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Solène LEROLLE

Identification and characterization of interferon-stimulated genes modulating Crimean-Congo hemorrhagic fever virus infection

Identification et caractérisation de gènes induits par l'interféron modulant l'infection par le virus de la fièvre hémorragique de Crimée-Congo

Devant le jury composé de :

MIRAZIMI, Ali, PR, Karolinska Institutet, Stockholm, Sweden	Rapporteur
JOUVENET, Nolwenn, DR, Institut Pasteur, Paris, France	Rapportrice
GOUJON, Caroline, DR, IRIM, Montpellier, France	Examinatrice
ETIENNE, Lucie, DR, CIRI, Lyon, France	Examinatrice
WILSON, Sam J., PR, University of Cambridge, Cambridge, United Kingdom	Examinateur
LEGROS, Vincent, MCU, CIRI, Lyon and VetAgro Sup, Marcy-l'Etoile, France	Examinateur
COSSET, François-Loïc, DR, CIRI, Lyon, France	Directeur de thèse

Summary

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne, emerging virus which poses a serious threat to public health due to its high epidemic potential. Indeed, this emerging virus is transmitted by hard ticks belonging to the *Hyalomma* genus, whose geographic distribution is in expansion. Currently, this virus is endemic in Africa, Middle East, southern Asia, southern, and eastern Europe. In humans, it causes a hemorrhagic disease with a mortality rate ranging from 5 to 30%, for which there are no specific treatment or vaccine available. Therefore, its handling is restricted to the highest biosafety-level containment facilities (BSL-4), and there is a dire need to gain more insight into CCHFV biology.

The interplays between CCHFV and innate immunity remain to date unwell characterized. Notably, type I interferon (IFN) has a crucial role in the establishment of an antiviral state against CCHFV, probably through the transcription of IFN-stimulated genes (ISGs) with antiviral properties. However, it remains to be determined which ISGs modulate CCHFV infection and their mechanisms of action.

To tackle these questions, we performed a gain-of-function screen using CCHFV virus-like particles competent for entry and transcription of a reporter minigenome (tc-VLPs), a BSL-2 model of CCHFV, and lentiviral vectors encoding >500 human, >350 simian and >250 bovine individual ISGs. We identified several ISGs that modulate either negatively or positively tc-VLPs infection and that were previously undescribed in the context of CCHFV infection. To define their mechanisms of action, we dissected their impact on the different steps of the viral life cycle using various assays, that were set up and optimized during my PhD. We identified ISGs inhibiting the step of viral entry, such as lysosomal-associated membrane protein 3 (LAMP3) and lymphocyte antigen 6 family member E (LY6E). Further, we pinpointed the antiviral activity of other ISGs such as poly(ADP)ribose polymerase family member 12 (PARP12), 2'-5'-oligo-adenylate synthase 2 (OAS2) or bovine hyaluronan synthase 3 (HAS3), that inhibited tc-VLPs infection through a yet undefined mechanism. Moreover, we highlighted the proviral activity of a GTP-binding protein, GTPBP1, that promoted tc-VLP replication and/or transcription. Finally, we could confirm the potent antiviral activity of PARP12, whose overexpression led to a reduction of the infection by a related orthonairovirus, Hazara virus (HAZV), and by authentic CCHFV. We also

observed that overexpression of GTPBP1 had no effect on HAZV infection, and unexpectedly inhibited authentic CCHFV, despite its ability to promote tc-VLPs replication and/or transcription. Studies are ongoing to further characterize both PARP12 and GTPBP1 activities in the context of CCHFV infection.

Collectively, these results provide novel insights into the interactions between CCHFV and components of the host innate immune response, and pave the way for future studies that will shed more light on the biology of this emerging and highly pathogenic virus.

Résumé

Le virus de la fièvre hémorragique de Crimée-Congo (CCHFV) est un virus émergent transmis par les tiques qui constitue une menace sérieuse pour la santé publique en raison de son fort potentiel épidémique. En effet, ce virus émergent est principalement transmis par des tiques dures appartenant au genre *Hyalomma*, dont la distribution géographique est en expansion. Actuellement, ce virus est endémique en Afrique, au Moyen-Orient, en Asie du Sud, en Europe du Sud et de l'Est. Chez l'humain, il provoque une maladie hémorragique avec un taux de mortalité allant de 5 à 30 %, pour laquelle il n'existe ni traitement spécifique ni vaccin. C'est pourquoi sa manipulation est confinée aux laboratoires de niveau de biosécurité le plus élevé (BSL-4) et qu'il est absolument nécessaire de mieux comprendre la biologie du CCHFV.

Les interactions entre le CCHFV et l'immunité innée demeurent mal caractérisées. En particulier, l'interféron (IFN) de type I, une cytokine dont la production et sécrétion sont induites à la suite de la détection d'une infection par un pathogène, joue un rôle crucial dans l'établissement d'un état antiviral contre le CCHFV. Cet état antiviral passe probablement par le biais de la transcription de gènes stimulés par l'IFN (ISGs) ayant des propriétés antivirales. Toutefois, il reste à déterminer quels ISGs modulent l'infection par le CCHFV et quels sont leurs mécanismes d'action.

Pour répondre à ces questions, nous avons étudié le CCHFV grâce à des pseudoparticules virales du CCHFV compétentes pour l'entrée et la transcription d'un minigénome rapporteur (tc-VLPs), un modèle BSL-2 du CCHFV. Nous avons tout d'abord vérifié que l'IFN de type I inhibait l'infection par les tc-VLPs dans une lignée cellulaire hépatique, les Huh7, comme observé pour le virus authentique. Puis, nous avons effectué un crible en gain de fonction en utilisant des vecteurs lentiviraux codant pour >500 ISG humains, >350 ISG simiens et >250 ISG bovins. Grâce à ce crible, nous avons identifié plusieurs gènes humains, macaques et bovins dont la surexpression inhibait l'infection par les tc-VLPs. Après avoir exclu les gènes dont la surexpression était toxique et ceux dont la surexpression induisait la réponse IFN, nous nous sommes concentrés sur les ISGs révélés par notre crible qui inhibaient ou promouvaient fortement l'infection par les tc-VLPs. Cette liste comprenait les top candidats à activité antivirale : la protéine transmembranaire induite par l'IFN 1 (IFITM1), la protéine associée aux membranes lysosomales 3 (LAMP3), la 2'-5'-oligo-

adénylate synthase 2 (OAS2), le membre 12 de la famille des poly(ADP)ribose polymérases (PARP12), le membre E de la famille 6 de l'antigène lymphocytaire (LY6E), le membre 3F de la famille des APOBEC (APOBEC3F), le membre F de la famille aux séquences de similarité 26 (FAM26F) et la hyaluronane synthase 3 bovine (HAS3). Nous nous sommes également concentrés sur l'activité provirale d'une protéine se liant au GTP, GTPBP1.

Pour définir leurs mécanismes d'action, nous avons disséqué leur impact sur les différentes étapes du cycle viral à l'aide de diverses expériences, qui ont été mises en place et optimisées au cours de ma thèse. Notamment, nous avons étudié l'impact des candidats sur l'expression des protéines virales, et avons pu identifier que IFITM1 et FAM26F inhibait l'expression des glycoprotéines Gc et Gn du CCHFV. Ainsi, nous avons émis l'hypothèse que ces protéines interféraient avec la production de nouvelles particules virales. Grâce à des pseudoparticules du virus de la stomatite vésiculeuse (VSV) pseudotypées avec les glycoprotéines du CCHFV, nous avons identifié l'effet antiviral de LY6E et LAMP3 spécifiquement contre l'entrée du CCHFV. Nous avons également observé que HAS3 et PARP12 inhibaient soit l'entrée du CCHFV et du VSV, soit la réplication du VSV. Enfin, nous avons pu constater que GTPBP1 promouvait la réplication et/ou la transcription virales. Grâce à la technologie CRISPR/Cas9, nous avons éteint l'expression de GTPBP1 dans les Huh7, mais cette extinction n'avait pas d'effet sur l'infection par les tc-VLPs, suggérant que GTPBP1 endogène jouait un rôle mineur dans l'infection par les tc-VLPs, ou alternativement, que son absence était compensée par une autre protéine cellulaire. Enfin, nous avons étudié l'effet de l'expression de ces ISGs sur un virus de la même famille que le CCHFV, le virus Hazara (HAZV), et sur le CCHFV authentique. Notamment, PARP12 possédait une forte activité antivirale contre le HAZV et le CCHFV authentique. Étonnamment, la surexpression de GTPBP1 n'avait pas d'effet sur l'infection par le HAZV, et inhibait l'infection par le CCHFV, malgré sa capacité à promouvoir la réplication et/ou la transcription des tc-VLPs. De études sont en cours afin de caractériser plus en détail les activités des gènes identifiés, notamment PARP12 et GTPBP1, dans le contexte de l'infection par le CCHFV.

Dans l'ensemble, ces résultats fournissent de nouvelles informations sur l'interaction entre le CCHFV et les composants de la réponse immunitaire innée de l'hôte, et ouvrent la voie à de futures études qui permettront de mieux comprendre la biologie de ce virus émergent et hautement pathogène.

Acknowledgements

I would like to start by thanking my jury members (Prof. Ali Mirazimi, Dr. Nolwenn Jouvenet, Dr. Caroline Goujon, Dr. Lucie Etienne, and Prof. Sam J. Wilson) for accepting to evaluate this work and taking part in my PhD defense jury. Besides my jury, I would like to extend my thanks to my thesis committee, Dr. Vincent Lotteau and Dr. Sébastien Nisole, who took time to go deeper in my projects and provided their insightful comments and advice.

I would like to thank my PhD director, Dr. François-Loïc Cosset, for his supervision and support throughout my PhD. Your support and guidance allowed me to grow scientifically and ameliorate both my theoretical and practical capacities. Thank you for your optimism and reassuring comments when I needed them. I also express my gratitude to my PhD co-supervisor, Dr. Vincent Legros. From being my professor at VetAgro Sup to being my director of veterinary thesis, and now as my PhD supervisor, your mentorship and dedication have been instrumental in my scientifical growth. Thank you for our endless scientific discussions on innumerable theories, your availability in rush periods, and your delicious waffles that even the walls of the laboratory will always remember.

I would also like to thank the members of the CCHFV group (especially Dr. Natalia Freitas, Maureen Ritter, and Thomas Vallet) for their insightful comments on my projects. My eternal gratitude goes to Thomas for your always enthusiastic technical support! Working with the members of the CCHFV group was an amazing experience. I am grateful to my fellow labmates, and especially “les Djeuns EVIR”, for the laughs that we had both in and out of the lab. I am looking forward to drinking beers and playing bowling with you. Special thanks to Audrey, my Maman Thésarde, for showing me how to survive to a PhD.

I would like to thank my childhood friend, Jeanne, for our strong and eternal friendship. I know that I can always count on you and that was a big help during my PhD. I am honored to be your witness and I look forward to living one of the most beautiful moments of your life very soon!

Of course, I am forever grateful to “les Limaçons”, Pauline and Marguerite, for our numerous adventures where everything goes always well (we are so lucky), for the innumerable hills that we have climbed and kilometers that we have walked, and for the memorable memes that always arise from all our adventures. I look forward to our next one! I would also like to thank my other friends from Ginette, Domitille, Marie, Philippe, Quentin, Lou, Inès, Marthe, Benoît, Jeanne, Lucie and Timothée. My thanks also go out to Mélanie. I look forward to seeing you in Nice and to rent a boat so that you can show me how well you drive them!

I would like to thank my family for their continuous support: my mother and my father for their love, my sister and brothers for our unbreakable bond, my beautiful nephews Leelo and Louis, Mimi for her kindness and my uncle for our delicious meals at “les Tontons Zingueurs”.

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Main abbreviations

APOBEC	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide
BSL	Biosafety level
BUNV	Bunyamwera virus
CCHF	Crimean-Congo hemorrhagic fever
CCHFV	Crimean-Congo hemorrhagic fever virus
CDS	Cytosolic DNA sensor
CLR	C-type lectin receptor
CRISPR	Clustered regularly interspaced short palindromic repeats
cRNA	Complementary RNA
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DUB	Deubiquitinase activity
ELISA	Enzyme-linked immunosorbent assay
FAM26F	Family with sequence similarity 26 member F
fLuc	Firefly luciferase
GPC	Glycoprotein precursor complex
gRNA	guide RNA
GTPBP1	GTP-binding protein 1
HAS3	Hyaluronan synthase 3
HAZV	Hazara virus
hpi	Hours post-infection
IFIH1	IFN-induced with helicase C domain-1
IFITM	IFN-induced transmembrane protein
IFN	Interferon
IFNAR	Interferon-α/β receptor
IRF	IFN-regulatory factor
ISG	Interferon-stimulated gene
ISRE	IFN-stimulated response elements
JAK	Janus kinase
KO	Knock-out
LACV	La Crosse virus
LAMP3	Lysosomal associated membrane protein 3
LY6E	Lymphocyte antigen 6 family member E
MAVS	Mitochondrial antiviral-signaling proteins
MDA5	Melanoma differentiation-associated gene 5
MLD	Mucin-like domain
mRNA	Messenger RNA
MX	Myxovirus resistance protein
NLR	NOD-like receptor
nLuc	Nanoluciferase
NP	Nucleoprotein
OAS	2'-5'-oligoadenylate synthase

ORF	Open reading frame
OTU	Ovarian-like tumor
PAMP	Pathogen-associated molecular pattern
PARP12	Poly-(ADP-ribose) polymerase family member 12
PKR	Protein kinase R
PRR	Pattern recognition receptors
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RVFV	Rift Valley fever virus
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SKI-I/S1P	Subtilisin kexin isozyme-1/site-1 protease
SLFN12	Schlafen family 12
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
SVP	Subviral particle
tc-VLP	Transcriptionnally- and entry-compentent virus-like particles
TLR	Toll-like receptor
UTR	Untranslated region
Ub	Ubiquitin
VEEV	Venezuelan equine encephalitis virus
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
VRP	Viral replicon particles
VSV	Vesicular stomatitis virus
WHO	World Health Organization

Preamble

I discovered the domain of virology during my studies of veterinary medicine, in VetAgro Sup, Lyon, during which the concept of "One Health" was presented as a sustainable, multidisciplinary, collaborative approach aiming at addressing and preventing health issues threatening the human-animal-environment interface. Intrigued by this topic, I did a summer internship in the group of Dr. El-Sayed Mohammed Abdel-Whab, in the Friedrich Löffler Institut, in Germany, in 2018, where I discovered the world of academic research. I participated in a project aiming at deciphering the importance of mutations in the neuraminidase of avian influenza viruses in crossing the species barrier. Motivated by this internship and convinced of the importance of studying emerging viruses to meet future health challenge, I followed a master's degree in fundamental virology at Pasteur Institute, in Paris, and I joined for my Master 2 internship the group of Dr. François-Loïc Cosset, in Lyon, to study the role of a secreted viral protein, GP38, in the pathogenesis of Crimean-Congo hemorrhagic fever virus (CCHFV). Following the obtention of my Master 2 and my doctorate in veterinary medicine, I was awarded a 3-year PhD scholarship from the "Fondation pour la Recherche Médicale" (FRM) to follow up on this project. However, after one year of several technical difficulties and inconclusive results that will not be presented in this manuscript, I decided to change the scope of my PhD and to focus on CCHFV interplays with the host innate immune system, and more specifically the interferon system, as the basis of these interplays were previously laid by other groups, opening for several interesting questions. Thus, I initiated a collaboration with the group of Prof. Sam J. Wilson and took advantage of the biosafety-level 2 (BSL-2)-adapted CCHFV transcriptionally- and entry-competent virus-like particle system (tc-VLPs) implemented in the laboratory to decipher which interferon-stimulated genes (ISGs) modulate CCHFV infection.

Thus, my PhD thesis will focus on the interplays between CCHFV and the host innate immune system and will give us more insights on how hosts defend themselves against CCHFV infections or, as part as the arms race between a virus and its host, how CCHFV uses the host innate immune system at his advantage. To do so, the introduction to my PhD will first focus on CCHFV biology, and then on what is currently known about the interplays between CCHFV and the innate immune system. Then, the results of my PhD will present what are the ISGs modulating

CCHFV infection, as well as some insights into their mechanisms of action. Finally, I will discuss the implications of these results and how they contribute to the global knowledge of host/CCHFV interactions.

Scientific contributions

This PhD manuscript highlights my main project, focusing on the interplays between Crimean-Congo hemorrhagic fever virus (CCHFV) and innate immunity. It consists of one manuscript currently in preparation, presented in Chapter 2 of the results. Furthermore, it includes supplementary data that explore the development of new assays to study CCHFV entry independently of its replication, that are presented in Chapter 1 of the results. In addition, I have contributed to other works from the laboratory: one already published research article (Freitas et al., 2020) and another one that is currently in preparation (Gautaum et al., in preparation). I also wrote a review article (Lerolle et al., 2021). The subsequent section provides a comprehensive view of my involvement in these publications.

Identification and characterization of interferon-stimulated genes modulating Crimean-Congo hemorrhagic fever virus infection

Solène Lerolle, Thomas Vallet, Emma L. Davies, Lola Canus, Alexandre Lalande, Cyrille Mathieu, Sam J. Wilson, François-Loïc Cosset and Vincent Legros

Manuscript in preparation
(F.-L. Cosset team members, PhD supervisor, PhD director)

In this manuscript, we have performed a gain-of-function screen using libraries of human, macaque or bovine ISGs and a BSL-2 model of CCHFV, the tc-VLPs. We have uncovered ISGs that modulate either positively or negatively tc-VLPs infection and dissected which step of the life cycle they target. We further characterized the mechanism of action of a proviral factor, GTPBP1, and discovered that it promoted viral reporter RNA transcription and/or replication. We investigated the role of endogenous GTPBP1 in tc-VLPs infection by CRISPR knock-out experiments. Finally, we tested the activity of the identified hits on a related orthonairovirus, HAZV, and on authentic CCHFV.
My contribution to this study was conceiving and designing experiments, conducting the majority of the experiments, and writing of the manuscript. Thomas Vallet performed some of the entry assays using VSV pseudotyped virus, some of the

infections with HAZV, most of the western blots, and provided technical help. Sam J. Wilson and Emma L. Davies generated the libraries for the initial screen and provided selected ISGs DNA for further experiments. Cyrille Mathieu, Lola Canus and Alexandre Lalande proceeded to infection and titration experiments using authentic CCHFV in BSL-4 settings. I performed the RNA extraction and RT-qPCRs from BSL-4 samples. François-Loïc Cosset and Vincent Legros provided scientific guidance and revised the manuscript.

Cooperation of cytoplasmic tail motifs of both CCHFV Gn and Gc glycoproteins determines their trafficking to virion assembly site and envelopment

Anupriya Gautam, Alexandre Lalande, Maureen Ritter, Natalia Freitas, **Solène Lerolle**, Lola Canus, Vincent Legros, François-Loïc Cosset, Cyrille Mathieu, and Bertrand Boson

Manuscript in preparation
(F.-L. Cosset team members, PhD supervisor, PhD director)

In this paper, we aimed at understanding how CCHFV Gn and Gc glycoproteins use host factors to reach the assembly site and envelopment of viral particles. We identified putative membrane trafficking motifs in the cytoplasmic tails of CCHFV glycoproteins and addressed how they can impact assembly/envelopment. Moreover, we identified host factors that contribute to CCHFV glycoproteins trafficking seemingly required for virus assembly and release of viral particles.

In this study, I produced tc-VLPs with glycoproteins mutated in their cytoplasmic tail domains and performed RNA immunoprecipitations.

The interplays between Crimean-Congo hemorrhagic fever virus (CCHFV) M segment-encoded accessory proteins and structural proteins promote virus assembly and infectivity

Natalia Freitas, Margot Enguehard, Solène Denolly, Camille Levy, Gregory Neveu, **Solène Lerolle**, Stephanie Devignot, Friedemann Weber, Eric Bergeron, Vincent Legros and François-Loïc Cosset

Plos Pathogens, 2020, DOI: 10.1371/journal.ppat.1008850
(F.-L. Cosset team members, PhD supervisor, PhD director)

Here, we examined the relevance of each M-segment-encoded non-structural proteins in virus assembly, egress, and infectivity. We identified the importance of GP160/85 and GP38 in Gc maturation, trafficking, and incorporation into particles, and NSm role in assembly and virion secretion.

For this work, I participated in the revisions of the manuscript, notably by performing western blot experiments.

Host cell restriction factors of Bunyaviruses and viral countermeasures

Solène Lerolle, Natalia Freitas, François-Loïc Cosset and Vincent Legros

Viruses, 2021, DOI: 10.3390/v13050784
(F.-L. Cosset team members, PhD supervisor, PhD director)

This review outlines the intrinsic and interferon-induced antiviral proteins found in humans which are known to inhibit members of the *Bunyavirales* order of medical or economic importance. We also explored the strategies developed by bunyaviruses to escape the antiviral state established by the infected cells. I conceptualized the review, wrote the first version of the manuscript, and created the figures. Natalia Freitas, François-Loïc Cosset, Vincent Legros and I revised the original manuscript.

Introduction

Chapter 1: Crimean-Congo hemorrhagic fever virus

I. Descriptive epidemiology of CCHFV: past and present of CCHFV distribution

1. Discovery of CCHFV

In 1944-1945, during World War II in Crimea, 200 Soviet soldiers developed symptoms corresponding to a hemorrhagic fever, with a 10% mortality rate. Notably, several of them had reported tick bites. The research of the scientist Mikhail Chumakov revealed the arboviral etiology of this fever: first, filtered blood from infected soldiers was inoculated to psychiatric patients who reproduced the same febrile symptoms than the soldiers, suggesting a viral etiology. Moreover, two healthy volunteers were inoculated with filtered suspensions of *Hyalomma marginatum* ticks in presence of antibiotics and developed after two days a mild clinical course of the same fever, demonstrating the tick-borne route of transmission of this virus. Thus, the virus was named "Crimean fever virus" (Hoogstraal, 1979; Whitehouse, 2004). Following this discovery, several outbreaks of the same or a related disease appeared in Central Asia, Soviet Union, and Bulgaria, but in absence of tools to perform serological and diagnostic studies, it remains unclear whether the same virus was at the origin of these diseases. A breakthrough was achieved in 1968 when Chumakov isolated the virus by inoculating intracerebrally newborn white mice (NWM) with blood from a patient named Drozdov, in Astrakhan (Russia). The isolation and the culture of the Drozdov strain has led to formidable advances in the CCHFV field, by allowing the production of antigens and antibodies useful for serological surveys and experimental studies.

During this time, in Congo, in 1956, Ghislaine Courtois followed the case of a 13-year-old boy with unspecific symptoms such as fever, headache, nausea, vomiting and joint pains, whose blood was inoculated into 3-day-old mice. One mouse having developed

symptoms, its brain was inoculated into 2-day-old mice, and this was repeated several times. This isolate was called V3011. Courtois then himself became ill with the same symptoms and the same procedure was applied as for V3011, leading to an isolate that Courtois called V3010. The virus was maintained for months by passages in infant mice due to the lack of dry ice and -70°C freezer. In 1957, infant mouse brains from the 35th passage for V3011 and 46th passage for V3010 were sent to the East African Virus Research Institute (EAVRI) in Entebbe, Uganda, where they were frozen. Only in 1961 did John P. Woodall dug them out for identification, but no known virus could be identified through the different tests that were available at the time (hemagglutination-inhibition, complement fixation, or neutralization tests) (Woodall, 2007). The virus was sent to New York, in the world reference center for arboviruses, and was named "Congo virus" at the demand of Courtois in 1967 (Simpson et al., 1967; Woodall et al., 1967).

In 1969, after Chumakov sent the Crimean fever virus to New York for identification, it was discovered that it was antigenically similar to Congo virus (Casals, 1969). Thus, in 1973, the virus was renamed Crimean-Congo hemorrhagic fever virus (CCHFV) (despite the recommendation of the International Committee for the Taxonomy of Viruses (ICTV) to call it "Congo-Crimean hemorrhagic fever virus" due to the principles of scientific nomenclature based on priority in publication, which made John P. Woodall wonder whether this was due to the fact that it was still the time of the Cold War and that the "Soviets got their way" (Woodall, 2007)).

2. Current geographic distribution of CCHFV

Nowadays, CCHFV is not only present in Crimea and in Congo but has an extensive geographical distribution. In fact, it is the most widespread tick-borne human disease (Kuehnert et al., 2021). It is currently present in numerous countries in Africa, Middle East, southern Asia, and southern and eastern Europe as shown by serological studies in humans or animals and reported human cases (Figure 1). As mentioned later in this manuscript, CCHFV has different lineages that are based on phylogenetic resemblances and approximate geographic distribution (Deyde et al., 2006). The groups include group I, West Africa; group II, Democratic Republic of the Congo; group III, South Africa and West Africa; group IV, Asia and the Middle East; group V: Europe and Turkey; group VI, Greece (but the virus from this group was recently classified as a distinct virus, Aigai virus); group VII, Mauritania.

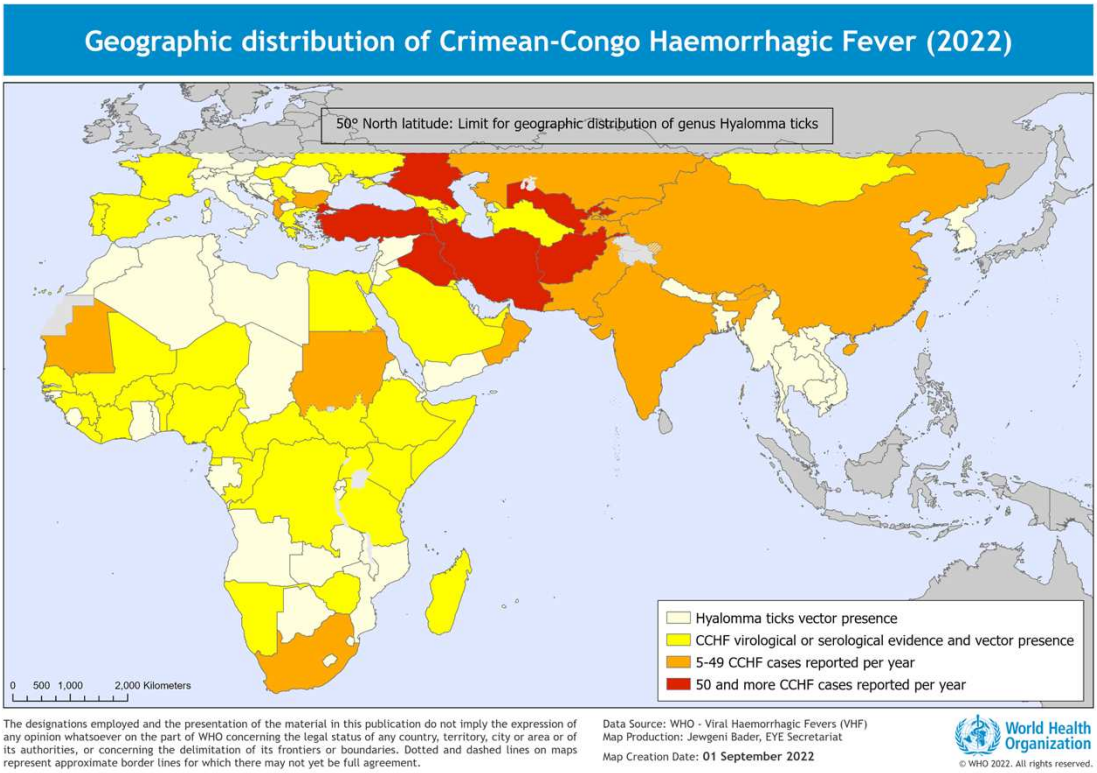

Figure 1. Geographic distribution of CCHFV (2022). From https://www.who.int/health-topics/crimean-congo-haemorrhagic-fever.

Most CCHF cases are sporadic in endemic zones, except for Turkey, where more than 50 cases per year occur (Ak et al., 2020) (Figure 2).

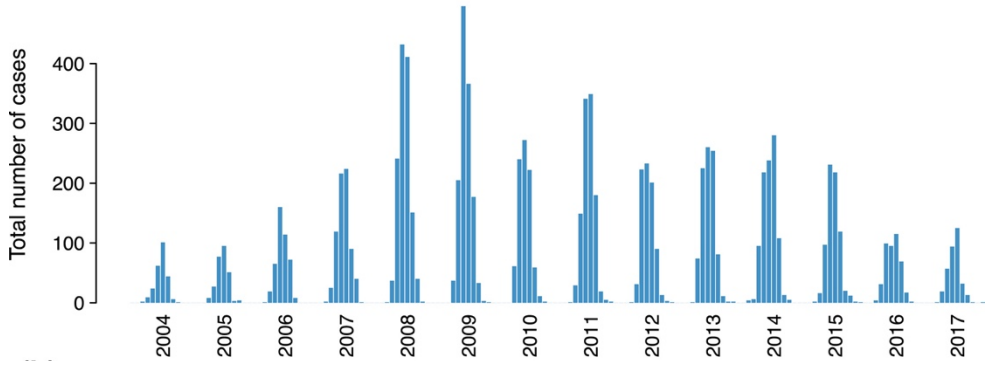

Figure 2. Monthly confirmed cases of CCHF between January 2004 and December 2017 in Turkey. From (Ak, Ergönül, and Gönen, 2020).

CCHFV distribution closely follows the range of its vector and reservoir host, the *Hyalomma* tick, and keeps increasing. It is expected that CCHFV distribution broaden due to climate change, that allows tick to survive in northerner latitudes, and due to the increase of human or animal movements.

II. Analytic epidemiology of CCHFV

1. *Hyalomma* ticks as vector and reservoir of CCHFV

As demonstrated by Chumakov in the 1940's, CCHFV is an arbovirus and hard ticks from the *Ixodidae* family and the *Hyalomma* genus were able to transmit the disease when crushed, filtered, and inoculated to volunteers. An important step in the history of CCHFV research was to identify if other arthropods might transmit the disease. The competence of an arthropod vector is defined by its capacity to gain, maintain, and transmit a pathogen. Thus, the mere isolation of the virus from an arthropod does not conclude on its ability to transmit it. So, even if CCHFV was isolated twice from mosquitoes from the *Culicoides* family (Causey et al., 1970; Lee, 1979), it was only probably due to a recent viremic blood meal that they ingested shortly before the capture. Experimentally, mosquitoes fed on CCHFV viremic blood or inoculated with CCHFV were not able to maintain the virus (Turell, 2007). On the contrary, CCHFV has been detected in more than 30 species of ticks, most of them hard ticks from the *Ixodidae* family (particularly *Hyalomma* sp., but also *Ixodes* sp., *Rhipicephalus* sp., *Amblyomma* sp., *Dermacentor* sp., and *Haemaphysalis* sp.). CCHFV was also found in soft ticks from the *Argasidae* family (*Argas* sp. and *Ornithodoros* sp.) (Gargili et al., 2017). However, experimental studies showed that these soft ticks could not maintain the virus nor transmit it, contrary to hard ticks (Durden et al., 1993; Shepherd et al., 1991, 1989).

As *Hyalomma* ticks feed only once after each molt (larva, nymph, and adult), a transstadial transmission of the pathogen is necessary for them to be a competent vector. CCHFV was found to persist throughout the tick life cycle, proving its transstadial transmission. In addition, CCHFV can be transmitted vertically to the next progeny (Gargili et al., 2017; Turell, 2007). More studies are needed to decipher if this vertical transmission occurs via the infected ovaries, (*i.e.,* transovarial transmission), or via infection of the developed eggs, (*i.e.,* transovum transmission). Moreover, one study showed that hypostomectomized male ticks could still transmit the virus to

intact female ticks, suggesting that venereal transmission of CCHFV occurs (Gonzalez et al., 1992). Finally, CCHFV can be transmitted from an infected tick to another tick through co-feeding on the same host, despite the absence of detectable viremia (Gordon et al., 1993).

As most hard ticks have a long-term survival (2 years), virus-carrying ticks allow it to persist through the winter and serve as a vehicle for its reintroduction the following spring. Thus, hard ticks, and especially *Hyalomma* ticks, are considered not only as CCHFV vector, but also as its reservoir. *Hyalomma* ticks are shown in Figure 3.

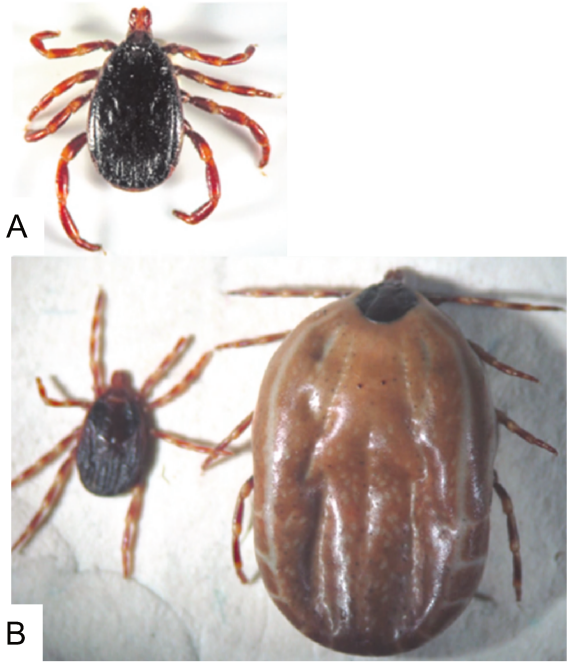

Figure 3. *Hyalomma marginatum* ticks. (A) Adulte male. **(B)** Unfed (left) and engorged (right) adulte females. From (Turell, 2007).

Hyalomma ticks have a two-host behavior. After hatching of the egg, the larva feeds on a host, typically species of the *Rodentia* or *Lagomorpha* order or of the *Aves* class, and molts while remaining attached to its host. The nymph feeds on the same host and returns to the ground for molting. As an adult, *Hyalomma* ticks find another host, preferentially large ungulates, but also species from the *Lagomorpha*, on which they will feed and multiply. Female ticks then return to the ground to lay more than 1000 eggs. Contrary to most ticks which are "ambush ticks", waiting passively to encounter a host, *Hyalomma* ticks are "hunting ticks" that can travel up to 400m to attach to a host (Bente et al., 2013).

2. Transmission of CCHFV to animals and humans

CCHFV circulates silently in the nature in a tick-vertebrate-tick cycle (Hoogstraal, 1979). Transmission to humans occurs through tick bites, but also from contact with fluids from infected, viremic animals, as shown in Figure 4.

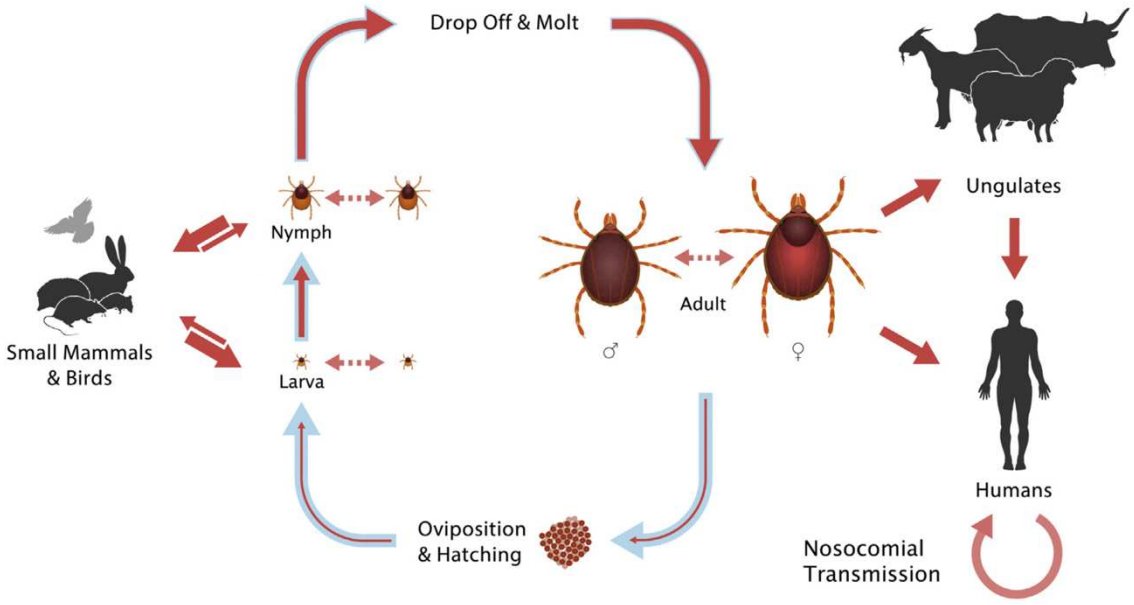

Figure 4. Transmission of CCHFV in ticks and mammals. The course of the tick life cycle is indicated with blue arrows. Solid red arrows indicate transmission opportunities of CCHFV from ticks to mammals, from mammals to ticks, or from mammals to mammals. The thickness of the red arrow indicates the efficiency of CCHFV transmission. CCHFV transmission from tick to tick through co-feeding is represented through dashed, red arrows. From (Bente et al., 2013).

Spread of CCHFV occurs mainly during spring and summer, when larvae and nymphs feed on their hosts and females prepare to lay eggs (Bente et al., 2013). Ticks become infected through feeding on their host, which are small mammals or birds for the larvae and nymphs, and larger hosts for adults, such as cattle or humans. Opportunities to transmit CCHFV to its host occur at each step of the tick life cycle.

3. Susceptible species for CCHFV infection

As CCHFV infection does not lead to symptomatic outcomes in most animals, serological surveys have been the main source of information to determine which animals can be infected by CCHFV. Historical testing methods for detection of

antibodies directed against CCHFV included agar gel diffusion precipitation (AGDP), reverse passive hemagglutination inhibition (RPHI), complement fixation (CF) and neutralization assays (Casals and Tignor, 1974; Faghih et al., 1975; Gaidamovich et al., 1973; Swanepoel et al., 1983). However, these methods were slowly replaced by more reliable and sensitive tests such as immunofluorescence assays (Saijo et al., 2002) or enzyme-linked immunosorbent assays (ELISAs), mainly using recombinant CCHFV nucleocapsid protein (NP) as target antigen. Many ELISAs have been developed, reviewed in (Raabe, 2020). Moreover, experimental infections of laboratory animals with CCHFV have helped identifying more precisely the range of hosts that CCHFV targets.

a. Infection of domestic animals

Sero-epidemiological surveillance of CCHFV occurs in regions where the virus is endemic. It reveals that many domestic animals are susceptible to CCHFV infection, although the infection remains asymptomatic in these species. A comprehensive review of sero-epidemiological studies of CCHFV in domestic animals recapitulates the main findings (Spengler et al., 2016a). Notably, high seroprevalence was found in species such as cattle and sheep (79,1% and 75% in Afghanistan, respectively) (Mustafa et al., 2011), goats (66% in Turkey) (Tuncer et al., 2014), horses (58,8% in Iraq) (Ceianu et al., 2012), or camels (26% in Kenya) (Morrill et al., 1990). Only one study, realized in Zimbabwe, explored the seroprevalence in companion animals such as dogs, and found through RPHI that 6% of the 1978 dogs tested had antibodies against CCHFV (Shepherd et al., 1987b). Three studies focused on pigs in Egypt, India and Russia; out of 222 animals tested, none were positive for the presence of CCHFV antibodies (Darwish et al., 1978; Mourya et al., 2012; Spengler et al., 2016a).

b. Infection of wild animals

Study of CCHFV seroprevalence in wild species is important to determine their role in the natural ecology of CCHFV. In coherence with the fact that large mammals are the preferred host of the adult *Hyalomma* tick, studies from South Africa and Zimbabwe found that large herbivores had the highest CCHFV antibody seroprevalence, such as giraffes (23 – 100%), rhinoceroses (40 – 68%), elands (46%), gemsboks (46,2%), waterbucks (44,4%), nyalas (40%), kudus (21,8 – 50%) or zebras (17%) (Burt et al., 1993; Shepherd et al., 1987b). However, all non-human primates that were sampled were negative.

As immature *Hyalomma* tick preferentially feed on small mammals, the latter also play a significant role in CCHFV ecology. Indeed, hares probably are amplifying hosts of ticks, and therefore of CCHFV with a seroprevalence of 3 – 22%, as well as several hedgehogs (with variable roles depending on the species) (Burt et al., 1993; Hoogstraal, 1979; Nalca and Whitehouse, 2007; Shepherd et al., 1987b; Spengler et al., 2016a). Bats were also found to be susceptible to CCHFV as detected in France in 1969 or Iran in 1975 (Hoogstraal, 1979; Saidi et al., 1975), and more recently, other viruses from the same family as CCHFV (nairoviruses) were discovered in bats (Dacheux et al., 2014; Ishii et al., 2014).

c. Infection of birds

Birds can convey numerous ticks and are thought to be in part responsible for their intercontinental transport and dissemination of CCHFV (Hoogstraal et al., 1963, 1961). For instance, CCHFV-positive ticks have been found on migratory birds in Turkey (Leblebicioglu et al., 2014), Greece (Lindeborg et al., 2012), Morocco (Palomar et al., 2013) or, more recently, in Italy (Mancuso et al., 2022). However, most birds are refractory to CCHFV infection. Russian studies in the 1970's stipulated that rooks and rock doves did not develop symptoms, nor viremia, nor seroconversion after CCHFV inoculation (Hoogstraal, 1979), but the question of CCHFV infection of birds was raised anew in 1975 by one study from Semashko that detected CCHFV antibodies with a low seroprevalence of 0.2% in chicken and ducks, and another one from Zarubinsky that detected antibodies in a magpie. Almost 20 years later, CCHFV antibodies were detected by ELISA in red-beaked hornbills, glossy starlings and guinea fowls in Senegal (Zeller et al., 1994a). More importantly, *Hyalomma* ticks fed on red-beaked hornbills or long-tailed glossy starlings experimentally inoculated with CCHFV were infected despite a lack of viremia in the birds. These ticks also were able to further transmit the virus to rabbits and to their progeny (Zeller et al., 1994b). These data prove that even if birds do not show detectable viremia, they play an important role in CCHFV dissemination.

Ostriches represent another interesting exception to the refractiveness of birds to CCHFV infection. Indeed, in November 1984, a worker was infected by CCHFV shortly after having slaughtered ostriches in South Africa. Following this case, sero-epidemiological studies in the neighboring farms detected CCHFV antibodies in the sera from ostriches, but not other birds (Shepherd et al., 1987a). Since then, another outbreak of 17 cases among workers at an ostrich slaughterhouse happened in 1996

(Capua, 1998) and 4 cases in 2007 in workers at an ostrich farm in Iran (Mostafavi et al., 2013). Moreover, experimentally infected ostriches developed viremia from day 1 to day 4 post-infection, virus was detectable in visceral organs until 5 days post-infection, and they developed antibodies directed against CCHFV (Swanepoel et al., 1998). Since then, it has been implemented in South Africa that ostriches be free of ticks 14 days before slaughter, to help prevent CCHFV transmission to humans.

4. Modes of transmission of CCHFV to humans

CCHFV is primarily transmitted to humans through bites of infected ticks, or by crushing engorged, CCHFV-infected ticks. Moreover, contact with infected animals represents another mode of transmission of CCHFV, through direct contact with tissues or blood of infected animals, both wild and domestic. Notably, contact with infected animal carcasses, particularly during slaughtering or butchering, poses a significant risk of transmission (Hoogstraal, 1979).

Human-to-human transmission of CCHFV is also possible. In healthcare settings, CCHFV can be transmitted from person to person through contact with infected blood or body fluids. In some cases, it is probable that CCHFV was transmitted through aerosols to health-care workers, when the patients were ventilated (Celikbas et al., 2014; Pshenichnaya and Nenadskaya, 2015).

Three cases of possible sexual transmission of CCHFV were reported (Pshenichnaya et al., 2016). Vertical transmission also occurs, as three cases of intrauterine or perinatal infections with CCHFV were described, all of them fatal for the baby (Ergönül et al., 2010).

Finally, in 1967, several cases of laboratory-acquired CCHF were described in Africa (Simpson et al., 1967; Woodall, 2007); thus, CCHFV has been classified as a biosafety level (BSL)-4 pathogen to help limit these infections.

III. Symptoms, lesions, diagnostic, treatments, and vaccination perspectives

1. Clinical and pathologic features of CCHFV infection

a. Clinical symptoms

While CCHFV infects many mammals, the infection is symptomatic only in humans. Among the CCHFV-infected people, 80% do not develop any clinical symptoms, whereas 20% of them develop a Crimean-Congo hemorrhagic fever (CCHF) (Goldfarb et al., 1980). CCHF develops in four phases, namely the incubation period, the pre-hemorrhagic period, the hemorrhagic period, and, in case of survival of the patient, convalescence (Ergönül, 2007) (Figure 5).

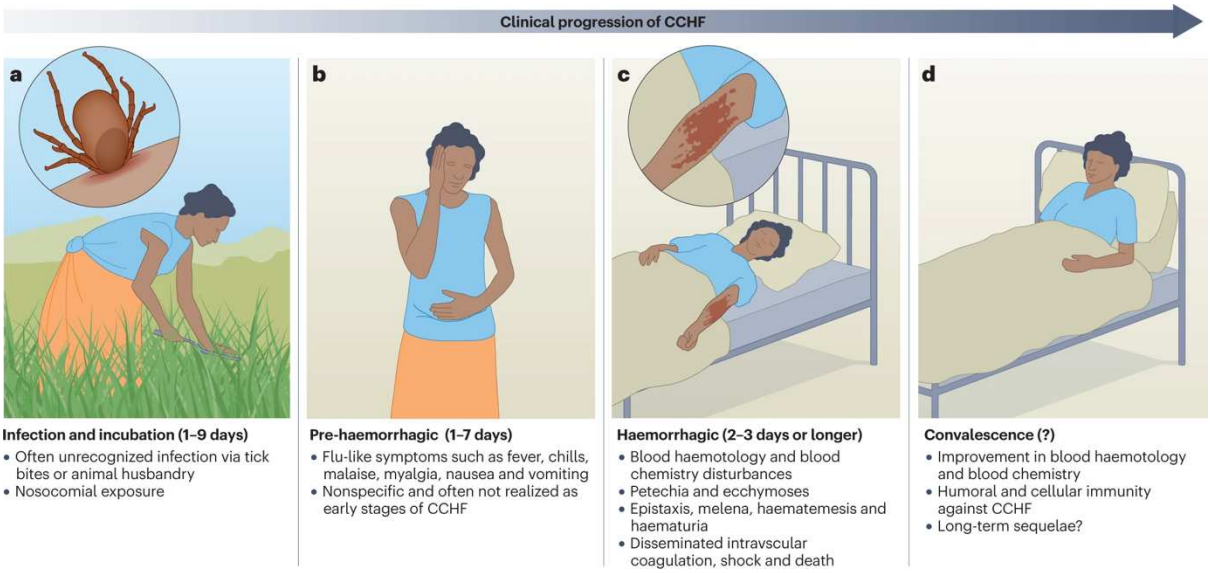

Figure 5. Clinical progression of CCHF. (a) The incubation period begins when CCHFV is transmitted to the patient, either through tick bites, animal husbandry, or through nosocomial exposure. This period lasts from 1 to 6 days. **(b)** The pre-hemorrhagic period includes non-specific, flu-like symptoms and lasts from 1 to 5 days. It is then followed by **(c)** the hemorrhagic stage, that lasts from 1 to 10 days or results in death at the peak of the period. During this stage, patients exhibit hemorrhages ranging from small petechiae to large ecchymoses as well as internal bleedings. **(d)** The convalescent phase begins around 10 to 20 days after onset of illness. From (Hawman and Feldmann, 2023).

The incubation period depends on the mode of transmission of the virus. After a tick bite from a CCHFV-infected tick or crushing a CCHFV-infected tick, the incubation varies from 1 to 6 days. Precise data are difficult to obtain, the exact dates of the tick bites being often not known. On the other hand, when the transmission occurs

through contact with tissues or blood from infected animals or humans, the incubation period is slightly longer (5 to 6 days) (Ergönül, 2007; Hoogstraal, 1979). The onset of the symptoms during the pre-hemorrhagic period is sudden. This period regroups unspecific symptoms such as fever, headache, myalgia, and dizziness (Ergönül, 2007; Hoogstraal, 1979; Swanepoel et al., 1989), and lasts from 1 to 5 days. It is then followed by the hemorrhagic period, which begins on day 3 to 5 of illness, and lasts from 1 to 10 days or results in death at the peak of the period. During this phase, patients harbor hemorrhages ranging from small petechiae to large hematomas on mucous membranes and skin, and bleedings from various sites (Figure 6). Bleedings from the respiratory tract (hemoptysis and epistaxis), the gastro-intestinal tract (hematemesis, melena, and intra-abdominal bleeding), the urinary tract (hematuria) and from injections sites are common, but vaginal, gingival, and cerebral hemorrhages also have been reported (Ergönül, 2007; Hoogstraal, 1979; Kleib et al., 2016). The convalescent phase begins about 10-20 days after onset of illness and is associated with less severe symptoms such as hypotension, asthenia, and labile pulse (Hoogstraal, 1979). To date, it remains unknown whether CCHFV infection leaves long-term sequelae.



Figure 6. Ecchymoses in a CCHF patient. From (Ergönül, 2007).

b. Histopathologic changes upon CCHFV infection

Autopsies of human patients infected with CCHFV revealed through immunohistochemistry and *in situ* hybridization that the primary targets of CCHFV are hepatocytes, mononuclear phagocytes, and endothelial cells (Burt et al., 1997). These observations were confirmed both *in vitro* (Connolly-Andersen et al., 2011, 2009; Lindquist et al., 2018; Peyrefitte et al., 2010) and *in vivo* (Welch et al., 2019; Zivcec et al., 2013) in murine models of infection. Additionally, immunohistochemistry and *in situ* hybridization performed in a mouse model of neurological infection (humanized NOD-SCID-γ (NSG)-SGM3 mice) showed that glial cells are also a target for CCHFV infection (Spengler et al., 2017). More recently, a recombinant fluorescent reporter virus in a murine model of infection allowed the identification of other organs that are targeted by CCHFV, such as the female reproductive tract (notably the ovaries and uteruses) (Welch et al., 2019). Furthermore, male cynomolgus macaques infected with CCHFV had detectable levels of viral RNA in their reproductive organs (Smith et al., 2019b), further suggesting that CCHFV is able to infect the reproductive tract of various mammals. This is could in part explain the vertical transmission of CCHFV in humans that has been mentioned earlier.

c. Biochemical changes upon CCHFV infection

During infection, numerous changes in laboratory variables appear and are associated with the severity of the cases (Bente et al., 2013). First, early leukopenia and thrombocytopenia are developed, followed by abnormalities in coagulation, including prolonged prothrombin time (PT), prolonged activated partial thromboplastin time (aPTT), and detection of fibrin degradation products, which are characteristics of disseminated intravascular coagulation (DIC). Moreover, elevation in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels are detected, markers of organ lesions (Figure 7). Finally, elevated levels of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) or interleukin-6 (IL-6) are associated with fatal cases (Bente et al., 2013; Ergönül, 2007; Ergönül et al., 2006).

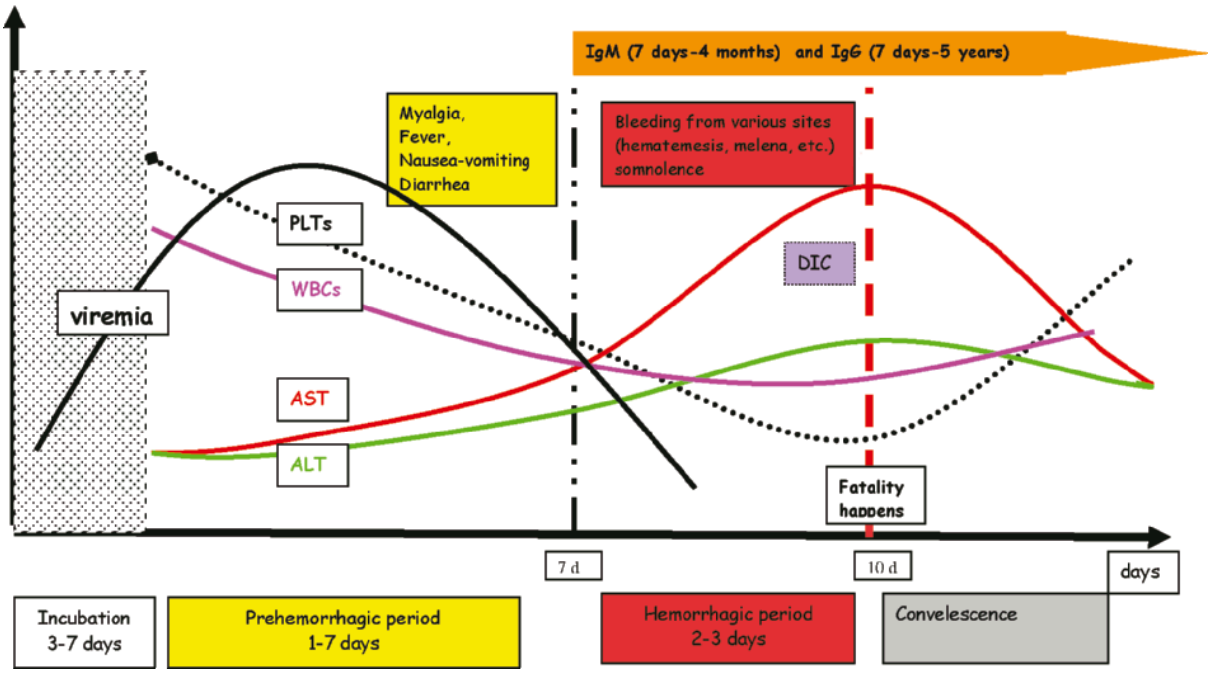

Figure 7. Biochemical and clinical changes upon CCHFV infection in patients. PLTs: platelets; WBCs: white blood cells; AST: aspartate aminotransferase; ALT: alanine aminotransferase; DIC: disseminated intravascular coagulation. Adapted from (Ergönül, 2007).

d. Mortality rates

Throughout the years, different mortality rates for CCHFV have been published, varying from 5 to 30% (Hoogstraal, 1979). These discrepancies can be explained by different hypotheses. First, distinct strains of CCHFV can have different virulence. For instance, the strain AP92 was considered to be of low virulence (Papa et al., 2014) until it was declassified and named Aigai virus, closely related but distinct from CCHFV (Papa et al., 2022). Another explanation is that these mortality rates are overestimated. Indeed, the access to health systems and their quality that differ between countries might explain these differences, as well as the general tolerance of the patients who may go to the hospital with milder symptoms than in other countries and are thus included in the number of diagnosed cases. Furthermore, the development and wider distribution of diagnostic methods also impact the mortality rate, more mild cases being diagnosed. Finally, potential co-existent infections are sometimes a reason for a higher mortality rate (Ergönül, 2007).

2. Diagnosis

a. Epidemiological elements and differential diagnosis

Due to the lack of specificity of the symptoms from the pre-hemorrhagic phase, it is difficult to diagnose CCHFV infections. Differential diagnosis includes infectious diseases, such as other viral hemorrhagic fevers (caused by viruses from the families *Arenaviridae, Filoviridae, Flaviviridae, Hantaviridae, Phenuiviridae* and *Reoviridae*), other viral infections (notably viral hepatitis), or bacterial or parasitic infections (such as leptospirosis or malaria). Non-infectious diseases must be considered as well, such as B12 vitamin deficiency (Ergönül, 2007). Thus, CCHF diagnosis must associate clinical and epidemiological elements, and the disease must be suspected if the patient lives in or traveled to an endemic region, if there was a tick bite or if there was exposure to animal blood or tissues (Ergönül, 2006).

b. Laboratory diagnosis

Experimental diagnosis is necessary to confirm the infection by CCHFV. This experimental diagnosis can be performed by either direct identification or indirect identification of CCHFV.

Direct identification includes viral isolation through the culture of the virus on susceptible cell lines; however, due to the constraints inherent to CCHFV culture in BSL-4 settings, this method is not used anymore. RT-PCR to detect viral RNA in the patient samples is quicker and sensitive, but this method is useful only when the patient is viremic, *i.e.,* during the first week of the disease (Raabe, 2020). As the severity of the disease is associated with elevated viral load, this method can be used as prognostic factor. However, some strains were sometimes not detected by this method due to nucleotide divergence between the strains. To counteract this, primers were developed that target the 5′ untranslated region (UTR) of the S segment, which is well conserved, to allow the detection of 18 different strains of CCHFV (Atkinson et al., 2012).

Indirect serological testing of CCHFV is useful after the first week of disease, when specific antibodies have been developed against the virus (Figure 7). Methods such as agar gel diffusion precipitation (AGDP), reverse passive hemagglutination inhibition

(RPHI) or complement fixation (CF) or enzyme-linked immunosorbent assays (ELISAs) can be used, the latter being the most common technique (Raabe, 2020).

3. Treatments

a. Symptomatic treatment

Asymptomatic or mild infections generally do not require treatment. On the contrary, treatment of more severe infections mainly relies on supportive therapy, like the administration of thrombocytes or fresh frozen plasma from healthy human donors.

b. Etiologic treatment

Currently, there are no etiologic treatments available against CCHFV. It was shown that a guanosine analog, ribavirin, inhibited CCHFV replication targeting the viral polymerase through inhibitory competition, both *in vitro* (Bergeron et al., 2010; Paragas et al., 2004; Watts et al., 1989) and *in vivo* (Bente et al., 2010; Tignor and Hanham, 1993). This broad-spectrum antiviral molecule was used to treat CCHFV-infected patients during more than 20 years, but its effectiveness was only described in observational studies, and not in randomized clinical trials, except for one trial that did not confirm its beneficial role (Koksal et al., 2010). More recently, studies performed in type I interferon-deficient mice showed that ribavirin was efficient to inhibit CCHFV infection at early time points, but was ultimately unable to prevent the disease, while favipiravir, a pyrazine-carboxamide derivative, inhibited CCHFV infection at all time-points (Hawman et al., 2018; Oestereich et al., 2014). The efficacy of favipiravir against CCHFV was confirmed in a cynomolgus macaque model of infection (Hawman et al., 2020).

Interferons (IFN) are potent cytokines inducing an antiviral state and can be used clinically against some viruses, such as hepatitis C virus. However, a clinical trial involving IFN as treatment against CCHFV did not show its beneficial effect (van Eeden et al., 1985).

4. Prevention

a. Control of ticks in the environment and control measures for humans

Control measures to ensure the limitation of CCHFV infections involve controlling the tick bites. Kinetics of CCHFV transmission from the tick to its host is unknown. For this reason, it is recommended to remove as quickly as possible any tick found on the body. To limit the exposure to the virus, it is essential to inform the public of its existence. The World Health Organization (WHO) recommends wearing protective clothes, using acaricides and avoiding zones where ticks are present. To limit nosocomial infections, full personal protective equipment is required to handle patients suspected to be infected by CCHFV.

b. Medical prophylaxis

To date, there are currently no vaccines approved against CCHFV. From 1974 to 1996, an inactivated suckling mouse brain-derived vaccine was used in Bulgaria for protection against CCHFV (Papa et al., 2011). However, lack of clinical trials and safety problems relative to this vaccine do not make it a viable solution.

