indeed, Mtr4 and Ski2, two Ski2-like helicases in the same family as SNRNP200, have been shown to play a major role in RNA surveillance owing to their ability to process RNA in the 3’-5’ direction, which is useful to recognize [160]. Human mutations in Ski2 (SKIVL2) have been shown to lead to the accumulation of endogenous RLR ligands produced by the activation of the UPR IRE1 pathway, which can activate RIG-I and lead to interferonopathies (autoimmune disorders) [373]. Additionally, recent evidence showed that the depletion of other adaptor proteins, such as STING, are essential to produce synergistic

type I interferon following the induction of UPR stress with thapsigargin, a small molecule that affects ER calcium homeostasis [371].

Lastly, SNRN200 could be involved in the regulation of the cytoplasmic splicing of ER stress response genes, such as XBP1. Cytoplasmic splicing is an emerging phenomenon that shows that many human transcripts have nuclear and cytoplasmic splicing signals (for review, see [374]). In short, this intron retention mechanism gives rise to an additional layer of transcriptional regulation that allows for a spatiotemporal regulation of splicing certain proteins. As an example, the UPR-stress response XBP1 is converted via cytoplasmic splicing from an inactive long transcript bearing a short 26-nucleotide intron to an active short transcript via the endoribonuclease activity of IRE1 [375]. Consequently, it will be interesting to explore the role of SNRNP200 in the regulation of UPR stress.

As a proof of concept, our preliminary data showed that SNRNP200 depletion, using shRNA and a firefly luciferase IFNB1 promoter assay, blocks the induction of IFNB1 by Golgicide A and tunicamycin, while thapsigargin triggers IFNB1 production both in the presence or absence of an SeV infection (Figure 29.)

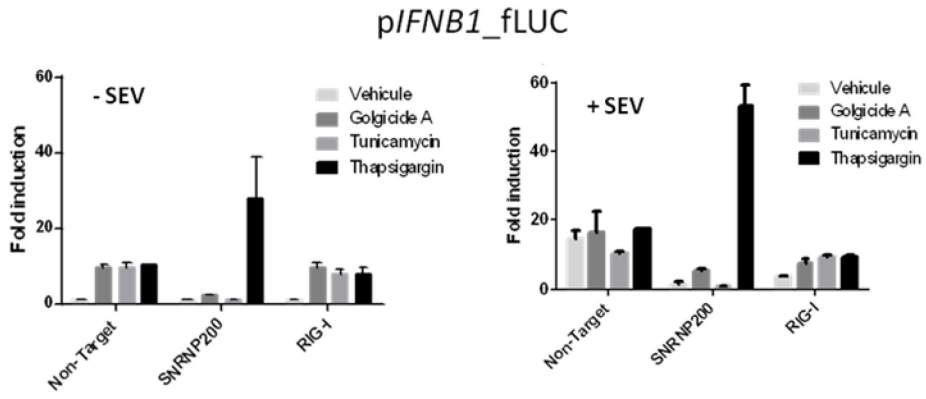


Figure 29. SNRN200 promotes IFNB1 production upon ER-stress response. SNRNP200 modulates IFNB1 production upon induction of stress response, in contrast to RIG-I. A549 cells were transduced with shRNA and targeting SNRNP200 or RIG-I for 72 hours, before being treated for 4 hours with Golgicide A [2.0ug/mL], Tunycamycin [2.5ug/mL], or Thapsigargin [500nM] in uninfected (right) and infected (left) cells.

As mentioned above, thapsigargin critically depends on STING for stress-induced IRF3 activation and type I interferon production, while tunicamycin promotes IRF3 phosphorylation through a distinct STING-independent mechanism. Thus, based on these results, it can be inferred that SNRNP200 might act as a negative or positive regulator of the type I interferon UPR stress response (e.g., competition/association with STING for IRF3 under conditions of cellular stress; gene regulation of the IRE1-XBP1 pathway; role in the elimination of cytoplasmic immuno-stimulatory RNA). Future research should aim to uncover SNRNP200's contribution to the UPR stress response and further our understanding of its cellular function beyond its traditional spliceosomal connotation.

3.5.6. Concluding Remarks

This dissertation mainly focused on the central theme of understanding the role of SNRNP200 in the antiviral response against RNA viruses. From the initial characterization of the phenotype to the identification of its with TBK1 and vRNA, we developed a research project that culminated in a proposed model where SNRNP200 serves as a vRNA sensor and adaptor protein of the RLR-pathway, which is required for IRF3 activation. These studies illustrated an unexpected role played by SNRNP200 in innate immunity and provided the first link between a spliceosomal protein and innate antiviral immunity, which is not based on the regulation of gene expression but on the upstream role of PRR, signal transduction and activation of effector proteins. Our studies also provide an interesting roadmap that will refine our understanding of many aspects of innate immunity, such as the regulation of TBK1-IRF3 interactions, preference of vRNA by non-classical RNA sensors and sentinels and the contribution of another Ski2-like helicase and adaptor protein of the RLR pathway to the synergy between UPR stress and type I interferon response.

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Annex A – Material and Methods

Ethics statement

This study was approved by Institutional Review Board (IRB) of the participating institution (McGill Children's Hospital, McGill University Health Centre and Centre Hospitalier Universitaire de l'Université de Montréal (CHUM)) and written informed consent was obtained from all participants before participation.

Expression vectors

SF3A1, *NHP2L1* and *PHF5A* cDNAs were purchased from GE Dharmacon/Open Biosystems. Following PCR-amplification, PCR products were cloned into pcDNA3.1-Hygro-MCS using EcoRV/HindIII[308]. SNRNP200 was subcloned from the pBluescriptSK-hBrr2 obtained from R. Lührmann[376] into pcDNA3.1-Hygro(+) (Life Technologies) using NotI and XhoI restriction sites. SNRNP200 deletion mutants and S1087L point mutation were generated by PCR. All constructs were verified by Sanger sequencing and subsequent western blot analysis. If necessary, validated constructs where subcloned into pcDNA3.1-MCS-FLAG. pIFNB1-LUC and p2xNF-κB-LUC luciferase reporter constructs were previously described[377-379]. Generation of stable HEK 293T cells harboring the pIFNB1-LUC and pEF1α-LUC promoters was previously described[289].

Cell lines and culture

Human embryonic kidney HEK 293T (ATCC), human epithelial adenocarcinoma HELA (ATCC) and human hepatoma cell lines Huh7 / Huh7.5 (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Wisent). Human lung adenocarcinoma epithelial A549 (ATCC) were cultured in Ham's F-12 medium (Life Technologies). Both media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (all from Wisent) at 37 °C in an atmosphere of 5% CO_2. Transient transfections were performed with lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh

heparinized peripheral blood samples by Ficoll-Histopaque gradient centrifugation (Sigma-Aldrich). Unfrozen PBMCs were washed twice in 10 ml of sterile RPMI 1640 and re-suspended in RPMI 1640 supplemented with 10 % FBS. PBMCs were counted using a haemocytometer and count were adjusted using trypan blue exclusion to plate $1x10^6$ PBMCs in 100 µl RPMI 1640 supplemented with 10 % FBS in 96 well plate. For monocyte-derived macrophage (MDM), PBMC were harvested has described above and monocyte were isolated using MACS Monocyte Isolation Kit II human (Miltenyi Biotec) as per manufacturer's protocol before differentiation into MDM for five days in the presence of 10 ng/mL granulocyte-monocyte colony stimulating factor (M-CSF, R&D).

shRNA and siRNA gene silencing

shRNAs from MISSION TRC shRNA lentiviral library (Sigma-Aldrich) were used as followed: shRNA targeting SNRNP200 (TRCN0000051831), SF3A1 (TRCN0000006597), PHF5A (TRCN0000074878), NHP2L1 (TRCN0000074799), or shRNA non-target (NT). shRNA were transfected in combination with a standard packaging mix (1.5 µg pMDLg/pRRE, 1.5 µg pRSV-REV and 3 µg pVSVg) as previously described[380]. siRNA ON-TARGETplus SMARTpool, Human SNRNP200 and siRNA non-targeting #1 Human, ON-TARGETplus (GE Healthcare, Dharmacon), Santa Cruz HELIC2 siRNA (h) (sc-75243) were transfected with lipofectamine RNAi Max (Life Technologies) for 48 hours according to manufacturer's protocol.

Firefly luminescence assay

For, assays in 96-well plates, cells were seeded in white 96-well plates at a density of 5,000 HEK 293T pIFNB1_LUC and 1,250 293T pEF1α-LUC in 100 µl of complete phenol-red free DMEM containing 4 µg/ml polybrene. Infection with lentivirus encoding shRNA were carried out immediately after cell seeding at a MOI of 10 (except when specified otherwise) and incubated for three days at 37 °C in an atmosphere of 5% CO_2. Cells were infected with 100 HAU/ml of SeV (Cantell Strain, Charles River Labs) for 16 hours before cell lysis and firefly luminescence reading in a 100 mM Tris acetate, 20 mM Mg acetate, 2 mM EGTA, 3.6 mM

ATP, 1% Brij 58, 0.7% β-mercaptoethanol and 45 μg/ml luciferine pH 7.9 buffer. All infections were performed in an enclosed in a class II cabinet.

Assays used in western blot or qRT-PCR experiments where scaled up accordingly and carried out in the maternal HEK 293T or appropriate cell line.

Influenza A/Gaussia luminescence assay

For influenza infection, $3x10^5$ HEK 293T pIFNB1_LUC cells were seeded in 6-well plates. The next day, cells were transfected with an influenza vRNA reporter plasmid[381] and infected with 0.1 ul of purified influenza virus (A/PR/8/34, from Charles River). Five days later, 20 ul of cell supernatant was used to quantify the Gaussia luciferase using a Gaussia Luciferase Assay HTS (Nanolight Technology). Cell lysates were used to quantify the IFNB1 induction according to the Firefly luminescence assay described above.

HCV/Renilla luminescence assay

J6/JFH(p7-Rluc2a) virus production was conducted as previously described[382]. Briefly, HCV DNA template used for *in vitro* transcription was linearized using XbaI and subsequently transcribed using TranscriptAid T7 High Yield Transcription Kit according to manufacturer protocol (Life Technologies). The resulting HCV RNA was then electroporated into Huh7.5 and virus-containing culture medium was collected, filtered (0.45 μm) and kept at -80°C. For infection, 100ul of virus was added to 5000 Huh7 cells that had been plated in 96-well white opaque plates one day before. Culture medium was replaced six hours later and Huh7 cells were transfected with pIFNB1-LUC (50 ug/well) the next day. Three days later Huh7 cells were washed twice with PBS, before Rluc and Fluc quantification using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer protocol.

Western immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS; Wisent), harvested and lysed in 10mM Tris-HCl, 100mM NaCl, 0.5% Triton X-100, pH7.6 with EDTA-free Protease Inhibitor Cocktail (Roche). Cell lysates were clarified by centrifugation at 13,000 g for 20 min at 4 °C and subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

Western blot analysis was performed using mouse anti-PHF5A (Abnova), anti-IRF3 (Santa Cruz), anti-TRAF3 (Santa Cruz), anti-RIG-I (Alexis Biochemicals), anti-ACTIN (Chemicon International), anti-TBK1 (Imgenex and Santa Cruz), anti-IKBKE (Santa Cruz), anti-TUBULIN (ICN), anti-GAPDH (RDI) and rabbit anti-SNRNP200 (Sigma-Aldrich), anti-SF3A1 (Santa Cruz), anti-RELA (Santa Cruz), anti-NHP2L1 (Abcam), anti-ISG56 (Novus Biologicals), anti-MDA5 (Alexis Biochemicals), anti-MAVS (Alexis Biochemicals), anti-IKBKE (eBioscience), STAT1 (ABCAM), STAT1 tyr701 (ABCAM), IFNAR1 (Santa Cruz) and anti-IRF3-P-ser386 (Abcam). HRP-conjugated secondary antibodies were from Bio-Rad. The chemiluminescence reaction was performed using the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer).

Co-immunoprecipitation

For co-immunoprecipitation, FLAG-tagged protein expressing cells were harvested and lysed as described above. Resulting cell extracts were adjusted to 1 mg/ml and subjected to IP as follows: preclearing of the lysates was done by incubating lysates with 40 µl of a 50:50 slurry of immunoglobulin G-Sepharose (GE Healthcare) prepared in the lysis buffer with IgG beads for 1 hour. Pre-cleared lysate were immunoprecipitated by adding 20 µl of M2 anti-FLAG affinity gel (Sigma-Aldrich) prepared in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) overnight as described by the manufacturer. Immunoprecipitates were washed five times in lysis buffer. For interaction analysis, elution was performed using 250 ng/µl purified FLAG peptide for 45 min at 4 °C (Sigma-Aldrich). Eluates were analyzed by western immunoblotting.

Microarray analysis

The microarray studies were performed with HEK 293T cells transduced with lentiviral-expressing shNT (control) or shSNRNP200 RNA targeting *SNRNP200* gene for three days following 16 hours infection with SeV (100 U/ml) or 16 hours of treatment with a mixture of IFN-α from human leukocytes (400 U/ml; Sigma-Aldrich). A total of 10 µg of RNA was reverse transcribed using oligo(dT) 16-18 primers and SuperScript® II Reverse Transcriptase (Life Technologies) according to the manufacturer's instructions. Following purification using QIAquick PCR Purification kit (Qiagen), up to 1 µg of purified cDNA was mixed with 5'-Cy3

labeled random nonamers (Trilink Biotechnology) and heated at 95 ºC for 10 minutes and transferred on ice for 10 minutes. Samples were mixed with 1 mM dNTP and 2 µl of 3'-5' exo-Klenow fragment (New England Biolabs) and incubated at 37 ºC for 2 hours. The labeling reaction was stopped using 50 µM EDTA and the DNA precipitated using 0.5 M NaCl and 1 volume isopropanol, washed with 80% ethanol and resuspended in water. Hybridizations were carried out using the Human GE 4x44K v2 Microarrays (Agilent Technologies) containing probes targeting 27,958 Entrez Gene RNAs. Arrays were scanned at 5 µm resolution using a GenePix4000B scanner (Molecular Devices). Data from scanned images were extracted using GenePix 6.1 (Axon) and processed and normalized using ArrayPipe (v2.0). Processed data was used as input for linear modeling using Bioconductor's limma package, which estimates the fold-change between predefined groups by fitting a linear model and using an empirical Bayes method to moderate standard errors of the estimated log-fold changes in expression values from each probe set. *P* values from the resulting comparison were adjusted for multiple testing according to the method of Benjamini and Hochberg. Subsequently, gene enrichment analysis were conducted using DAVID [383, 384], STRING [385, 386] and Gene network were constructed using GENEMANIA [386].

Biotin-RNA/Biotin-DNA pull-down

RNA pull-down assay was performed using Dynabeads M270 Streptavidin (Life Technologies). Dynabeads were incubated with biotin-labeled RNA (poly I:C (InvivoGen) and full-length Jc1 HCV) for 1 hours according to manufacturer's protocol. Biotin-HCV RNA was obtained by subjecting linearized HCV DNA to T7 reverse transcription (TranscriptAid T7 High Yield, Life Technologies) and biotin-dUTP (Enzo Life Sciences). Saturated beads were added to whole 100 µg cell lysate and incubated, in a cold room, on a rotating wheel. Beads were washed three times and RNA-bound proteins were eluted after boiling in 0.1% SDS and analyzed by western blot. Poly (dA:dT) and Poly(dG:dC) were purchased from Sigma and labeled using Label IT Nucleic Acid Labeling Kit (Mirus Bio) and biotin-DNA pull-down assays were performed as described above.

RNA extraction and qRT-PCR assays

Total cellular RNA was extracted with the RNeasy Mini kit (Qiagen). Reverse transcription was performed on 1 µg total cellular RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). In order to amplify only the cDNA, primers were located in the splicing junction between two exons. PCR reactions were performed using 1.5 µl of cDNA samples (15 ng), 5 µl of the Fast TaqMan PCR Master Mix (Applied Biosystems), 10 pmol of each primer (IDT) and 5 pmol of the UPL probe (Roche) in a total volume of 10 µl. The ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level and was programmed to an initial step of 3 minutes at 95 °C, followed by 40 cycles of 5 seconds at 95 °C, 30 seconds at 60 °C and 1 second at 72 °C. All reactions were run in duplicate on biological duplicate and the average values were used for quantification. ACTIN (β-actin) or GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and HPRT1 (hypoxanthine phosphoribosyltransferase 1) were used as endogenous controls. The relative quantification (RQ) of target genes was determined by using the ΔΔCt method with the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems).

Virus Plaque Assays

Plaque assays were conducted in VERO cells and MDCK.2 cells (ATCC) using a method described elsewhere ([387]). Briefly, supernatants were harvested from infected cells and used to inoculate in serial dilutions VERO (SeV) and MDCK.2 cells (FLUA) for 45 minutes and 1 hour, respectively. After infection, cells were washed with PBS and an overlay of 0,6% agarose was superimposed to 2X DMEM medium. At 72 hours post-infection, cells were stained with crystal violet, washed with PBS and the number of plaques (lysed cells) were counted to compute the viral titer.

ELISA assays

ELISA assays were carried out with 50 ul of cell culture supernatants using the VeriKine Human Interferon Beta Elisa Kit (PBL Assay Science) according to the manufacturer's protocol. Samples were run as technical duplicates on biological triplicates.

