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Interaction of Filovirus Proteins with Innate Immune Pathways

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To my loving husband.

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ABSTRACT

Ebola virus and Marburg virus are members of the Filoviridae family consisting of negative sense RNA viruses, known for their filamentous virion structure. These viruses have been attributed to cause large-scale, high mortality epidemics, which can cripple public health infrastructure, and lead to transnational issues reminiscent of the COVID19 pandemic. Rapid cellular multiplication, unrestrained inflammation and excessive interference with the antiviral innate immunity are the hallmarks of filoviral infections. The studies in this doctoral thesis have looked at the molecular pathways that VP35 and VP24 proteins of the filoviruses use in antagonising the RIG-I pathway signalling mechanisms and the downstream type I interferon-induced responses. Ebola virus VP35 has been shown to sequester the association of viral double-stranded RNA with PACT, which triggers the activation of RIG-I and phosphorylation of IRF3/IRF7 to induce IFN-α2 production. In addition,VP24 has been shown to bind to importin-α5 inhibiting translocation of interferon-induced, phosphorylated STAT1 to the nucleus and thereby inhibiting the expression of interferon-stimulated genes. New filoviruses have been discovered whose ability to inhibit or delay the innate immune responses is not yet fully understood. Study I in this thesis compares nine filovirus VP24 proteins for their inhibition of RIG-I pathway leading to IFN-β and IFN-λ1 promoter activation and IRF3 phosphorylation. Study II identifies the role of nine filoviral VP24 proteins in inhibiting interferon-induced pathway, while Study III expanded these findings by investigating the role of VP35 proteins of nine filoviruses on both RIG-I and interferon-induced pathway. The results obtained add to the knowledge of the innate immune inhibition capabilities of these viruses and could be utilised in the development of specific antiviral interventions that will assist the innate immune defences to reduce the pathogenicity of filoviruses.

KEYWORDS: Filovirus, RIG-I, VP24, VP35, IRF, IFN, Interferon-induced Signalling Pathway, Antivirals

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TIIVISTELMÄ

Ebolavirus ja Marburgvirus kuuluvat Filoviridae-heimoon. Filovirusten ominaispiirteisiin kuuluu negatiivinen, yksijuosteinen RNA-genomi, ja viruspartikkeleiden filamenttimainen rakenne. Filovirukset voivat aiheuttaa laajamittaisia, korkeaan kuolleisuuteen johtavia epidemioita, jotka voivat lamauttaa paikallisia terveydenhuoltojärjestelmiä ja johtaa Covid-19-pandemian kaltaisiin rajoja ylittäviin ongelmiin. Filovirusten nopea lisääntyminen, infektion aiheuttamat hallitsemattomat tulehdustilat ja filovirusten tehokkaat luontaisen immuunivasteen häirintämekanismit ovat filovirusinfektioiden tunnusmerkkejä. Tässä väitöskirjassa on tutkittu filovirusten VP35- ja VP24- proteiinien kykyä estää solun luontaisen immuniteetin RIG-I-välitteisiä ja interferonivälitteisiä signaalireittejä. Ebola-viruksen VP35 estää viruksen varhaisen tunnistuksen sitoutumalla viruksen kaksijuosteiseen RNA:han ja estämällä sen vuorovaikutuksen PACT-proteiinin kanssa, mikä normaalisti käynnistäisi RIG-I-reitin ja IRF3/IRF7:n fosforylaation johtaen IFN-α2:n tuottoon. Lisäksi Ebolaviruksen VP24-proteiinin on osoitettu sitoutuvan importiini-α5:een estäen fosforyloidun STAT1:n siirtymisen tumaan, mikä puolestaan estää interferonistimuloituvien geenien ilmentymistä. Uusia filoviruksia on löydetty mutta niiden kykyä estää tai viivästyttää luontaisen immuuniteetin vasteita ei vielä tunneta. Tämän väitöskirjan osajulkaisussa I vertaillaan yhdeksän filoviruksen VP24-proteiinin kykyä estää RIG-I signaalireittiä joka johtaa IRF3:n fosforylaation kautta IFN-β- ja IFN-λ1-promoottorien aktivaatioon. Osajulkaisussa II tutkitaan yhdeksän filoviruksen VP24-proteiinin kykyä estää interferonin indusoimaa signaalireittiä, ja osajulkaisussa III näitä havaintoja laajennettiin tutkimalla yhdeksän filoviruksen VP35-proteiinien kykyä estää sekä RIG-I-välitteistä että interferonivälitteistä signaalireittiä. Saadut tulokset lisäävät tietoa näiden virusten kyvystä estää luontaisen immuniteetin vasteita, ja tuloksia voitaisiin hyödyntää luontaista immuniteettia auttavien ja siten filovirusten patogeenisuutta vähentävien, antiviraalisten lääkeaineiden kehittämisessä.

AVAINSANAT: Filovirus, RIG-I, VP24, VP35, IRF, IFN, interferonivälitteinen signaalireitti, antiviraaliset lääkeaineet

Table of Contents

Abbreviations

ATP	Adenosine triphosphate
BDBV	Bundibugyo virus
BOMV	Bombali virus
BSA	Bovine serum albumin
CARD	Caspase Activation and Recruitment Domain
cDNA	Complementary DNA
CFR	Case Fatality Rate
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EBOV	Ebola virus
EDTA	Ethylenediaminetetraacetic acid
ESCRT	Endosomal Sorting Complex Required for Transport
EVD	Ebola virus disease
FBS	Fetal bovine serum
FCA	Freund's complete adjuvant
FIWIV	Fiwi virus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma-Activated Sequence
GP	Glycoprotein
GST	Glutathione S-transferase
HCV	Hepatitis C virus
HEK293	Human embryonic kidney 293 cell
HuH7	Human hepatoma 7 cell
HUJV	Huángjiāo virus
IFA	Immune fluorescent assay
IFN	Interferon
IFNAR	Interferon-alpha/beta receptor alpha chain
IFNGR1	IFN-γ receptor 1
IFNGR2	IFN-γ receptor 2
IFNLR1	Interferon Lambda Receptor 1

ISRE	Interferon-Stimulated Response Element
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IKK	Inhibitor of kappaB kinase
IL10R2	Interleukin-10 Receptor Subunit Beta
IMPA	Importin Alpha
IPTG	Isopropyl-β-thiogalactopyranoside
IRF	Interferon regulatory factor
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
ISG	Interferon-stimulated gene
JAK/STAT	Janus Kinase / Signal Transducer and Activator of Transcription
KDNV	Kander virus
KPN- α1	Karyopherin-α1
kD	Kilodalton
LGP2	Laboratory of Genetics and Physiology 2
LLOV	Lloviu virus
MARV	Marburg virus
MAVS	Mitochondrial antiviral signaling protein
MCP	Monocyte chemoattractant protein
MDA5	Melanoma differentiation-associated protein 5
MEGA 7	Molecular Evolutionary Genetics Analysis Computing Platform 7
MHC	Major histocompatibility complex
MLAV	Měnglà virus
MOA	Mechanism of Action
mRNA	Messenger ribonucleic acid
MUSCLE	Multiple Sequence Comparison by Log Expectation
MVD	Marburg virus disease
MxA	Myxovirus resistance protein
NES	Nuclear Export Signal (NES)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next-Generation Sequencing
NK	Natural killer cell
NLR	Nucleotide-binding oligomerisation domain-like receptors
NLS	Nuclear localisation Signal
NP	Nucleoprotein
NPC1	Niemann–Pick C1
OAS	Oligoadenylate Synthetase
OBLV	Oberland virus
PACT	PKR activating protein
PAMP	Pathogen-associated molecular pattern

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKR	Protein kinase R
PRR	Pattern recognition receptor
RAVV	Ravn virus
RdRp	RNA-dependent RNA polymerase
RESTV	Reston virus
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl suslphate polyacrylamide gel electrophoresis
sGP	Soluble glycoprotein
ss	Single-stranded
SsGP	Small soluble glycoprotein
STAT	Signal transducer and activator of transcription
SUDV	Sudan virus
TAPV	Tapajós virus
TAFV	Taï Forest virus
TBK1	TANK-binding kinase 1
TIM-1	T-cell immunoglobulin and mucin domain-1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
UTR	Untranslated region
VP	Viral protein
WB	Western blot
WHO	World Health Organisation
Wt	Wild type
XILV	Xīlǎng virus

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I. He, F. B., Khan, H., Huttunen, M., Kolehmainen, P., Melén, K., Maljanen, S., Qu, M., Jiang, M., Kakkola, L., & Julkunen, I. (2022). Filovirus VP24 proteins differentially regulate RIG-I- and MDA5-dependent type I and III interferon promoter activation. *Frontiers in Immunology*, 12, 694105. https://doi.org/10.3389/fimmu.2021.694105

II. Khan, H., Tripathi, L., Kolehmainen, P., Lundberg, R., Altan, E., Heroum, J., Julkunen, I., Kakkola, L., & Huttunen, M. (2023). VP24 matrix proteins of eight filoviruses downregulate innate immune response by inhibiting the interferon-induced pathway. *Journal of General Virology*, 104(8), 001888. https://doi.org/10.1099/jgv.0.001888

III. Khan, H., Mikkonen, U., Laakso, T., Syrjä, S., Julkunen, I., Huttunen, M., Kolehmainen, P., & Kakkola, L. (2026) *Inhibition of innate immune pathways by nine filovirus VP35 proteins* [Unpublished manuscript].

1 Introduction

The Filoviridae family has been of special scientific and public-health interest since the first outbreaks of the filoviruses reported in 1967 (Marburg virus) and in 1976 (Ebola virus). Both Ebola virus disease (EVD) and Marburg virus disease (MVD) have a high severity which is characterised by rapid onset, as well as high systemic inflammation, vascular leakage, coagulopathy and a high fatality rate (Umar & Diggle, 2025; Francis et al., 2025). The 2013–2016 West African Ebola virus epidemic was a turning point in filoviral ecological history as it illustrated the potential for a mere sporadic zoonosis to turn into a transnational emergency. Almost 27,000 people were infected and >11,000 died, showing the speed at which the filoviruses can spread when proper monitoring and early containment efforts fail (Spengler et al., 2016). Further outbreaks within the Democratic Republic of the Congo (DRC) and Uganda (Khan et al., 1995; Okware et al., 2002) continue to support the fact that the filovirus epidemics will not merely stand as historical events but tends to be a constant threat to public health.

The most noteworthy characteristic of filoviruses is their negative-sense single-stranded RNA genome which encodes seven structural and accessory proteins that have highly specialised functions during replication, assembly and immunosuppression. While other structural proteins are constitutive elements of the virion itself and assist in viral replication, the VP35 and VP24 proteins also have marked effects on survival by undermining the innate immune system on several organisational levels (Uwase et al., 2025).

The innate immune system is the first line of defence against virus infiltration which depends on germline-encoded pattern recognition receptors (PRRs) to perceive pathogen-associated molecular patterns (PAMPs) (Kawasaki & Kawai., 2014). Of these receptors, RIG-I-like receptor (RLR)-family comprises of RIG-I, MDA5 and LGP2; and plays a special role in the detection of negative-sense RNA viruses, including filoviruses. When filoviral signatures become traceable in the cell, RIG-I undergoes ATP-dependent conformational changes revealing its N-terminal CARD domain, which allows an interaction with the antiviral signalling protein MAVS, triggering a cascade of antiviral pathways (van Huizen & Gack, 2025).

Activation of MAVS on mitochondria and peroxisomes leads to the formation of protein complexes with adaptor proteins and kinases. These kinases phosphorylate transcription factors (IRF3/IRF7) that translocate to the nucleus and stimulate the transcription of type I interferons. Simultaneously, the nuclear factor kappa B (NF-κB) activation triggers the expression of pro-inflammatory cytokines, like interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and chemokines, which attract immune cells to site of infection. Secreted interferons increase the antiviral response via the JAK/STAT pathway and result in the transcription of interferon-stimulated genes (ISGs) that further inhibit viral replication, virion assembly and budding (Schoggins & Rice, 2011).

Ebola virus VP35 has been shown to be a versatile protein that is essential in viral RNA replication as a polymerase cofactor, and in immunosuppression as a potent antagonist of RIG-I signalling (Uwase et al., 2025). C-terminal interferon inhibitory domain (IID) of VP35 exhibits high affinity binding to the double-stranded RNA, thereby masking the detection of viral replication intermediates by RIG-I and MDA5 (Cárdenas et al., 2006; Leung et al., 2010). This is an extremely effective mechanism for inhibiting PRR activation, that in turn also inhibits the initial interferon triggering which detects viral entry.

Ebola virus VP24 has been shown to inhibit the cellular response to interferons that are produced by the infected cells. VP24 shows specificity to the JAK/STAT pathway by attaching to importin-α5 and preventing nuclear translocation of phosphorylated STAT1 (Reid et al., 2006). This inhibits the establishment of the ISGF3 complex (STAT1–STAT2–IRF9) that is necessary to activate the transcription of ISGs. In addition, VP24 and VP35 proteins also have a role in modulating antigen presentation, regulation of cytokine production and dendritic cell maturation, which lead to a severe immune paralysis (Ramanan et al., 2011; Lubaki et al., 2016).

The effects of VP35 and VP24 proteins lead to profound innate immune suppression and a high degree of viral replication before the adaptive immune system is able to generate a response. Despite investigation of the immunobiology of filoviral infections, a significant knowledge gap exists especially on the immunomodulatory effect of the all currently known mammal-infecting filoviruses (Lu et al., 2022; Uwase et al., 2025). This doctoral thesis investigated the role of all mammalian-infecting filovirus VP24 and VP35 proteins and provides in-depth insights into the mechanisms of interaction between filoviral proteins and innate immune pathways. The results obtained in this thesis provide, in addition to the understanding of filovirus interactions with immune responses, also knowledge for designing antiviral drugs and immunomodulating therapies for counteracting the spread and severity of filoviral infections.

2 Review of the Literature

2.1 Filoviruses

2.1.1 Historical perspective

Filoviridae is a family of filamentous and negative-sense, single-stranded, RNA viruses, which are clinically associated with repeated hemorrhagic fever outbreaks in sub-Saharan Africa (Biedenkopf et al., 2024; Languon & Quaye, 2021). The spread and pathogenesis of filoviruses illustrate fundamental challenges in sustainable global pathogen containment, as viral immune evasion and high case fatality rates intersect with delayed surveillance, healthcare system vulnerability, and limited access to effective countermeasures (Munyeku-Bazitama et al., 2024).

The first known filovirus outbreak was reported in 1967 when laboratory workers in Marburg (Germany), Frankfurt (Germany) and Belgrade (Serbia) simultaneously contracted a previously undocumented type of hemorrhagic fever while handling tissues from African green monkeys (*Chlorocebus aethiops*) imported from Uganda (Slenczka, 2017). In 1976, two more lethal haemorrhagic fever epidemics occurred in Yambuku (Zaire or Democratic Republic of the Congo) and Nzara (Sudan), which led to the discovery of Ebola virus (EBOV) and commenced the "Ebola" nomenclature (Breman et al., 2016).

2.1.2 Taxonomic Classification

According to the International Committee on Taxonomy of Viruses (ICTV) the Filoviridae family has eight genera (Table 1, Figure 1).

The emergence of novel filoviruses during the last two decades has highlighted a dynamic evolution of Filoviridae family. In addition, the incidences of Lloviu virus (LLOV) in Europe and Měnglà virus (MLAV) in China, have demonstrated geographic spread of filoviruses, and have challenged the initial presumption of their African-centric ecology (Yang et al., 2019; Kemenesi et al., 2018). Also, the identification of Bombali virus (BOMV) in bats (Goldstein et al., 2018) has pointed towards a hidden virosphere beyond human epidemics. This virosphere does not only mark the infectivity of filoviruses in organisms beyond human beings, but also as a

source of human exposure to these pathogens. Due to factors like deforestation, wildlife habitat loss, and bush-meat hunting, there has been a large increase in human exposure to zoonotic transmission (Mahanta & Sulabh, 2025).

Table 1 Key filoviral genera, species, host organisms (including natural hosts and laboratory animal models), causes of human exposure, and scientific publications. Data gathered from Biedenkopf et al., 2024 and ICTV webpages, available at: https://ictv.global/

Genus	Species	Host	Human Exposure	Publication
Orthoebolavirus	EBOV, BOMV, BDBV, RESTV, SUDV, TAFV	Bats, Domestic Pigs; Laboratory Exposure (Rodents, Nonhuman Primates, Carnivores (domestic ferrets), and Suids (domestic pigs)	Direct contact with blood, body fluids, or injured skin	Marsh et al., 2011; St Claire et al., 2017; Siragam et al., 2018; Kuhn et al., 2020
Orthomarburgvirus	MARV, RAVV	Egyptian Rousettes (Bats); Laboratory Exposure (Rodents, Nonhuman Primates)	Direct contact with blood, body fluids, or injured skin	Kuhn et al., 2020
Cuevavirus	LLOV	Bats (*Miniopterus schreibersii*)	Not reported	Kemenesi et al., 2018, Sun et al., 2022, Tóth et al., 2023
Dianlovirus	MLAV	Bats (*Rousettus sp.*)	Not reported	Yang et al., 2017, Yang et al., 2019, Makenov et al., 2023
Tapjovirus	TAPV	Snakes (*Bothrops atrox*)	Not reported	Horie 2021
Striavirus	XILV	Frogfish (*Antennarius striatus*)	Not reported	Shi et al., 2018
Thamnovirus	KDNV, FIWIV, HUJV	Horse-faced Filefish (*Thamnaconus septentrionalis*), European perch (*Perca fluviatilis*)	Not reported	Shi et al., 2018, Hume and Mühlberger 2019, Hierweger et al., 2021
Oblavirus	OBLV	European perch (*Perca fluviatilis*)	Not reported	Hierweger et al., 2021

2.1.3 Epidemiology of Filoviral Infections

Ebola, Sudan and Marburg virus infections have caused rare but high-fatality outbreaks in sub-Saharan Africa, mainly due to ecological disruption resulting in animal-human contact, bushmeat consumption, food and water contamination, poverty, and insufficient healthcare infrastructure (Stephens et al., 2022). The epidemiology and severity of major outbreaks mainly concern pathogenicity of viral species, transmission dynamics (ease of human exposure), and healthcare system resilience.

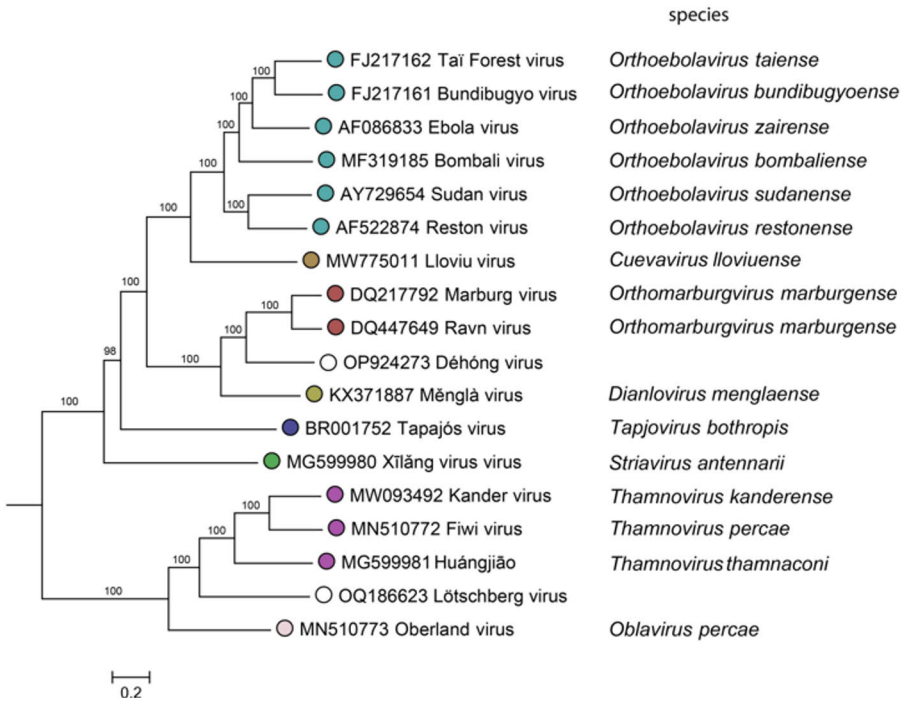


Figure 1 Taxonomic Classification of Filoviridae family. The different genera of this family are colour coded in the figure (Reproduced from "ICTV Virus Taxonomy Profile: Filoviridae 2024," by N. Biedenkopf et al., 2024, The Journal of General Virology, 105(2), Article 001955 (https://doi.org/10.1099/jgv.0.001955). CC BY 4.0.)

The first documented EVD outbreaks in 1976 in Yambuku (Zaire) and Nzara (Sudan), involved EBOV and Sudan virus (SUDV) with case fatality rate (CFRs) of 88% and 53% respectively, demonstrating virulence differences in the lineage (Breman et al., 2016). The clinically most significant EBOV outbreak in West Africa occurred between 2013 and 2016, infecting over 28,000 people and killing >11,000 people (Ohimain & Silas-Olu, 2021). The outbreak also transformed filovirus epidemiology from localised self-limiting clusters to a transnational public health concern exacerbated by urban transmission, global mobility, and delayed containment.