To develop a vaccine against CCHVF, several targets exist. The nucleoprotein (NP) is one of them; despite its intra-particular location, it was shown to be secreted (Zhou et al., 2011; Freitas et al., 2020), to possess numerous B and T epitopes (Liu et al., 2014; Moming et al., 2018; Shrivastava et al., 2020; Wei et al., 2010) and to be well-conserved (Hewson et al., 2004a). Other obvious targets are the envelope glycoproteins Gn and Gc, involved in viral entry. *In vitro*, several neutralizing antibodies have been described, targeting Gc (Bertolotti-Ciarlet et al., 2005). However, they were not protective in a murine model of infection (Golden et al., 2019). Another protein arisen from maturation of the glycoprotein complex (GPC), namely GP38, represents a promising vaccine target, as non-neutralizing antibodies, plasmid-derived DNA vaccines, or viral vectoral vaccines targeting this protein were protective in murine models of infection (Garrison et al., 2017; Golden et al., 2019; Scher et al., 2023; Suschak et al., 2021).

Several vaccinal approaches have been tested to develop a CCHFV-targeting vaccine, including plasmid-derived DNA vaccines, messenger RNA (mRNA) vaccines, viral pseudoparticles or viral vector vaccines, but to date, none of them are at the step of

human clinical trial. The advances in CCHFV vaccine development are reviewed in (Ozdarendeli, 2023; Tipih and Burt, 2020).

IV. Taxonomy and genetic diversity of CCHFV

1. Taxonomy

Among the *Bunyavirales* order are more than 500 viruses responsible for infections and diseases in numerous hosts, such as plants, animals, and humans. According to the International Committee on Taxonomy of Viruses (ICTV), there are 13 families of viruses among this order (Kuhn et al., 2022), and five of them are associated to diseases in humans: *Arenaviridae, Hantaviridae, Nairoviridae, Peribunyaviridae* and *Phenuiviridae.* Notably, the *Nairoviridae* family contains tick-borne, enveloped, single-stranded RNA virus with a tri-segmented genome of negative or ambisense polarity. This family is divided in 7 genera, the *Orthonairovirus* genus being the one that contains the most viruses with more than 40 species and viruses. The nomenclature of this genus is frequently modified, according to new discoveries and new classifications of viruses, such as Meram virus, which was discovered in 2020 (Ergünay et al., 2020), Estero Real virus which was previously classified as a member of the *Peribunyaviridae* family (Nunes et al., 2018), or Aigai virus, which was first identified as a low pathogenic strain of CCHFV, member of the genogroup VI, before it was classified as a distinct virus, member of the *Congoid orthonairovirus* species (Papa et al., 2022).

Orthonairoviruses infect many species, among which humans. To date, CCHFV is the most pathogenic orthonairovirus for humans, but not the only one, as Nairobi sheep disease virus (NSDV), Ganjam virus, Dugbe virus, Erve virus, Kasokero virus, Issyk-kul virus and Soldado virus are the suspected etiologic agents of febrile diseases, headaches, pruritus and diarrheas (Burt et al., 1996; Chastel, 1998; Dandawate et al., 1969; Kalunda et al., 1986; L'vov et al., 1984; Moussa et al., 1975; Rao et al., 1981; Treib et al., 1998). Other vertebrates are infected by orthonairoviruses, sometimes in an asymptomatic manner or on the contrary with very important symptoms. Indeed, NSDV and Ganjam virus are responsible in sheep and goats for a hemorrhagic gastroenteritis in Africa and Asia respectively, with a mortality rate of more than 90% (Davies, 1997). Similarly, immunocompetent laboratory mice infected with Leopard Hill virus, an orthonairovirus identified in bats, develop similar symptoms of gastroenteritis (Ishii et al., 2014).

2. Genetic diversity

Viral RNA-dependent RNA polymerases (RdRp) are prone to errors; thus, RNA viruses accumulate mutations and expose a high level of genome plasticity. The exact mutation rate of CCHFV L protein, which bears the RdRp activity, is unknown. However, nucleotide variations of 20, 31, and 22% for the S, M and L segments of CCHFV genome were respectively found, and amino acid variations vary from 8, 27, and 10% for the nucleoprotein (NP), the glycoprotein precursor complex (GPC) and the L protein, respectively. Thus, distinct virus groups were revealed, based on phylogenetic comparison of 15 CCHFV genomes and their approximate geographic distribution (Deyde et al., 2006), as mentioned earlier in the manuscript.

Moreover, CCHFV genome can undergo reassortments, a phenomenon possible due to the segmented nature of its genome (Hewson et al., 2004b; Zhou et al., 2013). Analysis of these reassortments have been used to study migration and geographical circulation of CCHFV.

V. CCHFV structure, genome, and viral proteins

1. CCHFV structure and genome

CCHFV was structurally characterized by electron microscopy and its structure was found to be typical of the *Bunyavirales* order, *i.e.* "spherical particles measuring about 90-150 nm with distinct surface projections approximately 8-10 nm in length", corresponding to the envelope glycoproteins, Gc and Gn (Korolev et al., 1976; Murphy et al., 1973) (Figure 8).

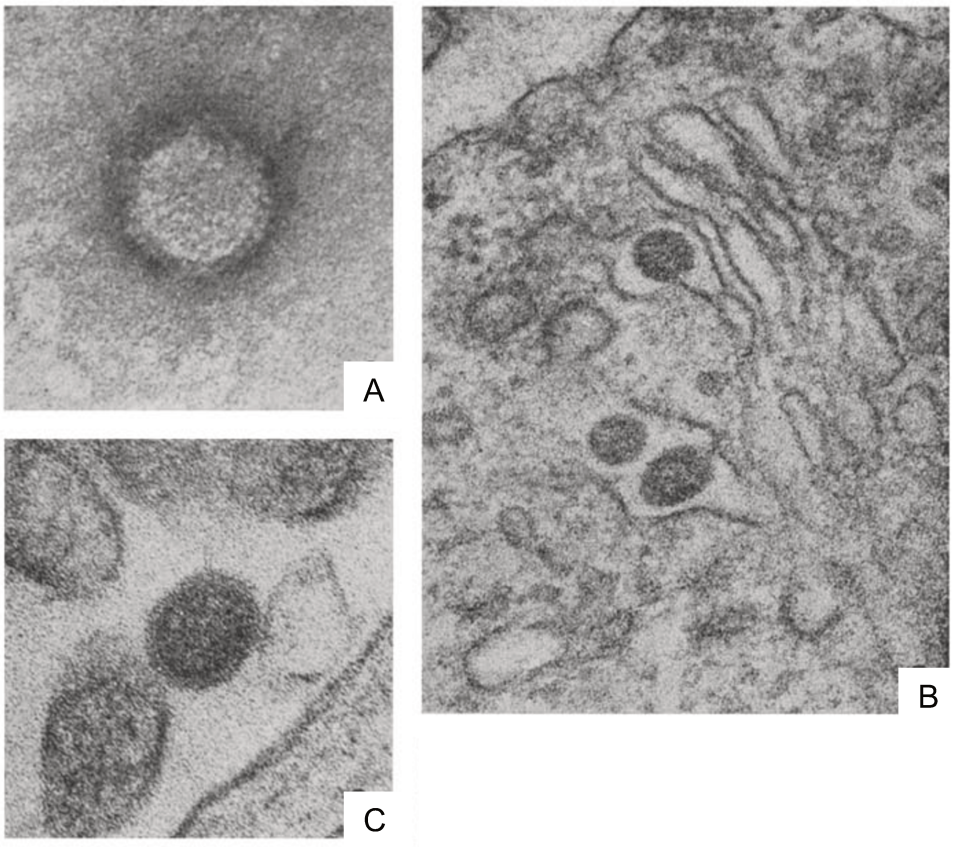

Figure 8. Electron microscopy of CCHFV particles. (A) Negatively stained virus particle from a porcine kidney cell culture preparation. Magnification x170,000. **(B)** CCHVF particles within cisternae of the Golgi complex. Magnification x60,000. (**C)** Extracellular viral particle. Magnification x130,000. From (Korolev et al., 1976).

As an orthonairovirus, CCHFV genome is constituted of three segments of single-stranded RNA (ssRNA). The smallest segment, the S segment, is of ambisense polarity and has a size of 1.6 kb in average. The medium segment (M segment) and large segment (L segment) are of negative polarity and measure 5.4 kb and 12.1 kb, respectively (Figure 9).

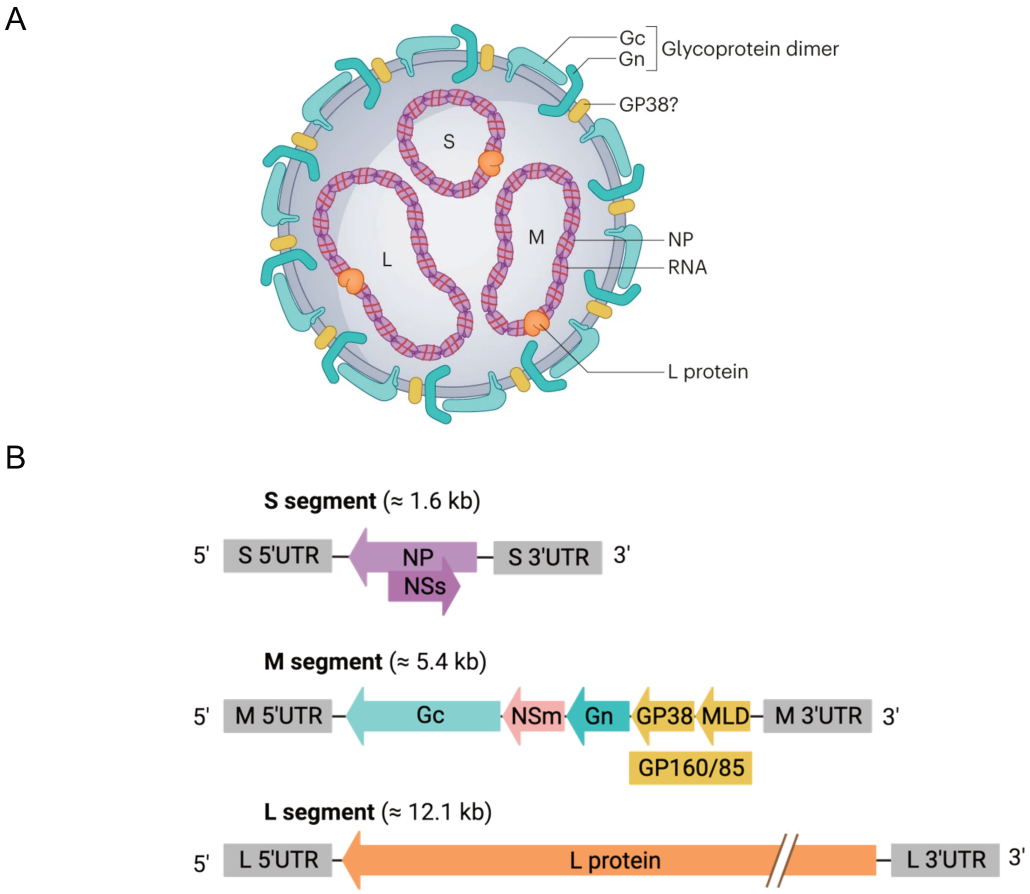

Figure 9. Schematic representation of CCHFV genome. (A) A typical CCHFV particle is represented. In this schematic representation, GP38 is represented in the viral envelope, but this remains to be formally demonstrated. **(B)** CCHFV genome is schematically represented. The viral S segment encodes two proteins, NP in negative-sense and NSs in an overlapping, positive sense. The viral M segment encodes a glycoprotein precursor (GPC) (not represented in the figure), which is further matured into different proteins (Gn, Gc, NSm, MLD, GP38) that are depicted here on the viral genome. The L segment encodes the L protein. All segments contain specific untranslated regions (UTRs). GPC: glycoproteins precursor; NP: nucleoprotein; NSm: non-structural protein M; NSs: non-structural protein S; MLD: mucin-like domain; UTR: untranslated region. Adapted from (Hawman et al., 2023).

Similar to other bunyaviruses, CCHFV segments all contain untranslated regions (UTRs) at their 5′ and 3′ ends, that flank the open reading frames (ORFs) and that are partially complementary, allowing the pseudo-circularization of the genome with the complementary UTRs shaped as panhandles (Jeeva et al., 2017b) (Figure 10).

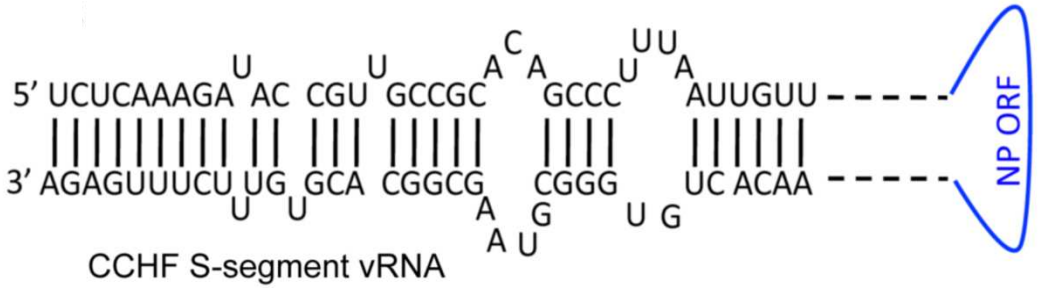

Figure 10. Schematic representation of the complementarity of CCHFV S segment 5′ and 3′UTRs. From (Jeeva et al., 2017b).

2. CCHFV encoded proteins

CCHFV encodes 10 viral proteins, as depicted in the Table 1 and Figure 9.

Table 1. CCHFV encoded viral proteins and their known roles

Segment	Viral protein	Known roles	References
S segment	Nucleoprotein (NP)	Formation of viral ribonucleoproteins (vRNPs)	(Jeeva et al., 2017b, 2019)
		Augmentation of viral mRNA translation	(Jeeva et al., 2017a)
		Endonuclease activity	(Guo et al., 2012)
		Inhibition of apoptosis	(Karlberg et al., 2015, 2011)
	Non-structural protein s (NSs)	Induction of apoptosis	(Barnwal et al., 2016)
M segment	Gc	Receptor binding and entry	(Mishra et al., 2022; Zivcec et al., 2016)
	Gn		
		Liaison with viral RNA	(Estrada and De Guzman, 2011)
	NSm	Promoting of GPC processing and virion assembly	(Freitas et al., 2020)
	Mucin-like domain (MLD)	Unknown	
	GP38, GP85 and GP160	Maturation of GPC	(Freitas et al., 2020)
		Presence at the surface of the virion and at the plasma membrane; biological function unknown	(Golden et al., 2019)
		Secreted proteins; biological function unknown	(Sanchez et al., 2006)
L segment	L protein	RNA-dependent RNA polymerase	(Honig et al., 2004)
		Endonuclease and cap-snatching activities	(Devignot et al., 2015; Holm et al., 2018)
		Formation of viral ribonucleoproteins (vRNPs)	(Macleod et al., 2015)
		Inhibition of innate immune responses through deubiquitylation and deISGylation	(Scholte et al., 2017)

a. S segment-encoded proteins

The S segment of CCHFV encodes in its negative polarity the nucleoprotein (NP). Crystal structure of NP shows that it has a globular domain, the head, and an arm or stalk domain (Carter et al., 2012a, 2012b; Guo et al., 2012; Wang et al., 2015, 2012) (Figure 11). NP oligomerizes through interactions between the head domain and the arm domain of another NP to form an antiparallel double superhelix polymer of nine NP per helix (Macleod et al., 2015; Wang et al., 2012), but monomeric forms of NP are also found in infected cells (Wang et al., 2016).

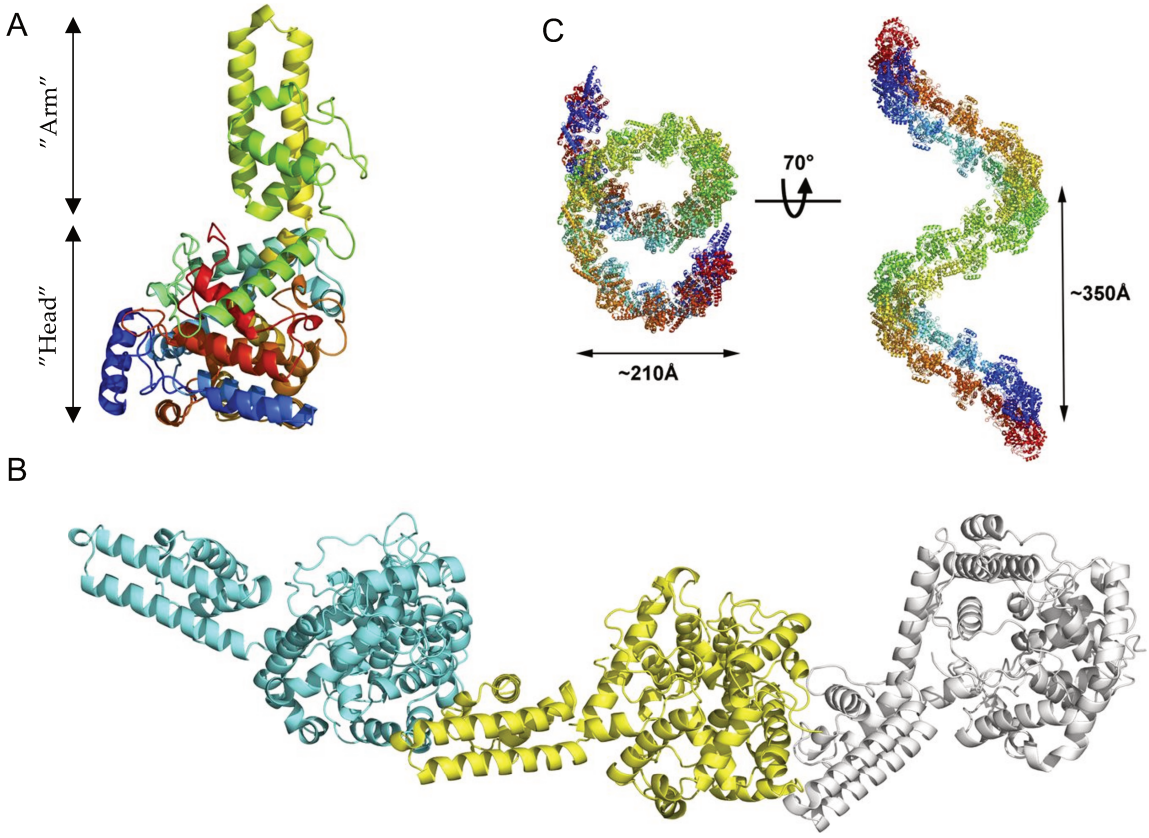

Figure 11. Crystal structure of CCHFV NP (strain IbAr10200) and its homo-oligomerization. (A) Crystal structure of NP. The structure is colored in rainbow from blue (N-terminus) to red (C-terminus)**. (B)** Head-to-tail oligomerization of three NP molecules (colored in blue, yellow, and white). **(C)** Antiparallel double superhelix polymer of nine NP per helix. Adapted from (Wang et al., 2012).

The primary function of NP is to encapsidate the viral genome and antigenome to form viral ribonucleoproteins (vRNPs). To date, it remains unclear how only viral genomic RNAs and not complementary, antigenomic RNAs are packaged into newly formed virions. NP binds with high affinity to the panhandle structure of the genome segments, in a sequence-dependent manner. On the contrary, NP also binds single-

stranded RNA (ssRNA), whether viral or non-viral, in a sequence-independent manner (Jeeva et al., 2017b). Truncation mutants revealed that NP binds double stranded RNA (dsRNA) structures such as the viral panhandle through its stalk domain, while it rather binds ssRNA through its globular domain (Jeeva et al., 2019). Interestingly, NP can bind simultaneously viral panhandle and ssRNA, and this dual binding results in a unique conformational change of NP. In particular, the highly conserved nine nucleotides from both 5′ and 3′ ends of the viral segments are bound by NP with a very strong affinity (Jeeva et al., 2017b). Interestingly, CCHFV NP can bind panhandle structures from another orthonairovirus, Hazara virus (HAZV), but not from an hantavirus (Andes hantavirus), suggesting that NP-panhandle interaction is genus-specific (Jeeva et al., 2019).

In addition to binding to genomic and antigenomic RNAs, NP also binds viral messenger RNAs (mRNAs) through their 5′ viral UTR, a phenomenon that preferentially promotes their translation. This phenomenon was likely not dependent on the canonical host translation machinery, as chemical inhibition of the eIF4F complex (composed of the three initiation factors eIF4A, eIF4G and eIF4E) did not impact the ability of NP to increase the translation of viral mRNAs. However, it was dependent on the integrity of the initiation factor eIF4G. More studies are needed to decipher how eIF4G and NP act together to specifically increase the translation of viral mRNAs (Jeeva et al., 2017a).

In addition, mapping of the interaction domains of NP revealed binding sites to the amino- and carboxy-terminal ends of the L protein (Macleod et al., 2015). These interactions suggest a role of NP-L protein complex in transcription and replication of viral genome, as seen for hantaviruses (Cheng et al., 2014).

The S segment also encodes, in its positive polarity, an ORF overlapping the one of NP, resulting in the translation of a non-structural protein called NSs. This protein is actively produced during infection, but undergoes active proteasomal degradation (Barnwal et al., 2016). Both NP and NSs modulates host cell apoptosis. Indeed, CCHFV infection induces host cell apoptosis both *in vitro* and *in vivo* (Karlberg et al., 2015; Lindquist et al., 2018; Rodrigues et al., 2012). NSs might play a role in the induction of apoptosis, as it disrupts the mitochondrial membrane potential (Barnwal et al., 2016). Conversely, NP is thought to suppress the induction of apoptosis by Bax and to inhibit the release of cytochrome c from mitochondria (Karlberg et al., 2015). Moreover, it possesses a DEVD site that is cleaved by caspase 3; thus, NP was presented as a

caspase 3 decoy to inhibit apoptosis (Karlberg et al., 2011). Of note, the DEVD cleavage site is inaccessible on the oligomeric forms of NP (Wang et al., 2012).

b. M segment-encoded proteins

CCHFV M segment encodes a unique protein, the glycoproteins precursor complex (GPC). CCHFV GPC has the most complex structure among bunyaviruses. GPC maturation, described later in this manuscript (Figure 13), ultimately results in the generation of Gn and Gc glycoproteins, as well as non-structural protein NSm, mucin-like domain (MLD), GP38 and GP160/85.

Gn and Gc are the envelope proteins of CCHFV and form at its surface a locally ordered lattice of heterodimers (Mishra et al., 2022). Gc is a class II membrane fusion protein and neutralizing antibodies arisen against CCHFV glycoproteins all target Gc (Bertolotti-Ciarlet et al., 2005; Fels et al., 2021).

Located between Gn and Gc and containing several transmembrane domains (Altamura et al., 2007), NSm promotes glycoprotein processing and virion assembly in a pseudoparticle context (Freitas et al., 2020). However, a recombinant virus lacking NSm could be generated, suggesting that NSm is not necessary for viral replication (Welch et al., 2020), at least in vertebrates. The N-terminal end of the GPC is cleaved several times to give rise to a GP160/GP85 protein that is further processed into a mucin-like domain (MLD) that is heavily glycosylated and a secreted viral protein, GP38 (Sanchez et al., 2006, 2002). GP38 is necessary to promote GPC processing and virion assembly (Freitas et al., 2020), and there is limited evidence that it is present at the surface of the virion and the plasma membrane (Golden et al., 2019). Moreover, GP38 is secreted either alone or linked to the MLD, as GP160/85 (Sanchez et al., 2006, 2002), but its biological role as secreted protein remains to be deciphered.

c. L segment-encoded protein

CCHFV L protein bears the RNA-dependent RNA polymerase (RdRp) activity necessary for viral transcription and replication (Honig et al., 2004). Moreover, it also bears an endonuclease domain responsible for snatching the cap from cellular mRNAs to initiate the transcription of viral mRNAs (Devignot et al., 2015).

With more than 12 000 nucleotides, CCHFV L protein is uniquely large among the *Bunyavirales* order. Indeed, it possesses an ovarian tumor (OTU) domain at its N-terminal end. While this domain is dispensable for L protein RdRp activity (Bergeron et al., 2010), its deubiquitylation (DUB) and deISGylation activities have a role in modulating the host innate immune response, that is discussed later in this manuscript (see Chapter 3, Part IV).

VI. CCHFV replication cycle

All viruses are obligatory intracellular parasites, but depending on their genome and structure, they have developed various way to replicate. CCHFV replication cycle takes place within the cytoplasm and its different steps are described in Figure 12 and in the text below.

1. Entry

The cycle of CCHFV infection begins, as for all viruses, with the step of viral entry. As an enveloped virus, CCHFV glycoproteins Gn and Gc are involved in the step of viral entry. Entry into host cells occurs through receptor-mediated endocytosis. The receptor of CCHFV is not yet known. Even if interactome studies revealed an interaction between Gc and nucleolin, the latter implication in the early steps of the viral cycle remains to be studied (Xiao et al., 2011). Following the interaction between the virus and its receptor, the virus is internalized through cholesterol- and clathrin-dependent endocytosis (Garrison et al., 2013; Simon et al., 2009). Rab5 protein as well as protein from the ESCRT machinery are necessary to transport CCHFV from the early endosomes to the multivesicular bodies (MVBs), suggesting that MVBs are the organelles where membrane fusion occurs (Shtanko et al., 2014). CCHFV is also thought to enter into the cytoplasm via early endosomes (Garrison et al., 2013). Within these organelles, the acidic environment triggers the dissociation of the heterodimer formed between Gc and Gn, and Gc changes its conformation into a trimer of hairpins to allow membrane fusion (Mishra et al., 2022). Following the fusion between viral and endosomal membranes, the vRNPs are released into the cytoplasm.

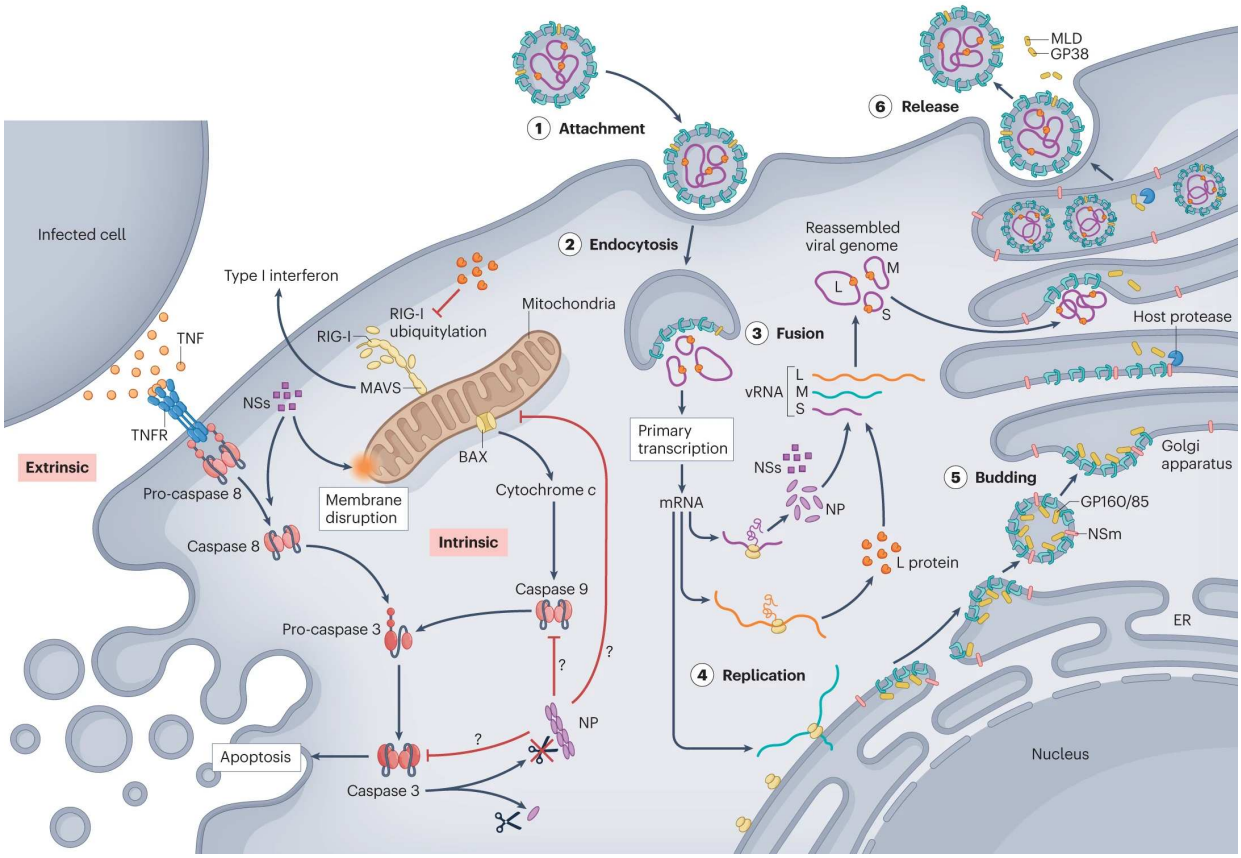

Figure 12. CCHFV replication cycle. (1) and (2) CCHFV enters the cell through receptor-mediated endocytosis. **(3)** The viral genome is released in the cytoplasm following membrane fusion. **(4)** The RdRp transcribes the viral genome into viral mRNAs, followed by translation and maturation of viral proteins. The RdRp also syntheses new negative-sense viral genome that are coated with NP and a bound L protein. **(5)** The GPC is translated into the endoplasmic reticulum and undergoes series of post-translational maturations and proteolytic processing that eventually lead to the production of the viral glycoproteins Gn and Gc as well as the non-structural proteins NSm, MLD, GP38 and GP160/85. Newly formed genomes are packaged into enveloped particles and bud within the Golgi. **(6)** These particles as well as GP38 and GP160/85 are released extracellularly. Moreover, NP blocks the intrinsic pathway of apoptosis and is cleaved by caspase 3 in its monomeric form. NSs promotes apoptosis through disruption of the mitochondrial membrane or extrinsic apoptotic pathways. The OTU domain of the L protein inhibits RIG-I-mediated IFN responses via its deubiquitylating function. From (Hawman and Feldmann, 2023).

2. Transcription and replication

After the release of the vRNPs into the cytoplasm, they are used as template for transcription and replication. Because of the negative polarity of its genome, CCHFV must carry the complete viral transcription machinery to perpetuate its cycle. The precise site of replication of CCHFV is yet to be described.

The RdRp activity of the L protein is necessary to transcribe the negative-sense genomic RNA into viral mRNA. Moreover, to initiate its transcription, CCHFV RdRp uses 9- to 16-nucleotide-long capped mRNA primers that are snatched from cellular mRNAs by the RdRp (Jeeva et al., 2017a). These snatched cellular caps are likely taken from mRNAs in P bodies located within the host cell, as described for hantaviruses (Mir et al., 2008). L protein also possibly uses a prime-and-realign mechanism similar to hantaviruses to initiate transcription (Garcin et al., 1995; Neriya et al., 2022). The cap-snatching activity of L protein relies on its 693rd residue, an aspartate, as it coordinates a Mn^{2+} that is critical for this activity (Morin et al., 2010). Mutating this residue into an alanine impairs the transcription activity independently of the replication activity of the L protein (Devignot et al., 2015).

For some bunyaviruses from different families (for instance orthobunyaviruses such as La Crosse virus (LACV) or Bunyamwera virus (BUNV) and phleboviruses such as Rift Valley Fever virus (RVFV)), the transcription is coupled to translation. Indeed, it was shown with translation inhibitors that protein synthesis is required for efficient transcription of viral mRNAs (Abraham and Pattnaik, 1983; Ikegami et al., 2005; Patterson and Kolakofsky, 1984; Raju and Kolakofsky, 1986). In fact, sequences of bunyaviruses segments possess transcription termination signals located throughout their genome. In absence of concomitant translation of the nascent viral mRNA, these transcription termination signals are read and lead to an RNA interaction involving the nascent strand, resulting in the termination of the transcription and the formation of truncated transcripts. However, translocating ribosomes on the nascent strand prevent formation of this RNA interaction, and allow the complete transcription of the mRNA by the polymerase (Barr, 2007). This phenomenon seems to be shared by different members of the *Bunyavirales* order, but whether CCHFV also requires a protein synthesis concomitant to its genome transcription remains to be elucidated.

The mechanisms for termination transcription for CCHFV remain unclear but is likely to end before the entire 5′ terminus of the viral RNA (vRNA) is transcribed. Indeed, the 3′ end of the S segment mRNA is truncated from 126 nucleotides (Jeeva et al., 2017a). Interestingly, BUNV and RVFV S and M segment-derived mRNAs also have truncated 3′ ends. For these segments, transcription-termination signals are located within the 5′ UTR of the viral genome and are surprisingly similar, suggesting a conserved mechanism across bunyaviruses to terminate their transcription. However, the L segment of phleboviruses do not contain this sequence motif and for this segment, mRNA termination occurs through a "runoff" transcript approach (Barr et

al., 2006; Blakqori et al., 2012; Ikegami et al., 2007; Lara et al., 2011). This may be a shared feature among bunyaviruses, as Sin Nombre virus, a hantavirus, also appears to have L-segment-derived mRNA of the same length as the antigenome (Hutchinson et al., 1996).

CCHFV replication occurs thanks to the L protein that is bound to the vRNP to result in the production of positive-sense, complementary RNA (cRNA) that is encapsidated by the NP to form the antigenome. This antigenome serves as template to form new negative-sense, genomic RNA (Bergeron et al., 2010). Studies suggested that L UTRs are more efficient to promote reporter replication (Bergeron et al., 2010; Devignot et al., 2015; Zhao et al., 2013), but another suggested that there is no difference between the segments (Zivcec et al., 2015). Study of the replication promoter of nairoviruses shows that UTRs of many nairoviruses contain terminal proximal nucleotides that are highly conserved within segments and nairoviruses, called promoter element 1 (PE1) (Neriya et al., 2022). PE1 is followed by PE2, a region that is less conserved and segment-specific, and, unlike other bunyaviruses, whose sequence (and not only base-pairing ability) is important for promoter activity, as shown in a minigenome reporter assay for HAZV (Matsumoto et al., 2019). Unique to nairoviruses, PE1 and PE2 are separated by a spacer region that lacks base-pairing ability. The inability of the spacer region to form dsRNA and its shortness (5 nucleotides for HAZV, 4 nucleotides for CCHFV) are essential for promoter activity (Matsumoto et al., 2019). Interestingly, hantaviruses and nairoviruses also differ from other bunyaviruses as their promoter starts with an uridine (U), whereas promoters from other bunyaviruses start with an adenosine (A) (Neriya et al., 2022). It is suggested that RNA replication of nairoviruses is internally initiated with 5'pppC at the +2 position of the promoter, using the G of 3'-AGA of the antigenome; then, a realignment occurs to the 3'-AGA and vRNA is further elongated to generate 5'pppCUCU. Finally, a cleavage of 5'pppC occurs, resulting in a 5'pU end.

3. Proteins translation and maturation

Viral mRNAs are used as template by the cellular translation machinery to produce viral proteins. Notably, GPC undergoes several cleavages through host furin-like and subtilisin/kexin-isozyme-1 (SKI-1) proteases and maturation steps as they traffic through the endoplasmic reticulum (ER) and Golgi apparatus to give rise to two individual structural proteins, Gn and Gc, and several non-structural proteins (Figure 13). GPC translation is directed through a signal peptide located at its N-terminus to

the ER. Then, the signal peptide is cleaved by a signal peptidase and GPC is translated and spans 5 times through the ER membrane with its transmembrane domains. Concomitantly to translation, GPC acquires N-glycosylations and disulfide bonds. Moreover, it is cleaved by signal peptidases to generate Gn and Gc precursors (Pre-Gn and Pre-Gc, respectively), as well as a non-structural, transmembrane protein called non-structural protein m (NSm) (Altamura et al., 2007; Sanchez et al., 2002). After trafficking to the Golgi network, PreGc is cleaved at its RKPL motif, probably by a SKI-1/site-1 protease (SKI-I/S1P)-like protease (Bergeron et al., 2007). PreGn acquires several O-glycosylations at its N-terminal mucin-like domain (MLD) and is cleaved at its RRLL motif by a SKI-1/S1P protease, generating Gn as well as two non-structural proteins, GP85 and GP160 (Vincent et al., 2003). The two latter proteins are further cleaved in the trans-Golgi network by a furin at a RSKR site, liberating the non-structural protein GP38 (Sanchez et al., 2006). GP38, GP85 and GP160 are secreted to the extracellular milieu, but what becomes of the MLD is unknown.

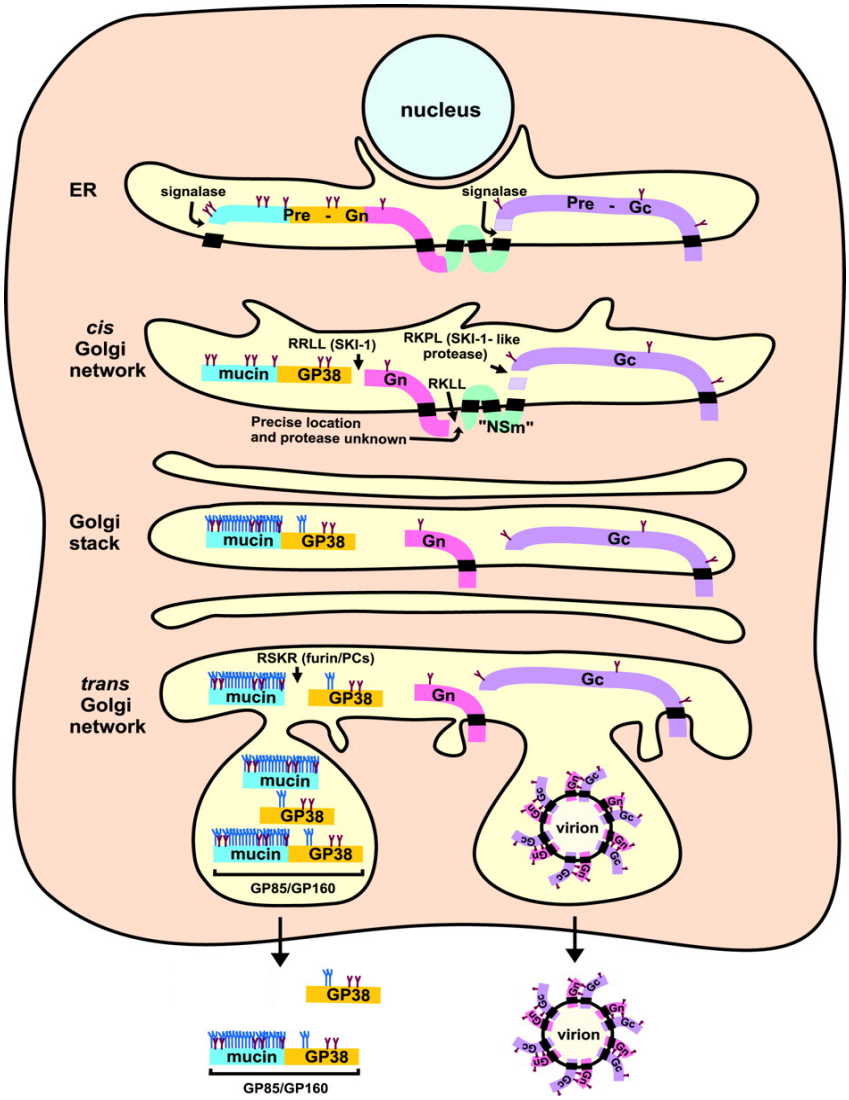

Figure 13. Complex maturation of CCHFV GPC. GPC contains 6 transmembrane domains, represented in black. GPC synthesis and maturation begins in the endoplasmic reticulum through a signal peptide that is cleaved. Concomitantly to translation, GPC acquires N-glycosylations (depicted in red) and disulfide bonds, and it is further cleaved by signal peptidases (or signalases) into Gn precursor (PreGn), Gc precursor (PreGc), and NSm, a non-structural protein. After trafficking to the Golgi network, several O-glycosylations (depicted in blue) of the mucin-like domain (MLD) of PreGn occur, as well as a cleavage at the RRLL motif by a proprotein convertase, the subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P), leading to the formation of Gn, GP160 and GP85. The two latter proteins are further cleaved in the trans-golgi network by a furin at a RSKR site, liberating GP38. Moreover, PreGc is cleaved into Gc by a SKI-1/S1P-like protease at a RKPL motif. GP38, GP85 and GP160 are secreted. Adapted from (Sanchez et al., 2006).

4. Assembly and budding

To date, little is known about CCHFV assembly and budding. Subcellular localization of the glycoproteins in the Golgi apparatus and the trans-Golgi network (Bertolotti-Ciarlet et al., 2005) as well as of NP in perinuclear regions, near the Golgi apparatus (Andersson et al., 2004b, 2004a) suggest that assembly occurs in the Golgi complex and/or trans-Golgi network. Newly formed virions are released by exocytosis from the basolateral membrane in polarized epithelial cells (Connolly-Andersen et al., 2007).

VII. Tools to study CCHFV

CCHFV study is significantly constrained by safety requirements and containment measures due to its classification as a BSL-4 virus. Nevertheless, innovative, powerful tools have opened new avenues for research. These approaches allow for the dissection of CCHFV lifecycle. Notably, reverse genetics systems allow the generation of reporter viruses as well as the study of the impact of specific mutations.

1. Pseudotyped viral particles

To study CCHFV entry independently of all other steps of CCHFV cycle, it is useful to use pseudotyped viruses such as lentiviruses whose envelope proteins are easily exchangeable. Lentiviruses pseudotyped with CCHFV glycoproteins have been generated and used for neutralization tests (Vasmehjani et al., 2020). Another promising platform is the vesicular stomatitis virus (VSV). Non-replicative VSV pseudotyped with CCHFV glycoproteins were generated from a VSV virus deleted for its glycoprotein G (VSV-dG) which was used to infect cells expressing CCHFV GPC with a large deletion of 53 amino acids at the cytoplasmic tail of Gc, increasing the localization of the glycoproteins at the plasma membrane (Suda et al., 2016). More recently, recombinant VSV expressing the CCHFV GPC in its genome allowed the production of replication competent VSV pseudotyped with GPC. In this system, GPC also presented several mutations, two of them resulting in the truncation of 14 amino acids at the cytoplasmic tail of Gc (Rodriguez et al., 2019).

2. The minigenome system

To study CCHFV transcription and replication independently of CCHFV entry, several systems have been implemented, all based on the expression of a minigenome containing a reporter molecule whose ORF is flanked by the UTRs of the viral segments (Bergeron et al., 2010; Flick et al., 2003; Zhao et al., 2013). The expression of the reporter molecule is dependent on the presence of the viral replication machinery, namely NP and L protein, that are provided *in trans* either by transfection of expression plasmids (Bergeron et al., 2010) or by superinfection with CCHFV in BSL-4 settings (Flick et al., 2003). Initial minigenomes used for these studies relied on the RNA polymerase I transcription system (Flick et al., 2003), but in the most widely used system, the minigenome is encoded in an expression plasmid under the control of a T7 promoter, and expression of the T7 RNA polymerase (either *in cellulo* by transfection of a T7 expression plasmid or *in vitro*) leads to the production of viral RNA that is supported by NP and L protein (Bergeron et al., 2010) (Figure 14). Monitoring of the expression of the reporter molecule allows to study the replication and transcription of CCHFV genome. Moreover, using the L protein that bears the D693A mutation, abrogating its cap-snatching activity, it is possible to monitor CCHFV replication independently of CCHFV transcription (Devignot et al., 2015; Klemm et al., 2013).

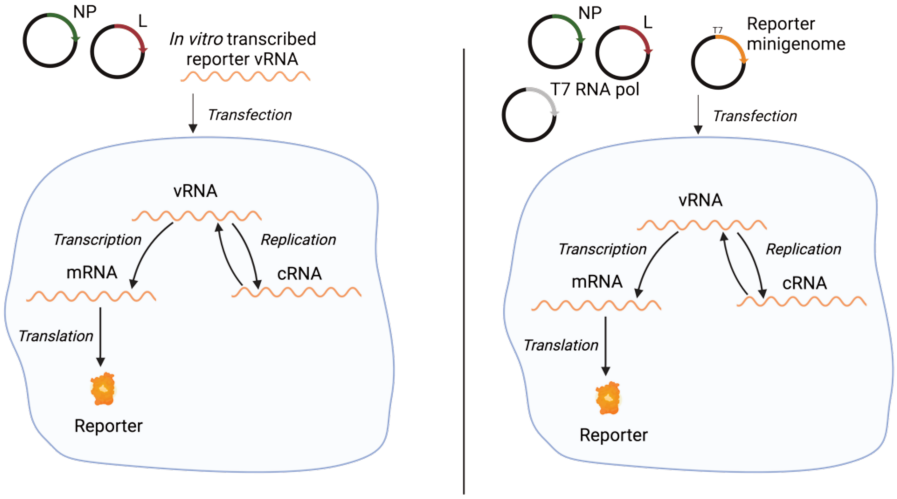

Figure 14. CCHFV minigenome assay. Cells are transfected with the replication machinery (NP and L protein), as well as a minigenome encoding a reporter molecule whose open reading frame is flanked by CCHFV untranslated regions (UTRs). Generation of viral RNA (vRNA) is achieved by *in vitro* transcription (left) or by transfection of the reporter minigenome plasmid under a T7 promoter as well as a T7 RNA polymerase helper plasmid (right). In the cell, vRNA is replicated into complementary RNA (cRNA) or transcribed into messenger RNA (mRNA) that is translated into the reporter molecule. Created with Biorender.

3. The transcriptionally- and entry-competent virus-like particles (tc-VLPs) system

A transcriptionally- and entry-competent virus-like particle (tc-VLPs) system has been designed, that allows to study concomitantly CCHFV entry and transcription/replication (Figure 15A) (Devignot et al., 2015; Zivcec et al., 2015). These particles mimic CCHFV particles, but do not encode any viral protein, rather a minigenome expressing a reporter molecule as in the abovementioned minigenome system. To produce these particles, helper plasmids expressing NP, L protein, GPC and T7 are expressed in producer cells alongside a minigenome plasmid under a T7 promoter, resulting in the encapsidation of the minigenome viral RNA flanked by CCHFV 5′ and 3′ UTRs and egress of newly formed virions thanks to the expression of the glycoproteins. These tc-VLPs can infect target cells, called "indicator cells", in which the viral RNA is replicated and transcribed using the viral replication machinery (Zivcec et al., 2015). Depending on the sensitivity of the reporter molecule, it is sometimes necessary for the indicator cells to express NP and L protein prior to infection, to easily monitor reporter expression (Devignot et al., 2015). Thanks to this system, it is possible to study entry, replication, and transcription of CCHFV minigenome. Moreover, the tc-VLPs can also infect "amplifier cells" that express NP, L protein and GPC prior infection; these cells can therefore produce newly formed virions and all steps of the viral life cycle are represented, while suitable in a BSL-2 facility (Devignot et al., 2015). By electron microscopy, it was found that these tc-VLPs were structurally and morphologically consistent with authentic CCHFV (Zivcec et al., 2015) (Figure 15B).

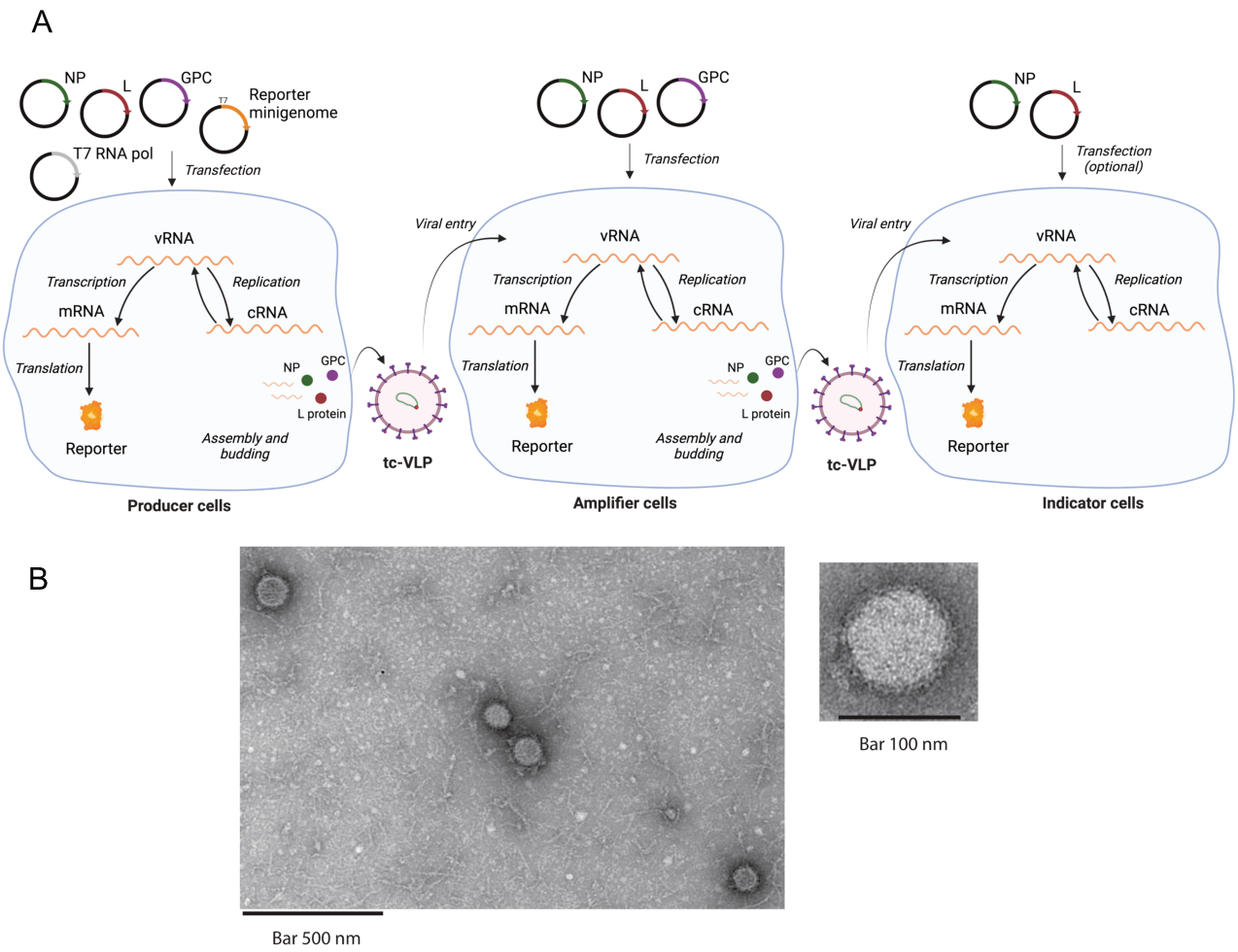

Figure 15. CCHFV tc-VLP assay. (A) Producer cells are transfected with the replication machinery (NP and L protein), the glycoprotein precursor complex (GPC), a minigenome encoding a reporter molecule whose ORF is flanked by CCHFV UTRs, and a T7 RNA polymerase. In the cell, viral RNA (vRNA) is replicated into complementary RNA (cRNA) or transcribed into messenger RNA (mRNA) that is translated into the reporter molecule. Moreover, assembly and budding of newly formed particles occur. Tc-VLPs are used to infect amplifier cells (that express NP, L protein and GPC) or indicator cells (that express NP and L protein, or that are left untransfected). Created with Biorender. **(B)** Supernatants from BSR-T7 cells producing tc-VLPs were subjected to electron microscopy. From (Zivcec et al., 2015).

4. The viral replicon particle (VRP) system

Viral replicon particles (VRPs) contain complete S and L genome segments but do not contain the M segment (Figure 16). The S segment and L segment are cloned in an expression plasmid under a T7 promoter, and VRPs are generated as in the tc-VLPs system, but with the S and L segments instead of the minigenome segment (Scholte et al., 2019b; Spengler et al., 2019). These particles perform authentic transcription and replication in infected cells, but do not produce the GPC necessary to the production of viral progeny. Thus, the infection occurs in a single-cycle manner, and can be studied in BSL-2 settings. VRPs expressing a reporter protein (ZsGreen) were also obtained by using a ZsGreen-S segment instead of the S segment, where ZsGreen is separated from the NP ORF by a P2A cleavage site (Scholte et al., 2019b), facilitating their monitoring.

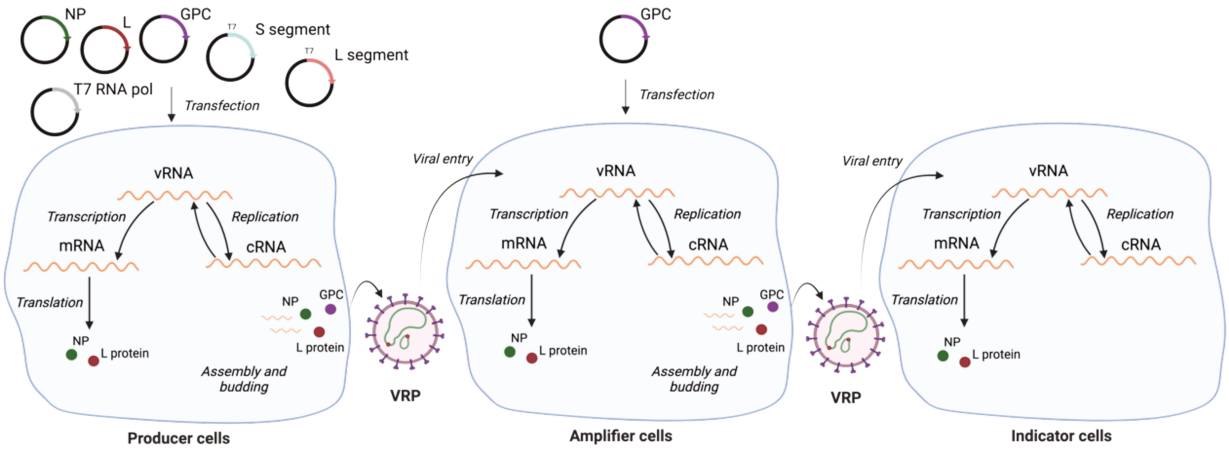

Figure 16. CCHFV VRP assay. Producer cells are transfected with the replication machinery (NP and L protein), the glycoprotein precursor complex (GPC), the S and L segments and a T7 RNA polymerase. In the cell, viral RNA (vRNA) is replicated into complementary RNA (cRNA) or transcribed into messenger RNA (mRNA) that is translated into NP and L protein. Moreover, assembly and budding of newly formed particles occur. VRPs are used to infect amplifier cells (that express GPC) or indicator cells (that are left untransfected). Created with Biorender.

5. Recombinant CCHFV

Finally, by adding the M segment to the VRP system, authentic, infectious, recombinant CCHFV (rCCHFV) particles are produced, that can infect cells in a multicycle manner (Bergeron et al., 2015) (Figure 17). Moreover, using the ZsGreen-S segment, fluorescent rCCHFV particles can be generated (Welch et al., 2017). These

particles authentically mimic CCHFV and can be used to easily introduce mutations in CCHFV genome to further dissect the role of each protein.

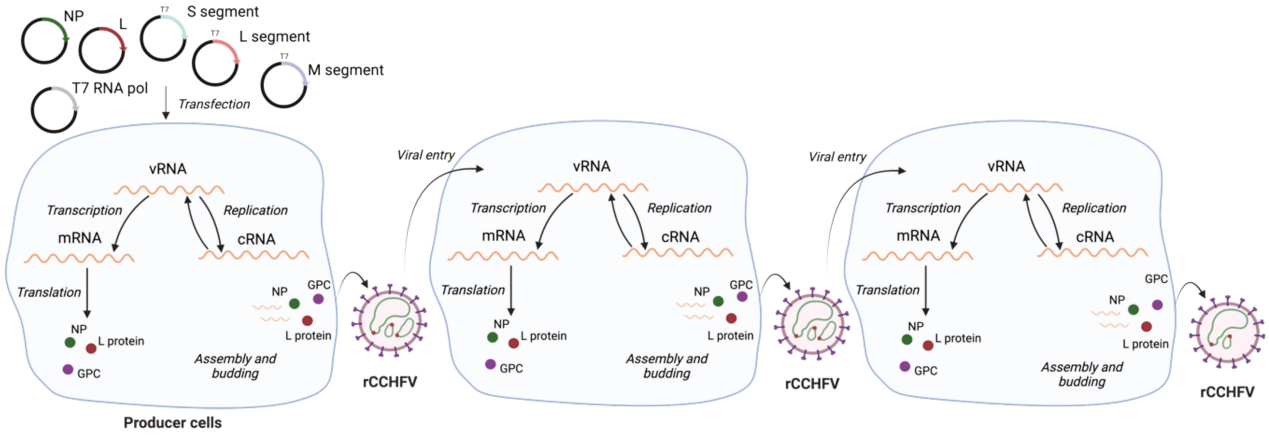

Figure 17. rCCHFV assay. Producer cells are transfected with the replication machinery (NP and L protein), the S, M and L segments and a T7 RNA polymerase. In the cell, viral RNA is replicated into complementary RNA (cRNA) or transcribed into messenger RNA (mRNA) that is translated into NP, L protein and GPC. Moreover, assembly and budding of newly formed particles occur. rCCHFV particles are used to infect cells in a multicycle manner. Created with Biorender.

6. Experimental infection studies and animal models

An important challenge in CCHFV study is to find a relevant animal model, that was cruelly lacking until 1968, when newborn white mice (NWM) were intracranially inoculated with CCHFV, allowing its isolation and the development of tools for its study (Hoogstraal, 1979). Throughout the years, many animals have been experimentally infected with CCHFV to determine their susceptibility to this virus. These experimentations are extensively reviewed in (Spengler et al., 2016b). Inoculation routes included subcutaneous, intravenous, intramuscular, intracranial, intraperitoneal, intradermal, or intracardiac routes. Several species were studied, such as wild mammals (hares, ground squirrels, hedgehogs, or small rodents), livestock (cattle, sheep, or equids), non-human primates or birds. However, none of these species developed symptoms that resembled those found in humans, and the only animal model available for CCHFV remained NWM.