Immunofluorescence analysis

HEK 293T were seeded in cover slip-containing 24-well plates and co-transfected with FLAG-SNRNP200 WT or S1087L mutant and MYC-TBK1 24 hours later. The following day, cells were infected or not with SeV for 16 hours before being washed twice with PBS, fixed with 4% paraformaldehyde-containing PBS during 20 minutes at room temperature and then permeabilized in 0.2% Triton X-100/PBS during 15 minutes. Blocking was made in PBS with 10% normal goat serum, 5% bovine serum albumin (BSA) and 0.02% sodium azide during 45 minutes at room temperature. Following three rapid washes, cells were labelled with rabbit anti-FLAG (Sigma-Aldrich) and mouse anti-MYC (Santa Cruz) primary antibodies diluted in 5% BSA/0.02% sodium azide/PBS for 2 hours. Slides were washed three times in PBS and then labeled with anti-rabbit or anti-mouse AlexaFluor 488, 594 or 647 secondary antibodies (Life Technologies) diluted in 5% BSA/0.02% sodium azide/PBS for 1 hour. Cells were extensively washed and incubated with Prolong Gold with DAPI (Life Technologies). Alternatively, nucleus were labeled with Syox Green (Life Technologies). Labelled cells were then examined by laser scanning microscopy using a TCS SP5 (Leica).

Annex B - Development of Panviral Therapeutics Requires a Better Understanding of Pathogen-induced Immune Response

Adapted from a review article published in Current Opinion in Virology.

EsSaad, S*., Tremblay, N*. Baril, M. & Lamarre, D. (2012) Regulators of innate immunity as novel targets for panviral therapeutics. Current Opinion in Virology 2(5): https://doi.org/10.1016/j.coviro.2012.08.009

*these authors contributed equally to the work

Editor: Daniel Lamarre and Mark A. Wainberg. Accepted: September 24, 2012

This review was updated in November 2017 to provide the reader with any development that occurred since the initial publication in 2012. Additional information is provided to put into perspective the concepts and ideas on which the original review was built upon.

B.1.1. Abstract

Interferons (IFNs) have long been used as an immunomodulatory therapy for a large array of acute and chronic viral infections. However, IFN therapies have been plagued by severe side effects. The discovery of pathogen recognition receptors (PRR) rejuvenated the interest for immunomodulatory therapies. The successes obtained with Toll-like receptor (TLR) agonists in activating immune cells and as adjuvant for prophylactic vaccines against different viruses paved the way to targeted immunomodulatory therapy. Better characterization of pathogen-induced immune disorders and newly discovered regulators of innate immunity have now the potential to specifically withdraw prevailing subversion mechanisms and to transform antiviral treatments by introducing panviral therapeutics with less adverse effects than IFN therapies.

B.1.2. Highlights

- IFN therapies have been plagued by severe side effects
- Discovery of PRRs rejuvenated interest for immunomodulatory therapies
- Panviral therapeutics will target key regulators of innate immune responses
- Future targeted immunomodulatory therapies will reduce side effects
- Panviral therapeutics in combination with DAAs to achieve viral eradication

B.1.3. Introduction

The innate immune system is the first line of defense for organisms that possess an adaptive immune system. It relies on the presence of specific receptors able to recognize recurring pattern in molecules associated with pathogens but not with host cells, allowing discrimination between self and non-self. These receptors are named pattern recognition receptors (PRR) and recognized pathogen-associated molecular patterns (PAMP) to induce the expression of cytokines and chemokines that restrict dissemination, eliminate pathogens and instruct pathogen-specific adaptive immune responses. In the recent years, tremendous advances in the characterization of PRR families, nucleic acid sensing, downstream signaling pathways and effector responses have revealed essential role of novel proteins and dynamic protein interactions network in the triggering of immune responses to intracellular pathogen such as viruses. In the near future, targeting specific regulators of PRR-mediated innate response to withdraw viral subversion mechanisms, and access to novel surrogate measurable effector markers, hold the promise of new panviral therapeutics that will minimize adverse effects associated with type I IFN therapy. This review briefly summarizes strategies and challenges of present and future targeted immunomodulatory therapies according to our increasing knowledge in regulation of innate immunity and of virus-induced immune host dysfunction.

B.2. Toward a better understanding of the innate immune response to viral infection

Signaling PPRs include the major families of Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).

Pathogen sensing takes place in all nucleated cells to generate cell-intrinsic innate immunity and in professional antigen presenting cells (APCs) to promote specific adaptive immune responses. While TLRs sense PAMPs in the extracellular space and endosomes, RLRs and NLRs function as pathogen sensors in intracellular compartments [388].

Interestingly, only a few of the known 13 TLRs have the ability to recognize viral molecules: TLR3 for viral dsRNA, TLR7/8 for viral ssRNA and TLR9 for viral unmethylated CpG DNA. Three cytosolic sensors of viral RNA have been characterized thus far: RIG-I for the sensing of 5' triphosphate structure and blunt-end base paring, MDA5 for the sensing of long dsRNA and LGP2 a CARDless regulator of its counterparts [389]. Following their activation, the CARD domain of RIG-I and MDA5 interacts with the CARD domain of the signaling adaptor MAVS (mitochondrial antiviral signaling protein) [191]. Both TLR and RLR viral sensing pathways converge to activate IFN regulatory factor IRF3- and IRF7-mediated type I IFN (α/β) antiviral response and NF-κB-mediated inflammatory pathway [390] (Figure 1). Recent studies aim at better defining innate immune responses have identified several novel signaling and regulatory molecules [391]. Global proteomic analysis has further revealed signaling modules with high interconnectivity and adaptor proteins regulating signalosome assembly upon antiviral response and type I IFN production [392].

2.2.1. PRR signaling in initiation of specific adaptive immune response

TLR- and RLR-mediated antiviral responses take place at the site of infection in nonimmune cells and resting immune cells, where secreted pro-inflammatory cytokines and type I IFNs increase expression of MHC class II antigens, CD40 and CD86 on APCs [393]. Cytokines produced at sites of infection play a key role in the activation and differentiation of dendritic cells (DC), macrophages, neutrophils and NK cells, all major players of the innate immune response [394] (Figure 30). When mature DCs detect virus derived antigens, they migrate to the lymph nodes to present antigens to CD4+ and CD8+ T cells and B cells, inducing their activation [395]. Thus, modulation of PRR-mediated antiviral responses can have important ripple effects on both qualitative and quantitative aspects of the specific adaptive immune responses to maximize the therapeutic potential of immunomodulatory drugs [396].

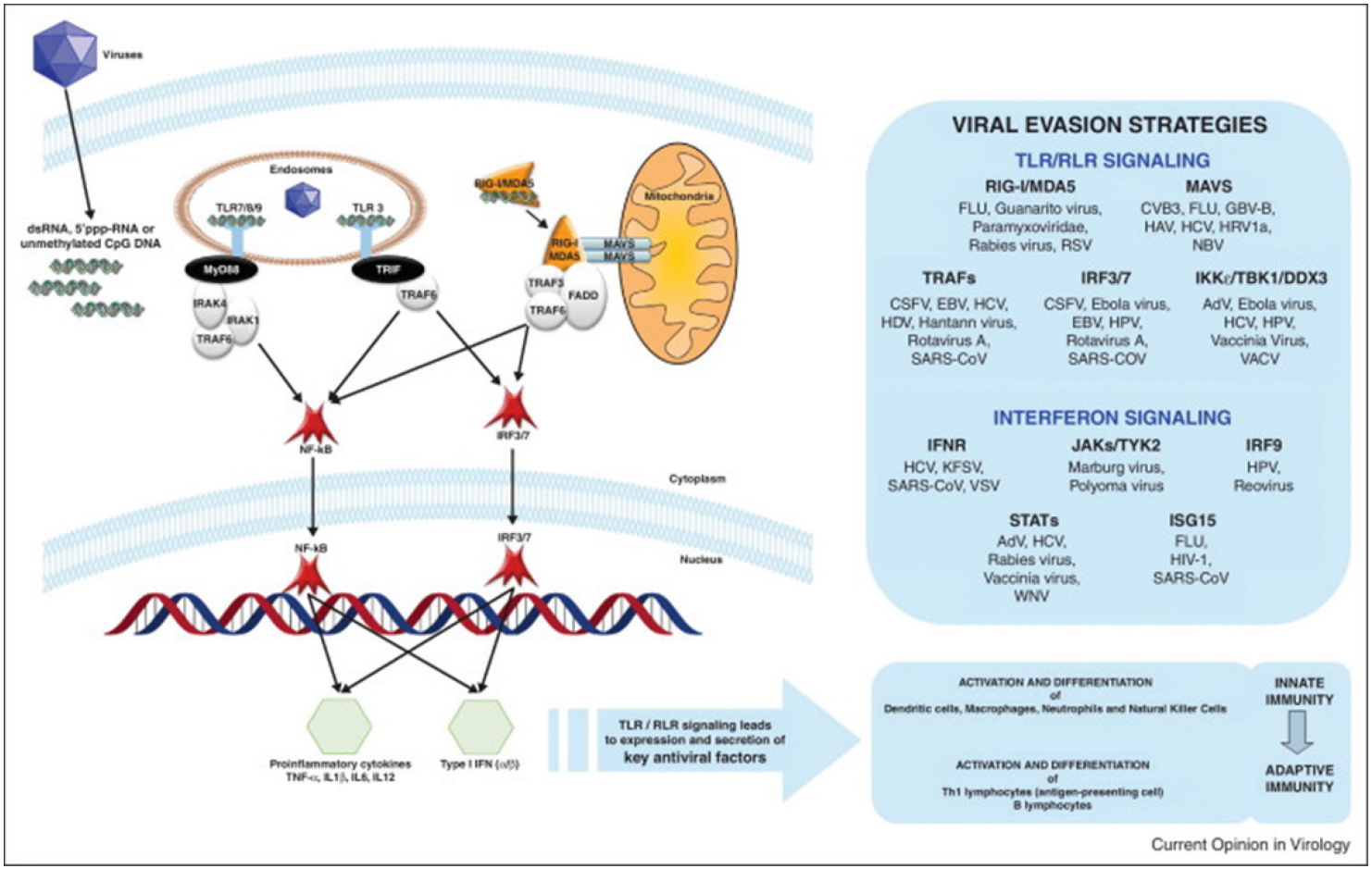

Figure 30. TLR and RLR signaling. Viral nucleic acids are recognized by endosomal and cytoplasmic PRRs. Activation of MYD88-dependant TLR7/8/9 signaling, TRIF-dependant TLR3 signaling and RIG-I/MDA5 signaling results in nuclear translocation of IRF3/7 and NF-κB transcriptional factors, leading to type I IFN and proinflammatory cytokines production. Effectors of innate immune response allow mounting of an optimal adaptive immune response. Viral evasion strategies are also identified that interfere with TLR/RLR and IFN signaling pathways.

2.2.2. Negative regulation of innate immune response and pathological consequences

Antiviral innate response must be tightly regulated in order to prevent uncontrolled production of cytokines that might have deleterious effects on the host. Type I IFN signature induced by PRR activation has been observed in diverse autoimmune disorders including diabetes, and is believed to play a role in the induction of chronic inflammatory disorders such as asthma and rheumatoid arthritis. In the recent years, a better picture has emerged in the biology of regulators illustrating the existence of numerous negative regulators that often play a nonredundant role and target the same positive regulator [391]. Many negative regulators have been characterized that are either involved in direct interaction with PRRs, dissociation of adaptors complexes, degradation of signal proteins or transcriptional regulation [12]. Post-translational modifications (phosphorylation and ubiquitination) have emerged as key mechanisms to regulate innate immune responses. Degradation of signal proteins mediated by the ubiquitin-proteasome and autophagy systems plays crucial roles in negative regulation of TLR signaling, and unlike disruption of adaptors contributes to termination of signaling as these degradations are irreversible [397]. Examples include proteins SOCS and PIN1 that promote polyubiquitination and proteasomal degradation of Mal adaptor and IRF3/7 respectively, to suppress type I IFN and antiviral responses. Recently, miRNAs have also emerged as fine tuners of innate immune responses, which target mRNAs encoding TLRs, intracellular signaling proteins and cytokines. Examples include miR-146 that targets IRAK1 and TRAF6, and miR-155 that targets MYD88, TAB2 and IKKε [398]. Thus, targeting specific negative regulator of the innate immune response may offer a new immunotherapeutic strategy to treat a range of infectious and inflammatory diseases [399].

2.2.3. Viral subversion mechanisms

Cellular defence have evolutionarily challenged viruses that in turn have developed strategies to counteract innate immune response. Indeed TLR and RLR sensing pathways are fundamental targets for virus-encoded immune suppression. These viral subversion mechanisms include recruitment of ubiquitin proteasome system, mimicry of the host cell components and sequestration and cleavage of key components of the immune system.

One notable example is MAVS adaptor that is targeted by numerous viruses through proteolytic cleavage by hepatitis C virus (HCV), hepatitis A virus (HAV), Coxsackievirus B3 (CVB3), human rhinovirus 1a (HRV1a) and GB virus B (GBV-B), through decrease of the mitochondrial membrane potential by influenza A virus (FLU) or through inhibition of its interaction with RIG-I by hepatitis B virus (HBV). Processes of viral evasion are varied and are beyond the scope of this review, but are recapitulated in Figure 1 [reviewed in 400]. Importantly, host proteins targeted by multiples viruses highlight key players of innate immunity, which represent potential therapeutic targets to restore antiviral response and eventually cure cells from viruses. However, these specific viral evasion strategies must also be taken into account when developing immunomodulatory therapeutics to provide the greatest clinical benefits.

2.2.4. IFNs: Pioneer of panviral therapies

Type I IFNs were rapidly used as a therapeutic agent against HBV and HCV, and demonstrated antiviral activity against infection with SARS-CoV [401], FLU [402], West Nile virus (WNV) [403], yellow fever virus (YFV) [404] and Ebola virus [405]. Refinement of therapies was explored with the development of improved IFN molecules like consensus interferon (CIFN: a completely synthetic interferon) [406], albinterferon (a fusion protein between IFNα2a and human albumin) [407] and Y shape interferon [408]. Recently, virus-induced type III IFNs (IFN-λ1-3: IL-29, IL28A, IL28B) have gained a lot of interest to treat viral infections since naturally occurring variants of the IL28B gene have been a major prediction factor in spontaneous and treatment-induced clearance of HCV [409, 410]. Early clinical trials of recombinant pegylated-IFN-λ1 in HCV-infected patients showed reduced adverse effects compared to IFN-α, likely linked to minimal expression of IFN-λ receptors in hematopoietic cells [411, 412]. However, subsequent clinical trials, failed to show the noninferiority of IFN-λ vs. to IFN-α, showed a higher rate of relapse and resulted in an increased of early treatment discontinuation suggesting that additional studies will be required to understand which cross-section of infected individuals would benefit from this novel treatment, if at all [413].

B.2.3. TLR targeted therapies (Table II)

The discovery of TLRs heralded the rebirth of interest in innate immunity. Their specificity in recognizing most classes of pathogens, as well as their role in the pathogenesis of multiple diseases represent the strongest evidences that TLRs are valuable therapeutic targets. TLR targeted drugs have been approved and small-molecule compounds are being investigated in the treatment of viral infections as stand-alone treatment or adjunct to direct acting antivirals (DAAs).

Imidazoquinolines: The most advanced examples of TLR agonists are Imiquimod (Aldara, 3M) and Resiquimod (R-848, 3M), which are members of the imidazoquinolinamines [414]. Imiquimod is the only approved TLR7 agonist and is use for topical treatment of external genital and perianal warts resulting from human papillomavirus (HPV) infection [415]. Resiquimod is a mixed TLR7/8 agonist that reached phase III trial for the treatment of genital herpes before being suspended due to a lack of efficacy [416].

Isatoribine: ANA-773 (Anadys Pharmaceuticals) is a second generation of orally bioavailable prodrug of isatoribine that signals through TLR7, which is expressed in B cells and DCs [417]. In HCV infected patients, ANA-773 was generally well tolerated and resulted in a significant -1.26 $\log_{10}$ decrease in HCV RNA levels following 10 days of treatments [418]. ANA-773 is now assessed in phase IIa, and its efficacy will be evaluated in combination with ribavirin and DAAs as an IFN replacement.

Immunomodulatory oligonucleotides: Synthetic cytosine-phosphate-guanine containing oligodeoxynucleotides (CpG-ODNs) are potent TLR9 agonists, which interact directly with DCs to stimulate cytokine release and induce adaptive immune responses [419]. In Phase I clinical trials, subcutaneously administration of IMO-2125 (Idera Pharmaceuticals) as monotherapy resulted in a more than -1 $\log_{10}$ decrease in HCV RNA levels in prior nonresponders to PEG-IFN/ribavirin after 4 weeks [420], and in combination with ribavirin to a -2.4 $\log_{10}$ decrease in HCV RNA in treatment-naïve patients at day 29 [421, 422]. Based on its efficacy, IMO-2125 could provide an alternative to IFNs for HCV therapy. However, Idera Pharmaceuticals delayed a phase II study after the observation of atypical lymphocytic proliferation in preclinical toxicology study.