The Marburg virus disease (MVD), that first emerged in 1967 in European laboratory workers, has also re-emerged irregularly in sub-Saharan Africa, particularly in Angola (2005), Uganda (2012, 2014, 2017), and Tanzania (2023) with very high CFRs of more than 80% (Srivastava et al., 2023). These outbreaks highlighted that ecological and infrastructural vulnerabilities exacerbate epidemics and influence filoviral transmission at a larger scale.

It has been known that zoonotic reservoirs and spillover dynamics (leading to human exposure) are important to filoviral spread. Studies of transmission have established that direct contact with infected bats or intermediary hosts like non-

human primates can lead to clinically relevant exposure, which can be followed by human-to-human transmission via body fluids or contaminated fomites (Hood & Carroll, 2024).

Infectious virus may persist in body fluids for over a year after recovery, demonstrating that transmission risk extends well beyond the acute phase. For example, the prolonged presence of virus in semen—up to 500 days after recovery underscores the need to consider long-term transmission risks in disease control strategies and highlights the role of behavioural factors in sustaining transmission beyond acute illness (Fischer et al., 2017; Keita et al., 2017).

2.2 Virion and Genome Organisation

2.2.1 Filovirus Morphology

Viruses in the Filoviridae family have unique virion morphology and genome structure. The virus particles are filamentous, pleomorphic and flexible, while typically 80 nm in diameter and 800–1,400 nm in length with forms up to 14,000 nm observed under electron microscopy (Geisbert & Jahrling, 1995).

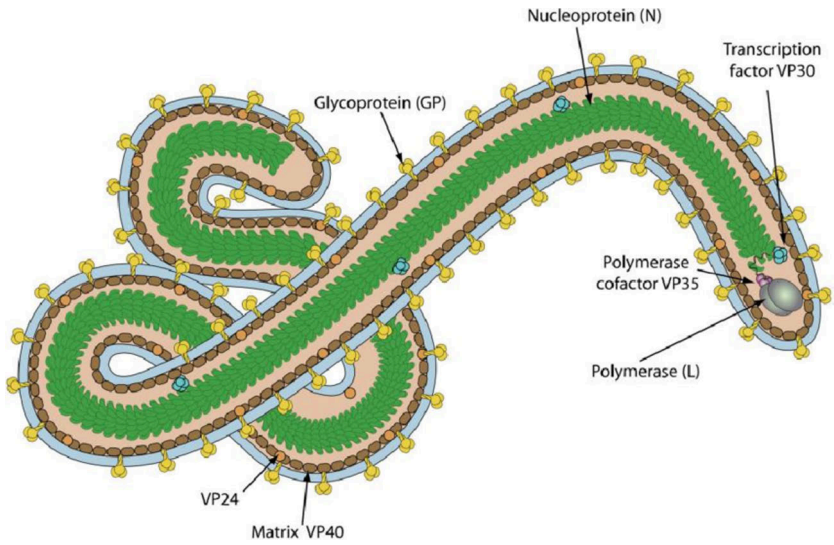


Figure 2 Morphological structure of the filovirus virion (Reproduced from ViralZone, (https://viralzone.expasy.org/23). CC BY 4.0.)

The virion also has a distinctive "shepherd's crook" or branching morphology (Figure 2) which is caused by filamentous nucleocapsid, wrapped in a lipid sheath containing viral glycoproteins (GPs). A matrix layer formed by viral proteins 40 and

24 (VP40 and VP24) mediates virion assembly and budding, while a central nucleocapsid complex contains the negative-sense RNA genome encapsidated by the nucleoprotein (NP) and associated with VP35, VP30, and the large (L) polymerase (Hu & Noda, 2023).

2.2.2 Filovirus Life Cycle

The filovirus life cycle in host cells (Figure 3) is tightly coordinated and dynamically regulated by integrating complex viral macromolecule-host cell machinery interactions to ensure efficient replication, assembly and dissemination (Ibrahim et al., 2024).

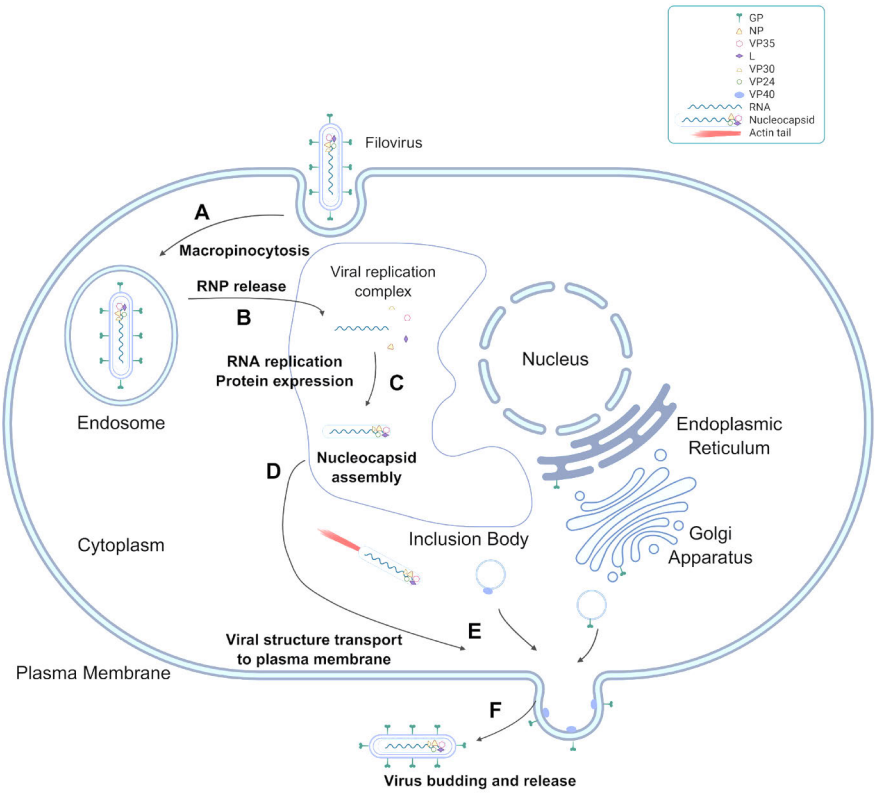


Figure 3 Schematic representation of the filovirus replication cycle. The filovirus infection cycle begins when filovirus particles enter the host cell (A). After entry, the ribonucleoprotein (RNP) complex is released (B). Inside the host cell, the virus replicates its RNA and expresses viral proteins (C). The viral protein VP24 mediates the nucleocapsid assembly (D). The nucleocapsid is then transported to the plasma membrane, while viral proteins VP40 and GP are delivered to the same site via vesicular transport (E). Finally, new viral particles bud and exit from the host cell (Reproduced from "Direct Intercellular Transport Mode of Filovirus Nucleocapsids" by Ibrahim, C.; Oda, H.; and Takamatsu, Y., Int. J. Mol. Sci. 2024, 26(17), 8485; used under CC BY 4.0.).

The viral surface glycoprotein (GP) is the main factor in filovirus tropism and infectivity upon entry into susceptible host cells. Host cell receptors, C-type lectins (DC-SIGN, L-SIGN), TIM-1, and Niemann–Pick C1 (NPC1), which is an endolysosomal cholesterol transporter necessary for membrane fusion, support the attachment of GP1 subunit (Alvarez et al., 2002; Kondratowicz et al., 2011; Carette et al., 2011).

Filoviruses use actin-dependent and non-clathrin-mediated macropinocytosis to internalise virions in endosomes after receptor engagement, after which the acidic endosomal environment causes cathepsins B and L to cleave GP1. This cleavage exposes receptor-binding regions that engage NPC1 and causes viral-endosomal membrane fusion (Côté et al., 2011). The cycle of replication begins after the successful entry of ribonucleoprotein complex (RNP) into the cytoplasm of the host cell (Dolnik & Becker, 2022).

Endosomal fusion results in uncoating and genome release, which involve regulated disintegration of the viral matrix protein VP40 and exposure of the nucleocapsid that contains nucleoprotein (NP), VP35, VP30 and the large RNA-dependent RNA polymerase (Winter et al., 2023). Filoviruses retain a partially compacted nucleocapsid shape following cytoplasmic release which often allows replication while conserving genetic integrity. VP35 stabilises nucleocapsids as a polymerase cofactor and interferon antagonist while the NP coordinated genomic encapsidation provides the scaffold for transcription (Noda et al., 2011).

The viral polymerase complex (L–VP35–VP30) mediates transcription and replication. The 19-kb negative-sense RNA genome is transcribed from the 3′ leader region to yield seven monocistronic mRNAs for the genes np, vp35, vp40, gp, vp30, vp24, and l (Mühlberger et al., 2007). Polymerase reinitiation inefficiency leads to a transcriptional gradient, which aligns with functional demand by producing more structural proteins than enzymatic proteins. Following the production, VP35 stabilises nascent transcripts while VP30 resolves secondary RNA structures at the GP gene to enable transcriptional elongation (Bach et al., 2021).

Transcription switches to replication after sufficient NP-encapsidation of nascent antigenomes, promoting the synthesis of full-length positive-sense antigenomic RNA and offspring negative-sense genomes. The replication complexes are located in cytoplasmic inclusion bodies that are virus-induced membrane-less organelles and concentrate viral and host components (Kolesnikova et al., 2000), optimising RNA synthesis and protecting replication intermediates from innate immune detection. Intracellular lifecycle concludes by the release of newly formed virions through the process of budding using filopodia (Hoenen et al., 2012).

2.3 Filoviral Proteins

2.3.1 Nucleoprotein (NP)

The structural and functional core of a filoviral nucleocapsid is the nucleoprotein (NP), which forms a helical ribonucleoprotein complex that scaffolds genome packaging, provides a transcriptional template, and assists viral replication by encapsulating the 19 kb negative-sense RNA genome. NP recruits the polymerase complex (L, VP35, and VP30) and is essential for nucleocapsid assembly on a functional level (Huang et al., 2002).

The α-helical core and extended N-terminal arm of NP facilitates oligomerisation and RNA binding via electrostatic interactions with the phosphate backbone (Hu et al., 2023). Unlike other Mononegavirales, filoviral NP forms a tightly packed helix that inhibits RNA accessibility, leading to a structural modification for minimising identification by cytosolic pattern recognition receptors like RIG-I and MDA5 (Hu et al., 2023).

Figure 4 Diagrammatic representation of the protein sequences in the EBOV genome.

2.3.2 Polymerase (L) and Cofactors VP30 and VP35

The RNA-dependent RNA polymerase (L) protein serves as the transcription and replication centre. This protein has conserved domains such an RNA polymerase domain, a capping domain with a methyltransferase activity, and an endonuclease-like module to coordinate viral mRNA synthesis and post-transcriptional processing (Li et al., 2025).

VP35 and VP30 are the structural stabilisers and transcriptional regulators within the filovirus–polymerase complex. VP35 is a cofactor for the activity of large (L) protein and VP35 also inhibits RIG-I–like receptor activation and thereby suppresses interferon synthesis (Bodmer et al., 2024) 7). In conjunction, VP30 facilitates initiation of viral genome transcription. This mediates the switch of polymerase between replication and transcription, as dephosphorylated VP30 stimulates mRNA transcription initiation at gene start sites, while phosphorylated VP30 represses them (Jain et al., 2021).

2.3.3 Glycoprotein (GP) and Secreted GP (sGP)

In EBOV, glycoprotein (GP) in filoviruses controls host cell entrance, tissue tropism and immunological recognition. During the infection cycle, several isoforms of the GP are produced by the via transcriptional editing, which results in the production of several soluble membrane-bound GPs (Jain et al., 2021). In contrast to other filoviruses, MARV does not employ RNA editing for GP expression, while encoding for a single GP product (Mehedi et al., 2023). During transcription of the GP gene, viral RNA-dependent RNA polymerase inserts an additional adenosine at a defined editing site, leading to the editing of the mRNA reading frame, which generates distinct mRNA species that encodes full-length transmembrane GP1,2 (Volchkova et al., 2011). These matured filoviral GPs are cleaved into GP1 and GP2 which form heterodimers that act as trimers to attach receptors, which form a trimeric spike on the virion surface that mediates receptor binding and membrane fusion (Rutten et al., 2020).

The functional components required for endosomal fusion of GP via NPC1 and cathepsin B/L pathway are produced by host furin's cleavage of GP1,2. Secreted glycoprotein (sGP) which is translated from the unprocessed GP-mRNA acts as an immune decoy by binding anti-GP antibodies in the circulation and diverting humoral reactions (Mohan et al., 2012). sGP functionally controls endothelial permeability and pro-inflammatory signalling by the inhibition of cytokine production from the macrophages (Bradley et al., 2018). Thereafter, RNA editing to co-express GPs and sGP exhibits as a complex mechanism for facilitating viral entry and immune evasion.

2.3.4 VP40 – Matrix Protein

VP40 matrix protein is a key structural component of virion morphology. It regulates the nucleocapsid-plasma membrane interface and virion assembly through self-oligomerisation and host endosomal sorting complex required for transport (ESCRT) machinery (Hoenen et al., 2010). VP40 binds viral RNA for a regulation of viral transcription, which establishes VP40 function as a structural scaffold and post-transcriptional modulator (Hoenen et al., 2010). Thus, its necessity for virion morphogenesis makes VP40 a key target for antiviral mechanisms that impair viral assembly.

2.3.5 Viral Proteins (VP) – VP24 and VP35

VP24 and VP35 are important proteins for innate immune antagonism and replication regulation. Filoviral VP24 is a minor structural protein but has key functions in nucleocapsid organization, virion assembly and immune antagonism. It has been shown that EBOV VP24 inhibits both type I and type II interferon-induced

signalling by preventing phosphorylated STAT1 nuclear translocation (Basler et al., 2009). Besides immune antagonism, VP24 helps nucleocapsid condensation and stability by creating lateral connections with NP to maintain genome complex helical architecture (Banadyga et al., 2017).

VP35, on the other hand, is a polymerase cofactor that binds with NP and L proteins and has strong antiviral innate response suppressing activity (Bodmer et al., 2024). The C-terminal interferon inhibitory domain (IID) of VP35 can bind double-stranded RNA with great affinity, protecting viral replication intermediates from cytosolic sensors (Leung et al., 2009). VP35 also inhibits IRF-3 phosphorylation, impairing cellular antiviral signalling pathways (Prins et al., 2009). Together, the mechanism of action (MOA) of VP24 and VP35 constitute an effective innate immune evasion of filoviruses.

2.4 Innate Immunity

The ability of the host to identify and eradicate infectious agents like filoviruses depends on the immune system, constituted by innate and adaptive immunity. The first line of defence is innate immunity, which uses physical barriers, pattern recognition receptors (PRRs), interferon production, and antiviral interferon-stimulated genes (ISGs) (Thompson et al., 2011; Wang et al., 2024). Early recognition activates inflammatory cascades and antiviral signalling pathways, limiting viral replication and alerting the adaptive immune system. T- and B-cells of adaptive immunity provide long-term antigen-specific protection due to immunological memory and clonal expansion, that allow the body to respond more precisely and effectively to the same pathogen (Alberts et al., 2002).

The innate immune system of the host primarily provides immediate, non-specific response to identify, restrict, and engulf pathogenic invaders via coordinated cellular, molecular, and signalling networks (Lu et al., 2022). It uses germline-encoded receptors and effector molecules that recognise pathogen-associated molecular patterns (Wang et al., 2024). The lack of specificity, however, makes it vulnerable to immune evasion strategies established by viruses which undermine immune sensing and inhibit antiviral signalling cascades (Weber, 2021).

2.4.1 Sensing Viral RNA - PRR, RLR Pathway and Upstream Events

2.4.1.1 RIG-I and MDA5

PRRs, e.g., RIG-I-like receptor (RLR) family, identify viral nucleic acids and activate antiviral signalling cascades in the innate immune system. Retinoic acid-

inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are key cytoplasmic members of the RLR family which sense viral RNA in the cytoplasm (van Huizen & Gack, 2025). These PRRs are important within the innate defence response because RIG-I detects short dsRNA or ssRNA with 5′-triphosphate or diphosphate moieties, while MDA5 recognises long dsRNA molecules that is commonly formed as an intermediate in viral replication (Figure 5).

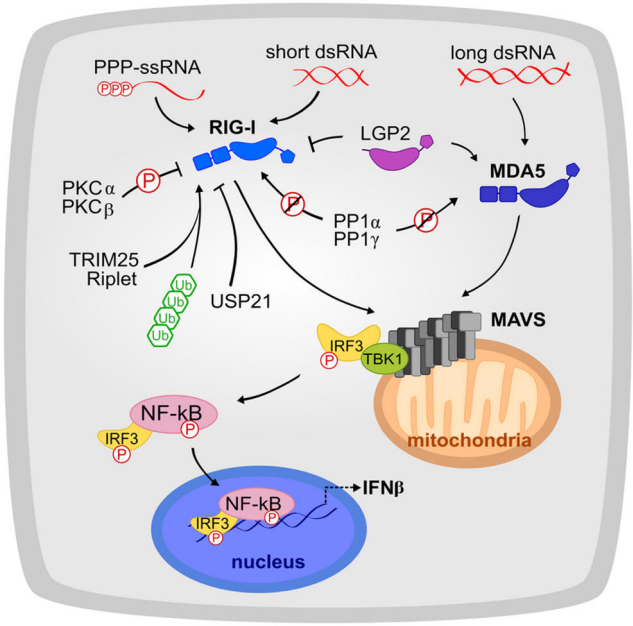


Figure 5 Schematic representation of RIG-I like receptor (RLR) pathway (Reproduced from "Pattern Recognition and Signaling Mechanisms of RIG-I and MDA5," by S. Reikine, J. B. Nguyen, and Y. Modis, 2014, Frontiers in Immunology, 5, Article 342 (https://doi.org/10.3389/fimmu.2014.00342). CC BY 4.0.)

Type I interferons and pro-inflammatory cytokines produced by triggering of these receptors support the host defence system against the RNA viruses such as filoviruses (Figure 5). Structurally, two N-terminal caspase activation and recruitment domains (CARDs), a central DExD/H-box RNA helicase domain, and a C-terminal ligand-specific repressor domain are conserved in RIG-I and MDA5 (Luo et al., 2021). The CARD domain of RIG-I is exposed for downstream signalling when ATP hydrolysis activates it upon viral RNA recognition. The CARD domains of MDA5 mediate antiviral signaling by interacting with the CARD domain of MAVS on mitochondria. Upon viral double-stranded RNA recognition, MDA5 CARDs oligomerize and activate downstream signaling, leading to type I interferon and pro-inflammatory cytokine production.

RIG-I detects the 5' phosphate groups of RNA whereas MDA5 forms filamentous structures along dsRNA molecules to induce antiviral signalling (Jiang et al., 2011). TRIM25 and Riplet are E3 ubiquitin ligases that positively regulate RIG-I mediated interferon production by catalyzing K63-linked ubiquitination of RIG-I, a modification required for its CARD-domain activation and downstream MAVS signaling (Versteeg et al., 2013; Oshiumi et al., 2009).

PRRs suppression is important to filovirus survival, as PRRs have a major antiviral role. In the cells where RIG-I or MDA5 are present but suppressed by the virus, filovirus replication is enhanced, resulting in increased production of infectious progeny virus and delayed induction of type I interferon responses. Contrarily, enhanced expression of RIG-I can cause hyper-inflammation and tissue damage (Adsonk et al., 2012; Shi et al., 2020). RIG-I and MDA5 not only induce interferon responses but also intersect with inflammasome and autophagy pathways, that reflects their role also in immune-metabolic regulation during infection (Deretic, 2021). This demonstrates that filoviruses have adapted not only to undermine antiviral cellular responses but also compromise mechanisms which can lead to programmed cell death that can interfere with the course of filoviral replication cycle.

2.4.1.2 Downstream Adaptors – MAVS, TRAFs and Kinases

Intracellular cascade involving both RIG-I and MDA5 merge on shared signalling mediator, mitochondrial antiviral signalling (MAVS) protein, which is the key adaptor linking viral RNA sensing to antiviral gene transcription. MAVS proteins are molecular scaffolds that combine numerous signalling modules on the outer mitochondrial membrane (Fitzgerald et al., 2003; Oganesyan et al., 2006; Guo & Cheung, 2007; Michallet et al., 2008).

The exposed domains of activated RIG-I or MDA5 engage with MAVS through CARD-CARD homotypic interactions, forming MAVS interactome that activates signal transduction. This interactome recruits downstream effectors like tumour necrosis factor (TNF) receptor-associated factors (TRAF3, TRAF6) and kinase complexes (TBK1 and IKKε) which phosphorylate nuclear transcription factors i.e., interferon regulatory factor-3 and -7 (IRF3, IRF7), and NF-κB (Jacobs & Coyne, 2013).