In the 2010's, it was shown that adult type I interferon receptor (IFNAR)-knock-out (*Ifnar*$^{-/-}$) mice were a severe/lethal model of disease after infection with CCHFV (Bereczky et al., 2010; Zivcec et al., 2013). These results were additionally confirmed in other immunodeficient mouse models such as *Stat-1*$^{-/-}$ mice (Bente et al., 2010), mice

transiently immunosuppressed thanks to an antibody directed against IFNAR1 (Garrison et al., 2017), or *Stat2*$^{-/-}$ hamsters (Ranadheera et al., 2020). Depending on the route of inoculation of the virus, these models were shown to be suitable to study either the acute phase of the disease or later stages of CCHF disease, such as convalescence. Indeed, *Ifnar*$^{-/-}$ mice inoculated subcutaneously with the CCHFV strain Hoti developed a disease from which they recovered, and developed CCHFV-specific B- and T-cell responses (Hawman et al., 2019). Furthermore, NOD-SCID-γ (NSG)-SGM3 mice (which are immunodeficient mice lacking mature T-, B-, and natural killer (NK) cells) humanized with human CD34^{+} stem cells were used in a model of CCHFV infection. These mice developed neurological symptoms (Spengler et al., 2017). Conversely, rare cases of neurological symptoms such as cerebral and cerebellar edema, encephalopathy, and subdural hematoma have been described in patients infected with CCHFV (Alavi-Naini et al., 2004; Kleib et al., 2016).

To counteract the lack of immunocompetent mouse model for CCHFV infection, a mouse-adapted variant of CCHFV was developed, that recapitulated in adult, immunocompetent mice the disease observed in humans (Hawman et al., 2021). This virus was obtained through chronic infection and serial passage of CCHFV within the livers of *Rag2*$^{-/-}$ mice, which retain intact innate immune responses while lacking adaptive immunity. This virus presented mutations within its structural as well as its non-structural proteins, whose role remain uncharacterized. However, it is tempting to speculate that these mutations may modulate the ability of the virus to escape the innate immune system.

Non-human primate (NHP) models for CCHFV infection remained for a long time unsuccessful using African green monkeys, baboons, rhesus macaques, or patas monkeys (Fagbami et al., 1975; Hoogstraal, 1979; Smirnova, 1979), and immunocompetent models were still lacking for CCHFV. However, in 2018, immunocompetent cynomolgus macaques infected with CCHFV (strain Hoti or Afg09-2990) developed viremia as well as a severe/fatal disease with symptoms representative of CCHF (Haddock et al., 2018; Hawman et al., 2020; Smith et al., 2019b, 2019a). However, in another study, CCHFV (Hoti strain)-infected cynomolgus macaque did not develop a severe disease, but only mild clinical symptoms (Cross et al., 2020).

Chapter 2: Innate immunity

I. General concepts of immunity

Immunity refers the host ability to resist and defend itself against pathogens. It generally can be categorized into four different types of immunity: (i) the host anatomical barriers; (ii) intrinsic immunity; (iii) innate immunity; and (iv) adaptive immunity.

The physical and anatomical barriers, such as the skin for instance, represent a continuous barrier always present, except if damaged (for instance by a lesion or a tick bite). Intrinsic immunity refers to the action of restriction factors that are always present and that directly act to prevent infection and viral replication. This immunity does not need to be induced to be active. On the contrary, innate immunity needs to be induced (see below), is rapid and non-specific to restrict viral replication. Moreover, it stimulates and primes the implementation of the adaptive immunity, which is slow to execute but is very specific and long-lasting, with a memory that allows the host to mount a quicker and more effective immune response if exposed to the same pathogen again.

II. Innate immunity

1. Immune sensing and pattern recognition receptors

Innate immunity is induced upon the recognition of a pathogen by pattern recognition receptors (PRRs), that detect specific features from pathogens, called pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) or homeostasis-altering molecular processes (HAMPs). PRRs are structurally composed of a ligand recognition domain, an intermediate domain, and an effector domain. After binding of respective PAMPs, PRRs recruit adaptor molecules through their effector domain, resulting in downstream signaling pathways to produce and express cytokines, among which interferons (IFNs) that are presented below, as well as molecules stimulating the adaptive immune response. PRRs

expression differs depending on the cell type, and is particularly high in antigen-presenting cells, immune cells, and epithelial cells.

PRRs are present at diverse localizations within the cell. Membrane-bound PRRs include the Toll-like receptors (TLRs) or C-type lectin receptors (CLRs), while cytosolic sensors include the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and cytosolic DNA sensors (CDS).

a. Toll-like receptors

TLRs are type I transmembrane glycoproteins. Their extracellular region contains leucine-rich repeats (LRRs) that are responsible for the recognitions of ligands, while their intracellular domain, also known as the Toll/IL-1R (TIR) domain, is responsible for the initiation of signal transduction through its binding to adaptor proteins. In humans, 10 different TLRs have been described (TLR1-10) and are expressed in the form of heterodimers or homodimers. Their subcellular localization varies and determines the type of PAMPs that they recognize. Indeed, plasma membrane localized TLRs (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) recognize membrane components of pathogens, such as lipids, lipoproteins, or proteins, while TLRs anchored at the membrane of endosomes (TLR3, TLR7, TLR8 and TLR9) recognize nucleic acids of pathogens (Figure 18). After recognition of PAMPs by TLRs, the TIR domain initiates a downstream signaling cascade through its binding to different receptor adaptor proteins. These pathways can be divided into myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways, depending on the adaptor protein. These pathways lead to the nuclear translocation of transcription factors such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) or interferon regulatory factors (IRFs), that induce the expression and secretion of pro-inflammatory cytokines and IFNs, respectively (reviewed in (Li and Wu, 2021)).

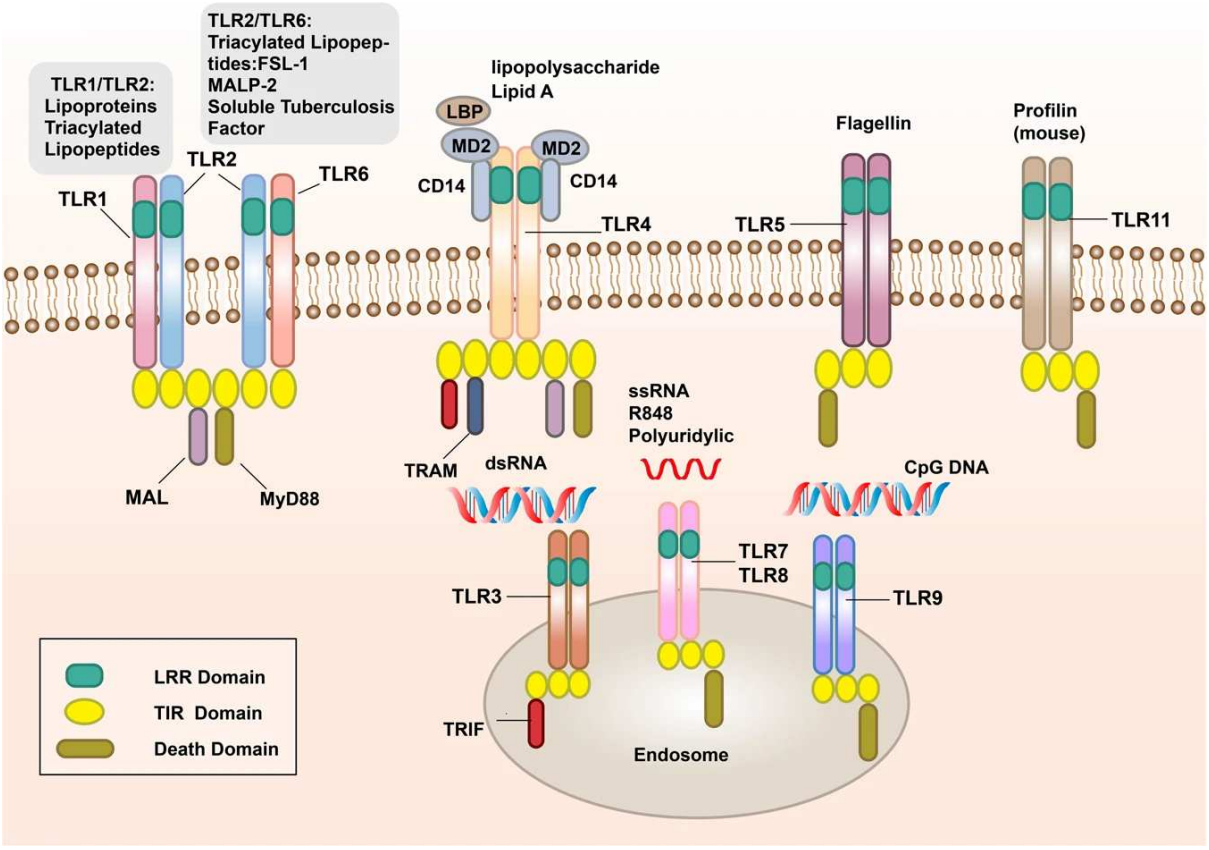

Figure 18. The TLRs and their signaling pathway. TLRs are membrane-bound proteins that homo- or oligo-dimerize and recruit adaptor molecules with the same TIR domain to transmit signals upon recognition of a specific ligand by the LRR domain. From (Li and Wu, 2021).

b. RIG-I-like receptors

RLRs are essential intracellular sensors involved in the recognition of many RNA viruses (reviewed in (Rehwinkel and Gack, 2020)). This family of PRRs is expressed in most cell types and encompasses three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5), also known as IFN-induced with helicase C domain-1 (IFIH1), and laboratory of genetics and physiology 2 (LGP2). They are localized within the cytosol, but studies show a localization of functional RIG-I within the nucleus as well (Liu et al., 2018). They are composed of a central DexD/H helicase domain and a carboxy-terminal domain (CTD) involved in the recognition of viral PAMPs, namely vRNA. RIG-I and MDA5 also have two caspase activation and recruitment domains (CARD) at their amino-terminus, responsible for the transmission of downstream signaling. Additionally, RIG-I contains a repressor domain (RD) at its C-terminal end, that has a self-inhibitory function. LGP2 lacks a CARD and is therefore unable to transmit downstream signals. It negatively regulates RIG-I signaling, but promotes MDA5 binding to RNA (Bruns and Horvath, 2015) (Figure 19).

RIG-I and MDA5 recognize RNA of different viruses to induce IFN responses (Andrejeva et al., 2004; Yoneyama et al., 2004), but despite their similar structure, they recognize different types of RNA. RIG-I mainly recognizes short dsRNA of less than 1000 bp, while MDA5 recognizes longer dsRNA chains (Kato et al., 2008). Moreover, RIG-I can sense the 5′ triphosphate end of RNA viruses (Hornung et al., 2006; Pichlmair et al., 2006).

On its resting conformation, the CARD, CTD and helicase domain of RIG-I are folded, until the CTD recognizes immunostimulatory RNA, leading to a conformational change exposing the CARDs that dimerize and recruit linker molecules such as mitochondrial antiviral-signaling proteins (MAVS), that also contain a CARD. MAVS relay the signal to TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε), that in turn activate IRF3 and IRF7. After translocation of the latter into the nucleus as well as of NF-κB, expression of IFNs and pro-inflammatory cytokines is induced (Figure 19).

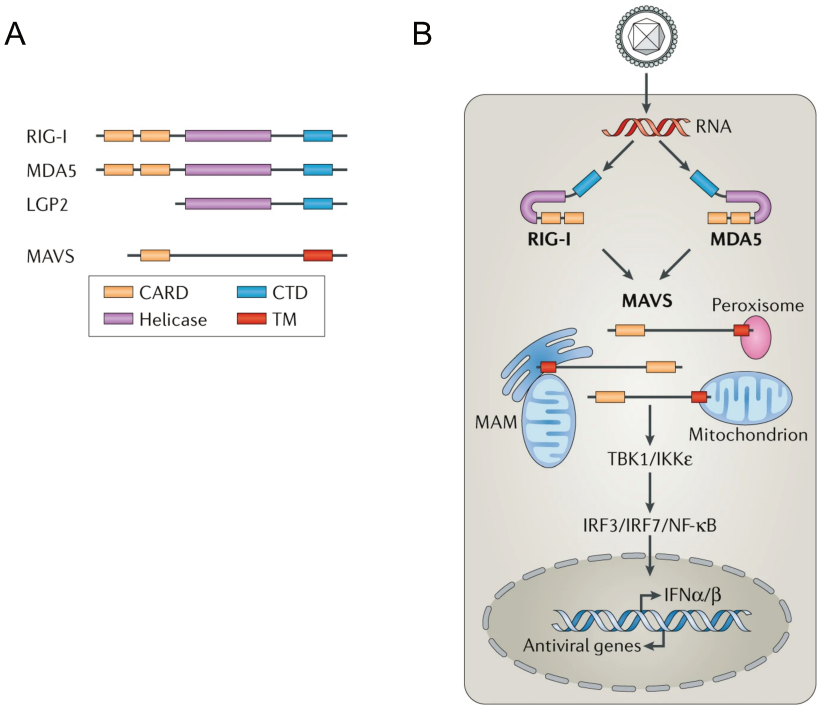

Figure 19. The RLRs and their signaling pathway. (A) The domain architecture of RIG-I, MDA5, LGP2 and MAVS are represented. **(B)** The signaling pathway induced by RIG-I and MDA5 activation is shown. Recognition of immunostimulatory RNA by RIG-I or MDA5 leads to their conformational change and multimerization of their CARDs (not shown), which allow homotypic interactions with MAVS. MAVS transmit the signal to TBK1/IKKε, that in turn activate IRF3, IRF7 and NF-κB. The latter proteins translocate to the nucleus to induce the expression of type I IFN and other genes. From (Rehwinkel and Gack, 2020).

c. NOD-like receptors

NLRs are intracellular cytosolic sensors that contain three domains. At the C-terminal end, LRRs are involved in the identification of ligands; in the central part of the proteins, a nucleotide-binding domain (NBD, also known as NACHT) is involved in nucleic acid binding and oligomerization of the protein; finally, at the N-terminal end is located the effector domain. Depending on the nature of the effector domain, several subfamilies of NLRs exist: NRLC subfamily have a CARD, NLRP have a pyrin domain (PYD), NLRB contain baculovirus inhibitor of apoptosis protein repeats, NLRA have an acidic activation domain, and NLRX subfamily members have other NLR effectors domain. On their resting conformation, LRRs fold to cover the central NACHT domain, that is exposed only upon a conformational change following LRRs interaction with a PAMP. Exposed NACHT oligomerize and recruit through homotypic interactions downstream adaptor proteins to lead to the production of cytokines (reviewed in (Li and Wu, 2021)).

d. Cytoplasmic DNA sensors

Cytoplasmic DNA is strongly immunostimulatory, whether derived from pathogens or from host genome (for instance, damaged mitochondrial DNA). There are several DNA sensors within the cytoplasm, including members of the absent in melanoma 2 (AIM2)-like receptors (ALRs), namely AIM2, IFN-induced protein 16 (IF16), IFN-induced protein X (IFIX) or myeloid nuclear differentiation antigen (MNDA). Many other DNA sensors also exist, such as DNA-dependent activator of IRFs (DAI), RNA polymerase III, or DExD/H box helicases. However, these PRRs are cell-type or DNA-sequence specific and therefore do not constitute universal cytosolic DNA sensors (reviewed in (Unterholzner, 2013)), unlike the cyclic GMP-AMP (cGAMP) synthase (cGAS), which recognizes DNA independently of its sequence and which is expressed in various cell types (Sun et al., 2013). cGAS binding to DNA triggers cGAS dimerization and production of the second messenger 2'-3'-cGAMP, that binds to stimulator of IFN genes (STING). STING then recruits TBK1 and IKK, that ultimately leads to the nuclear translocation of IRF3 to the nucleus to turn on the transcription of IFNs and other immunostimulatory genes (reviewed in (Yu and Liu, 2021)).

e. C-type lectins receptors

C-type lectins receptors (CLRs) are phagocytic receptors expressed mostly at the surface of macrophages and dendritic cells. The CLR family comprises many proteins that contain at least one domain that recognizes carbohydrates, the carbohydrate recognition domain (CRD). Through CRD, CLRs interact with glucan carbohydrates, mannose and fucose on pathogens. This recognition leads to phagocytosis of the pathogen for direct degradation and elimination to prevent infection, and for antigen-presentation. Thus, CLRs also modulate the response of the adaptive immune system (reviewed in (Geijtenbeek and Gringhuis, 2009)).

2. Interferons

a. Definition of interferons

Interferons (IFNs) are cytokines that were discovered in 1957 by Isaacs and Lindenmann (Isaacs and Lindenmann, 1957), when they noticed the presence of an element in the supernatant that interfered with the replication of influenza virus. IFNs are cytokines that can be divided into 3 groups: type I, type II and type III IFNs, discovered in 1957, 1965 and 2003, respectively (Kotenko et al., 2003; Sheppard et al., 2003; Wheelock, 1965). In humans, type I IFNs include several subtypes of IFN-α (α1, α2, α4, α5, α6, α7, α8, α10, α13, α14, α16, α17, and α21), but also IFN-β, IFN-ε, IFN-κ and IFN-ω. IFN-δ and IFN-τ have only been described in pigs and cattle, respectively, and do not have human homologues (La Bonnardière et al., 1991; Martal et al., 1979). Type II IFNs are represented by only one subtype of IFN, IFN-γ, and type III IFNs include IFN-λ1 (IL-29), -λ2 (IL-28A), -λ3 (IL-28B) and -λ4 (Prokunina-Olsson et al., 2013).

IFNs are produced upon the recognition of PAMPs by some PRRs. Some cell types are particularly efficient in the production of type I IFN, such as plasmacytoid dendritic cells. IFNs are then secreted into the extracellular medium, where they act in an autocrine and paracrine manner to implement an antiviral state within the cells. They are recognized by specific receptors. Type I IFNs are recognized by the IFN-α/β receptor (IFNAR), composed of a dimer of IFNAR1 and IFNAR2; type II IFNs by the IFN-γ receptor (IFNGR), composed of a tetramer of IFNGR1 and IFNGR2; and type III IFNs bind a dimer of IFN-λ receptor 1 (IFNLR1) and interleukine-10 receptor 2 (IL10R2) (Aguet and Blanchard, 1981; Branca and Baglioni, 1981; Kotenko et al., 2003;

Orchansky et al., 1984; Sheppard et al., 2003), resulting in signaling cascades described thereafter.

b. IFN signaling cascade

Despite their different receptors, type I IFN and type III IFN recognition converges towards the same canonical signaling pathway: the Janus activated kinase/Signal transducer and activator of transcription (JAK/STAT) pathway (Figure 20). IFNAR1 and IFNLR1 are associated with tyrosine kinase 2 (TYK2), and IFNAR2 and IL10R2 with Janus activated kinase 1 (JAK1). Binding of type I and type III to their cognate receptors triggers phosphorylation of JAK1 and TYK2, which leads to the subsequent tyrosine phosphorylation of STAT1 and STAT2, leading to the formation of a complex between STAT1, STAT2 and IFN-regulatory factor 9 (IRF9), also known as IFN-stimulated gene factor 3 (ISGF3). Translocation of this complex to the nucleus and binding to IFN-stimulated response elements (ISREs) in DNA initiate the transcription of multiple IFN-stimulated genes (ISGs) (Figure 20). As for type II IFN, binding of these cytokines to their receptor leads to the phosphorylation of JAK1 and JAK2, leading to the formation of phosphorylated STAT1 homodimers, also known as IFN-γ-activated factor (GAF) that binds to IFN-γ-activated site (GAS) elements after its translocation into the nucleus (Mesev et al., 2019; Platanias, 2005; Zhou et al., 2007).

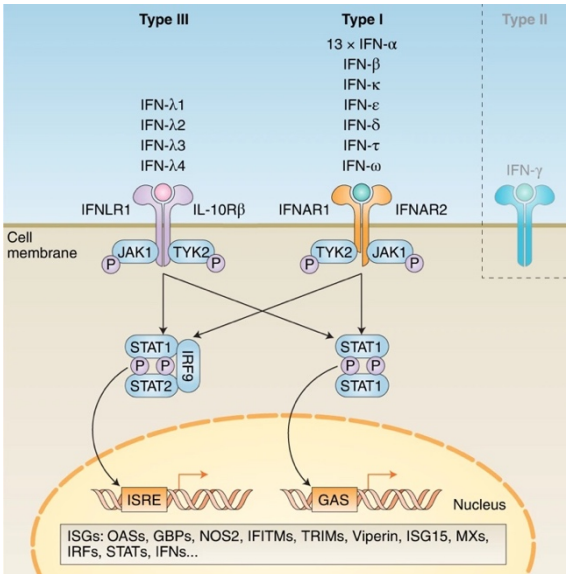

Figure 20. The type I and type III IFN canonical signaling cascades. Type I IFN and type III bind to different receptors (a heterodimer of IFNAR1 and IFNAR2, and IFNLR1 and IL-10R2, respectively) but they induce a convergent signaling cascade. Binding of type I and type III IFN to these receptors leads to the phosphorylation of TYK2 and JAK1, that further phosphorylate STAT1 and STAT2. The phosphorylated STAT1-STAT2 heterodimer interacts with IRF9 to form the ISGF3 complex that translocates to the nucleus. There, it acts as a transcription factor that binds to ISRE or GAS promoters and induces ISG expression. From (Mesev et al, 2019).

IFN signaling pathway induces a strong and rapid implementation of an antiviral state through the expression of a given repertoire of ISGs, that depends on the type and subtype of IFN and the cell type (Moll et al., 2011). Type I IFN response is thought to induce more potently and rapidly the expression of ISGs than type III (Zhou et al., 2007).

c. Interferon-stimulated genes

Basically, an IFN-stimulated gene (ISG) is a gene whose expression is induced by the presence of IFN, resulting from the signaling cascade leading to the binding of ISGF3 or GAF on their upstream promoter (Schoggins, 2019). While some ISGs are not detected at a basal level, others are basally expressed even in absence of IFN (Mostafavi et al., 2016). Still, the underlying reality is more complex. Indeed, ISG expression might be induced in some cell types or tissues, but not in others. Moreover, some ISGs are transcription factors that positively regulate the expression of ISGs, such as IRF3 or IRF7. In addition, some ISGs are known PRRs or genes that belong to the IFN signaling cascade, further amplifying the IFN-based response, such as MDA5. To counteract this, repressors of the IFN pathway are also themselves ISGs and negatively control this biological process (Porritt and Hertzog, 2015). Suppressor of cytokine signaling 3 (SOCS3) is one well-described example of negative regulator of the IFN response, that induces the degradation of components of the JAK/STAT pathway (Babon et al., 2012). Of note, IFNs do not only induce the transcription of mRNAs that are translated into effector proteins, but also of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (Forster et al., 2015; Peng et al., 2010) that can have immunomodulatory functions. Finally, IFNs also repress the expression of genes that are called IFN-repressed genes (IRGs or IRepGs), such as family with sequence similarity 117 member B (FAM117B) and lysine demethylase 5B (KDM5B) (Shaw et al., 2017).

Importantly, the repertoire of ISGs differs from species to species. In a study comparing the interferome of 10 different species (*Homo sapiens* (human), *Rattus norvegicus* (rat), *Bos taurus* (cow), *Ovis aries* (sheep), *Sus scrofa* (pig), *Equus caballus* (horse), *Canis lupus familiaris* (dog), *Myotis lucifugus* (little brown bat, microbat), *Pteropus vampyrus* (large flying fox, fruit bat), and *Gallus gallus* (chicken)), it was found that there is a core of 62 ISGs that are common between all species and that are thought to mediate the ancestral functions of the IFN system involved in antigen presentation, IFN induction or suppression, ubiquitination and protein degradation, cell signaling

and apoptosis and antiviral responses (Shaw et al., 2017). Apart from these 62 ISGs, many species-specific ISGs were described. These specificities might be explored to understand differences in susceptibility to infections between different species.

ISGs are mostly studied for their antiviral action and a major goal is to understand the molecular mechanisms involved in this action. On one hand, some well-described ISGs inhibit rather non-specifically viral replication by implementing a cellular translational shutdown. For instance, protein kinase R (PKR) is a basally expressed ISG that detects notably dsRNA, inducing its dimerization. Dimerized PKR phosphorylates the translation initiation factor 2α (eIF2α), inducing a global translational shutdown (Pindel and Sadler, 2011). The translation machinery on which viruses depend for the translation of their own mRNAs is thus rendered inactive. However, cellular mRNAs are also targeted by this defense mechanism. On the other hand, some ISGs specifically inhibit viral replication by targeting specific steps of the viral cycle. Classical examples are the IFN-induced transmembrane proteins (IFITMs), that are localized either at the plasma membrane or at the membrane of endosomes and that inhibit entry of many enveloped viruses (Brass et al., 2009; Schoggins, 2019); or tetherin, that specifically inhibits egress of human immunodeficiency virus 1 (HIV-1) by anchoring it at the surface of the plasma membrane (Neil et al., 2008; Van Damme et al., 2008). The majority of studied ISGs exert their inhibitory effects on the initial phases of the viral life cycle. This can be attributed to the challenges associated with independently studying the later stages of the cycle or perhaps by the absence of such later stages inhibition (Schoggins, 2019).

In a constant arms race, viruses have evolved to counteract the action of ISGs. For instance, tetherin action is directly inhibited by the viral protein Vpu (Neil et al., 2008; Van Damme et al., 2008). Another example is the NSs protein of Rift Valley fever virus (RVFV), that induces proteasomal degradation of PKR (Habjan et al., 2009; Ikegami et al., 2009). Viruses have also evolved to inhibit more generally the induction of IFN, either by limiting their recognition by PRRs or by interfering with components of the different signaling pathways (reviewed in (García-Sastre, 2017)).

ISGs do not always exert antiviral roles against viruses. Most of them do not have any effect on viral replication, and sometimes, some of them have an unexpected proviral activity. For instance, mucolipin-2 (MCOLN2) is a cation channel that enhances viral entry of many RNA viruses by increasing trafficking efficiency from early to late endosomes, accelerating the release of viral genome into the cytosol (Rinkenberger

and Schoggins, 2018). Pro- or antiviral activity of ISGs might differ depending on the virus. Indeed, while lymphocyte antigen 6 family member E (LY6E) is a known restriction factor against coronaviruses by impairing membrane fusion (Pfaender et al., 2020), it conversely promotes viral entry of HIV-1, of multiple flaviviruses, and of influenza virus A (IAV) (Yu et al., 2017; Hackett and Cherry, 2018; Mar et al., 2018). For the latter virus, LY6E promotes the uncoating of its viral genome within the cytosol.

III. Methods to uncover ISGs modulating viral infection

1. Comparison of gene expression profiles in permissive and non-permissive cell lines

A classic way of identifying genes modulating viral infection is to compare the gene expression profiles between permissive and non-permissive cell lines through comparative transcriptomic or proteomic studies. These methods have been notably used to determine restriction factors modulating HIV-1 infection. Indeed, HIV-1 encodes several accessory proteins that counteract these restriction factors, rendering their identification particularly easier by comparing cell lines where the presence of the accessory protein is necessary for efficient HIV-1 infection with cell lines where it is dispensable. Several ISGs restricting HIV-1 were therefore identified, such as for instance APOBEC3G (Sheehy et al., 2002), tetherin (Neil et al., 2008), SAMHD1 (Laguette et al., 2011) or MX2 (Goujon et al., 2013; Kane et al., 2013).

2. Gain-of-function approaches

The expression of ISGs is induced by IFNs. A way to identify ISGs modulating viral infection is to mimic this phenomenon by ectopic expression of ISGs into permissive cells followed by infection with viruses. Indeed, expression of antiviral ISGs into permissive cells will render them non-permissive (Figure 21).

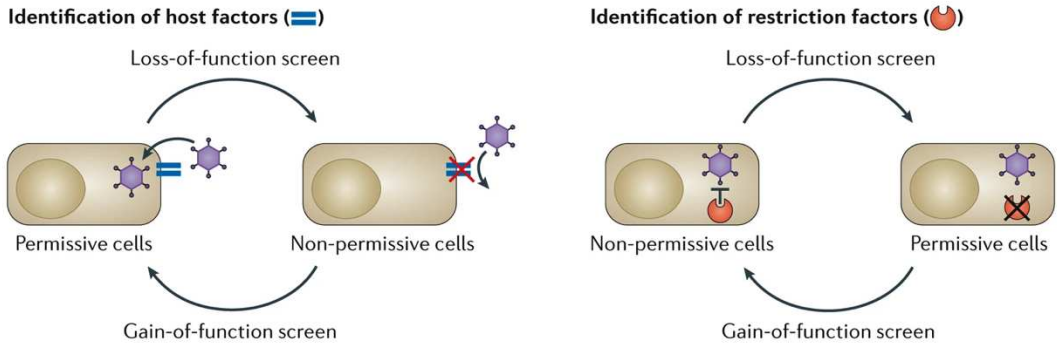

Figure 21. Identification of host restriction factors. To identify restriction factors, several strategies can be used. In a gain-of-function screen, overexpression of a restriction factor will render a permissive cell non-permissive. By contrast, in a loss-of-function screen, downregulation of a restriction factor in a non-permissive cell will enable virus infection. From (Puschnik et al., 2017).

Various numbers of chosen human ISGs were studied in a gain-of-function, arrayed manner, ranging from 7 genes (Zhang et al., 2007) to large-scale ISG screens with 300-500 ISGs (Schoggins et al., 2011; Wilson et al., 2012; Kane et al., 2016; Wickenhagen et al., 2021). Since 2011, these ISGs have been tested against many RNA and DNA viruses, allowing notably the cross-comparison of the susceptibility to ISG-mediated inhibition of positive- or negative-sense RNA viruses (Schoggins, 2019; Schoggins et al., 2014).

Physiologically, IFNs induce the simultaneous expression of numerous ISGs, and it is very likely that screens where ISGs are overexpressed alone will overlook the genes that need cofactors. Combinatorial approaches where several ISGs are overexpressed help tackle this limitation (Schoggins et al., 2011; Dang et al., 2018). Another way of circumventing this limitation is to use loss-of-function approaches, as described thereafter.

Because ISG sets differ between species (Shaw et al., 2017), it is necessary to study ISGs in a species-specific manner. To this end, libraries of macaque (Kane et al., 2016), bovine (Hardy et al., 2023) or bat (Boys et al., 2020) ISG cDNAs were generated, and are used to study species-specific effects of ISGs as well as putative evolutionary relationships.

However, ISG cDNA screens are technically demanding regarding cloning and validation of each individual cDNA. To overcome this limitation, clustered regularly interspaced short palindromic repeats (CRISPR) activation (CRISPRa)-based

approaches are useful. Guide RNAs (gRNAs) are used to direct endonuclease-deficient Cas9 (dCas9) fused to transcriptional activators to genes of interests to induce their expression. This method has the advantage to facilitate multiplexing and combinatorial studies. A CRISPRa screen was performed using a given ISG-focused set of pooled gRNAs (Danziger et al., 2022), but whole-genome CRISPRa screens also are potent tools to uncover ISGs modulating viral replication (Dukhovny et al., 2019).

However, both ISG cDNA or CRISPRa approaches typically include only one isoform per gene, and it is therefore possible to miss isoform-specific antiviral activities, as for instance nuclear receptor coactivator 7, whose only the short IFN-inducible isoform (NCOA7-alternative start (NCOA7-AS)) exerts an antiviral activity against influenza A virus (Doyle et al., 2018). Finally, the overexpression of ISGs may result in misleading expression patterns or functionalities due to potential artefacts.

3. Loss-of-function approaches

Loss-of-function approaches are useful to decipher which genes are necessary to contribute to IFN-mediated inhibition of viral infection in the context of the IFN response. Moreover, they help to mitigate potential artefacts associated with gene overexpression approaches and facilitate the identification of potential genes that require ISGs as cofactors. Loss-of-function can be achieved with small interfering RNAs (siRNA) or short hairpin RNAs (shRNA) targeting specific, chosen ISGs (Lesage et al., 2022; Li et al., 2013; Metz et al., 2012; Subramanian et al., 2018; Zhang et al., 2007) or targeting the whole genome (Brass et al., 2009; Fusco et al., 2013; Zhao et al., 2012). Moreover, CRISPR-Cas9 knock-out (KO) screens have helped discovering ISGs with antiviral activities. Here again, ISG-specific libraries of sgRNAs have uncovered ISGs with antiviral activities (OhAinle et al., 2018; Roesch et al., 2018; McDougal et al., 2023; Itell et al., 2023), as well as genome-wide CRISPR screens (Mac Kain et al., 2022; Richardson et al., 2018). Especially, the study from McDougal et al. as well as from Itell et al. determined that a limited set of ISGs is responsible for the restriction of a given virus. However, one caveat of pooled genome-wide CRISPR screens might be that genes involved in the IFN signaling pathways will likely rank high in screens involving an IFN response, as they are essential for the latter (Bonaventure et al., 2022).

Chapter 3: Innate immunity and CCHFV interactions

I. CCHFV and IFN

The role of IFN in constraining CCHFV pathogenicity in vertebrates is highlighted by the observed lethality of CCHFV infection in mice deficient in the IFN pathway, while having no such effect in wild-type mice. In addition, treatment of different cell types with IFN prior infection limits CCHFV replication. Treatment of cells with 1000 U/mL of IFN-α prior infection led to a reduction in CCHFV titers of one log in Huh7 cells and more than two logs in primary human umbilical vein endothelial cells (HUVECs) and A549 cells (Andersson et al., 2008, 2006; Karlberg et al., 2010). Similar restriction of CCHFV by type I IFN was observed in HEK293 cells (Mo et al., 2023), as observed by reverse transcription – quantitative polymerase chain reaction (RT-qPCR) in the infected cells. Interestingly, IFN-λ1 and -λ3 treatment (100 ng/mL) of HEK293 cells also led to an inhibition of CCHFV replication, although to a lesser extent than type I IFN (Mo et al., 2023). Of note, IFN-α administered 6 hours after infection had no effect on CCHFV replication, suggesting that CCHFV might have developed measures to counteract its effect (Andersson et al., 2008).

CCHFV infection induces an IFN response both *in vitro* and *in vivo*. Indeed, infection of A549 and SW13 cells with CCHFV induced the production and secretion of IFN-β of 150 pg/mL at 48 hours post-infection (hpi) (Spengler et al., 2015). Moreover, RNA-seq studies performed in HEK293 cells showed that differentially regulated genes were members of type I and type III IFN pathways. ISGs such as MxA, ISG15, OAS1, IFIT1 and IFTIM1 were upregulated in these cells (Mo et al., 2023). In hepatic cell lines such as Huh7 and HepG2 cells, RNA-seq revealed that the IFN signaling pathway was one of the top pathways identified (Kozak et al., 2020). Quantitative proteomic analyses performed in HepG2 showed an accumulation of STAT1 protein upon CCHFV infection (Fraisier et al., 2014). However, in this study, the phosphorylation status of this protein was not considered; thus, the accumulation of the protein can be due either to a cell response against CCHFV infection to control its replication, or to an inhibition of the JAK/STAT pathway by CCHFV. *In vivo*, infection of immunocompetent mice with the mouse-adapted CCHFV induced the production and secretion of IFN-α in male and female mice within 24 hpi and of IFN-β 3 days

post-infection in male mice (Hawman et al., 2021). Additionally, in cynomolgus macaques infected with Hoti strain or Afg09 strain of CCHFV, early differentially expressed genes were ISGs including IFIT proteins (IFIT1, IFIT2 and IFIT3), oligo-adenylate synthase 1 (OAS1), IFI44 and MxA (Arnold et al., 2021). In human patients infected with CCHFV, most of the genes of the IFN signaling pathways were upregulated, as shown in transcriptomic studies realized in peripheral blood mononuclear cells (PBMCs) harvested from patients in the acute or in the convalescent phase of CCHFV. Upregulated genes included *IFI27, ISG15, MX1, MX2* and several other ISGs. Interestingly, upregulation of several ISGs during the acute phase of infection was correlated with a successful disease outcome (Neogi et al., 2022).

Overall, these data suggest that CCHFV induces an IFN response, but it was shown *in vitro* that this response was delayed (Andersson et al., 2008). Indeed, infection with CCHFV delayed nuclear translocation of IRF3 compared to UV-inactivated virus, suggesting that CCHFV encodes a protein or several proteins that delay innate immune responses.

II. CCHFV sensing

The PAMP at the origin of CCHFV sensing by the host remains to date uncharacterized. One study showed that IFN-β promoter activity was not triggered upon transfection of RNA isolated from CCHFV particles in HEK293T cells, contrary to RNA from RVFV (Habjan et al., 2008). Indeed, CCHFV genome does not contain an immunostimulatory 5'triphosphate group that is normally present at the 5'end of viral RNA produced from RNA polymerases, while RVFV genome does. The strategy used by CCHFV to process its genome remains to date a mystery. Moreover, it was shown in this study by RNA immunoprecipitation that CCHFV RNA did not bind RIG-I, while 5'ppp-containing RVFV RNA did. On the contrary, another study revealed that CCHFV RNA increased IFN-β promoter activity in a RIG-I-dependent manner in HEK293T cells. CCHFV 5'monophosphate group might still be immunostimulatory, as removing all phosphate groups from CCHFV RNA abolished IFN responses (Spengler et al., 2015). Moreover, when digested with RNase V1, CCHFV RNA ability to induce IFN responses was decreased, suggesting that dsRNA also represents a putative PAMP (Spengler et al., 2015). In this study, the implication of MDA5 in recognizing CCHFV was ruled out by knock-down experiments in A549 cells. Overall,

these data suggest that CCHFV RNA is a PAMP, but it cannot be ruled out that other structures such as the viral envelope or the nucleocapsid might also be recognized.

III. ISGs modulating CCHFV and other bunyaviruses infection

1. ISGs modulating bunyaviruses infection

Within the *Bunyavirales* order, research has elucidated the interplays between ISGs and several viruses, shedding light on the mechanisms of viral inhibition. This subject has been extensively described in a review that I wrote (Lerolle et al., 2021) (Annex 1). Bunyaviruses are inhibited by many ISGs that act at every step of the viral cycle, as described in Figure 22. Non-exhaustive examples of ISGs inhibiting bunyaviruses are depicted hereafter.

a. ISGs targeting bunyaviruses entry

Entry of bunyaviruses is targeted by several ISGs, the most extensively described being IFN-induced transmembrane proteins (IFITMs). Indeed, among the 5 IFITMs that have been described in humans (IFITM1, -2, -3, -5 and -10), three of them (IFITM1, -2, and -3) possess a broad-spectrum antiviral activity, bunyaviruses included (reviewed in (Lerolle et al., 2021)). More precisely, it was suggested that IFITM2 and IFITM3 regulate RVFV membrane fusion with endosomal membranes, a mechanism similar as those described for Influenza A virus (IAV) or VSV (Mudhasani et al., 2013). Other ISGs targeting bunyaviral entry are γ-IFN-inducible lysosomal thiol reductase (GILT), which inhibits Lassa virus (LASV) entry through its thiol reductase activity (Chen et al., 2019), and cholesterol-25-hydrolase (CH25H), that modulate cellular membrane composition in cholesterol and thus impairing membrane fusion of RVFV and that induces changes in the glycosylation of the glycoprotein of LASV, inhibiting LASV attachment to its entry receptor (Shrivastava-Ranjan et al., 2016).

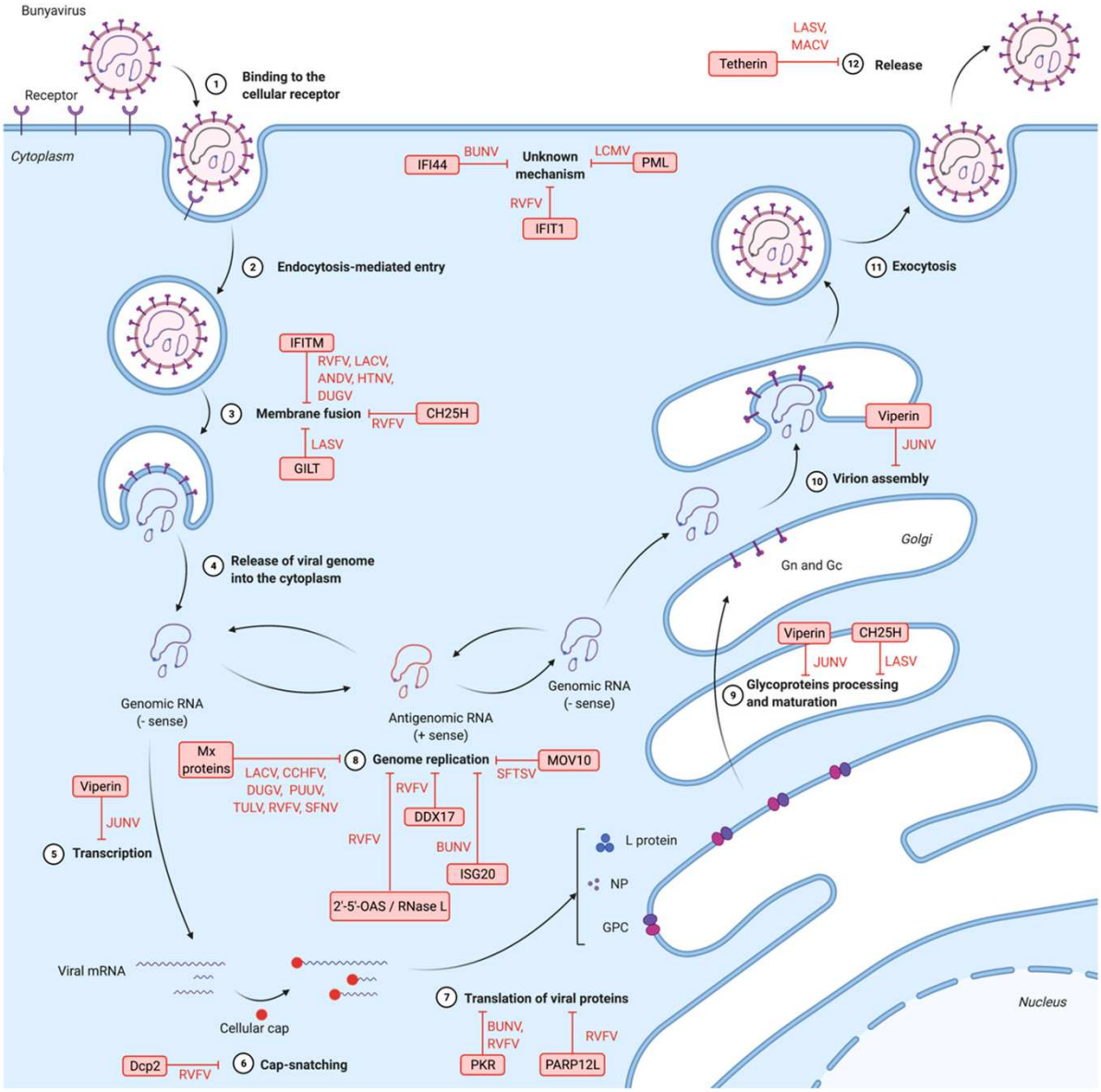

Figure 22. Host cell restriction factors against bunyaviruses. The steps of a typical bunyavirus cycle is represented, with (1) binding to the receptor, (2) endocytosis-mediated entry, (3) membrane fusion, (4) release of viral genome into the cytoplasm, (5) and (6) cap-snatching and transcription, (7) and (9) translation and maturation of viral proteins, (8) genome replication, (10) viral assembly and (11) and (12) egress. Each step of the cycle can be inhibited by restriction factors, which are written in red boxes. ANDV: Andes virus; BUNV: Bunyamwera virus; CCHFV: Crimean-Congo hemorrhagic fever virus; DUGV: Dugbe virus; HTNV: Hantaan virus; JUNV: Junín virus; LACV: La Crosse virus; LASV: Lassa virus; LCMV: lymphocytic choriomeningitis virus; MACV: Machupo virus; PUUV: Puumala virus; RVFV: Rift Valley fever virus; SFNV: Sandfly fever Naples virus; SFTSV: severe fever with thrombocytopenia syndrome virus; TULV: Tula virus. 2′-5′-OAS: 2′-5′-oligoadenylate synthetase; CH25H: cholesterol-25-hydrolase; Dcp2: Decapping 2; IFI44: interferon (IFN)-induced protein 44; IFIT1: IFN-induced protein with tetratricopeptide repeats 1; IFITM: IFN-induced transmembrane protein; ISG20: IFN-stimulated gene 20; GILT: γ-IFN-inducible lysosomal thiol reductase; MOV10: Moloney leukemia virus 10 protein; Mx: myxovirus resistance protein; PARP12L: long isoform of poly(ADP-ribose) polymerase 12; PKR: protein kinase R; PML: promyelocytic leukemia protein. From (Lerolle et al., 2021).

b. ISGs interfering with genomic transcription, protein translation and genomic replication of bunyaviruses

Several ISGs target bunyavirus genomic replication, among which Mx resistance proteins. These proteins are members of the family of large dynamin-like GTPases with a globular domain at their amino-terminus that bears the GTPase activity. In humans, two Mx proteins, namely MxA and MxB (also known as MX1 and MX2) are known to possess potent antiviral activity against a broad range of viruses (Haller et al., 2015), among which bunyaviruses (Frese et al., 1996). The interactions between MxA and La Crosse virus (LACV), an orthobunyavirus from the *Peribunyaviridae* family, is the most extensively researched within the bunyaviral order (Lerolle et al., 2021). It was shown that MxA interacts with LACV nucleoprotein and sequesters it within perinuclear complexes, making it inaccessible for the packaging of vRNA, thereby disrupting genome replication (Kochs et al., 2002; Reichelt et al., 2004). ISG20 is another protein targeting bunyaviral replication or transcription, as discovered through a gain-of-function screen of 488 ISGs against BUNV. Its antiviral activity was confirmed for other related peribunyaviruses as well as the orthonairovirus Dugbe virus (DUGV). ISG20 antiviral activity against BUNV was dependent on its exonuclease activity, and it targeted all viral segments (S, M or L) as well as all RNA species of the S segment of BUNV (genomic RNA, antigenomic RNA and mRNA) (Feng et al., 2018).

Bunyaviral translation is inhibited by a well-described protein, protein kinase R (PKR). This constitutively expressed, IFN-induced protein is present in a latent, inactive form, and is activated upon recognition of dsRNA or 5'ppp ssRNA. Following this recognition, PKR autophosphorylates and dimerizes, resulting in the phosphorylation of the eukaryotic translation initiation factor eIF2 α (eIF2α), which stops the translation process of both viral and cellular proteins. BUNV is targeted by this mechanism (Carlton-Smith and Elliott, 2012; Streitenfeld et al., 2003). Moreover, RVFV encoding an incomplete NSs gene (Clone 13) also is targeted by PKR, unlike wild-type RVFV, suggesting that NSs can counteract PKR-mediated viral restriction (Habjan et al., 2009; Ikegami et al., 2009).

c. ISGs blocking viral assembly and egress of bunyaviruses

Fewer ISGs have been described to target later stages of viruses, either because it is harder to study these steps independently of earlier steps, or because they are fewer

ISGs that target these steps (Schoggins, 2019). Still, both viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) and tetherin inhibit the later stages of bunyaviruses. Viperin inhibits Junín virus (JUNV), an arenavirus, by mislocalizing viral glycoproteins and altering lipogenesis, preventing JUNV to use the lipid rafts for virion budding (Peña Cárcamo et al., 2018). Tetherin, also known as bone marrow stromal antigen 2 (BST-2), prevents the release of many enveloped viruses, among which arenaviruses such as LASV or JUNV (Radoshitzky et al., 2010; Zadeh et al., 2020), or peribunyaviruses (Varela et al., 2017).

2. Activity of some ISGs against CCHFV

To date, very few ISGs have been studied in the context of CCHFV infection. Results from these studies are described below.

a. Mx proteins

Overexpression of MxA inhibited CCHFV replication in Vero cells (Andersson et al., 2004a) by 1000-fold compared to control cells as seen by RT-qPCR. The importance of MxA in controlling CCHFV infection was confirmed by knock-down experiments of MxA in Huh7 cells, that led to a significant rescue of the virus (Andersson et al., 2006). In MxA-overexpressing Vero cells, MxA colocalized with CCHFV NP and could co-immunoprecipitated it, suggesting that MxA interacted with NP. Moreover, MxA antiviral activity was dependent on its 645th amino acid, a glutamic acid, as proven by the absence of antiviral activity of the overexpressed MxA$_{E645R}$ mutant that also lacks antiviral activity against LACV, but not Influenza A virus (Kochs et al., 2002). MxA$_{E645R}$ did not interact anymore with LACV NP as well as CCHFV NP. Similarly, Dugbe virus (DUGV), another orthonairovirus, was also inhibited in MxA-overexpressing Vero cells (Bridgen et al., 2004). However, overexpression of human MxA in *Ifnar*$^{-/-}$ mice did not confer any protection against DUGV infection and mice succumbed to the disease (Boyd et al., 2006), suggesting that MxA alone is not sufficient to inhibit DUGV infection *in vivo*. *In vivo* studies in downregulation context remain to be performed, and whether CCHFV behaves the same as DUGV remains to be deciphered.

b. IFITM proteins

As abovementioned, IFITMs have been described to inhibit entry of several bunyaviruses. Intriguingly, IFITM1, -2 and -3 all lack antiviral activity against CCHFV (Mudhasani et al., 2013), highlighting species-specific differences among bunyaviruses regarding their susceptibility to ISG-mediated antiviral activities.

IV. CCHFV escape of innate immunity

As mentioned earlier, CCHFV delays the IFN response, suggesting that CCHFV somehow escapes and inhibits the implementation of the IFN-induced antiviral state (Andersson et al., 2008). Several studies explored the role of CCHFV-encoded proteins regarding this phenomenon. Interestingly, to date, no role of the non-structural proteins encoded by the M segment in escaping innate immunity has been described.

1. NP-mediated evasion from IFN responses

To date, only one study reports the effect of NP on IFN induction and observed that the NP from the Hoti strain of CCHFV slightly reduced the activity of IFN-β promoter by 30%, while the IbAr10200 NP stimulated it by 3 fold (Fajs et al., 2014). Thus, the delay of the IFN immune responses would not be due to expression of NP for the latter strain. In the absence of other studies to corroborate these findings, the putative role of NP in modulating IFN responses must be further investigated.

2. L protein OTU domain-mediated suppression of IFN responses

Among the *Bunyavirales* order, nairoviruses have the largest L segment (12 kb). Study of orthonairovirus L protein sequence reveals the presence of an ovarian tumor (OTU)-like protease motif at the amino-terminus, which is absent in other bunyaviruses (except in one plant virus from the *Phenuiviridae* family, the rice stripe tenuivirus) (Honig et al., 2004; Kinsella et al., 2004). Nairoviruses from other genus than orthonairoviruses, associated with millipedes and spiders (*Shaspivirus, Striwavirus* genus from the *Nairoviridae* family) lack this domain. Several biochemical and structural analyses of these orthonairovirus OTU domains showed that they possessed a deubiquitinase (DUB) activity (Akutsu et al., 2011; Bakshi et al., 2013;

Capodagli et al., 2011, 2013; Deaton et al., 2016; Dzimianski et al., 2019a; Frias-Staheli et al., 2007; Holzer et al., 2011; Kocabas and Aslan, 2015; van Kasteren et al., 2013). Mutation of the catalytic domain of CCHFV OTU protease (C40A) did not prevent L protein RdRp activity, as shown in a minigenome system (Bergeron et al., 2010). Interestingly, comparison of the DUB activity of the OTU from 12 orthonairoviruses showed that they could be divided into groups of OTU with high, moderate or low DUB activity. Notably, CCHFV OTU domain had a high DUB activity (Dzimianski et al., 2019a). These DUB activities were particularly efficient toward K6-, K11-, K48- and K63-linked ubiquitin (Ub).

Interestingly, IFN pathways are highly regulated thanks to post-translational modifications, among which ubiquitination. Notably, the ubiquitination of many components of the IFN signaling pathway is required for their activation, such as RIG-I (Gack et al., 2007). As expected, overexpression of isolated orthonairovirus OTU domains reduced the RIG-I-mediated IFN response (Bakshi et al., 2013; Frias-Staheli et al., 2007; Holzer et al., 2011). However, isolated OTU domains have a different localization than the full-length L protein. To decipher the role of the OTU domain in a context of infection, a reverse genetics system of CCHFV was used. Mutation of the catalytic domain of the CCHFV OTU (CCHFV-C40A) prevented rescuing the virus; but altering OTU activities by disrupting its binding to Ub (Q16R) allowed the generation of a recombinant CCHFV. Rescuing of a double mutant virus (CCHFV-C40A/Q16R) also proved successful, suggesting that the catalytic site of CCHFV OTU interfered with viral replication due to its binding of cellular Ub (Scholte et al., 2019a). Infection with wild-type CCHFV induced immune responses, and infection with CCHFV-Q16R mutant resulted in a strong increase in immune activation, which was dependent on RIG-I, suggesting that the OTU domain DUB activity was essential to dampen RIG-I-mediated innate immune responses (Scholte et al., 2017). Similarly, CCHFV-C40A/Q16R mutant was unable to grow in IFN-competent cells, and knock-down of RIG-I restored viral growth, confirming these previous results (Scholte et al., 2019a).

However, the OTU domain has not only a role in inhibiting innate immune responses, but also in CCHFV viral replication, as suggested by the inability to rescue CCHFV-C40A mutant, but not C40A/Q16R mutant and by the fact that neutralizing IFN failed to revert the attenuation of C40A-mutant tc-VLPs (Devignot et al., 2020). Moreover, stable occupancy of the OTU domain with a high-affinity synthetic Ub variant (UbV-CC4) led to a blocking of CCHFV OTU activity and a restoration of cellular RIG-I

signaling, and strikingly, also led to a blocking of CCHFV in RIG-I-deficient cells, suggesting that UbV-CC4 antiviral activity was not due to the immunosuppressive function of the OTU domain. Rather, it interfered with CCHFV viral transcription and/or replication, probably by interfering with the formation of vRNPs, as less NP was associated to L protein in presence of UbV-CC4 (Scholte et al., 2019a) and as elongation of RNA synthesis was not affected by UbV-CC4 (Tchesnokov et al., 2020).

In addition to their DUB activity, orthonairovirus OTU domains also display an activity of deISGylation (Akutsu et al., 2011; Bakshi et al., 2013; Capodagli et al., 2011, 2013; Deaton et al., 2016; Dzimianski et al., 2019a; Frias-Staheli et al., 2007; Holzer et al., 2011; Kocabas and Aslan, 2015; van Kasteren et al., 2013). ISG15 is a member of the Ub family and can be covalently conjugated onto target proteins via an enzymatic cascade called ISGylation. ISG15 is known to antagonize viral replication during infections, but its activity is diverse, pathogen- and host-species-dependent (Perng and Lenschow, 2018). Interestingly, ISGylation decreased the levels of expression of mutants of CCHFV L protein that lack deISGylase activity (L-C40A and L-A129R), but not wild-type L or L-Q16R, suggesting that deISGylation by the OTU domain was necessary to stabilize the protein (Scholte et al., 2017). As seen for OTUs DUB activity, deISGylase activity varied among members of the genus (Dzimianski et al., 2019b). Notably, deISGylase activity was present in orthonairoviruses that are associated with mammalian host species (human, sheep, cattle) and many OTUs displayed robust activity towards ISG15 from these species. ISG15 activity was predominantly restricted by specific orthonairovirus lineage which reflected the host-virus interaction or arms race, suggesting that this diverse deISGylase activity reflected the adaptation of the virus to its host, in a co-evolution process. Additionally, structural and mutational analysis identified key residues influencing ISG15 species specificities among nairovirus OTUs. For instance, HAZV OTU interacted exclusively with Ub, and not with ISG15, but structure-based mutagenesis proved successful to flip HAZV OTU substrate preference toward ISG15 (Dzimianski et al., 2020).

Objectives

CCHFV is a widespread virus responsible for a severe hemorrhagic fever. Given its potential to cause a public health emergency, CCHFV is listed among the Blueprint list of the World Health Organization (WHO), which aims at accelerating research and development for specific diseases. Indeed, understanding the virus and its biology is crucial to prevent and manage outbreaks in new regions and help limiting infections in endemic regions, and to develop effective therapies, vaccines, or diagnostic tools to improve patient outcome. Notably, there is a dire need to implement knowledge about the molecular mechanisms involved in virus/host interactions in the context of CCHFV infection, and more particularly, its interaction with the type I IFN pathway.

The objectives of this study were to gain more insight into the interactions between CCHFV and the type I IFN pathway. More especially, we aimed at identifying ISGs that modulate either positively or negatively CCHFV infection, and at characterizing which step of the viral lifecycle is targeted by the identified candidates.

To tackle these questions, I took advantage of the transcriptionally- and entry-competent virus-like-particles (tc-VLPs) system that was already implemented in the laboratory to screen libraries of lentiviral vectors individually expressing ISGs of human, macaque, or bovine origin. These libraries were provided through a collaboration that I initiated with Prof. Sam J. Wilson.

Thus, in the first part of the results of this manuscript, we aimed at optimizing the screening strategy as well as optimizing an entry assay to allow the study of CCHFV entry independently of its replication.

The second part of this study presents the results from the screen as well as the validation of the top candidates. We studied the steps that were targeted by the different ISGs identified, with a focus on GTP-binding protein 1 (GTPBP1), which was identified as a proviral gene in our screen. Through a collaboration with Dr. Cyrille Mathieu, we also validated some candidates identified in our screen with authentic CCHFV in BSL-4 conditions. These results are presented in the form of an article.

Results

Chapter I. Optimization of the gain-of-function screen and of the entry assays

I. Optimization of the gain-of-function screen strategy

To identify ISGs that modulate CCHFV infection, we chose to implement a gain-of-function approach using the well-described and established library of SCRPSY lentivectors (Kane et al., 2016; Wickenhagen et al., 2021; Galão et al., 2022; Hardy et al., 2023). These lentivectors individually express ISGs from human (*Homo sapiens*), macaque (*Macaca mulatta*) or bovine (*Bos taurus*) along with a TagRFP reporter molecule, in a 96-well plate format (see Chapter 2, Figure S1 for more information about the library). To adapt this system to the tc-VLPs, some optimization was necessary.

1. Choice of the cell line used to perform the screen

We aimed at identifying a suitable cell line to perform the screen using CCHFV tc-VLPs. Given that this cell line will be infected with CCHVF tc-VLPs, it should (i) be permissive and susceptible to CCHFV infection and (ii) have good transfection efficiencies to be able to perform multi-cycle screens or screens using tc-VLPs with a GFP-encoding minigenome, for which it is necessary to add NP and L protein *in trans* to detect a signal. Moreover, we reasoned that performing the screen in cells that were competent for IFN should allow the identification of ISGs which need other putative ISG cofactors for their activity, whose expression could be induced by tc-VLPs infection. Different cell lines were treated with type I IFN for 24 hours before infection with CCHFV tcVLPs encoding a nanoluciferase (nLuc) minigenome (nLuc tc-VLPs) (Figure 23).