Compound	Class	Viral disease	Target	Company	Clinical status
Antiviral treatments					
Aldara (Imiquimod)	small molecule of imidazoquinoline class	HPV	TLR7	3M Pharma	Marketed
Resiquimod	small molecule of imidazoquinoline class	HCV, HPV	TLR7/TLR8	3M Pharma	Suspended in phase III
ANA773	small molecule (prodrug of isatoribine)	HCV	TLR7	Anadys Pharmaceuticals	Phase IIa
CPG10101 (Actilon)	CpG ODN	HCV	TLR9	Coley pharmaceutical	Suspended in phase II
IMO-2125	CpG ODN	HCV	TLR9	Idera Pharmaceuticals	Phase I
Vaccine adjuvants					
Fendrix	MPLA + HBV antigen	HBV	TLR4	GlaxoSmithKline	Marketed
Cervarix	MPLA + alum + HPV 16 & 18 antigen	HPV	TLR4	GlaxoSmithKline	Marketed
Heplisav (ISS-1018)	CpG ODN + Hepatitis B surface antigen (HBsAg)	HBV	TLR9	Dynavax Technologies	Phase III
VAX125	Flagellin + influenza H1N1 hemagglutinin (HA) antigen	Influenza	TLR5	VaxInnate Corporation	Phase II
VAX102	Flagellin + influenza H1N1 matrix protein 2 (M2e) antigen	Influenza	TLR5	VaxInnate Corporation	Phase I

Table II. Development status of drugs targeting TLRs for treatment of viral infections.

2.3.1. Vaccine adjuvants using TLR agonists:

TLR agonists have been an extensively explored area in the development of vaccine adjuvants for prophylactic and therapeutic applications by linking innate and adaptive immune systems. The proof-of-concept of this approach was made with the AS04 adjuvant system that combines monophosphoryl lipid A (MPLA), an agonist of the TLR4 receptor, and aluminium salt [423-425]. AS04 has been approved in prophylactic vaccine against HBV (Fendrix, GlaxoSmithKline) [426] and HPV 16 and 18 (Cervarix, GlaxoSmithKline) [427].

The mechanism of action of AS04 is mediated by a transient and local activation of NF-kB activity and cytokine production, thus providing an innate immune signal for optimal activation of APCs [428]. Other notable examples of adjuvants in clinical development are Heplisav and VaxInnate. Heplisav is a HBV vaccine comprised of an immunostimulatory sequence (ISS-1018, Dynavax Technologies) that targets TLR9 receptor and HBV surface antigen. In phase III clinical trials, Heplisav demonstrated earlier and higher protection with fewer doses than currently licensed vaccines [429].]. As of now, Heplisav is all set for approval by the U.S. FDA with an expected date of November 10, 2017 and the first available dose in early 2018. VaxInnate Corporation is developing vaccines using highly conserved influenza immunogens fused to TLR5 agonist Salmonella typhimurium flagellin type 2 as an adjuvant to potentially

protect against all strains of seasonal and pandemic FLU strains (VAX102, VAX125, VAX128 and VAX168) [430-432]. As of now, VaxInnate Corporation was dissolved and no further studies were registered for a VAX-based technology. However, new studies are being conducted using fusion proteins or nanoparticles using a different influenza antigen, HA1-2, in combination with *s. typhimurium* fliC, giving a new momentum to the initial observation [433-435].

2.3.2. Future immunomodulatory targeted therapy and panviral approaches (Table III)

In the past decade, many newly emerging or re-emerging virus infections and fear of future pandemics have accentuated the need for novel antiviral therapy. Panviral therapeutics with a targeted therapy approach would be an ideal treatment for acute and chronic viral infections, either as a standalone treatment or in combination with DAAs. The major challenge in developing future immunomodulatory therapy will be to minimize adverse effects.

Description	Current work	Challenges and future work	Applications
Toll-like receptors (TLR3/7/8/9) [reviewed in 27]			
Recognition of extracellular PAMPs Activation leads to pro-inflammatory cytokines and type I IFNs production Set the pace for an adaptive immune response via T and B cells.	Extensive structural and functional studies available [49] TLR agonists marketed or in phase I-III clinical trials	Optimize the DC and NK cells mediated activation of adaptive immunity Minimize inflammatory responses and adverse effects Restore immunity by counteracting viral evasion processes	small molecules vaccine adjuvant
RIG-I like receptors (RIG-I, MDA5) [reviewed in 50]			
Recognition of intracellular PAMPs Activation leads to pro-inflammatory cytokines and type I IFNs production Modulation of the adaptive immunity through DC and NK cells	Structure recently identified [51,52] ssRNA RIG-I ligand reduces infection with FLU [53] RIG-I agonist as adjuvant in FLU vaccine [54]	Assess the relative contribution of RLRs in the regulation of immune cells Minimize inflammatory responses and adverse effects Restore immunity by counteracting viral evasion processes	small molecules vaccine adjuvant [55]
miRNA interference [reviewed in 12, 56]			
Highly conserved small untranslated RNA species Induction of gene silencing Involved in a wide variety of biological processes	Regulation of innate immune responses miRNAs can be induced in viral infections Role in the resolution of inflammation	Each miRNA targets multiple genes Minimize off target effects Develop better delivery systems	Antagomirs LNA

Table III. Current and future development of immunomodulatory targeted therapy

The aggravation of psoriatic plaques in HPV-infected patients treated with Imiquimod illustrates that triggering innate immune responses can lead to uncontrolled activation of the inflammatory response. Furthermore, immunomodulatory molecules, such as peptidoglycans, that bind to multiple PRRs (TLR2, NOD proteins and peptidoglycan recognition proteins) increase the risk of undesired side effects.

Development of therapeutics will require more extensive structural information of receptor-ligand interaction to maximize the specificity and avoid undesired interactions.

The selection of specific targets will require a comprehensive knowledge of innate immunity signaling pathways and regulators that are induced by and common to numerous viral infections. The mapping of an innate immune protein interaction network regulating IFNB1 has revealed signaling modules with high interconnectivity including MAVS, TBK1 and IRAK [392]. Each module interacts with many signaling proteins of the pathway offering multiple drug targets with specific immune effector function. Using a genome-wide RNAi screen assessing virus-induced IFNB1 transcription in human cells, we identified novel proteins and pathways capable of negatively and positively regulating innate immune responses (unpublished data). Comprehensive epistasis analysis of the various regulators acting at different steps of the antiviral responses from virus sensing, signal propagation/amplification up to feedback regulation, offers valuable information for selection of drug targets. In principle, strategies of targeted therapy could include small molecule-mediated activation of positive regulators or inhibition of negative regulators. An example of targeting a negative regulator could be the immuno-miRNA miR-155, which is induced by virus infection and down regulate MYD88, IRAK3, TAB2 and IKKε gene expression to suppress TLR signaling [12]. Silencing miR-155 function using antagomirs or locked nucleic acid (LNA) in infected cells could potentially restore TLR signaling. Another example of targeting a positive regulator could be via the master transcriptional regulator of ISGs YPEL5. Indeed, we recently reported that that YPEL5 , which is contained in a locus linked to a network of ISGs in mice, is a negative regulator of IFNB1 production via a functional association with the TBK1/IKBKE [436]. Thus, silencing YPEL5 using therapeutic 5'ppp-siRNA in infected cells could lead to an increase in RLR-signaling and, in correlation, to a faster and better viral restriction and clearance.

All-in-all, a better knowledge of surrogate end points measurable makers of immune effector function (correlating with pan antiviral efficacy) in relevant infected biological material will undoubtedly enhance selection process and therapeutic value of drug targets. Indeed, microarray analysis of infected primary cells can be used to identify early and late response innate immune genes, as well as virus-mediated inhibition of these genes [437-439].

Finally, the knowledge of virus-induced immune host dysfunction and of immune proteins targeted by multiples viruses will validate key viral host interfaces, leading to hypothesis-driven selection of therapeutic targets intended to restore innate immune responses. Indeed, by understanding common viral evasion strategies, we can identify important nodes of antiviral signaling and focus on the development of innovative therapeutics.

As an example, we recently completed the functional characterization of newly identified host interactors of HCV proteins [254]. Using a comprehensive microscopy-based high-content screening approach combined to the gene silencing of nuclear transport factors, we showed that NS3/4A-interacting proteins control the nucleocytoplasmic trafficking of IRF3 and NF-κB upon SeV infection. Notably, we showed that importin β1 (IMPβ1) is a hub protein that is highly targeted by several viruses. Indeed, upon silencing of IMPβ1, we observe a stark decrease of the nuclear translocation of IRF3 and NF-κB that correlates with a decrease in IFNB1 and IFIT1 production and a rapid increase of viral proteins and virus-mediated apoptosis.

Additionally, we showed that HCV NS3/4A triggers the cleavage of IMPβ1 and inhibits nuclear transport to disrupt IFNB1 production. Importantly, mutated IMPβ1 resistant to cleavage completely restores signaling, like the treatment with the BILN 2061 protease inhibitor, correlating with the disappearance of cleavage products. Overall, we believe that the data indicate that HCV NS3/4A targeting of IMPβ1 and related modulators of IRF3 and NF-κB nuclear transport constitute an important innate immune subversion strategy and will inspire new avenues for broad-spectrum antiviral therapies.

B.2.4. PRR-targeting therapies in cancer immunotherapy

Beside pan-antiviral applications, PRR-targeting therapies have gained a great momentum in the field of cancer immunotherapy. Indeed, recent reports have shown that RIG-I and cGAS signaling can induce tumor cell death directly, via the production of IFN, or indirectly via the activation of cytotoxic CD8 T cells and natural killer (NK) cells or via DC-mediated antigen presentation cross presentation of tumors associated antigens to CD8 T cells [187, 440]. Additionally, the modulation of certain TLR, such as TLR3/4/5/7, can be leveraged as anticancer therapies since their signaling can increase cytotoxic T cell activity and directly induce cancer cell death via apoptosis, pyroptosis, and autophagy.

Additionally, recent advances in our understanding of innate antiviral immunity has given a new momentum towards the development of therapeutic agents targeting RIG-I and cGAS-STING, in addition to the TLR pathway. As such, the three pathways are being targeted using a wide variety of synthetic ligands across many cancer types such as melanoma, pancreatic cancer, prostate cancer, ovarian cancer, colon cancer and gliomas (Table IV). At this time, all these strategies are still in pre-clinical or early phase 1 studies, and, as such, it will be interesting to see if any of these therapies can be translated into effective, safe and tolerable anti-cancer treatments.

Promising Agents	Receptors	Cancer Types	References
Bacillus Calmette-Guérin (BCG)	TLR2/4	Bladder cancer	[441]
monophosphoryl lipid A(MPL)	TLR4	Cervical cancer	[442]
Imiquimod	TLR7	Breast cancer	[443]
Flagellin-derived CBLB502 (Entolimod)	TLR5	Hepatoma	[444]
852A	TLR7	Hematologic malignancy	[445]
CpG ODN	TLR9	Glioblastoma	[446]
poly(I:C)/poly-ICLC	TLR3	Multiple cancer types	[447]
5′ ppp-siRNA for Bcl-2	RIG-I	Melanoma	[448]
5′ ppp-siRNA for TGF-β	RIG-I	Pancreatic cancer	[449]
HVJ-E	RIG-I	Prostate cancer, gliomas	[450, 451]
poly(I:C)	MDA5	Ovarian cancer, Pancreatic cancer	[452, 453]
cGAMP	STING	Colon cancer	[454]
c-di-GMP	STING	Melanoma	[455]
STINGVAX	STING	Melanoma	[456]

Table IV. Overview of promising agents that trigger the Toll-like receptors (TLR), RIG-I-like receptors (RLR) and stimulator of interferon gene (STING) pathway for cancer immunotherapy. Adapted from [187]. Used under a Creative Commons Attribution (CC BY) license 4.0.

B.2.5. Conclusions

TLRs agonists reflect substantial promise as therapeutic targets and demonstrate the huge potential of targeting innate immunity in fighting viral infections. More recently, this potential has been extended to RLR and cGAS agonist based on a better understanding of the mechanisms underlying the function of their respective signaling pathways. Indeed, due to a large unmet medical need in the field of cancer, the development of PRR ligands for cancer immunotherapy has gained a huge momentum. Luckily, this provides additional resources to explore the potential of PRR ligands as antiviral agents. In the future, integration of structural, proteomics and functional genomics data will pave the way to the identification of key regulators of innate immunity. Targeting immune regulators that promote PRR signaling to maintain transient activation of innate immune responses upon viral infection should pioneer the discovery of panviral therapeutics. Such targeted immunomodulatory therapy approach could change the way we treat infectious diseases by allowing a single treatment to be effective against numerous viruses, with minimal viral breakthrough. Soon, the increasing availability and potency of new targeted immunomodulatory panviral therapeutics could allow the re-thinking of temporal aspects of treatments that, in combination with available DAAs, could achieve viral eradication. The goal is to shape TLR-dependent, RLR-dependent and cGAS-STING-dependent innate immune responses to restore antiviral effects and to generate an optimal global immune response, while controlling inflammation.

B.2.6. Perspective: A proof-of-concept study for the discovery of pan-antiviral molecules

To test general therapeutic concept suggested by this review, we conducted a phenotypic screening to identify immunomodulator molecules with pan-antiviral like properties. Briefly, we screened three publicly available chemical libraries using the same HTS assays than for our genome-wide RNAi screen to identify selective small-molecule modulators of IFN-β (Figure 31a). The objective being to identify cellular targets of small-molecule modulators by deconvolution of newly identified regulators of the innate antiviral immunity identified in our RNAi screen.

Briefly, we screened the potential of 3266 small molecules to increase the expression of IFN-β by at least 50% in two cell lines (293T and A549). Then, we assessed the ability of the immuno-modulator compounds to restrict viral replication by at least 50%, as measured by a reporter system, using Influenza (A/PR8/34) and HCV (JC1(2a)) viruses (Fig. 31b). Overall, we identified a very limited number of compounds are immunomodulators with pan-antiviral like properties (Fig31c). Among them, we found the most commonly prescribed drug: statins. Indeed, statins are cholesterol-lowering agents that acts via the inhibition of the HMG-CoA reductase in the cholesterol pathway.

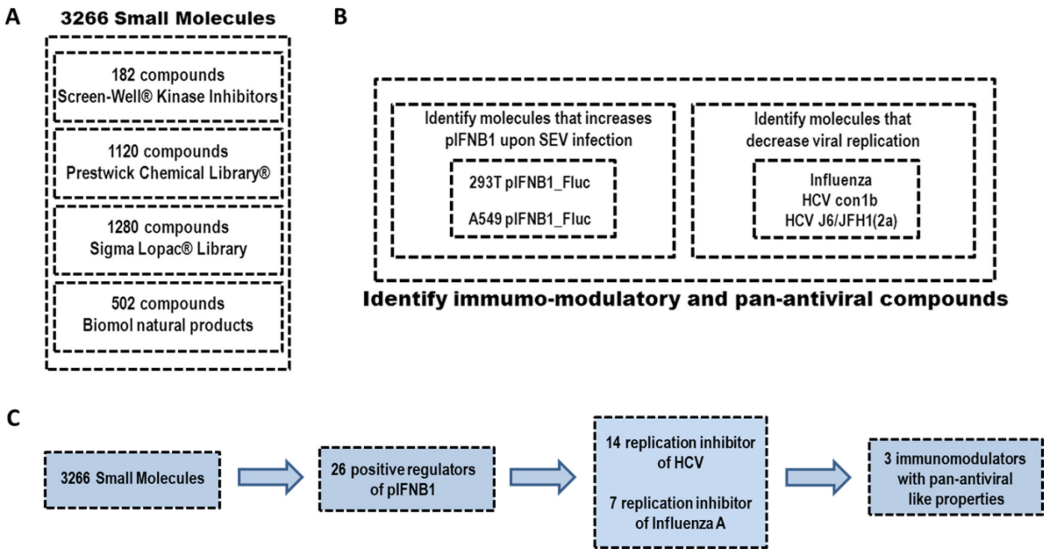

Figure 31. Summary of our proof-of-concept study for the discovery of pan-antiviral molecules. (A) Description of the chemical libraries screened in functional assays. (B) Description of the assays used to characterize the potential of a small molecule to promote IFNB1 transcription and restrict viral replication. (C). Breakdown of the number of molecules identified after each step of the chemical screen.

For statins, their antiviral potential has been reported *in vitro*, but their efficacy *in vivo* has been limited and has led to a decrease interest for their therapeutic application as antiviral drugs [457-463]. However, subsequent functional follow-up studies have identified that an upstream regulator of HMG-CoA reductase, SKI-1/S1P, has great *in vitro* antiviral activity for HCV and is currently being investigated as potential host target for the development of indirect-acting antiviral agents against other Flaviviridae viruses such as the emerging Dengue virus [464-467]. Thus, combinatorial ap-traproaches that leverages from chemical and functional screens is a sound way to identify small molecules with immuno-regulatory and antiviral potential.

Of note, while we did not proceed to the second part of the project (genomic shRNA screen deconvolution) due to the limited number of hits among publicly available libraries (3; with known targets), it would be interesting to see if this pipeline is a potent tool to map the target of a small molecules and accelerate broad-spectrum immunomodulators and antivirals by screening significantly larger libraries via an industrial partnership.

Annex C – Importinβ1 targeting hepatitis C virus NS3/4A protein restrict IRF3 and NF-κB signaling of IFNB1 antiviral response

Gagné B, **Tremblay N**, Park AY, Baril M, Lamarre D (2017) Importin β1 targeting by hepatitis C virus NS3/4A protein restricts IRF3 and NF-κB signaling of IFNB1 antiviral response. **Traffic** 18:362–377.