MAVS signalling is closely monitored at the molecular and organelle levels to ensure that immune activation does not come at the expense of mitochondrial stress or cell death. Spatial localisation of MAVS on mitochondria, peroxisomes, and mitochondria-associated membranes (MAMs) determines the nature and timing of antiviral responses i.e., peroxisomal MAVS signalling drives rapid interferon-

independent responses, while mitochondrial MAVS activates a sustained type I interferon production for a long-term antiviral defence (Castanier et al., 2012).

In addition, MAVS-dependent pathways are controlled by host post-translational processes such as ubiquitination, phosphorylation, and proteolytic cleavage. E3 ubiquitin ligases, TRAF3 and TRAF6, promote Lys63-linked polyubiquitination of MAVS and TBK1, facilitating downstream signal transmission (Liu et al., 2013). MAVS ubiquitination, phosphorylation and proteolytic cleavage serve as regulatory mechanisms that fine-tune antiviral innate immune signaling by controlling MAVS activation, aggregation, and degradation (Dong & Shen, 2024; Zhao et al., 2025).

2.4.1.3 Activation of IRF3, IRF7 and NF-κB

The transcription factors IRF3, IRF7, and NF-κB are activated at the end of RLR-MAVS signalling, leading to the transcription of antiviral and interferon genes. TBK1 and IKKε phosphorylate serine residues in the C-terminal regions of cytoplasmic transcription factors IRF3 and IRF7 (Liu et al., 2015). This modification increases the dimerisation, nuclear translocation, and DNA binding to target gene promoters of these transcription factors. IRF3 mediates the initial interferon response while IRF7 amplifies it in a feed-forward loop. Both promote the transcription of IFN-α, -β, and -λ, and also ISGs which guide antiviral activity in infected and neighbouring cells (Bourdon et al., 2025).

Following their activation, phosphorylated IRF3 and IRF7 undergo dimerisation and are translocated into the nucleus through importin-dependent nuclear transport mechanisms. Once in the nucleus, these dimers bind interferon-stimulated response elements (ISREs) within the promoters of target genes, initiating transcription of type I and type III interferons and downstream interferon-stimulated genes (Lin et al., 2000; Hiscott, 2007).

2.4.2 Interferons in Antiviral Defence

2.4.2.1 Types of Interferons and Their Receptors

Interferons are important to the innate antiviral defence because they mediate communication between infected and uninfected cells. These highly conserved immune system components are divided into three main classes i.e., type I, type II and type III IFNs based on molecular structure, receptor utilisation, cellular targets, and functional results (Wang et al., 2024). These classes constitute a complex and dynamic signalling network that allows the host to respond to viral infection.

In humans, type I Interferons consist of IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω subtypes. After viral infection, nearly all nucleated cells release some of these

cytokines, which bind to heterodimeric receptor complex comprising IFNAR1 and IFNAR2 subunits. The IFNAR complex activates Janus kinases (JAK1 and TYK2) which initiate phosphorylation cascades that activate STAT proteins, particularly STAT1 and STAT2 (Figure 6). The STAT proteins along with IRF9 create the ISGF3 complex that translocates into the nucleus to stimulate ISGs by binding to ISREs (Lazear et al., 2019). The ISGs encode many antiviral effectors including myxovirus resistance protein A (MxA), oligoadenylate synthetase (OAS), and protein kinase R (PKR) which directly block viral replication from genome transcription to protein synthesis (Sadler & Williams, 2008; Schoggins & Rice, 2011).

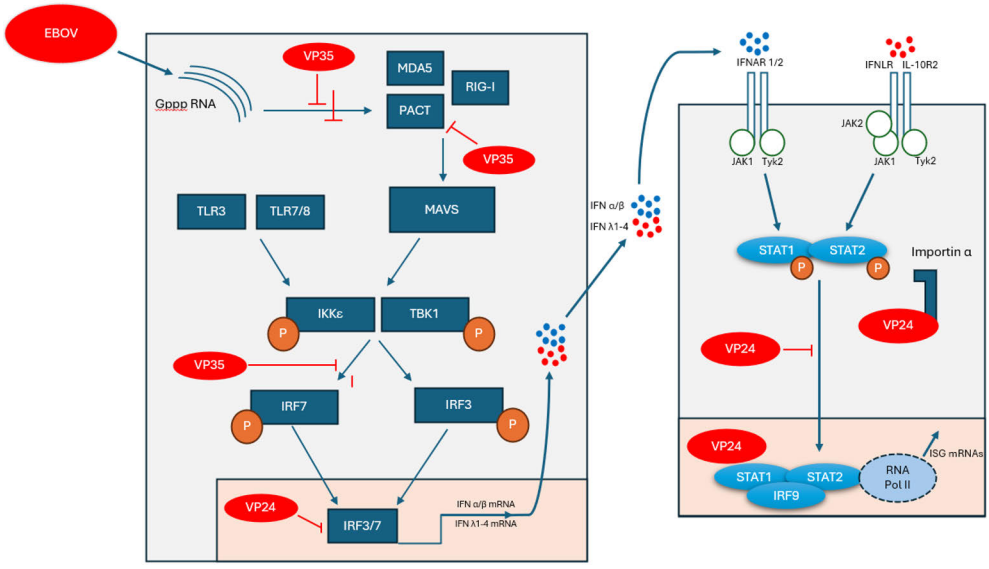


Figure 6 Mechanisms of type I and type III interferon production inhibition by EBOV via the actions of VP24 and VP35 filoviral proteins. VP35 blocks RIG-I signalling via dsRNA, PACT, degradation of IRF3/7 and phosphorylation of IKKε. VP24 inhibits IFN production downstream of IRF3/7, in addition to blocking STAT1-STAT2 nuclear import by binding to importin-α and inhibiting ISGs. This figure is adapated from Figure 3 in He et al., 2019.

Type II interferon (IFN-γ) is primarily released by activated T and NK cells, bridging innate and adaptive immunity via a receptor complex comprising IFN-γ receptor 1 and 2 (IFNGR1,2). It activates the JAK1/JAK2–STAT1 pathway and leads to expression of genes that improve antigen presentation, macrophage activation, and microbial clearance. IFN-γ has less direct antiviral activity than type I IFNs but it plays an important role in establishing the immunological milieu that impacts infection outcomes promoting pro-inflammatory macrophage polarisation (Tugal et al., 2013) and limiting viral transmission (Verhoeven et al., 2016).

Type III interferons (IFN-λ) are a relatively recently identified class of IFNs (Kotentko et al., 2003) that use the interferon lambda receptor 1 (IFNLR1) and interleukin-10 receptor subunit beta (IL10R2) receptor complex. Unlike type I IFNs, type III IFNs only act on epithelial and mucosal cells due to tissue-specific IFNLR1 expression. The binding of IFN-λ to its receptor activates the similar JAK-STAT dependent signaling cascade as type I IFNs, leading to expression of ISGs (de Weerd et al., 2024).

2.4.3 Modulation of IFN Gene Expression by Filovirus VP35

VP35 is a key interferon antagonist encoded by filoviruses. EBOV and MARV VP35 has been shown to act as a viral polymerase cofactor for replication and an immune evasion protein that suppresses IFN expression at several levels (Leung et al., 2010). Structurally, VP35 contains an N-terminal coiled-coil domain responsible for oligomerization and interaction with NP, and a C-terminal interferon inhibitory domain (IID) that binds double-stranded RNA with high affinity. Through this dsRNA-binding activity, VP35 masks viral RNA from cytosolic pattern recognition receptors such as RIG-I and MDA5, thereby preventing initiation of downstream antiviral signaling and inhibiting interferon gene expression (Kimberlin et al., 2010).

The basic patch on IID of VP35 binds to the phosphate backbone of dsRNA blocking RIG-I and MDA5 recruitment preventing MAVS activation and IRF3 phosphorylation which are essential steps for IFN-β production (Cárdenas et al., 2006). VP35 also directly inhibits RIG-I activation by binding to RIG-I cofactor PACT (cellular RNA-binding protein), which inhibits RIG-I ATPase activity and downstream signalling, further suppressing interferon induction (Luthra et al., 2013). Besides hiding viral RNA, VP35 suppresses innate immune pathway by inhibiting kinases TBK1 and IKKε, thereby blocking IRF3 and IRF7 phosphorylation. Kinase inhibition prevents nuclear transport of IRFs, resulting in suppression of IFN-β gene transcription (Prins et al., 2009).

2.4.4 Interferon Signalling and Antiviral Effector Pathways

2.4.4.1 JAK/STAT pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is the main route via which IFNs perform their antiviral, immunomodulatory and replication-suppressive actions. This pathway converts extracellular interferon signals into a precise transcriptional program that promotes hundreds of antiviral ISGs (Nan et al., 2017). The effectiveness of antiviral activity depends on the integrity of the JAK/STAT signalling cascade; disruption of this

cascade by viral antagonists undermines induction of interferon-stimulated genes (Nan et al., 2017).

The JAK/STAT pathway is initiated with the binding of type I (α/β) and type III (λ) IFNs to their receptor complex (IFNAR1 and IFNAR2, and IFNLR1 and IL10R2, respectively) on the cell surface. The ligand interaction causes conformational rearrangement that reciprocally phosphorylates receptor-associated tyrosine kinases JAK1 and TYK2 (Ank et al., 2006; Shemesh et al., 2021). Activated JAK1 and TYK2 phosphorylate specific tyrosine residues on the cytoplasmic tails of IFNAR1 and IFNAR2. STAT2 binds directly to these receptor phosphotyrosines via its SH2 domain, and STAT1 is recruited through interaction with STAT2 at the receptor complex, where both STATs are subsequently phosphorylated by the JAK kinases (Platanias L. C., 2005).

JAKs phosphorylate STATs that dimerize and form the ISGF3 complex, which includes the transcription factors (STAT1/STAT2) and interferon regulatory factor 9 (IRF9) (Darnell, 1997; Schindler et al., 2007). The nuclear import of ISGF3 complex is preceded by the phosphorylation of STAT1/STAT2, which exposes functional NLS, thereby enabling ISGF3 to interact with importin-α/β and translocate through the nuclear pore complex. Once inside the nucleus, ISGF3 binds to ISREs in the promoters of the corresponding genes which are then activated. When expressed these genes mediate antiviral, antiproliferative and immunomodulatory effects (Hu et al., 2021).

2.4.4.2 IFN-stimulated genes

ISGs are the effector arm of IFN system i.e., ISGs translate upstream signalling into antiviral, immune-modulatory, and apoptotic effects that limit viral replication. ISGs are the functional endpoints of JAK/STAT pathway, forming a broad repertoire of antiviral effectors that limit viral replication. ISREs or Gamma-Activated Sequence (GAS) promoter elements regulate over 400 genes in the ISG landscape and their coordinated activity dictates suppression of viral infection through the course of viral life cycle (Schoggins & Rice, 2011).

The coordination of ISG induction balances antiviral activity and host survival. ISGs inhibit viral life cycle phases via multiple ways depending on the individual. MxA, one of the ISGs that is a GTPase, has a defence mechanism that is effective against many negative-sense RNA viruses, including filoviruses. It halts viral replication by interfering with nucleocapsid assembly and genome replication (Nigg & Pavlovic, 2015). Another important ISG; PKR, phosphorylates eukaryotic translation initiation factor 2A (eIF2α), which causes translational failure, resulting in the inhibition of viral propagation by blocking viral and host protein synthesis (Harding et al., 2000).

The antiviral OAS1–RNase L pathway is activated upon detection of cytosolic double-stranded RNA. OAS1 synthesizes 2′–5′ oligoadenylates that bind to and activate RNase L, which subsequently degrades viral and cellular RNA, thereby restricting viral replication (Malathi et al., 2007). Filoviruses counteract this pathway by masking viral dsRNA through the action of VP35, preventing efficient OAS activation (Schwartz et al., 2020). The binding of VP35 to dsRNA also inhibits PKR which is an ISG that phosphorylates eIF2α to limit translation initiation (Feng et al., 2007; Cárdenas et al., 2006). Early-response ISGs like IFIT1 and OAS1 stop viral replication rapidly, whereas the late-response genes like ISG20 and tetherin (BST2) sustain defence and limit viral release (Evans et al., 2010).

Interferon-induced transmembrane proteins (IFITMs) include IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10 genes in humans (Yánez et al., 2020). IFITM3, as relevant for this doctoral thesis, has several key antiviral MOAs. First, it acts as a direct antiviral restriction factor by inhibiting viral entry, primarily blocking membrane fusion of enveloped viruses within endosomes before viral genomes reach the cytoplasm (Feely et al., 2011). In addition, IFITM3 alters endosomal and cellular membrane properties, including reducing membrane fluidity and disrupting cholesterol homeostasis, thereby preventing formation of fusion pores after hemifusion (Desai et al., 2014).

Next, IFITM3 redirects incoming virions toward late endosomes and lysosomes, where viruses are trapped and degraded, a process facilitated by its localisation and post-translational modifications such as S-palmitoylation (Spence et al., 2019). Finally, beyond intracellular restriction, IFITM3 contributes to immune protection at the organismal level by safeguarding immune and tissue-resident cells and modulating cytokine signaling and T-cell responses, thereby limiting viral pathogenesis in-vivo (Everitt et al., 2012). Filoviruses avoid IFIT-mediated restriction by encoding multifunctional polymerase complexes that catalyze mRNA capping and cap-methylation in a manner that closely mimics host RNA polymerase II transcripts (Valle et al., 2020).

ISGs are also associated with pleiotropic effects via the process of ISGylation. ISG15 protein covalently binds to host or viral proteins to modify their function and stability through ISGylation, which stabilises STAT1 and TBK1 (Liu et al., 2022). Filoviruses have evolved mechanisms via which they decrease ISGylation enzyme production and destroy ISGylated proteins (Okmura et al., 2008).

2.4.4.3 VP24 inhibits STAT Nuclear Import

Filoviruses have developed several methods to break these interferon-induced signalling networks. Whereas EBOV VP35 reduces interferon induction at the sensing and transcriptional levels, VP24 blocks the signalling cascade that transmits

the interferon activity. EBOV VP24 inhibits nuclear translocation of phosphorylated STAT1 preventing interferon-stimulated gene transcription (Xu et al., 2014). EBOV VP24 is a 251-amino acid protein which competes with nuclear import machinery by binding to karyopherin α (Reid et al., 2006). IFN binding to IFNAR receptors phosphorylates STAT1 and STAT2, creating the ISGF3 complex that translocates to the nucleus via karyopherin α binding (Hu et al., 2021). C-terminal domain of karyopherin-α2 (also known as importin-α1) is directly bound by VP24 by using the same interface as phosphorylated STAT1. In this way, the competitive binding stops STAT1 from docking to its nuclear transporter and entering the nucleus (Reid et al., 2006).

The ISG transcription is blocked due to the inability of ISGF3s to bind with ISREs in gene promoters. Binding by VP24 only specifically targets phosphorylated STAT1 which manifests as an evolutionary adaptation for solely but potently inhibiting IFN-mediated innate immunity (Mateo et al., 2010). VP24 expression reduces IFN responsiveness in innate immune cells, which consequently further amplifies immunosuppressive effects of VP24 (Ilinykh et al., 2015). VP24-mediated STAT1 inhibition allows immunological escape and contributes to deregulated inflammation in EBOV infection (Reid et al., 2006).

Structural studies using X-ray crystallography have revealed a cluster of hydrophobic and electrostatic contacts that stabilise the complex between VP24 and karyopherin-α1 (KPN-α1) (also known as importin α5). Mutations at key interface residues (e.g. W42 and K142) reduce KPN-α1 binding of VP24, weaken its binding affinity, and restore STAT1 nuclear translocation in infected cells (Schwarz et al., 2017). These important structural insights show that VP24-mediated immune suppression can be reversible with small-molecule importin binding inhibitors.

Viruses of the Filoviridae family suppress STAT-dependent interferon signaling, disrupting immune regulation and indirectly promoting excessive pro-inflammatory cytokine release from uninfected bystander cells, a process that contributes to endothelial dysfunction, vascular leakage, and multi-organ failure (Zhang et al., 2012; Jain et al., 2020).

2.5 Current Gaps and Challenges

The molecular and functional synergism between VP24 and VP35 and host signalling pathways is under explored. It is well established that EBOV VP35 blocks RIG-I activation and EBOV VP24 blocks STAT1 nuclear translocation (Basler & Amarasinghe, 2009; Reid et al., 2006), however, the functions of these viral proteins (VP24/VP35) have been mainly studied only for EBOV and MARV. Significant data gaps have been identified in recently published literature pertaining to filoviral VPs which points out that, although significant advances have been made in the field of

filoviral research, further research is needed to explain host-VP interactions, involvement of host cellular components in replication cycle, species-based differences in structural and functional organisation, and interaction with key components of innate immunity (Liu et al., 2022; Uwase et al., 2025)

- Recent discoveries of new filoviruses raise the question if these new filoviral VP24 and VP35 proteins exhibit the same or different molecular patterns of innate immune evasion that have been identified in the literature for EBOV and MARV.
- This can potentially also:
    - Highlight additional immune evasion functions and key relevant molecular determinants that are currently unknown.
    - Elucidate any differences in the extent of inhibiting key molecular elements of innate immunity, and,
    - Conservation of structural (i.e., in the virions) and functional organisation (i.e., for innate immune evasion) in the filoviral species under the remit of this doctoral thesis.

As it has been highlighted earlier that innate immune evasion is the key step in filoviral infection and replication cycle, identification of shared molecular pathways will help to identify targets for drug and vaccine development in addition to improving the understanding of filoviral infection and innate immune modulation.

3 Aims

This doctoral thesis attempts to clarify the signalling pathways that filoviral proteins VP35 and VP24 target to disrupt the host innate immune responses. Even though earlier studies have strived to understand the immune evasion mechanisms of Ebola and Marburg viruses, this doctoral thesis broadens this knowledge and determines how VP24 and VP35 proteins of all mammalian-infecting nine filovirus species within four genera inhibit innate immune responses.

The specific research objectives were:

1. To compare the ability of VP24 proteins of nine mammalian-infecting filovirus species to interfere with RIG-I signalling and interferon gene expression (Study I).
2. To identify which VP24 proteins from nine filovirus species can downregulate innate immune responses by inhibiting the interferon-induced pathways (Study II).
3. To compare the ability of VP35 proteins of nine filovirus species to interfere with RIG-I and interferon-induced pathways (Study III).

4 Materials and Methods

4.1 Cell Culture, Transfections and Cell Stimulations

Human embryonic kidney (HEK293) and hepatoma-derived Huh7 cell lines were selected for their proven efficacy in analysing viral-host interactions. HEK293 cells are optimal models for reporter assays and immunoblot studies with their high transfection efficiency and strong exogenous gene expression (Fliedl and Kaisermayer, 2011). Huh7 cells are often chosen in studies for imaging-based tests because their large cytoplasmic volume and distinct nuclear morphology allow the visualisation of intracellular viral protein localisation.

HEK293 and Huh7 cells were kept at 37 °C with 5% CO_2 and were cultured in Dulbecco's Modified Eagle Medium (DMEM- Lonza Biowhittaker). These cells were split on every alternate day to achieve 80-90% cellular confluency (between 16–24 passage numbers). 25 mM HEPES buffer (MP Biomedicals), 10% heat-inactivated foetal bovine serum (FBS; Integro), 1% penicillin/streptomycin (Lonza Biowhittaker), and 1× GlutaMAX supplement (Thermo Fisher Scientific) were added to the media. The sub-cultured cells were reseeded in 12-well or 96-well plates for the assays.

4.1.1 Transfection Reagents

The experimental objectives, plasmid size, and downstream assay requirements determined the use of three high-efficiency lipid-based transfection reagents; TransIT-LT1 (Mirus Bio) and Lipofectamine 2000 and Lipofectamine 3000 (Thermo Fisher Scientific) to transiently produce viral and host proteins.

4.1.2 Cell Stimulation Conditions

In Study I, II and III, first the expression plasmids and the reporter plasmids were transfected using TransIT-LT1 (Mirus Bio LCC, Madison). Subsequently, RIG-I pathway was stimulated by transfecting low molecular weight poly: IC (5 ug/ml) (Invivogen) with Lipofectamine 2000 (Invitrogen). In Study II and III, the interferon-

induced pathway was stimulated by adding peginterferon alfa-2a (IFN-α−2a; Pegasys, F. Hoffman-La Roche) at a concentration of 10 ng/ml for overnight. In Study III, RIG-I signalling was activated with poly (I:C) (5ug/ml), which is a synthetic double-stranded RNA analogue, or by infecting with a multiplicity of infection (MOI) 2.5 with Sendai virus which has a single stranded RNA genome and is a commonly used robust inducer of RIG-I and IFN pathway.

Cells expressing constitutively active ΔRIG-I or pathway intermediates MAVS, TBK1, IKKε or constitutively active IRF3-5D did not require additional stimulation because they independently stimulate downstream interferon expression.

4.2 Filovirus Plasmid Constructs

4.2.1 Gene Sequences and Cloning

The selected VP24 coding sequences were EBOV (Genbank accession number KM233113), BOMV (Genbank accession number MF319185), BDBV (Genbank accession number KC545394), RESTV (Genbank accession number KY798006), SUDV (Genbank accession number KC545389), TAFV (Genbank accession number KU182910), LLOV (Genbank accession number NC016144.1), MARV (Genbank accession number KC545387.1) and MLAV (Genbank accession number KX371887).