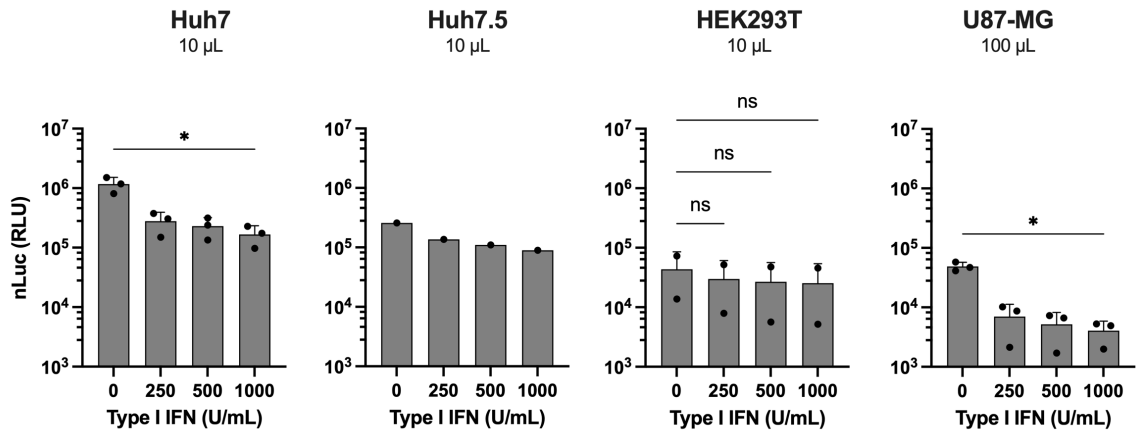

Figure 23. Effect of type I IFN on nLuc tc-VLPs infection in different cell lines. Huh7, Huh7.5, HEK293T and U87-MG cells were treated with increasing doses of type I IFN prior infection with nLuc tc-VLPs (10 µL for all cell lines except U87-MG that were infected with 100 µL). 24 hpi, cells were lysed, and luminescence was read. Mean and standard deviation (SD) of three (Huh7, U87-MG) and two (HEK293T) independent experiments are shown. For Huh7, HEK293T and U87-MG, statistical significance was analyzed using a Kruskal-Wallis test followed by post-hoc analysis using Dunn's multiple comparisons test (ns: non-significant, *: $p < 0.05$).

Huh7 cells, a hepatocyte-derived cellular carcinoma cell line, proved to meet all criteria with a high susceptibility and permissiveness to CCHFV nLuc tc-VLPs as well as a dose-dependent inhibition of infection upon IFN treatment. Of note, we also verified that the expression of one ISG, MX1, was induced upon type I IFN treatment of these cells by RT-qPCR (see Chapter 2, Figure 1). Thus, they were selected for further experiments.

2. Choice of the method of expression of the ISGs

In the literature, overexpression of the genes was achieved either by transduction with lentiviral vectors or by transfection of the pSCRPSY plasmid into target cells in 96-well plate format. To choose between these different methods, Huh7 cells were either directly transfected with the pSCRPSY vector or transduced with a selection of lentivectors expressing ISGs (Figure 24).

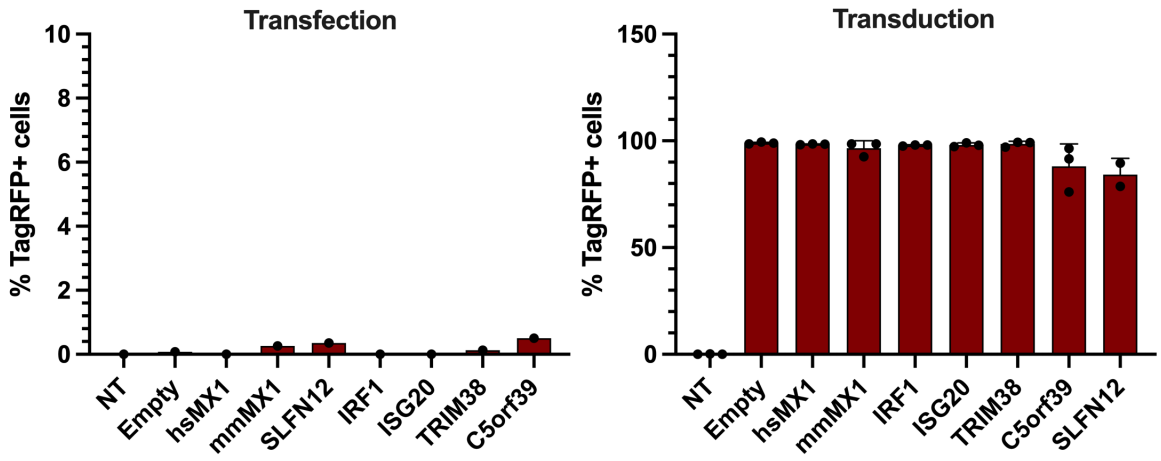

Figure 24. Comparison of transfection or transduction method for ISG expression in Huh7 cells. Huh7 cells were transfected with pSCRPSY (25 ng) (left) or transduced with SCRPSY lentiviral vectors (100 µL of vectors produced in 96 well-plate format) (right) expressing ISGs. 48 hours post-transfection or transduction, cells were fixed and TagRFP expression was analyzed by flow cytometry. For the transduction experiment, means and SD of three independent experiments are shown, except for SLFN12 which was performed twice.

Transfection of Huh7 cells in a 96-well plate format proved highly inefficient, while transduction of Huh7 cells with selected lentivectors allowed to achieve high levels of TagRFP expression. Thus, we opted for transducing the ISGs for our screen.

3. Choice of the type of tc-VLPs used for the screen

To identify ISGs modulating CCHFV infection, we took advantage of the tc-VLPs system that was already available in the laboratory. These tc-VLPs were at the time used in the laboratory in a single-cycle manner (Figure 15). nLuc tc-VLPs were used to infect either cells that do not express viral proteins or cells that express NP and L protein, and GFP tc-VLPs were used to infect only cells that express NP and L protein. Indeed, infecting cells lacking these proteins with GFP tc-VLPs resulted in the absence of any detectable GFP signal following infection, while nLuc signal could be measured in the same cells. Either way, because infected cells do not express GPC, no new viral particles are formed, allowing to study CCHFV early steps of lifecycle in a single-cycle manner.

However, by infecting cells that express NP, L protein and GPC, new viral particles are produced, as demonstrated with Renilla luciferase tc-VLPs (Devignot et al., 2015) and our own confirmations for GFP tc-VLPs and nLuc tc-VLPs (Figure 25A and 25C).

Infection of cells expressing NP + L + GPC (amplifier cells) led to a strong increase in GFP signal in GFP tc-VLPs infected cells compared to NP + L-infected cells, in which the signal stagnated over time. The same tendency was seen for nLuc tc-VLPs. These results suggested that newly formed virions were produced and subsequently infected neighboring cells, in a multi-cycle manner. Furthermore, supernatants from amplifier cells harvested 48 hpi were used to infect cells that express NP + L (indicator cells) and led to productive infection of indicator cells (Figure 25B and 25D). However, the GFP signal in indicator cells was low (Figure 25B), which was unexpected given the high percentage of GFP-positive cells in amplifier cells at the time of harvest. This suggested that amplifier cells produced much less tc-VLPs than producer cells.

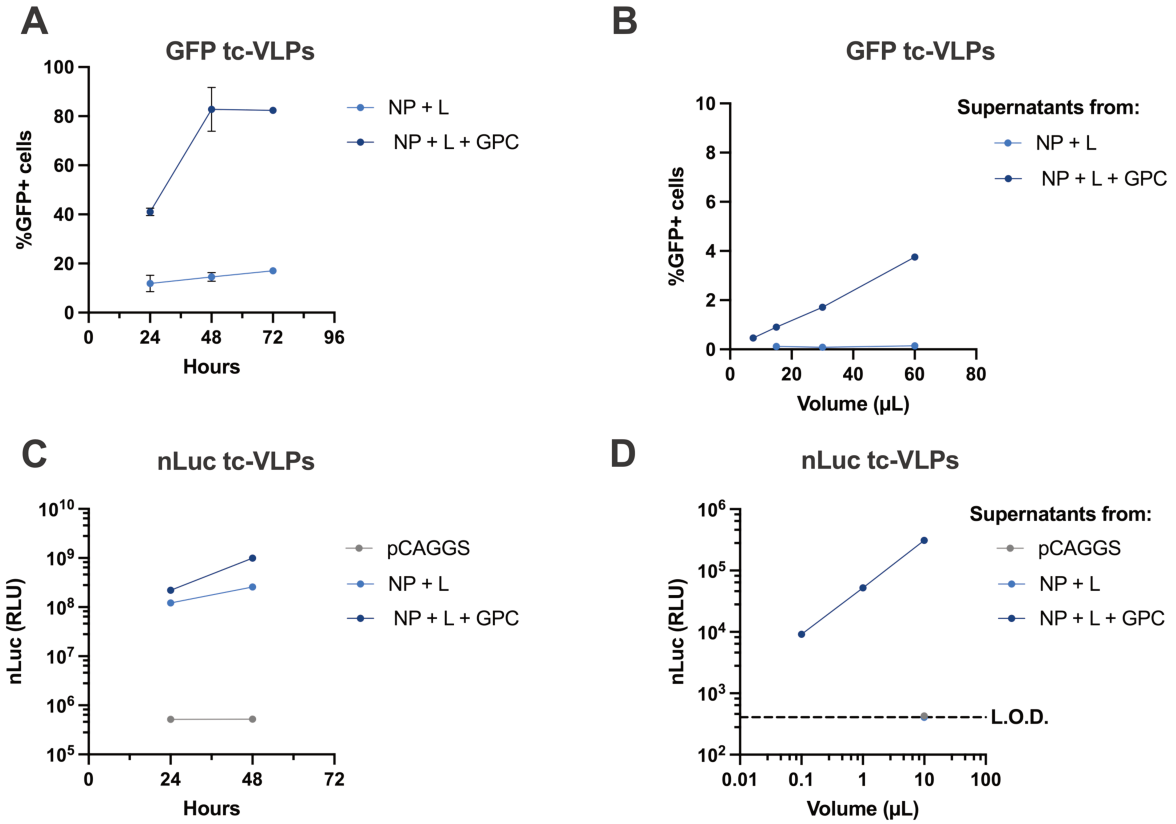

Figure 25. Amplification of GFP and nLuc tc-VLPs in a multi-cycle manner. (A) Huh7 cells were transfected with NP and L protein or with NP, L protein and GPC prior infection with GFP tc-VLPs. 3 hpi, medium was replaced. At the indicated time-points, the supernatants were harvested, and cells were fixed and analyzed by flow cytometry. Means and SD of two independent experiments are shown. **(B)** Supernatants from (A) harvested at 48 hpi were used to infect Huh7 cells expressing NP and L protein. 24 hpi, cells were fixed and analyzed by flow cytometry. **(C)** Huh7 cells were transfected with a control vector (pCAGGS), with NP and L protein or with NP, L protein and GPC prior infection with nLuc tc-VLPs. 3 hpi, medium was replaced. At the indicated time-points, the supernatants were harvested, cells were lysed, and luminescence was read. **(D)** Supernatants from (C) harvested at 48 hpi were used to infected Huh7 cells. 24 hpi, cells were lysed, and luminescence was read. L.O.D. = limit of detection.

Then, cells expressing no viral proteins, expressing NP + L or NP + L + GPC were transduced with selected SCRPSY lentivectors prior infection with nLuc or GFP tc-VLPs (Figure 26A and 26C). These vectors encoded human MX1 (hsMX1), IFN-regulatory factor 1 (IRF1), IFN-stimulated gene 20 (ISG20), Tripartite Motif Containing 38 (TRIM38), C5orf39, Schlafen family member 12 (SLFN12), as well as a macaque MX1 (mmMX1). hsMX1 and mmMX1 were chosen given that MX1 was identified as inhibiting CCHFV infection (Andersson et al., 2006, 2004a), and ISG20 due to its ability to restrict related viruses such as BUNV (an orthobunyavirus) or Dugbe virus (an orthonairovirus) (Feng et al., 2018). As members of the IFN signaling cascade, IRF1 and TRIM38 were chosen due to their role in inducing innate immune responses. Finally, C5orf39 and SLFN12 were included as they were found as antiviral ISGs in several screens against different viruses (Kane et al., 2016; Galão et al., 2022).

Expression of IRF1, C5orf39 and SLFN12 in non pretransfected cells led to a decrease in nLuc tc-VLPs infection relative to cells transduced with an empty lentivector ("Empty"), that was accentuated by the expression of viral proteins (NP + L and NP + L + GPC). Expression of ISGs in GFP tc-VLPs-infected cells also allowed the identification of putative antiviral ISGs, independently of GPC expression. Supernatants harvested from NP + L + GPC-expressing cells were used to infect cells. As expected, supernatants from IRF1-, C5orf39- and SLFN12-expressing cells were less infectious than the control supernatant, both for nLuc and GFP tc-VLPs (Figure 26B and 26D). However, consistent with our observation that amplifier cells do not produce high amounts of tc-VLPs, signals in these infected cells were very low.

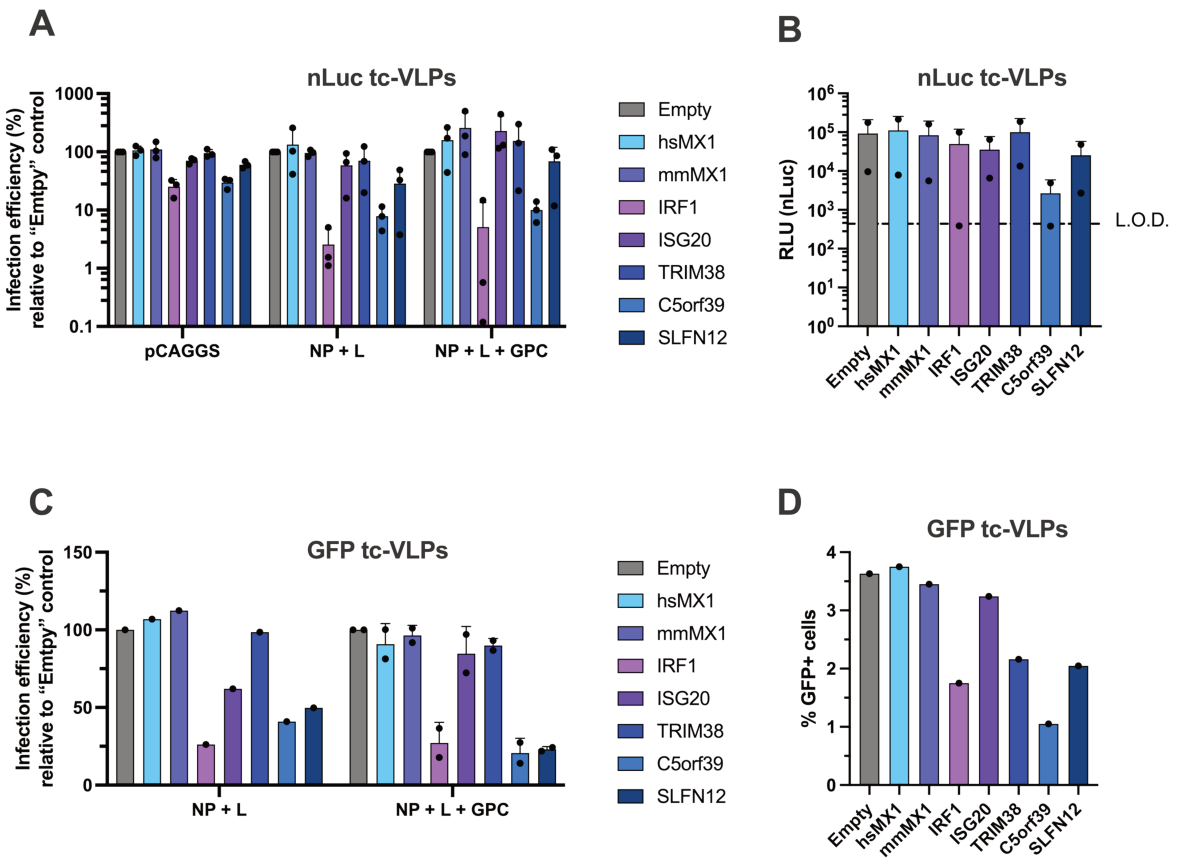

Figure 26. Optimization of the screen. (A) Huh7 cells were transfected with pCAGGS, with NP and L protein or with NP, L protein and GPC. 6 hours post-transfection, they were transduced with SCRPSY lentivectors. 48 hours post-transduction, they were infected with nLuc tc-VLPs. 3 hpi, medium was replaced. Supernatants from NP + L + GPC-expressing cells were harvested 30 hpi and luminescence was read on lysed cells. Results are expressed in relative infection efficiency compared to the "Empty" control of each cell type. **(B)** Supernatants from (A) (NP + L + GPC-expressing cells) were used to infect Huh7 cells. 24 hpi, cells were lysed, and luminescence was read. L.O.D. = limit of detection. **(C)** Huh7 cells were transfected either with NP and L protein or with NP, L protein and GPC. 6 hours post-transfection, they were transduced with SCRPSY lentivectors. 48 hours post-transduction, they were infected with GFP tc-VLPs. 3 hpi, medium was replaced. 30 hpi, supernatants from NP + L + GPC-expressing were harvested and GFP expression in cells was read by flow cytometry. Results are expressed in relative infection efficiency compared to the "Empty" control of each cell type. **(D)** Supernatants from (C) (NP + L + GPC-expressing cells) were used to infect NP + L-expressing Huh7 cells. 24 hpi, cells were fixed and analyzed by flow cytometry. (A), (B) and (C) Means and SD from two or three independent experiments are shown, except for the NP + L condition of (C) which was performed once.

Given the results of our optimization screens, NP + L-expressing as well as NP + L + GPC-expressing cells were found to be suitable to identify ISGs modulating CCHFV tc-VLPs infection. We chose to use GFP tc-VLPs for our screen given that this method allowed the identification of ISGs at a single-cell level by flow cytometry, contrary to nLuc tc-VLPs that only allowed to study infection efficiency at a bulk population level. This was of particular interest given that transduction levels of ISGs, assessed by the

percentage of TagRFP-positive cells, might differ between ISGs during the screen. Moreover, we chose to perform the screen in amplifier cells expressing NP + L + GPC. Indeed, in these cells, all the steps of the viral lifecycle are represented, from entry to assembly and budding of newly formed viral particles. Performing the screen in these cells could therefore help in the identification of putative ISGs modulating later stages of the viral lifecycle, contrary to NP + L-expressing cells.

II. Entry assay optimization

At the time of my arrival in François-Loïc Cosset laboratory, the system of tc-VLPs for the study of CCHFV was already implemented and commonly used. While this system allowed the study of the early steps of CCHFV replication cycle among which entry, this was also dependent on CCHFV transcription and replication. Thus, we aimed at developing a system to study CCHFV entry independently of CCHFV transcription and replication.

1. CCHFV GPC-pseudotyped lentiviruses

Pseudotyped viruses are viral particles bearing glycoproteins from another enveloped virus. They allow the study of the mechanisms that involve the glycoproteins originated from the other virus, such as, but not limited to, entry mechanisms, kinetics, tropism, serological investigation, etc. Lentiviruses can be pseudotyped with different viral glycoproteins and thus allow the study of the entry related to the virus with which they have been pseudotyped. A study published in 2020 reports the efficient generation of lentiviruses displaying CCHFV glycoproteins (Vasmehjani et al., 2020), and we decided to implement this system in our laboratory.

To do so, HEK293T cells were transfected with a lentivector expressing GFP (pCSWG-GFP), Gag-Pol (psPAX2) and with CCHFV glycoproteins. We tested different constructs of CCHFV glycoproteins, that bear mutations or deletions in Gc cytoplasmic tail, as current work in our laboratory shows that these mutations/deletions have an impact on Gc intracellular localization and in particular its accumulation at the plasma membrane (Gautam et al., in preparation). Notably, Gc that bears a mutation in the first tyrosine of its cytoplasmic tail (GPC_{Y1635A}) or which lacks 53 amino acids ($GPC_{\Delta 53}$) or the total cytoplasmic tail ($GPC_{\Delta 64}$) have been shown by us to predominantly localize at the plasma membrane, and by others to ensure

efficient incorporation into pseudotyped VSV (Suda et al., 2016). In a replication-competent VSV pseudotyped with CCHFV glycoproteins system, CCHFV GPC bears several mutations in GP38 as well as in Gc (Rodriguez et al., 2019). Mutations in Gc encompass 2 mutations and a deletion of 14 amino acids at the cytoplasmic tail of Gc and were introduced in a mutant of GPC called $GPC_{L1638R\text{-}R1648Q\text{-}\Delta14}$. These different constructs were tested to pseudotype lentiviruses (Figure 27).

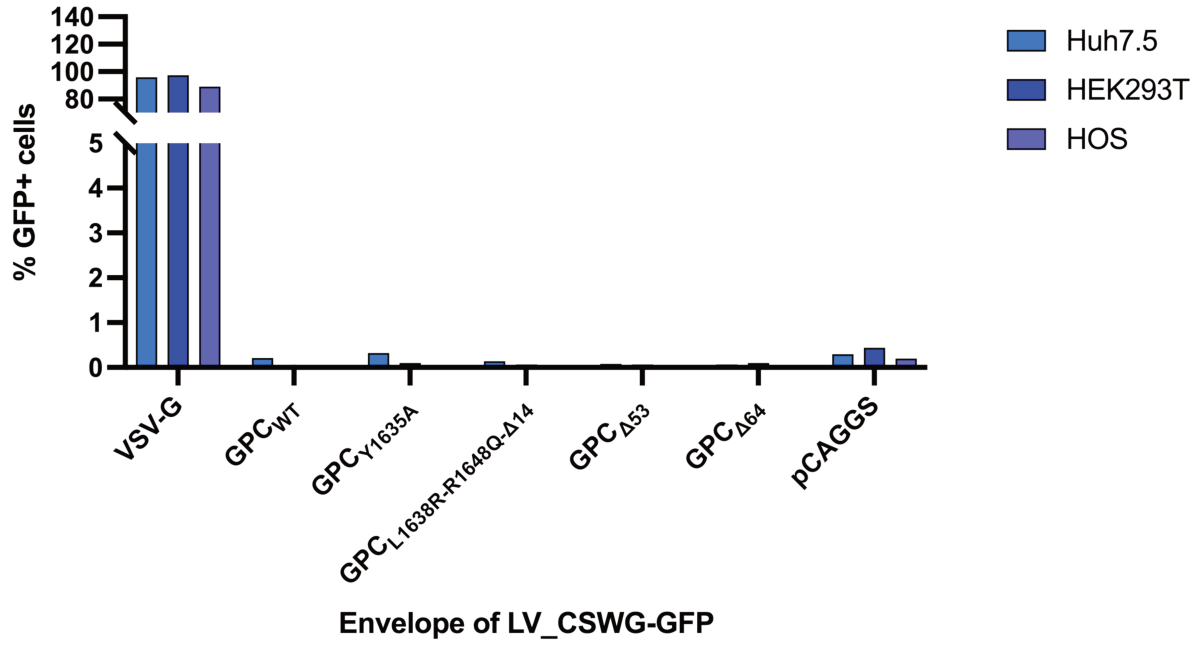

Figure 27. Inefficient pseudotyping of lentiviruses with CCHFV GPC. HEK293T cells were transfected with pCSWG-GFP, psPAX2, and different GPC constructs in a ratio of 2:2:8 (except for VSV-G envelope where a ratio of 2:2:0.5 was used). Supernatants were harvested 48 hours post-transfection and used to infect Huh7, HEK293T or HOS cells. 72 hpi, cells were fixed and GFP expression was analyzed by flow cytometry.

Infection of CCHFV permissive, susceptible cells (Huh7 cells), non-susceptible, permissive cells (HEK293T cells) or non-permissive, non-susceptible cells (human osteosarcoma HOS cells) with VSV-G-pseudotyped lentiviruses led to efficient and strong signal in GFP by flow cytometry. However, we were unable to detect any signal using CCHFV-GPC-pseudotyped lentiviruses. Different quantities of glycoproteins were also tested (data not shown), without success. We were therefore unable to produce lentiviruses bearing CCHFV glycoproteins.

2. Pseudotyped VSV

VSV pseudotyping is a largely and widely used technique to study viral entry independently of the viral replication. Two published works present the successful pseudotyping of VSV with CCHFV glycoproteins. The first one (Suda et al., 2016) exposes the generation of replication-deficient VSV pseudotyped with CCHFV $GPC_{\Delta 53}$ and $GPC_{\Delta 64}$ while the second reports the generation of replication-competent VSV (Rodriguez et al., 2019). In the latter virus, VSV-G coding sequence was replaced by CCHFV GPC, and after several passages, GPC had acquired several mutations, as aforementioned. We thus tested these different constructs as well as the GPC_{Y1635A} mutant and a mutant that lacks the endoplasmic retrieval motif ($GPC_{\Delta 5}$) to pseudotype replication-deficient VSV expressing GFP instead of VSV-G (rVSV-dG/GFP). Pseudotyped viruses were used to infect either permissive, susceptible cells (Huh7 cells) or non-susceptible, permissive cells (HEK293T cells) (Figure 28A).

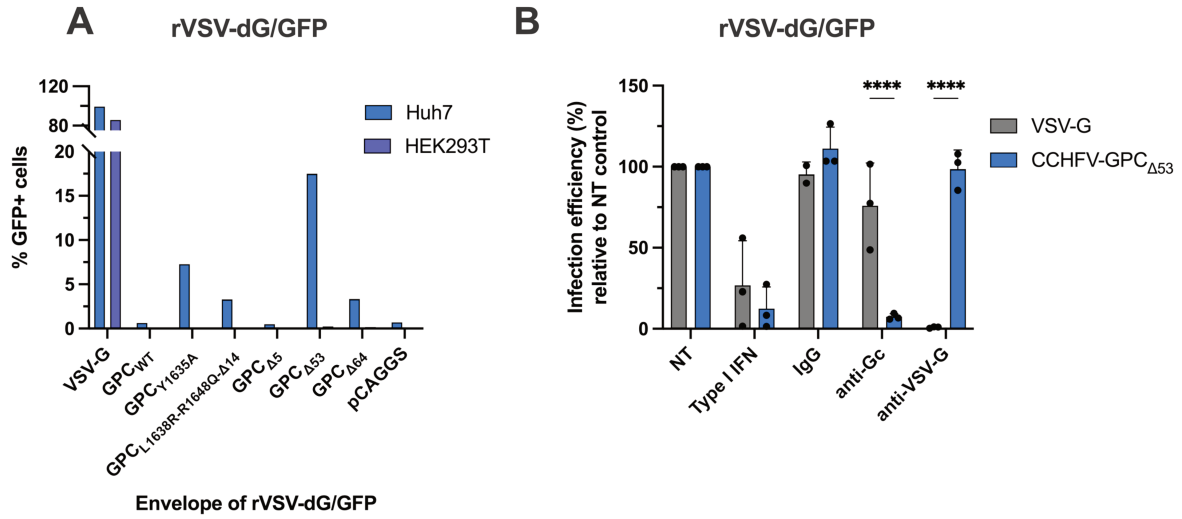

Figure 28. Efficient pseudotyping of VSV with CCHFV GPC. (A) HEK293T cells transfected with plasmids expressing VSV-G or GPC mutants were infected with rVSV-dG/GFP for 1 hour before the medium was replaced with medium containing an anti-VSV-G antibody. Supernatants harvested 24 hours post-transfection were used to infect Huh7 or HEK293T cells. 24 hpi, cells were fixed and GFP expression was analyzed by flow cytometry. **(B)** Huh7 cells were infected with VSV viruses pseudotyped with VSV-G (rVSV-dG/GFP*VSV-G) or $GPC_{\Delta 53}$ (rVSV-dG/GFP* $GPC_{\Delta 53}$) that were incubated with 10 µg/mL of anti-Gc antibody, anti-VSV-G antibody, or control mouse IgG prior infection. Alternatively, Huh7 cells that were treated for 24 hours with type I IFN (1000 U/mL) were also infected with pseudotyped viruses. Data are expressed as relative infection efficiency compared to a non-treated (NT) control. Means and SD from three independent experiments are shown. Statistical significance was analyzed using a two-way ANOVA with Šidák multiple comparisons test (****: $p < 0.0001$).

Infection of Huh7 and HEK293T cells with VSV-G-pseudotyped viruses led to a robust and strong signal in GFP (Figure 28A). Infection of Huh7 cells with $GPC_{\Delta 53}$ led to GFP expression in 17.5% of cells. To a lesser extent, GPC_{Y1635A}, $GPC_{\Delta 64}$ and $GPC_{L1638R\text{-}R1648Q\text{-}\Delta 14}$ also led to a signal in less than 10% of the cells. Infection of HEK293T cells with these viruses was not productive, suggesting that the pseudotyping was efficient. Moreover, treatment of viruses with neutralizing anti-Gc or anti-VSV-G antibodies decreased the infectivity of CCHFV-$GPC_{\Delta 53}$- or VSV-G-pseudotyped viruses, respectively (Figure 28B). Finally, type I IFN pre-treatment of Huh7 cells led to an inhibition of infection with both pseudotyped viruses, suggesting that type I IFN inhibits CCHFV-GPC-mediated and VSV-G-mediated entry and/or VSV-driven expression of GFP.

3. tc-VLPs with HiBiT-tagged viral proteins

While the VSV system is efficient to study entry of viruses, its limitation is that it also relies on VSV-driven expression of reporter molecule, a step that could be inhibited by anti-VSV as well as broad-spectrum antiviral proteins, as seen for type I IFN pre-treatment (Figure 28B). To counteract this, we aimed at developing an assay that could detect CCHFV entry independently of the replication of any virus.

Thus, we generated a mutant of CCHFV NP that expresses HiBiT at its N-terminus domain (HiBiT-NP). HiBiT is a small, 11-amino-acid-long fragment of nLuc that is complementary to a larger nLuc fragment called LgBiT. Infection of LgBiT-expressing cells with tc-VLPs containing this HiBiT-NP would therefore lead to a nLuc signal upon release of the vRNPs into the cytoplasm, *i.e.*, only after viral and host membrane fusion is mediated by viral glycoproteins in the endosomes. Such systems of HiBiT-containing virus-like particles have already been developed for other viruses, such as SARS-CoV-2 (Miyakawa et al., 2020; Kumar et al., 2021).

Thus, Huh7 cells were transfected to produce GFP tc-VLPs either with wild-type NP (WT-NP) or HiBiT-NP. As a control, Huh7 cells were also transfected with plasmids to produce GFP tc-VLPs with HiBiT-NP, but without GPC. Western blot of pelleted fractions of tc-VLPs-containing supernatants revealed that WT-NP and HiBiT-NP were detectable in the pelleted fractions (Figure 29A). Moreover, HiBiT signal was detectable at a size corresponding to NP. In the conditions without envelope, HiBiT signal was also detected in the pelleted fraction, suggesting that HiBiT-NP is secreted

independently of GPC expression. This was not unexpected, as NP is secreted independently of the glycoproteins (Freitas et al., 2020). Through infection of Huh7 cells with GFP tc-VLPs WT-NP or HiBiT-NP, we could demonstrate that HiBiT-NP did not impair tc-VLPs infectivity (Figure 29B). Because HiBiT signal was present extracellularly even in the absence of envelope, we measured the quantity of extracellular HiBiT with or without treatment with a detergent. Indeed, we reasoned that if pelleted HiBiT-NP is within viral particles, we should see higher signal in extracellular HiBiT in the presence of detergent than in absence of detergent. We could not detect extracellular HiBiT signal in GFP tc-VLPs WT-NP supernatants, and we could detect more signal in GFP tc-VLPs HiBiT-NP with envelope than in GFP tc-VLPs HiBiT-NP with no envelope. However, Triton 0.1% treatment did not increase the signal of extracellular HiBiT, suggesting that most HiBiT-NP were not contained within viral particles, or that this method was not sensitive enough to detect the amount of HiBiT-NP within particles (Figure 29C). Combined with the observation that GFP tc-VLPs HiBiT-NP showed conserved infectivity, it could be further hypothesized that NP within particles represents an insignificant fraction of secreted NP in the tc-VLPs system.

Then, we proceeded to the infection on Huh7 cells stably expressing LgBiT with these tc-VLPs. Prior infection, cells were treated for 1 hour with 50 nM of Bafilomycine A or DMSO. Alternatively, viruses were incubated for 1 hour with 10 μg/mL of neutralizing anti-Gc antibody or control mouse IgG. Infection of NP + L-expressing Huh7 cells followed by flow cytometry 24 hpi revealed that Bafilomycine A treatment of cells or anti-Gc treatment or viruses abrogated infection (Figure 29D). To study the entry of HiBiT into cells, we performed a kinetics by reading nLuc signal in live Huh7_LgBiT cells (Figure 29E, F and G). Infection of cells with GFP tc-VLPs WT-NP gave no luminescence signal, while infection of cells with GFP tc-VLPs HiBiT-NP had a high luminescence signal that increased over time to reach a plateau at 2 hpi. However, GFP tc-VLPs HiBiT-NP with no envelope also gave rise to a high luminescence signal, suggesting that the signal that was seen was not specific to GPC-mediated entry of GFP tc-VLPs. Moreover, these luminescence signals could be inhibited by neither Gc neutralization (Figure 29F) nor by Bafilomycine A treatment (Figure 29G). Overall, these data suggested that tc-VLPs HiBiT-NP were not suitable to study CCHFV entry.

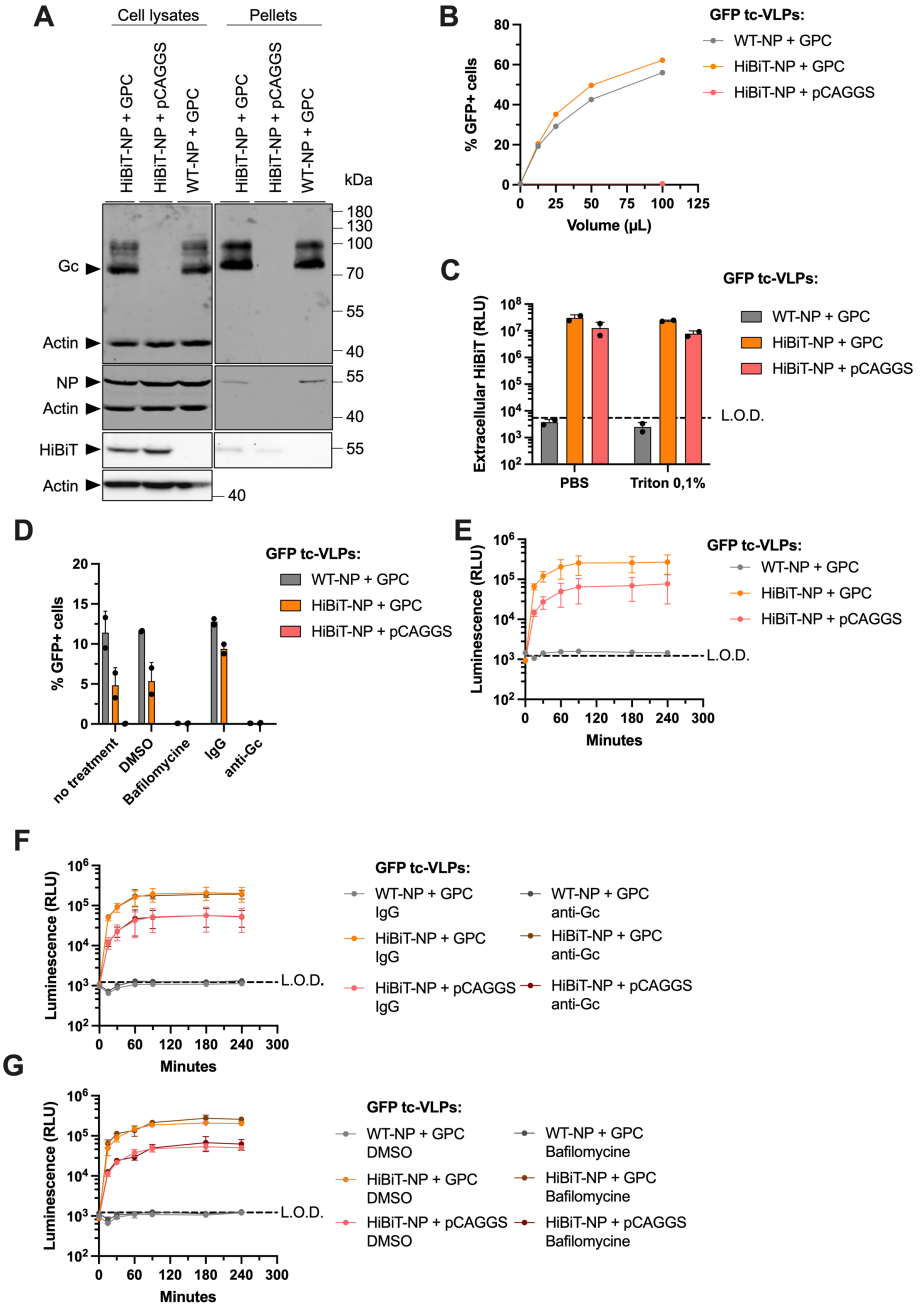

Figure 29. Production and characterization of GFP tc-VLPs HiBiT-NP. (A) Huh7 cells were transfected with L protein, T7, CCHFV GFP minigenome plasmids, with GPC plasmid or pCAGGS, and with NP-WT or NP-HiBiT plasmids. 72 hours post-transfection, cells were lysed and subjected to western blot using anti-Gc, anti-NP, anti-actin antibodies or LgBiT protein. Supernatants were harvested and ultracentrifuged before western blot analysis (pellets). **(B)** Huh7 cells expressing NP and L protein were infected with GFP tc-VLPs expressing NP-WT, NP-HiBiT, or NP-HiBiT without envelope. 24 hpi, cells were fixed and GFP expression was analyzed by flow cytometry. **(C)** GFP tc-VLPs-containing supernatants were treated 1h at 37°C with PBS or Triton 0.1%. Extracellular HiBiT was quantified by luminometry. L.O.D. = limit of detection. **(D)** Huh7 cells expressing NP and L protein were either treated with DMSO or 50 nM Bafilomycine A for 1h at 37°C. Alternatively, GFP tc-VLPs were treated 1h at 37°C with anti-Gc antibody or control mouse IgG. Then, the cells were infected with the tc-VLPs and GFP expression was analyzed by flow cytometry 24 hpi. **(E)** Huh7 cells stably expressing LgBiT (Huh7_LgBiT cells) were infected with GFP tc-VLPs expressing NP-WT, NP-HiBiT, or NP-HiBiT without envelope. **(F)** GFP tc-VLPs were treated 1h at 37°C with anti-Gc antibody or control mouse IgG before infection of Huh7_LgBiT cells. **(G)** Huh7_LgBiT cells were treated with DMSO or 50 nM Bafilomycine A for 1h at 37°C prior infection with the different GFP tc-VLPs. **(E), (F) and (G)**, luminescence was analyzed in live cells over time using Vivazine. For (C), (D), (E), (F), (G), means and SD from two independent experiments are shown.

Consequently, we tried to insert HiBiT in the glycoproteins rather than in CCHFV NP. HiBiT was inserted at the cytoplasmic tail of Gc (Gc-HiBiT) so that it is located inside viral particles. Thus, it should give a nLuc signal only after viral and endosomal membrane fusion, upon encountering cytoplasmic LgBiT. We tried to produce GFP-minigenome-containing tc-VLPs with Gc-HiBiT. Alternatively, because CCHFV Gc (in the context of GPC transfection of cells) is secreted even in the absence of L protein, NP or minigenome, we produced subviral particles (SVPs). Production of GFP tc-VLPs bearing only the HiBiT version of glycoproteins was unsuccessful, as the tc-VLPs were not infectious (data not shown). However, this was circumvented by the combination of different ratios of Gc wild-type (Gc-WT) and Gc-HiBiT (either 75% Gc-WT and 25% Gc-HiBiT, or 50% Gc-WT and 50% Gc-HiBiT). Western blot analysis of pelleted fractions of tc-VLPs or SVPs-containing supernatants revealed that Gc-WT and Gc-HiBiT were detectable in the pelleted fractions (Figure 30A and 31A). Moreover, HiBiT signal at the size of Gc was seen in the pelleted fractions of HiBiT-Gc-containing tc-VLPs or SVPs. As seen by infecting NP + L-expressing Huh7 cells with the different tc-VLPs, Gc-HiBiT incorporation resulted in a decreased infectivity of viral particles (Figure 30B). To check whether HiBiT was inside or outside viral particles, we treated the particles with detergent and measured the quantity of extracellular HiBiT. Triton 0.1% treatment led to a slightly higher signal of extracellular HiBiT for GFP tc-VLPs that contain Gc-HiBiT (Figure 30C), suggesting that some HiBiT could indeed be inside tc-VLPs particles. However, this was not seen for SVPs (Figure 31B). As performed for tc-VLPs HiBiT-NP, we proceeded to the infection on Huh7 cells stably expressing LgBiT with these tc-VLPs. Bafilomycine A or anti-Gc treatment was efficient to abrogate tc-VLPs infection (Figure 30D). Because SVPs do not contain any minigenome, we could not check whether these treatments were efficient to inhibit SVPs entry. Analysis of nLuc signal in live cells over time revealed too little signal to work with for both tc-VLPs or SVPs (Figure 30E, 30F, 30G and Figure 31C, 31D and 31E).

Overall, these data suggest that the HiBiT system for detection of CCHFV entry independently of viral replication has yet to be optimized, by increasing the production of HiBiT-containing particles. For instance, ultracentrifugation of supernatant through a 20% sucrose cushion could help getting rid of all extracellular HiBiT that is not within tc-VLPs, and help concentrating the HiBiT-containing particles.

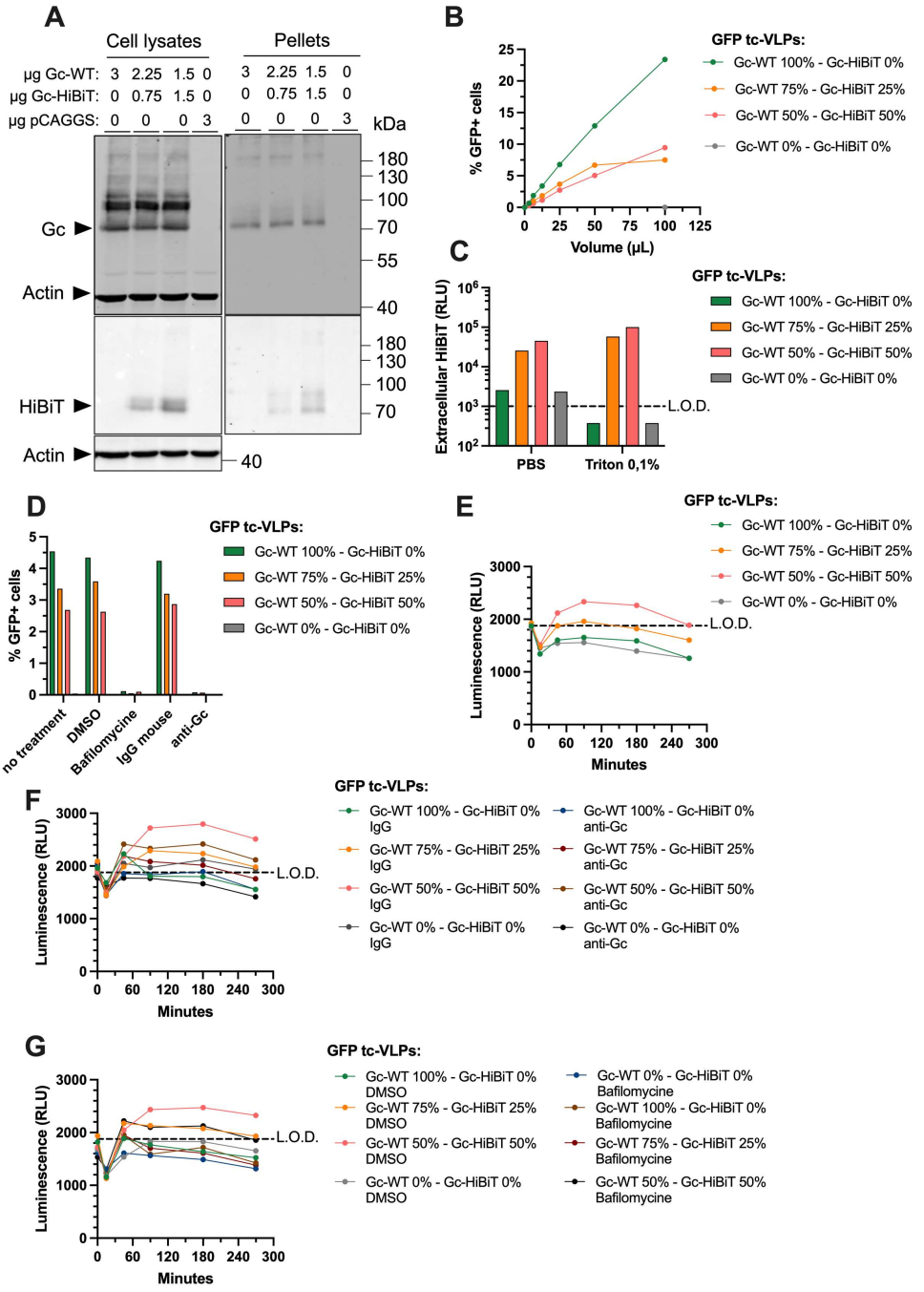

Figure 30. Production and characterization of GFP tc-VLPs Gc-HiBiT. (A) Huh7 cells were transfected with L protein, T7, NP and CCHFV GFP minigenome plasmids, and with different quantities of GPC-Gc-WT, GPC-Gc-HiBiT or pCAGGS. 72 hours post-transfection, cells were lysed and subjected to western blot using anti-Gc and anti-actin antibodies or LgBiT protein. Supernatants were harvested and ultracentrifuged before western blot analysis (pellets). **(B)** Huh7 cells expressing NP and L protein were infected with the different GFP tc-VLPs. 24 hpi, cells were fixed and GFP expression was analyzed by flow cytometry. **(C)** GFP tc-VLPs-containing supernatants were treated 1h at 37°C with PBS or Triton 0.1%. Extracellular HiBiT was quantified by luminometry. L.O.D. = limit of detection. **(D)** Huh7 cells expressing NP and L protein were either treated with DMSO or 50 nM Bafilomycine A for 1h at 37°C. Alternatively, GFP tc-VLPs were treated 1h at 37°C with anti-Gc antibody or control mouse IgG. Then, the cells were infected with the tc-VLPs and GFP expression was analyzed by flow cytometry 24 hpi. **(E)** Huh7 cells stably expressing LgBiT (Huh7_LgBiT cells) were infected with the different GFP tc-VLPs. **(F)** GFP tc-VLPs were treated 1h at 37°C with anti-Gc antibody or control mouse IgG before infection of Huh7_LgBiT cells. **(G)** Huh7_LgBiT cells were treated with DMSO or 50 nM Bafilomycine A for 1h at 37°C prior infection with the different GFP tc-VLPs. **(E), (F) and (G)**, luminescence was analyzed in live cells over time using Vivazine.

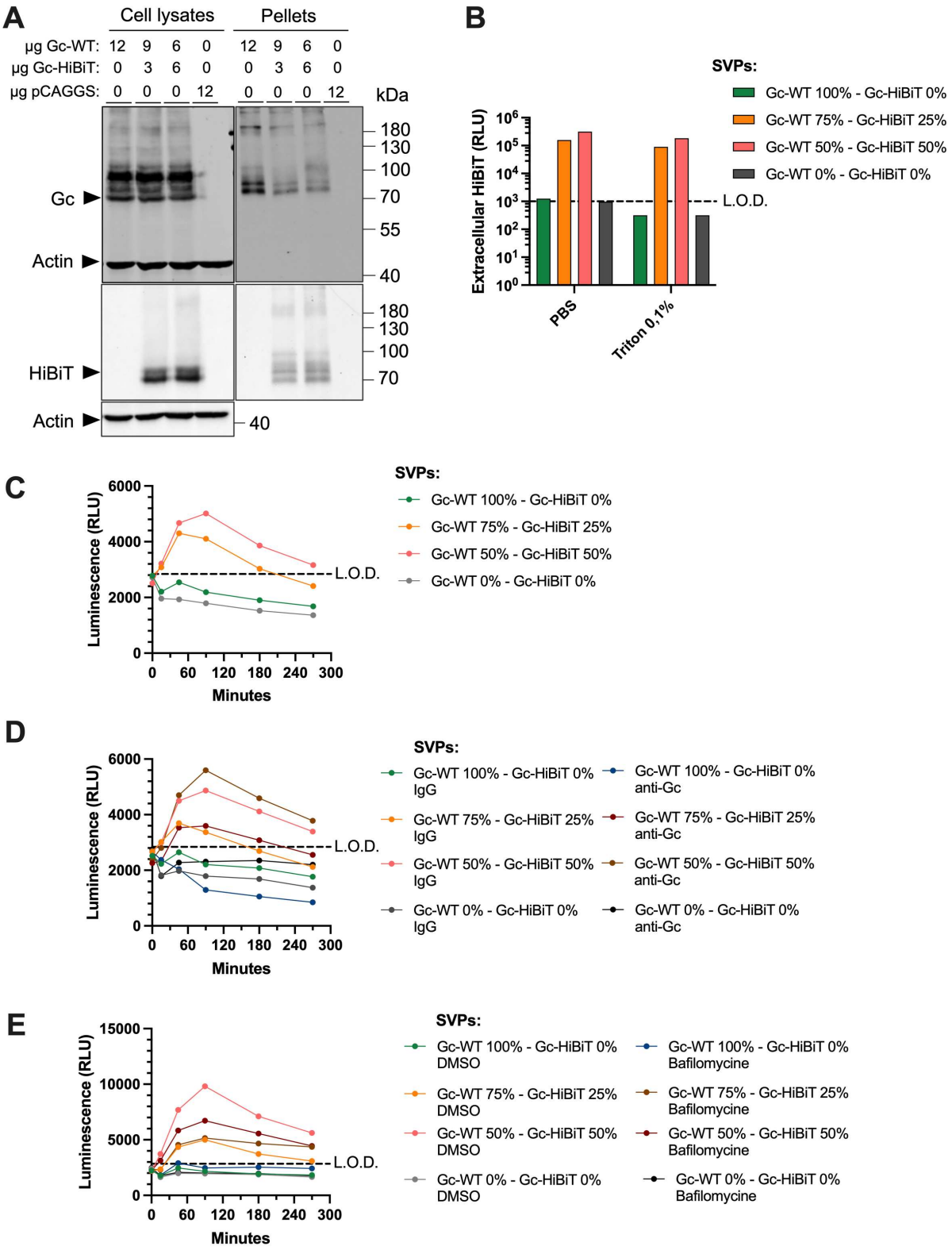

Figure 31. Production and characterization of SVPs Gc-HiBiT. (A) Huh7 cells were transfected with different quantities of GPC-Gc-WT, GPC-Gc-HiBiT or pCAGGS. 72 hours post-transfection, cells were lysed and subjected to western blot using anti-Gc and anti-actin antibodies or LgBiT protein. Supernatants were harvested and ultracentrifuged before western blot analysis (pellets). **(B)** SVPs-containing supernatants were treated 1h at 37°C with PBS or Triton 0.1%. Extracellular HiBiT was quantified by luminometry. L.O.D. = limit of detection. **(C)** Huh7 cells stably expressing LgBiT (Huh7_LgBiT cells) were infected with the different SVPs. **(D)** SVPs were treated 1h at 37°C with anti-Gc antibody or control mouse IgG before infection of Huh7_LgBiT cells. **(E)** Huh7_LgBiT cells were treated with DMSO or 50 nM Bafilomycine A for 1h at 37°C prior infection with the different SVPs. **(C), (D) and (E)**, luminescence was analyzed in live cells over time using Vivazine.

III. Material and methods

Cell lines

All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) complemented with 10% of fetal calf serum (FCS), 100 U/mL of penicillin and 100 µg/mL streptomycin (Gibco) and incubated at 37°C with 5% CO_2 unless otherwise stated. Human hepatocarcinoma (Huh7) cells were a kind gift of David Durantel. U87-MG were a kind gift of Olivier Reynard. Huh7.5 and HOS cells were propagated from laboratory stocks. Human embryonic kidney (HEK293T) cells were propagated from laboratory stocks and maintained in DMEM complemented with 10% FCS, 100 U/mL of penicillin and 100 µg/mL streptomycin, and geneticin (200 µg/mL). Huh7 cells stably expressing LgBiT were maintained in DMEM complemented with 10% FCS, 100 U/mL of penicillin and 100 µg/mL streptomycin, and geneticin (1 mg/mL).
The cells were regularly tested for mycoplasma contamination.

Plasmids and constructs

The constructs encoding wild-type CCHFV strain IbAr10200 V5-tagged L polymerase (pCAGGS-V5-L), CCHFV NP (pCAGGS-NP), CCHFV-specific negative-sense eGFP-expressing minigenome (pT7RiboSM2_vL_eGFP), CCHFV-specific negative-sense nLuc-expressing minigenome (pSMART-LCK_T7-vL-nLuc), CCHFV GPC (pCAGGS-GPC), T7 RNA polymerase (pCAGGS-T7) and an empty vector (pCAGGS) were previously described (Devignot et al., 2015; Zivcec et al., 2015; Freitas et al., 2020; Suda et al., 2016). The plasmids pCAGGS-CCHFV-$GPC_{\Delta 64}$, -GPC_{Y1635A}, -$GPC_{\Delta 5}$, -$GPC_{\Delta 53}$, -$GPC_{L1638R\text{-}R1648Q\text{-}\Delta 14}$, -GPC-HiBiT and pCAGGS-HiBiT-NP were generated through mutagenesis and overlap extension polymerase chain reactions (PCR). pSCRPSY was previously described (Kane et al., 2016). pcDNA3.1-FRB-LgBiT was a gift from Masaharu Somiya (Addgene plasmid #182650). pWPIR-LgBiT-IRES-neo was obtained through PCR and cloning.

Reagents

Universal type I IFN was obtained from PBL Assay Science (11200). Mouse monoclonal antibodies targeting CCHFV strain Ibar10200 anti-PreGc (clone 11E7 and clone 30F7) and anti-NP (clone 9D5) were obtained from the Joel M. Dalrymple—Clarence J. Peters USAMRIID Antibody Collection through BEI Resources, NIAID, NIH. Mouse monoclonal antibody targeting nLuc was obtained from R&D Systems (MAB10026-100). Bafilomycine A was obtained from Sigma (19-148). Anti-VSV-G 41A.1 was already described (Perez-Vargas et al., 2019).

CCHFV transcription- and entry-competent virus-like particle (tc-VLP) assay

CCHFV tc-VLPs were produced in Huh7.5 producer cells seeded in a 10 cm dish ($1.2x10^6$ cells) and transfected with 1.2 μg of pCAGGS-NP, 3 μg of pCAGGS-GPC, 3.6 μg of pCAGGS-V5-L, 3 μg of pT7RiboSM2_vL_eGFP, and 3 μg of pCAGGS-T7, using GeneJammer transfection reagent (Agilent). 6 hours post-transfection, medium was changed, and tc-VLP-containing supernatants were harvested 72 hours later, cleared by centrifugation for 5 minutes at 750 g and filtrated. In some experiments, pCAGGS-GPC-Gc-HiBiT or pCAGGS-HiBiT-NP were used to produce tc-VLPs.

Huh7 cells seeded in a 10 cm dish ($1.2x10^6$ cells) were transfected with 1.2 μg of pCAGGS-NP, 3 μg of pCAGGS-GPC, and 3.6 μg of pCAGGS-V5-L. Alternatively, they were transfected with 1.2 μg of pCAGGS-NP and 3.6 μg of pCAGGS-V5-L, or with an empty control vector (pCAGGS). Transfected DNA amounts were kept constant between conditions using pCAGGS. 6 hours post-transfection, the cells were seeded in 96 well-plates ($2x10^4$ cells per well) or in 24 well-plates ($8x10^4$ cells per well), depending on the experiment. In experiments performed with interferons, increasing amounts of universal type I IFN (PBL Interferon Source) were added to the cells 24 hours post-transfection. 24 hours post-transfection of the cells, (or 24 hours following IFN treatment in specific experiments), tc-VLPs-containing supernatants were transferred to the cells. 3 hpi, the cells were washed with PBS and fresh medium was added to the cells. 24-48 hpi, the cells were trypsinized and fixed in 1% paraformaldehyde (PFA) and the percentage of infected (GFP-positive) cells was determined by flow cytometry using a MACSQuant VYB flow cytometer (Miltenyi Biotec). In case the infected cells were transfected with pCAGGS-GPC, supernatants were harvested and used to infect pCAGGS-NP, pCAGGS-V5-L-transfected Huh7 cells. These cells were fixed 24 hpi and the percentage of infected (GFP-positive) cells was determined by flow cytometry using a MACSQuant VYB flow cytometer (Miltenyi Biotec).

For nLuc tc-VLPs, the same procedure has been applied to Huh7 cells. Producer cells were transfected with pSMART-LCK_T7-vL-nLuc instead of pT7RiboSM2_vL_eGFP. Following infection of Huh7 cells (expressing viral proteins or not), cells were washed once with PBS, lysed with Passive Lysis Buffer 1x and reporter activities were measured with Nano-Glo Luciferase assay system (Promega) following the manufacturer instructions and a Mithras LB 940. In case the infected cells were transfected with pCAGGS-GPC, supernatants were harvested and used to infect naïve Huh7 cells. These cells were lysed 24 hpi and luminescence activity was measured with Nano-Glo Luciferase assay system (Promega) following the manufacturer

instructions and a Mithras LB 940. The limit of detection (L.O.D.) was set at the level of RLU from non-infected cells.

Optimization of the screen of ISGs

The optimization of the screen was performed with a small number of pSCRPSY-ISG plasmids.

For transfection experiments, Huh7 cells were seeded in 96-well plate ($2x10^4$ cells/well) before transfection of 25 ng of pSCRPSY-ISG plasmid using GeneJammer transfection reagent (Agilent). 6 hours post-transfection, medium was changed. 48 hours post-transfection, cells were fixed, and the percentage of TagRFP-positive cells was analyzed by flow cytometry using a MACSQuant VYB flow cytometer (Miltenyi Biotec).