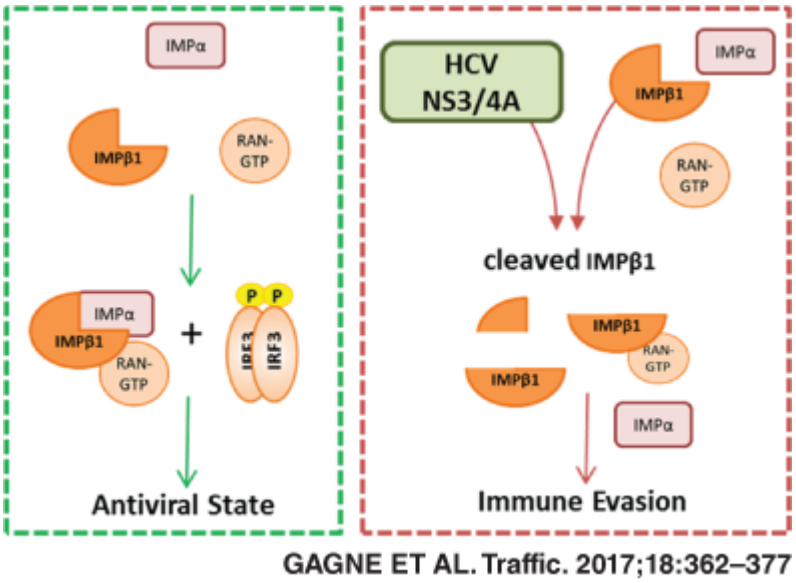

Figure 32. Hepatitis C virus (HCV) hijacks the cellular host machinery to promote replication and to evade immune response. Using a microscopy-based High Content Screening (HCS) assay, we demonstrated that HCV-host interactors involved in nuclear transport are crucial for IRF3 and NFκB-p65 signaling of IFNB1 response. To subvert innate immunity, NS3/4A interacts and cleaves IMPβ1, a key nucleocytoplasmic transport receptor, to prevent nuclear translocation of IRF3 and NFκB-p65 transcription factors. Future studies will confirm if IMPβ1 is a prime target for several viruses to evade host defense.

Received: 3 October 2016 | Accepted: 9 March 2017 | Uncorrected manuscript published: 13 March 2017 | Published on: 2 May 2017
DOI: 10.1111/tra.12480

WILEY Traffic

ORIGINAL ARTICLE

Importin β1 targeting by hepatitis C virus NS3/4A protein restricts IRF3 and NF-κB signaling of IFNB1 antiviral response

Bridget Gagné[1,2] | Nicolas Tremblay[1,2] | Alex Y. Park[1,3] | Martin Baril[1] | Daniel Lamarre[1,2]

[1]Centre de Recherche du CHUM (CRCHUM), Montréal, Canada

[2]Département de Médecine, Faculté de Médecine, Université de Montréal, Montréal, Canada

[3]Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Montréal, Canada

Correspondence
Daniel Lamarre, CRCHUM, Montréal, Canada.
Email: daniel.lamarre@umontreal.ca

Funding information
Canadian Institutes for Health Research, Grant/Award number: CIHR-MOP-115010 and CIHR-CI6-103135; Novartis/Canadian Liver Foundation Hepatology Research Chair; FRQ-S PhD scholarship.

In this study, newly identified host interactors of hepatitis C virus (HCV) proteins were assessed for a role in modulating the innate immune response. The analysis revealed enrichment for components of the nuclear transport machinery and the crucial interaction with NS3/4A protein in suppression of interferon-β (IFNB1) induction. Using a comprehensive microscopy-based high-content screening approach combined to the gene silencing of nuclear transport factors, we showed that NS3/4A-interacting proteins control the nucleocytoplasmic trafficking of IFN regulatory factor 3 (IRF3) and NF-κB p65 upon Sendai virus (SeV) infection. Notably, importin β1 (IMPβ1) knockdown—a hub protein highly targeted by several viruses—decreases the nuclear translocation of both transcription factors and prevents IFNB1 and IFIT1 induction, correlating with a rapid increased of viral proteins and virus-mediated apoptosis. Here we show that NS3/4A triggers the cleavage of IMPβ1 and inhibits nuclear transport to disrupt IFNB1 production. Importantly, mutated IMPβ1 resistant to cleavage completely restores signaling, similar to the treatment with BILN 2061 protease inhibitor, correlating with the disappearance of cleavage products. Overall, the data indicate that HCV NS3/4A targeting of IMPβ1 and related modulators of IRF3 and NF-κB nuclear transport constitute an important innate immune subversion strategy and inspire new avenues for broad-spectrum antiviral therapies.

KEYWORDS

HCV, importin-α, importin-β, IMPβ1/KPNB1, innate antiviral immunity, interferon-β, IRF3, microscopy-based high-content screening, NF-κB p65, NPC, NS3/4A protein, nuclear pore complex, nuclear translocation, nucleocytoplasmic transport, RNAi screen, SeV, virus-host interaction

1 | INTRODUCTION

Several viruses interact with components of the nucleocytoplasmic trafficking to facilitate viral replication. Indeed, many proteomic and functional RNAi genetic screens revealed that modulation of nuclear pore complexes (NPCs), nuclear transport receptors and the RAN-GTPase system have profound effects on viral replication.[1–5] For instance, interactions between viral proteins, nucleoporins (Nups), RAN, importin-α (IMP-α), importin-β (IMP-β) and exportin (EXP) have been observed with hepatitis C virus (HCV),[1,6,7] picornavirus,[8] and human immuno-deficiency virus (HIV)-1.[9,10] In HCV infection, viral proteins (core, NS2, NS3 and NS5A) harbor a nuclear localization signal (NLS) and/or nuclear export signal (NES)[11,12] suggesting that nuclear-cytoplasmic shuttling is important for it virus life cycle that is mostly within the cytoplasm.[6] Indeed, Nups were reported to accumulate in virus-induced endoplasmic reticulum (ER)-derived membranous structures where HCV replication occurs (so-called membranous webs), indicating that Nups can gate these cytoplasmic compartments to promote viral RNA replication and to prevent cytosolic RIG-I-like receptor (RLR) sensing of viral RNA.[6,7,13] This is further supported by the findings that silencing IMPβ1, TNPO1 (KPNB2) and RAN, all involved with nucleocytoplasmic transport and elucidated to interact with NS3/4A protein, decreases HCV replication.[1] For many negative-sense RNA viruses (NSV) including Sendai virus (SeV), the

nuclear trafficking of the matrix protein has been reported to be critical for efficient production of viruses and to their pathogenesis.[14] Viruses also co-opt nucleocytoplasmic trafficking to facilitate transport of viral RNAs. Influenza A virus, one of the few NSV to replicate in the nucleus, uses NS1 to form an inhibitory complex with nuclear RNA export factor 1 (NXF1) and NTF2-related export protein 1 (NXT1/p15) to block mRNA export,[15] while interaction of its nucleoprotein (NP) with NXT1 promotes the nuclear export of viral ribonucleoproteins (vRNPs) in an EXP1-dependent manner.[16]

Viruses also interact with nucleocytoplasmic trafficking of transcription factors (TFs) of the immune responses as a viral strategy to promote viral growth. Highly pathogenic mononegavirale members such as the Nipah virus interacts with IMPα4 (KPNA3) and IMPα3 (KPNA4) for its viral protein nuclear localization to inhibit the activation of the IRF3-responsive promoter,[17] while Ebolavirus interacts with IMPα5 (KPNA1), IMPα6 (KPNA5) and IMPα7 (KPNA6) to disrupt their interaction with tyrosine-phosphorylated STAT1 for nuclear import, inhibiting IFN-stimulated gene (ISG) production.[18,19] Similarly, positive-sense RNA poliovirus and rhinovirus are shown to disrupt nucleocytoplasmic trafficking by proteolytic cleavage of specific Nups that inhibit host antiviral defense pathways.[20–23] Nidovirales like porcine reproductive and respiratory syndrome virus (PRRSV) degrades IMPα5 to block IFN-stimulated gene factor 3 (ISGF3) nuclear import, while SARS coronavirus (SARS-CoV) tethers IMPβ1 to ER and Golgi membranes, blocking STAT1 nuclear import.[24,25] Finally, HCV NS3/4A interacts with IMPβ1 to prevent nuclear translocation of STAT1 and subsequent induction of ISGs.[1] Thus, viruses have evolved multiple strategies to exploit nucleocytoplasmic transport pathways to avert the host innate immune response and facilitate their replication.

NPCs are ~60 to 120 MDa structures involved in the trafficking of macromolecules between the nucleus and the cytoplasm[26] that are composed of at least 30 different Nups.[27–29] These include the transmembrane Nups (POMs) that anchor to the NPC in the nuclear envelope, structural Nups that form the skeleton of the NPC, and the intrinsically disordered Nups that constitute the permeability barrier with their disordered phenylalanine-glycine-rich regions (FG-Nups). The translocation of macromolecules (>40-60 kDa) across the NPC is facilitated by the IMP-β family proteins, which are nucleocytoplasmic transport receptors (NTRs) that primarily carry nuclear proteins and small RNAs as their cargoes through the nuclear pores. The human genome encodes 20 species of IMP-β family NTRs, of which 10 are nuclear import receptors, 7 are export receptors, 2 are bi-directional receptors and RanBP6 that the function is undetermined.[30] IMPβ1 is one of the best-studied member of the IMP-β protein family that mediate import of proteins into the nucleus either directly or indirectly through binding of adaptor proteins that belong to the IMP-α family.[30] These adaptors recognize proteins that contain a classical NLS and connect IMPβ1 and cargo molecules for their translocation in the nucleus. As such, IMPβ1 contains 19 tandem HEAT sequence repeats, which binds cargo-IMP-α complex through HEAT repeats 7-19,[31] RAN via HEAT repeats 1-3, 6, 7, 13 and 14[32,33] and FG-Nups via HEAT repeats 5 and 6 for the translocation of cargo through NPCs.[34] Many proteins are also recognized directly by specific NTRs of the IMP-β family (TNPO1, TNPO2, TNPO3/IPO12) without the intervention of IMP-α, and often contain a proline-tyrosine NLS (PY-NLS) motif.[27] Conversely, EXP-1/CRM1, through interaction with NES, and others such as EXP-2/CSE1L, -5, -6, -7, -t and RanBP17 facilitate protein export to the cytoplasm. Finally, the direction of cargo transport is mainly controlled by GTP-binding nuclear protein Ran (RAN) across the nuclear envelope. RAN in the nucleus dissembles NTR-cargo import complexes, releasing the cargo and allowing NTRs to translocate back to the cytoplasm,[35] whereas RAN bound EXP-cargo complexes in the cytoplasm are dissembled when RANGTP is hydrolyzed to RANGDP by GTPase-activating protein 1 - (RANGAP1) and RAN binding proteins.[36]

In this study, HCV-host interactors previously identified by liquid chromatography-mass spectroscopy (LC-MS/MS) analysis are investigated for their effect on *IFNB1* transcription in response to SeV infection by gene silencing. Factors with the greatest inhibitory effects are functionally associated with protein transport by Gene Ontology (GO) analysis. Notably, all candidates were determined to interact with NS3/4A protein, which is well-known for its role in evading the antiviral response. The effects of silencing proteins associated to nucleocytoplasmic trafficking were investigated for IFNB1 production through the nuclear translocation of IRF3 and nuclear factor-kappa B (NF-κB) p65 TFs upon viral infection. By a microscopy-based high-content screening combined to gene silencing, we identified several proteins that reduce the number of IRF3- and p65-positive nuclei within a 10 hours period post-infection decreasing IFNB1 production. We also identified proteins that delayed, increased or had a differential effect on IRF3 and NF-κB p65 nuclear translocation, and similarly reduced IFNB1 production upon gene knockdown. Depletion of the main import carrier IMPβ1 showed the most significant decrease in IFIT1 (ISG56) expression, which correlated with rapid increase of viral protein levels and virus-mediated apoptosis. We found that expression of NS3/4A triggers the cleavage of IMPβ1 to evade IFNB1 production in cells that are knockout (KO) for mitochondrial antiviral-signaling protein (MAVS) and reconstituted with an NS3/4A cleavage-resistant MAVS variant (C508R). Moreover, expression of cleavage-resistant IMPβ1 variant fully restores signaling and IFNB1 induction, similar to the treatment of infected cells by BILN 2061 protease inhibitor, correlating with the disappearance of IMPβ1 cleavage products. The data underline the targeting of key regulators of IRF3 and NF-κB nuclear trafficking of IFNB1 antiviral response as a viral subversion strategy to promote viral replication and pathogenesis.

2 | RESULTS

2.1 | Effect of silencing HCV-interacting proteins on IFNB1 antiviral response

We previously identified hundreds of human proteins interacting with HCV by an immunoprecipitation (IP)-based approach using a 3X-FLAG peptide in fusion with viral proteins and LC-MS/MS analysis.[1] This HCV interactome study led to the identification of selective host interactors to one of the viral proteins Core, NS2, NS3/4A, NS4B, NS5A and NS5B. The biological significance of these host proteins with an RNAi silencing screen further revealed that majority of these interactors are not affecting virus replication. However, HCV replication

is often monitored in the RIG-I deficient Huh7.5 cell line such that one cannot assess if the viral-host protein interactions benefit the virus through subversion of the innate immune response resulting in increased viral replication and pathogenesis. To test this hypothesis, we silenced 132 selective HCV-host interactors (Figure S1, Supporting Information) using ~5 independent short hairpin RNA (shRNA)-expressing lentiviruses, and measured the induction of IFNB1 promoter-driven firefly luciferase upon SeV infection of A549 human lung carcinoma cells and Human Embryonic Kidney (HEK)293T cells. We previously showed that SeV infection predominantly activates the RLR pathway in these cells, leading to the nuclear translocation of NF-κB and IRF3 TFs, induction of IFNB1 mRNA and secretion of IFN-β cytokine.[37] In parallel, cells harboring a luciferase gene driven by the nonimmune endogenous elongation factor 1 alpha (EEF1A1) promoter were used to assess the effects of shRNAs on basal transcription. The gene silencing screens revealed 35 modulators of antiviral response in HEK293T cells and 29 modulators in A549 cells (Figure 1). In total, 53 HCV-host interactors, with 11 identified in both cell lines, specifically altered IFNB1 reporter expression by at least 2 independent shRNAs without affecting the constitutive expression of the EEF1A1 promoter (Table S2). The effect of silencing these 53 genes on modulation of HCV replication and antiviral response is summarized in Figure S2. Notably, 12 proteins significantly affected both viral replication and IFNB1 induction. Overall, the data provide evidence that viruses can hijack cellular processes that contribute to the innate immune response and viral replication giving a dual growth advantage.

2.2 | GO analysis

GO enrichment was performed to determine whether these 53 virus-host interactors associated with one another through a particular complex or cell process. Table 1 lists 10 statistically significant enriched terms ($P < .05$) in order of association, as well as the respective genes. The 2 most enriched terms are "protein import into the nucleus," "docking" (enriched 72-fold with 4 proteins: IMPβ1/KPNB1, TNPO1, EXP1/XPO1 and EXP2/CSE1L, and "nuclear pore" (enriched 23-fold with 6 proteins: IMPβ1, TNPO1, lamin-B receptor [LBR], EXP1, EXP2 and RAN). As previously indicated, HCV proteins such as NS3 contain an NLS that contributes to the compartmentalization of viral replication within NPC-containing virus-induced membranous webs to limit access of pathogen-recognition receptors (PRRs) to these sites. Moreover, except for LBR, all 5 proteins associated with the nucleocytoplasmic transport have been shown to interact with NS3/4A, which is the main player in the evasion of the innate immune response through the cleavage of important signaling adaptors, such as MAVS and TRIF in the RLRs and Toll-like receptors

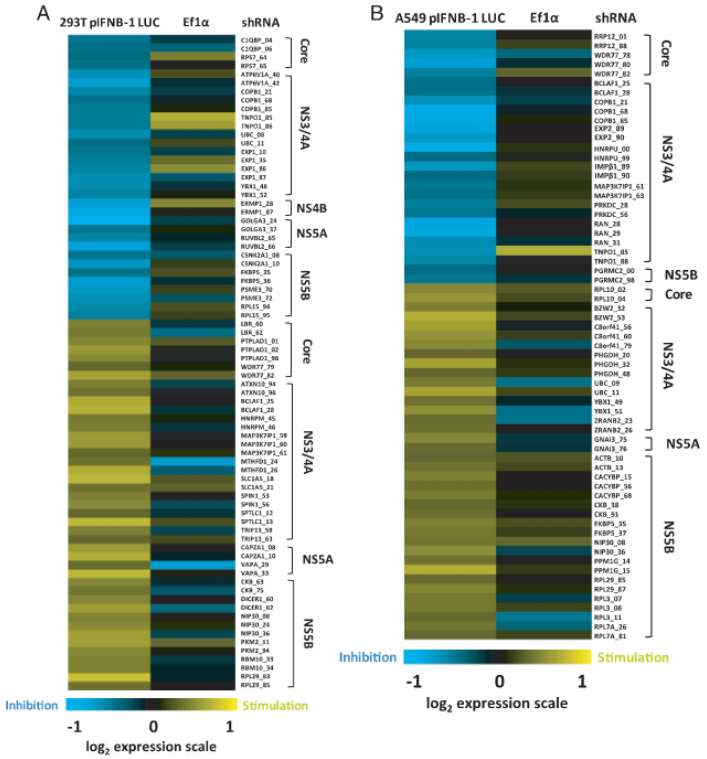

FIGURE 1 Modulation of virus-mediated interferon-β (IFNB1) production by gene silencing of host interactors of hepatitis C virus (HCV) proteins. Heat map visualization of the *IFNB1* promoter activity upon silencing expression of 53 HCV-host interactors in Sendai virus (SeV)-infected cells (log_2 scale). The screens are performed with Human Embryonic Kidney (HEK)293T (A) and A549 (B) cells stably expressing the firefly luciferase under the control of the *IFNB1* promoter and transduced with lentivirus-encoding short hairpin RNA (shRNA). Results were normalized according to cells treated with control shRNA non target (NT) (set to 1—black) based on an average of 2 independent experiments. The following criteria were applied to select modulator hits: at least 2 shRNAs per gene with >25% effect on *IFNB1* promoter activity without affecting the nonimmune elongation factor 1α (EF1α) promoter-driven luciferase activity. Hits are clustered by their corresponding HCV binding partners, and the last 2 digits correspond to the shRNA number of The RNAi Consortium (TRC).