The selected VP35 coding sequences were: EBOV (Genbank accession number AIG96587.1), BOMV (Genbank accession number ASJ82202), BDBV, (Genbank accession number AGL73458.1), RESTV (Genbank accession number ARU80313.1), SUDV (Genbank accession number AGL73423.1), TAFV (Genbank accession number ALT19759.1), LLOV (Genbank accession number YP_004928136.1), MARV (Genbank accession number YP_001531154.1), and MLAV (Genbank accession number YP_010087184.1).

For some genes (BDBV and MARV VP24 for Study I and Study II, and EBOV, RESTV and MARV VP35 for Study III), codon optimisation was required to enhance translation in HEK293 and Huh7 cells. All of the genes (irrespective of codon optimisation) were cloned into mammalian plasmid pEBB-HA-N, which has an N-terminal HA epitope tag and the recombinant protein expression is under strong CMV promoter by GeneArt (ThermoFisher Scientific).

4.2.2 NLS-Mutant VP24 Construct

A nuclear localisation signal (NLS) mutant VP24 construct was created to investigate the molecular mechanism of VP24-mediated suppression of interferon signalling. Phusion Site-Directed Mutagenesis Kit (ThermoFischer Scientific) was

used to create the mutant VP24 construct according to the manufacturer's instructions, disrupting importin-α binding residues in the VP24 NLS motifs. The constructs retained same HA-tag and expression backbone (pEBB-HA-N) as the wild-type VP24 plasmid.

4.3 Immunofluorescence

4.3.1 Sample Preparation, Fixation and Permeabilisation

Huh7 cells were grown on glass coverslips in 12-well plates overnight in DMEM with 10% FBS, 1× GlutaMAX and antibiotics at 37 °C with 5% CO_2. The cells were transfected with N-terminal HA-tagged VP24 and VP35 expression plasmids by Lipofectamine 3000 following the protocol provided by manufacturer. After 24 hours, the cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT). After fixation, the cells were permeabilised with 0.1% Triton X-100 in PBS.

4.3.2 Primary and Secondary Antibodies and Staining Conditions

After permeabilisation, the samples were treated for 1 hour at RT with primary mouse monoclonal anti-HA antibody (1:1000) (Biolegend) or in-house anti-MxA antibody pool raised in three guinea pigs (1:1000) or anti-IRF3 antibody raised in rabbit (1:200) (Cell Signaling Technology) diluted in 3–5% BSA/PBS, followed by staining with anti-mouse- Alexa568 or anti-rabbit- Alexa488 antibodies (Li-Cor Biosciences). The samples on the coverslips were washed with 0.5% BSA/PBS and mounted with Moviol® 4-88ProLong Gold Antifade mountant (Thermo Fisher Scientific). Dapi (1:2500) (Thermo Fisher Scientific) diluted in 5% PBS was used to stain the nuclei. Mitotracker was used according to manufacturer's instructions (MitoTracker® Red CMXRos, Cell Signaling Technology).

4.3.3 Imaging Systems and Quantification

Fluorescence imaging was performed for the cover slips using a Leica DFC7000 T microscope with a 63× oil-immersion objective. The cells on 96-well plates were imaged with an Evos microscope with a 20× objective (Thermo Fisher Scientific). The images were manually quantified with Image J software. A Zeiss Axio Imager microscope with a 63× oil objective was employed to visualize the coverslips for high-resolution imaging of nuclear and cytoplasmic compartments of the cells.

Leica LAS X-processed Images were manually analysed using ImageJ/Fiji (NIH) software and statistical differences between the groups were analysed using Chi-squared test. Dual immunofluorescence labelling was used to assess relationship of VP24/IRF3 or VP35/MAVS and mitochondrial structures for colocalisation studies.

4.4 Reporter Gene Assays

4.4.1 Luciferase assay

Filovirus VP24 and VP35 proteins were tested for their inhibitory effects using reporter gene assays. HEK293 cells were used because of their well-characterised PRRPRR signalling responsiveness (Fliedl & Kaisermayer, 2011). Cells were transfected in 96-well plates at 80–90% confluency with expression and reporter constructs using Lipofectamine 3000 according to manufacturer's instructions (Thermo Fisher Scientific). IFN-β, IFN-λ1, MxA, or IFITM3 promoter-luciferase constructs were co-transfected with wtRIG-I, ΔRIG-I, MDA5, MAVS, TBK1, IKKε or IRF3-5D expression plasmids.

The positive control plasmid, expressing the RIG-I-inhibiting HCV NS3/4A protease, was used to assess interference. All the transfection mixtures contained the RSV-Renilla luciferase plasmid as an internal normalisation control to account for transfection efficiency. Transfected and stimulated cells were lysed and processed using the Twinlite Dual Luciferase Reporter Gene Assay System (PerkinElmer) or Dual-Luciferase® Reporter Assay System (Promega), following the protocol provided by the manufacturer. Luminescence was measured with a PerkinElmer Victor Nivo Multimode Plate Reader and the Renilla luciferase levels were utilised to normailize the firefly luciferase data. Each of the condition was tested in triplicate wells with three separate replicates (n = 9) for statistical reliability.

4.5 Immunoblotting

4.5.1 Protein Extraction and Lysis Conditions

The cells were cultured in 12-well plates and transfected with different expression plasmid combinations (depending on experimental purpose). HEK293 cells were transfected with 200–2000 ng/well VP24 constructs to assess dose-dependent effects on signalling components and co-transfected with 500 ng/well wtRIG-I, ΔRIG-I or MDA5, and 250 ng/well IRF3 expression plasmids (Study I). HEK293 and Huh7 cells were transfected with 1200–1500 ng/well VP24 constructs to assess expression levels on signalling components (Study II), and for VP35 expression plasmids, doses

of 40, 133 and 400 ng/well, equivalent to 3, 10, and 30 ng/well in 96-well luciferase experiments were used. The cells were co-transfected with 500 ng/well ΔRIG-I, MAVS, TBK1, IRF3-5D, IKKε expression plasmids (Study III).

The cells were lysed under freezing conditions after overnight incubation (16–18 hours) post-transfection. The cell lysis for immunoblotting was performed by using Passive Lysis Buffer of the Dual Luciferase Reporter Gene Assay Kit (Promega) which was supplemented with Complete Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche) (Study I–II) to preserve protein structure and avoid phosphorylation. For VP35 analysis, a native lysis buffer with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 2% NP-40 was used (Study III).

All lysis stages were performed at 0–4 °C for 30 min in ice-cold lysis buffer. The samples were centrifuged at 13,000×g for 10 minutes at 4°C and supernatants were collected. The Bradford or BCA protein assays were used to assess protein concentration to standardise loading volumes. Protein denaturation and disulphide bond reduction were achieved by mixing lysates with 4× Laemmli sample buffer and heating at 95°C for 5 minutes before electrophoresis.

4.5.2 SDS-PAGE and Transfer of proteins to membranes

Protein samples were resolved using 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) or 4–12% gradient gels (Bio-Rad), depending on molecular weight and resolution requirements.

Following electrophoresis, wet transfer system was used to transfer proteins onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes (at 100 V overnight at 4 °C). PVDF membranes (Amersham, Merck KGaA) were chosen for their protein-binding capacity and fluorescence compatibility, while Amersham Protran 0.2 μm nitrocellulose membranes (GE Healthcare) were selected for comparative evaluation of protein expression. PVDF membranes were pre-activated with 100% methanol for 1 minute to improve protein binding before transfer.

After transfer, the membranes were blocked for 1 hour at room temperature with 5% non-fat dried milk or 3% BSA in Tris-buffered saline with 0.1% Tween-20 (TBS-T) to inhibit non-specific antibody binding.

4.5.3 Antibody Panels

Mouse monoclonal anti-HA1.1 antibody (1:1000; BioLegend) was used to detect the N-terminal HA-tag linked VP24 and VP35 proteins. Mouse anti-Tubulin (1:700; clone 6C5, Santa Cruz Biotechnology) and mouse anti-GAPDH (1:700; 6C5, Santa Cruz Biotechnology) were used as loading controls.

Rabbit anti-IRF3 (1:200) and rabbit anti-phospho-IRF3 (Ser396; 1:200; Cell Signalling Technology) were utilised to measure IRF3 activation after RIG-I stimulation. Rabbit anti-RIG-I (1:200) and rabbit anti-MDA5 (1:200) confirmed cytoplasmic RNA sensor expression.

Anti-MAVS (rabbit) 1:500 (Cell Signaling Technology), anti-TBK1 (rabbit) 1:500 (Cell Signaling Technology), anti-flag (mouse) 1:1000 (Sigma), anti-IKKE (rabbit) (Abcam) 1:1000, and anti-IRF3 (rabbit) 1:500 (rabbit animal identification number: KCVD8) were used to investigate the expression of pathway components.

After the overnight incubation with primary antibody (1:1000) (LI-COR Biosciences) at 4 °C, the membranes were washed three times with TBS-Tween and probed with secondary antibodies, IRDye 680RD goat anti-mouse IgG (1:15,000) (LI-COR Biosciences) and IRDye 800CW goat anti-rabbit IgG (1:15,000) (LI-COR Biosciences) diluted in 5% (w/v) non-fat dry milk/BSA in TBS-T for 1 hour at room temperature.

4.5.4 Detection via LI-COR Odyssey Imaging

Membranes were washed in TBS-Tween to remove unbound antibodies and imaged with the LI-COR Odyssey Fc Imaging System. The target and loading control proteins were visualised simultaneously in dual channels (700 nm and 800 nm) for the two IRDye-conjugated secondary antibodies.

Using LI-COR Image Studio, the band intensities were normalised against internal loading controls (GAPDH or Tubulin). Relative expression ratios were calculated to compare viral protein expression across doses and assess the effects of VP24 or VP35 on host protein expression levels.

4.6 Phylogenetic Analysis

4.6.1 Sequence alignment using MUSCLE

The Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm in MEGA7 software suite was used to generate multiple sequence alignments (MSA) of filovirus VP24 and VP35 proteins to establish their evolutionary relationships. MUSCLE was selected for its accuracy and computational efficiency in matching vast and varied viral genomes including protein-coding regions with different evolutionary rates (Edgar, 2004). The NCBI GenBank database provided complete genome sequences or individual VP24 and VP35 gene sequences for filoviruses. The accession numbers for each sequence were documented to maintain reference log and traceability. Open reading frame verification were performed on the acquired

nucleotide sequences to align homologous areas, however, complete VP24 and VP35 coding areas were used to avoid alignment bias among viral species.

Furthermore, multiple sequence alignments were carefully checked to correct terminal residue misalignments, while amino acid alignments were back translated to nucleotide sequences and codon structure for evolutionary reconstruction.

4.6.2 Tree construction with MEGA7

Aligned VP24 and VP35 sequences were phylogenetically reconstructed using Maximum Likelihood (ML) technique implemented in MEGA version 7 (Study I and II) and version 11 (Study III) respectively. The ML approach, using General Time Reversible model with gamma distribution and possibility of evolutionary invariability for some sites, was selected for its statistical strength and ability to represent varied nucleotide substitution rates across locations, which aligns with high genetic variety and adaptive evolution of filoviruses (Kumar et al., 2016, Tamura et al., 2021).

ML trees were built with 100 bootstrap replicates to evaluate branch topology statistical confidence, such that strong phylogenetic support was indicated by bootstrap values above 70% (Lemoine & Gascuel, 2024). MEGA visualised or annotated the trees for highlighting branch lengths proportional to site substitutions.

4.7 Importin Binding Assays

Glutathione S-transferase (GST) fusion proteins were generated in *E.coli* to study the interaction between filovirus VP24 proteins and host importin α5 in pull-down binding assays (Study I and II). The human importin α5 cDNA was cloned into a pGEX expression vector and converted into competent *E. coli* BL21 (DE3) cells while the protein expression was stimulated using 1 mM IPTG for 4 hours at 30 °C, resulting in high levels of soluble GST-importin α5 fusion protein.

Immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100) with 5 mg/mL lysozyme (Sigma-Aldrich) was used to lyse bacterial cell walls. The lysates were incubated at room temperature for 30 minutes, sonicated briefly to shear DNA and then centrifuged at 13,000 rpm for 5 minutes to clear the supernatant. GST importin α5 was immobilised on GE Healthcare Glutathione Sepharose 4B beads after 1 hour incubation at 4 °C with gentle stirring. The Coomassie Brilliant Blue staining of 12% SDS-PAGE confirmed the purity and quantity of bound protein fractions.

VP24 expression constructs from various filoviruses were transiently expressed in HEK293 cells using TransIT-LT1 transfection reagent (Mirus Bio). The cells were lysed 24 hours post-transfection in IP buffer followed by centrifugation at 10,000 × g

for 5 min at 4 °C to obtain the supernatant for pull-down assays. The TnT® Quick Coupled Transcription/Translation System (Promega) was used to translate and radiolabel BDBV, MLAV and MARV VP24 proteins that could not be produced in mammalian cells. The proteins were labelled with [^{35}S]-methionine/cysteine (PerkinElmer EasyTag Express) for autoradiography.

Equivalent amounts of lysate or radiolabeled protein were incubated with 25 µl Glutathione Sepharose-bound GST or GST-fusion proteins in IP buffer for 1 hour at 4 °C to conduct binding assays. The boiling in Laemmli sample buffer eluted bound complexes. The eluates were separated on 12% SDS-PAGE gels, transferred to PVDF membranes (Millipore), and probed with mouse anti-HA1.1 antibody (1:1000; BioLegend) and HRP-conjugated anti-mouse secondary antibody for HEK293 expressed proteins. The gels with [^{35}S]-labelled proteins were processed with Amplify reagent (Amersham Biosciences) and visualised using autoradiography on HyperMax films.

5 Results

5.1 Phylogenetic analysis of evolutionary relationships (Study I and III)

Filovirus VP24 and VP35 proteins have a broad range of functions, which are key to viral replication as well as the suppression of host defense pathways for effective viral immune evasion. Phylogenetic analysis of filovirus VP24 and VP35 proteins showed evolutionary relationships within the Filoviridae family. Both the VP24 and VP35 results showed monophyletic clustering of the *Orthoebolavirus*, *Cuevavirus*, and *Orthomarburgvirus* genera, indicating a high degree of evolutionary conservation. In contrast, *Marburgvirus* and *Dianlovirus* formed a strongly supported monophyletic clade that was separate from the other filoviruses.

The phylogenetic analysis of VP24 sequences showed virus species of *Orthoebolavirus* genus, EBOV, SUDV, BOMV, BDBV, TAFV and RESTV, being grouped into a single, compact branch with short node lengths, while LLOV (genus *Cuevavirus*) takes a position between the Orthoebolaviruses and Orthomarburgviruses. Despite clear genetic divergence, LLOV more closely resembles the former in both complete genome and VP24 amino acid sequences (Figure 7A and 7B). In conjunction, MLAV (genus *Dianlovirus*) and MARV (genus *Orthomarbugvirus*) form their distinct group which is genetically segregated from the *Orthoebolavirus* genus. In addition, these viruses show similar patterns of evolutionary divergence for both complete genome and VP24 amino acid sequences.

Phylogenetic analysis of VP35 amino acid sequences (Figure 8) revealed a pattern consistent with that observed for VP24. EBOV, SUDV, BOMV, BDBV, TAFV, and RESTV clustered together, however, the branching relationships differed slightly. In contrast to the VP24 phylogeny, SUDV clustered with TAFV and BDBV clustered with RESTV, whereas in the VP24 analysis SUDV and BDBV grouped with RESTV and TAFV, respectively.

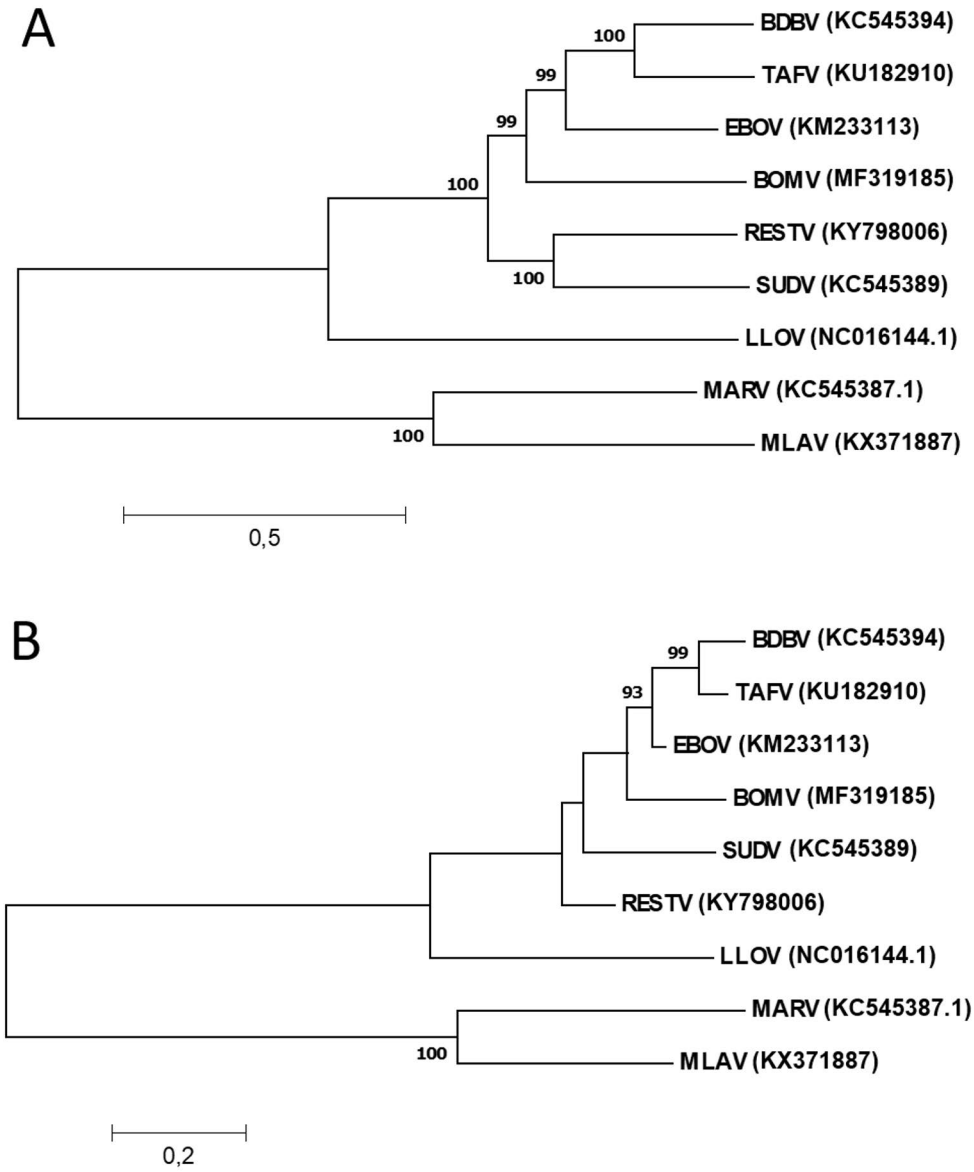


Figure 7 Phylogenetic analysis of the nine filoviruses used in the VP24 studies in this thesis. (A) entire genomes and (B) VP24 amino acid sequences. This figure is a modified version of Figure 1, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

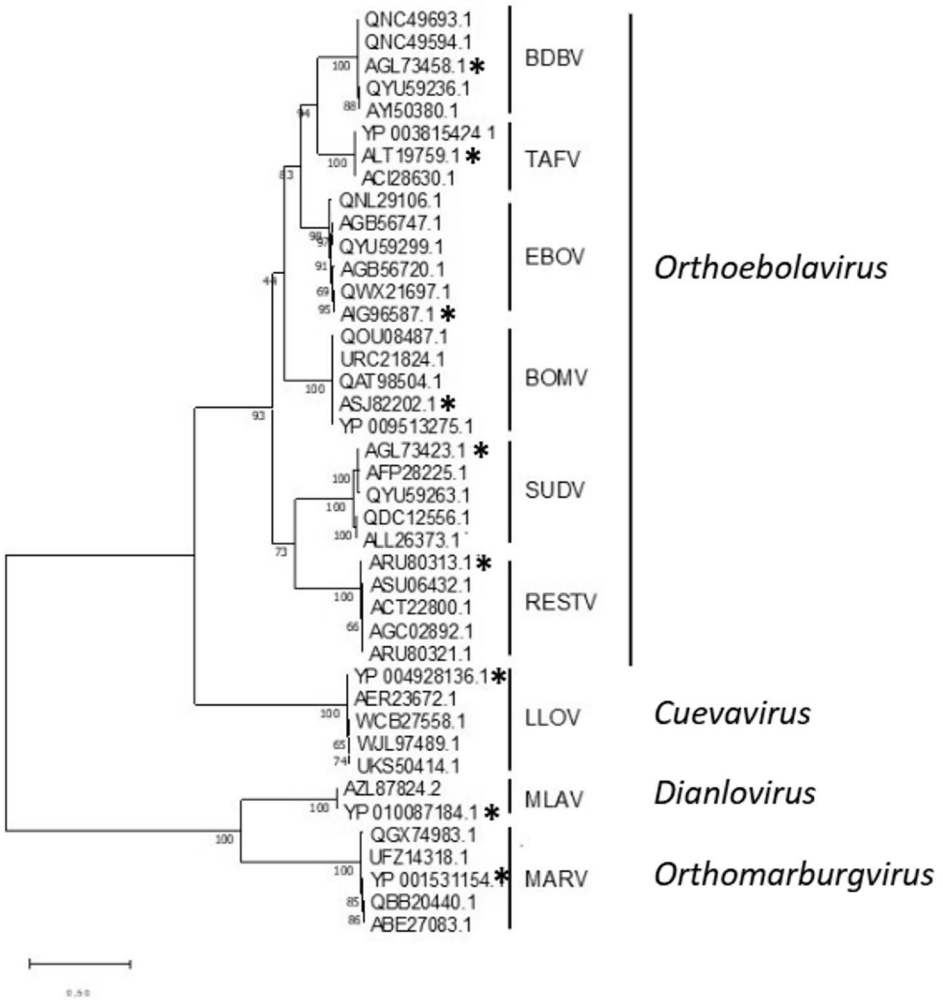


Figure 8 Phylogenetic analysis of VP35 amino acid sequences. The black boxes indicate the nine filoviral VP35 amino acid sequences selected for the studies in this thesis. This figure is reproduced from Figure 1 (Unpublished data, Study III).