Lentivirus vectors expressing ISGs were generated in a 96-well plate format as previously described (Schoggins et al., 2011). Briefly, $3.5x10^4$ HEK293T cells were transfected with 125 ng of the pSCRPSY-ISG construct and plasmids expressing HIV Gag-Pol (NLGP) and VSV-G in a ratio of 25:5:1. The medium was changed 24 hours post-transfection, and supernatants were harvested at 48, 72 and 96 hours and stored at -80°C. 100 µL of lentiviral vector ISG libraries were used to reverse-transduce Huh7 cells not expressing viral proteins, expressing NP and L protein, or expressing NP, L protein and GPC ($2,5x10^4$ cells/well in a 96-well plate) for 48 hours. Then, cells were infected with GFP tc-VLPs or nLuc tc-VLPs for 3 hours, then washed once with PBS and the medium was replaced. 30 hpi, cells were fixed (for GFP tc-VLPs) or lysed (for nLuc tc-VLPs) for reporter expression analyses as described before. Moreover, supernatants were harvested and used to infect Huh7 expressing NP and L protein (for GFP tc-VLPs) or naïve Huh7 cells (for nLuc tcVLPs). 24 hpi, cells were fixed (for GFP tc-VLPs) or lysed (for nLuc tc-VLPs) for reporter expression analyses as described before.

Pseudotyped lentiviruses assay

$8x10^5$ HEK293T cells in a 6-well plate format were transfected with 2 µg pCSWG-GFP, 2 µg psPAX2, and 8 µg of GPC mutants or 0.5 µg pHCMV-VSV-G using Lipofectamine 2000 (Thermo Fisher Scientific). Supernatants were harvested 48 hours post-transfection and used to infect Huh7, HEK293T, or HOS cells. 72 hpi, cells were fixed with 2% PFA and the number of GFP-positive cells was analyzed by flow cytometry as described before.

VSV-pseudotyped assay

$8x10^5$ HEK293T cells in a 6-well plate format were transfected with 2 μg of GPC mutants or pHCMV-VSV-G using PEI MAX (Polysciences). 24 hours post-transfection, cells were infected with a recombinant replication-deficient vesicular stomatitis virus (VSV) expressing GFP instead of VSV-G (a kind gift from Olivier Reynard) with a multiplicity of infection (MOI) of 5 for 1 hour. Then, medium was replaced by fresh medium containing an anti-VSV-G antibody. Supernatants were harvested 24 hpi, centrifuged, filtered through a 0.45 μm filter and stored at -80°C. Pseudotyped virions were used to infect Huh7 cells or HEK293T for 1 hour. For some experiments, they were incubated for 1h at 37°C with 10 μg/mL of mouse IgG, mouse anti-Gc antibody (30F7) or anti-VSV-G antibody prior infection. Alternatively, cells were treated with 1000 U/mL of type I IFN prior infection. 24 hpi, cells were fixed in 2% PFA and analyzed by flow cytometry using a MACSQuant VYB flow cytometer (Miltenyi Biotec).

HiBiT System

Huh7 stably expressing LgBiT (Huh7_LgBiT) were obtained by transduction of Huh7 cells with lentiviral vectors with the pWPIR-LgBiT-IRES-neo backbone. Efficient expression of LgBiT was checked through intracellular staining of LgBiT with an antibody against nLuc (dilution 1:200) using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer instructions.

Tc-VLPs were produced as described before, with pCAGGS-GPC-Gc-HiBiT or pCAGGS-HiBiT-NP depending on the experiment. To produce SVPs, Huh7.5 cells seeded in 10-cm dishes ($1.2x10^6$ cells) were transfected with different combinations of pCAGGS-GPC-WT and -GPC-HiBiT or pCAGGS for a total amount of 12 μg of DNA per well. 72 hours post-transfection, supernatants were proceeded as described to produce tc-VLPs. Alternatively, they were concentrated by ultracentrifugation through a 20% sucrose cushion (SW41 rotor at 28000 rpm for 2 hours at 4°C). Infection experiments were performed as described before for GFP tc-VLPs. Alternatively, Huh7_LgBiT seeded in 96-well were treated with Nano-Glo® Vivazine Live Cell Substrate (Promega) according to the manufacturer instruction and infected with different tc-VLPs or SVPs. Luminescence was read on a Mithras LB 940.

Western blotting

For western blot analysis, cells were washed once with PBS and lysed using cold cell lysis buffer (20 mM Tris pH 7.5, 1% Triton, 0.05% SDS, 0.5% sodium deoxycholate, 150

mM NaCl) containing protease inhibitors (Roche Applied Science). They were precipitated by centrifugation at 12,000 g for 30 min. For detection of NP, samples were denatured at 95°C for 5 min, in reducing loading buffer (375 mM Tris HCl pH 6.8, 12% SDS, 60% Glycerol, 0.04% Bromophenol blue, 500 mM β-mercaptoethanol) and electrophoresed on 10% SDS-polyacrylamide gels. Alternatively, for Gc detection, samples were processed in non-reducing loading buffer (375 mM Tris HCl pH 6.8, 12% SDS, 60% Glycerol, 0.04% Bromophenol blue) and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane by electroblotting (BioRad) for 1h30 at 100V. After saturation in TBST (20 mM Tris HCl, pH 7.5, 150 mM NaCl and 0.05%-Tween)-milk 10% for 1h, membranes were incubated with primary antibodies for the detection of CCHFV proteins (mouse monoclonal 11E7 anti-Gc 1:500 and mouse monoclonal 9D5 anti-NP 1:1000), in TBST-milk 10% overnight at 4°C. After 3 washes of 10 min using TBST, membranes were then incubated with IRDye secondary antibodies (Li-Cor Biosciences) at 1:20000 dilution in TBST for 30 min at room temperature, followed by imaging with Odyssey infrared imaging CLx system (Li-Cor Biosciences). Quantification of proteins was performed with Odyssey imaging CLx system software. Alternatively, HiBiT signal was detected using the Nano-Glo® HiBiT Blotting System (Promega) according to the manufacturer instructions and revealed with a ChemiDoc Imaging System (BioRad).

Chapter 2. Identification and characterization of interferon-stimulated genes modulating Crimean-Congo hemorrhagic fever virus infection

Identification and characterization of interferon-stimulated genes modulating Crimean-Congo hemorrhagic fever virus infection

Solène Lerolle[1], Thomas Vallet[1], Emma L. Davies[2], Lola Canus[1], Alexandre Lalande[1], Cyrille Mathieu[1], Sam J. Wilson[2], François-Loïc Cosset[1] and Vincent Legros[1,3]

[1]Centre International de Recherche en Infectiologie, Université de Lyon, Université Claude Bernard Lyon 1, INSERM, U1111, CNRS, UMR5308, ENS de Lyon, Lyon, France
[2]Medical Research Council, University of Glasgow, Centre for Virus Research, Institute of Infection, Inflammation and Immunity, University of Glasgow, UK
[3]Vetagro Sup, Université de Lyon, Lyon, France

Abstract

As a tick-borne, zoonotic, emerging virus, Crimean-Congo hemorrhagic fever virus (CCHFV) poses a serious threat to public health. Indeed, it is responsible in humans for a hemorrhagic disease with a mortality rate of 10-40%, for which there is no specific treatment or vaccine, justifying the need to study this virus under conditions of maximum biosafety level (BSL-4). To date, the interactions between CCHFV and innate immunity, one of the host main lines of defense against viruses, are poorly understood. Notably, type I interferon (IFN) has a crucial role in the establishment of an antiviral state against CCHFV, probably through the transcription of IFN-stimulated genes (ISGs) with antiviral properties. However, it remains to be determined more precisely which ISGs modulate CCHFV infection as well as their mechanisms of action and respective contributions.

To answer those questions, we performed a gain-of-function screen using CCHFV virus-like particles competent for entry and transcription of a reporter minigenome (tc-VLPs), a BSL-2 model of CCHFV, and lentiviral vectors encoding >500 human, >350 simian and >250 bovine individual ISGs. We could identify the antiviral activity of poly-(ADP-ribose) polymerase family member 12 (PARP12) against CCHFV as well as other antiviral effectors, such as 2'-5'-oligoadenylate synthase 2 (OAS2) or bovine hyaluronan synthase 3 (HAS3). Moreover, we uncovered an unexpected dual activity of GTP-binding protein 1 (GTPBP1) demonstrating that this protein specifically promotes the transcription/replication of CCHFV RNA genome while having an antiviral activity against authentic CCHFV. Taken together, these data will pave the way for further studies on the intricacies between CCHFV and innate immunity.

Introduction

The *Bunyavirales* order is a large order of viruses that encompasses, among others, the *Nairoviridae* family. Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the *Orthonairovirus* genus and represents a growing threat to human public health, as it is nowadays endemic in Africa, Asia, Middle East, and new cases are described in Europe. While asymptomatic in many mammal species, this tick-transmitted infection is responsible in humans for a hemorrhagic disease with a 5-30% mortality rate, for which there is currently no treatment and no vaccine available (Hawman and Feldmann, 2023). To this date, the mystery persists as to why CCHFV remains pathogenic exclusively for humans.

CCHFV is an enveloped virus with a tri-segmented RNA genome of negative or ambisense polarity. The small (S) segment encodes in negative polarity the nucleoprotein (NP), which encapsidates viral RNAs (vRNAs) to form viral ribonucleoproteins (vRNPs), and a small non-structural protein (NSs) in positive polarity, on an open-reading frame overlapping the one of NP. The medium (M) segment encodes the glycoprotein precursor complex (GPC) which undergoes several proteolytic processes and post-translational maturation to yield the envelope glycoproteins Gn and Gc as well as several non-structural proteins (NSm, MLD, GP38, GP160/85). Finally, the large (L) segment encodes the L protein that bears the RNA-dependent RNA polymerase (RdRp) activity responsible for the transcription and replication of vRNA (Hawman and Feldmann, 2023). Upon entry of target cells (hepatocytes, endothelial cells, or monocyte-derived macrophages), CCHFV genome is released into the cytoplasm. Transcription of viral mRNA is initiated by L protein in a manner dependent on its endonuclease activity, which snatches the cap from cellular RNAs (Devignot et al., 2015). Replication of the genomic RNA leads to the formation of antigenomic RNA of positive polarity, which serves as template to promote the synthesis of genomic, negative-sense RNA. Assembly of newly formed viral proteins and vRNA occurs in the Golgi as well as the budding of the new viral particles.

The innate immunity represents one of the earliest barriers against pathogen invasion of organisms and relies on the activation of inducible pathways upon recognition of these pathogens. Interferons (IFNs) are cytokines that are largely involved in restricting numerous viruses. Notably, type I and type III IFNs have antiviral roles by inducing the Janus kinase/signaling transducers and activators of transcription

(JAK/STAT) pathway, which ultimately leads to the transcription of thousands of IFN-stimulated genes (ISGs) with immunomodulatory functions (Schoggins, 2019).

The interplays between CCHFV and innate immunity are currently poorly characterized. Type I IFN pretreatment of target cells leads to an inhibition of CCHFV infection, suggesting that type I IFN induces the expression of ISGs with antiviral activities targeted against CCHFV (Andersson et al., 2006). However, while CCHFV infection in humans leads to the upregulation of numerous ISGs (Neogi et al., 2022), it was shown that this virus hinders the prompt initiation of IFN-mediated antiviral responses (Andersson et al., 2008), suggesting that it encodes one or several putative counteracting factors. Notably, CCHFV L protein bears an ovarian tumor (OTU) domain at its amino-terminal end that inhibits IFN-mediated responses through its deubiquitylation and deISGylation activities (Scholte et al., 2017). The study of one particular ISG, Mx resistance protein A (MxA, also known as MX1), in the context of CCHFV infection showed that its overexpression inhibited CCHFV infection by 1,000-fold (Andersson et al., 2004a), while its knock-down rescued it (Andersson et al., 2006). MxA interacted with CCHFV NP and sequestered it to perinuclear regions, probably rendering NP unavailable for crucial viral life cycle processes, such as vRNPs formation or polymerase activity. However, the contribution of other known ISGs to IFN-mediated antiviral activity against CCHFV remains largely unknown (Lerolle et al., 2021).

Due to the lack of treatment or vaccine and to its highly pathogenic nature, CCHFV manipulation is restricted to biosafety level-4 (BSL-4) facilities, largely challenging its study. Yet, a well-established transcriptionally- and entry-competent virus-like particles (tc-VLP) system allows its study in BSL-2 settings. It relies on the encapsidation of a minigenome expressing a reporter molecule flanked by viral untranslated regions (UTRs) into CCHFV-like particles harboring Gn and Gc at their surface (Devignot et al., 2015; Zivcec et al., 2015; Freitas et al., 2020) (Figure 1A). Tc-VLPs entry into target cells depends on Gn and Gc glycoproteins, while transcription and replication of the minigenome depend on the expression of NP and L protein. Production of new tc-VLPs occurs when the cells express GPC *in trans*. Thus, the tc-VLP system allows the study of all viral cycle steps in BSL-2 settings.

Gain-of-function screens based on ectopic expression of ISGs followed by infection of viruses allowed the identification and characterization of numerous ISGs targeting a broad range of viruses spanning various branches of the mammalian viral phylogeny

(Schoggins et al., 2011, 2014; Kane et al., 2016; Hardy et al., 2023). Notably, such a screen successfully identified the antiviral activity of ISG20 against Bunyamwera virus (BUNV), a member of the *Bunyavirales* order from the *Peribunyaviridae* family (Feng et al., 2018). Another screen using a similar BSL-2 system developed for Ebola virus (EBOV), another BSL-4 virus, was used to study the impact of TRIM25 and ZAP on the vRNPs (Galão et al., 2022). Thus, these tools are useful to identify and gain more insight into the IFN-mediated restriction of highly pathogenic viruses such as CCHFV.

Here, we used CCHFV tc-VLPs to screen a library of lentiviral vectors encoding human, macaque or bovine ISGs. We identified several ISGs that were not previously known to modulate CCHFV infection. Taken together, these data pave the way to the study of newly identified antiviral and proviral factors against highly pathogenic, emerging CCHFV.

Results

CCHFV tc-VLP replication is sensitive to type I interferon.

The CCHFV tc-VLP system is a reverse genetics system of particles enveloped by CCHFV glycoproteins (Gn and Gc) and containing a reporter (GFP or nanoluciferase (nLuc)) minigenome flanked by the untranslated regions (UTRs) of the L segment (Devignot et al., 2015; Freitas et al., 2020; Zivcec et al., 2015). Tc-VLP stocks are produced from Huh7.5 producer cells that are transfected with plasmids expressing NP, L protein and GPC, as well as the minigenome and the T7 DNA-dependent RNA polymerase. Newly produced tc-VLPs are then used to infect either Huh7 amplifier cells that express CCHFV NP, L protein and GPC, Huh7 indicator cells that express CCHFV NP and CCHFV L protein, but lack the GPC, or Huh7 cells that do not express any viral proteins. Tc-VLPs replication is assessed by measuring reporter gene expression in infected cells (Figure 1A).

CCHFV is sensitive to type I IFN pretreatment of Huh7 cells (Andersson et al., 2006). To confirm that the BSL-2 model of CCHFV tc-VLPs can reproduce this phenotype, we first tested the sensitivity of tc-VLPs to type I IFN. Huh7 cells expressing NP, L protein and GPC were treated for 24 hours with three doses of universal type I IFN (IFN-α) and infected with equal doses of CCHFV tc-VLPs with a GFP reporter minigenome (GFP tc-VLPs). The efficacy of the type I IFN treatment was assessed by measuring 24 hours after the type I IFN treatment the transcription levels of the *MX1*

gene (Figure 1D). The percentage of GFP-positive, infected cells was measured 48 hpi. We found that type I IFN treatment led to a robust dose-dependent decrease of the percentage of infected cells (Figure 1B). However, it did not impact the mean fluorescence intensity (MFI) of GFP in infected cells (Figure 1C), suggesting that type I IFN is efficient in preventing early stages of infection such as viral entry, but not later stages within infected cells.

The decrease of infected cell numbers upon type I IFN treatment could be explained by a putative effect of type I IFN on the expression of CCHFV proteins added *in trans*. To check whether type I IFN influences the expression or stability of these proteins, we performed western blot analyses of NP, Gn and Gc intracellular levels in type I IFN pre-treated, transfected Huh7 cells. Despite the V5-tag at the amino-terminal of the plasmid-expressed L protein, we were unable to detect it by western blot analyses. Yet, we found that NP, Gn and Gc levels were not affected by type I IFN pre-treatment (Figure 1E), indicating that their expression or stability are not modified by type I IFN treatment.

Overall, these data suggested that type I IFN induces the expression of interferon-stimulated genes (ISGs) that inhibit tc-VLPs entry, minigenome replication, gene expression and/or tc-VLP assembly and secretion. We therefore sought to identify these ISGs.

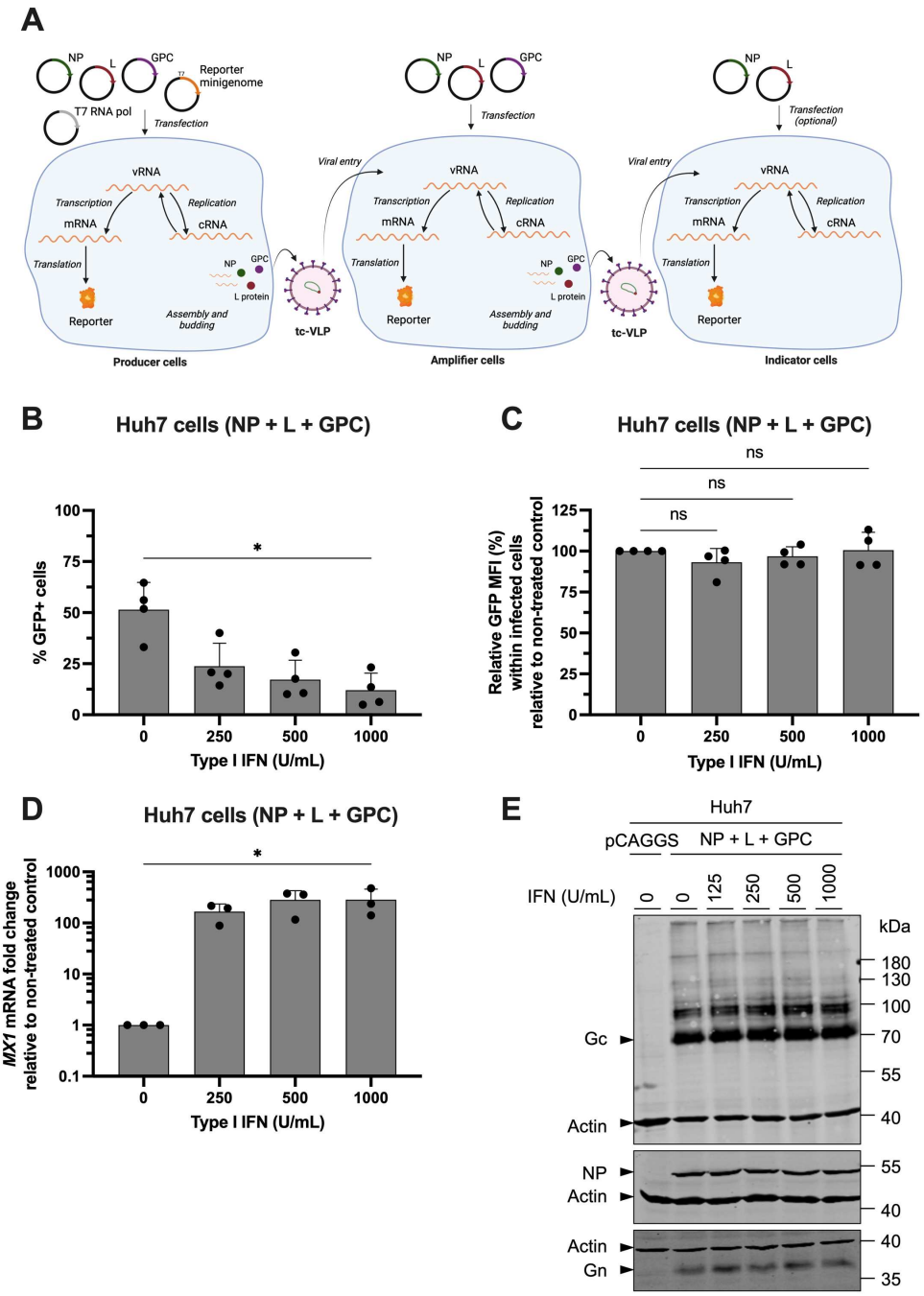

Figure 1. CCHFV tc-VLPs are inhibited by type I IFN pretreatment. (A) Schematic representation of CCHFV tc-VLP assay. Tc-VLPs are produced from producer cells transfected with helper plasmids and a plasmid encoding a reporter minigenome. Tc-VLPs are used to infect either amplifier cells or indicator cells. Tc-VLPs replication is measured by analysis of reporter expression in producer or infected cells. **(B) and (C)** Huh7 cells expressing NP, L protein and GPC were pre-treated with increasing doses of type I IFN before infection with GFP tc-VLPs. **(B)** The percentage of GFP positive cells and **(C)** mean fluorescence intensity (MFI) were analyzed by flow cytometry. **(D) and (E)** Huh7 cells expressing NP, L protein and GPC, or a control plasmid (pCAGGS) were treated with increasing doses of type I IFN and were harvested for **(D)** RT-qPCR analysis targeting the *MX1* gene or **(E)** western blot analysis with antibodies targeting Gc, NP and actin, and rabbit sera targeting Gn. (A) was created with Biorender. (B), (C) and (D) Means and standard deviations (SD) of (B) and (C) four and (D) three independent experiments are shown. (E) A representative blot from three independent experiments is shown. (B), (C) and (D) Statistical significance was analyzed using a Kruskal-Wallis test followed by post hoc analysis using Dunn's multiple comparisons test (ns: non-significant, *: $p < 0.05$).

An arrayed ISG screen reveals ISGs modulating CCHFV replication.

We then screened an arrayed library of lentiviral vector-encoded ISGs in a 96-well plate format (one ISG per well) using a library of >500 human (*Homo sapiens*), >350 rhesus macaque (*Macaca mulatta*) and >250 bovine (*Bos taurus*) ISGs (Kane et al., 2016; Hardy et al., 2023) (Supplementary Figure S1A). Briefly, Huh7 cells expressing NP, L protein and GPC were transduced with lentivectors co-expressing individual ISGs and a red fluorescent protein (TagRFP). Cells were infected 48 hours later with GFP tc-VLPs. GFP expression among TagRFP-positive cells was measured 33 hours post-infection (hpi) by flow cytometry (Supplementary Figure S1A).

Cells lowly expressing TagRFP, *i.e.,* with poor transduction level (less than 30%), were discarded from our analysis. Yet, we identified several ISGs that inhibited tc-VLPs replication by more than 60% compared to a lentiviral control "Empty" (Figure 2A). Interestingly, some ISGs were identified in the human, macaque, and bovine screens, suggesting a conserved antiviral activity across host species (IFN-induced with helicase C domain 1 (IFIH1), IFN-regulatory factor 1 (IRF1), 2'-5'-oligoadenylate synthase 2 (OAS2), IFN-induced transmembrane protein 1 (IFITM1), poly(ADP)ribose polymerase family member 12 (PARP12) or LY6E lymphocyte antigen 6 family member E (LY6E), while others had a distinct phenotype depending on its species of origin (Figure 2B). For instance, we could confirm the antiviral activity of human MX1, as previously described in the literature (Andersson et al., 2004a, 2006), though, interestingly, bovine MX1 did not exhibit an antiviral activity against CCHFV tc-VLPs (Figure 2B). Moreover, we could identify genes with antiviral activities that are ISGs only in one species, such as bovine hyaluronan synthase 3 (HAS3), which exhibited a profound inhibition of tc-VLPs infection. Outstandingly, we could also pinpoint the proviral activity of human GTP-binding protein 1 (GTPBP1), promoting a 3-fold increase of GFP tc-VLPs replication.

To confirm their pro- or antiviral activity against tc-VLPs, the impact of the 40 top human, macaque and bovine hits was evaluated in three independent experiments (Figure 2C, 2D and 2E). When available in the libraries, top hit homologs were also included. For some ISGs, the transduction efficiency was not sufficient to analyze their activity on tc-VLPs replication (Supplementary Figure S1B, S1C and S1D). For others, their antiviral activity was not confirmed (Figure 2C, 2D and 2E). However, antiviral activity could be validated for human IFIH1, Schlafen family member 12 (SLFN12), C5orf39, IRF1, lysosomal associated membrane protein 3 (LAMP3), family with sequence similarity 26, member F (FAM26F), LY6E, Mohawk homeobox (MKX), IFN-

induced transmembrane protein 1 (IFITM1), OAS2 and apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 3F (APOBEC3F) (Figure 2C); macaque IFNB1, SLFN12, IRF1 and C9orf52 (Figure 2D); and bovine HAS3, IRF1, TRAF Interacting Protein with Forkhead Associated Domain (TIFA) and PARP12 (Figure 2E). Notably, bovine HAS3 led to a profound and robust decrease in infection efficiency by more than 2 logs. Moreover, the proviral activity of human GTPBP1 could be established.

From this list of interest, three genes were removed, namely C5orf39, MKX and SLFN12. Indeed, C5orf39 is a known proapoptotic factor and was already found to have a cytotoxic effect upon overexpression (Xiong et al., 2013; Galão et al., 2022). MKX also was previously shown to be cytotoxic upon overexpression (Wickenhagen et al., 2021). Moreover, we experienced difficulties to produce lentiviral vectors expressing SLFN12 with sufficient titers. Indeed, SLFN12 was shown to inhibit retroviral replication (Kane et al., 2016). We then checked that the overexpression of the remaining genes was not cytotoxic using a lactate dehydrogenase (LDH) release assay (Figure 2E). Moreover, we also ruled out ISGs that can induce expression of a reporter gene under the control of an IFN-stimulated response element (ISRE) (human IRF1, IFIH1 and IRF7; macaque IRF1 and IFNB1; bovine IRF1 and Zinc finger CCCH-type antiviral protein 1 (ZC3HAV1)), which are known members of the IFN signaling cascade (Figure 2F).

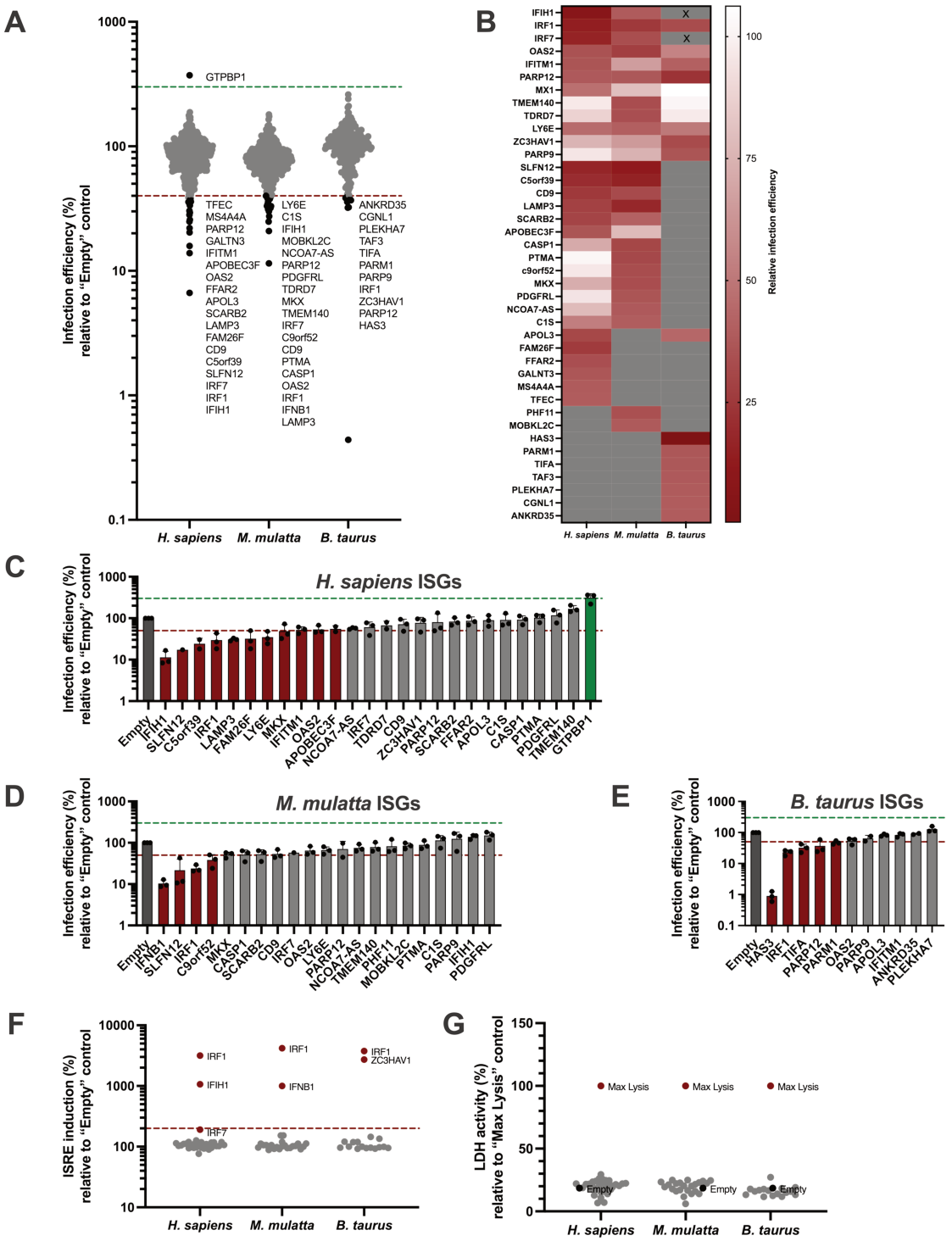

Figure 2. An ISG screen reveals ISGs modulating CCHFV tc-VLPs replication. (A) The screen was conducted as described in Figure S1A. Results are expressed in relative infection efficiency compared to the Empty control set at 100%. Antiviral hits are under the threshold of 40% (red dotted line) and proviral hits are above the threshold of 300% (green dotted line). **(B)** Heatmap of the top antiviral hits from the screen. Grey cases are used when ISG was not present in the ISG library of the species. Grey cases with a cross represent low transduction levels of the ISG. **(C), (D) and (E)** Miniscreens were then conducted in triplicate experiments using chosen ISGs from **(C)** human, **(D)** macaque and **(E)** bovine species. Antiviral hits (in red) are under the threshold of 50% (red dotted line) and proviral hits (in green) are above the threshold of 300% (green dotted line). **(F)** ISRE induction upon ISG overexpression in Huh7 cells was measured by luminescence and is expressed as fold change to Empty control set at 100%. Genes above the threshold of 200% are above the red dotted line. **(G)** Cytotoxicity upon ISG overexpression in Huh7 cells was assessed by measuring the activity of lactate dehydrogenase (LDH) in the supernatant. Results are expressed in percentage compared to a control where all cells are lysed (Max lysis). (C), (D) and (E) Means and SD of three independent experiments are shown.

LY6E and LAMP3 inhibit CCHFV entry while GTPBP1 augments CCHFV replication.

We then focused on human ISGs (IFITM1, LAMP3, OAS2, PARP12, LY6E, APOBEC3F, FAM26F and GTPBP1) identified in our screen and included bovine HAS3 for further analyses. To study CCHFV entry independently of CCHFV replication, we produced recombinant replication-deficient vesicular stomatitis virus (VSV) expressing GFP instead of VSV-G and pseudotyped with CCHFV glycoproteins, using a GPC bearing a large deletion at the cytoplasmic tail of Gc to ensure CCHFV glycoproteins incorporation to VSV virion (rVSV-dG/GFP*CCHFV-$GPC_{\Delta 53}$) (Suda et al., 2016). A rVSV-dG/GFP*VSV-G was used as control. Infection of CCHFV-GPC-pseudotyped viruses was abrogated by a neutralizing anti-Gc antibody while not affected by an anti VSV-G neutralizing antibody (Figure S2A). Interestingly, infection of ISG-overexpressing cells with these viruses showed a CCHFV-specific inhibition of viral entry in LAMP3- and LY6E-overexpressing cells (Figure 3A). APOBEC3F overexpression led to an increase of VSV-G-dependent entry while it had no effect on CCFHV glycoproteins-dependent entry. PARP12 and HAS3 overexpression led to a decrease in infection of both viruses. Of note, PARP12 was already known to inhibit VSV infection (Liu et al., 2012). Finally, IFITM1, OAS2, FAM26F and GTPBP1 had no effect on viral entry, independently of the glycoproteins that were expressed at the virion surface. Overall, these data suggested that LAMP3 and LY6E specifically inhibit CCHFV entry. Moreover, they suggested that PARP12 and HAS3 either inhibit entry of both viruses and/or specifically inhibit VSV-driven reporter expression.

To study CCHFV replication independently of viral entry, we took advantage of a CCHFV minigenome assay (Bergeron et al., 2010). Briefly, Huh7 expressing NP and L protein, but lacking the GPC, were transfected with an *in vitro* transcribed minigenome, an RNA of negative polarity expressing nLuc. In this RNA, the nLuc open-reading frame (ORF) is flanked by CCHFV L 3′ and 5′ UTRs. Thus, nLuc luminescence signal derives from NP- and L protein-driven replication and transcription, followed by cellular translation of the messenger RNA (mRNA) that is produced. To check whether overexpressed ISGs could have an impact on general RNA expression and/or transfection efficiency, we concomitantly transfected an *in vitro*-transcribed, capped and polyadenylated RNA expressing firefly luciferase (fLuc). Importantly, HAS3 overexpression led to an important decrease in fLuc signal, suggesting that it inhibited RNA transfection efficiency or had a general impact on RNA expression; thus, it was excluded from further analyses (Figure S2B).

Transfection of CCHFV genomic nLuc RNA in Huh7 cells that were transfected with an empty control plasmid (pCAGGS) led to an absence of nLuc signal (Figure 3B, dark grey bar), while NP and L protein transfection induced nLuc expression (Figure 3B, light grey bar), suggesting that viral reporter RNA is transcribed and replicated. ISG overexpression had no effect on nLuc signal, at the notable exception of GTPBP1-overexpression which led to an increase of 40-fold of nLuc/fLuc ratio. Overall, these data suggested that GTPBP1 specifically promotes CCHFV minigenome transcription and/or replication and/or translation of reporter molecule.

We then checked whether overexpression of the ISGs induced a modulation in plasmid-driven viral protein expression. Interestingly, IFITM1 and FAM26F overexpression led to a decrease in Gc and Gn expression compared to transduction of the Empty control lentivector, suggesting that these proteins could interfere with formation of viral progeny by lack of glycoproteins. No effect of ISGs overexpression could be seen on NP expression (Figure 3C). By flow cytometry, ISG overexpression did not increase the percentage of NP-expressing cells (Figure 3D). NP mean fluorescence intensity (MFI) within transfected and transduced cells was slightly increased in PARP12-expressing cells (Figure 3E). Overall, these results suggested that IFITM1 and FAM26F could interfere with formation of viral progeny. Moreover, they showed that the increase in CCHFV replication upon GTPBP1 overexpression is likely not due to an increase of NP protein levels expression. Of note, the expression levels of L protein upon GTPBP1 overexpression could not be analyzed.

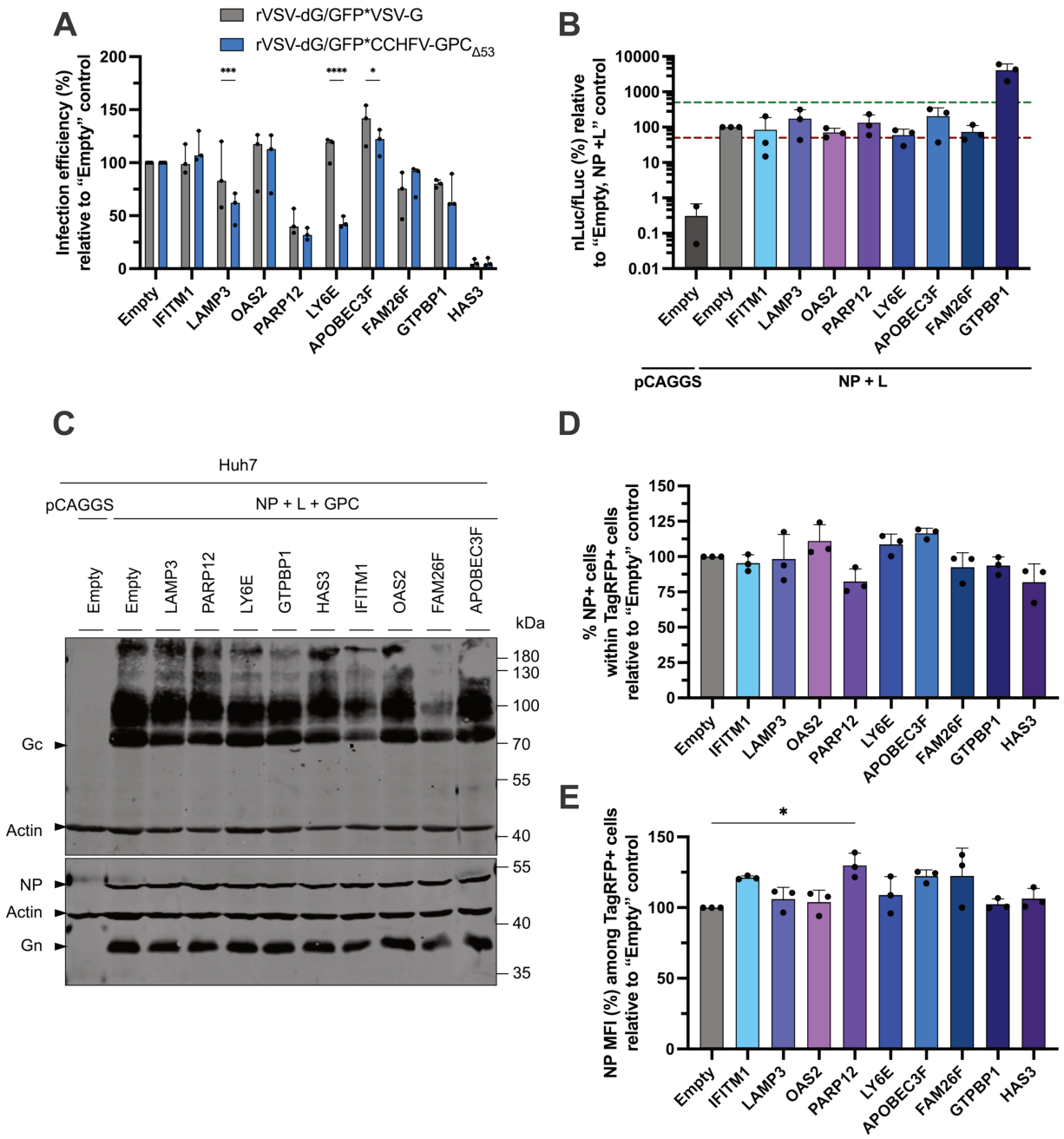

Figure 3. Role of selected ISGs on CCHFV entry and CCHFV minigenome replication. (A) Huh7 cells transduced with ISG lentivectors (MOI = 3) prior infection with VSV viruses pseudotyped with VSV-G (rVSV-dG/GFP*VSV-G) or GPC$_{\Delta 53}$ (rVSV-dG/GFP*-CCHFV-GPC$_{\Delta 53}$). Data are expressed as relative infection efficiency compared to the Empty control set at 100%. Statistical significance was analyzed using an ordinary two-way ANOVA with Šidák multiple comparisons test (*: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$). **(B)** Huh7 cells transfected either with a control plasmid (pCAGGS) or NP and L protein (NP + L) and transduced with ISG lentivectors (MOI = 3) were transfected with *in vitro* transcribed RNAs of control fLuc and genomic viral RNA expressing nLuc. 24 hours post-transfection, cells were lysed, and luminescence was read. Data are expressed as a ratio of nLuc/fLuc and normalized to the Empty, NP + L condition set at 100%. **(C)** Huh7 cells transfected either with a control plasmid (pCAGGS) or NP, L protein and GPC (NP + L + GPC) transduced with ISG lentivectors (MOI = 3) were lysed and analyzed by western blot using antibodies against NP, Gc, and actin, and anti-Gn mouse sera. A representative western blot from four independent experiments is shown. **(D)** and **(E)** Alternatively, the cells from (C) were fixed, permeabilized, stained for NP and analyzed by flow cytometry. **(D)** represents the percentage of NP-positive cells among transduced cells and **(E)** the mean fluorescence intensity (MFI), expressed relatively to Empty control set at 100%. (B), (D) and (E) Statistical significance was analyzed using a Kruskal-Wallis test followed by post-hoc analysis using Dunn's multiple comparison test (*: $p < 0.05$). (A), (B), (D) and (E) Means and SD of three independent experiments are shown.

GTPBP1 proviral activity is dependent on NP and L protein and modulates viral transcription/replication.

Reporter molecule signal in nLuc tc-VLPs-infected Huh7 cells that do not express any viral protein is solely dependent on the NP and L proteins that are already present in the virion and brought to cells upon infection. On the other hand, in Huh7 cells that express NP and L protein, reporter molecule signal upon nLuc tc-VLPs infection is dependent on both virion-incoming and plasmid-expressed NP and L. Finally, in Huh7 cells that express NP, L protein as well as GPC, reporter molecule signal is dependent on the above-mentioned signals as well as on a GPC-driven amplification of the signal due to new infection of neighboring cells. We took advantage of these different properties to infect Huh7 cells transfected with a control plasmid (pCAGGS), Huh7 cells that express NP and L protein or Huh7 that express NP, L protein and GPC with nLuc tc-VLPs. Interestingly, GTPBP1 had a proviral effect in cells that overexpress NP and L or NP, L and GPC (4.1- and 5.0-fold increase, respectively) while its proviral effect in control-transfected cells was very moderate (1.2-fold increase) (Figure 4A). Further, the supernatants from NP, L protein and GPC-expressing, infected cells were used to infect naïve cells (Figure 4B). Infection was 3.64-fold more efficient with supernatants coming from GTPBP1-overexpressing cells, suggesting that the latter cells produced more tc-VLPs. These data suggested that GTPBP1 proviral effect is highly dependent on NP and L protein presence and abundance.

To further study GTPBP1 proviral effect, we generated different RNA species: genomic CCHFV nLuc RNA of negative polarity (as above-mentioned), antigenomic CCHFV nLuc RNA of positive polarity, or CCHFV nLuc messenger RNA (mRNA), which is capped (Figure 4C). We transfected these RNAs in cells that express a control plasmid (pCAGGS) or NP and L protein plasmids. Moreover, we also studied the impact of NP expression alone, as it was previously reported that NP augments viral mRNA translation (Jeeva et al., 2017a). Of note, cells transduced with GTPBP1 lentivectors had higher levels of *GTPBP1* RNA as well as higher levels of GTPBP1 protein (Figure S3A and S3B).

Intriguingly, transfection of antigenomic CCHFV nLuc RNA gave rise to a higher nLuc signal than genomic RNA, in both NP- and L protein-overexpressing and non-expressing cells (Figure 4D, grey, light blue and middle blue bars). This was counterintuitive, as antigenomic RNA must theoretically undergo a replication step to generate genomic RNA that can be transcribed into viral mRNA. Thus, we hypothesized that despite being not capped, antigenomic RNA might be directly

translated by the cellular machinery. mRNA transfection gave rise to a high signal in cells that do not express NP and L, hence reflecting its translation without prior replication or transcription. However, expression of NP did not increase its expression (light blue bars), contrarily to previously published data (Jeeva et al., 2017a).
Expression of NP and L increased nLuc signal in Empty-transduced cells that were transfected with genomic or antigenomic RNA, but not with viral mRNA (middle blue bars). This expression was abrogated by ribavirin (RBV) treatment of the cells, a known CCHFV replication inhibitor (dark blue bars) (Bergeron et al., 2010). Importantly, RBV treatment of the cells did not impact general transfection efficiency nor the translation of control fLuc mRNA (Figure S3C). Transduction of GTPBP1 increased nLuc signal by 34.5-fold, 29.5-fold and 2.9-fold compared to control-transduced cells expressing NP and L for genomic RNA, antigenomic RNA and viral mRNA, respectively, but not in cells that do not express NP and L protein. This suggested again that GTPBP1 proviral activity is dependent on NP and L protein expression. Moreover, its proviral effect was abrogated by RBV treatment for the genomic and antigenomic RNA, demonstrating that this proviral activity depends on replication and/or transcription of viral RNA. Interestingly, GTPBP1-dependent increase of nLuc expression in mRNA-transfected cells could also be inhibited by RBV, suggesting that despite its cap, this RNA might still be available for viral transcription and/or replication. Of note, this viral mRNA is non-polyadenylated. To check whether GTPBP1 could specifically promote the translation of non-polyadenylated RNAs compared to polyadenylated ones, we generated capped fLuc mRNAs with or without 3' polyA-tail (Figure 4C). While the presence of a polyA-tail was not necessary to generate fLuc signal, it increased its signal. However, GTPBP1 overexpression did not specifically increase the translation of the non-polyadenylated mRNA (Figure 4E).

Taken together, these data suggested that GTPBP1 has a specific proviral effect that is dependent on NP and L protein, and on L protein replication and/or transcription activity.

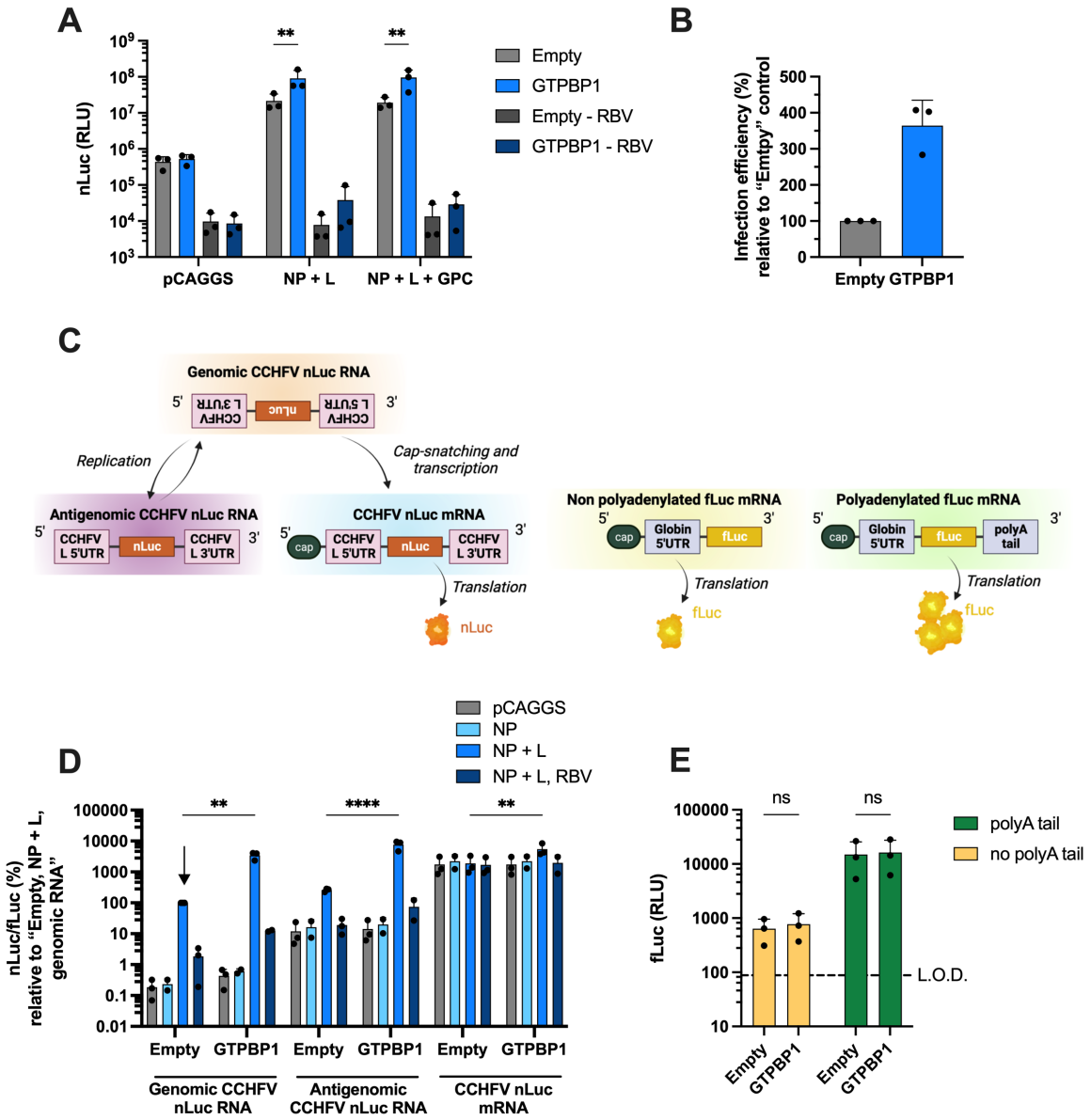

Figure 4. GPTBP1 promotes CCHFV minigenome replication and/or transcription. (A) Huh7 cells transfected either with a control plasmid (pCAGGS), NP and L protein (NP + L) or NP, L and GPC (NP + L + GPC) were transduced with ISG lentivectors (MOI = 3) prior infection with nLuc tc-VLPs. In the RBV conditions, RBV (50 ng/mL) was added 1 hour before infection. Luminescence was read 24 hpi. Statistical significance was analyzed using a two-way ANOVA with Dunett's multiple comparison's test (**: $p < 0.01$). **(B)** Supernatants from the cells expressing NP + L + GPC (without RBV treatment) from (A) were used to infect naïve Huh7 cells. 24 hpi, luminescence was read. Data are expressed as relative infection efficiency compared to the Empty control set at 100%. Statistical significance was analyzed using a Mann-Whitney test and was non-significant. **(C)** Schematic diagram of the different RNA species. Created with Biorender. **(D)** Huh7 cell transfected either with a control plasmid (pCAGGS), NP, or NP and L protein (NP + L) and transduced with ISG lentivectors (MOI = 3) were transfected with *in vitro* transcribed RNAs of control fLuc and genomic, antigenomic or messenger RNA expressing nLuc. In the RBV conditions, RBV (50 ng/mL) was added 1 hour before transfection. 24 hours post-transfection, cells were lysed, and luminescence was read. Data are expressed as a ratio of nLuc/fLuc and normalized to the Empty, NP + L, genomic RNA condition set at 100% (the arrow represents the condition to which data are normalized). Statistical significance was analyzed using a two-way ANOVA with Tukey's multiple comparison test (**: $p < 0.01$, ****: $p < 0.0001$). **(E)** Huh7 cell transduced with ISG lentivectors (MOI = 3) were transfected with *in vitro* transcribed RNAs of polyadenylated or non-polyadenylated fLuc. 24 hours post-transfection, cells were lysed, and luminescence was read. L.O.D. = limit of detection. Statistical significance was analyzed using a two-way ANOVA with Šidák multiple comparisons test (ns: non-significant). (A), (B), (D) and (E) Means and SD of three independent experiments are shown.

Endogenous expression of GTPBP1 does not have a significant role in promoting CCHFV tc-VLPs infection.

GTPBP1 expression is induced in THP-1 cells by type II IFN, but not in Jurkat or HeLa cells (Senju and Nishimura, 1997; Kudo et al., 2000). To check whether GTPBP1 is induced by type I IFN in Huh7 cells, we analyzed GTPBP1 protein levels in type I IFN-pretreated or control Huh7 cells. Interestingly, in these cells, GPTBP1 protein levels are not increased by type I IFN (Figure 5A).

To complement the data obtained with our overexpression approach and evaluate the contribution of GTPBP1 to tc-VLPs replication in a physiological context, we knocked-out GTPBP1 expression using CRISPR/Cas9. We checked by western blot analyses the efficiency of the knock-outs (KO) (Figure 5B) and further infected the cells with nLuc tcVLPs. Luminescence analyses revealed that GTPBP1 KO had no effect on nLuc expression following infection, independently of type I IFN treatment (Figure 5C). Alternatively, we transfected the cells with NP, L protein and GPC and infected them with GFP tc-VLPs. Here, we could observe a small inhibition of reporter expression in the type I IFN-treated condition for one sgGTPBP1 (sgGTPBP1#1), but which was non consistent between all sgCTRL RNAs nor with all sgRNAs directed against GTPBP1, despite a similar reduction of GTPBP1 protein expression levels by western blot (Figure 5D). Importantly, we checked if GTPBP1 KO influenced NP expression. NP staining and flow cytometry analyses showed a small reduction of NP staining for the sgGTPBP1#1, this time in the PBS-treated condition (Figure 5E). Overall, these data suggested that endogenous GTPBP1 has no role in tc-VLPs replication or NP expression. Several hypotheses can be formulated to explain this phenotype. Indeed, GTPBP1 endogenous levels of expression might be too low to exert a significant proviral activity. Alternatively, the absence of GTPBP1 might be compensated by a putative protein with a redundant role.

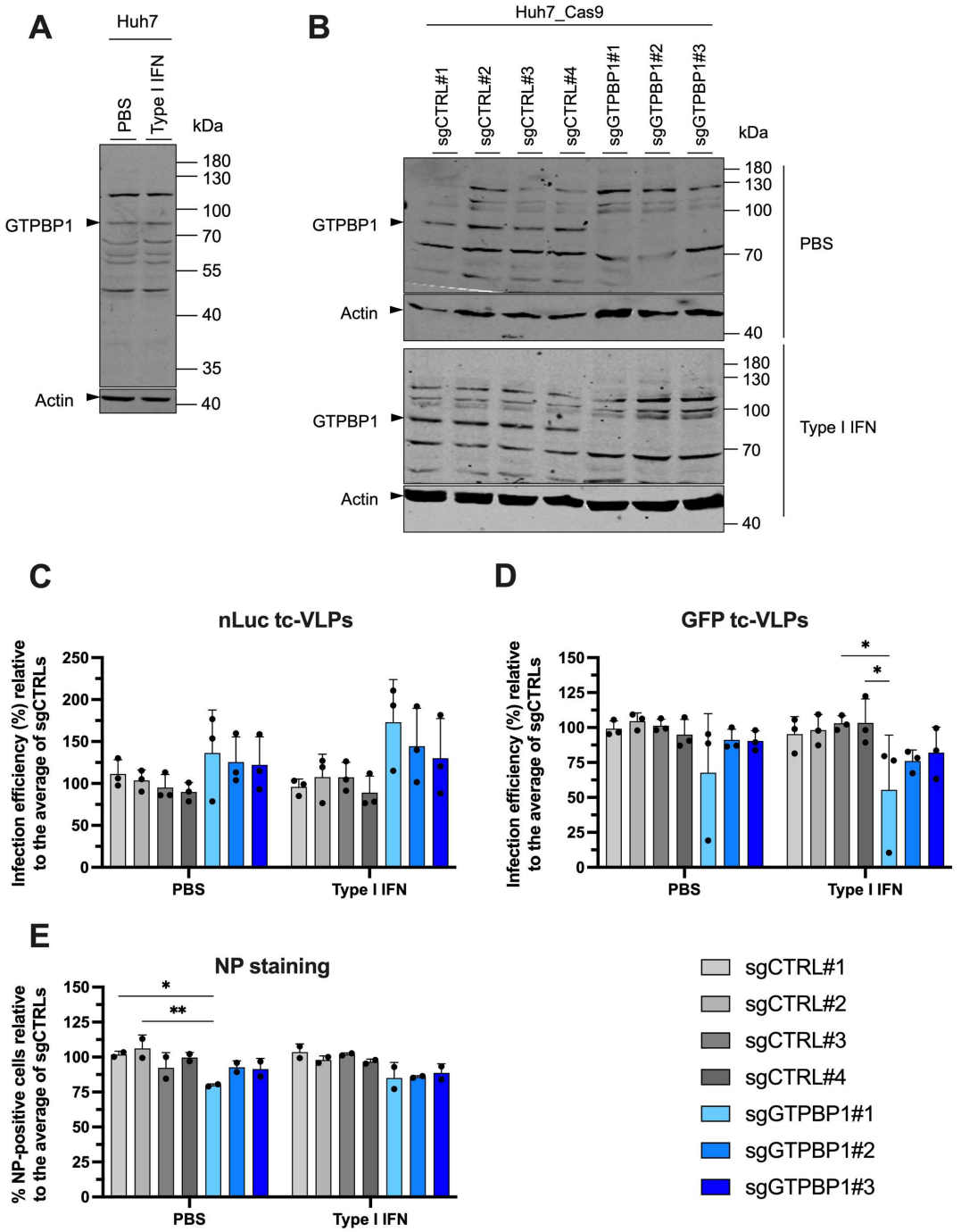

Figure 5. Endogenous GTPBP1 does not impact tc-VLPs infection nor NP expression. (A) Huh7 cells were treated with type I IFN (1000 U/mL) for 24 hours before lysis and western blot analysis using antibodies against GTPBP1 and actin. **(B)** Huh7 cells stably expressing Cas9 were transduced with single guide RNAs (sgRNAs) control (sgCTRL#1 to #4) or targeting GTPBP1 (sgGTPBP1#1 to #3). Cells were treated with type I IFN (1000 U/mL) or PBS for 24 hours before lysis and western blot analysis using anti-GTPBP1 and anti-actin antibodies. A representative western blot from three independent experiment is shown. **(C)** Huh7 cells from (B) were infected with nLuc tc-VLPs. Luminescence was read 24 hpi. **(D)** Huh7 cells from (B) were transfected with NP, L protein and GPC expression plasmids prior infection with GFP tc-VLPs. 48 hpi, the cells were fixed and GFP expression was analyzed by flow cytometry. **(E)** Huh7 cells from (B) were transfected with NP, L protein and GPC expression plasmids. 24 hours post-transfection, cells were fixed, permeabilized and stained for NP prior flow cytometry analysis. **(C), (D)** Data are expressed as relative infection efficiency or **(E)** relative percentage of NP-positive cells compared to the average of all sgCTRL RNAs for each condition (PBS or IFN-treated) set at 100%. Means and SD of three independent experiments are shown. Statistical significance was analyzed using a two-way ANOVA with Tukey's multiple comparisons test (*: $p < 0.05$, **: $p < 0.01$).

PARP12 and HAS3 broadly inhibit nairoviruses while OAS2 and GPTBP1 specifically inhibit authentic CCHFV infection.

Hazara virus (HAZV) is an orthonairovirus closely related to CCHFV, but which is non-pathogenic in humans. Thus, we studied the effect of the overexpression of chosen ISGs on a recombinant HAZV whose S segment expresses GFP separated from the NP ORF by a P2A site (rHAZV-GFP) (Fuller et al., 2019) (Figure 6A). HAZV infection was profoundly inhibited by RBV treatment of the cells (99,54%), as well as by HAS3 (90,11%) and PARP12 (74,70%) overexpression. LY6E and FAM26F overexpression also inhibited HAZV infection, although to a lesser extent. However, OAS2, GTPBP1 and LAMP3 overexpression did not impact HAZV infection. We also studied the role of endogenous GTPBP1 in HAZV infection using CRISPR/Cas9-mediated KO of this protein (Figure 6B). No effect of the KO could be observed on HAZV infection. These data suggested that HAS3, PARP12, FAM26F and LY6E have a broad-spectrum antiviral activity against nairoviruses, while OAS2 and LAMP3, and GTPBP1 might have specific antiviral or proviral roles for CCHFV tc-VLPs, respectively.