TABLE 1 Gene ontology (GO) terms enrichment of HCV-host interactors affecting virus-mediated IFNB1 induction

GO term	Gene count	Genes	Fold enrichment	*P* value
Protein Import into Nucleus, Docking (BP)	4	EXP2. IMPβ1, TNPO1, EXP1	72	.00002
Nuclear Pore (CC)	6	EXP2, IMPβ1, LBR, RAN, TNPO1, EXP1	23	.000005
Translation Elongation (BP)	7	RPL10, RPL15, RPL29, RPL3, RPL7A, RPS7, UBC	21	.00000073
Protein Import into Nucleus (BP)	5	EXP2, IMPβ1, RAN, TNPO1, EXP1	18	.00016
Ribonucleoprotein Complex (CC)	11	ACTB, DICER1, RPL10, RPL15, RPL29, RPL3, RPL7A, RPS7, UBC, EXP1, YBX1	6.5	.0000038
RNA Processing (BP)	6	DICER, RBM10, RPS7, WDR77, YBX1, ZRANB2	3.4	.029
Nucleoplasm (CC)	8	ACTB, CSNK2A1, IMPβ1, PRKDC, RAN, RUVBL2, UBC, EXP1	2.8	.021
Cell Cycle (BP)	7	PHGDH, PPM1G, PSME3, RAN, SPIN1, TRIP13, UBC	2.8	.035
Membrane-enclosed Lumen (CC)	13	ACTB, C1QBP, CACYBP, CSNK2A1, IMPβ1, PRKDC, RAN, RPL3, RPS7, RRP12, RUVBL2, UBC, EXP1	2.1	.011
Nucleotide Binding (MF)	14	ACTB, ATP6V1A, CSNK2A1, CKB, DICER1, GNAI3, MTHFD1, PHGDH, PKM2, PRKDC, RAN RBM10, RUBVL2, TRIP13	1.9	.018

GO biological process (BP), molecular functions (MF) and cellular compartment (CC) terms significantly enriched ($P < .05$) are indicated with their respective list of genes and fold enrichment. The GO terms incorrectly list EXP1/XPO1 as being involved in protein import, when it is the main carrier for protein export.

(TLRs) pathways, respectively.[38–40] In addition, NS3/4A protein is known to interact with IMPβ1 to prevent STAT1 nuclear translocation in response to type I IFN-mediated JAK-STAT pathway activation in HeLa human cervical cancer cells.[1]

2.3 | Microscopy-based high content screening of nuclear transport of IRF3 and NF-κB p65 upon viral infection

The interaction of a set of nuclear transport factors with HCV proteins and their combined effects on increased viral replication and subversion of innate immune response encourage further mechanistic studies. Owing to their physiological function, we hypothesized that the observed effects on IFNB1 induction are associated with the nuclear translocation of IRF3 and NF-κB p65 in response to a phosphorylation event upon viral infection.[41,42] The nucleocytoplasmic shuttling of IRF3 and NF-κB have been reported to be mediated via IMP-α/β and EXP1-dependent pathways,[43–46] while a more recent study indicated that the import of NF-κB p65 mainly relies on the canonical IMPα1/IMPβ1 pathway following tumor necrosis factor-α (TNF-α) stimulation.[46] The Nups involved in these processes, however, are yet to be determined. To test our hypothesis, we developed a microscopy-based screen to quantify the translocation of IRF3 and NF-κB p65 to the nucleus in a kinetic analysis of SeV infection. Figure 2A details the RNAi microscopy screening methodology. Briefly, nucleocytoplasmic trafficking genes are silenced in A549 cells cultured in transparent 96-well plates by lentiviruses encoding shRNA for 4 days to allow for the efficient knockdown of the targeted gene. Five independent lentiviruses per gene (1 per well) are used at a multiplicity of infection (MOI) of 10. A control shRNA expressing a non-target sequence (shRNA NT) is included in each 96-well plate. Knockdown cells are then infected with SeV for 1, 3, 5, 8 and 10 hours, fixed, and permeabilized prior to nuclear labeling with Hoechst and IRF3 or NF-κB p65 immunostaining. Images are captured in 9 pre-determined fields per well, under an Operetta High Content Screening (HCS) microscope. Images are then processed using the Harmony software to delimitate the nuclear region and to measure the fluorescence intensity of IRF3 or NF-κB p65 within the nucleus. For each 96-well plate, a fluorescence cut-off is set to allow the automated discrimination of cells with (green) or without (red) IRF3 or NF-κB p65 nuclear staining (Figure 2A). This information allowed us to calculate the percentage of cells with IRF3 or NF-κB p65 nuclear staining, and to evaluate the effect of each shRNA on SeV infection-induced nuclear translocation of TFs.

A representative time-course experiment performed with control shRNA NT-transduced cells showed the nuclear translocation of both TFs over a 10-hour SeV infection (Figure 2B). Graphical representations of these results are plotted using the percentage of positive nuclear staining for IRF3 or NF-κB p65 (Figure 2C). Over the course of a 10-hour SeV infection, we observed an increase in both IRF3 and NF-κB p65 nuclear staining culminating to ~75% positive cells at 5 hours post-infection, followed by a decrease to ~30% at 10 hours. The microscopy data are confirmed biochemically with cell fractionation and western blot analysis (Figure 2D). As expected, we observed that IRF3 phosphorylation on Ser386 began at 1 hour, culminated at 5 hours and then decreased at 10 hours post-infection, consistent with the amount of total nuclear IRF3 observed at these time points. The phosphorylated forms of IRF3 could also be observed using the anti-IRF3 antibody in both cytoplasmic and nuclear fractions. Moreover, phosphorylation of the NF-κB negative regulator NFKBIA on Ser32—which triggers its ubiquitination and degradation—gradually increased from 1 to 10 hours post-infection. NFKBIA degradation then allowed for NF-κB p65 nuclear translocation, which began at 1 hour, culminated at 5 hours and subsequently decreased at 10 hours post-infection. Cytoplasmic and nuclear fraction purity was confirmed by exclusive histone 1 (H1) staining in the

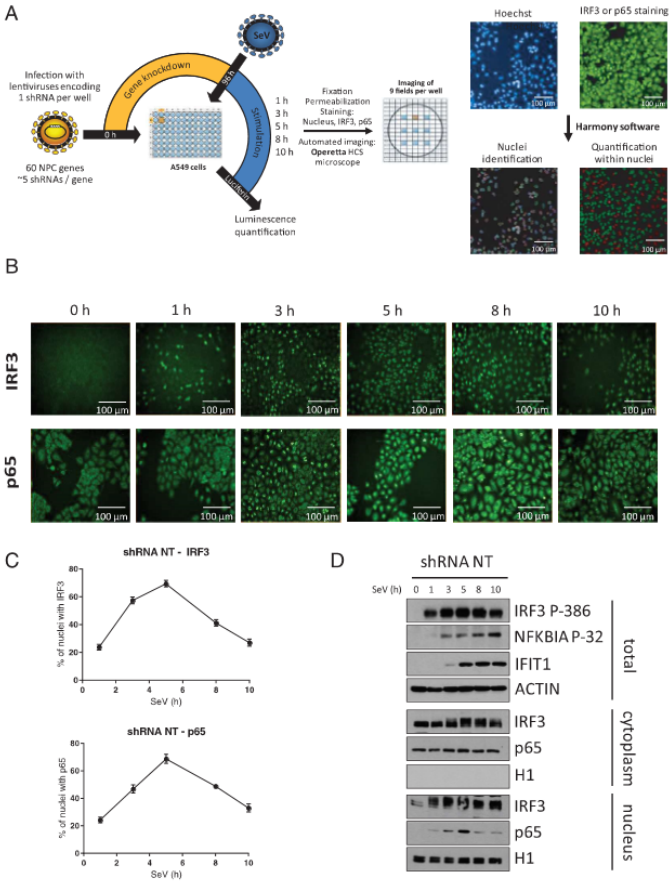

FIGURE 2 Microscopy-based High Content Screening (HCS) of IFN regulatory factor 3 (IRF3) and NF-κB p65 nuclear translocation. A, Overview of the microscopy-based gene silencing screen. A549 cells plated in 96-well plates are transduced with 5 independent lentivirus-encoding short hairpin RNA (shRNA) per gene (1 shRNA per well) at a multiplicity of infection (MOI) of 10 for 4 days to silence expression of 60 nuclear transport factors. A control shRNA NT is included in each 96-well plate. Cells are infected with Sendai virus (SeV) for 1, 3, 5, 8 or 10 hours before fixation, permeabilization, Hoechst nuclear labeling and antibody staining of IRF3 or NF-κB p65 with Alexa Fluor 488 (green). Images of cells are captured in 9 pre-determined fields for each well using an Operetta HCS Microscope. Images are processed using Harmony software to delimitate the nuclear region and measure the fluorescence intensity of IRF3 or NF-κB p65 within the nucleus. For each 96-well plate, a fluorescence cut-off is set to allow automated discrimination of cells with (green) or without (red) IRF3 or NF-κB p65 nuclear staining and to calculate the percentage of cells for each shRNA-mediated gene knockdown. Scale bar is equal to 100 µM. B, Representative time course imaging performed with the control shRNA NT showing the nuclear translocation of IRF3 or NF-κB p65 over a 10-hour Sendai virus (SeV) infection (1 representative of 9 field images). Scale bar is equal to 100 µM. C, Graphic representation of the microscopy image-based analysis showing an increase in the percentage of cells with positive nuclear staining for IRF3 or NF-κB p65 culminating with ~75% of positive cells at 5 hours post-infection followed by a decrease to ~30% of positive cells at 10 hours. D, Immunoblot analysis of total cell lysates, cytoplasmic and nuclear extracts of A549 cells infected with lentivirus-encoding shRNA NT at a MOI of 10 for 3 days and infected with SeV for 0, 1, 3, 5, 8 and 10 hours prior to cell harvesting

nucleus and constant cytoplasmic NF-κB p65 levels (coupled with the absence of NF-κB p65 in the nucleus in the absence of SeV infection). With respect to gene expression, IRF3 and NF-κB p65 nuclear translocation led to the induction of antiviral response genes, including ISG56 (IFIT1) protein, which are detectable starting 3 hours post-infection. Collectively, these biochemical observations correlated with the results of HCS imaging analysis, thus validating our approach.

Applying the screening methodology, we were able to directly measure the effect of silencing 60 proteins associated with the NPC, nuclear transporters and RAN system. Each independent shRNAs targeting a specific gene was compared with that of control cells transduced with lentivirus-expressing shRNA NT. To facilitate their visualization and analysis, results obtained with control shRNA NT were normalized to 0 for every time point, allowing the effect of independent shRNA to be represented as relative percentage of each TF in the nucleus of viable cells. The shRNA screen revealed 33 proteins for which knockdown significantly affected IRF3 or NF-κB p65 nuclear translocation with at least 2 independent shRNAs following SeV infection (Table 2). Notably, knockdown led to decreased translocation for 25 proteins, delayed translocation for 4 proteins (IMPα4, NUP43, NUPL2 and TNPO2), increased translocation for 2 proteins

TABLE 2 Identification and functional classification of nuclear transport genes affecting IFNB1 production

Protein	Role		IRF3	p65
IMP-α adaptors				
IMPα1	Dual	Negative	−20	−30
IMPα3	Dual	Positive	30	10
IMPα4	Dual	Negative	−20	−30
IMPα5	p65	Negative	0	−30
IMPα6	Dual	Negative	−20	−40
IMPα6[1]	IRF3	Positive	30	0
IMPα7	p65	Negative	0	−20
IMP-β import receptors				
IMPβ1	Dual	Negative	−40	−35
IPO4	Dual	Negative	−10	−10
IPO7	Dual	Negative	−15	−15
IPO8	Dual	Negative	−15	−35
TNPO1	Dual	Negative	−40	−30
TNPO2	Dual	Negative	−5	−5
IPO12	Dual	Negative	−20	−20
IMP-β export receptors				
EXP1	Dual	Positive	30	20
EXP2	Dual	Negative	−40	−40
RANBP3	p65	Negative	0	−25
RANBP3[1]	IFR3	Positive	20	0
mRNA export				
NXT1	Dual	Negative	−25	−15
NXT2	Dual	Negative	−10	−20
NXF1	Dual	Negative	−30	−40
NXF2	Dual	Negative	−25	−25
RAN gradient				
RAN	Dual	Negative	−40	−30
NUTF2	Dual	Negative	−25	−30
RCC1	Dual	Negative	−30	−30
FG-Nups				
RANBP2	Dual	Negative	−25	−25
NUP214	Dual	Negative	−25	−25
NUPL2	Dual	Negative	−20	−15
Outer-ring Nups				
NUP160	Dual	Negative	−35	−30
NUP107	Dual	Negative	−25	−25
NUP43	Dual	Negative	−20	−30
Linker Nups				
NUP93	Dual	Negative	−15	−25
NUP88	Dual	Negative	−20	−25
Central FG-Nups				
NUP54	Dual	Negative	−20	−30
NUP35	Dual	Negative	−10	−20
NUPL1	Dual	Negative	−25	−25

[1] At 8 h post-infection.

(IMPα3 and EXP1), and to differential effects on IRF3 and NF-κB p65 translocation for 2 proteins (IMPα6 and RANBP3). We further divided these 33 proteins into subgroups based on their function and localization, respectively.

2.4 | Analysis of IMP-β NTRs and Nups knockdown on virus-mediated IRF3/NF-κB p65 nuclear translocation and IFNB1 production

The analysis revealed that the silencing of IMP-α proteins (KPNA1-6) led to a distinct phenotype for 5 of the 6 adaptors, with a major decrease in NF-κB p65 nuclear translocation at 3 hours post-SeV infection, the only exception being with IMPα3 (KPNA4) knockdown cells (Figures 3 and S3). Notably, the effect of these adaptors on IRF3 was more variable—but IMPα3 also increased IRF3 translocation; whereas IMPα6 (KPNA5) knockdown resulted in delayed IRF3 and diminished NF-κB p65 nuclear translocation. Alternatively, functionally grouped NTRs mostly attenuated nuclear translocation (Figures 4 and S4), which are expected of IMP-β nuclear import receptors; however, TNPO2 knockdown showed delayed translocation for both TFs, and differed from TNPO1's knockdown phenotype (Figures 4B and S5). Moreover, knockdown of the main export carrier EXP1 increased nuclear translocation of TFs, in contrast to EXP2 (CSE1L), with transient and sustained kinetics for IRF3 and NF-κB p65, respectively (Figures 4C and S6). Interestingly, IMPα6 and RANBP3 knockdown both caused a delay in IRF3 translocation and a transient decrease in NF-κB p65, suggesting that RANBP3 utilizes IMPα6 as an adaptor (Figures 4C and S6). Figure 5 displayed a decrease of IRF3 and NF-κB p65 nuclear translocation for the knockdown of most Nups that are categorized by their localization within the NPC, with the exception of NUPL2 (Figures 5A and S9) and NUP43 (Figures 5B and S10). Specifically, NUPL2 knockdown cells increased IRF3 translocation at very early stages of infection, and correlated with EXP1 knockdown effect, which is expected as NUPL2 promotes EXP1 function.[47] Furthermore, NUP43 knockdown cells showed a similar nuclear translocation phenotype for both factors, as seen with IMPα6 and RANBP3 knockdown, suggesting that NUP43 plays a functionally significant role in RANBP3-IMPα6 import complex through the NPC.

In parallel to the microscopy-based assay, the effect of silencing each protein was also determined on IFNB1 promoter activity following SeV infection. Our results showed that the majority of nuclear transport factors that hindered IRF3 and NF-κB p65 nuclear translocation decreased IFNB1 promoter-driven reporter activity at 6 hours post-SeV infection, including EXP1 and IMPα3 knockdown that both increased IRF3 and NF-κB p65 nuclear staining. Results of the IFNB1 reporter assay are presented in histograms alongside nuclear translocation curves in Figures S3-S12 (complete data can be found in Table S4). The histograms also show the effect of each protein knockdown on cellular proliferation and survival, as determined by relative number of nuclei per independent shRNA and compared with the control shRNA NT within the same 96-well plate. This measure allowed us to better discriminate between non-specific effects of shRNA expression on cellular fitness versus an actual function in innate antiviral immunity. More importantly, our data demonstrate that nuclear transport factors interacting with HCV NS3/4A protein modulate IRF3 and NF-κB p65 signaling of IFNB1 antiviral response.