5.2 Comparative Analysis of Filovirus VP24 and VP35 amino acid sequences

5.2.1 Sequence Conservation and Amino Acid Comparison (Study I – III)

The VP24, which is a minor matrix protein, and VP35, a viral polymerase cofactor, both act also as important multifunctional interferon antagonists and demonstrated similar conservation within and divergence between genera. The comparison of

VP24 amino acid sequences representing eight Filoviridae species revealed high intra-species conservation. The alignment of the VP24 sequences showed that virus species in *Orthoebolavirus* genus (EBOV, BOMV, BDBV, RESTV, SUDV, and TAFV) share 69–89% amino acid identity amongst themselves and 34–36% with MARV of *Orthomarburgvirus* genus (Table 2). LLOV of genus *Cuevavirus* share 56–59% identity with *Orthoebolavirus* members but only 36% with MARV of genus *Ortomarburgvirus* and 33% with MLAV of genus *Dianlovirus*. The large pairwise amino acid differences of *Orthoebolaviruses* indicate that they are different from LLOV, MLAV and MARV. LLOV displayed the intermediate identity levels to *Orthoebolaviruses* and low identity levels to MARV supporting its classification into a separate *Cuevavirus* genus.

Table 2 VP24 amino acid sequence pairwise identity between eight filoviral strains. This table is reproduced from Figure 1, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

VP24 amino acid sequence pairwise identity

	MLAV	BOMV	LLOV	TAFV	RESTV	SUDV	MARV	BDBV
EBOV	38 %	82 %	59 %	88 %	82 %	75 %	36 %	86 %
MLAV		37 %	33 %	37 %	38 %	35 %	57 %	37 %
BOMV			57 %	79 %	76 %	74 %	34 %	75 %
LLOV				57 %	58 %	59 %	36 %	56 %
TAFV					76 %	72 %	36 %	89 %
RESTV						75 %	34 %	76 %
SUDV							35 %	69 %
MARV								35 %

The VP35 phylogenetic comparison gave similar results (Table 3) as *Orthoebolavirus* VP35 amino-acid sequences clustered with 65–77% sequence identity among EBOV, BOMV, BDBV, TAFV, RESTV and SUDV. LLOV VP35 had 49–51% similarity with Orthoebolaviruses. MARV VP35 showed only 31-33% identity to the Orthoebolaviruses, while MLAV VP35 shared 55% amino acid identity with MARV demonstrating a relatively high degree of genetic diversity between genera.

Table 3 VP35 amino acid sequence pairwise identity between nine filoviral strains. This table is reproduced from Table 2 (Unpublished data, Study III).

	BOMV	SUDV	RESTV	MLAV	MARV	LLOV	EBOV	TAFV	BDBV
BOMV									
SUDV	65 %								
RESTV	67 %	67 %							
MLAV	36 %	35 %	35 %						
MARV	34 %	33 %	33 %	55 %					
LLOV	53 %	53 %	52 %	34 %	35 %				
EBOV	73 %	68 %	67 %	35 %	35 %	54 %			
TAFV	73 %	68 %	67 %	37 %	34 %	52 %	76 %		
BDBV	71 %	67 %	65 %	35 %	34 %	52 %	75 %	77 %	

The intra-species amino acid variation in VP24 sequences was analysed in 112 isolates of nine species from infectious outbreaks from years 1967 to 2021. Analysis of isolates collected across multiple years, and geographic regions revealed only limited amino acid variation in VP24, indicating strong evolutionary constraint on this protein. MARV and RESTV showed the highest variability, having up to five and six amino acid sequence alterations in their 250–253 amino acid residues, respectively. EBOV isolates collected over 54 years (years 1967–2021) exhibited only 0–2 amino acid changes, which further reflected the exceptional stability of the protein across isolates. MLAV and TAFV have only one VP24 sequence each in GenBank, which prevented intra-species comparison (Table 4).

Table 4 Amino acid sequence comparison of VP24 proteins across 112 filovirus isolates representing nine filovirus species. This table is reproduced from Table 1, from original publication II which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)

Species	No. of sequences	Collection years	No. of amino acid changes in VP24	Amino acid change %
EBOV	20	1976–2021	0–2	0–0.8
BOMV	8	2016–2019	0–1	0–0.4
BDBV	20	2007–2012	0–2	0–0.8
MLAV	1	2015	0	0
RESTV	20	1989–2018	0–6	0–2.4
SUDV	20	1976–2012	0–2	0–0.8
TAFV	1	1994	0	0
LLOV	2	2003–2019	0–3	0–1.2
MARV	20	1967–2012	0–5	0–2.0

The intra-species amino acid variation analysis of VP35 sequences of 41 isolates of nine filovirus species showed that EBOV VP and SUDV VP35 exhibited 0–10 and 0–11 number of amino acid changes respectively, while BOMV, BDBV, MLAV, RESTV, TAFV, LLOV and MARV have comparatively less with 0–2, 0–2, 0, 0–4, 0, 0–2 and 0–6 amino acid residue changes, respectively (Table 5). The sequence comparison analysis showed that VP24 and VP35 have high intra-species stability, VP35 somewhat less, reflecting their essential roles in host immune evasion, whereas the inter-species variability between the genera could imply distinct functions.

Table 5 Comparison of 41 filovirus VP35 amino acids. Sequences from nine species showing the proportion of amino acid variations through years 1976–2021. This table is reproduced from Table 1 (Unpublished data, Study III).

Species	Number of Sequences	Collection Year	Number of amino acid changes	Amino acid Change %
EBOV	6	1976-2021	0-10	0-2.5
BOMV	5	2016-2019	0-2	0-1.4
BDBV	5	2007-2012	0-2	0-0.5
MLAV	2	2015	0	0
RESTV	5	1989-2015	0-4	0-1.1
SUDV	5	1976-2015	0-11	0-3.1
TAFV	3	1994	0	0
LLOV	5	2003-2021	0-2	0-0.5
MARV	5	1980-2021	0-6	0-1.7

5.2.2 Nuclear localisation signal of VP24 proteins (Study II)

Filovirus VP24 proteins contain a nuclear localisation signal (NLS). The sequence alignment of cluster 3 amino acids of NLSs highlighted that BOMV, BDBV and TAFV VP24s are identical to EBOV in this region but RESTV, SUDV and LLOV have a conservative substitution (leucine to glutamine (LLOV) or valine (RESTV, SUDV), pink in Figure 9). MLAV and MARV amino acid sequences in this NLS region remarkably differ from the EBOV sequence. The differences in amino acid sequences could lead to differences in protein functions. The cluster 3 is important for importin interaction and to further analyze the function of NLS of VP24s, the critical amino acids were mutated in the chosen four VP24s into alanine as shown in Figure 8, and the resulting VP24 mutants were used in the subsequent analyses.

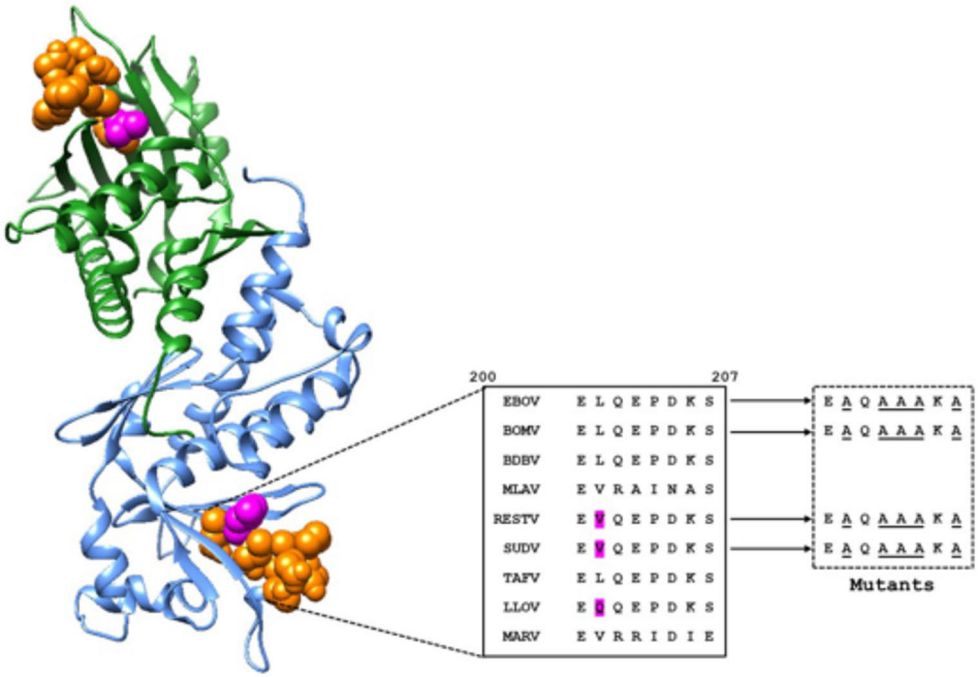


Figure 9 EBOV VP24 dimer structure, represented in blue and green ribbons. The orange circles indicate nuclear localization signals of VP24, and the pink circles represent the amino acids of RESTV, SUDV, and LLOV that differ from EBOV. Alignment box shows the amino acids of each cluster-3 nuclear localization signals (pink boxes correspond to pink circles). VP24 proteins with mutated NLSs were made by introducing the indicated alanine changes. This figure is reproduced from Figure 1, from original publication II which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

5.3 Filovirus VP24 proteins

5.3.1 Expression and subcellular localisation (Study I and II)

To confirm VP24 expression, HEK293 and Huh7 cells were transfected with nine wildtype VP24 and four NLS-mutated constructs. After 24 hours, proteins in cell lysates were separated on SDS-PAGE and immunoblotted for HA-tagged VP24 expression. The non-codon optimised BDBV and MARV VP24 initially demonstrated low expression levels. Synthesizing codon-optimised BDBV and MARV VP24s constructs to match human codons increased expression levels (Figure 10). Therefore, codon-optimised BDBV and MARV VP24s were used for all functional and localisation investigations (Study I).

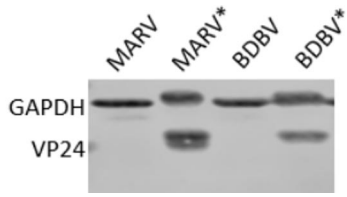


Figure 10 Codon optimised expression constructs of MARV and BDBV show better expression of VP24 proteins as compared to non-optimised MARV and BDBV. This figure is reproduced from Figure 2, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

HEK293 cells expressed all wild-type and NLS-mutated VP24 proteins (Figure 11a and 11b respectively), although the expression levels varied by filoviral proteins i.e., EBOV, BOMV, TAFV, MLAV and LLOV showed less expression as compared to BDBV, RESTV and SUDV filoviruses (Study II). For Huh7 cells, all wild-type and NLS-mutated VP24 proteins were expressed, however, expression levels were low for BOMV, MLAV and LLOV (Figure 11c and 11d). Overall, despite low expression for some filoviral VP24 proteins, all of these showed detectable expression in both cell lines.

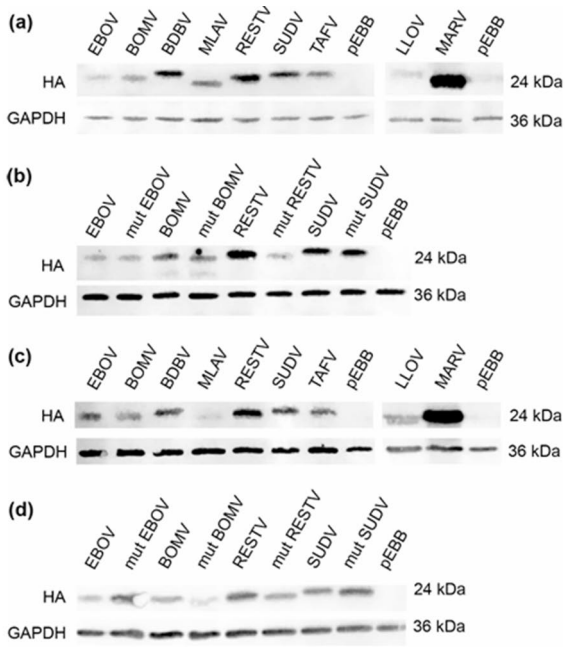


Figure 11 HEK293 (a, b) and Huh7 (c, d) cells express VP24 proteins that are N-terminally HA-tagged and four were NLS-mutated (b and d). Controls were empty vector-transfected (pEBB) cells and loading control was GAPDH. This figure is reproduced from Figure 2, from original publication II which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

In addition to immunoblotting, the expression of VP24 proteins was verified with immunofluorescence, also allowing for analysis of sub-cellular localisation. Immunofluorescence labelling of transfected Huh7 cells revealed that all HA-tagged VP24 proteins, including codon-optimised proteins, were successfully expressed but with different subcellular localisation patterns (Figure 12A). The quantitative imaging showed that most VP24 proteins localised in both the nucleus and cytoplasm with cytoplasmic and nuclear enrichment for EBOV (95%), BOMV (96%), SUDV (92%), LLOV (96%) and BDBV (95%); whereas the nuclear distribution was less for RESTV (44%), TAFV (29%) and MLAV (47%) leading to their combined cytoplasmic/nuclear enrichment values of 56%, 71%, and 53% respectively (Figure 12B.).

The native MARV VP24 localised mostly in the cytoplasm (91%) but its codon-optimised form (MARV*) demonstrated nuclear and cytoplasmic distribution of 93%, reflecting that codon optimisation enhances expression and affects nuclear localisation. RESTV VP24 had a mitochondrial-like granular cytoplasmic pattern that was clearly different from other filovirus VP24 proteins (Figure 12C). However, MitoTracker labelling showed that RESTV VP24 granules (Figure 12D) were segregated from mitochondria, establishing that its localisation pattern was not connected with mitochondrial membranes.

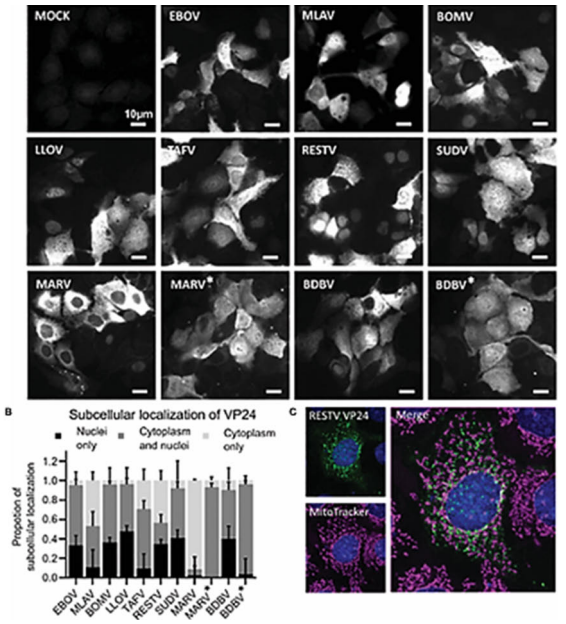


Figure 12 (A) Subcellular localisation of nine filovirus VP24 proteins expressed in Huh7 cells. BDBV* and MARV* are codon optimised expression constructs. (B) Quantification of filovirus VP24 subcellular localisation. (C) RESTV VP24-expressing Huh7 cell labeled with anti-HA antibodies (VP24) and MitoTracker. This figure is reproduced from Figure 2, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

5.3.2 Importin Binding for nuclear transport of filoviral VP24 proteins (Study II)

NLS is required for binding to importins and translocation to the nucleus. Selected NLS-mutated VP24 constructs were created by replacing lysine and arginine in cluster 3 with alanine (Figure 9). To analyze the effect of NLS mutations on subcellular distribution, immunofluorescence was used to compare wt- and NLS-mutated VP24s of EBOV, SUDV, RESTV and BOMV in transfected Huh7 cells (Figure 13). The wt- and NLS-mutated VP24s were readily expressed, and wt-VP24s localised mostly in the nuclei, while NLS-mutated VP24s redistributed throughout the cytoplasm and exhibited dispersion between both nuclear and cytoplasmic regions (Figure 13A).

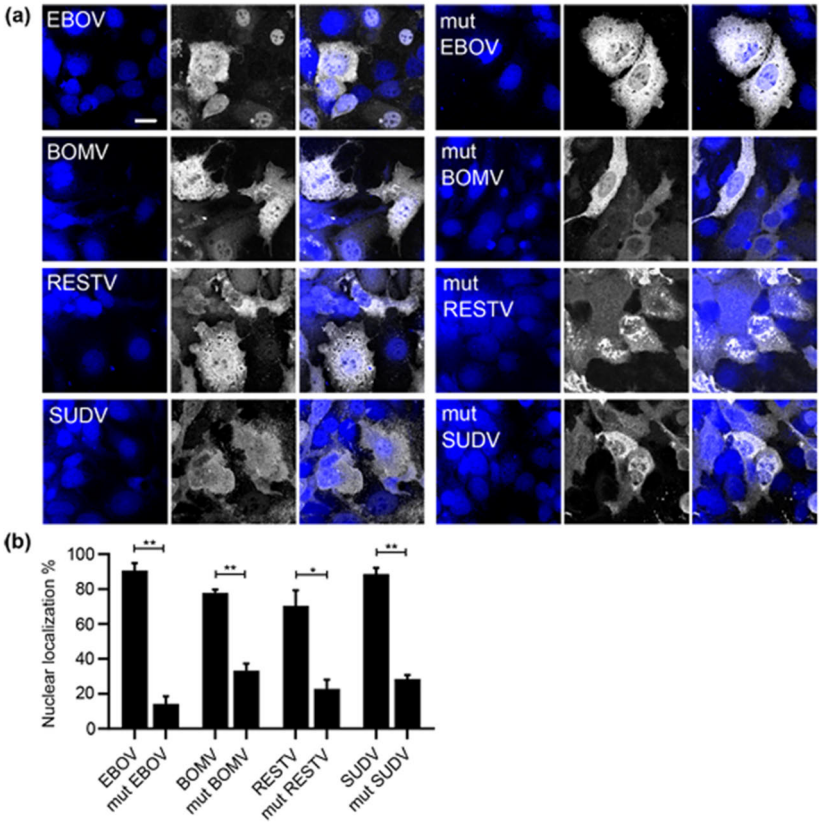


Figure 13 Nuclear localisation of NLS-mutated VP24 proteins. (a) Immunofluorescence was used to examine the subcellular location of NLS-mutated VP24s (EBOV, BOMV, RESTV, SUDV) and their wild-type counterparts in transfected Huh7 cells. HA-tagged VP24s were stained with anti-HA (white), and nuclei were stained with DAPI (blue). (b) Quantification of the nuclear localisation of wt- and NLS-mutated VP24s. This figure is reproduced from Figure 3, from original publication II which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

Nuclear accumulation was quantified and the results verified those observed visually; all four NLS-mutated VP24s had significantly lower nuclear accumulation than their wt counterparts (Figure 13B). Since this phenomenon was observed for all four studied filovirus species, it indicates that the cluster 3 of NLS is essential for nuclear localisation of VP24, and disruption of NLS markedly reduced this process.

To determine the ability of wt and NLS-mutated VP24s to bind importin α5, *in vitro*-translated or lysates from HEK293 cells expressing wt or NLS-mutated VP24s were allowed to interact with immobilised GST-tagged importin α5. All nine wt-VP24s bound to importin α5 (Figure 14, low aggregating/in-vitro expressing VP24s were [35S]Met/Cys-radiolabelled), however, the binding of MLAV and MARV VP24s was remarkably less potent compared to the others.