Finally, we sought to confirm the effects observed using tc-VLPs with authentic CCHFV. Infection in BSL-4 settings using CCHFV was carried out in ISG-overexpressing Huh7 cells. RBV treatment almost completely inhibited CCHFV infection at 48 hpi, as determined by RT-qPCR on infected cells (Figure 6D) and harvested supernatants (Figure 6C). A robust inhibition of CCHFV infection of almost one log in the infected cells and in the supernatants (94.06% and 92.58%, respectively) was also observed at 24 hpi upon PARP12 overexpression. Astonishingly, GTPBP1 overexpression also led to a diminution of CCHFV infection at 24 hpi, suggesting that this protein is rather an antiviral factor rather than a proviral one in the context of authentic infection. A discrepancy between tc-VLPs infection and authentic virus infection was also observed for LAMP3 overexpression, which appeared to be slightly proviral for authentic virus rather than antiviral at 24 hpi and had no effect anymore at 48 hpi. Finally, HAS3 overexpression was slightly antiviral at 24 hpi, but not at 48 hpi, and LY6E overexpression had no impact on CCHFV infection. Supernatants from PARP12- and GTPBP1-overexpressing cells harvested 24 hpi were used for titration assays on VeroE6 cells (Figure 6E). Confirming the results obtained by RT-qPCR, overexpression of both PARP12 and GTPBP1 led to a reduction of viral titers by 88.1% and 89.9%, respectively.

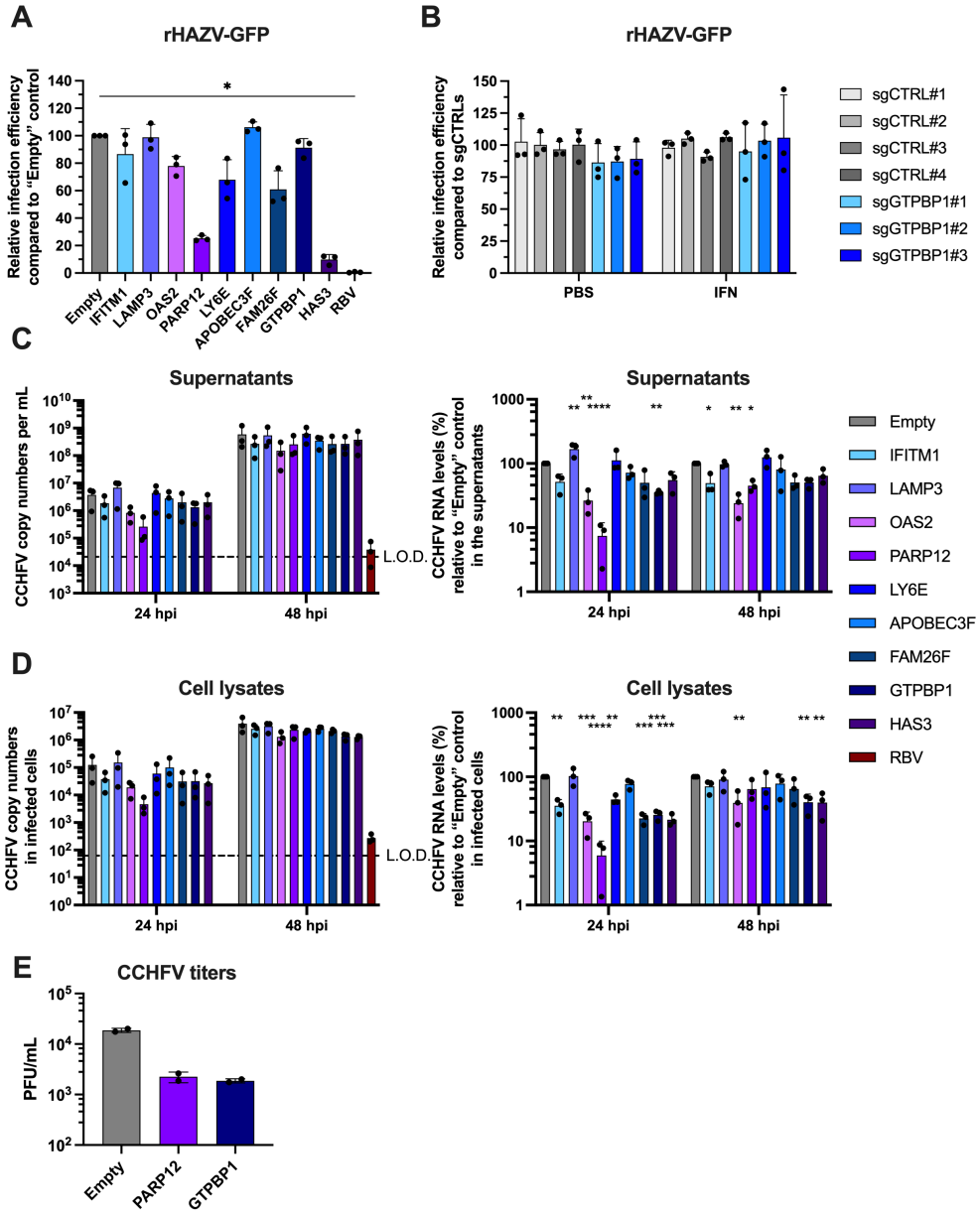

Figure 6. PARP12 and HAS3 broadly inhibit nairoviruses while GTPBP1 and OAS2 specifically inhibit authentic CCHFV infection. (A) Huh7 cells transduced with ISG-lentiviruses (MOI = 3) were infected with recombinant Hazara virus expressing GFP (rHAZV-GFP) (MOI = 0.3) for 16 hours before fixation and flow cytometry analysis. Data are expressed as relative infection efficiency compared to the Empty control set at 100%. Statistical significance was analyzed using a Kruskal-Wallis test followed by post-hoc analysis using Dunn's multiple comparison test (*: $p < 0.05$). **(B)** Huh7 cells stably expressing Cas9 were transduced with single guide RNAs (sgRNAs) control (sgCTRL#1 to #4) or targeting GTPBP1 (sgGTPBP1#1 to #3). Cells were treated with IFN (1000 U/mL) or PBS for 24 hours before infection with rHAZV-GFP (MOI = 0.3) for 16 hours. Cells were fixed and analyzed by flow cytometry. Data are expressed as relative infection efficiency cells compared to the average of all sgCTRL RNAs for each condition (PBS or IFN-treated) set at 100%. Statistical significance was analyzed using a two-way ANOVA with Tukey's multiple comparisons test and was non-significant. **(C) and (D)** Huh7 cells transduced with ISG lentivectors (MOI = 3) were infected with authentic CCHFV (MOI = 0.01) for 24 or 48 hours. In ribavirin (RBV) conditions, RBV (50 ng/mL) was added 1 hour before infection. **(C)** Supernatants were harvested and treated with Trizol-LS and **(D)** cells were lysed with Trizol before RT-qPCR analysis targeting CCHFV L 3'UTR. In the right panels, data are expressed as relative CCHFV RNA levels compared to the Empty control from each time-point set at 100%. Statistical significance was analyzed using a two-way ANOVA with Dunnett's multiple comparisons test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$). **(E)** Alternatively, supernatants from CCHFV-infected cells were used for titration in VeroE6 cells. (A), (B), (C), (D) Means and SD of three independent experiments are shown. (E) Means and SD of one experiment performed in technical duplicates are shown.

Discussion

Here, we unveiled human, macaque and bovine ISGs with antiviral effects against emerging, highly pathogenic CCHFV, using the tc-VLPs as model. We first demonstrated that tc-VLPs are inhibited by type I IFN, as seen for authentic CCHFV. We also confirmed the antiviral activity of a known antiviral ISG, namely human MX1. Interestingly, MX1 antiviral activity was not conserved in the bovine species (Figure 2B). Bovine MX1, which exhibits 73% similarity with human MX1, was shown to potently inhibit VSV or rabies virus (Baise et al., 2004; Leroy et al., 2006), but not paramyxoviruses such as respiratory syncytial or Sendai viruses (Leroy et al., 2005). Species-specific differences in MX1 antiviral activity were notably already described for duck and chicken MX1 that are inactive against influenza A virus (IAV) while human MX1 has a potent, very well described antiviral activity against this virus (Bernasconi et al., 1995; Benfield et al., 2008; Daviet et al., 2009; Schusser et al., 2011). Further studies of the differences of phenotype between bovine and human MX1 are necessary to fully understand why bovine MX1 lacks antiviral activity against CCHFV. Moreover, this demonstrated the power of performing screens of ISGs from different species to highlight potential species-specific pro- or antiviral activities.

As expected, our screens revealed several ISGs involved in the IFN pathway as antiviral factors against CCHFV. Notably, IFIH1, also known as melanoma differentiation-associated protein 5 (MDA5), was a potent inhibitor of CCHFV (Figure 2A, 2B and 2C). This pattern recognition receptor member of the RIG-I-like family of proteins is involved in the sensing of many viruses (Rehwinkel and Gack, 2020). However, knock-down of IFIH1 in A549 cells showed that this PRR is not involved in the establishment of the IFN response upon CCHFV infection (Spengler et al., 2015). It is likely that the antiviral effect of IFIH1 identified in our screen is due to its overexpression that stimulates the transcription of elements under an IFN-stimulated response element (ISRE) promoter (Figure 2F) but not to a putative involvement of IFIH1 in the sensing of CCHFV.

Further, we identified PARP12, whose all homologs (human, macaque, and bovine) strongly inhibited CCHFV tc-VLPs (Figure 2A), HAZV (Figure 6A) and authentic CCHFV infection (Figure 6C, 6D and 6E). PARP12 is a member of the PARP family with well described antiviral activities (Malgras et al., 2021) and exists in 2 isoforms: the long isoform, PARP12, and the short isoform (PARP12S) that lacks the C-terminal PARP domain. An antiviral function for PARP12 has been reported against

Venezuelan equine encephalitis virus (Atasheva et al., 2012, 2014) VSV (Liu et al., 2012), Zika virus (ZIKV) (Li et al., 2018, 2021), SARS-CoV-2 (Lee et al., 2021) or Chikungunya virus (CHIKV) (Krieg et al., 2023). Specifically, PARP12 inhibits ZIKV infection by targeting its non-structural proteins NS1 and NS3 to proteasomal degradation, and this activity is dependent on its PARP domain (Li et al., 2018). Alternatively, PARP12 induces the mono(ADP)ribosylation of nsP2 of CHIKV, hindering its protease activity necessary for genome replication (Krieg et al., 2023). Here, we showed that PARP12 did not impact NP nor Gc expression levels upon transfection; however, this remains to be studied in the context of viral infection. Further, we did not assess putative changes in the ADP-ribosylation status of these proteins, that might explain PARP12 antiviral activity against CCHFV, as seen for CHIKV and nsP2 (Krieg et al., 2023). Thus, the effect of PARP12 and, more specifically, its PARP domain, on the expression of CCHFV structural proteins as well as non-structural proteins must be studied to decipher the molecular mechanisms involved in its antiviral activity.

Additionally, our screen identified GTPBP1 as a proviral factor for CCHFV tc-VLPs (Figure 2A, 2C and 4A). This GTP-binding protein has a translation elongation activity and additionally plays an important role in mRNA surveillance and ribosome-associated quality control (Woo et al., 2011; Zinoviev et al., 2018). Moreover, it was shown to resolve paused ribosomes (Terrey et al., 2020). Here, GTPBP1 was found to specifically increase the replication/transcription/translation of CCHFV RNAs (genomic and antigenomic), and its effect was inhibited by ribavirin. It also increased translation of a CCHFV-specific mRNA; however, this effect was abrogated by ribavirin, suggesting that GTPBP1 activity is still dependent on viral replication and/or transcription and not on cellular translation (Figure 4C). Further studies should determine whether viral transcription, replication or both are enhanced by GPTBP1. However, KO of GTPBP1 did not impair CCHFV replication (Figure 5), suggesting that endogenous GTPBP1 has no role on CCHFV replication, or that it is not expressed in Huh7 cells at sufficient levels to promote viral replication. Alternatively, its absence could be compensated by a protein with a redundant role. Of note, a previous report showed that GTPBP2 might have a redundant role (Senju et al., 2000). However, human GTPBP2 present in our screen did not increase tc-VLPs infection efficiency. Astonishingly, GTPBP1 overexpression had no effect on HAZV and had an antiviral effect against authentic CCHFV. These discrepancies can be explained by the limitations of our tc-VLPs system, *i.e.*, the absence of some non-structural proteins, the differences of expression of viral proteins compared to authentic infection, or

differences between the sequences or secondary structures of the viral segments and the different reporter segments. Moreover, in the minigenome assay, the reporter molecule is flanked with the UTRs of the L segment of CCHFV. It remains to be studied whether GTPBP1 also has a proviral effect on the other UTRs, namely S and M UTRs.

Bovine hyaluronan synthase 3 (HAS3) was previously shown to inhibit blue tongue virus (BTV) infection (Hardy et al., 2023). Here, we show that its overexpression also potently inhibits CCHFV and HAZV (Figure 2A, 2B, and 2E). This protein is responsible for the synthesis of hyaluronan. It is thus tempting to speculate that it inhibits viral entry by inducing the formation of a shield of hyaluronan polymers, components of the extracellular matrices. Interestingly, HAS3 is an ISG in the bovine species but not in the human or macaque species (Shaw et al., 2017). Upregulation of the expression of this gene in the bovine species could therefore in part explain why this species remains asymptomatic upon CCHFV infection. To verify HAS3 contribution to the pathogenesis of viruses that infect bovines, further analyses such as KO of this gene in bovine cells should be performed.

We also identified LAMP3 that specifically inhibited CCHFV entry, as showed with pseudotyped VSV (Figure 3A). Of note, it was shown that LAMP3 expression is induced in CCHFV-infected patients (Neogi et al., 2022), and is increased in Sjögren syndrome (SS) patients, a chronic and progressive autoimmune disease. LAMP3 expression results in lysosomal membrane permeabilization, destabilization of the autophagic flux and activation of caspases (Tanaka et al., 2020, 2022). It remains to be deciphered whether LAMP3 antiviral activity against CCHFV tc-VLPs is related to this apoptotic activity. Intriguingly, we could not confirm LAMP3 antiviral activity against HAZV or authentic CCHFV, suggesting that these viruses might have evolved to counteract this factor.

In this study, human, macaque and bovine LY6E, a transmembrane protein located to the cell surface, inhibited CCHFV tc-VLPs (Figure 2A and 2B). Human LY6E was found to inhibit CCHFV entry (Figure 3A). While we were able to confirm LY6E antiviral activity against HAZV (Figure 6A), it had surprisingly no effect on authentic CCHFV (Figure 6C and 6D). As LY6E has been shown to promote viral infection of flaviviruses or IAV by promoting viral uncoating after membrane fusion (Mar et al., 2018)., but to inhibit viral entry of coronaviruses by impairing membrane fusion (Pfaender et al., 2020; Mar et al., 2023) , it can be suggested that LY6E might have an

antiviral activity at the early stages of CCHFV cycle (that can be detected using CCHFV tc-VLPs and CCHFV-pseudotyped VSV) and a proviral activity at later stages of CCHFV cycle, but this remains to be studied. A putative viral counteracting factor could also explain the discrepancy.

We also identified the antiviral role of OAS2 against CCHFV (Figure 2A, 6C and 6D). Further studies are needed to decipher if its antiviral mechanism is linked to the production of 2'-5'-oligoadenylates that could activate RNase L, resulting in the degradation of single-stranded RNAs, as described for many viruses (reviewed in (Sadler and Williams, 2008)).

Finally, the impact of the overexpression and KO of the identified candidates on CCHFV infection should be studied in biologically relevant primary cells, such as primary human hepatocytes (PHH), human umbilical vein endothelial cells (HUVEC), or monocyte-derived macrophages.

Overall, this work lay the basis for a better comprehension of the complex intricacies between CCHFV and the innate immune system. It paves the way to better understand the biology of the virus and identify targets for the development of therapeutics to fight CCHFV infection.

Materials and methods

Cell lines

All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) complemented with 10% of fetal calf serum (FCS), 100 U/mL of penicillin and 100 µg/mL streptomycin (Gibco) and incubated at 37°C with 5% CO_2 unless otherwise stated. Human hepatocarcinoma (Huh7) cells were a kind gift of David Durantel. Huh7.5 and VeroE6 cells were propagated from laboratory stocks. Human embryonic kidney (HEK293T) cells were propagated from laboratory stocks and maintained in DMEM complemented with 10% FCS, 100 U/mL of penicillin and 100 µg/mL streptomycin, and geneticin (200 µg/mL). Huh7 cells stably expressing Cas9 were maintained in DMEM complemented with 10% FCS, 100 U/mL of penicillin and 100 µg/mL streptomycin, and blasticidin (10 µg/mL).
The cells were regularly tested for mycoplasma contamination.

Plasmids and constructs

The constructs encoding wild-type CCHFV strain IbAr10200 V5-tagged L polymerase (pCAGGS-V5-L), CCHFV NP (pCAGGS-NP), CCHFV-specific negative-sense eGFP-expressing minigenome (pT7RiboSM2_vL_eGFP), CCHFV-specific negative-sense nLuc-expressing minigenome (pSMART-LCK_T7-vL-nLuc), CCHFV GPC (pCAGGS-GPC), CCHFV-$GPC_{\Delta 53}$ (pCAGGS- $GPC_{\Delta 53}$), T7 RNA polymerase (pCAGGS-T7) and an empty vector (pCAGGS) were previously described (Devignot et al., 2015; Zivcec et al., 2015; Freitas et al., 2020; Suda et al., 2016). CCHFV-specific positive-sense nLuc-expressing minigenome was cloned by overlap polymerase chain reaction (PCR) and enzymatic digestion into pSMART-LCK to generate (pSMART-LCK_T7-vL-antigenomic-nLuc). pSCRPSY was previously described (Kane et al., 2016). pISRE-fLuc was a kind gift of Pierre-Olivier Vidalain. pZ-A2-T7-fLuc was a kind gift of Lionel Condé. For CRISPR experiments, pXPR_047 was a gift from David Root (Addgene plasmid #107145); pLX311-Cas9 was a gift from William Hahn & David Root (Addgene plasmid #118018); lentiGuide-Puro was a gift from Feng Zhang (Addgene plasmid #52963); LentiGuide-Puro-CTRLg1 (CG509) and LentiGuide-Puro-CTRLg2 (CG510) were a gift from Caroline Goujon (Addgene plasmids #139455 and #139456). The plasmids encoding pMK-RQ-S(eGFP), pMK-RQ-L and pMK-RQ-M for HAZV rescue were a kind gift from John N. Barr (University of Leeds, United Kingdom).

Reagents

Universal type I IFN was obtained from PBL Assay Science (11200). Ribavirin was obtained from Sigma (R9644). Mouse monoclonal antibodies targeting CCHFV strain Ibar10200 anti-PreGc (clone 11E7 and clone 30F7) and anti-NP (clone 9D5 and clone 2B11) were obtained from the Joel M. Dalrymple—Clarence J. Peters USAMRIID Antibody Collection through BEI Resources, NIAID, NIH. Rabbit polyclonal anti-Gn antibody has been kindly given by Ali Mirazimi (Karolinska Institute, Sweden). Alternatively, mouse polyclonal anti-Gn antibody was obtained through DNA immunization of mice. Anti-VSV-G 41A.1 was already described (Perez-Vargas et al., 2019). Rabbit polyclonal antibody targeting GTPBP1 was obtained from Thermo Fisher Scientific (PA566710).

CCHFV transcription- and entry-competent virus-like particle (tc-VLP) assay

CCHFV tc-VLPs were produced in Huh7.5 producer cells seeded in a 10 cm dish ($1.2x10^6$ cells) and transfected with 1.2 µg of pCAGGS-NP, 3 µg of pCAGGS-GPC, 3.6 µg of pCAGGS-V5-L, 3 µg of pT7RiboSM2_vL_eGFP, and 3 µg of pCAGGS-T7, using GeneJammer transfection reagent (Agilent). 6 hours post-transfection, medium was changed, and tc-VLP-containing supernatants were harvested 72 hours later, cleared by centrifugation for 5 minutes at 750 g and filtrated. Huh7 cells seeded in a 10 cm dish ($1.2x10^6$ cells) were transfected with 1.2 µg of pCAGGS-NP, 3 µg of pCAGGS-GPC, and 3.6 µg of pCAGGS-V5-L. 6 hours post-transfection, the cells were seeded in 96 well-plates ($2x10^4$ cells per well) or in 24 well-plates ($8x10^4$ cells per well), depending on the experiment. In experiments performed with type I IFN, increasing amounts of universal type I IFN (PBL Interferon Source) were added to the cells 24 hours post-transfection. 24 hours post-transfection, (or 24 hours following type I IFN treatment in specific experiments), tc-VLPs-containing supernatants were transferred to the cells. 3 hpi, the cells were washed with PBS and fresh medium was added to the cells. In experiments performed with ribavirin, ribavirin (50 ng/mL) was added 1 hour before infection and was kept at 50 ng/mL after each medium change. 24-48 hpi, the cells were trypsinized and fixed in 2% paraformaldehyde (PFA) and the percentage of infected (GFP-positive) cells was determined by flow cytometry using a MACSQuant VYB flow cytometer (Miltenyi Biotec).

For nLuc tc-VLPs, the same procedure has been applied to Huh7 cells. Producer cells were transfected with pSMART-LCK_T7-vL-nLuc instead of pT7RiboSM2_vL_eGFP. Following infection of Huh7 cells (expressing viral proteins or not), cells were washed once with PBS, lysed with Passive Lysis Buffer 1x and reporter activities were

measured with Nano-Glo Luciferase assay system (Promega) following the manufacturer instructions and a Mithras LB 940.

Generation of human, macaque and bovine lentivector library

Lentivirus vectors expressing ISGs from human, macaque or bovine origin were generated in a 96-well plate format as previously described (Schoggins et al., 2011), with one ISG per well. Briefly, $3.5x10^4$ HEK293T cells were transfected with 125 ng of the pSCRPSY-ISG construct and plasmids expressing HIV Gag-Pol (NLGP) and VSV-G in a ratio of 25:5:1. The medium was changed 24 hours post-transfection, and supernatants were harvested at 48, 72 and 96 hours and stored at -80°C. Independent stock of lentiviruses were generated for validation assays.

Arrayed ISG expression screening

ISG screening was conducted on Huh7 cells expressing NP, L and GPC in a 96-well plate format, using libraries of human, simian and bovine ISGs encoded by pSCRPSY (Kane et al., 2016). 100 µL of lentiviral vector ISG libraries were used to reverse-transduce the cells ($2.5x10^4$ cells/well in a 96-well plate) for 48 hours. Transduced cells were then infected with GFP tc-VLPs (MOI = 0.3). 3 hours later, the cells were washed once with PBS and medium was changed. 33 hpi, the cells were fixed in 1% PFA. The percentage of transduced (TagRFP-positive) and infected (GFP-positive) cells were determined by flow cytometry. ISGs with transduction levels (measured by RFP expression) below 30% were excluded from downstream analyses. Data were analyzed using FlowJo software.

The initial ISG screen was performed in a single biological experiment. ISG confirmatory miniscreens were executed in two technical duplicates in three independent experiments.

Cytotoxicity and ISRE-fLuc assay

For the cytotoxicity assay and the ISRE-fLuc assay, Huh7 cells were transfected with pISRE-fLuc (Stratagene) and a control plasmid expressing nanoluciferase. 24 hours post-transfection, cells were transduced with 100 µL of the miniscreen library of selected ISGs from the initial screen. Then, 48 hours post-transduction, supernatants were harvested to measure the toxicity of the expressed ISGs using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer instructions. Moreover, cells were washed once with PBS, lysed with Passive Lysis Buffer 1x and reporter activities were measured with Nano-Glo Dual-Luciferase

reporter assay system (Promega) following the manufacturer instructions to measure ISRE induction (fLuc-positive cells) as a surrogate for IFN induction.

VSV-pseudotyped assay

$8x10^5$ HEK293T cells in a 6-well plate format were transfected with 2 µg of pCAGGS-$GPC_{\Delta 53}$ or pHCMV-VSV-G using PEI MAX (Polysciences). 24 hours post-transfection, cells were infected with a recombinant replication-deficient vesicular stomatitis virus (VSV) expressing GFP instead of VSV-G (a kind gift from Olivier Reynard) with a multiplicity of infection (MOI) of 5 for 1 hour. Then, medium was replaced by fresh medium containing an anti-VSV-G antibody. Supernatants were harvested 24 hpi, centrifuged, filtered through a 0.45 µm filter and stored at -80°C. Pseudotyped virions were used to infect Huh7 cells for 1 hour. For some experiments, virions were incubated for 1h at 37°C with 10 µg/mL of mouse IgG, mouse anti-Gc antibody (30F7) or anti-VSV-G antibody prior infection. 24 hpi, cells were fixed in 2% PFA and analyzed by flow cytometry using a MACSQuant VYB flow cytometer (Miltenyi Biotec).

***In vitro* transcription of viral RNA**

pZ-A2-T7-fLuc was linearized by digestion with EcoRI (for the polyadenylated version of the RNA) or XbaI (for the non-polyadenylated version of the RNA), and pSMART-LCK_T7-vL-nLuc and pSMART-LCK_T7-vL-antigenomic-nLuc by BamHI (the enzymes were obtained from New England Biolabs). DNA was precipitated and digested with Mung Bean Nuclease, precipitated, and *in vitro* transcribed using the T7 RiboMAX™ Express Large Scale RNA Production System according to the manufacturer instruction (Promega). For some RNAs, 3′-O-Me-m7G(5')ppp(5')G RNA Cap Structure Analog (ARCA) (New England Biolabs) was added to the mix. DNA template was removed by DNase treatment (Thermo Fisher Scientific), and RNA was precipitated using phenol/chloroform. RNA quality was checked on an agarose gel.

CCHFV minigenome assay

Huh7 cells seeded in a 10 cm dish ($1.2x10^6$ cell) were transfected with 1.2 µg of pCAGGS-NP and 3.6 µg of pCAGGS-V5-L. 6 hours post-transfection, they were reverse-transduced into 24-well plates ($8x10^4$ cells) with control lentivectors or ISGs lentivectors (MOI = 3). 16 hours post-transduction, the medium was replaced and 48 hours post-transduction, the cells were transfected with 50 ng of RNA expressing fLuc and 250 ng RNA expressing genomic, antigenomic or viral mRNA of CCHFV-specific nLuc. 24 hours post-transfection, cells were washed once with PBS, lysed with Passive

Lysis Buffer 1x and reporter activities were measured with Nano-Glo Dual-Luciferase reporter assay system (Promega) following the manufacturer instructions and using a Mithras LB 940.

Western blotting

For western blot analysis, cells were washed once with PBS and lysed using cold cell lysis buffer (20 mM Tris pH 7.5, 1% Triton, 0.05% SDS, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors (Roche Applied Science). They were precipitated by centrifugation at 12,000 g for 30 min. For detection of Gn, NP or GTPBP1, samples were denatured at 95°C for 5 min, in reducing loading buffer (375 mM Tris HCl pH 6.8, 12% SDS, 60% Glycerol, 0.04% Bromophenol blue, 500 mM β-mercaptoethanol) and electrophoresed on 10% SDS-polyacrylamide gels. Alternatively, for Gc detection, samples were processed in non-reducing loading buffer (375 mM Tris HCl pH 6.8, 12% SDS, 60% Glycerol, 0.04% Bromophenol blue) and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane by electroblotting (BioRad) for 1h30 at 100V. After saturation in TBST (20 mM Tris HCl, pH 7.5, 150 mM NaCl and 0.05%-Tween)-milk 10% for 1h, membranes were incubated with primary antibodies for the detection of CCHFV proteins (mouse monoclonal 11E7 anti-Gc 1:500, rabbit polyclonal anti-Gn 1:5000 or mouse polyclonal anti-Gn 1:400, and mouse monoclonal 9D5 anti-NP 1:1000), in TBST-milk 10% overnight at 4°C. After 3 washes of 10 min using TBST, membranes were then incubated with IRDye secondary antibodies (Li-Cor Biosciences) at 1:20000 dilution in TBST for 30 min at room temperature, followed by imaging with Odyssey infrared imaging CLx system (Li-Cor Biosciences). Quantification of proteins was performed with Odyssey imaging CLx system software.

Knock-out experiments using CRISPR/Cas9

Lentiviral vectors expressing Cas9 were produced by transfecting HEK293T cells seeded in 10 cm dishes with 8.6 µg of the vector (pLX311-Cas9), 8.6 µg of Gag-Pol (psPAX2) and 2.7 µg of pHCMV-VSV-G. Supernatant was harvested 48 hours post-transfection and used to transduce Huh7 cells and the blasticidin selection was added. To check the expression and activity of the Cas9, Huh7_Cas9 cells were transduced with lentiviral vectors expressing GFP and a single-guide RNA against GFP and the fluorescence was analyzed by flow cytometry 10 days post-transduction. To design sgRNAs targeting GTPBP1, CRISPick was used (Broad Institute). The sequences for sgGTPBP1#1, sgGTPBP#2 and sgGTPBP#3 are 5'-GGACCAGGTAATCACGCACG-3',

5′-TGATGACACCTACTCCGTCC-3′ and 5′-ATGACCAGCCAAGTCGATGA-3′, respectively. The sequences for 2 additional control sgRNA (sgCTRL#3 and sgCTRL#4) are 5′-TTAATTTGGGTGGGCCCTGC-3′ and 5′-TTGGATATTAATTAGACATG-3′. These sequences were cloned into lentiGuide puro using BsmBI sites. Lentiviral vectors were produced and used to transduce Huh7_Cas9 cells that were maintained under puromycin selection.

HAZV rescue and infection

HAZV was rescued as previously described (Fuller et al., 2019). Briefly, Huh7.5 cells were seeded into 6-well plates ($2x10^5$ cells per well) before transfection with 1.2 µg pMK-RQ-S(eGFP), 1.2 µg pMK-RQ-M, 1.9 µg pMK-RQ-L and 0.6 µg pCAGGS-T7. Supernatants were harvested 72 hours post-transfection. Viral amplification was performed on Huh7.5 cells seeded in T75 flasks and infected with rescued virus. Supernatants were harvested 72 hpi.

For infection experiments, Huh7 cells seeded in 24-well plates were infected with HAZV (MOI = 0.3) for 1 hour before the medium was replaced. The cells were fixed 16 hpi using 2% PFA and analyzed by flow cytometry.

CCHFV infection in BSL-4 facility

CCHFV (strain IbAr10200, obtained from Pasteur Institute) was amplified on VeroE6 cells. Huh7 cells transduced with ISG lentivectors were infected with CCHFV (MOI = 0.01) for 1 hour before the medium was replaced. At the indicated time-points, supernatants were harvested and treated with TRIzol™ LS Reagent (Thermo Fisher Scientific), and the cells were lysed using TRIzol™ Reagent (Thermo Fisher Scientific).

CCHFV titration in BSL-4 facility

10-fold serial dilutions (from 200 to 0.002 µL) of supernatants from infected cells were used to infect 80% confluent VeroE6 cells in 24 well-plates for 1 hour at 37°C. Then, the cells were washed once with PBS and the medium was replaced with a mix of DMEM 5% FCS / carboxymethyl cellulose (CMC) viscosity 3.2% (1:3, vol:vol). Cells were incubated for 120 hours at 37°C, then washed 3 times with PBS, fixed with PFA 4% for 20 min, and permeabilized in PBS/Triton 0.5%/Glycine 0.5% for 5 min at room temperature (RT). After three washes, cells were incubated with 200 µL of mouse anti-NP (mouse monoclonal 2B11 anti-NP,1:500) in PBS/0.1% Triton X100/3% bovine serum albumin (BSA) and incubated overnight at 4°C. Then, after three washes, cells were incubated with goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Sigma, A3562, 1:2000) in PBS. After three washes in PBS, staining was

revealed by adding 200 µL of 1-Step NBT/BCIP (Thermo Fisher Scientific) and foci were count.

Determination of viral RNA by RT-qPCR

RNAs were extracted, reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad) and quantified using the FastStart Universal SYBR Green Master kit (Roche Applied Science). The following primers were used for CCHFV detection: 5′-CCCCACACCCCAAGATAATA-3′ and 5′-ACTACTCTGCATTCTCCTCA-3′; for GAPDH detection: 5′-TGGAAGATGGTGATGGGATTTC-3′ and 5′-AGGTGAAGGTCGAGTCAACG-3′, for MX1 detection: 5′-GGCTGTTTACCAGACTCCGACA-3′ and 5′-CACAAAGCCTGGCAGCTCTCTA-3′, for GTPBP1 detection: 5′-CCTTCATCGACTTGGCTGGTCA-3′ and 5′-CCAGGTGTTCTTTGGTCATCCC-3′. As an internal control of extraction, an exogenous RNA from the linearized Triplescript plasmid pTRI-Xef (Invitrogen) was added into the supernatant prior to extraction and quantified with specific primers (5′-CGACGTTGTCACCGGGCACG-3′ and 5′-ACCAGGCATGGTGGTTACCTTTGC-3′). For CCHFV L 3′UTR and GTPBP1 detection, they were performed on a QuantStudio™ 6 Flex Real-Time PCR System apparatus (Thermo Fisher Scientific) and for MX1 detection, they were performed on an Applied StepOne real-time PCR apparatus (Thermo Fisher Scientific).

Supplementary figures

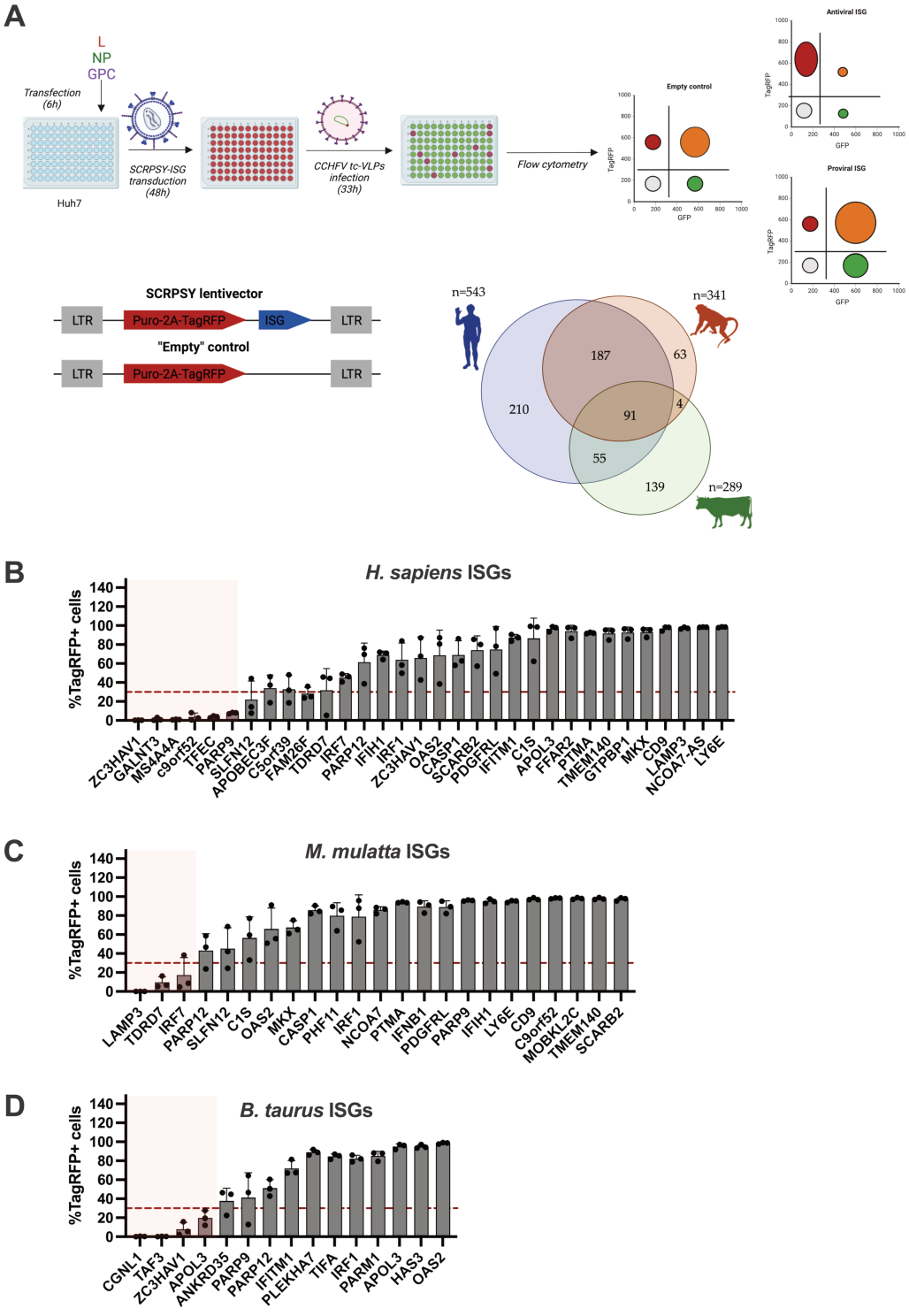

Figure S1. Schematic representation of the ISG screen and transduction efficiencies of the miniscreens. (A) Huh7 cells expressing NP, L and GPC were transduced with SCRPSY lentivectors before infection with GFP tc-VLPs. Transduction efficiencies and tc-VLPs replication were assessed by flow cytometry. The libraries encompass ISGs from human, macaque, or bovine origin. For the miniscreens, transduction efficiencies of **(B)** human, **(C)** macaque or **(D)** bovine ISGs into Huh7 cells expressing NP, L protein and GPC was assessed by flow cytometry. The red dotted line represents a threshold of 30% of transduction efficiency. ISGs discarded from the miniscreen analysis due to low transduction efficiencies are highlighted with red boxes. Means and SD of three independent experiments are shown.

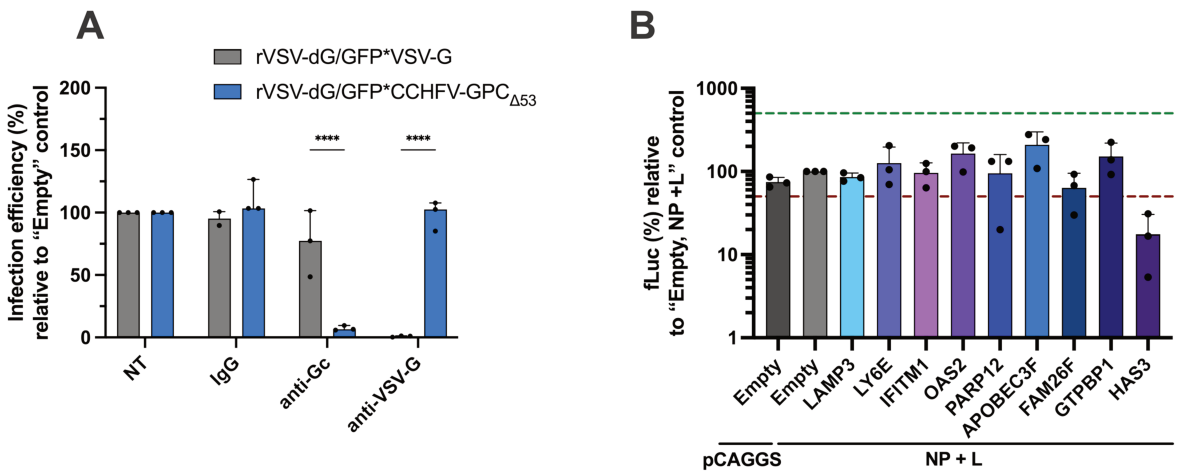

Figure S2. Control of efficient pseudotyping of VSV viruses and of transfection of RNA into Huh7 cells. (A) Huh7 cells were infected with VSV viruses pseudotyped with VSV-G (rVSV-dG/GFP*VSV-G) or GPC$_{\Delta 53}$ (rVSV-dG/GFP*CCHFV-GPC$_{\Delta 53}$) that were incubated with 10 µg/mL of anti-Gc antibody, anti-VSV-G antibody, or control mouse IgG prior infection. NT = non-treated. Data are expressed as relative infection efficiency compared to a non-treated (NT) control. Statistical significance was analyzed using a two-way ANOVA with Šidák multiple comparisons test (****: $p < 0.0001$). **(B)** Huh7 cells transfected either with a control plasmid (pCAGGS) or NP and L protein (NP + L) and transduced with ISG lentivectors (MOI = 3) were transfected with an *in vitro* transcribed capped and polyadenylated RNA expressing fLuc. 24 hours post-transfection, the luminescence was read. Data are expressed as fLuc signal relative to the "Empty, NP + L" control set at 100%. The red and green dotted line indicate thresholds of 50% diminution and 500% augmentation of fLuc signal, respectively. Statistical significance was analyzed using a Kruskal-Wallis test followed by post hoc analysis using Dunn's multiple comparisons test and was non-significant. (A) and (B) Means and SD of three independent experiments are shown.

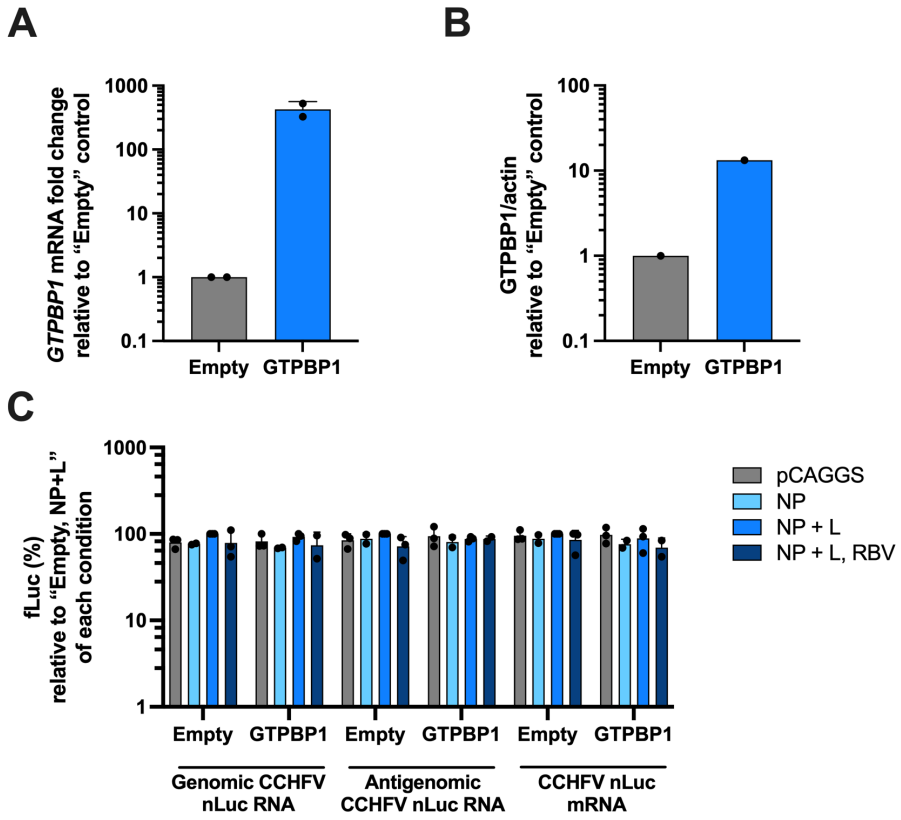

Figure S3. GTPBP1 is overexpressed in transduced Huh7 cells and RBV treatment does not impact fLuc expression. (A) and (B) Huh7 cells were transduced with a control lentivector "Empty" or with a GTPBP1 lentivector (MOI = 3). 48 hours post-transduction, cells were **(A)** lysed and the levels of *GTPBP1* RNA were assessed by RT-qPCR or **(B)** analyzed by western blot using anti-GTPBP1 and anti-actin antibodies and quantified. **(C)** Huh7 cell transfected either with a control plasmid (pCAGGS), NP, or NP and L protein (NP + L) and transduced with ISG lentivectors (MOI = 3) were transfected with *in vitro* transcribed RNAs of control fLuc and genomic, antigenomic or messenger RNA expressing nLuc. In the RBV conditions, RBV (50 ng/mL) was added 1 hour before transfection. 24 hours post-transfection, cells were lysed, and luminescence was read. Data are normalized to the Empty, NP + L condition of each RNA species set at 100%. Statistical significance was analyzed using a two-way ANOVA with Tukey's multiple comparisons test and was non-significant. (A) and (C) Means and SD of (A) two and (C) three independent experiments are shown.

Discussion and perspectives

I. Perspectives and future studies of the identified hits

Overall, the screen that we performed identified numerous genes with pro- or antiviral activities against CCHFV tc-VLPs, HAZV and authentic CCHFV, that were briefly described in the discussion part of the Chapter 2 of the results. In this part, more light will be shed on two candidates identified, namely PARP12 and GTPBP1, as well as future perspectives to help identifying their mechanisms of action in the context of CCHFV infection.

1. PARP12

a. PARP12 and viruses

PARP12 is a member of the poly(ADP)ribose polymerase family, that catalyze the transfer of negatively charged ADP-ribose subunits to target proteins or nucleic acids using NAD^+ as a substrate, a phenomenon called mono-ADP-ribosylation (MAR) or poly-ADP-ribosylation (PAR), depending on how many ADP-ribose are transferred to the target. PARP proteins are involved in several biological processes, such as chromatin modification, translation or transcription regulation, or regulation of cellular death (Schreiber et al., 2006). Human genome encodes 17 PARPs that all possess a PARP domain. 10 of them are classified in four subfamilies based on the other structural domains that they contain (Schreiber et al., 2006): the DNA-dependent PARPs (PARP1 and PARP2), the tankyrases (tankyrase-1 and tankyrase-2), the CCCH-PARPs (PARP7 (also known as tiPARP), PARP12 and PARP13 (also known as ZAP)), and the MacroPARPs (PARP9, PARP14, and PAR15). Other PARPs (PARP4, PARP6, PARP8, PARP10, PARP11, PARP16) do not enter in these categories, based on their architectural domains (Figure 32).

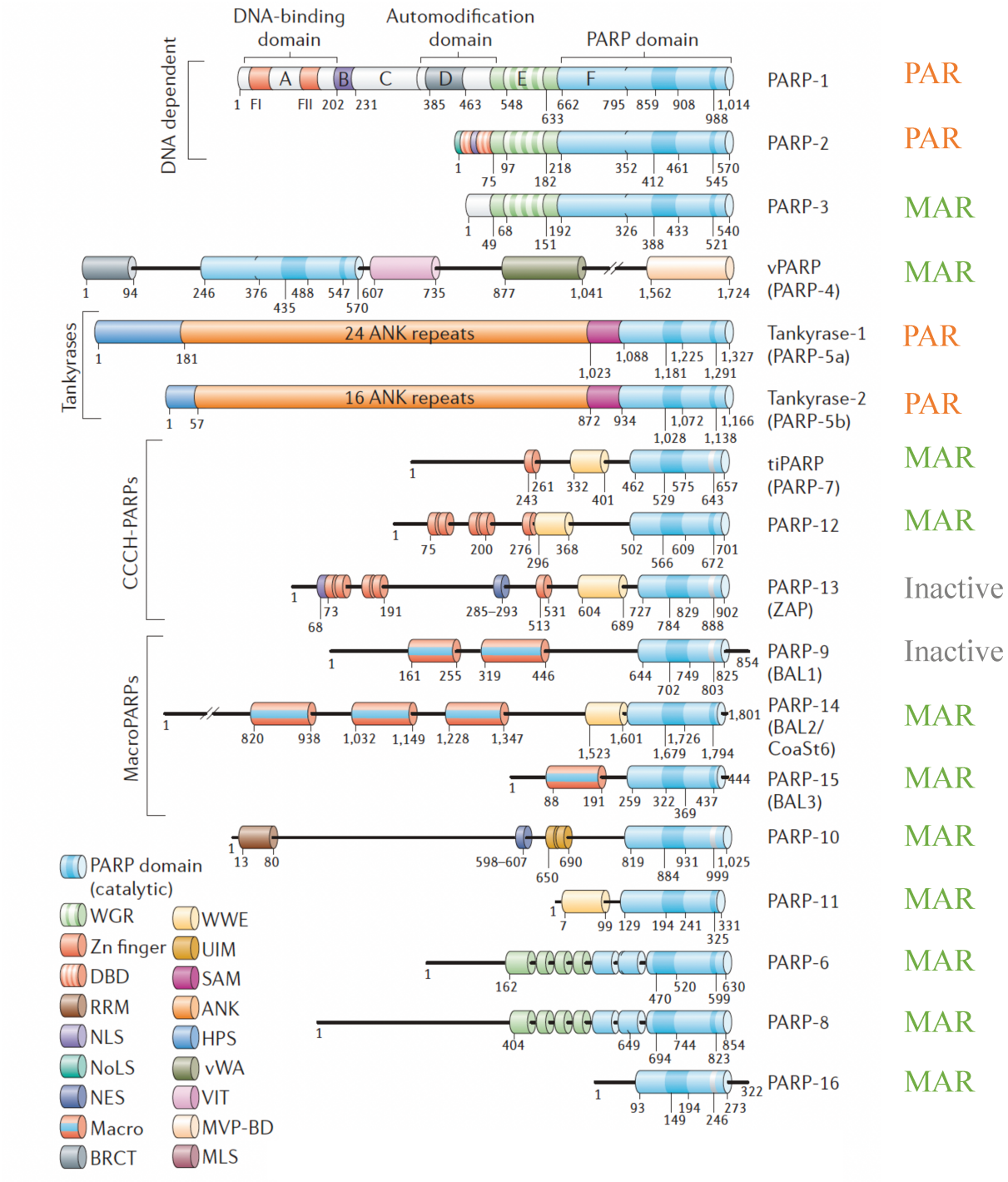

Figure 32. The PARP superfamily. The 17 members of the PARP superfamily are represented with their domain architecture. Splicing variants are not represented. BRCT: BRCA1 C terminus; SAM: sterile α-motif; ANK: ankyrin; vWA: von Willebrand factor type A; VIT: vault inter-α-trypsin; MVP-BD: major vault protein binding site; MLS: mitochondrial localization signal; NES: nuclear export signal; NoLS: nucleolar localization signal; NLS: nuclear localization signal. The activity of each PARP (MARylation, (MAR), PARylation (PAR) or inactive) is indicated on the right. Adapted from (Schreiber et al., 2006).

PARP12, with tiPARP and PARP13, contain one (for tiPARP) or five (for PARP12 and PARP13) N-terminal $CX_8CX_5CX_3$-like zinc-finger motifs (ZnF) able to bind RNA, a central WWE domain which is a putative protein-protein interaction motif, and the C-terminal PARP domain which bears the catalytic activity (Figure 32). Of note, the catalytic residue responsible for the MAR and PAR activity in PARP1 is not conserved within tiPARP, PARP13, PARP9, PARP10 and PARP16. While this probably explains why PARP9 and PARP13 are devoid of MAR and PAR activity, PARP10 and tiPARP still can be active in MARylation (Vyas et al., 2014). Importantly, it was shown that most PARPs are responsible for MARylation, while only PARP1, PARP2 and the tankyrases are responsible for PARylation (Vyas et al., 2014). Catalytic activity of PARP12 resides in its H574 and/or G575 amino acids (Welsby et al., 2014), and PARP12 autoribosylates at its D600, D611, C474 and C584 amino acids (Vyas et al., 2014). By comparison with PARP10 for which E882 mutation resulted in loss of autoribosylation, E569A mutants of PARP12 were generated and are supposed to be devoid of autoribosylation activity (Atasheva et al., 2014). However, this was not formally proved.

PARP12 exists in 2 isoforms, generated through alternative splicing. The short isoform, PARP12S, lacks the PARP domain and has to date no known antiviral activity. However, the long isoform of PARP12 was found to be antiviral through a gain-of-function screen against VSV (Liu et al., 2012). Moreover, by transfecting the genome of Venezuelan equine encephalitis virus (VEEV) in which PARP12 sequence was inserted, followed by superinfection with different viruses, PARP12 was found to downregulate VEEV, Sindbis virus (SINV), encephalomyocarditis virus (EMVC), and one bunyavirus, Rift Valley fever virus (RVFV) infections (Atasheva et al., 2012). More precisely, PARP12 expression from this replicon affected translation of both VEEV proteins and cellular proteins (Atasheva et al., 2014). This effect was dependent on its PARP domain. Mutations inactivating the protein catalytic activity (H574A) or affecting its auto-ribosylation (E569A) reduced the ability of PARP12 to cause a translational shutoff, but these mutants remained efficient in inhibiting VEEV, suggesting that PARP12 might inhibit VEEV replication through more than one mechanism. Mass spectrometry analyses and fractionation experiments found that PARP12 interacted both with polysomes (through its PARP domain) and RNA (through its ZnF domain), which could explain how PARP12 exerts antiviral activity through several mechanisms (Atasheva et al., 2014). All these studies were performed in a context of overexpression and would need to be performed in a knock-down or

KO context to validate the role of endogenous PARP12 in the control of the replication of these viruses.

However, more recently, a CRISPR/Cas9 KO screen in the context of Zika virus (ZIKV) infection found PARP12 as one of the top candidates (Li et al., 2018). Interestingly, PARP12 overexpression decreased the levels of non-structural proteins (NS) NS1 and NS3, while its KO led to increased protein expression. More specifically, PARP12 mediated the ADP-ribosylation of NS1 and NS3, triggering their proteasomal degradation. It was further discovered that PARP11 promoted PARP12-mediated degradation of NS1 and NS3 (Li et al., 2021), both in overexpression or downregulation approaches. PARP11 and PARP12 were found to interact through their WWE domains.

Further, PARP12 and PARP10 both inhibit Chikungunya virus (CHIKV) (Krieg et al., 2023). Antiviral activity of PARP12 against a CHIKV replicon was dependent on its catalytic activity, while its activity against fully infectious CHIKV was not, suggesting once again that PARP12 might contribute to viral restriction through several different mechanisms. Interestingly, PARP10 antiviral activity was dependent on its catalytic activity against both CHIKV replicon and infectious CHIKV. More precisely, PARP10 and PARP12 overexpression led to increased MARylation of non-structural protein (nsP) nsP2, which inhibited its proteolytic activity necessary for viral replication, but without affecting its levels of expression nor promoting its degradation (contrary to what was seen for the non-structural proteins of ZIKV). Of note, nsP2 MARylation was counteracted by CHIKV nsP3 protein. Indeed, nsP3, and more specifically, its macrodomain which bears de-MARylation activity, removed the PAR10- and PARP12-catalyzed mono(ADP-ribose) from nsP2 that hinder its protease activity.

Other macrodomains from different viruses also counteract PARP12-mediated antiviral activities. Indeed, coronaviruses encode a non-structural protein, nsp3, whose macrodomain bears ADP-ribosylhydrolase activities. More specifically, macrodomain-deficient mutant viruses are more restricted by type I IFN (Kuri et al., 2011), and macrodomain-deficient mouse hepatitis virus (MHV) infection is inhibited by PARP12, as shown by PARP12 downregulation experiments that led to a partial rescue of the virus, both *in vitro* and *in vivo* (Grunewald et al., 2019; Kerr et al., 2023). Finally, in a study revealing the RNA interactome of SARS-CoV-2, knock-down of PARP12 led to a rescue of genomic RNA levels of SARS-CoV-2 in Calu-3-infected cells (Lee et al., 2021).

PARP12 also might have a less direct antiviral role, by modulating innate immune responses. Indeed, upon different stimuli, PARP12 is relocalized either to stress granules, where it mediates the blocking of mRNA translation, or to stress-granules-unrelated structures, the latter correlating with increased NF-κB signaling (Welsby et al., 2014). Thus, PARP12 could have a role in modulating cellular translation, and in enhancing inflammatory responses.

b. PAR(P)ticipation of PARP12 in CCHFV restriction

PARP12 is listed among the core of 62 ISGs that are common between all species (Shaw et al., 2017). Accordingly, PARP12 was a top hit in all the three species that were studied in our initial screen (relative infection efficiencies of 37.7%, 36.96% and 22.7% for the human, macaque or bovine PARP12, respectively). Human and macaque PARP12 are very similar (97.86% identity and 99% similarity), while bovine PARP12 and human PARP12 are less similar (74.01% identity and 84.23% similarity). The catalytic site of these homologs is well conserved.

Results from our confirmatory miniscreens were more mitigated, and only bovine PARP12 antiviral activity could consistently be confirmed in three independent experiments. However, human PARP12 exhibited a robust antiviral activity against HAZV (74.70% inhibition) and, importantly, against authentic CCHFV, with almost a ten-fold reduction in viral titers. Importantly, PARP12 did not increase the transcription of ISRE-regulated reporter molecule, suggesting that PARP12 antiviral role does not rely on the induction of IFN responses. PARP12 overexpression led to a decrease in GFP expression following infection with VSV pseudotyped with CCHFV glycoproteins or VSV-G. However, given that PARP12 overexpression inhibits VSV replication (Liu et al., 2012), these results were expected and cannot lead us to conclude nor exclude that PARP12 has a role on CCHFV entry. In our minigenome assay, no variation of the minigenome-derived nLuc signal was noted upon PARP12 overexpression, suggesting that PARP12 might not have a role on CCHFV replication/transcription nor on cellular translation. Finally, PARP12 did not impact the expression on plasmid-expressed Gc, Gn or NP.

In an experiment performed once, KO of human PARP12 in Huh7 cells was performed followed by infection with rHAZV-GFP or nLuc tc-VLPs, or transfection with NP, L protein and GPC and infection with GFP tc-VLPs and NP staining. We were unable to

detect PARP12 with one commercially available antibody and were thus unable to check the efficiencies of the KO. Results are presented in Figure 33.