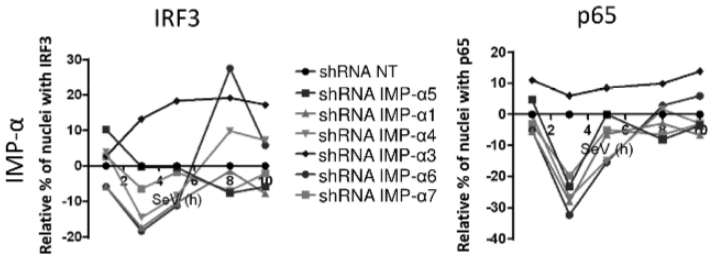

FIGURE 3 Effect of silencing importin (IMP)-α adaptors on IFN regulatory factor 3 (IRF3) and NF-κB p65 nuclear translocation. IMP-α adaptors are silenced in Sendai virus (SeV)-infected cells. Relative percentage of cells containing IRF3 (left) and NF-κB p65 (right) in the nucleus after normalization of the control short hairpin RNA (shRNA) NT to 0 for all time points. Results are presented as average of all shRNAs for each IMP-α. Individual shRNA results on IRF3 and NF-κB p65 nuclear translocation, *IFNB1* promoter activity and cell proliferation and survival are described in Figure S3

2.5 | HCV NS3/4A-mediated cleavage of IMPβ1 inhibits nuclear transport of IRF3 and NF-κB p65 to evade IFNB1 production

Because knockdown of the NS3/4A-interacting nuclear transport factors all negatively affected IFNB1 reporter activity, we wanted to assess their independent effects on viral replication. For this, A549 knockdown cells for IMPβ1, TNPO1, EXP1, EXP2 and RAN were infected with SeV for 8 and 24 hours (Figure 6). Notably, IMPβ1 knockdown led to a drastic and rapid increase in SeV protein levels when compared with those of control cells transduced with shRNA NT. This is further accompanied by nearly a complete inhibition of the antiviral response at 8 hours as measured by IFIT1 (ISG56) protein induction. Similar data are observed with HEK293T (Figure S13). These results suggest that IMPβ1 is targeted by HCV NS3/4A protein to disrupt IFNB1 production, reminiscent to a similar mechanism employed by HCV to prevent the nuclear translocation of STAT1 and type I IFN signaling.[1] Using 3 independent shRNAs specific to IMPβ1, we confirmed that its depletion significantly hindered IFNB1 induction of SeV-infected cells correlating with the reduced nuclear translocation of IRF3 and NF-κB p65 when compared with the shRNA NT (Figure 7A). Indeed, we showed that IMPβ1 knockdown led to a strong decrease in IRF3 nuclear translocation at 3 and 5 hours post-infection, before returning to normal levels at 8 and 10 hours post-infection. A similar pattern was observed for NF-κB p65 nuclear translocation, although with a less drastic decrease at 3 and 5 hours post-infection, and culminated with increased nuclear NF-κB p65 staining at 8 and 10 hours post-infection compared with that in presence of shRNA NT. In addition, we used caspase-mediated poly [ADP-ribose] polymerase 1 (PARP1) cleavage—that generates 89- and 24-kDa fragments—to assess virus-mediated apoptosis. Correspondingly, we observed that the rapid increase in SeV protein levels of IMPβ1-depleted A549 cells was accompanied by an accelerated induction of apoptosis that started 1 hour post-infection instead of 3 hours post-infection in control shRNA NT expressing cells (Figure 7B).

Because all proteins associated with nuclear transport interact with NS3/4A, we examined its interaction with the critically important IMPβ1 more carefully. Co-immunoprecipitation experiments were run to validate the NS3/4A-IMPβ1 interaction (Figure 7C). BILN 2061, a potent NS3/4A-specific protease inhibitor (PI), was used as an additional control.[48] Interestingly, aside from the expected interaction, western blot analysis revealed the recognition of an additional IMPβ1 fragment only with protease active NS3/4A and in the absence of BILN 2061 PI. An amino acid sequence within IMPβ1 C-terminus was found to be concordant with the cleavage consensus of NS3/4A protease. The resulting sizes of predicted fragments agreed with the molecular weights of bands observed in western blot analysis: 97 kDa for full-length IMPβ1 vs 89 kDa for cleaved IMPβ1 (Figure 7C). The cleavage at this site could rationalize the NS3/4A interaction-mediated prevention of IRF3/NF-κB p65 as well as STAT1 nuclear translocation.[1,49] To assess a role of NS3/4A-mediated cleavage of IMPβ1 in subverting IRF3/NF-κB p65 nuclear translocation, we investigated the IFNB1 promoter-driven luciferase upon SeV infection in cells that expressed a NS3/4A protease cleavage-resistant MAVS variant, mutated at Cys-508 for an arginine ($MAVS^{C508R}$),[50] and MAVS wild-type (WT) as control. We engineered MAVS KO cells using GeneArt gRNA, Platinum Cas9 nuclease and Lipofectamine CRISPRMAX Cas9 Transfection Reagent from Thermo Fisher Scientific[51] to reduce competition between endogenous and ectopically expressed MAVS proteins. We first demonstrated that expression of NS3/4A completely blocks SeV-mediated IFNB1 production of MAVS KO cells reconstituted with the WT protein, which is completely restored by BILN 2061 PI (Figure 8A). More importantly, NS3/4A is still able to inhibit IFNB1 of SeV-infected cells that are reconstituted with the $MAVS^{C508R}$ variant, blocking up to 70% of the IFNB1-driven luciferase induced by control cells in the absence of active NS3/4A protease. Furthermore, BILN2061 PI restores IFNB1 and IFIT1 induction, correlating with the disappearance of IMPβ1cleavage (Figure 8A,B). To confirm this, we constructed an IMPβ1 variant that is mutated at multiple residues within the cleavage consensus of NS3/4A protease (D812A, C817S and A818G), including Cys-817 as the major determinant to produce a cleavage resistant IMPβ1 ($IMPβ1^{CR}$). The expression of $IMPβ1^{CR}$ but not of IMPβ1 WT completely restores IFNB1 production of $MAVS^{C508R}$-reconstituted cells expressing NS3/4A protease upon infection, similar to the treatment with BILN 2061 PI (Figure 8C). The IMPβ1-dependent nuclear transport of IRF3 was also investigated by immunofluorescence microscopy in MAVS KO cells reconstituted with the $MAVS^{C508R}$ variant and kinetic analysis of SeV infection

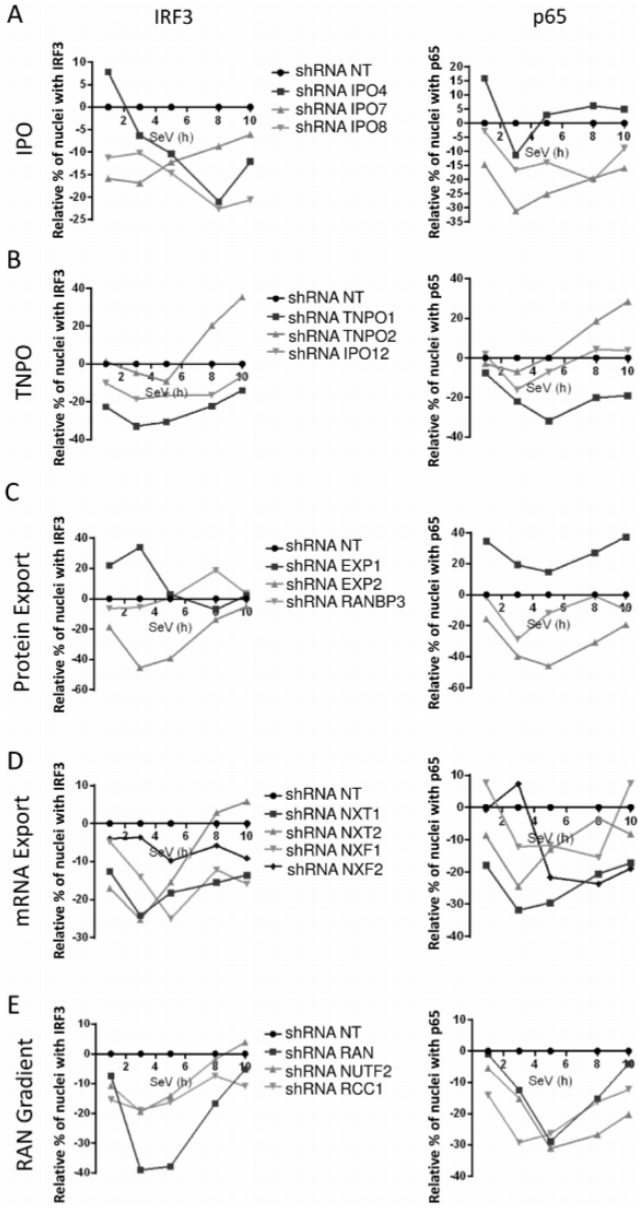

FIGURE 4 Effect of silencing importin-β (IMP-β) nucleocytoplasmic transport receptor (NTR) and RAN components on IFN regulatory factor 3 (IRF3) and NF-κB p65 nuclear translocation. Importins (IPO4, IPO7, IPO8), transportins (TNPO1/IMPβ2, TNPO2/IPO3, TNPO3/IPO12), as well as proteins involved in protein export (EXP1/XPO1, EXP2/CSE1L, RANBP3), mRNA export (NXT1, NXT2, NXF1, NXF2) and RAN gradient (RAN, NUTF2, RCC1) are silenced in Sendai virus (SeV)-infected cells. Results are presented as the average of all short hairpin RNAs (shRNAs) in relative percentage of cells containing IRF3 and NF-κB p65 in the nucleus after normalization of the control shRNA NT to 0 for all time points. Individual shRNA results on IRF3 and NF-κB p65 nuclear translocation, *IFNB1* promoter activity and cell proliferation and survival are described in Figures S4-S8

(Figure 8D). NS3/4A protease expression greatly hindered IRF3 and IMPβ1 from being transported to the nucleus with a major reduction at 4 hour post-infection. Remarkably, complete restoration of IRF3 nuclear positive cells are observed upon expression of IMPβ1^{CR}, similar to the treatment with BILN 2061 PI (Figure 8E,F). Altogether, the data demonstrate that NS3/4A protease-mediated cleavage of IMPβ1 inhibits innate immune response to viral infection by restricting nuclear translocation of IRF3 and NF-κB.

3 | DISCUSSION

The goal of the study was to elucidate novel proteins, families or processes that affected innate immune response from a subset of human proteins previously elucidated to be interacting with HCV proteins. We found that majority of host interactors did not affect HCV replication,[1] although their knockdown was performed in the Huh7.5 cell line that is deficient for the RLR signaling pathway. Thus, we

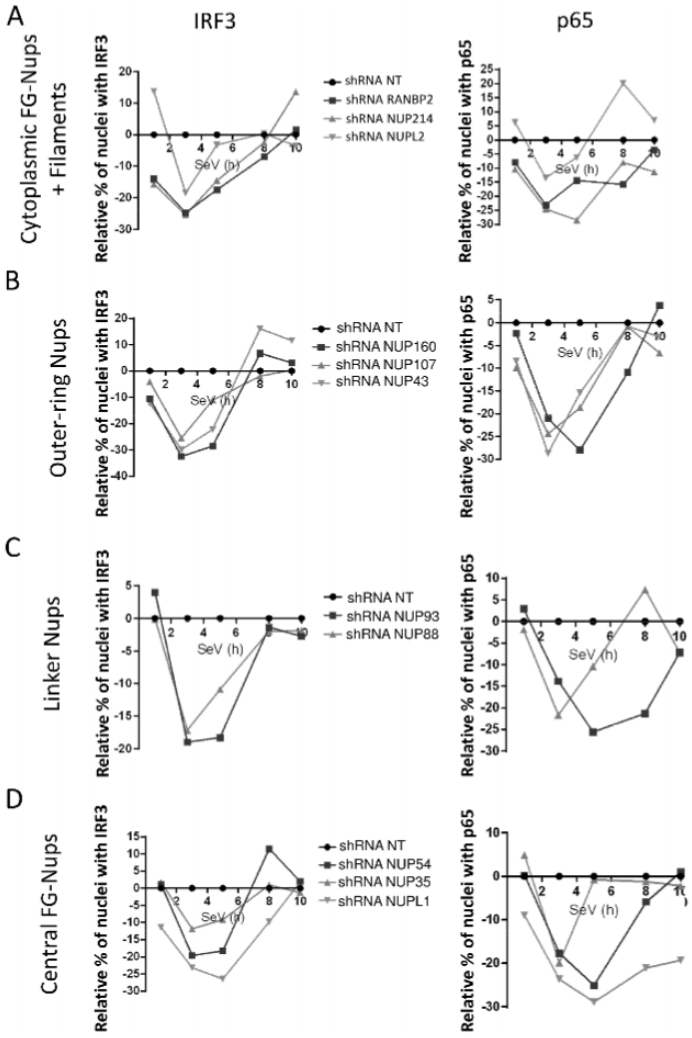

FIGURE 5 Effect of silencing nucleoporins (Nups) on IFN regulatory factor 3 (IRF3) and NF-κB p65 nuclear translocation. Cytoplasmic FG-Nups and filaments (RANBP2, NUP214, NUPL2), outer-ring Nups (NUP43, NUP107, NUP160), linker Nups (NUP93, NUP88) and central FG-Nups (NUP54, NUP35, NUPL1) are silenced in Sendai virus (SeV)-infected cells. Results are presented as the average of all short hairpin RNAs (shRNAs) in relative percentage of cells containing IRF3 and NF-κB p65 in the nucleus after normalization of the control shRNA NT to 0 for all time points. Individual shRNA results on IRF3 and NF-κB p65 nuclear translocation, *IFNB1* promoter activity and cell proliferation and survival are described in Figures S9-S12

postulated that these host interactors may counteract viral replication by modulating antiviral response thereby providing a plausible explanation for their targeting by viral proteins. To test this hypothesis, we used a SeV model of infection and RIG-I competent cells, and found that silencing expression of 53 of 132 interactors had a significant effect on IFNB1 production (Figure 1). Moreover, depletion of 12 of these 53 interactors was previously reported to reduce HCV replication in RLR-deficient Huh7.5 cells (Figure S2), providing strong evidence that viruses through host interactions can hijack cellular processes involved in the modulation of innate immune response and additionally contributing to the virus replication life cycle giving a dual growth advantage. The GO term analysis of the 53 interactors revealed enrichment in nuclear protein import and export processes (Table 1). Interestingly, the interactors associated to the nucleocytoplasmic trafficking of proteins (IMPβ1, TNPO1, EXP1, EXP2 and RAN) exhibit 2 main characteristics—a potential to interact with NS3/4A (Figure S1), and a functional relevance to SeV-induced *IFNB1* promoter inhibition (Figure 1). Correspondingly, IMPβ1, TNPO1 and RAN decrease HCV replication upon their depletion in the Huh7.5 cell line,[1] confirming the targeting of key players of the nuclear transport cell process to concurrently evade innate immune response and to promote virus replication. To gain mechanistic insight of the modulation of IFNB1 antiviral response by these interactors, we developed a microscopy-based high-content screening assay that directly evaluates the nuclear translocation of IRF3 and NF-κB p65 over a 10-hour viral infection time course. The assay was validated biochemically using nuclear/cytoplasmic fractionation (Figure 2D), and supported by the kinetics of phosphorylation of IRF3 and of the NF-κB inhibitor NFKBIA upon virus-mediated induction of antiviral response. The combined high-content microscopy and medium throughput gene

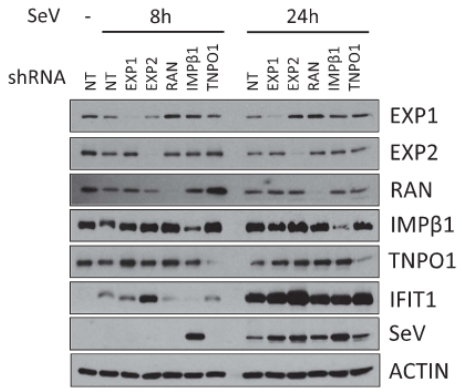

FIGURE 6 Effect of silencing NS3/4A-interacting nuclear transport factors on Sendai virus (SeV) infection and ISG56 (IFIT1) induction. Immunoblot analysis of A549 cells infected with SeV for 8 or 24 hours following the silencing of EXP1/XPO1, EXP2/CSE1L, RAN, importin β1 (IMPβ1)/KPNB1 and TNPO1/IMPβ2 for 3 days. Short hairpin RNA (shRNA) NT is used as a control

silencing screen approach was used to measure the dynamics and phenotype of IRF3 and NF-κB p65 nuclear translocation as a valuable readout of the early antiviral response. We expanded the silencing screen to study genes involved in the NPC from the different sections of the pore (Figures 5, S9 to S12) as well as those involved in protein import, protein and mRNA export and RAN energy gradient (Figures 3, 4, S3 to S8). The screen provides a comprehensive understanding of the nuclear trafficking of IRF3 and NF-κB p65 at different early time points of a viral infection. Moreover, the study has linked 33 nuclear transport factors that modulate the nuclear translocation of IRF3 and NF-κB p65 as cargo proteins to the induction of an antiviral cellular phenotype.