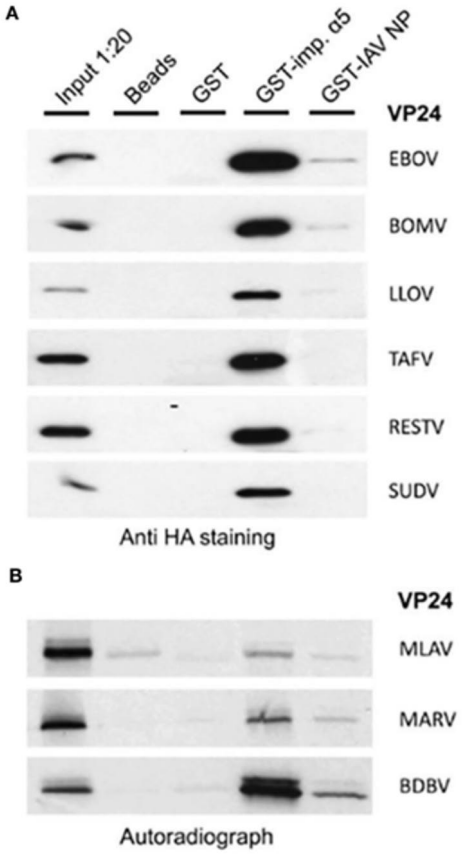


Figure 14 GST pull-down experiments for VP24 expression plasmid transfected cells for *in-vitro* translated VP24 proteins. A) Soluble cell extracts of HEK293 cells were analysed using anti-HA staining and, B) [35S]Met/Cys-labeled and *in vitro*-translated BDBV, MARV and MLAV VP24 proteins were analysed with autoradiography. This figure is reproduced from Figure 3, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

For the NLS-mutated VP24s, the binding to importin α5 clearly decreased compared to wt-VP24s (Figure 15). The bound VP24s from EBOV, BOMV, RESTV, and SUDV wt- and mutant forms to importin-α5 were detected with immunoblotting (Figure 15A). It was demonstrated that importin α5 binding was significantly reduced in the NLS-mutated constructs (Figure 15B). MARV VP24 showed no binding to importin α5, confirming the predominantly cytoplasmic subcellular localisation and the absence of a filovirus-like NLS motif (Figure 15C).

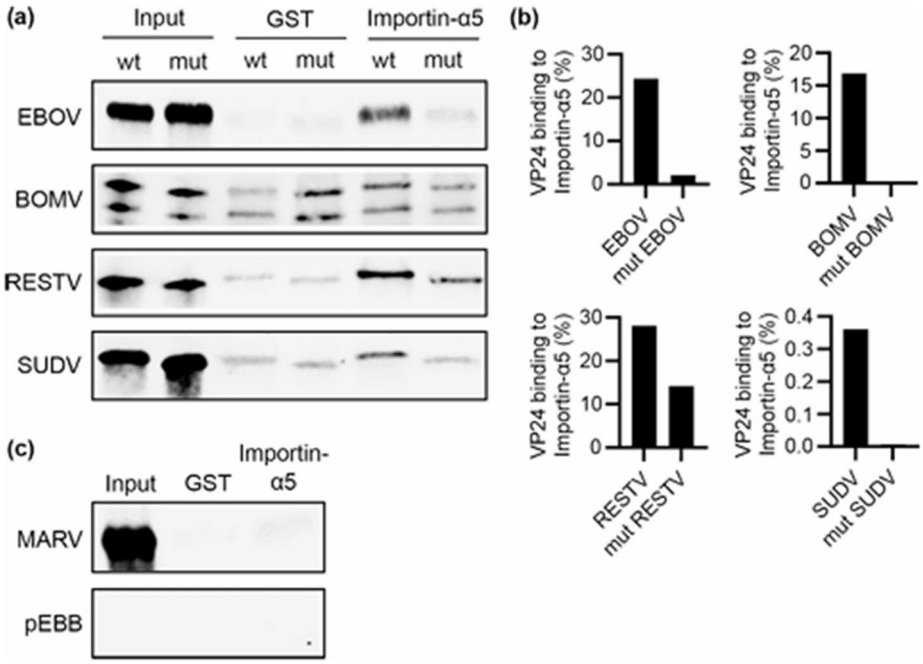


Figure 15 VP24 binding to importin α5 in HEK293 cells, (a) Expression of wild-type and NLS-mutated EBOV, BOMV, RESTV, and SUDV VP24s. (b) For quantification, band intensities for bound VP24 were compared to input for bound VP24. Unspecific GST binding was subtracted. 1/10 of the sample was used for the input. This figure is reproduced from Figure 4, from original publication II which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

These importin-binding experiments highlighted that the cluster 3 in NLS is an active importin-binding site in VP24. The NLS-mutated VP24s showed reduced nuclear localisation and decreased binding to importin α5, indicating that these residues are crucial for nuclear import.

5.3.3 VP24 mediated effects on the activation of the RIG-I pathway (Study I)

5.3.3.1 Inhibition of IFN-λ1 and IFN-β Promoter Activation by Filovirus VP24 Proteins (Study I)

The effects of VP24 proteins on the interferon beta (IFN-β) and interferon lambda-1 (IFN-λ1) promoters were examined to elucidate the impact of these proteins on the activation of the production of type I and type III interferons, respectively. The aim was to examine whether the filovirus VP24 proteins broadly suppress RIG-I–dependent induction of interferon production or differentially affect type I versus type III interferon pathways, and to analyze whether the phenomenon is common or unique to any filovirus VP24. ΔRIG, a constitutively active form of RIG-I, can signal even in the absence of viral RNA (Saito et al., 2007) and directly activates the RIG-I pathway. Due to expression of ΔRIG-I, strong activation of IFN-β and IFN-λ1 promoters is anticipated, unless an interferon antagonist, like VP24, inhibits this signaling. Thus, ΔRIG is used as a controlled and robust positive stimulus in reporter assays.

HEK293 cells were co-transfected with expression plasmids encoding ΔRIG-I and the luciferase reporter constructs driven by either the IFN-λ1 or IFN-β promoters, together with an RSV-Renilla reporter to normalize for transfection efficiency. The inhibitory effect of VP24 proteins was examined at increasing concentration to analyse dose-dependent effect on RIG-I signalling based interferon promoter activation. Hepatitis C virus non-structural protein 3/4A (HCV NS3/4A) was used as a control since, as a protease, it inhibits RIG-I signalling by cleaving MAVS (Ferreira et al., 2016). Therefore, HCV NS3/4A expression significantly reduced IFN-λ1 and IFN-β promoter-driven luciferase expression and established the sensitivity of reporter systems (Figures 16A and 16B).

Seven out of nine filovirus VP24 proteins inhibited IFN-λ1 and IFN-β promoter activation in response to ΔRIG-I stimulation. EBOV, LLOV, TAFV, RESTV, SUDV, MARV and BDBV VP24s showed significant dose-dependent suppression of promoter activity. However, the inhibitory dose-dependent effects were more pronounced for IFN-λ1 promoter activation as compared to IFN-β promoter activation (Figure 16A and 16B). In conjunction, no inhibition of ΔRIG-I induced promoter activation was observed for MLAV and BOMV, even at the highest tested doses. For MLAV VP24, this is in good concordance with the results of the weak importin-α5 binding, however, BOMV VP24 shows adequate importin- α5 binding, but lacks IFN promoter inhibition.

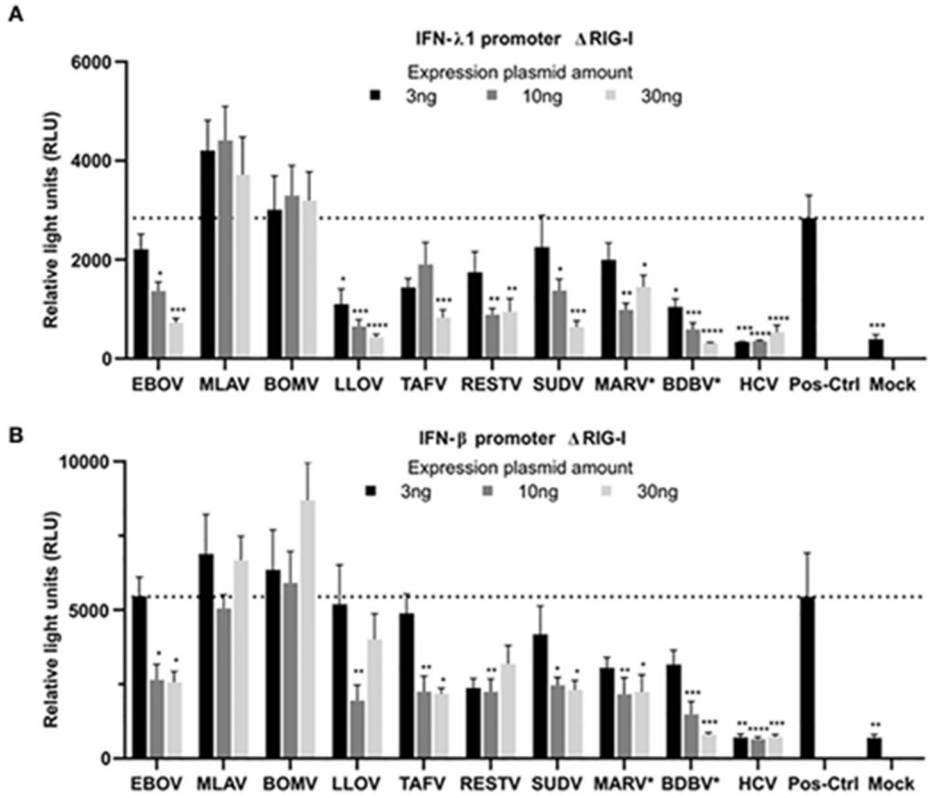


Figure 16 VP24 proteins-based inhibition of ΔRIG-I driven IFN-λ1 and IFN-β promoter activation. Filovirus VP24 expression plasmids (*codon optimised) were transfected into HEK293 cells at 3, 10, or 30 ng/well, along with ΔRIG-I and reporter plasmids, IFN-λ1 (A) or IFN-β (B) promoter-luciferase and RSV-Renilla. Luciferase values were normalised with RSV-Renilla expression control. The results show mean of three experiments conducted in replicated (n=9), and standard errors are shown as error bars. Statistical significance of the results was calculated with Ordinary one-way ANOVA with Dunnett's multiple comparisons test with a single pooled variance (positive control). This figure is reproduced from Figure 6, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

5.3.3.2 The effect of filovirus VP24 proteins on IRF3 Phosphorylation and IRF3 nuclear translocation (Study I)

IRF3 phosphorylation is the downstream outcome of RIG-I pathway. It is known that EBOV VP24 does not inhibit IRF3 phosphorylation (He et al., 2017). The inhibition of IRF3 phosphorylation by selected four mammalian-infecting filovirus VP24 proteins was investigated to further explore the impact on antiviral signalling. HEK293 cells were co-transfected with ΔRIG-I and IRF3 expression plasmids and increasing amounts of VP24 constructs of EBOV, SUDV, RESTV, and LLOV (Figure 17). ΔRIG-I readily induced phosphorylation of IRF3, whereas

none of the four VP24 proteins inhibited IRF3 phosphorylation, even at highest VP24 expression levels. These results suggest that the inhibitory action of VP24s on the induction of interferon promoter act downstream or independently of IRF3 activation.

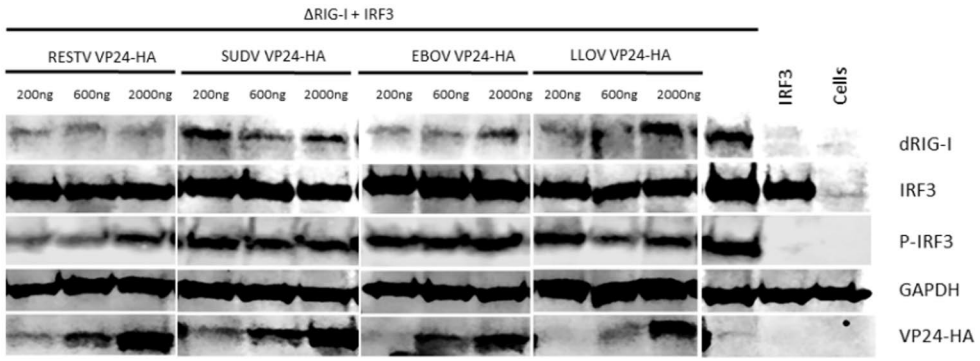


Figure 17 HEK293 cells were transfected with increasing amounts of VP24s expression plasmids, ΔRIG-I and IRF3, followed by immunoblotting based assessment of IRF3 phosphorylation. RESTV, SUDV, EBOV, and LLOV VP24 were shown to lack inhibition of IRF3 phosphorylation, indicating further downstream mode of inhibitory action. This figure is reproduced from Figure 8, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

To activate IRF3 and explore the VP24 effects on IRF3 nuclear import, Huh7 cells were transfected with wt-EBOV VP24 or NLS-mutated EBOV VP24 constructs and stimulated with polyI:C. Immunofluorescence demonstrated that IRF3 efficiently accumulated in nuclei of both control and VP24 expressing cells (Figure 18A). IRF3 nuclear localisation was similar in wt- and NLS-mutant EBOV VP24-expressing cells, confirming that EBOV VP24 does not interfere with nuclear translocation of IRF3 (Figure 18B).

To sum up the sections 5.3.3.1 and 5.3.3.2, all nine VP24 proteins were adequately expressed in both HEK293 and Huh7 cells but the expression levels differed between filoviruses, with the lowest expression level observed for MLAV. In addition, all nine VP24 proteins bind to importin-α5, however, the binding for MLAV and MARV were notably less than the other tested filoviruses. All filoviruses VP24 proteins were localised in the nucleus except MLAV, RESTV, TAFV and non-codon optimised MARV. NLS-mutated RESTV, EBOV, BOMV and SUDV did not bind to importin and did not localise in the nucleus. Amongst the tested filoviruses, MLAV and BOMV did not inhibit IFN-promoter activation, while all others showed statistically significant inhibition. In conjunction, RESTV, SUDV, EBOV and

LLOV filoviral VP24 proteins did not inhibit IRF3 phosphorylation, while EBOV VP24 did not inhibit IRF3 nuclear localisation.

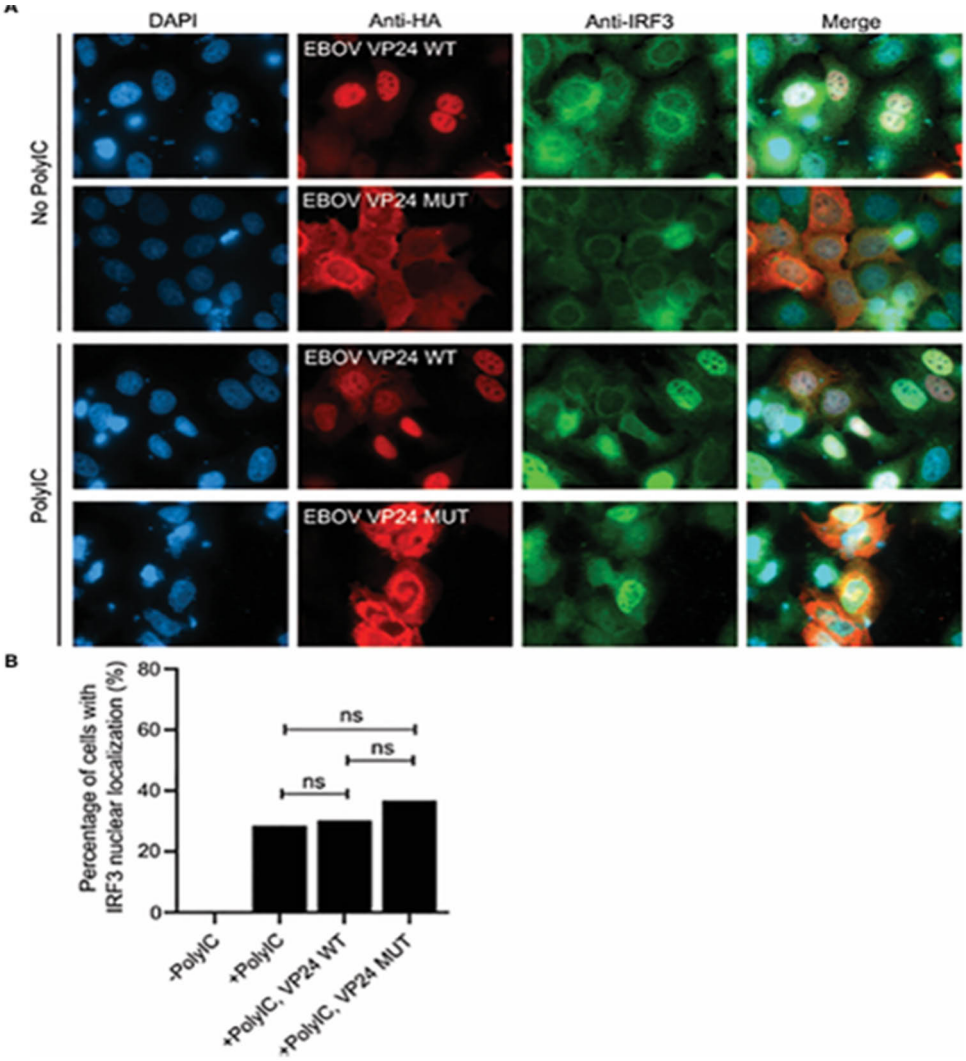


Figure 18 (A) Effect of EBOV VP24 on IRF3 nuclear import. Anti-HA, anti-IRF3 and fluorescent secondary antibodies were used to stain Huh7 cells., (B) Quantification of nuclear translocation of IRF3. This figure is reproduced from Figure 9, from original publication I which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

5.3.4 Filovirus VP24 proteins suppress activation of MxA and IFITM3 promoters (Study II)

To explore the potential of VP24 proteins in suppressing IFN-induced signalling, HEK293 cells were co-transfected with MxA or IFITM3 promoter-driven luciferase constructs and expression plasmids expressing VP24 proteins of nine filoviruses i.e., EBOV, BOMV, BDBV, MLAV, RESTV, SUDV, TAFV, LLOV, and MARV (Figure 19A and 19C), and NLS-mutated VP24s of EBOV, BOMV, RESTV and SUDV (Figure 19B and 19D). Transfected cells were stimulated with IFN-α2a to activate the interferon-induced pathways.

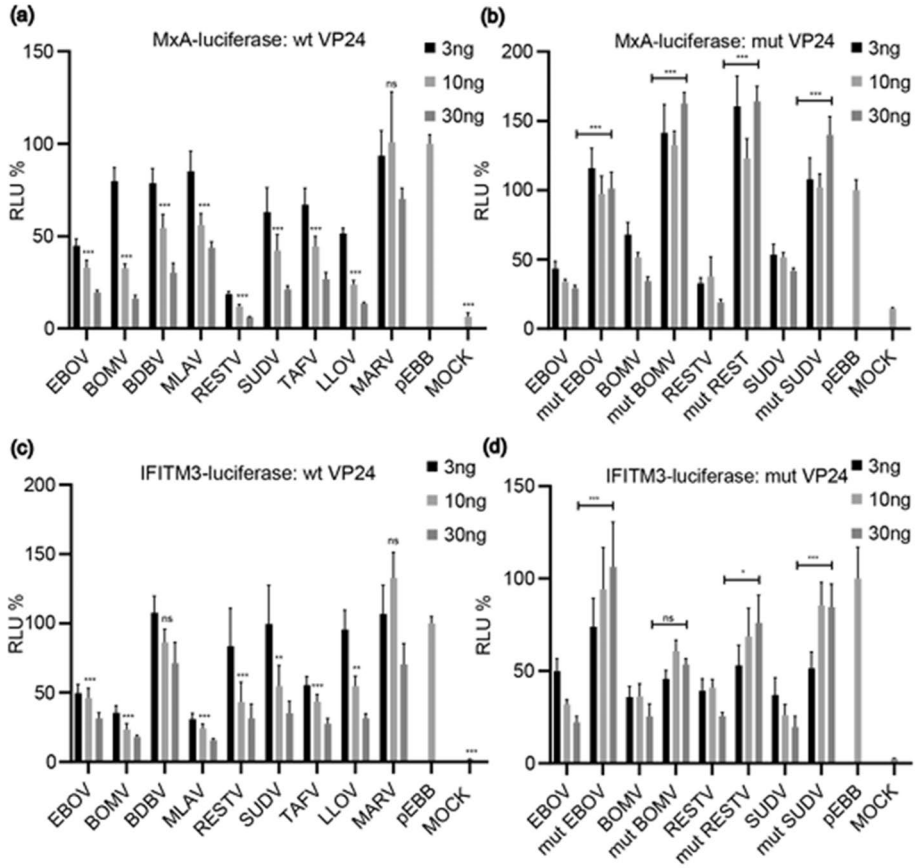


Figure 19 The effect of nine filovirus VP24 proteins and NLS-mutant VP24s from EBOV, BOMV, RESTV, and SUDV on IFN-α2a–stimulated activation of the MxA (a, b) and IFITM3 (c, d) promoters. Cells transfected with an empty expression vector and stimulated with IFN-α2a (pEBB-HA) served as the positive control, while non-transfected cells (MOCK) were used as the negative control. This figure is reproduced from Figure 6, from original publication II which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

The results of the luciferase-promoter reporter assays revealed that eight out of the nine VP24 proteins (EBOV, BOMV, BDBV, MLAV, RESTV, SUDV, TAFV, and LLOV) strongly inhibited IFN-induced MxA promoter activation in a dose dependent manner, while the IFN-α2a treatment in the absence of VP24s (experimental control, pEBB vector) strongly induced MxA promoter activation. MARV VP24 did not affect MxA promoter activation even at high expression levels (Figure 19A). NLS-mutated VP24 constructs of EBOV, SUDV, BOMV, and RESTV were also tested, and it was demonstrated that all NLS-mutated VP24s lost their capacity to suppress MxA promoter activation due to perturbed importin-binding (Figure 19B).