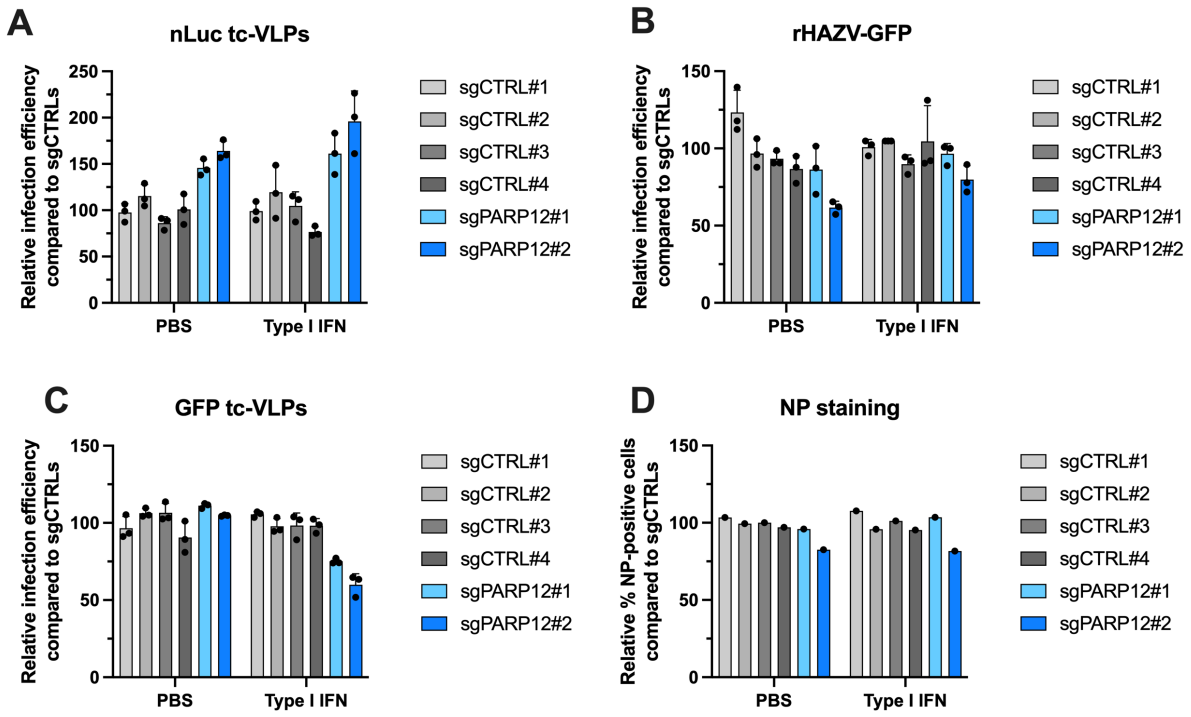

Figure 33. Endogenous PARP12 does not impact tc-VLPs infection, HAZV infection nor NP expression. Huh7 cells stably expressing Cas9 were transduced with single guide RNAs (sgRNAs) control (sgCTRL#1 to #4) or targeting PARP12 (sgPARP12 #1 and #2). They were treated with type I IFN (1000 U/mL) or PBS for 24 hours. **(A)** The cells were infected with nLuc tc-VLPs and luminescence was read 24 hpi. **(B)** The cells were infected with rHAZV-GFP (MOI = 0.3) for 16 hours and GFP expression was analyzed by flow cytometry. **(C)** The cells were transfected with NP, L protein and GPC expression plasmids prior infection with GFP tc-VLPs. 48 hpi, the cells were fixed and GFP expression was analyzed by flow cytometry. **(D)** The cells were transfected with NP, L protein and GPC expression plasmids. 24 hours post-transfection, cells were fixed, permeabilized and stained for NP prior flow cytometry analysis. (A), (B), (C), Data are expressed as relative infection efficiency or (D) relative percentage of NP-positive cells compared to the average of all sgCTRL RNAs for each condition (PBS or type I IFN) set at 100%. Means and SD of one experiment performed in technical triplicates are shown.

The results were contradictory, with PARP12 KO resulting in a rescue of nLuc tc-VLPs infection, but in a diminished infection with rHAZV-GFP or GFP tc-VLPs. Thus, this experiment must be repeated to draw conclusions on the effect of endogenous PARP12 in the context of CCHFV tc-VLPs or HAZV infection.

c. Future studies on PARP12 and CCHFV

Our results set the stage for more studies of the interactions between CCHFV and PARP12. Several questions must be answered and represent ongoing work in the laboratory.

Does PARP12 induce CCHFV non-structural proteins degradation? Are CCHFV proteins target of PARP12-mediated MARylation?

We showed that PARP12 has no effect on Gc, Gn or NP expression. Due to the difficulty to see L protein by western blot, PARP12 effect on this protein remains to be elucidated. Moreover, because PARP12 was reported to promote the degradation of non-structural proteins of ZIKV (Li et al., 2018, 2021), it would be interesting to check whether PARP12 overexpression or downregulation influence CCHFV non-structural proteins, namely GP38, GP160/85, NSm or NSs. Of note, GP38 and GP160/85 are secreted proteins and are not detected intracellularly by western blot (Freitas et al., 2020). Whether PARP12 modulates the secretion of these proteins remains unknown and should be investigated. To date, no antibodies exist against NSm or NSs, but tagged versions of NSs were successfully generated and could be used to detect a putative modulation by PARP12 (Barnwal et al., 2016). Alternatively, CCHFV structural and non-structural proteins might represent a target for PARP12-mediated MARylation, which could hinder their activities, as seen for nsP2 of CHIKV (Krieg et al., 2023). Thus, MARylation status of CCHFV proteins should be verified by western blot. Indeed, antibodies are commercially available to detect MAR by immunoblot and immunofluorescence (Vivelo and Leung, 2015; Krieg et al., 2023). Alternatively, purified viral proteins could be subjected to an *in vitro* ADP-ribosylation assay using ^{32}P-NAD^{+} and purified PARP12 (either its catalytic domain or the full-length protein), as performed for CHIKV nsP2 and PARP10 (Krieg et al., 2023). Because purification of some viral proteins can prove difficult, another strategy would be to use macrodomains or other ADP-ribose binding proteins as bait to pull-down and identify by mass spectrometry whether viral proteins are enriched. One caveat of this approach is that these molecules usually do not discriminate between ADP-ribosylated proteins from non-covalent binders.

What are the domains of PARP12 responsible for its antiviral activity against CCHFV?

As seen in the literature, PARP12 exerts its antiviral activity through different mechanisms, involving either its PARP domain or its RNA-binding domain. Catalytic

activity of PARP12 is also, but not always, involved in its antiviral activity. Thus, truncation (PARP12$_{\Delta ZnF}$, PARP12$_{\Delta WWE}$, PARP12$_{\Delta PARP}$) and catalytic mutant PARP12$_{G575A}$ (described in (Welsby et al., 2014; Li et al., 2018)) are in the process of being generated to elucidate which are important for PARP12 antiviral activity against CCHFV.

Does PARP12 rely on cofactors to exert its antiviral role against CCHFV?

PARPs with antiviral activities sometimes rely on cofactors to exert their antiviral activities. This is notably the case for PARP13, also known as zinc finger antiviral protein (ZAP) or zinc finger CCCH-type antiviral protein 1 (ZC3HAV1), which possesses antiviral activities against numerous viruses through binding to viral RNA via its zinc finger domain. More precisely, it binds directly to CpG dinucleotides-rich RNAs and destabilizes them (Gao et al., 2002; Takata et al., 2017; Meagher et al., 2019). Because ZAP does not have functional enzymatic activity, its antiviral function relies on cofactors such as TRIM25 or KHNYN (Li et al., 2017; Zheng et al., 2017; Ficarelli et al., 2019; Galão et al., 2022). Whether PARP12 also relies on putative cofactors for its antiviral activity against CCHFV should be investigated. Notably, considering the synergy observed between PARP11 and PARP12 for restricting ZIKV, experiments on the activity of PARP12 against CCHFV in PARP11- (or other PARPs)-KO cells should be performed. Alternatively, PARP12 interactome should be investigated through APEX or TurboID proximity labelling followed by mass spectrometry analysis, in the context of tc-VLPs infection or HAZV infection (Rhee et al., 2013; Cho et al., 2020).

Is the molecular mechanism of PARP12 antiviral activity conserved across species?

PARP12 is one of the core ISGs (Shaw et al., 2017), suggesting that it has conserved antiviral activity across species. Accordingly, macaque and bovine PARP12 were found as antiviral factors against CCHFV tc-VLPs. Experiments to investigate PARP12 mechanism of action should therefore be confirmed with macaque and bovine PARP12, as well as other PARP12 from other species, to decipher whether its activity and its molecular mechanism are conserved.

Does CCHFV encode a counteracting factor?

In a constant arm race, viruses evolve to counteract restriction activities from host proteins. PARP12 activity is notably restricted by macrodomains of nsp3 from coronaviruses or alphaviruses (Grunewald et al., 2019; Krieg et al., 2023; Kerr et al., 2023). Whether CCHFV also encodes an ADP-ribosylhydrolase is to date unknown.

Is PARP12 activity against CCHFV conserved in biologically relevant, primary cells, and/or *in vivo*?

Throughout the manuscript, the studies were performed in Huh7 cells, a hepatoma cell line. While this cell line is relevant to study CCHFV, due to its hepatic tropism, it is crucial to validate our findings within more biologically relevant models, such as primary human hepatocytes (PHH) or human umbilical vein endothelial cells (HUVEC), through overexpression or downregulation approaches. Of course, these approaches should be performed for all ISGs that were identified in our study, and not only for PARP12.

Moreover, *Parp12*$^{-/-}$ mice were recently generated by (Kerr et al., 2023). Mouse PARP12 and human PARP12 share 80.17% identity and 88.19% similarity. These *Parp12*$^{-/-}$ mice developed normally, except for the testes of male mice that were smaller than wild-type mice. Percentages of innate and adaptive immune cells were similar between *Parp12*$^{-/-}$ and wild-type mice within the spleen. However, male *Parp12*$^{-/-}$ mice were unable to produce litters, suggesting a defect in their reproductive system. If mouse PARP12 exhibits the same *in vitro* antiviral activity against CCHFV than human PARP12, these immunocompetent mice could be infected with the mouse-adapted variant of CCHFV (Hawman et al., 2021), to decipher whether PARP12 exerts a role in controlling CCHFV infection in *in vivo* models of infection. Of course, these experiments impose that they be performed within BSL-4 animal facilities.

2. GTPBP1

a. GTPBP1 biological roles

GTPBP1 was first identified in monocytes (THP-1) treated by IFN-γ as a protein bearing GTP-binding motifs (Senju and Nishimura, 1997). Its discovery was closely followed by the identification of GTPBP2, another IFN-γ-stimulated gene with 49% identity and 66% similarity (Kudo et al., 2000; Senju et al., 2000; Watanabe et al., 2000). Mouse and human GTPBP1 are highly conserved (97% identity). GTPBP1 induction by IFN-γ depends on the cell type, as it was not induced in HeLa nor Jurkat cells, contrary to GTPBP2. Both proteins have GTP-binding motifs sharing common characteristics with elongation factors (EF) families. Accordingly, GTPBP1 harbors a GTP-binding activity, a GTPase activity, as well as a translation activity, however at a much slower rate than eEF1H (Zinoviev et al., 2018). Considering the slow rate of this translation activity, GTPBP1 rather has a role in mRNA surveillance and ribosome-associated quality control mechanisms. In agreement, rat GTPBP1 targets specific

RNAs (such as the RNA of Aanat, an enzyme involved in melatonin synthesis in the pineal gland) to the RNA exosome complex for degradation. Rat GTPBP1 did not interact directly with the mRNA, but mainly via the RNA exosome (Woo et al., 2011). Thus, one hypothesis for rat GTPBP1 activity is that it retains aminoacyl-tRNA for a long time due to its slow translation activity; thus, it elicits quality control programs and directs the exosome toward mRNA targets. The slow translation activity of GTPBP1 would result in ribosome stalling if not for the large excess of eEF1A compared to GTPBP1. Interestingly, GTPBP2 does not interact with the exosome nor stimulates exosomal mRNA decay (Woo et al., 2011; Zinoviev et al., 2018). However, both *Gtpbp1* and *Gtpbp2* deficiency in C57BL/6J mice (which have a defect in expression of neuronal $tRNA^{Arg}_{UCU}$ gene, leading to accumulation of a precursor, translational-incompetent neuronal Tr20) led to neurodegeneration, linked to ribosome stalling at AGA codons (Ishimura et al., 2014; Terrey et al., 2020). Thus, GTPBP1 and GPTBP2 both have roles in resolving paused ribosomes, that are likely disjoint, as mice KO for *Gtpbp1* and *Gtpbp2* exhibited the same onset and progression of neurodegeneration as compared to single KO.

To our knowledge, no interactions between viruses and GTPBP1 have been described. Of note, GTPBP1-like factors are encoded by giant viruses, more specifically Mimiviruses (Zinoviev et al., 2019).

b. GTPBP1 and CCHFV

We were thus intrigued by the discovery of GTPBP1 proviral activity in our screen to unravel ISGs modulating CCHFV infection. GTPBP1 was proviral independently of the minigenome used (GFP or nLuc). Using pseudotyped VSV, we demonstrated that GTPBP1 does not promote GPC-driven viral entry. Moreover, it did not increase the expression of plasmid-expressed NP, Gn or Gc. Its effect on other proteins remains to be deciphered.

Importantly, using a minigenome assay, we demonstrated that GTPBP1 promoted viral replication and/or transcription and/or translation. Given that GTPBP1 has an elongation activity, though weak, one straightforward hypothesis would be an increase of translation associated with GTPBP1 overexpression. However, GTPBP1 had no effect on the translation of a control mRNA (fLuc) and did not increase the translation of non-polyadenylated RNAs. GTPBP1 overexpression slightly increased the translation of viral mRNA, but this effect was surprisingly abrogated by ribavirin,

suggesting that GTPBP1 effect on viral mRNA was probably due to a limitation of our system to mimic viral mRNAs and that these mRNAs might still be available for replication, an hypothesis that remains to be confirmed. Overall, our data suggested that GTPBP1 proviral activity was dependent on replication and/or transcription of RNAs flanked with CCHFV UTRs.

We then tried to verify whether endogenous GTPBP1 had an impact on CCHFV replication. In our cells, GTPBP1 was not induced by type I IFN treatment. However, we did not test the ability of other types of IFN to induce GTPBP1 expression in Huh7 cells. Nonetheless, cell-specific differences in the IFN inducibility of GTPBP1 were not unexpected, given that it is induced by type II IFN only in THP-1, but not in HeLa nor Jurkat cells (Kudo et al., 2000). Downregulation of GTPBP1 had no effect on tc-VLPs infection, nor HAZV infection, suggesting that endogenous GTPBP1 had either no role or a minor role in promoting CCHFV replication. Alternatively, it could mean that its absence is compensated by another protein with a similar role. An obvious candidate being GTPBP2, downregulating both GTPBP1 and GTPBP2 in the same cells would be a way to explore a potential compensation of one protein by the other. However, in our screen, GTPBP2 overexpression led to a relative infection efficiency of 72.8%, ruling out its putative proviral effect, and further corroborating that GTPBP1 and GPTBP2 have roles that are likely disjoint.

Importantly, overexpression of GTPBP1 had no impact on HAZV replication. Thus, we first hypothesized that GTPBP1 effect was specific to CCHFV viral UTRs. However, we were astonished to observe that GTPBP1 overexpression had an antiviral effect on authentic CCHFV. Of course, the system of tc-VLPs is an incomplete model to study CCHFV. It notably lacks NSs, which is expressed upon infection with authentic CCHFV, but not in the tc-VLPs system. To decipher whether NSs could inhibit the proviral effect of GTPBP1, we tried in a preliminary experiment to express it in the minigenome assay (Figure 34).

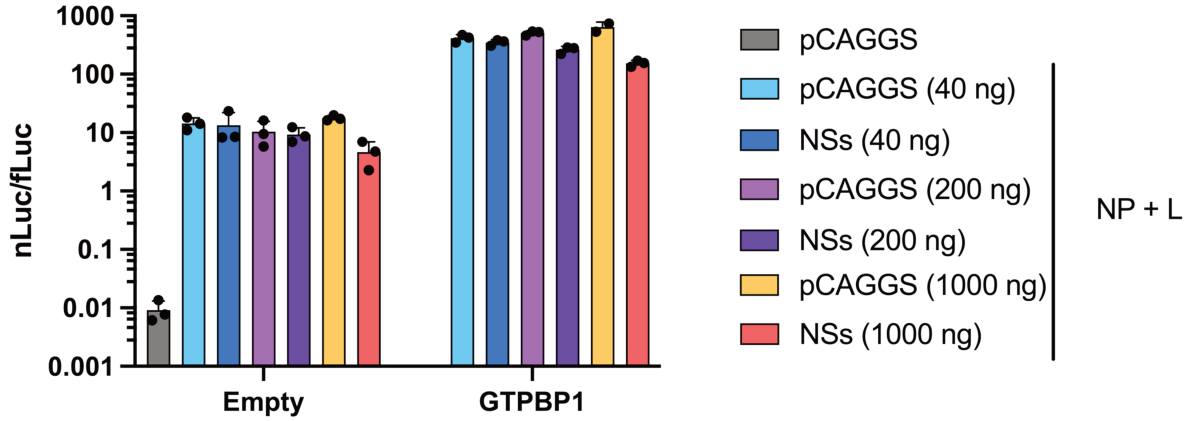

Figure 34. NSs does not impact GTPBP1 proviral effect on minigenome replication/transcription. Huh7 cells were transfected with pCAGGS, or with NP and L protein. Moreover, cells were transfected with increasing amounts of NSs or pCAGGS control plasmid. Then, cells were transduced with ISG lentivectors (MOI = 3) and transfected with *in vitro* transcribed genomic viral RNA encoding nLuc or control mRNA encoding fLuc. 24 hours post-transfection, cells were lysed, and luminescence was read. Means and SD of one experiment performed in technical triplicates are shown.

The physiological quantities of NSs in infected cells are unknown. We then decided to test different quantities of NSs and observe its effect on CCHFV replication. High amounts of NSs led to a decrease of replication in both Empty- or GTPBP1-transduced cells. It also led to a decrease of fLuc signal, suggesting a general effect of high amounts of NSs, probably due to its apoptotic role (Barnwal et al., 2016). Lower amounts of NSs had no impact on viral replication, independently from the expression of GTPBP1. Thus, this suggests that the absence of NSs alone does not explain the discrepancies observed between the tc-VLPs and CCHFV regarding GTPBP1 pro- or antiviral effect.

c. Future studies on GTPBP1 and CCHFV

Overall, our data raise a lot of questions concerning the interplays between CCHFV and GTPBP1, how cellular factors can influence viral replication or transcription, and, more generally, about entanglement between translation, replication, and transcription for bunyaviruses. Some experiments, that represent ongoing work in the laboratory, might help shedding more light on these interactions, described below.

Does GTPBP1 have a role on transcription or replication?

Our data suggest that GTPBP1 has a role on transcription and/or replication of a minigenome flanked with CCHFV UTRs. To decipher whether it rather promotes

transcription or replication, an interesting experiment would be to perform the assay of replication in cells that express NP, GPC, and L protein, in comparison with cells that express NP, GPC and a mutant of the L protein that lacks the endonuclease domain responsible for the cap-snatching activity, L-D693A (Devignot et al., 2015), or with cells that express NP, GPC and a mutant of the L protein that lacks replication/transcription activity, L-ΔDD (Bergeron et al., 2010). The cells expressing NP, GPC and L-WT would thus: (i) display reporter activity, as L-WT would transcribe vRNA into mRNA and (ii) produce infectious tc-VLPs, that will be used to infect cells expressing NP and L-WT and lead to reporter signal in these cells. The cells expressing NP, GPC and L-D693A would: (i) not display any reporter activity, as L-D693A would not transcribe vRNA into mRNA; and (ii) still produce infectious tc-VLPs, that will be used to infect cells expressing NP and L-WT and lead to reporter signal in these cells. Finally, the cells expressing NP, GPC and L-ΔDD would: (i) not display any reporter activity and (ii) not produce any infectious tc-VLPs. Thus, this system allows the study of replication independently of transcription and was already described for other bunyaviruses (Klemm et al., 2013). It can therefore be used to decipher GTPBP1 effect on replication. Alternatively, studying the relative levels of different RNA species by strand-specific RT-qPCR could prove informative regarding the role of GTPBP1 in promoting either replication and/or transcription. Of note, such a system was implemented for Bunyamwera virus to gain more insight into ISG20-mediated restriction of replication (Feng et al., 2018).

Does GTPBP1 have a role on translation?

Though we have demonstrated that GTPBP1 proviral activity is dependent on CCHFV RNA replication and/or transcription, we have not formally shown that it has no effect on translation. The fact that for bunyaviruses, transcription is coupled to translation (Barr, 2007), reinforces the idea that an effect of GTPBP1 on translation could have an impact on transcription and/or replication. To decipher if GTPBP1 has a role on replication, transcription or translation, cells treated with translation inhibitors will be transfected with the replication machinery in presence or absence of GTPBP1, followed by strand-specific RT-qPCRs to detect the amounts of viral RNA (genomic, antigenomic or mRNA). Moreover, puromycilation experiments will help uncovering potential differences in translation activities between control-transduced cells and GTPBP1-overexpressing cells.

Does GTPBP1 interact with viral components?

Our data suggest that GTPBP1 expression has no impact on Gn, Gc nor NP expression. However, it remains to be studied whether it has an impact on L protein or non-structural proteins that were unstudied.

Furthermore, studying viral components localization upon GTPBP1 overexpression by confocal microscopy would help deciphering whether GTPBP1 promotes replication and/or transcription by relocalizing the replication machinery to specific places. Additionally, confocal (super resolution) microscopy combined with RNA fluorescence *in situ* hybridization (FISH) could give more insight into potential colocalization between GTPBP1 and viral components. Pull-down of GTPBP1 followed by western blot analyses or RT-qPCR would also give more insight into GTPBP1 putative interactions with viral components. Should we identify a specific interaction between GTPBP1 and viral UTRs, we could perform individual nucleotide-resolution cross-linking and immunoprecipitation (iCLIP), as was performed for OAS1 to highlight its interaction with specific stem loops from the 5'UTR of SARS-CoV-2, mediating its antiviral activity (Wickenhagen et al., 2021). Alternatively, if interactions between GTPBP1 and viral proteins are observed, directed mutagenesis of specific residues essential to this binding might give more insight into the role of GTPBP1 in tc-VLPs or CCHFV infection.

Does GTPBP1 need a cofactor?

GTPBP1 has been reported to interact with the exosome complex, or with ribosomes (Terrey et al., 2020; Woo et al., 2011; Zinoviev et al., 2018). To gain more insight into the interactome of GTPBP1, it would be interesting to perform immunoprecipitation of GTPBP1 in conditions of infection with tc-VLPs or not, followed by mass spectrometry analyses. Deciphering the putative remodeling of the ribosome interactome in the context of infection with tc-VLPs and in presence or absence of GTPBP1 would also prove informative (Simsek et al., 2017).

Why does GTPBP1 promote tc-VLPs replication but inhibit CCHFV infection?

The discrepancies observed between tc-VLPs and CCHFV are a crucial point to disentangle. Two main hypotheses could explain these observations:

(i) The length and the structure of the RNA segments

In our minigenome assay and tc-VLPs experiments, viral RNA is relatively short. Indeed, counting the viral UTRs, the GFP and nLuc minigenomes measure around 1 kb and 0.8 kb, respectively. Even if these sizes are close to the one of the S segment (1.6 kb), they are far from the sizes of the M segment (5.4 kb) and L segment (12.1 kb).

In addition, internal RNA structures might differ between minigenomes and viral segments, governing sensitivity to GTPBP1 activity.
Thus, to decipher if this is the case, the minigenome assay should be performed on viral segments (S segment, M segment or L segment). The M segment is unavailable in the laboratory for biosafety reasons, but not the S or L segment. However, transcription and translation of these viral segments give rise to NP and L protein, that are indistinguishable from the plasmid-derived NP and L protein necessary for the minigenome assay. Thus, we propose to perform the experiment on segments that have been engineered to also express a small reporter molecule, such as HiBiT for instance, to monitor translation.

(ii) The UTRs

The UTRs present in our tc-VLPs and minigenome assays correspond to the UTRs of the L segment. Replacing these UTRs with the ones of the M and S segments should give more insight into a putative segment-specific role of GTPBP1.

II. Methods to unravel ISGs modulating infections by highly pathogenic, zoonotic, tick-borne viruses

1. Gain-of-function or loss-of-function screen?

To identify ISGs modulating CCHFV infection, we relied on a well-described medium-throughput screening platform of ISGs (Schoggins et al., 2011; Kane et al., 2016). This method was successfully used to gain more insight into the interplays of many viruses with several ISGs throughout the years. Indeed, it allows the functional screening in an arrayed manner of many ISGs and is a powerful way to identify ISGs that are sufficient to modulate viral replication on their own.

However, gain-of-function screens also presents limitations, as stated in the introduction. One caveat of this approach is the potential irrelevance of the effect of some genes resulting from overexpression. This is why genes identified through a gain-of-function approach must be validated by KO experiments, to verify their role at an endogenous level. Moreover, for ISGs, their role must be validated in the context of the IFN response. For instance, IFIH1 was our top candidate identified in our screen. However, KO of IFIH1 does not rescue virus infection (our data with tc-VLPs (not shown) and (Spengler et al., 2015) with authentic CCHFV). Moreover,

overexpression of IFIH1 strongly increased the establishment of IFN responses, as shown by our ISRE induction assay. Thus, the antiviral activity of IFIH1 in the context of this study is probably only due to an induction of IFN responses through overexpression, and not to an actual sensing of CCHFV.

Another important caveat of these gain-of-function approaches is that they might not identify antiviral genes that need cofactors for their pro- or antiviral activities. Combinatorial approaches counteract this limitation (Schoggins et al., 2011; Dang et al., 2018). Moreover, this limitation highlights the dire need to perform the screen in relevant cell lines. For instance, ZAP antiviral activity is highly dependent on TRIM25, and in some cases, ZAP antiviral activity relies on a TRIM25 from the same or related species due to a functional co-evolution of the two genes (Gonçalves-Carneiro et al., 2021). In this work, we have screened ISGs in a human hepatoma cell line, target for CCHFV infection. While these cells are relevant to screen the human library of ISGs, this is not necessarily the case for macaque and bovine libraries, and we cannot exclude that some pro- or antiviral macaque or bovine factors that inhibit CCHFV tc-VLPs were missed due to this limitation.

In the literature, gain-of-function screens of ISGs have revealed numerous ISGs with antiviral activities, but few with proviral activities. One trivial explanation is that they are few proviral ISGs, but another explanation could be that overexpressing a non-limiting proviral factor will not enhance viral replication. To identify proviral ISGs, loss-of-function screens are more appropriate (Lesage et al., 2022).

Recently, loss-of-function screens targeted against HIV-1 or VEEV followed by combinatorial KO of the top ISG candidates have revealed that only a set of few ISGs is sufficient to restrict the viruses (Itell et al., 2023; McDougal et al., 2023). While this raises the question of the necessity to screen bigger ISGs libraries in gain-of-function approaches, it is important to underline the fact that gain-of-function screens are particularly efficient to highlight the antiviral activities of some ISGs that, in downregulation experiments, have only a weak impact on viral replication. This is the case for many ISGs, such as OAS1. Indeed, while OAS1 overexpression led to a very potent inhibition of SARS-CoV-2 replication, its KO did not lead to a rescue of the virus (Wickenhagen et al., 2021).

Overall, both gain-of-function and loss-of-function screens are useful to fully unravel the landscape of restriction of viruses. Indeed, the respective limitations of both

methods explain why there is often little overlap between gain-of-function and loss-of-function screens top hits.

2. Relevance of BSL-2 models for BSL-4 viruses to identify ISGs modulating their infection

Targeted or whole-genome screens are highly valuable in uncovering the intricate network of interactions between hosts and viruses. However, applying such screens to study BSL-4 viruses comes with hurdles, due to the technical constraints relative to BSL-4 laboratories and the potential lack of some equipment. Two whole-genome loss-of-function screens were performed in the context of Ebola virus (EBOV) in BSL-4 laboratories (Filone et al., 2015; Flint et al., 2019). These two latter screens had a viability-based read-out. Such screens could be conducted in the context of CCHFV infection, as infection of Huh7 cells with CCHFV leads to cytopathic effects (Rodrigues et al., 2012).

While these loss-of-function screens revealed important host factors for EBOV replication, none of them revealed antiviral factors able to restrict its infection. Due to the obvious challenges to studying viruses in BSL-4 settings, studies focusing on ISGs modulating BSL-4 viruses, such as CCHFV, EBOV or Lassa virus (LASV), were initially limited to specific ISGs that were previously found to inhibit other viruses, such as MxA for CCHFV (Andersson et al., 2004a, 2006), ZAP for EBOV (Müller et al., 2007) or 25-hydroxycholesterol (25-HC) for LASV (Shrivastava-Ranjan et al., 2016).

To address this data gap, gain-of-function screens such as the one that we have performed in this manuscript offer valuable insights into the landscape of host restriction factors against highly pathogenic viruses. Indeed, two gain-of-function screens using ISG libraries were performed with BSL-2 models of EBOV. One of them relied on the use of BSL-2-contained EBOV whose VP30 gene, an essential transcription factor, had been replaced by a Renilla luciferase reporter molecule (Kuroda et al., 2020). These reporter viruses are infectious only in VP30-expressing cells, but otherwise have similar growth kinetics and morphology as full EBOV (Halfmann et al., 2008). Using this virus, the authors found several genes inhibiting different step of EBOV lifecycle. Among others, they identified CCDC92, which inhibits viral mRNA transcription and NP incorporation into VLPs. Another gain-of-function screen was performed using EBOV transcription- and replication-competent virus-like particles (trVLP), a system very similar to CCHFV-specific tc-VLPs (Galão

et al., 2022). This assay relied on the transfection into producer cells of an EBOV minigenome containing the matrix protein VP40, the glycoprotein GP, the nucleocapsid component VP24 as well as a Renilla luciferase reporter gene. Other proteins necessary for production of trVLPs (namely the nucleoprotein NP, the polymerase L, and the transcription factors VP30 and VP35) were added *in trans*. The trVLPs produced were used to infect target cells that overexpress the viral proteins added *in trans*. Using this system, the authors successfully identified TRIM25 as an EBOV restriction factor, in synergy with ZAP. Indeed, TRIM25 promoted the ubiquitination of incoming NP and its dissociation from the trVLP genome. Thus, these systems have shed more light on the landscape of restriction factor against a highly pathogenic virus, EBOV, for which little was hitherto known. Of course, these results have yet to be validated with authentic EBOV, as stated by the authors of the two studies. Using the BSL-2 CCHFV tc-VLPs system, we also have uncovered several ISGs restricting CCHFV tc-VLPs infection. This screen and initial characterization of these ISGs performed in BSL-2 settings allowed us to perform BSL-4 validations of targeted, selected ISGs, largely alleviating the amount of work that would have represented a gain-of-function screen performed with the complete libraries on authentic CCHFV. Moreover, tc-VLPs are a minimal model of CCHFV and might lack putative counteractive factors present in authentic CCHFV, enabling the identification of ISGs that inhibit the virus, but which are counteracted by these counteractive factors. For instance, using tc-VLPs and VSV pseudotypes, we identified LAMP3 as an inhibitor of CCHFV entry; however, LAMP3 had no effect on HAZV nor CCHFV infection, maybe due to a potential counteractive factor.

Of course, the system of CCHFV VRP described in the introduction and based on the production of virus-like particles encapsidating the two segments L and S, with transcomplementation of GPC, represents a more reliable tool to study CCHFV (Scholte et al., 2019b) (Figure 16). In this system, L and S are expressed from genomic segments, and not from plasmids as in the tc-VLPs system. Thus, quantities of viral proteins expressed are more representative of what happens during an infection with authentic CCHFV. However, we tried unsuccessfully to reproduce this system in the laboratory, and it has yet to be optimized. Inter-laboratories differences in cell types could explain this discrepancy. Thus, we chose to perform the screen with the tc-VLPs, because we previously showed that we could identify genes modulating their replication in the optimization steps.

3. Zoonotic and arthropod-borne viruses: towards screening the non-human hosts and vectors

CCHFV is a zoonotic virus that infects many mammals, including cattle. Thus, gaining more insight into CCHFV and its non-human hosts is crucial. Our screen aimed at shedding the light into the cattle host factors/CCHFV interplays, by screening a library of bovine ISGs with CCHFV tc-VLPs. We could notably identify bovine HAS3 as a very potent inhibitor or CCHFV tc-VLPs infection in human cells. Its antiviral activity could be confirmed against HAZV as well as, to a lesser extent, authentic CCHFV. Bovine HAS3 was also recently identified in a screen directed against blue tongue virus (BTV) performed in HEK293T cells (Hardy et al., 2023). Nonetheless, the role of endogenous bovine HAS3 in restricting BTV or CCHFV has yet to be demonstrated through downregulation approaches in relevant bovine cell lines, such as bovine endothelial cells (BovEC). Moreover, the role of human HAS3 (which was absent from our libraries due to its non-inducibility by IFNs) in CCHFV infection must also be studied.

Very interestingly, in this publication (Hardy et al., 2023), the authors conducted a comparative analysis of growth kinetics and interferon responses between species infected with BTV, focusing on those that exhibit symptoms (ovine) and those that are disease-tolerant (bovine). Thus, they aimed at identifying the tipping point between asymptomatic and symptomatic progression of the disease. They found that BTV was equally restricted by the identified ISGs (IFIT1, viperin and HAS3) in bovine and ovine cells, suggesting that the ISG landscape alone is not responsible for the symptomatic or asymptomatic outcome of the disease. Rather, they identified that the growth kinetics of the virus within these cells determined the implementation of innate immune responses. Indeed, BTV grew faster in ovine cells, leading to a premature translational shut-off in these cells compared to bovine ones and thus a diminished expression of specific ISGs. Alternatively, the virus is more efficiently detected in bovine cells, leading to a quicker establishment of the IFN response. Thus, for BTV, the timing of type I IFN response is essential in controlling viral disease. It would be interesting to perform similar experiments with CCHFV, by comparing growth kinetics in human endothelial cells such as human umbilical vein endothelial cells (HUVEC) and BovEC.

In parallel to these gain-of-function studies, loss-of-function screens performed in relevant animal cell lines would give valuable insights into host-virus interactions. For

instance, libraries of sgRNAs have been described and used for whole-genome CRISPR/Cas9 screen for green monkeys against SARS-CoV-2 (Wei et al., 2021; Rebendenne et al., 2022), swine using PigGecKO, PK15-GeCKO, or SsCRIPSRko.v1 libraries against influenza virus, pseudorabies, Japanese encephalitis virus (JEV) or porcine reproductive and respiratory syndrome virus (PRRSV) (Zhao et al., 2020; Hölper et al., 2021; Zhou et al., 2021; Jiang et al., 2022), or bovine using btCRIPSRko.v1 against bovine herpes virus type I (BHV-1) (Tan et al., 2020). It is anticipated that an expanding number of libraries, designed to encompass a broader spectrum of species, will significantly contribute to elucidating the intricate web of interactions between viruses and their animal hosts. Such screens could be performed in the context of CCHFV infection.

Finally, it is also of the utmost importance to gain more insight into CCHFV interplays with its tick vector. Recent advances in tick genome annotation and genome editing of ticks will likely lead to new discoveries regarding CCHFV/tick interactions. Indeed, the first report of successful gene editing in ticks from the *Amblyomma americanum* species using RNAi marked a pivotal milestone in the advancement of tick research (Aljamali et al., 2002). This method has since then been often used to discover genes that impact tick function and tick/virus or tick/bacteria interactions (reviewed in (de la Fuente and Kocan, 2022)). Another breakthrough was achieved when a group reported the Cas9-mediated gene editing in *Ixodes scapularis* ticks, by embryo injection or by Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) (Sharma et al., 2022). The latter approach facilitates the delivery of the Cas9 RNP complex to the developing germline via the hemocoel, allowing targeted and heritable mutations to be generated by adult tick injection which is a more practical alternative to the challenging process of embryo tick injection. Moreover, *I. scapularis* genome editing was successfully achieved in tick cells through nucleofection of the Cas9 (Singh et al., 2023). Thus, these methods could be applied to tick cells or ticks themselves to gain more insight into CCHFV interplays with its vector.

General conclusion

Throughout this manuscript, we have gained more insight into the interplays between CCHFV and innate immunity. More specifically, using a gain-of-function screen targeting human, macaque, and bovine ISGs, as well as tc-VLPs as a model for CCHFV infection, we have uncovered several ISGs that modulate CCHFV infection, either negatively or positively. Among them, we identified IFITM1, LAMP3, OAS2, PARP12, LY6E, FAM26F, GTPBP1 and bovine HAS3.

We have successfully pinpointed specific steps that some of these ISGs target, such as LY6E and LAMP3 affecting viral entry, or GTPBP1 impacting transcription and/or replication. Further studies are needed to gain a comprehensive understanding of the underlying molecular mechanisms driving their antiviral or proviral activities. For other top candidate ISGs identified in this study, we have yet to discover which specific stage of the CCHFV lifecycle they modulate and elucidate their corresponding molecular mechanisms of action.

Tc-VLPs serve as a valuable and useful model to study CCHFV. As we progressed to validate our findings observed with CCHFV tc-VLPs using authentic CCHFV, we could confirm that PARP12 acts as a potent antiviral factor against CCHFV. However, the specific stage of the viral lifecycle at which PARP12 exerts its effects remains unidentified, and ongoing studies aim to elucidate the molecular mechanisms underlying its antiviral activity. Surprisingly, we were unable to confirm the proviral role of GTPBP1 in the context of CCHFV infection. Indeed, the overexpression of GTPBP1 resulted in a drastic reduction in CCHFV titers and viral RNA copies, both intracellularly and extracellularly. These results are intriguing, and further investigations are underway to uncover the mechanisms by which GTPBP1 exhibits antiviral activity against CCHFV, despite its potential to increase NP and L protein-mediated transcription and/or replication of viral reporter RNAs.

These results await validation through downregulation approaches, to highlight the contribution of the endogenous top candidates in the restriction of CCHFV induced by IFN. Furthermore, it is important to investigate the roles of these various candidates within primary and biologically relevant target cells during CCHFV infection.

More generally, our studies pave the way for a better understanding of the intricate interactions between CCHFV and the host innate immune system. The roles of many other candidate genes in the context of CCHFV infection remain to be explored. Gaining deeper insight into the interplay between zoonotic, emerging arboviruses and innate immunity is of the utmost importance, given the substantial threat that they pose to public health.

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Annex

viruses

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Review

Host Cell Restriction Factors of Bunyaviruses and Viral Countermeasures

Solène Lerolle [1], Natalia Freitas [1], François-Loïc Cosset [1,*] and Vincent Legros [1,2,†]

[1] CIRI—Centre International de Recherche en Infectiologie, Université de Lyon, Université Claude Bernard Lyon 1, Inserm, U1111, CNRS, UMR5308, ENS Lyon, 46 allée d'Italie, 69007 Lyon, France; solene.lerolle@ens-lyon.fr (S.L.); natalia.bezerra-de-freitas@ens-lyon.fr (N.F.); vincent.legros@ens-lyon.fr (V.L.)
[2] Université de Lyon, VetAgro Sup, 69280 Marcy-l'Étoile, France
* Correspondence: flcosset@ens-lyon.fr
† Co-senior author.

Abstract: The *Bunyavirales* order comprises more than 500 viruses (generally defined as bunyaviruses) classified into 12 families. Some of these are highly pathogenic viruses infecting different hosts, including humans, mammals, reptiles, arthropods, birds, and/or plants. Host cell sensing of infection activates the innate immune system that aims at inhibiting viral replication and propagation. Upon recognition of pathogen-associated molecular patterns (PAMPs) by cellular pattern recognition receptors (PRRs), numerous signaling cascades are activated, leading to the production of interferons (IFNs). IFNs act in an autocrine and paracrine manner to establish an antiviral state by inducing the expression of hundreds of IFN-stimulated genes (ISGs). Some of these ISGs are known to restrict bunyavirus infection. Along with other constitutively expressed host cellular factors with antiviral activity, these proteins (hereafter referred to as "restriction factors") target different steps of the viral cycle, including viral entry, genome transcription and replication, and virion egress. In reaction to this, bunyaviruses have developed strategies to circumvent this antiviral response, by avoiding cellular recognition of PAMPs, inhibiting IFN production or interfering with the IFN-mediated response. Herein, we review the current knowledge on host cellular factors that were shown to restrict infections by bunyaviruses. Moreover, we focus on the strategies developed by bunyaviruses in order to escape the antiviral state developed by the infected cells.

Keywords: bunyaviruses; restriction factors; innate immunity; interferon; viral countermeasures

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1. Introduction

Viral infections are often detrimental to the host, disrupting their homeostasis and jeopardizing their survival. In reaction to this, hosts have developed several strategies to recognize viral pathogens and restrict their replication. Restriction factors represent crucial factors of the host intrinsic and innate immunity involved in antagonizing viral invasion and replication. This set of proteins, whether constitutively expressed or induced by the interferons (IFNs), are key factors of the innate immune response and utilize diverse mechanisms to inhibit specific steps of the viral life cycle: entry, replication, transcription, translation and/or egress. However, viruses enter into an arms race to escape host immunity by directing some of their proteins to counteract these restriction factors in a more or less specific manner.

The *Bunyavirales* order comprises a large number of emerging, medically and/or economically important viruses known as bunyaviruses. The molecular biology of bunyaviruses is an emerging field of active research. In recent years, numerous studies have focused on proteins restricting the bunyavirus life cycle and on their viral countermeasures. In this review, we aim at discussing the restriction factors which have been shown to inhibit

bunyaviruses replication at distinct stages of their life cycles, including viral entry, replication and egress. Additionally, the strategies developed by these viruses to circumvent and limit the impact of these restriction factors are underlined.

2. Medical Importance of Viruses in the *Bunyavirales* Order

The *Bunyavirales* order was established in 2017 and contains more than 500 viruses classified into 12 families. Previously, viruses from the *Bunyavirales* order were classified into two families: the *Bunyaviridae* and the *Arenaviridae* families. However, structural and functional similarities between members of the two families led to the establishment of the new *Bunyavirales* order, containing viruses with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes [1]. Notably, five families within the *Bunyavirales* order (*Arenaviridae*, *Hantaviridae*, *Nairoviridae*, *Peribunyaviridae* and *Phenuiviridae*, on which we focus in this review, as listed in Table 1) contain viruses of medical or economic importance, as they are able to cause diseases in mammalian hosts (including humans and livestock) as well as in plants.

Members of the *Arenaviridae* family (arenaviruses), are associated with rodent-transmitted disease in humans. They are commonly divided into Old-World and New-World arenaviruses. Notably, Lassa virus (LASV), Junín virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV) or Sabiá virus (SBAV) are responsible for viral hemorrhagic fevers in humans. With a prevalence of 300,000 cases per year, LASV is the most prevalent and threatening [2]. The prototypic Old-World arenavirus lymphocytic choriomeningitis virus (LCMV) is responsible for a mild febrile illness that can develop to a neurological disease. Like arenaviruses, members of the *Hantaviridae* family (hantaviruses) are also transmitted by rodents to humans. They are responsible for two types of diseases in humans: the hemorrhagic fever with renal syndrome (HFRS), and the hantavirus cardio-pulmonary syndrome (HCPS). Notably, Hantaan virus (HTNV) and Seoul virus (SEOV) are of significant medical importance, with mortality rates reaching up to 15% [3]. The *Nairoviridae* family contains tick-transmitted viruses (nairoviruses) of both medical and economic importance. Some are human pathogens, with the most important to date being the Crimean-Congo hemorrhagic fever virus (CCHFV), which is responsible for a viral hemorrhagic fever with a mortality rate reaching 30%. Others are pathogenic to livestock, and are therefore at the origin of important economic losses, such as Nairobi sheep disease virus (NSDV) [4]. In the *Peribunyaviridae* family are arboviruses (peribunyaviruses) which are also responsible for diseases in humans and in livestock. In humans, Oropouche virus (OROV) or La Crosse virus (LACV) are responsible for fever-like symptoms that can lead to an encephalitis. In livestock, Schmallenberg virus (SBV) is notably responsible for abortions and newborn malformations, leading to important economic losses in endemic regions. Bunyamwera virus (BUNV) is considered the prototype virus of this family [5]. Finally, members of the *Phenuiviridae* family are arboviruses (phenuiviruses) such as the Rift valley fever virus (RVFV) or the severe fever with thrombocytopenia syndrome virus (SFTSV). RVFV is a mosquito-borne virus responsible for abortions and important economic losses in livestock, as well as for febrile illness in humans. Hemorrhagic fevers were additionally described in 1% of the RVFV infection cases in humans [6].

This non-exhaustive list of diseases caused by bunyaviruses reveals the economic and medical importance of these viruses. The absence of etiologic treatments or prophylactic strategies against some, such as CCHFV and LASV, makes the research on these viruses a priority. Thus, it is of significant importance to increase the research on bunyaviruses to understand their pathogenicity and to pave the way to new treatments and vaccines. To this end, three diseases caused by bunyaviruses, namely Crimean-Congo hemorrhagic fever, Lassa fever and Rift valley fever, are listed among the nine medically and economically important diseases registered in the R&D Blueprint program of the World Health Organization (WHO), which aims at prioritizing the research in public health emergency contexts [7].

Table 1. Taxonomical classification of bunyaviruses discussed in this review.

Family	Genus	Species	Virus (Abbreviation)
Arenaviridae	*Mammarenavirus*	*Argentinian mammarenavirus*	Junín virus (JUNV)
		Brazilian mammarenavirus	Sabiá virus (SBAV)
		Guanarito mammarenavirus	Guanarito virus (GTOV)
		Lassa mammarenavirus	Lassa virus (LASV)
		Lymphocytic choriomeningitis mammarenavirus	Lymphocytic choriomeningitis virus (LCMV)
		Machupo mammarenavirus	Machupo virus (MACV)
Hantaviridae	*Orthohantavirus*	*Andes orthohantavirus*	Andes virus (ANDV)
		Hantaan orthohantavirus	Hantaan virus (HTNV)
		Prospect Hill orthohantavirus	Prospect Hill virus (PHV)
		Puumala orthohantavirus	Puumala orthohantavirus (PUUV)
		Seoul orthohantavirus	Seoul virus (SEOV)
		Sin Nombre orthohantavirus	New York virus (NYV)
			Sin Nombre virus (SNV)
		Tula orthohantavirus	Tula virus (TULV)
Nairoviridae	*Orthonairovirus*	*Crimean-Congo hemorrhagic fever orthonairovirus*	Crimean-Congo hemorrhagic fever virus (CCHFV)
		Dugbe orthonairovirus	Dugbe virus (DUGV)
		Nairobi sheep disease orthonairovirus	Nairobi sheep disease virus (NSDV)
		Thiafora orthonairovirus	Erve virus (ERVEV)
Peribunyaviridae	*Orthobunyavirus*	*Akabane orthobunyavirus*	Akabane virus (AKAV)
		Bunyamwera orthobunyavirus	Bunyamwera virus (BUNV)
		Cache Valley orthobunyavirus	Cache Valley virus (CVV)
		La Crosse orthobunyavirus	La Crosse virus (LACV)
		Oropouche orthobynyavirus	Oropouche virus (OROV)
		Schmallenberg orthobunyavirus	Sathuperi virus (SATV)
			Schmallenberg virus (SBV)
Phenuiviridae	*Banyangvirus*	*Guertu banyangvirus*	Guertu virus (GTV)
		Heartland banyangvirus	Heartland virus (HRTV)
		Huaiyangshan banyangvirus	Severe fever with thrombocytopenia syndrome virus (SFTSV)
	Phlebovirus	*Punta Toro phlebovirus*	Punta Toro virus (PTV)
		Rift Valley fever phlebovirus	Rift Valley fever virus (RVFV)
		Sandfly fever Naples phlebovirus	Sandfly fever Naples virus (SFNV)
			Toscana virus (TOSV)
		Uukuniemi phlebovirus	Uukuniemi virus (UUKV)

3. Morphology of Bunyavirus Particles and Genome Organization

Typical bunyavirus particles have a lipid envelope containing two viral glycoproteins, termed Gn and Gc. The viral negative-sense RNA genome is tri-segmented, and the three segments are termed according to their size: small (S), medium (M) and large (L) segment [8–10] (Figure 1). Unusually, arenaviruses have a bi-segmented RNA genome with only the S and L segments [11].

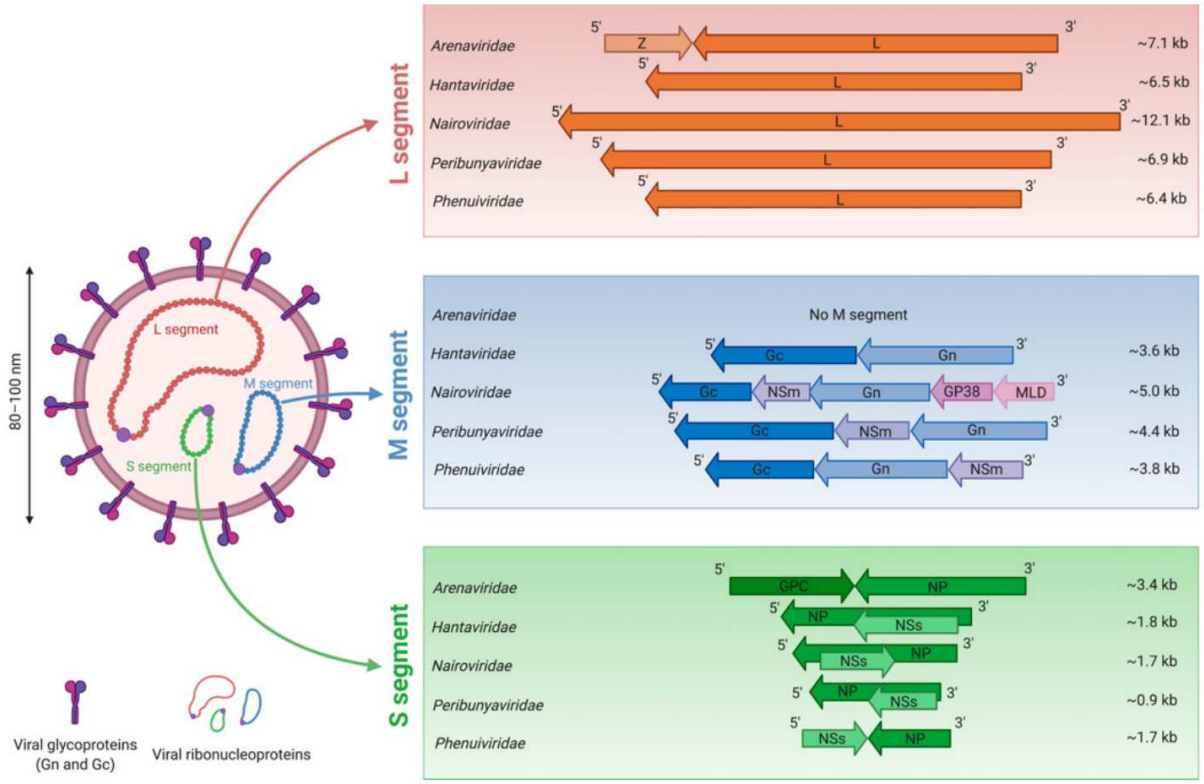

Figure 1. Schematic representation of viral genomes from the *Bunyavirales* order. A typical bunyaviral particle is represented on the left. The particularities of the genome from each family are represented on the right. The nucleotide lengths are displayed and based on full-length sequences available in GenBank. The viral M segment encodes a glycoprotein precursor (GPC) (not represented in the figure), which is further matured into different proteins (Gn, Gc, and sometimes NSm, MLD, GP38) that are depicted here on the viral genome. GPC: glycoproteins precursor; NP: nucleoprotein; NSm: non-structural protein M; NSs: non-structural protein S; MLD: mucin-like domain; Z: zinc-binding matrix protein.

The S segment encodes the nucleoprotein (NP) involved in the formation of viral ribonucleoparticles (RNPs) [12]. Moreover, the S segment of some peribunyaviruses and hantaviruses also has an overlapping functional open reading frame (ORF) which encodes a non-structural protein termed NSs as the result of alternate AUG-initiation codon selection [8,13]. Some phenuiviruses and nairoviruses' S segments have the particularity to encode a protein called NSs in the opposite orientation relative to the NP gene, in an ambisense strategy [14,15]. The ORF of CCHFV NSs overlaps the NP ORF which is in contrast to that of phenuiviruses, where the viral proteins NP and NSs coding regions are separated by an intergenic region.

The M segment encodes the glycoproteins precursor (GPC), which is ultimately matured as Gn and Gc glycoproteins. Additionally, for some viruses, the maturation of the GPC leads to the maturation of non-structural proteins, such as NSm for RVFV, NSm for some peribunyaviruses, and NSm and GP38 associated to a mucin-like domain (MLD) for CCHFV [5,16].

Finally, the L segment encodes a unique protein, the L protein, which possesses the RNA-dependent RNA polymerase (RdRP) activity [8–10].

As previously mentioned, arenaviruses have only two segments, termed S and L. Both segments encode two genes in an ambisense orientation separated by an intergenic region [17]. The S segment encodes the NP and the GPC, which is further matured into the

glycoproteins Gn and Gc. In addition to the L protein, the L segment encodes a zinc-binding matrix protein termed Z protein [11].

4. Bunyavirus Cell Cycle

Despite the high number of virus species in the *Bunyavirales* order, their cycles share similarities. The typical bunyaviruses cell cycle is briefly described here [3,10]. The viral cycle is initiated upon the interaction between one or two glycoproteins located at the surface of the virions with a cellular receptor. As far as we know, entry of all bunyaviruses into cells is mediated by endocytosis. Following fusion between virion and endosomal membranes, the viral genome is liberated into the cytoplasm. The protein L, which is part of the RNPs and contains a RdRP domain, uses the segments as templates for the production of messenger RNAs (mRNAs), which are translated into proteins thanks to the cellular translation machinery. Bunyaviruses have developed a cap-snatching mechanism, by which they steal the cap of cellular mRNAs in order to initiate the translation of viral mRNAs [18]. Replication of the viral genome also is ensured by the RdRP domain of the L protein. Viral genomic RNA is used as a template to generate a complementary RNA intermediate known as antigenomic RNA, which is then encapsidated by the NP. These antigenomic RNPs are later used as a template for the synthesis of new genomic RNPs. Once the new RNPs and viral proteins are synthetized, assembly and budding occurs at membranes of the Golgi apparatus. New virions are thought to leave the infected cell by exocytosis.

5. Induction of Type I Interferons and Innate Immunity Following Bunyavirus Infection

Upon infection, pathogens and their molecular associated patterns (PAMPs) are recognized by a large variety of cellular proteins known as pattern recognition receptors (PRRs) [19,20]. PRRs can be found in the cytoplasm, such as in the family of the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the family of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). More precisely, two RNA helicases among the RLR family, RIG-I and the melanoma differentiation-associated protein 5 (MDA5), are the main intracellular receptors of viral RNA. Indeed, RIG-I is activated upon binding to short double-stranded (ds) RNA molecules and to the triphosphate group at the 5′ end of viral single-stranded (ss) RNA, while MDA5 activation is dependent on longer dsRNA structures [20,21]. Upon activation of these PRRs, signaling cascades are triggered, involving the activation of TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε), which in turn phosphorylate the IFN regulatory factors 3 and 7 (IRF3 and 7). IRF3 and IRF7 then translocate into the nucleus to induce the transcription of genes encoding type I and type III IFN [20], key factors of the innate immunity.

The activation of RIG-I is at the origin of the production of type I IFN upon infection of some bunyaviruses such as JUNV [22] and SFTSV [23]. Moreover, isolated genomic RNAs from particles of LASV or RVFV strongly activate the production of type I IFN in a RIG-I-dependent manner [24]. Conversely, genomic RNAs isolated from CCHFV and HTNV particles do not induce a RIG-I-dependent IFN production. Notably, the genomic RNAs of both viruses are monophosphorylated at their 5′ end, instead of having a triphosphorylated 5′ end (5′ppp). Thus, suggestions were made that the 5′ppp ssRNA of bunyaviruses genomes are the PAMPs recognized by RIG-I. Additional data identified NP associated with a 5′ppp dsRNA panhandle, as seen in the genome of bunyaviruses, as a potent RIG-I activator [25]. Moreover, it was shown that despite having a 5′p end, HTNV replication is still impaired by RIG-I, and that HTNV NP stimulates RIG-I signaling [26]. As seen in HTNV, another study showed that CCHFV still activates RIG-I despite its modified 5′ end [27]. However, the implication of CCHFV NP as component of a putative PAMP recognized by RIG-I was not assessed and further studies are warranted to decipher the nature of CCHFV's RIG-I-activating PAMP.

MDA5 also is implicated in the detection of bunyaviruses, like LCMV, as shown by in vitro studies and in vivo studies in MDA5-knock-out (KO) mice. LCMV RNA was

sufficient to induce in vitro production of type I IFN and interacts with MDA5, defining it as putative PAMP [28]. However, live LCMV did not induce production of type I IFN, suggesting that other LCMV components than LCMV RNA interfere with the production of type I IFN, as later discussed in this review.

Other PRRs are also found at membranes, such as the plasma or endosomal membranes, like the family of Toll-like receptors (TLRs). TLR3 implication in the induction of IFN-mediated responses upon HTNV infection was shown in vitro but remains to be confirmed in vivo [29]. Another TLR, TLR7, is implicated in LCMV-induced production of type I IFN in plasmacytoid dendritic cells [30]. Finally, JUNV glycoproteins were found to activate TLR2-mediated innate immune responses in vitro [31].

After their production, type I and type III IFN are secreted and act in an autocrine and paracrine manner upon recognition by their receptors (IFN-α/β receptor (IFNAR1/2) and IFN-λ receptor 1/interleukin-10 receptor 2 (IFNLR1/IL10R2) respectively), through the activation of the janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) pathway. Binding of these IFNs to their cognate receptors triggers phosphorylation of janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which leads to the phosphorylation and association in a heterodimer of signal transduced and activator of transcription proteins 1 and 2 (STAT1 and 2). After the recruitment of IRF9 and translocation into the nucleus, this heterodimer activates the transcription of IFN-stimulated genes (ISGs), with antiviral, immunomodulatory and antiproliferative properties (reviewed in [32]). The ISGs induced by type III IFN overlap those induced by type I IFN, with type I IFN acting in a more potent and rapid manner [33].

6. Inhibition of Bunyavirus by IFN-Stimulated Genes and Other Antiviral Factors

Upon bunyavirus infection and sensing by the PRRs, type I IFNs are produced. The importance of the induction of type I IFN synthesis has been shown for some bunyaviruses, like CCHFV, LACV, BUNV, HTNV, RVFV or Punta Toro virus (PTV). Indeed, for these viruses, infection of IFN-deficient animal models leads to the development of a severe disease, while the infection of immunocompetent animal models is less severe or even asymptomatic [34–41]. This is a solid evidence for the importance of the IFN-induced antiviral state against bunyaviruses, suggesting that some ISGs are restriction factors for bunyaviruses. Recently, a mouse-adapted variant of CCHFV that recapitulates the disease in immunocompetent mice was reported [42], but how the mutations found in this variant enable the virus to escape the immune system of the immunocompetent mice remains to be characterized. Moreover, bunyavirus infection induces the upregulation of the expression of several known ISGs, as shown by different transcriptome analyses performed on RVFV-infected human kidney or airway epithelial cells [43,44], on SBV-infected primary bovine fibroblasts [45], or on LASV-exposed human or non-human primate peripheral blood mononuclear cells [46,47]. In addition to ISGs, and independently of type I IFNs, alternative host cell factors interacting with bunyaviruses also have an antiviral action. The restriction factors currently known to exert an antiviral activity against bunyaviruses will be described hereafter, along with their mechanism of action, when it is known. Figure 2 represents these restriction factors as well as the step of the viral cycle which is inhibited.