Several NTRs were previously associated to the nuclear trafficking of NF-κB p65 while there are virtually no reports for IRF3 during viral infection. In our study, the depletion of IMP-α family members had varying effects on IRF3/NF-κB p65 nuclear localization but IFNB1 production was significantly decreased when IMPα1, IMPα4 and IMPα6 are individually silenced (Figures 3 and S3), which could be attributed to these 3 genes decreasing both IRF3 and NF-κB p65 nuclear translocation at 3 hours post-infection. NF-κB p65 was reported to be transported by IMPα3 and IMPα4 while a more recent study identified IMPα1 as the most critical adaptor for its nuclear translocation upon tumor necrosis factor-α (TNF-α) treatment.[43,44,46] Our results largely support these studies with a predominant role of IMPα1 and IMPα4, except for the depletion of IMPα3 that may be over-compensating by other adaptors causing the increase of NF-κB p65 during viral infection. In addition, the need for fast activation upon viral infection must not require a process that is dependent on IMPα3 as its knockdown has no impact on IFNB1 production. Our data also demonstrate that IMPβ1 is one of the main import receptor for IRF3 and NF-κB p65 import upon viral infection (Figure 7), as previously reported for NF-κB p65.[46] However, other carriers have been identified for NF-κB p65 import including IPO8,[46] which transports this factor in a NLS-independent fashion after stimulation TNF-α. In our study, knockdown of IPO8 resulted in a decrease IRF3 and NF-κB p65 nuclear translocation during the entire time course of infection and reduction of IFNB1 production (Figures 4A and S4), suggesting that an NLS-independent import process for IRF3 and NF-κB p65 may take place during viral infection. We also identified the import carriers TNPO1 and IPO7 as their knockdown reduces nuclear translocation of both TFs (Figures 4A,B, S4, S5), which supports the finding of a PY-NLS that is recognized by TNPO1.[52] Finally, knockdown of IPO4, TNPO2 and IPO12 affect irregularly or at later time NF-κB p65 and IRF3 nuclear translocation (Figures 4, S4 and S5), suggesting a weak if any contribution to these processes in a virus-dependent manner. IMP-β family NTRs involved in protein export have significant effects for IRF3 and NF-κB p65 nuclear translocation and IFNB1 production, but also have pleiotropic effects on cell survival. Nevertheless, EXP1 was previously shown to bind IRF3 NES, and the use of EXP1 inhibitor leptomycin B further demonstrated an accumulation of IRF3 in the nucleus.[45] Our results support EXP1 as the main carrier for IRF3 export causing nuclear accumulation of IRF3 during the early phase of the infection upon it knockdown (Figures 4 and S6). This is further supported by RANBP3 knockdown, an EXP1 cofactor for protein export,[53,54] which increases nuclear translocation of IRF3 at 8 hours of SeV infection but surprisingly without affecting IFNB1 production (Figure S6). Among all EXP family members, only EXP1 systematically exported NF-κB p65 at all time confirming a previous study.[46] Despite the increased nuclear levels of IRF3 and NF-κB p65, EXP1 knockdown decreased *IFNB1* promoter activity following SeV infection (Figure S6). This may be explained by an effect on cell survival and/or by the fact that residual NF-κB p65 can re-associate with IκBα to reduce it transcriptional activity when sequestered in the nucleus.[55,56] Interestingly, EXP2 knockdown causes a dramatic decrease of nuclear IRF3 and NF-κB p65 during the entirety of the SeV infection time course leading to a significant reduction of IFNB1 and cell fitness (Figure S6). This is possibly due to its major role for the export of IMP-α adaptors to the cytoplasm as a recycling mechanism of import complexes.[57] We also identified NXF1 and NXT1 proteins that significantly reduced nuclear import of IRF3/NF-κB p65 and IFNB1 production (Figures 4D and S7). These proteins are exploited by several viruses to promote viral mRNA export and inhibit host mRNA trafficking.[20] Further studies will be required to demonstrate if the diminished amounts of IRF3 and NF-κB p65 proteins translated and able to enter the nucleus are due to the reduced export of their mRNAs to prevent antiviral response. The silencing of RAN, NUTF2 and RCC1 equally affected IRF3 and NF-κB p65 nuclear translocation and IFNB1 production, while only RAN silencing affected cells survival (Figures 4E and S8). As NUTF2 is responsible for recycling RANGDP back to the nucleus and RCC1 for exchanging the GDP to GTP, these data confirm that the lack of RANGDP or RANGTP in the nucleus affect nuclear transport with IRF3 being less affected than NF-κB p65. Nevertheless, the silencing of RAN components NUTF2 and RCC1 decreasing IFNB1 production without affecting cell fitness (Figure S8) further corroborate that this process is critical for ensuring proper nucleocytoplasmic transport required for antiviral response. Finally, multiple Nups had a similar silencing phenotype with the reduced nuclear translocation of IRF3 and NF-κB

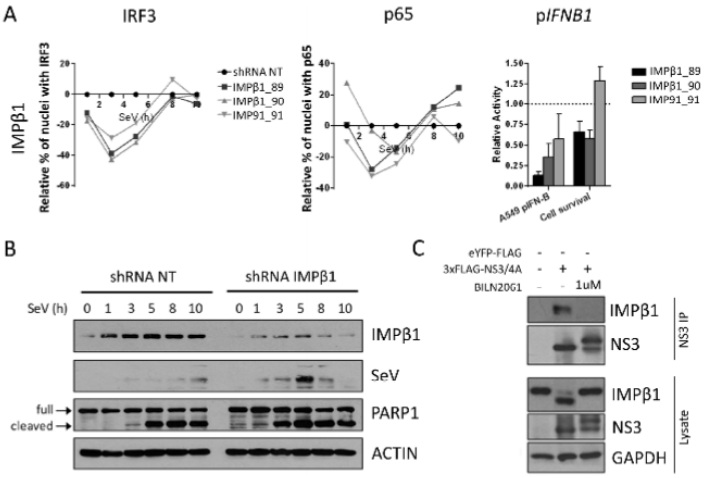

FIGURE 7 Importin β1 (IMPβ1) silencing impairs IFN regulatory factor 3 (IRF3) and NF-κB p65 nuclear translocation, *IFNB1* induction, and increases Sendai virus (SeV) protein expression and cell apoptosis. A, A total of 3 independent short hairpin RNA (shRNA) targeting IMPβ1 significantly affected nuclear translocation of both IRF3 (left panel) and NF-κB p65 (middle panel) when compared with the shRNA NT. Relative percentage of cells containing IRF3 and p65 in the nucleus are illustrated after normalization of the control shRNA NT to 0 for all time points. The effect of shRNA-mediated knockdown on SeV-induced *IFNB1* production is measured in A549 cells stably expressing the firefly luciferase under the control of the *IFNB1* promoter (right panel). In addition, the effects of each shRNA on cell proliferation and survival are evaluated using images from the microscopy screen by dividing the total number of nuclei for a given shRNA and dividing it by the total number of nuclei for the shRNA NT control (right panel). B, Immunoblot analysis of A549 cells infected with SeV for 1, 3, 5, 8 or 10 hours following transduction with shRNA NT (control) or shRNA 89 targeting IMPβ1 for 3 days. PARP1 cleavage (arrows) is used as apoptosis readout. C, Human Embryonic Kidney (HEK)293T cells are transfected with 3×FLAG-NS3/4A expression vector and treated with 1 μM of BILN 2061 PI. At 48 hours post-transfection, cells are harvested and co-immunoprecipitation using anti-FLAG coated beads is performed on cell lysates. NS3 and IMPβ1 interaction is resolved using immunoblot. NS3, NS3/4A precursor, IMPβ1 and cleaved IMPβ1 are resolved using immunoblotting analysis of cell lysates

p65 at early time of SeV infection (Figure 5). The silencing of cytoplasmic FG-Nups (RANBP2 and NUP214) and of outer-ring Nups (NUP107-160 complex) regulating the diameter of NPCs[58] further correlated with a decrease of IFNB1 production (Figures 5A,B, S9 and S10). The linker Nups, which are extremely important for the proper assembly of the NPC and recruitment of NUP62 for transport competency, also contribute to IRF3/NF-κB p65 nuclear transport (Figures 5C and S11). Indeed, NUP93 and NUP88 knockdown decrease the translocation of both TFs and IFNB1 production as previously reported.[37,59] At last, central FG-Nups NUP54 and NUPL1 (encoding NUP58 and NUP45) also significantly reduce IFNB1 production, in contrast to the weak phenotype of NUP35 (Figures 5D and S12).

Very importantly, our data revealed a unique phenotype of IMPβ1 knockdown by the complete inhibition of IFIT1 induction at early time point post-infection, correlating a rapid and drastic increased of SeV protein levels (Figures 6 and S13). Mechanistically, inhibition of the nuclear translocation of both TFs explains the strong reduction of antiviral response in IMPβ1-depleted cells. Interestingly, their nuclear localization are inhibited at early time points but return to normal levels despite the silencing of IMPβ1 (Figure 7A), demonstrating that the transient reduction of IRF3 and NF-κB p65 in the nucleus is sufficient to delay IFNB1 response to foster viral replication and cell apoptosis (Figure 7B). Remarkably, HCV NS3/4A protein interaction with IMPβ1 can counteract IFNB1 production by disrupting IRF3 and NF-κB p65 nuclear transport upon induction of a cellular antiviral state. We previously reported that the silencing of IMPβ1 phenocopied HCV infection in preventing STAT1 from accumulating in the nucleus, thus providing strong evidence that the interaction between NS3/4A and IMPβ1 mediates the blocking of STAT1 nuclear accumulation to promote HCV replication.[1] We now provide evidence that IMPβ1 targeting by NS3/4A can hinder nuclear translocation of IRF3 and NF-κB p65. We demonstrated a cleavage of IMPβ1 in cells expressing NS3/4A protein and inhibition of this cleavage by treatment with BILN2061 PI. An amino acid sequence within IMPβ1 C-terminus was found to be concordant with the cleavage consensus of NS3/4A protease, and the resulting sizes of predicted fragments agreed with the molecular weights of bands observed in western blot analysis (Figure 7D). More interestingly, the cleavage at this putative site (cysteine 817) occurs within HEAT repeat 18, which is required for interaction with adaptor proteins of the IMP-α family through an IMP-β binding (IBB) domain.[31,60] This could therefore rationalize the NS3/4A-dependent prevention of IRF3/NF-κB p65 as well as STAT1 nuclear translocation.[1,49] By using MAVS KO cells reconstituted with a $MAVS^{C508R}$ variant to prevent interference of NS3/4A with early MAVS signaling, we confirm the NS3/4A-

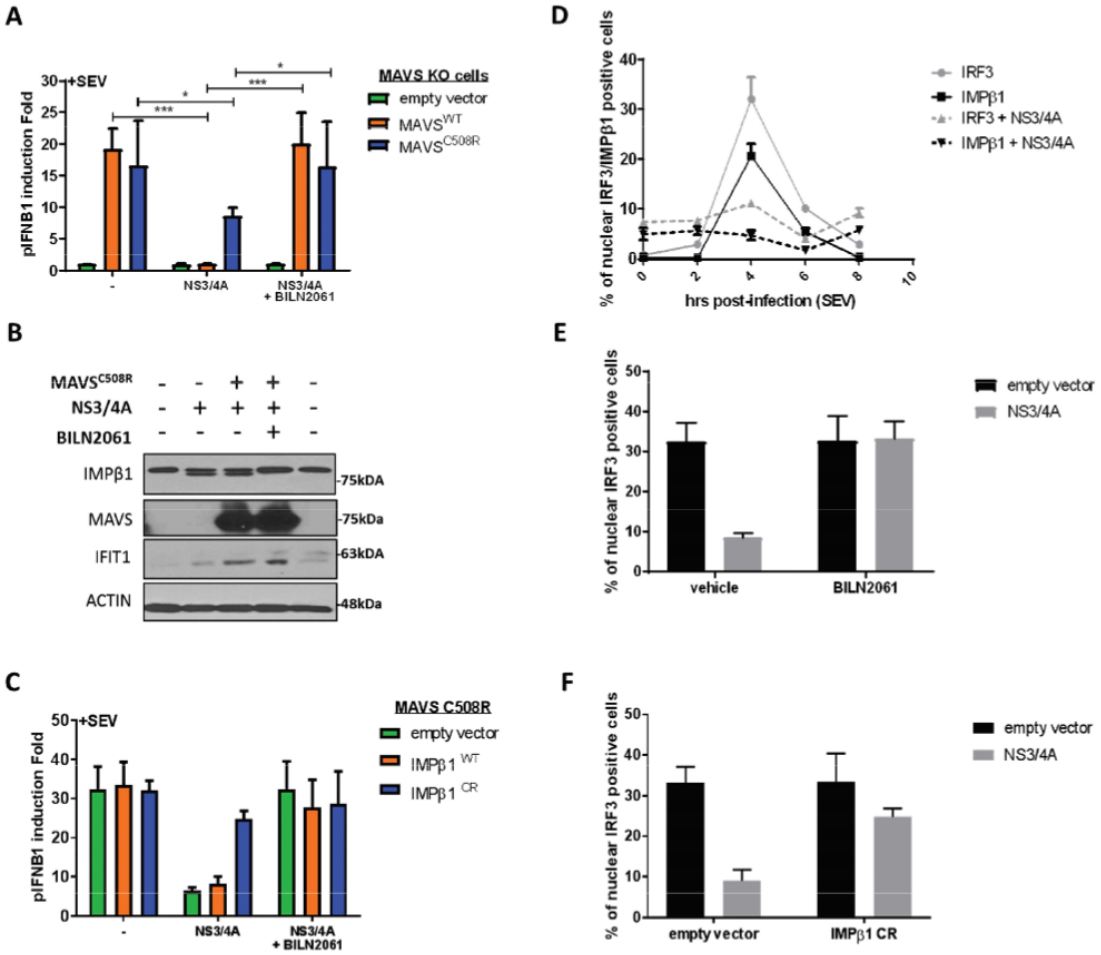

FIGURE 8 NS3/4A-mediated cleavage of importin β1 (IMPβ1) and interferon-β (IFNB1) inhibition are completely restored by expression of NS3/4A cleavage-resistant IMPβ1 variant (IMPβ1^{CR}) and treatment with BILN 2061 PI. A, Human Embryonic Kidney (HEK)293T MAVS knockout (KO) cells were transfected with an empty vector or an NS3/4A, MAVS^{WT}, cleavage resistant MAVS^{C508R} and pIFNB1-LUC expression plasmids, as indicated, for a total of 48 hours. BILN2061 was used as a positive control to inhibit protease activity of NS3/4A protein. At 32 hours post-transfection, cells were infected with Sendai virus (SeV) for 16 hours and luciferase signal was resolved. Average pIFNB1 induction folds are from 6 biological replicates. Data were analyzed using 1-way ANOVA and significance using Bonferroni post hoc test. B, HEK293T MAVS KO cells were transfected with cleavage resistant MAVS^{C508R}, an empty vector or NS3/4A, as indicated, for a total of 48 hours. BILN2061 was used as a positive control to inhibit protease activity of NS3/4A protein. At 32 hours post-transfection, cells were infected with SeV for 16 hours and were harvested for western blot analysis. Immunoblots of IMPβ1, MAVS, IFIT1 and Actin are presented to complement luciferase results from (A). C, HEK293T MAVS KO cells reconstituted with cleavage resistant MAVS^{C508R} were transfected with an empty vector or NS3/4A, IMPβ1^{WT}or cleavage resistant IMPβ1^{CR} and pIFNB1-LUC expression plasmids, as indicated, for a total of 48 hours. BILN2061 PI was used to inhibit protease activity of NS3/4A protein. At 32 hours post-transfection, cells were infected with SeV for 16 hours and luciferase signal was resolved. Average pIFNB1 induction folds are from 6 biological replicates. Data were analyzed using 1-way ANOVA and significance using Bonferroni post hoc test. D, A549 cells were transfected with MAVS^{C508R}, an empty vector or NS3/4A, for a total of 48 hours, as indicated. At 40 hours post-transfection, cells were either uninfected (time 0) or infected with SeV for 2, 4, 6 and 8 hours. Cells were fixed, permeabilized and stained for IRF3 and IMPβ1, and nuclear positive cells were resolved using immunofluorescence. E, A549 cells were transfected with MAVS^{C508R}, an empty vector or NS3/4A, for a total of 48 hours, as indicated. Concurrently, cells are treated with DMSO (vehicle) or BILN2061 PI. At 44 hours post-transfection, cells were infected with SeV for 4 hours. Cells were fixed, permeabilized and stained for IRF3, and nuclear positive cells were resolved using immunofluorescence. F, A549 cells were transfected with MAVS^{C508R}, an empty vector or cleavage resistant IMPβ1^{CR} for a total of 48 hours, as indicated. At 44 hours post-transfection, cells were infected with SeV for 4 hours. Cells were fixed, permeabilized and stained for IRF3, and nuclear positive cells were resolved using immunofluorescence.

mediated cleavage of IMPβ1 as a novel HCV strategy to evade IFNB1 production (Figure 8). Indeed, expression of IMPβ1^{CR} completely restores SeV-mediated IFNB1 production, similar to treatment with BILN 2061 PI, correlating the nuclear translocation of IRF3 and the disappearance of IMPβ1 cleavage. Interestingly, the truncated IMPβ1 protein still interact with NS3/4A protein (Figure 7C), which may promote viral replication by its relocalization to the membranous webs. Future studies are required to validate the phenotype of IRF3/NF-κB p65 localization seen in NS3/4A-expressing cells in the context of HCV infection, and the contribution of the cleaved N-terminal IMPβ1 fragment (aa1-817) at Nups-containing cytoplasmic compartments to promote viral RNA replication. Nevertheless, such viral strategy for evading IRF3/NF-κB signaling of IFNB1 production while also disrupting STAT1-mediated IFN signaling might be more common than previously believed as predicted for several viruses: SARS-CoV ORF6 targets IMPβ1 to block STAT1 nuclear import[25]; EMCV Leader protein binds RAN and completely disrupts nucleocytoplasmic transport[8]; FMDV 3C protease induces IMPα5 degradation to block STAT1/2 nuclear import[61]; Nipah virus W protein interacts with IMPα3 and IMPα4 to inhibit the activation of the IRF3-responsive promoter[17]; and Ebola virus VP24 protein interacts with IMPα6, blocking STAT1 nuclear import and inhibiting ISG production.[18,19] In this context, our work does not only allow insight into this complex nucleocytoplasmic transport machinery by elucidating components involved in the nuclear translocation of IRF3 and NF-κB but also identifies host proteins targeted by multiple viruses to evade immune response as potential therapeutic targets to treat a broad range of viral infections.