IFITM3 promoter activation showed a similar trend as eight filovirus VP24s (except MARV) inhibited IFITM3 promoter activity in a dose-dependent manner (Figure 19C), albeit BDBV VP24 related inhibition was inferred to be statistically insignificant. MARV VP24 and NLS-mutated constructs of EBOV, SUDV, BOMV, and RESTV (Figure 19D) demonstrated minimal or no inhibition, which reflected that proper nuclear localisation and importin interaction are necessary for VP24 to suppress interferon signalling.

To assess the effect of VP24s on the expression of endogenous MxA, Huh7 cells were transfected with VP24 expression constructs and induced with IFN-α2a. The expressed MxA and VP24s were visualised with immunofluorescence in co-stained cells. Those VP24 proteins, which suppressed MxA promoter activation, also inhibited IFN-α induced expression of endogenous MxA protein (Figure 20A). Additionally, NLS-mutated constructs of four filovirus VP24 constructs (EBOV, SUDV, BOMV, and RESTV) lost the inhibitory effects on the endogenous MxA protein expression (Figure 8 in Study II). Quantification analysis demonstrated that nearly 90% of IFN-induced control cells expressed MxA, while the percentage of expression dropped significantly to 20–80% for VP24-expressing cells (Figure 20B).

These results revealed that eight out of nine mammalian filovirus VP24s efficiently suppress IFN-α induced activation of MxA and IFITM3 promoters, and reduced IFN-induced endogenous MxA protein levels. In conjunction, MARV VP24 showed no suppression of IFN-induced responses which differentiated it from VP24s of other mammalian-infecting filoviruses included in the study II.

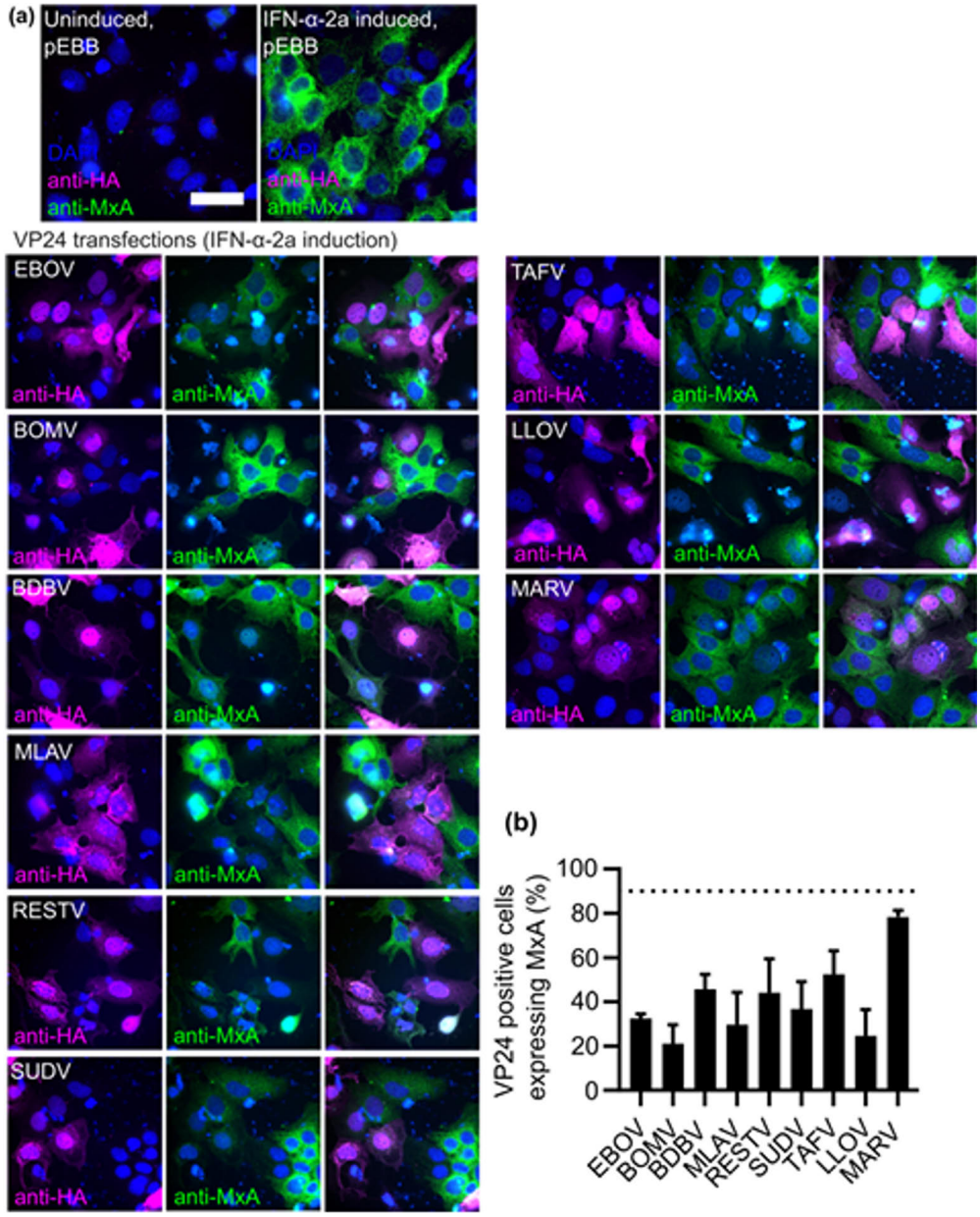


Figure 20 Filovirus VP24s inhibit IFN-α−2a mediated MxA protein expression (A) Immunofluorescence images of empty plasmid vector (pEBB) and nine filovirus VP24 proteins, (B) Quantification of Huh7 cells expressing endogenous MxA for nine filoviral VP24 proteins. This figure is reproduced from Figure 7, from original publication II which is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

5.4 Filovirus VP35 proteins (Study III)

5.4.1 Expression and subcellular localisation of VP35s (Study III)

HEK293 and Huh7 cells were transfected with plasmids encoding HA-tagged VP35s from EBOV, BOMV, BDBV, RESTV, SUDV, TAFV, LLOV, MARV and MLAV to explore the expression and distribution of VP35 proteins of the mammalian-infecting filoviruses. Immunoblotting of HEK293 cells transfected with VP35 expression plasmids showed VP35 protein expression increasing proportionally with plasmid concentration (Figure 21), establishing effective transcription and translation of all constructs. VP35 was detected at predicted molecular weights in all viral species, confirming protein integrity. The loading control tubulin showed equal protein loading, while mock-transfected and empty vector (pEBB) controls revealed no VP35 signal which ensure the experimental reliability. To ensure strong protein production, codon-optimised EBOV, RESTV, and MARV VP35 constructs were used in subsequent experiments. The expression efficiency of these optimised constructs was similar to other EBOV VP35s which allowed standardised comparative analysis across all nine species.

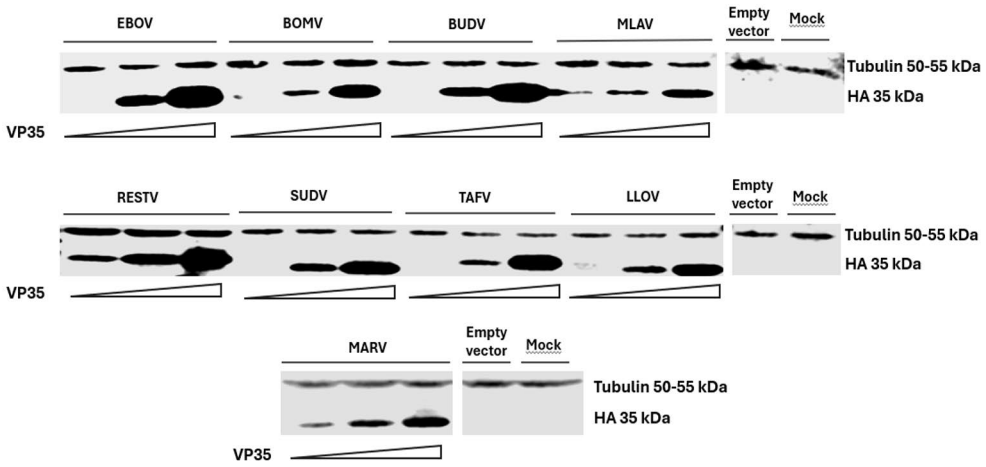


Figure 21 HEK293 cells express VP35 proteins that are N-terminally HA-tagged in a expression plasmid concentration dependent manner. Tubulin served as a loading control, while empty vector (pEBB)-transfected and non-transfected (Mock) cells served as controls. This figure is reproduced from Figure 2 (Unpublished data, Study III).

To analyse the subcellular localisation of VP35s, Huh7 cells were transfected with the VP35 expression plasmids and examined by immunofluorescence microscopy. It was illustrated that all VP35 proteins were highly expressed and

localised predominantly in the cytoplasm (Figure 22). There was no nuclear accumulation of VP35 protein and expression was homogeneous across cells, while the empty vector transfected and mock controls showed no HA-specific staining, confirming antibody specificity and absence of background signal.

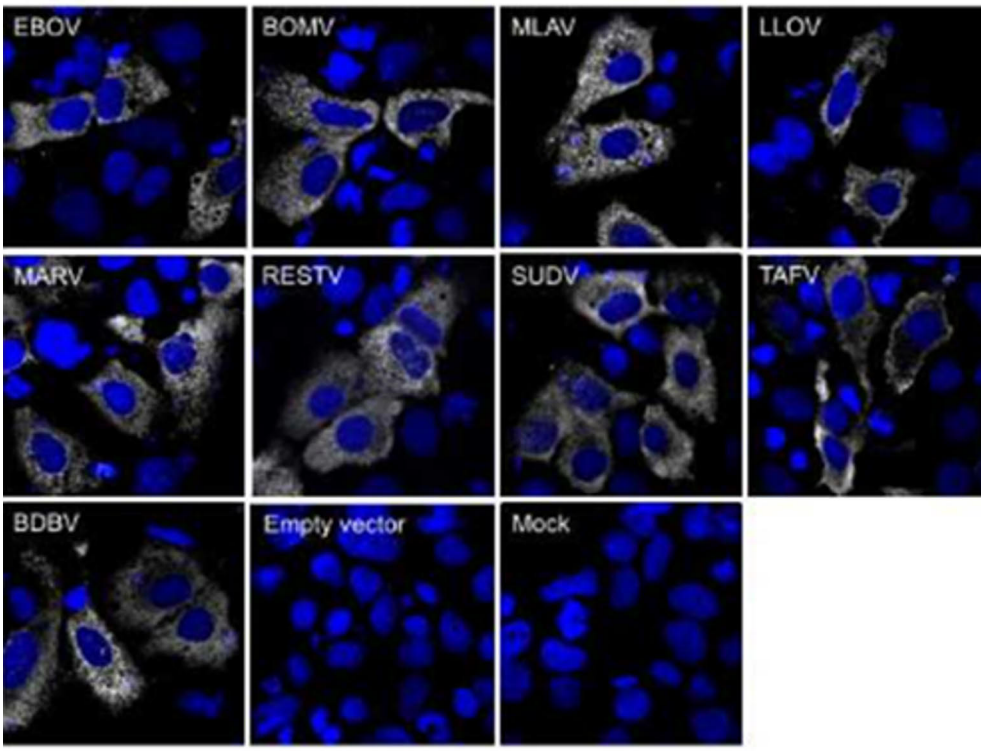


Figure 22 Subcellular localization of nine VP35s (EBOV, BOMV, MLAV, LLOV, MARV, RESTV, SUDV, TAFV, and BDBV) were analysed with immunofluorescence in transfected Huh7 cells. Anti-HA (staining HA-tagged VP35s, white) was used to label cells, while DAPI (blue) was used to stain nuclei. This figure is reproduced from Figure 3A (Unpublished data, Study III).

Mitochondria are central to antiviral signaling through MAVS-dependent RIG-I signaling. To determine if VP35s co-localization within mitochondria, VP35-expressing Huh7 cells were stained with Mitotracker. None of the VP35 proteins co-localised with mitochondrial structures (Figure 23). Thus, all nine filovirus VP35 proteins are efficiently expressed in mammalian cells, are localised in the cytoplasm, and seem not to directly interact with mitochondrial antiviral signalling complexes.

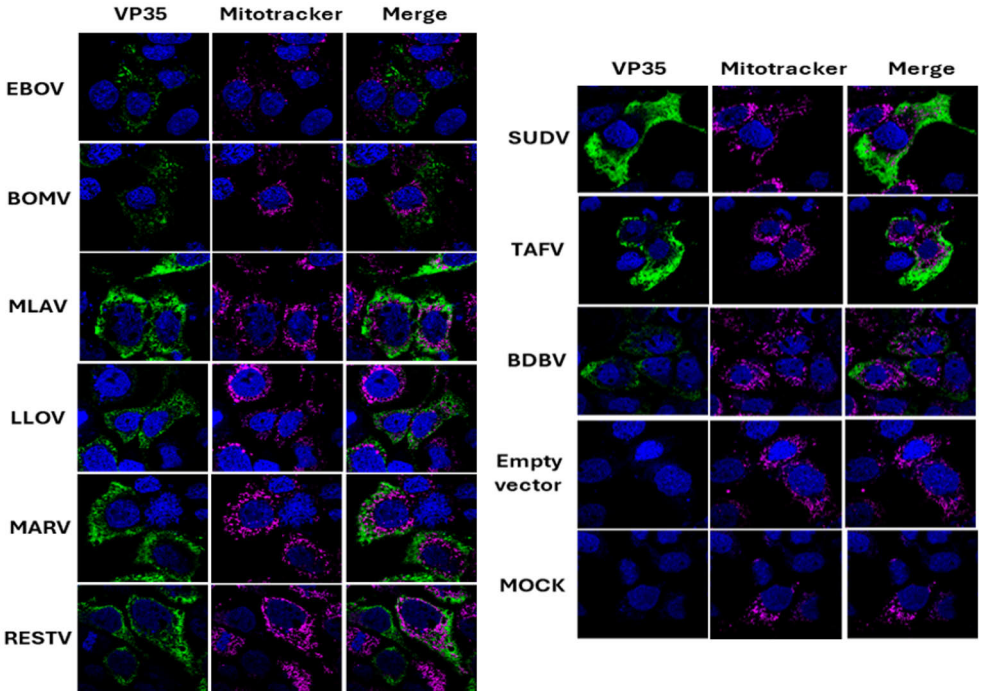


Figure 23 Mitotracker was used to assess mitochondrial co-localization with VP35 proteins. VP35 proteins are expressed in Huh7 cells, and those do not co-localize with the mitochondria. This figure is reproduced from Figure 3B (Unpublished data, Study III).

5.4.2 VP35s inhibition of endogenous RIG-I (Sendai virus/PolyIC induced) signalling is not mediated by downstream pathway components (Study III)

To analyze the effect of nine VP35 proteins, representing mammalian-infecting filovirus species, on the RIG-I-mediated antiviral signalling pathway, HEK293 cells were co-transfected with IFN-λ1-promoter-luciferase reporter construct and VP35 expression plasmids. The transfected cells were stimulated overnight either with polyI:C, a synthetic double-stranded RNA analogue to viral RNAs, or with Sendai virus, a negative sense, single stranded RNA virus, which both strongly stimulate the RIG-I pathway (Figure 24). A dual-luciferase reporter gene assay was used to quantify firefly luciferase activity which was normalised to Renilla luciferase that was used as an internal transfection control.

PolyI:C stimulation strongly enhanced IFN-λ1-promoter activity in empty vector–transfected cells by activating the innate antiviral system and the positive control, the HCV NS3/4A protease, resulted in a strong inhibition of the promoter activation (Figure 24A). The co-expression of all nine filovirus VP35 proteins (EBOV, BOMV, BDBV, RESTV, SUDV, TAFV, LLOV, MARV and MLAV)

significantly reduced IFN-λ1-promoter activity, establishing that all VP35s inhibit endogenous RIG-I signalling, although a clear dose dependency could not be observed in the analysis.

Infection of the VP35 transfected cells with Sendai virus resulted in similar but clearer inhibitory pattern of IFN-λ1 promoter activation (Figure 24B); empty vector-transfected cells showed maximum IFN-λ1 promoter activation, while VP35 protein-expressing cells showed significantly lower IFN-λ1 promoter activity. Hence, all VP35 proteins showed statistically significant inhibition of polyI:C or Sendai virus induced IFN-λ1 promoter activation.

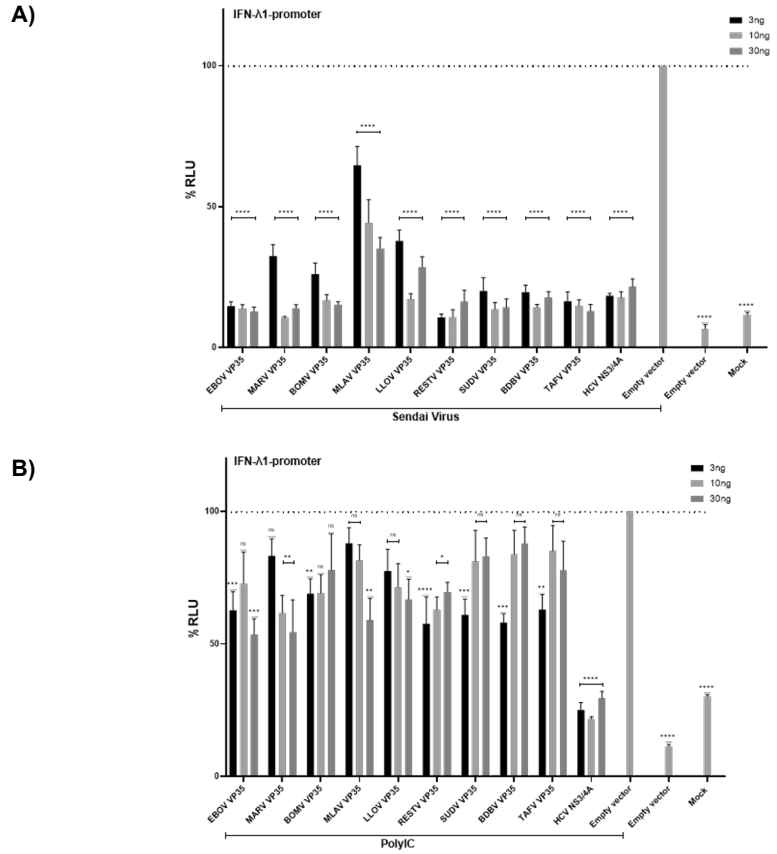


Figure 24 Inhibitory effects of filovirus VP35 proteins on IFN-λ1 induced gene expression. Increasing amounts (3, 10 or 30 ng/well) of VP35 expression plasmids together with expression plasmid wt-RIG-I and reporter plasmids IFN- λ1 promoter-luciferase (30 ng/well) and RSV-Renilla (50 ng/well) were used to transfect HEK293 cells, induced through polyI:C (A) or Sendai virus (B). Mean values of three independent experiments with three replicates (n=9) are represented by bars and standard errors are shown with error bars. Ordinary one-way ANOVA with Dunnett's multiple comparisons test and a single pooled variance were used to calculate statistical significance. Each column value was compared to the positive control. P values, *p = < 0.05, **p = < 0.005, ***p = < 0.0005, ****p = < 0.0001. This figure is reproduced from Figure 4 (Unpublished data, Study III).

Three VP35s were selected to analyze at which step of the RIG-I pathway VP35s exert the inhibitory effect. EBOV and MARV VP35s were selected since those represent the most studied filoviruses, while BOMV VP35 was chosen as the most recently discovered filovirus. The HEK293 cells were co-transfected with VP35 expression plasmids, along with individual components of the RIG-I pathway, i.e. ΔRIG-I (constitutively active form), MAVS, TBK1, IKKε or IRF3-5D (constitutively active form), and the IFN-λ1-promoter-luciferase reporter construct. The overexpression of any of these pathway components leads to the activation of the IFN-λ1 promoter. As in the previous experiments, HCV NS3/4A and empty vector transfected cells were used as controls. The luciferase-reporter promoter analysis demonstrated that, while expression of pathway components activated the IFN-λ1 promoter and HCV NS3/4A inhibited the activation at and before MAVS, VP35 proteins did not inhibit promoter activation induced by the overexpression of any of the downstream signalling pathway components (Figure 25A).