6.1. Restriction Factors Targeting Bunyaviruses Entry

As mentioned before, bunyaviruses are enveloped viruses that enter the cells via endocytosis. Viral and cellular membrane fusion can occur in the early and late endosomes or lysosomes, depending on the virus. To date, several proteins have been described to interfere and inhibit the entry of bunyaviruses: the IFN-induced transmembrane (IFITM) proteins, the γ-IFN-inducible lysosomal thiol reductase (GILT) and the cholesterol-25-hydrolase (CH25H).

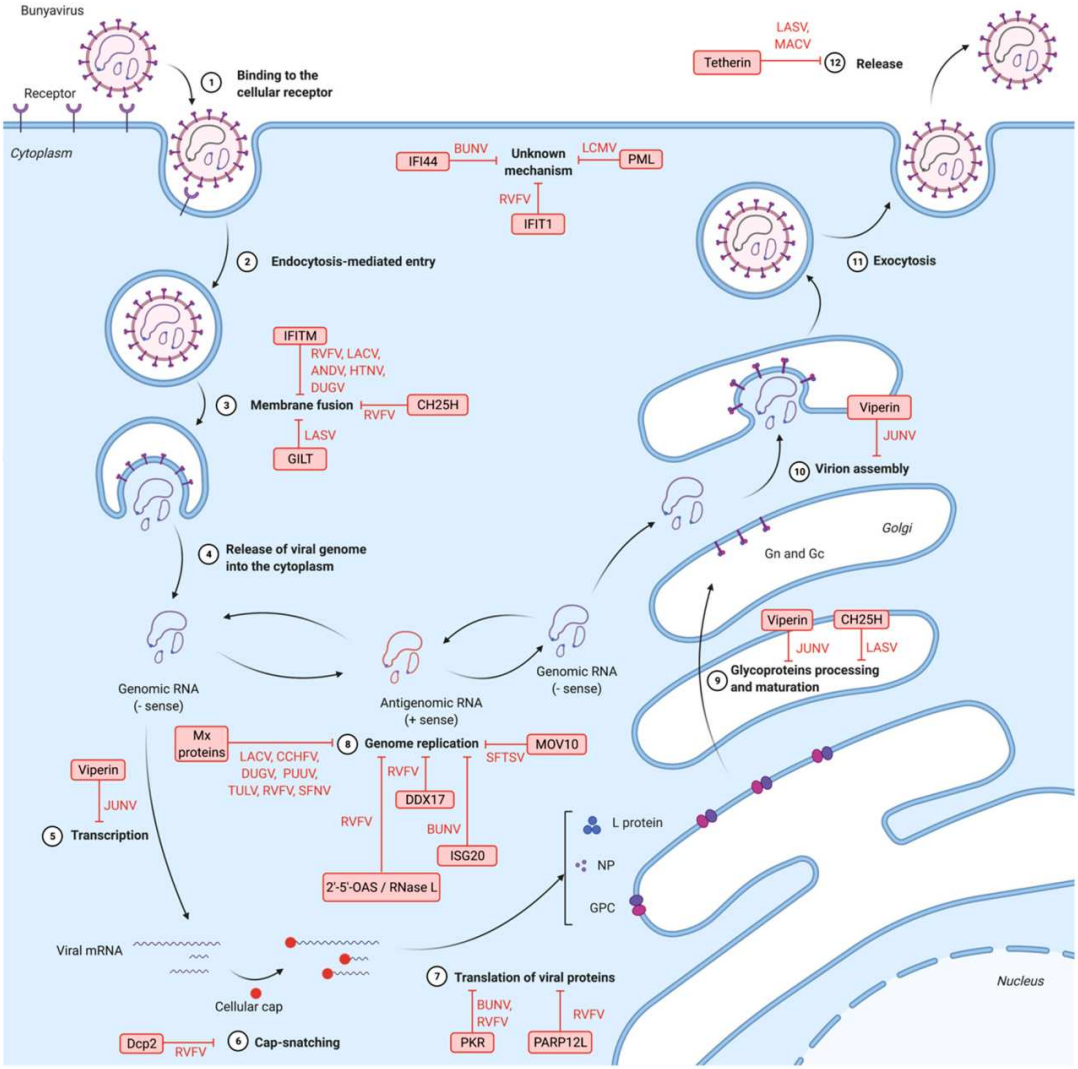

Figure 2. Steps of the bunyavirus cycle inhibited by host restriction factors. Bunyaviruses bind to the receptor (1), then enter the cell and release their genome into the cytoplasm (2, 3 and 4). Genomic RNA is then transcribed into mRNA (5 and 6), later translated into viral proteins (7 and 9). Moreover, genomic RNA is replicated (8). New virions assemble and exit the cell (10, 11 and 12). Each step of the cycle can be inhibited by host restriction factors, which are written in red boxes. ANDV: Andes virus; BUNV: Bunyamwera virus; CCHFV: Crimean-Congo hemorrhagic fever virus; DUGV: Dugbe virus; HTNV: Hantaan virus; JUNV: Junín virus; LACV: La Crosse virus; LASV: Lassa virus; LCMV: lymphocytic choriomeningitis virus; MACV: Machupo virus; PUUV: Puumala virus; RVFV: Rift Valley fever virus; SFNV: Sandfly fever Naples virus; SFTSV: severe fever with thrombocytopenia syndrome virus; TULV: Tula virus. 2′-5′-OAS: 2′-5′-oligoadenylate synthetase; CH25H: cholesterol-25-hydrolase; Dcp2: Decapping 2; IFI44: interferon (IFN)-induced protein 44; IFIT1: IFN-induced protein with tetratricopeptide repeats 1; IFITM: IFN-induced transmembrane protein; ISG20: IFN-stimulated gene 20; GILT: γ-IFN-inducible lysosomal thiol reductase; MOV10: Moloney leukemia virus 10 protein; Mx: myxovirus resistance protein; PARP12L: long isoform of poly(ADP-ribose) polymerase 12; PKR: protein kinase R; PML: promyelocytic leukemia protein.

6.1.1. IFN-Induced Transmembrane Proteins (IFITMs)

In humans, five IFITMs have been described (IFITM1, IFITM2, IFITM3, IFITM5 and IFITM10). They comprise two anti-parallel transmembrane domains, separated by a short cytoplasmic domain. They are basally expressed, but their expression is robustly

induced by type I IFN. IFITM proteins have been widely studied for their roles in many biological processes, such as development and immune cell signaling [48]. Interestingly, an antiviral role of IFITM1/2/3 has been described against a broad range of viruses, such as influenza A virus (IAV) and human immunodeficiency virus type I (HIV-I) [49]. The mechanisms of IFITMs restriction activity are still not well characterized, but seem to be corroborated with their subcellular localization and appear to inhibit viral and cellular membrane fusion. Indeed, these transmembrane proteins are located mostly in early or late endosomes and lysosomes for IFITM2/3, whereas IFITM1 is located at the plasma membrane. Correspondingly, IFITM2/3 efficiently restrict viruses entering the cytoplasm via late endosomes, while IFITM1 more efficiently restricts viruses that enter the cytoplasm via direct fusion with the plasma membrane or with the membrane of early endosomes [49].

To date, IFITMs have been shown to inhibit the in vitro infection of several bunyaviruses from different families, including RVFV, LACV, Andes virus (ANDV) and HTNV [50,51] and Dugbe virus (DUGV) [52,53]. Some specificities exist: for example, only IFITM2/3 restrict RVFV, only IFITM1/3 restrict DUGV, while all three IFITMs inhibit LACV, ANDV and HTNV. For RVFV, a study suggested that IFITM2 and IFITM3 inhibit fusion between viral and endosomal membranes, and consequently the release of RNPs into the cytoplasm, as observed for other viruses such as IAV [50].

Although IFITM proteins inhibit some medically important bunyaviruses, its specificity remains to be understood. Indeed, IFITMs seem to have no effect on some other bunyaviruses, such as CCHFV [50]. A study using pseudoparticles expressing the envelope proteins of LCMV, LASV and MACV [54] revealed that they were not affected by IFITM expression, suggesting that all three viruses are refractory to IFITMs restricting activity, as CCHFV. Mechanisms of escape to IFITMs restricting activity have been described for other viruses. For instance, IAV suppresses the expression of IFITM3 by activating p53 or degrading eukaryotic translation initiation factor B (eIF4B) [55,56]. For CCHFV, LCMV, LASV and MACV, it remains to be understood how these viruses are able to escape IFITMs restriction.

6.1.2. γ-IFN-Inducible Lysosomal Thiol Reductase (GILT)

The γ-IFN-inducible lysosomal thiol reductase (GILT) is a protein located in the lysosomes of dendritic cells, macrophages or B lymphocytes, which has a role in the degradation of cathepsin, as well as in facilitating antigen presentation by major histocompatibility complexes (MHC) I and II. Because of its lysosomal localization, its restriction activity is thus limited to viruses invading the cytoplasm via lysosomes, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Ebola virus (EBOV), and from the *Bunyavirales* order, LASV, as shown by a study using pseudotyped retroviral particles expressing the envelope glycoproteins of these viruses [57]. The restriction activity of GILT is notably linked to its thiol reductase activity, as mutations of the reductase motif abolished GILT-mediated restriction of viral entry. Importantly, GILT reduces the proteolytic activity of cathepsin L, a lysosomal protease essential for the infectious entry of SARS-CoV and EBOV. GILT inhibition of cellular entry of SARS-CoV and EBOV could therefore be linked to the interference with cathepsin L metabolism in a thiol reductase-dependent manner. However, LASV entry does not rely on cathepsin L, explaining the necessity to further study the mechanism of action of GILT restriction activity against this virus.

6.1.3. Cholesterol-25-hydrolase (CH25H)

The cholesterol-25-hydrolase (CH25H) and its enzymatic product 25-hydroxycholesterol (25HC) are important regulators of cholesterol homeostasis [58]. In brief, CH25H and 25HC inhibit the transcription factor sterol regulatory element-binding protein (SREBP) and activate the transcription factor liver X receptor (LXR). These two transcriptional factors have a great importance in modulating cellular cholesterol homeostasis. Moreover, 25HC regulates the location of cholesterol molecules in membranes. The CH25H gene is an ISG in mice and rhesus monkeys; yet whether CH25H is encoded by a human ISG is still debated [58].

Recently, CH25H and 25HC have been shown to exert an antiviral activity against a large number of highly pathogenic viruses, such as HIV-1 or EBOV. To date, two members among the *Bunyavirales* order are known to be inhibited by CH25H: RVFV [59] and LASV [60]. CH25H induces cellular membrane changes that impair viral entry at the step of membrane fusion for RVFV [59]. Of note, CCHFV entry is dependent on cholesterol [61], and CH25H could have an impact on this step, which has hitherto not been studied. For LASV, an alternative mechanism has been observed that differs from RVFV. Indeed, 25HC induces changes in the glycosylation of the GP1 glycoprotein of LASV. GP1 is still incorporated to newly formed virions, but its binding to α-dystroglycan, the LASV attachment and entry receptor, is impaired [60]. The mechanism by which 25HC induces these changes in GP1 glycosylation is yet to be defined.

6.2. Restriction Factors Interfering with Genomic Transcription, Protein Translation and Genomic Replication

Following the entry steps, the viral genome is released into the cell cytoplasm and a first step of transcription occurs, followed by a step of replication. Numerous host factors are known to interfere with these steps, as described in this review.

6.2.1. Myxovirus Resistance Protein A (MxA)

The Mx family GTPases is composed of MxA and MxB in humans (and Mx1 and Mx2 in rodents). These type I and type III IFN-induced proteins have a well-described antiviral activity against a broad range of viruses, with both RNA and DNA viruses being susceptible to their antiviral action. They have a large GTPase domain at their amino-terminus, followed by a central interacting domain and a carboxy-terminal leucine zipper domain, and are located near the smooth endoplasmic reticulum [62].

The antiviral action of MxA against LACV is the most extensively described in the *Bunyavirales* order, both in vitro and in vivo [39,63–67]. Its potential mechanism of action relies on its interaction with LACV NP to sequester it in perinuclear complexes, rendering it unavailable for the encapsidation of viral RNA, and thus interfering with genome replication [64,65]. CCHFV and DUGV are also inhibited in vitro by MxA [68,69]. For CCHFV, as seen for LACV, MxA induces a perinuclear re-localization of CCHFV NP. However, this antiviral activity of MxA was not confirmed in vivo for DUGV. Indeed, transgenic *Ifnar*$^{-/-}$ mice constitutively expressing MxA were as sensitive to DUGV infection as *Ifnar*$^{-/-}$ mice, suggesting that the presence of MxA alone does not confer any protection [38]. For hantaviruses, the role of MxA is also controversial. MxA gene expression is upregulated during infection with hantaviruses [70–72]. While some studies revealed an inhibition of genome replication for HTNV, Puumala virus (PUUV) and Tula virus (TULV) when MxA is overexpressed [63,73], another study showed that *MxA* silencing by knock-down (KD) did not impair the IFN-induced inhibition of HTNV [74]. Finally, two members of the *Phenuiviridae* family, RVFV and sandfly fever Naples virus (SFNV), are also both inhibited upon MxA overexpression [63,67,75], and a study relying on the formation of RVFV virus-like particles (VLPs) demonstrated that the viral primary transcription is the cycle step targeted by MxA [76].

6.2.2. Protein Kinase R (PKR)

The protein kinase R (PKR) is a serine-threonine kinase that is constitutively expressed, albeit in a latent and inactive form. Its expression is upregulated by type I IFNs. To be activated, it needs to encounter, notably, dsRNA or 5′ppp ssRNA. Upon binding to one of these triggers, PKR auto-phosphorylates and dimerizes. Once dimerized, active PKR phosphorylates the α-subunit of the eukaryotic translation initiation factor eIF2 (eIF2α), which stops the translation process [62]. Because viruses need the cellular translation machinery to translate their proteins, PKR is considered as a restricting factor for a high number of viruses, including BUNV [77,78]. Indeed, BUNV infection leads to PKR-dependent phosphorylation of eIF2α and in vivo studies performed on PKR-deficient laboratory mice revealed a higher susceptibility to BUNV infection compared to wild-type mice [78]. As for

RVFV, a natural isolate with a large depletion in the NSs gene (Clone 13) is susceptible to PKR restriction, both in vitro and in vivo [79,80]. Compared to Clone 13, RVFV expressing a complete NSs is much less susceptible to PKR restriction. These results suggest an antagonism function of RVFV NSs protein against PKR-mediated viral restriction, which is later discussed in this review.

However, PKR does not restrict all bunyaviruses. Indeed, infection of PKR-deficient laboratory mice with DUGV did not have an impact on their survival rate, suggesting that PKR alone does not restrict the replication of this virus [38]. Moreover, while PKR was activated in human cells upon infection by some arenaviruses (more precisely JUNV and MACV), it did surprisingly not affect their viral replication. In the same study, LASV infection of human cells did not activate PKR [81].

6.2.3. IFN-Induced Protein with Tetratricopeptide Repeats 1 (IFIT1)

The IFN-induced protein with tetratricopeptide repeats 1 (IFIT1) is a protein whose ability to bind the 5′ppp end of RNA was discovered by co-immunoprecipitation of agarose beads coupled to 5′ppp RNA in uninfected human embryonic kidney cells (HEK293) followed by mass spectrometry experiments [82]. Following this discovery, the authors performed KD experiment by small interfering (si) RNA of IFIT1 in HeLa cells and assessed its effect in a context of infection. This KD led to an increased replication of NSs-deficient RVFV Clone 13, suggesting that IFIT1 is a restriction factor against RVFV [82]. The mechanism of action of this restricting activity might be linked to IFIT1 binding to 5′ppp RNA, but this remains to be confirmed.

6.2.4. Long Isoform of Mono-ADP-ribosyltransferase 12 (PARP12L)

The poly (ADP-ribose) polymerases (PARPs) are enzymes catalyzing the transfer of ADP-ribose units to target proteins using a nicotinamide adenine dinucleotide (NAD^+) as substrate. More specifically, PARP12 is a mono-ADP-ribosyltransferase whose expression is IFN-induced and whose long isoform, PARP12L, was discovered to have an antiviral effect against a virus from the *Alphaviridae* family, namely Venezuelan equine encephalitis virus (VEEV). This antiviral activity was found to be broad-spectrum, since the replication of RVFV was also inhibited by PARP12L in an overexpression system of PARP12L [83]. For VEEV, it was shown that PARP12L inhibits cellular and viral translation, like PKR. However, it appears that PARP12L uses a different mechanism than PKR to inhibit translation, involving its binding to polysomes [84]. The same mechanism for RVFV PARP12L restricting activity could be proposed but needs confirmation.

6.2.5. Decapping Protein 2 (Dcp2)

Decapping 2 (Dcp2) is a protein located in the P-bodies which is involved in the mRNA decapping machinery. This machinery is essential to ensure the cellular RNA turnover, by removing the protecting 7-methylguanylate (7 mG) cap present at the 5′ end of cellular mRNAs. mRNAs ripped of their 5′ cap expose a 5′ monophosphate, which is target for cellular exonucleases, thus begetting RNA degradation.

A genome-wide RNA interference (RNAi) screen performed in *Drosophila* cells, which are used as an insect cell model useful to study arboviruses interactions with their vector, identified Dcp2 as a potential host restriction factor for RVFV. Depletion or overexpression experiments of Dcp2 in *Drosophila* cells led respectively to an increase and decrease of RVFV transcription. These results were confirmed in a RNAi performed in adult flies: Dcp2-depleted flies succumbed to RVFV infection, whereas control flies presented little mortality. These results were extended to another bunyavirus transmitted by mosquitoes, LACV [85]. A similar finding was described in plant cells for the tomato spotted wilt virus (TSWV), a plant bunyavirus from the *Tospoviridae* family [86]. Notably, bunyaviruses have developed a cap-snatching mechanism in order to protect their mRNAs and initiate their translation [18,87]. Indeed, several strategies have been developed by viruses to ensure the stability of their mRNA. All rely on the protection of their 5′ ends from cellular

exonucleases [88], whether it is by encoding their own capping machinery, by attaching a small, viral-encoded protein to the 5′ end, or by stealing the 5′ end of host mRNAs thanks to a viral-encoded endonuclease. This last strategy, called cap-snatching, ensures that viral mRNAs become indistinguishable from endogenous mRNAs. Thus, it is thought that a competition between the viral endonuclease responsible for cap-snatching and Dcp2 for cellular mRNA caps is at the origin of the restricting activity of Dcp2. However, these results remain to be confirmed in mammalian cells.

6.2.6. DDX17

DDX17 is a DEAD-box RNA helicase that has a major role in RNA metabolism. DDX17 is the human homolog of Rm62, whose role in antiviral defense against both RVFV and LACV was identified through RNAi screens in *Drosophila cells* and in adult flies [89]. Conservation of the antiviral role of Rm62 in human cells was observed, as *DDX17* silencing in human cells led to an increased viral replication of both RVFV and LACV. More precisely, DDX17 can interact with RVFV RNA, likely via the recognition of defined structured viral RNA elements, notably the hairpin-like structures present in the intergenic region on the S segment (between N and NSs) and in the 5′ non-coding region of the S segment. It remains to be understood how this binding mediates an antiviral activity, but it can be hypothesized that DDX17 acts as a sensor protein bringing viral RNA to the degradation machinery, as it is known to interact with exonucleases or proteins involved in the decapping machinery, like Dcp2. As a helicase, DDX17 may also unwind viral RNA to facilitate its degradation. A recent biophysical study obtained evidence in support of the latter hypothesis. Indeed, it was observed in vitro that DDX17 helicase domain interacts with and unwinds the intergenic region of RVFV S segment along with S 5′ non-coding region [90].

6.2.7. IFN-Induced Protein 44 (IFI44)

Little is known about the IFN-induced protein 44 (IFI44, previously known as MTAP44). It was initially discovered in the context of hepatitis C virus (HCV) infection, for which it does not have any antiviral activity described. Interestingly, its overexpression leads to BUNV restriction in vitro, but the involved mechanism is yet to be understood [77]. However, in another study, IFI44 silencing by siRNA negatively affected LCMV replication, suggesting that IFI44 is not a host restriction factor, but rather a host dependency factor for LCMV [91].

6.2.8. Promyelocytic Leukemia Protein (PML)

Promyelocytic leukemia protein (PML) nuclear bodies (NBs) are nuclear structures containing several proteins, among which PML, that contribute to the regulation of a large number of key cellular processes, such as cell cycle progression, DNA damage response, transcriptional regulation and apoptosis. PML expression is induced by type I IFNs.

In a context of LCMV infection, it was shown that PML-deficiency in murine embryonic fibroblasts leads to an increased LCMV replication and that overexpression of PML results in diminished LCMV replication [92]. This LCMV-directed antiviral activity of PML was confirmed in vivo in PML-deficient LCMV-infected laboratory mice, which exhibited an accelerated onset of symptoms and higher viral loads compared to control mice [93]. Upon LCMV infection, it was shown that PML NBs are relocated to the cytoplasm following an interaction of PML with the arenaviral Z protein [94]. However, the consequences of this re-localization as well as the exact mechanism by which PML interferes with LCMV replication remain to be understood.

6.2.9. Moloney Leukemia Virus 10 Protein (MOV10)

Moloney leukemia virus 10 protein (MOV10) is an RNA helicase with an important role in regulating cellular mRNA stability and translation. Moreover, it has an antiviral activity against a high number of viruses, including retroviruses [95], hepatitis B virus

(HBV) [96], HCV [97] and some RNA viruses (vesicular stomatitis virus (VSV) [98] and IAV [99]). For retroviruses like HIV-1, MOV10 is known to interfere with viral reverse transcription, which could explain its antiviral activity. However, for some other viruses, like hepatitis D virus, MOV10 has a proviral rather than an antiviral activity [100].

The role of MOV10 during bunyaviral infections was only recently studied [101]. It was shown that SFTSV infection specifically upregulates MOV10 expression in vitro and that MOV10 inhibits the replication of SFTSV and other related viruses (Heartland virus (HRTV) and Guertu virus (GTV)) in infected cells. This antiviral activity of MOV10 was confirmed in vivo in *MOV10*-KD mice, in which SFTSV viral loads were significantly lower than in control, SFTSV-infected mice. The helicase function of MOV10 was found to be dispensable for MOV10 SFTSV-directed antiviral activity. However, MOV10 interacts with the NP of SFTSV, and this interaction was found to be essential for MOV10 antiviral activity, as it impairs NP oligomerization and NP interactions with viral RNA and with the L protein. Thus, the assembly of RNPs is inhibited. As the NP of SFTSV shows only low sequence identity with other bunyaviruses, it remains to be further investigated whether MOV10 has an antiviral activity against other members of the order.

6.2.10. IFN-Stimulated Gene 20 (ISG20)

IFN-stimulated gene 20 (ISG20) is an exonuclease known as a restriction factor for multiple viruses, including for instance HBV and HCV [102,103]. In a flow-cytometry-based gain-of-function screen of 488 ISGs, ISG20 was notably found to be a potent restriction factor against BUNV [53]. As seen for HBV, ISG20 antiviral activity against BUNV is linked to its exonuclease activity. Indeed, mutations in its active exonuclease site abrogated its antiviral effects against BUNV. The antiviral effect against BUNV of endogenous ISG20 was confirmed by short hairpin (sh) RNA-mediated KD of *ISG20*. Moreover, in vitro gain-of-function experiments conducted upon other bunyaviral infections demonstrated a specific effect of ISG20. Indeed, while DUGV and PUUV also were inhibited by the overexpressed ISG20, phenuiviruses appeared resistant to ISG20 antiviral activity.

6.2.11. 2′-5′-Oligoadenylate Synthetase (2′-5′-OAS)/RNase L

The 2′-5′-oligoadenylate synthetase (2′-5′-OAS) is a constitutively expressed enzyme whose production is upregulated by IFN and which is activated by dsRNA. It catalyzes the synthesis of 2′-5′-oligoadenylates, which bind and activate the latent endoribonuclease RNase L. Once activated, RNase L degrades ssRNAs and therefore inhibits viral replication [62]. As such, it is considered as a potent restriction factor for a broad range of viruses. Concerning bunyaviruses, only a few studies have attempted to determine the implication of 2′-5′-OAS/RNase L system as a host restriction factor. Some microarray experiments performed on cells infected with hantaviruses revealed an upregulation of the expression of the 2′-5′-OAS [70,71], however further studies are needed to determine the consequences of this upregulation. Moreover, the depletion of 2′-5′-OAS by RNAi led to an increase in the viral replication of a NSs-depleted RVFV, suggesting a potential restricting role of 2′-5′-OAS that still needs to be characterized [104]. However, the 2′-5′-OAS/RNase L system does not seem to restrict BUNV replication, as shown by in vivo experiments conducted in RNase L-deficient mice [78].

6.3. Restrictions Factors Blocking Viral Assembly and Egress

The last steps of the viral cycle lead to the clustering of virus assembly factors at the site of assembly, the assembly and envelopment of viral particles, and the egress of newly formed virions. Relative to other viral cycle steps, only few restriction factors have been described to play a role at this stage. Among them, viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) and tetherin have both been identified as restriction factors of some viruses from the *Bunyavirales* order.

6.3.1. Viperin

Viperin is encoded by an ISG and localizes in the endoplasmic reticulum and in lipid droplets. Its antiviral action has been described for a large number of viruses.

Notably, viperin is upregulated upon LCMV infection [105]. Similar findings were reported for JUNV, and viperin overexpression led to a strong antiviral effect against JUNV by inhibiting infectious virus release [106]. This phenomenon may be explained by two mechanisms. First, viperin overexpression leads to a mislocalization of viral glycoproteins, hence perturbing viral assembly or virion morphogenesis. Second, it leads to an altered lipogenesis that could prevent JUNV to use the lipid rafts for virion budding. However, viperin could also have an antiviral role at an earlier step of JUNV cycle. Indeed, it has been shown that viperin overexpression in JUNV-infected cells leads to a decreased viral RNA transcription. Moreover, JUNV NP interacts with viperin. Taken together, these data suggest that viperin might interact with JUNV NP to counteract its function on genome viral transcription and replication [106].

BUNV has also been shown to be regulated by viperin [77]. However, unlike JUNV, viperin expression is not upregulated upon BUNV infection, except when a recombinant virus whose NSs has been deleted is used, suggesting a role of NSs to counteract the regulatory action of viperin. The exact mechanism of viperin restriction activity for BUNV remains to be elucidated.

6.3.2. Tetherin

Tetherin, also known as bone marrow stromal antigen 2 (BST-2), is encoded by an ISG. This protein is a type II transmembrane protein whose ectodomain structure is linked to an amino-terminal transmembrane region and a glycosylphosphatidylinositol (GPI) anchor at its carboxy-terminus. In humans, tetherin is located at the *trans*-Golgi network (TGN), endosomes and within lipid rafts at the plasma membrane.

Tetherin was initially found to be a potent antiviral factor of HIV-1 that is counteracted by the viral protein Vpu. Its name reflects its ability to restrict the release of HIV-1 by retaining the viral particle at the cell membrane, like an anchor. This protein has a propension to form homodimers, and it was also shown that it can be incorporated into the viral membrane of HIV-1 [107]. Therefore, a model of antiviral activity has been proposed for this protein in which homodimerization between tetherin monomers from cellular and viral membranes is at the origin of the retention of the virion at the cell membrane.

Since then, tetherin was found to inhibit a large number of enveloped viruses [108], including bunyaviruses such as arenaviruses like LASV, MACV and JUNV [109]. Notably, the release of LASV and JUNV VLPs based on the expression of its matrix protein Z [110] as well as infectious LASV, JUNV and MACV [109,111] is restricted by tetherin. The mechanism does not involve tetherin dimerization [112], in contrast to what had first been proposed for HIV-1. Moreover, further studies are needed to know whether tetherin is incorporated into the membrane of arenaviruses, as seen for HIV-1. Notably, RVFV was found to be refractory to tetherin restriction activity [109].

Tetherin demonstrates species-related specificities, which have repercussions on its ability to inhibit some viruses from the *Bunyavirales* order, such as peribunyaviruses. This family contains viruses which are pathogenic for humans (like OROV and LACV), for ruminants (like SBV, Akabane virus (AKAV) and Sathuperi virus (SATV)), or both (like Cache Valley virus (CVV)). Interestingly, SBV, AKAV and SATV (which are viruses with a ruminant tropism) are restricted by human tetherin, but not by sheep tetherin. For SBV, human tetherin was found to induce a reduction of the incorporation of the Gc glycoprotein into SBV virions. Conversely, OROV and LACV (which are viruses with a human tropism) are restricted by sheep tetherin, but not by human tetherin. CVV, which has both a human and a ruminant tropism, was restricted by neither human tetherin nor sheep tetherin. Thus, the species-related specificities of tetherin might in part explain the host species range of these pathogens [113].

7. Escape of Antiviral Responses by Bunyaviruses

Bunyaviruses have developed numerous strategies to escape the antiviral responses. As seen previously, a large number of known bunyaviruses restricting factors are dependent on the IFN response. Thus, to escape efficiently host antiviral responses, bunyaviruses either inhibit the production of IFN or diminish the IFN-induced response. But some restricting factors also are counteracted directly by bunyaviruses, such as PKR and tetherin. The different strategies developed to escape host antiviral responses are schematically summarized in Figure 3.

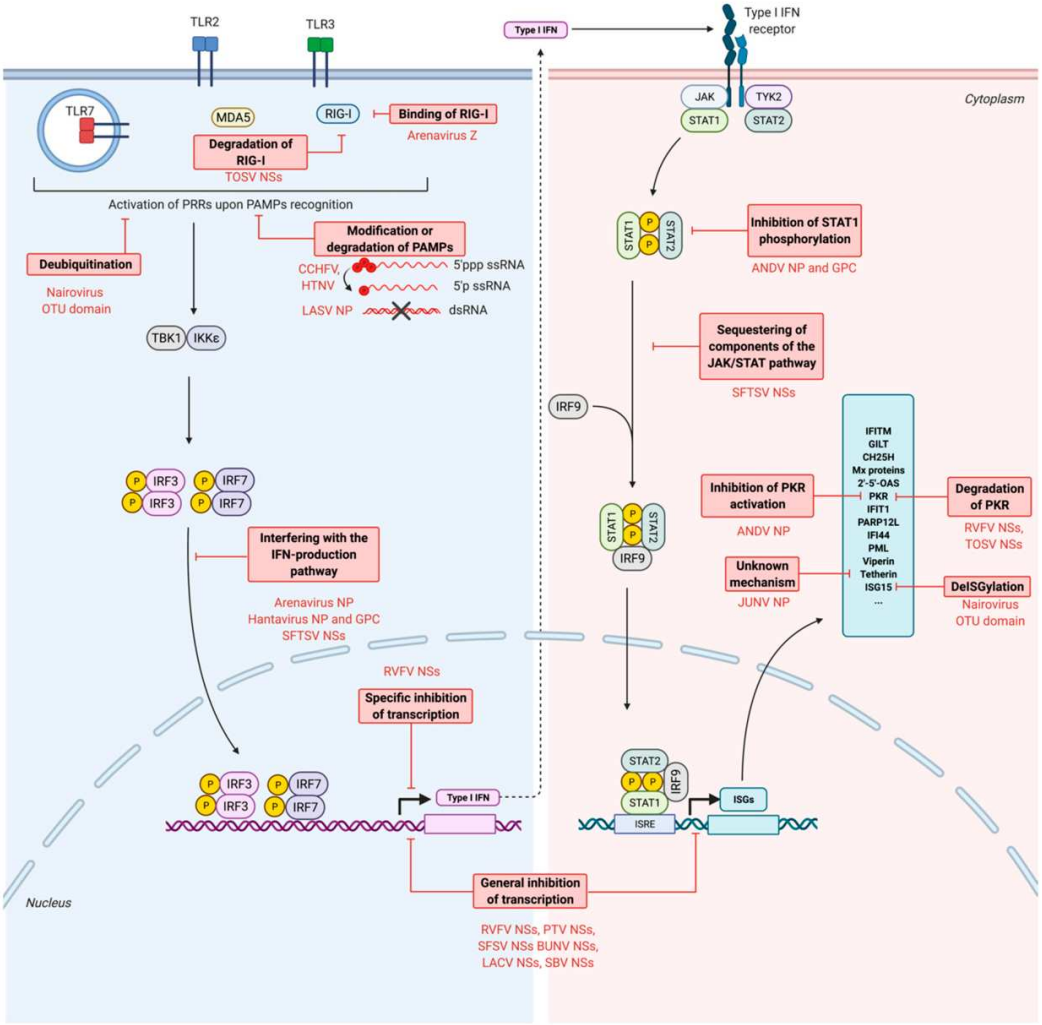

Figure 3. Viral strategies counteracting the host intrinsic and innate immunity system. Upon detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), pathways are activated leading to the production of type I interferon (IFN). One of them involves the phosphorylation, dimerization and nuclear translocation of IFN regulatory factors 3 and 7 (IRF3 and IRF7). Type I IFN are secreted and act in an autocrine and paracrine manner. Upon recognition by the type I IFN receptor, the signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2) are phosphorylated and associate as a heterodimer, as well as with the IFN regulatory factor 9 (IRF9). This complex translocates to the nucleus to activate the transcription of IFN-stimulated genes (ISGs). Viral countermeasures developed by viruses to inhibit these pathways at different steps are written in red boxes. GPC: glycoproteins precursor; NP: nucleoprotein; OTU domain: ovarian tumor domain. IKKε: IκB kinase-ε; IRF: IFN regulatory factor; ISG: IFN-stimulated gene; ISRE: IFN-stimulated response element; JAK: janus kinase; MDA5: melanoma differentiation-associated protein; RIG-I:

retinoic acid-inducible gene-I; STAT: signal transducer and activator of transcription; TBK1: TANK-binding kinase 1; TLR: Toll-like receptor; TYK2: tyrosine kinase 2. ANDV: Andes virus; CCHFV: Crimean-Congo hemorrhagic fever virus; JUNV: Junín virus; HTNV: Hantaan virus; LACV: La Crosse virus; LASV: Lassa virus; LCMV: lymphocytic choriomeningitis virus; PTV: Punta Toro virus; RVFV: Rift valley fever virus; SBV: Schmallenberg virus; SFTSV: severe fever with thrombocytopenia virus; SFSV: Sandfly fever Sicilian virus; TOSV: Toscana virus.

7.1. Escape from the PRRs Recognition

In order to inhibit the production of IFN which leads to an antiviral state, some viruses act at the very beginning of the IFN production signaling cascade. They avoid being recognized by the PRRs through strategies involving the modification or degradation of PAMPs.

7.1.1. Modification of PAMPs: Processing of the Genome

Bunyaviruses do not produce high amounts of dsRNA, which is an important PAMP [114]. However, their ssRNA genome possesses a 5′ppp end, which is a potent trigger of RIG-I-mediated IFN production [24]. Yet, as already described, both nairoviruses and hantaviruses have developed an alternative strategy that is supposed to avoid recognition by RIG-I: during replication, the 5′ end of their genome is processed to be monophosphorylated rather than tri-phosphorylated [24]. The exact mechanism by which these 5′p ends are generated remains to be fully understood. However, despite this strategy, both CCHFV and HTNV still induce a RIG-I-dependent IFN production [26,27], suggesting that an alternative PAMP is at the origin of a RIG-I-mediated detection of viral RNAs by the host cell. The role of HTNV NP in RIG-I activation was already mentioned in this review [26]. Moreover, the complementary antigenomic RNA could be a PAMP itself, as it does interact with less affinity than genomic RNA with the NP, as shown for hantaviruses [115], and could thus be more accessible to RIG-I sensing by lack of NP-mediated protection from detection. Alternatively, the production of 5′ monophosphorylated ends might involve the generation of 5′ppp intermediates or truncated defective interfering (DI) particles, which could be putative PAMPs. More studies are necessary to determine the PAMPs at the origin of the PRR-mediated IFN production.

7.1.2. Degradation of PAMPs

dsRNA is an important PAMP and is recognized by RIG-I and PKR. Even though bunyaviruses do not produce high amounts of dsRNA, it can still trigger the activation of these sensors. To inhibit the dsRNA-mediated activation of RIG-I and PKR, LASV NP exhibits a dsRNA-specific exonuclease activity. This activity was shown to inhibit the accumulation of viral PAMPs, and consequently enables the virus to escape PRR recognition [116–119].

7.2. Inhibition of the Production of IFNs

Inhibiting the production of IFNs is an important and widely developed strategy by viruses to avoid the establishment of an antiviral state. Bunyaviruses inhibit the production of IFNs by either inducing a cellular transcriptional and translational shutoff or interfering with the signaling cascade leading to the production of IFN.

7.2.1. Global Cellular Transcriptional and Translational Shutoff

The S segment of bunyaviruses, with the exception of arenaviruses, encodes a non-structural protein termed NSs, which has already been mentioned as a modulator of several restriction factors. The NSs protein of RVFV was the first one to be identified as an IFN-antagonist [35,120]. Since then, the NSs proteins from other phenuiviruses (PTV [121], Toscana virus [122], Sandfly fever Sicilian virus (SFSV) [123], SFTSV [124]), from peribunyaviruses (LACV [37], BUNV [125,126], OROV [127], SBV [128]), or from hantaviruses (TULV and PUUV [129], ANDV [130]) also were found to be IFN-antagonists.

RVFV NSs counteraction of the IFN-mediated response is the most extensively studied and relies on its ability to induce a global cellular transcriptional shutoff [131]. Indeed, it was shown that NSs interacts with the p44 component of the transcription factor II H (TFIIH), a general transcription factor that recruits the RNA polymerase II to the promoters, and sequesters it into nuclear filamentous structures to prevent the assembly of TFIIH [132]. Moreover, it also interacts with the p62 subunit of TFIIH [133] and promotes its proteasomal degradation [134] by recruiting the F-box protein FBXO3 [135], a substrate recognition component of modular E3 ubiquitin ligases of the Skp1, Cullin1, F-box (SCF) protein type. Finally, RVFV NSs expression was also reported to correlate with a block of nuclear host mRNA export [136]. As a result of these events, IFN production is negatively impacted. However, NSs also specifically targets IFN synthesis by interacting with a component of a host transcriptional suppressor complex specifically regulating IFN-ß expression, the Sin3A-associated protein (SAP30). Indeed, a recombinant RVFV virus in which NSs is deleted for the region of interaction with SAP30 was found to be unable to inhibit IFN response and to be avirulent in mice [137]. The interaction of NSs with SAP30 leads to the formation of a repression complex on the IFN-ß promoter. Consequently, the IFN-ß production is negatively impacted.

Some differences in NSs functions are found among phenuiviruses. For example, as RVFV NSs, PTV and SFSV NSs inhibit host transcription. On the contrary, TOSV NSs does not affect cellular transcription [122]. Finally, UUKV NSs was found to only have a weak IFN-antagonist effect [138].

Notably, phenuiviruses NSs is expressed in an ambisense coding strategy from the S segment. Thus, it is expected to be expressed only at later stages of infection, during the secondary transcription round. This is contradictory with its IFN-antagonism activity, which happens at early stages of infection. However, it was reported that antigenomic RNA segments, like genomic segments, are packaged into the virions for both RVFV and UUKV and are therefore transcribed during primary transcription [139–141]. Thus, NSs protein is also likely produced at early stages of infection.

As for peribunyaviruses, NSs induces a massive and rapid inhibition of cellular mRNA transcription. Indeed, NSs of both BUNV and LACV directly interfere with the cellular RNA polymerase II, by reducing the phosphorylation of its carboxy-terminal domain for BUNV (which is necessary for mRNA elongation) [126] and by additionally degrading its large subunit RPB1 for LACV by a mechanism involving the cellular E3 ubiquitin ligase subunit, Elongin C [142,143]. Like LACV, SBV NSs also induces RPB1 degradation [128,144].

CCHFV was also reported to express a NSs protein that undergoes rapid proteasomal degradation [15]. However, an IFN-antagonist activity was not described for this protein. To date, whether other members of the *Nairoviridae* family encode a functional NSs is unknown. It is therefore expected that nairoviruses have developed an NSs-independent strategy to escape the innate immune response, as mentioned hereafter.

7.2.2. Interference with the IFN Production Pathway

- Alteration of post-translational modifications of key factors from the IFN production pathway: deubiquitination and deISGylation

The type I IFN response is highly regulated and relies, notably, on post-translational modifications of several key actors of the type I IFN response by ubiquitin (Ub) [145,146]. For instance, K63-linked polyubiquitination is necessary for the activation of several components of the pathway, including RIG-I [147]. Global ubiquitination is regulated by a number of cellular deubiquitinases (DUB), among which the ovarian tumor protease (OTU) subfamily [148,149].

Relative to other bunyaviruses, nairoviruses encode a particularly large L protein of roughly 12 kb. Studies showed that several nairoviruses have an OTU-like protease motif at the N-terminus of their L protein [150–152], which is absent in the L proteins of other bunyaviruses, except for one plant virus from the *Phenuiviridae* family, the rice stripe tenuivirus

(RSV), which also encodes an OTU domain [153,154]. The similarity of this domain with cellular OTU proteins suggests that it may be involved in deubiquitination. Indeed, several biochemical and structural analysis of OTU domains of different nairoviruses corroborate this hypothesis [155–159]. Biochemical and structural studies of nairoviral OTU domains first focused on CCHFV or closely CCHFV-related nairoviruses, including NSDV, Ganjam virus (GANV), DUGV and Erve virus (ERVEV) [160–164]. However, recently, two global studies comparing the DUB activity of 12 nairoviruses [165] showed that nairoviral OTUs differ in their DUB activity and can therefore be divided into groups possessing high, moderate or low deubiquitinase activity. These differences can notably be explained by the fact that there is a high degree of diversity between nairoviral OTUs, with sometimes less than 25% amino acid identity with CCHFV OTU domain [165]. However, globally, a higher activity toward K6-, K11-, K48- and K63-linked Ub was observed. By disrupting these ubiquitinase functions, nairoviral OTUs dampen antiviral responses.

By removing post-translational modifications essential for the innate immune signaling pathways, the nairoviral OTU domains are able to dampen antiviral responses, as shown by several studies relying on the overexpression of the isolated OTU domains [155–157] (Figure 4). Moreover, the immunomodulatory effects of DUB activities of the CCHFV OTU domain have been observed in a reverse genetic system. Indeed, a reduced activation of key components of the innate immune response (including RIG-I) was observed upon infection with wild-type CCHFV, while infection with a mutated CCHFV whose OTU lacked DUB activity led to the establishment of an antiviral state in infected cells [159]. However, when associated with the full-length L protein, the ability of the OTU domain to suppress the type I IFN-mediated immune response seems to be reduced [155–157] and a study using a CCHFV VLP system with a protease-negative OTU mutant in immunocompetent cells revealed that IFN induction was not impacted upon infection [166], adding more complexity to the understanding of the contribution of the OTU domain in the antiviral response.

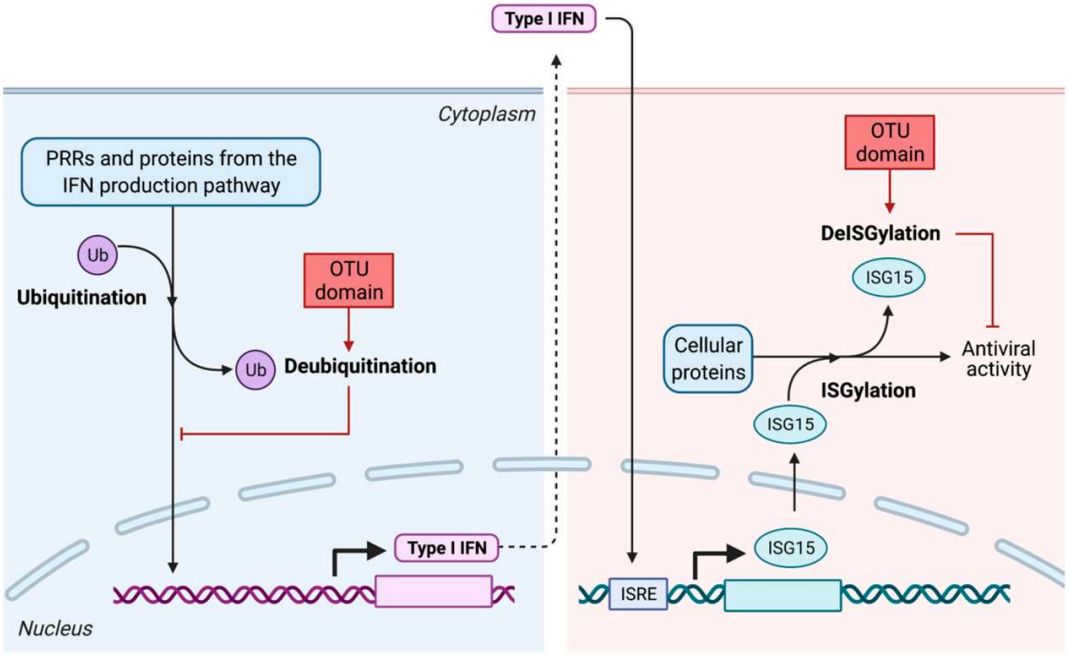

Figure 4. Deubiquitination and deISGylation activity of the nairovirus OTU domain. IFN: interferon; ISG15: IFN-stimulated gene 15; ISRE: IFN-stimulated response element; OTU domain: ovarian tumor domain; Ub: ubiquitin.

It is worth noting that studies on nairoviral OTU domains also highlighted an additional activity of viral OTUs compared to cellular DUB proteins of the OTU family, namely

the deISGylase activity [155–159] (Figure 4). Type I IFN induces the expression of a 15 kDa protein, the IFN-stimulated gene 15 (ISG15), which is a member of the ubiquitin family [167]. ISG15 can be covalently conjugated onto target proteins via an enzymatic cascade called ISGylation [168]. This protein, conjugated or in its free intracellular or extracellular form, is known to antagonize viral replication during acute and latent infections, although its activity, being diverse, pathogen- and host species-dependent [169,170], is not well understood. Interfering with ISG15 conjugation could enable nairoviruses to escape the innate immune response. As for the DUB activity of nairoviral OTUs, the deISGylase activity of nairoviral OTUs also is diverse among nairoviruses, which could reflect an adaptation of the virus to its host. Indeed, ISG15 is a protein that is found only in vertebrates and that is highly divergent between species. The preferences toward the ISG15 from one or another species generally reflects the preferential hosts infected by the virus [171], hinting that a co-evolution process may have contributed to these specificities.

- Inhibition of the activation of RIG-I

RIG-I is one of the main sensors of bunyaviruses infection at the origin of IFN production. Some viruses from this order have therefore developed strategies to inhibit its activation. For instance, TOSV NSs interacts with RIG-I and triggers its proteasomal degradation [172].

As for arenaviruses, transfection of LCMV NP and LCMV RNA led to a decrease in LCMV RNA-induced activation of the type I IFN in vitro [28]. Moreover, LCMV NP was found to bind both RIG-I and MDA5. Yet, two NP mutants that do not inhibit the type I IFN signaling pathway also bind both RLRs, suggesting that RIG-I or MDA5 binding by NP is not sufficient to inhibit their activation and suggesting the necessity of another viral factor to efficiently counteract the RLR-mediated antiviral effects for these viruses [28]. In fact, the Z protein encoded by the L segment of arenaviruses was reported to inhibit the activation of RIG-I. Indeed, the Z proteins of four New-World arenaviruses, Guanarito virus (GTOV), JUNV, MACV and Sabiá virus (SBAV) were reported to bind RIG-I by co-immunoprecipitation experiments, which resulted in the downregulation of the IFN response [173]. However, in this study, this property was not shared by Old-World arenaviruses like LCMV or LASV, whereas in another study, all pathogenic arenaviruses, including LCMV and LASV, were found to have a Z-mediated inhibition of RLR-induced IFN production [174]. These controversial results can be explained by differences in the experimental systems, such as for instance the Z expression levels. The study of Z proteins in an infectious context is therefore necessary to better understand whether they are able to counteract the innate immune responses.

- Interference with the IFN production pathway

Another strategy for viruses to escape the host innate immunity is to inhibit the production of IFN by disrupting the signaling cascade leading to its production. Bunyaviruses have developed several ways to achieve this.

For instance, the arenaviral NPs inhibit the nuclear translocation and the transcriptional activity of IRF3 and of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), both key factors of the IFN production pathway [28,175–177]. More specifically, LCMV and JUNV NPs associate with IKKε and block its ability to phosphorylate IRF3 [178].

Hantaviruses NPs are also known to antagonize type I IFN responses [179–181]. More precisely, HTNV NP reduces the nuclear translocation of NF-κB, by interacting with importin α proteins involved in the nuclear shuttling of NF-κB, thus inducing a sequestration of NF-κB into the cytoplasm and inhibiting IFN production [179–182]. However, the NP proteins of other hantaviruses (ANDV, SNV, New York virus (NYV) and PHV) have no effect on NF-κB activation [183]. But recent studies revealed that ANDV NP is able to inhibit TBK1 and IKKε activation by an as yet unknown mechanism [183–185]. This activity of ANDV NP seemed not to be shared by other viruses, like SNV, NYV and PHV. Additionally, ANDV GPC was also found to inhibit the type I IFN induction, as well as SNV GPC [185]. Moreover, several reports showed that the ectopic expression of the cytoplasmic tail of the

glycoprotein Gn of some hantaviruses (ANDV, NYV and TULV) leads to the inhibition of IRF3 activation [186–188]. This shows that glycoproteins can also be involved in the antagonism of the cellular antiviral responses by blocking IFN production. Furthermore, a study focusing on ANDV NSs protein showed that it acts as an IFN-antagonist by suppressing signals downstream of the RIG-I/MDA5 pathway but upstream of TBK1 [130].

Like the NSs of other phenuiviruses, SFTSV NSs has an immunomodulatory role, but its mechanism is different than the other phenuiviruses NSs. Indeed, SFTSV NSs is concentrated in cytoplasmic structures where it interacts with and sequesters several components of the RIG-I pathway, like RIG-I itself, tripartite motif-containing 25 protein (TRIM25) (a RIG-I-activating E3 ubiquitin ligase), TBK1 and IRF7 [23,124,189–194]. The IFN production pathway is therefore disrupted.

7.3. Inhibition of the Production of ISGs

Another strategy developed by viruses to inhibit the antiviral effects of IFN is to inhibit the production of ISGs, by interfering with the JAK/STAT pathway. For ANDV, this mechanism relies on the NP- and GPC-induced inhibition of STAT1 phosphorylation and nuclear translocation, thus inhibiting the production of ISGs [185,195]. For SNV, another hantavirus, while NP was not able to inhibit the IFN-induced transcription of ISGs, GPC was [185].

NSs of SFTSV was already mentioned for its ability to induce the sequestration of key components of the IFN production pathway. In addition, it was also found to sequester STAT1 and STAT2 into inclusion bodies, therefore interfering with the production of ISGs [196].

7.4. Inhibition of the Activity of Restriction Factors

Finally, some aforementioned restriction factors are directly inhibited by bunyaviral components and are consequently unable to exert their restricting activity.

7.4.1. Inhibition of PKR

As previously mentioned, RVFV NSs has an important role in inhibiting IFN production by inducing a global as well as an IFN-specific transcriptional shutoff. However, IFN is still produced during RVFV infection and leads to the expression and activation of ISGs, like PKR. RVFV NSs notably counteracts the aforementioned PKR-induced inhibition of translation by inducing its proteasomal degradation [79,80,131]. This degradation is initiated by the recruitment by NSs of two F-box proteins, FBXW11 and β-TRCP1 [197,198]. Once more, differences exist between the NSs of phenuiviruses. Indeed, TOSV NSs efficiently induces PKR degradation [199], while PTV and SFSV NSs do not affect cellular levels of PKR [123,199].

Instead of degrading PKR, ANDV NP has developed another strategy. Indeed, in an overexpression system, ANDV NP inhibited PKR dimerization which is necessary for its activation [200], as seen for HCV NS5A protein [201]. HCV NS5A was found to bind to the dimerization site of PKR; in contrast, ANDV NP did not bind PKR. Thus, it remains to be understood how NP inhibits PKR dimerization.

7.4.2. Inhibition of Tetherin

As mentioned above, JUNV egress is restricted by tetherin, as shown by the retention of JUNV VLPs based on the expression of the Z protein by tetherin [111]. Interestingly, JUNV NP seems to antagonize tetherin's antiviral action, as overexpression of NP was able to rescue JUNV VLPs production. Moreover, JUNV NP tetherin antagonism was shown to be broad-spectrum. Indeed, its overexpression also led to the production of EBOV VLPs based on the expression of the matrix protein VP40, which are sensible to tetherin restriction activity. However, exactly how JUNV NP antagonizes tetherin remains to be determined.

8. Concluding Remarks

A plethora of host factors have been described to inhibit bunyaviruses infections, through mechanisms that vary depending on the host proteins but also on the targeted virus. Many restriction factors display a broad-spectrum antiviral activity, such as IFITMs, PKR, viperin or tetherin, as they target conserved features of virus replication such as virus entry, genomic replication, protein translation and virus egress. However, the role of hundreds of ISGs and host factors that are activated upon bunyavirus infection is still unknown. In the future, further genome-wide approaches, such as RNAi screens or CRISPR/Cas9 activation or knock-out screens will help to define important restriction factors that target bunyaviruses.

Gaining more knowledge about the strategies developed by bunyaviruses to escape the host antiviral state might be helpful to determine important and promising viral targets. Indeed, their inhibition could lead to the establishment of an efficient antiviral state that counteracts infection. The development of reverse genetic systems enabling the production of recombinant viruses or VLPs allowed the discovery of the role of some viral proteins, such as NSs of RVFV or the OTU domain of nairoviruses, which have a role in counteracting the innate immune responses. For phenuiviruses such as RVFV, whose NSs role in IFN-antagonism has been widely described, live attenuated virus vaccines in which NSs is deleted were developed and are much less virulent than the wild-type virus. The naturally attenuated mutant Clone 13 of RVFV has a deletion of 70% of its NSs gene and as many studies showed its immunogenicity and innocuity, it is therefore used as a live attenuated vaccine in livestock [202]. Other live attenuated vaccines are widely and safely used, such as the 17D strain of the yellow fever virus. One major obstacle of using live attenuated vaccines is the risk of their reversion to virulence, as seen for circulating vaccine-derived polioviruses (cVDPVs) [203]. However, Clone 13 does not revert to virulence upon experimental animal passage, suggesting that this risk under natural conditions is very low [204]. For CCHFV, it was recently shown that the stable occupancy of the CCHFV-encoded DUB with a Ub variant, blocking OTU DUB activity, blocked viral infection in addition to enhancing host antiviral responses [205]. Therefore, small antiviral molecules targeting its OTU domain could represent a promising area of antiviral research. These two examples provide elegant proofs of the great potential of targeting viral IFN-antagonists to design specific therapeutics or new efficient vaccines, and this area of research should be further developed.

Dedication

The authors dedicate this paper to the memory of our colleague and friend Professor Renaud Mahieux (1968–2020).

Author Contributions: Conceptualization, S.L.; writing—original draft preparation, S.L.; writing—review and editing, V.L., F.-L.C., N.F. and S.L.; supervision; F.-L.C. and V.L. All authors have read and agreed to the published version of the manuscript.

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Summary

As a tick-borne, zoonotic, emerging virus, Crimean-Congo hemorrhagic fever virus (CCHFV) poses a serious threat to public health. It is responsible in humans for a hemorrhagic disease with a mortality rate of 10-40%, with no specific treatment or vaccine. To date, the interactions between CCHFV and innate immunity are poorly understood. Notably, type I interferon (IFN) has a crucial role in the establishment of an antiviral state against CCHFV, probably through the transcription of IFN-stimulated genes (ISGs) with antiviral properties. However, it remains to be determined which ISGs modulate CCHFV infection and their mechanisms of action.

To address these questions, we performed a gain-of-function screen using CCHFV virus-like particles competent for entry and transcription of a reporter minigenome (tc-VLPs), a BSL-2 model of CCHFV, and lentiviral vectors encoding >500 human, >350 simian and >250 bovine individual cDNAs of ISGs. We identified several ISGs that modulate either negatively or positively tc-VLPs infection and that were previously undescribed in the context of CCHFV infection. To define their mechanisms of action, we dissected their impact on the different steps of the viral life cycle using various assays. We identified ISGs inhibiting the step of viral entry and a GTP-binding protein that promotes tc-VLP replication and/or transcription. Moreover, the effects of the overexpression of the ISGs were studied in the context of wild-type virus infection in BSL-4 conditions.

Collectively, these results provide new data on the interaction of CCHFV with host innate immune response components and pave the way for therapeutic countermeasures.

Résumé

Le virus de la fièvre hémorragique de Crimée-Congo (CCHFV) est un virus émergent zoonotique transmis par les tiques responsable chez l'homme d'une maladie hémorragique ayant un taux de mortalité de 10 à 40 %, sans traitement ni vaccin spécifique. À ce jour, les interactions entre le CCHFV et l'immunité innée sont mal connues. Notamment, l'interféron de type I (IFN) joue un rôle crucial dans l'établissement d'un état antiviral contre le CCHFV, probablement par la transcription de gènes stimulés par l'IFN (ISGs) présentant des propriétés antivirales. Cependant, il reste à déterminer quels ISGs modulent l'infection par le CCHFV et leurs mécanismes d'action.

Nous avons réalisé un crible en gain de fonction en utilisant des pseudoparticules virales compétentes pour l'entrée et la transcription d'un minigénome rapporteur (tc-VLPs), un modèle d'étude du CCHFV, et des vecteurs lentiviraux codant individuellement >500 ISGs humains, >350 ISGs macaques et >250 ISGs bovins. Nous avons identifié plusieurs ISGs qui modulent négativement ou positivement l'infection par les tc-VLPs. Pour définir leurs mécanismes d'action, nous avons disséqué leurs impacts sur les différentes étapes du cycle viral à l'aide de diverses méthodes. Nous avons identifié des ISGs inhibant l'entrée virale et une protéine de liaison au GTP qui promeut la réplication et/ou la transcription des tc-VLPs. De plus, les effets de la surexpression de ces ISGs ont été étudiés dans le contexte de l'infection par le virus sauvage.

Dans l'ensemble, ces résultats fournissent de nouvelles données sur l'interaction du CCHFV avec la réponse immunitaire innée de l'hôte et ouvrent la voie à des stratégies thérapeutiques.