In summary, this study led to the identification of cellular interaction partners of HCV that are hijacked to evade innate immune response and are associated to nucleocytoplasmic transport pathways. Using a microscopy-based RNAi screen, we identified 33 proteins involved with the nuclear transport of IRF3 and NF-κB p65 as cargo and induction of IFNB1 upon virus infection. More specifically, we showed that IMPβ1 and related TNPO1, EXP1, EXP2 and RAN, elucidated as HCV NS3/4A interactors, mechanistically control the nuclear translocation of IRF3 and NF-κB. Furthermore, NS3/4A is able to cleave IMPβ1, restricting IRF3 and NF-κB p65 from being transported to the nucleus, in a novel viral strategy to abolish IFNB1 production. Overall, the data support an innate evasion "hot spot" that underlines the importance and redundancy of nucleocytoplasmic trafficking proteins for optimal antiviral response. Thus, they are a valuable stepping stone in understanding the dynamics of the early antiviral response with the goal of validating therapeutic targets for a broad-spectrum of viral infection.

4 | MATERIAL AND METHODS

4.1 | Cell culture

HEK293T cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Wisent). A549 (human lung adenocarcinoma epithelial) cell lines were cultured in Ham's F-12 medium (Invitrogen). Both media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine (all from Wisent) and 1% nonessential amino acids (Invitrogen) at 37°C in an atmosphere of 5% CO_2. Cell populations of HEK293T and A549 stably harboring the pIFNB1-LUC and of HEK293T stably harboring the pEF1α-LUC used in the screens were produced after selection with 200 μg/mL of hygromycin B (Wisent). Transfections were performed with linear 25 kDa polyethylenimine (PEI) (Polysciences, Inc) at 3 μg PEI to 1 μg DNA ratio.

4.2 | Expression vectors

IMPβ1 (KPNB1) and MAVS C508R cDNAs were purchased from DNASU and Addgene, respectively. Following polymerase chain reaction (PCR)-amplification, PCR product was cloned using PfI23II/NotI enzymes into pcDNA3.1_FLAG-MCS(MB) expression vector.[38] 3×FLAG-NS3/4A, FLAG-eYFP and pIFNB1-LUC have been described before.[1] IMPβ1 mutants were generated via site-directed mutagenesis within the expression vectors using the Q5 kit using the Forward primer GGATCACACAaATGGAGTAGTAGCTaGTGgTGCTGGACTAATAGGGG and the Reverse primer CCCCTATTAGTCCAGCACcACtAGCTACTACTCCATt TGTGTGATCC (NEB). All constructs were verified using Sanger sequencing at the genomics platform at the Institute of Research in Immunology and Cancer (Montreal, Québec, Canada).

4.3 | Lentiviral shRNA library production

From MISSION TRC lentiviral library (Sigma-Aldrich), 132 MS hits were selected and shRNA were produced as follows: 5 different shRNA-expressing lentiviruses per gene were produced individually in HEK293T cells (2×104) that are plated 1 day prior to transfection. Transfections were performed using a Biomek FX (Beckman Coulter) enclosed in a class II cabinet according to MISSION Lentiviral Packaging Mix protocol (SHP001). Viruses were collected at 24 and 48 hours post-transfection and were pooled prior to freezing. A non-target sequence (NT) shRNA-expressing control lentivirus and 4% of random samples of each plate were used to measure lentiviral titers for quality control purposes. Titers were determined by limiting dilution assays using HeLa cells. Briefly, samples were diluted in complete DMEM (1:400 or 1:10 000) and added to HeLa cells. Media was changed at day 3 and 5 with complete DMEM containing 1 μg/mL puromycin (Wisent). After 4 days of selection, cells were stained with 1.25% crystal violet and plaque-forming units (PFU) were counted to determine viral titer.

4.4 | Large-scale shRNA production

The 293T cells were transfected with pRSV-REV, pMDLg/pRRE, pMD2-VSVg and various shRNA-expressing pLKO.1-puro constructs (Sigma-Aldrich) using linear 25 kDa PEI (Polysciences, Inc.) at 3 μg PEI to 1 μg DNA ratio as previously described.[37] After 48 hours of transfection, cell media was collected, filtered (0.45 μm filter), and aliquoted. MOI was determined using limiting dilution assays as described in previous section.

4.5 | Firefly luminescence assay

For screening, cells were seeded in white 96-well plates at a density of 5000 HEK293T pIFNB1_LUC, 5000 A549 pIFNB1_LUC and 1250 HEK293T pEF1α-LUC in 100 μL of complete phenol-red free DMEM containing 4 μg/mL polybrene. Infection with lentivirus encoding shRNA were carried out immediately after cell seeding at a MOI of 10 and incubated for 3 days at 37°C in an atmosphere of 5% CO_2. Cells were infected with 100 HAU/mL of SeV (Cantell Strain, Charles River Labs) for 6 hours for A549 and 16 hours for HEK293T cells before cell lysis and firefly luminescence reading in a 100 mM Tris acetate, 20 mM Mg acetate, 2 mM EGTA, 3.6 mM ATP, 1% Brij 58, 0.7% β-mercaptoethanol and 45 μg/mL luciferine pH 7.9 buffer. All the infections were performed in an enclosed class II cabinet.

4.6 | Operetta microscopy

A549 cells were plated on clear 96-well plates at a density of 1000 cells in 100 μL complete Ham's F-12 medium containing 4 μg/mL polybrene. Infection with lentivirus encoding shRNA (5 individual shRNAs per gene) were carried out immediately after cell seeding at a MOI of 10 and incubated for 4 days at 37°C in an atmosphere of 5% CO_2. As control the MISSION shRNA NT clone (Sigma SHC002) was included in each 96-well plate. Cells were infected with 100 HAU/mL of SeV (Cantell Strain, Charles River Labs) for 0, 1, 3, 5, 8 or 10 hours before being fixed with 4% paraformaldehyde-containing PBS for 20 minutes at room temperature and then permeabilized in 0.2% Triton X-100/PBS for 15 minutes. Blocking was made in PBS with 10% normal goat serum, 5% bovine serum albumin (BSA) and 0.02% sodium azide for 45 minutes at room temperature. Following 3 rapid washes, cells were labeled with mouse anti-IRF3 (SL-12; Santa Cruz Biotechnology) or rabbit anti-p65 (C-20; Santa Cruz Biotechnology) primary antibodies diluted in 5% BSA/0.02% sodium azide/PBS for 2 hours. Wells were washed 3 times in PBS and then labeled with anti-mouse AlexaFluor 488 or anti-rabbit AlexaFluor 488 secondary antibodies (Invitrogen) diluted in 5% BSA/0.02% sodium azide/PBS for 1 hour. Cells were extensively washed and incubated with Hoechst dye (Invitrogen) at a final concentration of 1 μg/mL in PBS. Images of cells were captured in 9 predetermined fields for each well (Operetta High Content Screening Microscope; Perkin Elmer) and images were processed using Harmony (Perkin Elmer). Cut-off for nuclear staining for IRF3 and p65 were between 230 to 300 and 515 to 735, respectively. Percentage of cells with IRF3 or p65 nuclear staining was calculated by dividing the number of nuclei where nuclear fluorescence was higher than the cut-off for IRF3 or p65 staining by the total number of nuclei stained by Hoechst for the 9 fields. The effect of a shRNA on cell proliferation and survival was evaluated by dividing the total number of nuclei in the 9 fields of the 5 time points and dividing it by the total number of nuclei in the 9 fields of the 6 time points of the shRNA NT control wells included in every 96-well plate.

4.7 | Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS; Wisent), harvested and lysed in 10 mM Tris-HCl, 100 mM NaCl, 0.5% Triton X-100, pH 7.6 with ethylenediaminetetraacetic acid (EDTA)-free Protease Inhibitor Cocktail (Roche). Cell lysates were clarified by centrifugation at 13 000*g* for 15 minutes at 4°C and subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Western blot analysis was performed using the following antibodies: ACTIN was purchased from Chemicon International (Billerica, MAB1501R); FLAG was purchased from Sigma (St. Louis, F3165); EXP2/CSE1L, IRF3 P-386, IMPβ1/KPNB1 and TNPO1 were purchased from Abcam (Toronto, ab96755, ab76493, ab2811 and ab10303); H1, p65, RAN and EXP1/XPO1 were purchased from Santa Cruz Biotechnology (sc-8030, sc-8008, sc-58467 and sc-74454); IRF3 was purchased from Phoenix Airmid Biomedical (18781); IFIT1/ISG56 was purchased from Novus Biologicals (NBP1-32329); NFKBIA P-32 was purchased from Cell Signaling Technology, Inc., (2859). The antibody for PARP1 and SeV was a kind gift from MJ. Hébert and M. Servant, respectively. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad. The chemiluminescence reaction was performed using the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer).

4.8 | Co-immunoprecipitation

For co-immunoprecipitation (co-IP), FLAG-tagged protein expressing cells were harvested and lysed as described above. Resulting cell extracts were adjusted to 1 mg/mL and subjected to IP as follows: pre-clearing of the lysates was done by incubating lysates with 40 μL of 50:50 slurry of immunoglobulin G-Sepharose (GE Healthcare) prepared in the lysis buffer with IgG beads for 1 hour. Pre-cleared lysate were immunoprecipitated by adding 20 μL of M2 anti-FLAG affinity gel (Sigma-Aldrich) prepared in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) overnight as described by the manufacturer. Immunoprecipitates were washed 5 times in lysis buffer. Elution was performed using 250 ng/μL purified FLAG peptide for 45 minutes at 4°C (Sigma-Aldrich). Eluates were analyzed using western blotting.

4.9 | Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic extraction was performed on 4 000 000 A549 cells that were previously plated on 100 mm plates, infected with lentivirus encoding shRNA NT or IMPβ1 (TRCN0000123189) at a MOI of 10 for 3 days and infected with SeV for 0, 1, 3, 5, 8 and 10 hours prior to harvesting. Nuclear and cytoplasmic fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's protocol.

4.10 | Functional enrichment analysis

DAVID database was used for functional annotation.[62,63] DAVID functional annotation chart tool was used to perform GO biological process and InterPro protein domain analysis. Terms with a *P*-value lesser than 5×10^{-2} were considered as significantly overrepresented.

ACKNOWLEDGEMENTS

We thank K. Audette and J. Duchaine of the IRIC's screening facility. This work was supported by grants from the Canadian Institutes for Health Research (CIHR-MOP-115010 and CIHR-CI6-103135), the Novartis/Canadian Liver Foundation Hepatology Research Chair to D.L. and FRQ-S PhD scholarship to N.T.

AUTHORS CONTRIBUTIONS

B.G., M.B. and D.L. were involved in conceptualization. B.G. was involved in methodology. Investigation was done by B.G., A.Y.P. MB and N.T. Writing—Original Draft was carried out by B.G., M.B., N.-T. and D.L. Writing—Review & Editing was carried out by N.T. and D.L.; B.G. and N.T. were involved in Visualization. Supervision, administration and funding by D.L.

The Editorial Process File is available in the online version of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Gagné B, Tremblay N, Park AY, Baril M and Lamarre D. Importin β1 targeting by hepatitis C virus NS3/4A protein restricts IRF3 and NF-κB signaling of IFNB1 antiviral response. *Traffic*. 2017;18:362–377. https://doi.org/10.1111/tra.12480

Annex D – Author's Contributions 2012 - 2017

D.1. Peer Reviewed Articles

1. Gagné B, **Tremblay N**, Park AY, Baril M, Lamarre D (2017) Importin β1 targeting by hepatitis C virus NS3/4A protein restricts IRF3 and NF-κB signaling of IFNB1 antiviral response. **Traffic** 18:362–377. **Contribution:** 45%: I have created a clear and concise graphical abstract that shows that IMPβ1 mediates the nuclear translocation of IRF3, essential to produce IFN-β, upon viral infection. In HCV infection, IMPβ1 is cleaved by HCV-NS3/4a protease as a novel mechanism of immune evasion. To elucidate the contribution of this mechanism to the RLR-pathway, I have generated a MAVS knock out (KO) cell line using CRISPR-Cas9 genome editing, along with various NS34/a cleavage-resistant MAVS and IMPβ1 clones. I then conducted various experiments (reporter assays, western blots and confocal microscopy) and showed that NS3/4A-mediated cleavage of importin β1 (IMPβ1) and interferon-β (IFNB1) inhibition are completely restored by expression of NS3/4A cleavage-resistant IMPβ1 variant (IMPβ1CR) and treatment with BILN 2061 NS3/4a protease inhibitor (Figure 8). In addition, I have written and formatted the manuscript for publication and drafted Table 1 and Table 2 that summarize the gene enrichment analysis and the functional classification of the genes that affects the nuclear trafficking of IRF3 and p65, respectively.

2. Jeidane S*, Scott-Boyer, MP*, **Tremblay, N** et al. (2016) Association of a Network of Interferon-Stimulated Genes with a Locus Encoding a Negative Regulator of Non-conventional IKK Kinases and IFNB1. **Cell Rep** 17:425–435. **Contribution:** 20%: I planned and supervised the first author while performing the rescue experiments. I also supervised/performed the western blots, the western blots quantitative analysis and the co-immunoprecipitations to show that YPEL5 is a regulator of the RLR-pathway via the TBK1/IKBKE protein kinases that are essential for the activation of IRF3 (Figure 4, 5, 6, 7).

3. **Tremblay N** et al. (2016) Spliceosome SNRNP200 Promotes Viral RNA Sensing and IRF3 Activation of Antiviral Response. PLoS Pathog 12: e1005772. *Erratum in PLoS Pathog 2017 Jan 24;13(1):e1006174.* **Contribution: 60**%: I have used western blots, Elisa, RT-QPCR, viral plaque assays and rescue experiments to show that SNRNP200 is required to initiate a type I interferon response upon infection with several RNA viruses (Figure 1, 2). I have analyzed the data from microarray experiments to identify genes that are essential for the early (IRF3-dependent) and late (IFN-α/β dependent) antiviral response and affected by the depletion of SNRNP200 (Figure 3). I have mapped the functional domains that are required for SNRNP200 antiviral activity and mapped the interaction with the TBK1 protein kinase using cloning and directed mutagenesis in combination with western blots, co-immunoprecipitation, Elisa, reporter assays and confocal microscopy (Figure 4, 6, 7). I have used DNA/RNA-coupled to streptavidin beads to assess the role of SNRNP200 in viral nucleotide sensing using co-immunoprecipitation, western blot and RT-QPCR (Figure 5). I have used western blots, RT-QPCR and ELISA to confirm the role of SNRNP200 in innate antiviral immunity in primary cells (macrophages) and PBMCs of RP33 patients that are carrying mutations in SNRNP200 (Figure 9). I have made significant intellectual contributions to the proposed model that recapitulates the role of SNRNP200 in innate antiviral immunity (Figure 10.)

D.2. Book Chapter and Review Articles

1. Said E*, Tremblay N*, Al-Balushi M, Al-Jabri AA, Lamarre D (2017) Viruses Seen by our Cells: The Role of Viral RNA Sensors. Journal of Immunology Research. [in revision] **Contribution: 45%**: I have drafted the outline of the review article and written the first part of the review on the role of RNA sensors and sentinels in innate antiviral immunity.

2. **Tremblay N**, Park AY, Lamarre D (2016) HCV NS3/4A Protease Inhibitors and the Road to Effective Direct-Acting Antiviral Therapies in **Hepatitis C Virus II** (Springer Japan) **Contribution: 65%**: I have drafted the outline of the review article and written the book chapter and designed and prepared the 2 figures.

3. Es-Saad S*, Tremblay N*, Baril M, Lamarre D (2012) Regulators of innate immunity as novel targets for panviral therapeutics. Curr Opin Virol 2:622–8. **Contribution: 45%**: I have drafted the outline of the review article designed and written the first part of the review on the role of RLR-pathway and its possible targets for pan-viral therapeutic agents.

D.3. Extracurricular research publications

1. Chartrand C, **Tremblay N**, Renaud C, Papenburg J (2015) Diagnostic Accuracy of Rapid Antigen Detection Tests for Respiratory Syncytial Virus Infection: Systematic Review and Meta-analysis. J Clin Microbiol 53:3738–49. **Contribution:** 30%: As the second reviewer of the meta-analysis, I have manually extracted clinical parameters from about 100 reports and I have contributed to the statistical analysis of the data using STATA (METENDI) by running the analyses and presenting the results according the standards of the field.

2. Chartrand C, Renaud C, **Tremblay N** (2016) Rapid influenza diagnostic tests: clinical usage and significance in A Practical Guide to Global Point-of-Care Testing (CSIRO PUBLISHING). **Contribution: 40%**: I have drafted the outline of the book chapter and written the sections pertaining to the epidemiology, economic impacts, clinical presentation and diagnosis/treatment of an influenza infection. Additionally, I have spearheaded the publication of the book chapter by liaising with the editorial office.