Moreover, immunoblotting (Figure 25B) demonstrated that VP35 protein expression did not affect the quantity of ΔRIG-I, MAVS, TBK1, IKKε or IRF3-5D which further showed that the inhibitory impact on IFN-λ1 promoter activation was not caused by protein degradation or suppression of expression of the pathway components (Figure 25). As expected, HCV NS3/4A that was used as a control, cleaved MAVS. These results affirmed that filovirus VP35 proteins suppress IFN-λ1 promoter activation upstream of constitutively active form of RIG-I. The results have highlighted that all nine VP35 proteins suppress IFN-λ1-promoter activation but do not affect downstream signalling components of RIG-I pathway. The absence of inhibition in ΔRIG-I--mediated activation indicated that VP35 operates upstream of RIG-I signalling rather than targeting effector kinases or transcription factors.

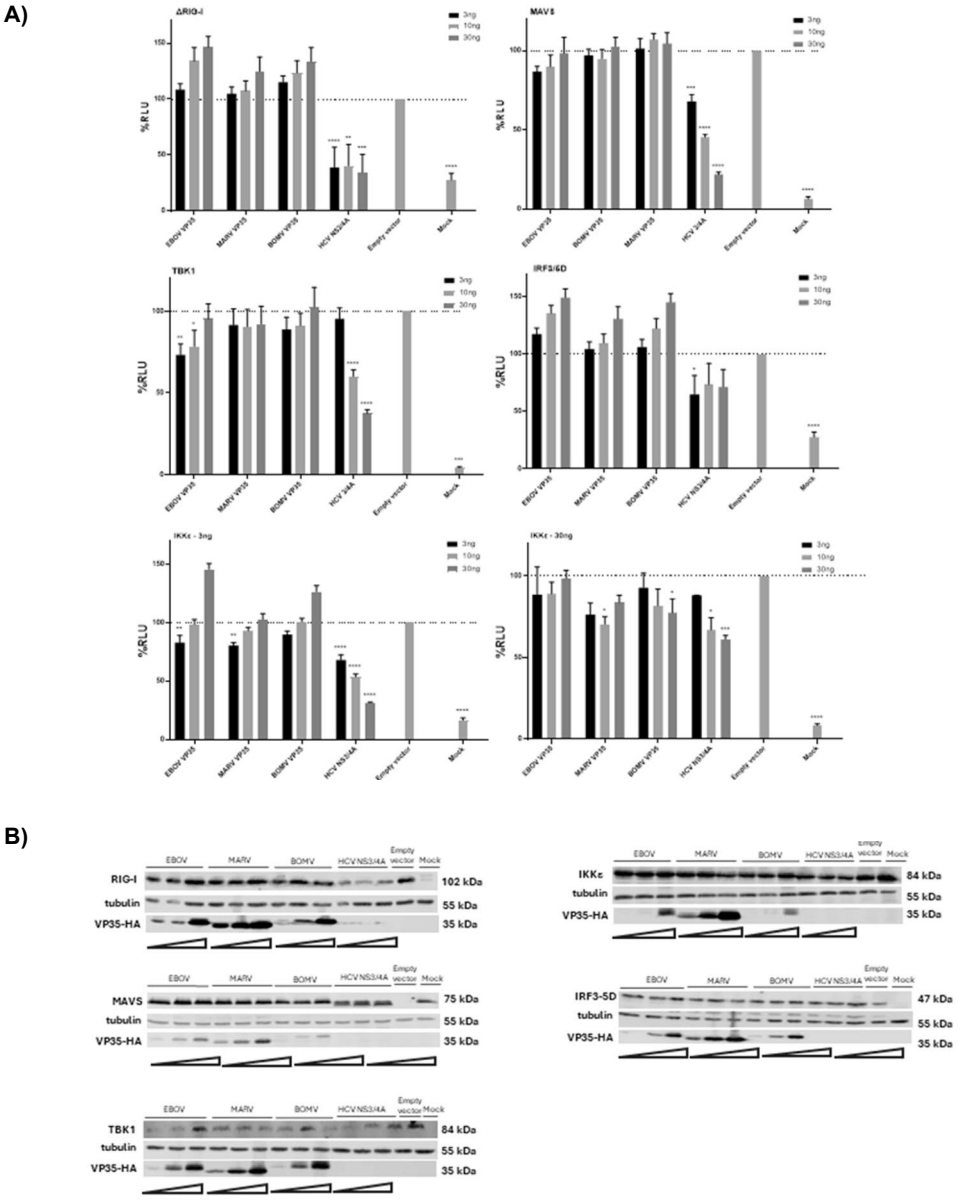


Figure 25 (A) Luciferase assay results showing no statistically significant evidence for EBOV, BOMV or MARV VP35 proteins mediated inhibition of MAVS, TBK1, IKKe, and IRF3-5D. (B) Immunoblots of HEK293 cells co-transfected with EBOV, MARV or BOMV VP35 expression plasmids and ΔRIG-I, MAVS, TBK1, IKKε, or IRF3-5D. HCV NS3/4A was used as a control and tubulin as a loading control. This figure is reproduced from Figure 5 (Unpublished data, Study III).

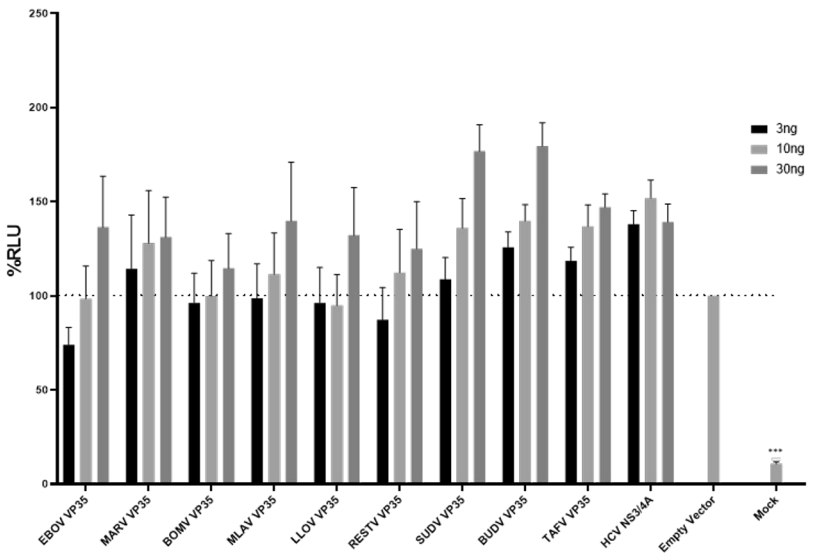
5.4.3 VP35 mediated effects on the IFN-Induced Pathway are not mediated by MxA and IFITM3 promoters (Study III)

The effect of nine filovirus VP35 proteins on IFN-induced pathway was assessed, which results in the activation of MxA or IFITM3 promoters. HEK293 cells were co-transfected with MxA, or IFITM3-promoter-luciferase reporter constructs and VP35 protein expression plasmids. Following transfection, cells were treated overnight with IFN-α2a to activate the IFN-induced JAK–STAT signalling pathway, which promotes transcription of interferon-stimulated response elements (ISREs)-regulated genes. Luminescence activity, indicating the activation of IFN-induced MxA- or IFITM3-promoters, was measured using dual-luciferase reporter assay.

The results showed that IFN-α2a stimulation significantly activated MxA and IFITM3 promoters in empty vector-transfected control cells devoid of VP35 expression. As expected, HCV NS3/4A lacked any significant inhibitory effect on IFN-induced activation of the promoters. Unlike VP24 proteins, none of the nine VP35 proteins inhibited IFN-induced promoter activation (MxA-promoter in Figure 26A and IFITM3-promoter in 26B).

As a conclusion, all nine VP35 proteins similarly inhibit RIG-I antiviral signalling but are unable to affect interferon-induced activation of the promoters of known ISGs. This data supports the role of VP35 as a cytoplasmic innate immune response antagonist that targets RIG-I activation without disrupting the IFN-mediated responses.

A) MxA



B) IFITM3

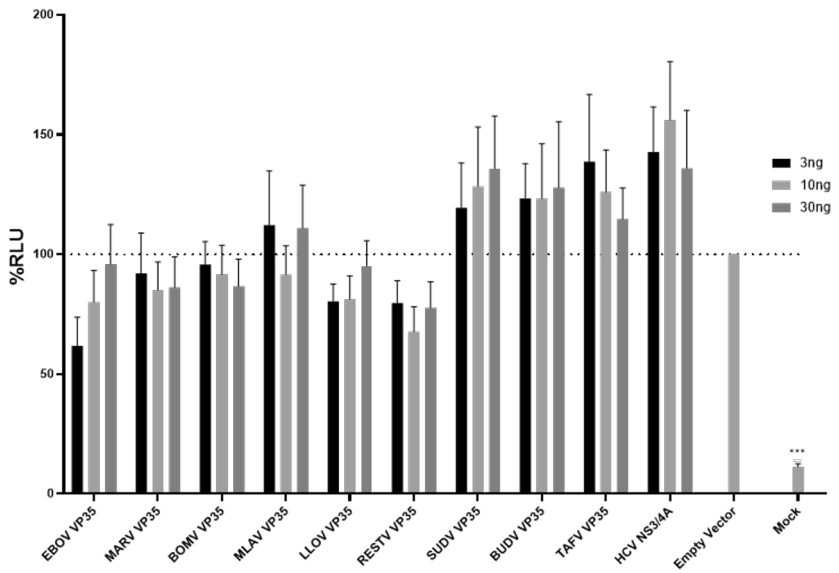


Figure 26 Filovirus VP35 proteins-based inhibition of the IFN-induced MxA (A) and IFITM3 (B) promoter activation. HEK293 cells were transfected with increasing amounts (3, 10 or 30 ng/well) of VP35 expression plasmids together with MxA or IFITM3-promoter-luciferase constructs) and RSV-Renilla. Transfected cells were induced with IFNα2a. This figure is reproduced from Figure 6 (Unpublished data, Study III).

6 Discussion

The thesis examines mammalian-infecting filoviruses that deploy VP24 and VP35 to evade host innate immune responses. These proteins operate as dual molecular antagonists to inhibit distinct but interrelated IFN pathway components, allowing the virus to multiply and spread in the host while evading antiviral defenses.

The first study revealed that nine filovirus VP24 proteins affected the activation of type I and III interferon promoters, triggered by RIG-I, and the inhibition took place downstream of IRF3. The second study confirmed that eight out of nine filoviral VP24 proteins inihibit IFN-induced pathway through MxA and IFITM3 promoters, establishing their dependancy on functional cluster 3 NLS. However, MARV VP24 has weak or lack of inhibition of IFN-induced pathway, although similar subcellular localisation as with other VP24 proteins.

The final study of this doctoral thesis examined the effects of nine filoviral VP35 proteins on the RIG-I and IFN-induced pathway and documented that these proteins inhibit the RIG-I pathway induced through PolyI:C or Sendai virus, pointing towards an upstream mode of inhibition as compared to VP24 proteins. Overall, the findings support the dual-tiered immune evasion model, i.e.,VP35 stops cytoplasmic sensors from detecting viral RNA while VP24 suppresses nuclear antiviral transcriptional responses.

In my results, VP24 and VP35 sequences have been found to be highly conserved within species, indicating strong evolutionary linkages and functional importance. This doctoral thesis, thereby, identifies VP24 and VP35 as key factors in Filoviridae pathogenesis and adaptive evolution.

6.1 Comparative Evolutionary Perspective

The comparison of VP24 and VP35 functions across nine Filoviridae species demonstrated cladic evolutionary conservation amongst filoviruses. Filoviral immune evasion depend on molecular determinants that suppress host innate immune signalling (Basler & Amarasinghe, 2009). Differences in the magnitude of immune antagonism across filoviral species likely reflect evolutionary constraints and adaptation to distinct host environments.

According to studies conducted for this doctoral thesis, BOMV and MLAV showing low IFN-suppression is significant due to their evolutionary position in Filoviridae family i.e., these viruses attain evolutionarily intermediate lineages. BOMV (*Orthoebolavirus*) and MLAV (*Dianlovirus*) have reduced comparative ability to inhibit RIG-I (VP35) or IFN-induced signalling (VP24). The reduced ability aligns with the existing literature that efficient immune evasion is necessary for systemic viral propagation and human pathogenesis (Messaoudi et al., 2015). It has also been demonstrated that BOMV replicates in mammalian cells similar to EBOV but does not induce clinical disease, for which specific mechanisms of different pathogenic potential are not fully known (Bodmer et al., 2023). Also, despite its strong genetic relationship with MARV, MLAV is connected with bat reservoirs and has not been linked to human disease, possibly due to low direct transmission under natural conditions (Zhao et al., 2022).

The phylogenetic evidence from the current study is consistent with an association between immune evasion efficacy and pathogenic potential. VP24 sequence alignment has shown that *Orthoebolavirus* species use conserved amino acid motifs in the cluster 3 region of the NLS for importin α5 binding and STAT1 antagonism, but MARV and MLAV have different residues in this area, reducing their capacity to block interferon-stimulated gene activation (He et al., 2017).

VP35 proteins from all nine species show statistically significant suppression of RIG-I-mediated IFN promoter activation, which is correlated with moderate structural differences. Crystallographic investigations have already cited that pathogenic *Orthoebolavirus* VP35s have higher electrostatic complementarity for dsRNA binding than *Orthomarburgvirus* VP35s (Edwards et al., 2016). The phylogenetic reconstruction from the current study supported this evolutionary gradient by displaying that *Orthoebolavirus* and *Cuevavirus* VP24 and VP35 proteins form compact branches, indicating strong selective conservation while *Orthomarburgvirus* proteins form distinct branches nodes.

The important evolutionary topology highlighted functional specialisation within genera supporting earlier evidence i.e., *Orthoebolavirus* species have converged to optimise replication in primate hosts, but also circulate in bats, while *Orthomarburgvirus* species have diverged due to long-term co-evolution within bat reservoirs (Towner et al. 2009). Thereafter, the intermediate grouping of LLOV between EBOV and MARV and its moderate suppression of interferon responses illustrates an evolutionary gradient between immune evasion efficiency and host adaptation. On the other hand, it has also been documented in the literature that a correlation between genotype (EBOV) and severity/lethality of disease could not be established in animal models (Marzi et al., 2018).

Comparative analyses help us in understanding the evolutionary concept in the existing literature by demonstrating that differential immune suppression of VP24

and VP35 directly reflects their host adaption and pathogenicity. However, BOMV and MLAV have weaker immune antagonism, supporting the principle that insufficient adaptation to human interferon pathways limits zoonotic spillover potential while EBOV and SUDV have greater suppressive efficiency aligning with their reported epidemic potential and mortality. The structural and phylogenetic similarities between functional potency and evolutionary conservation supports the co-evolution of immune evasion mechanisms and virulence in Filoviridae (Shi et al., 2018; Ramanan et al., 2012). This co-adaptive process highlights the evolutionary adaptation of pathogenic Orthoebolaviruses in humans, but emerging or divergent species can be limited by sub-optimal molecular interfaces with host immune systems which determine their pathogenic potential.

The phylogeny results demonstrated that *Orthoebolavirus* VP24s form a strict evolutionary cluster with good sequence conservation (69-88% identity). MARV and MLAV VP24s are on different branches with only 34-38% similarity with Orthoebolaviruses. According to previous studies, MARV coevolved within *Rousettus aegyptiacus* bats by adopting replication mechanisms that would rely less on STAT1 antagonism and more on modulation of other cellular pathways (Towner et al., 20 09; Basler et al., 2019). The genomic differences between MARV and EBOV VP24s reflected as different host adaptation strategies as EBOV VP24 preserved a strong importin-targeting mechanism to overcome IFN-induced gene expression, whereas MARV VP24 lacks effective STAT nuclear import antagonism, due to structural differences in the cluster 3 region between EBOV and MARV VP24 proteins (Zhang et al., 2014).

6.2 VP24 proteins suppress RIG-I/MDA5 dependent Type I/III IFN-promoter activation

The study data revealed that VP24 proteins EBOV, SUDV, RESTV, TAFV, LLOV, BDBV, and MARV, effectively suppressed RIG-I/MDA5-driven activation of IFN-λ1 and IFN-β promoters. Previous studies have highlighted that *Orthoebolavirus* VP24 inhibits interferon signalling by competing with phosphorylated STAT1 binding to importin α5, preventing nuclear translocation of the ISGF3 complex and transcription of interferon-stimulated genes (Reid et al., 2006; Xu et al., 2014; Ramanathan et al., 2023).

Comparative analysis across filoviruses highlights functional diversity in VP24 protein activity. All tested VP24 proteins were expressed in HEK293/Huh7 cells but with different expression levels, while only seven VP24 proteins (6 Ebolaviruses and LLOV) showed importin-α5 binding efficiency. MARV and MLAV VP24 proteins were not only expressed in low levels, but also exhibited weak importin-α5 binding,

and predominantly localised outside of the nucleus, in contrast to nuclear localisation of other tested VP24 proteins.

Correspondingly, MLAV VP24 protein also failed to inhibit IFN-λ1 and IFN-β promoter activation, while codon-optimised MARV retained this activity. BOMV VP24 similarly lacked inhibitory activity despite retaining sequence similarity to EBOV VP24 and possessing NLS. These findings indicate that sequence conservation alone is insufficient to confer functional interferon antagonism, and that additional factors—such as protein stability, expression efficiency, and precise structural features of the importin-binding interface—are critical determinants of VP24 function.

The current data demonstrates that RESTV, SUDV, EBOV and LLOV do not inhibit IRF3 phosphorylation while EBOV VP24 does not inhibit IRF3 nuclear localisation. VP24-mediated inhibition downstream of IRF3 phosphorylation aligns with mechanistic model consistent across the existing literature for EBOV, as VP24 does not disrupt antiviral signalling pathways involving TBK1 or IKKε kinases due to continued phosphorylation and nuclear accumulation of IRF3 (He et al., 2017). However, nuclear antagonistic MOA of VP24 still targets host transcriptional machinery downstream of IRF3-dependent gene activation, which is established in the present studies, by the loss of IFN-β and IFN-λ1 promoter activation despite intact IRF3 activity.

According to previous reports, type III IFNs like IFN-λ1, provide mucosal antiviral protection via IFNLR1/IL10R2 receptor complexes while type I IFNs regulate systemic antiviral states through IFNAR1/2 receptors (de Weerd et al., 2024). The dose-dependent suppression of RIG-I-induced IFN-λ1 promoter activity by VP24 aligns with existing studies for EBOV (He et al., 2017; Ramananthan et al., 2023). Thus, the understated molecular effects of VP24 occur during interferon transcriptional amplification (post-IRF3 nuclear import but pre-IFN gene transcription). As a rationale, a positive feedback loop of IFN-mediated autoinduction of transcription is plausible which is hampered by classical VP24-mediated effects on STAT1, but further investigation would be needed to provide deeper insights into the specific mechanistic basis of this observation.

Moreover, the absence of BOMV and MLAV VP24 inhibition revealed the functional diversity among Filoviridae species. In conjunction, diversity in the filovirus mediated inhibitory effects may also be traceable to dose effects i.e., the amounts of filoviral VP24 proteins required to cause a significant level of inhibition may vary across species. Current studies have indicated that VP24s share conserved nuclear localisation signals (NLS), but despite sequence similarities to EBOV VP24, BOMV VP24 fails to block IFN-β and IFN-λ1 promoter activation. This indicates that sequence conservation alone does not ensure functional equivalency.

The protein structure, expression stability, and post-translational regulation also affect interaction of VP24 with host nuclear import proteins. Although MLAV VP24 lacks inhibitory effect; it is similar to MARV in terms of functional divergence of VP24 without the importin-binding interface. The inability of BOMV and MLAV VP24s to suppress IFN induction suggests lower immune evasion and possibly lower virulence, which is consistent with the evolutionary and epidemiological observations that link strong interferon antagonism with increased pathogenicity among *Orthoebolavirus* lineages. The datasets of current studies established that most of the Filoviridae species suppress RIG-I/MDA5-driven interferon induction via VP24 proteins. In conjunction, VP24 protein inhibits downstream of IRF3 activation, demonstrating its nuclear rather than cytoplasmic effects.

6.3 VP24-Mediated Suppression of IFN-Induced Pathway

The research studies for this thesis have found that eight of the nine VP24 proteins tested (EBOV, BOMV, BDBV, MLAV, RESTV, SUDV, TAFV and LLOV) inhibited IFN-α-induced activation of MxA and IFITM3 promoters, while MARV displayed no such inhibition. The finding supports previous literature which establishes that EBOV, but not MARV, VP24 proteins inhibit STAT1 nuclear import and suppress IFN-stimulated gene (ISG) expression, inhibiting the interferon (IFN) response (Reid et al., 2006; Reid et al., 2007; Valmas et al., 2010; Schwarz et al., 2017).

In a key earlier study, it has been shown that MARV inhibits both type I and type II interferon responses by a mechanism that is distinct from EBOV, as mediated by VP40 proteins rather than EBOV VP24. MARV VP40 inhibits both IFN-α/β and IFN-γ by preventing tyrosine phosphorylation of Janus kinases (JAK) and STAT transcription factors, which are essential for downstream IFN signaling. MARV VP40 also inhibits IL-6 – induced STAT1 and STAT3 phosphorylation, indicating a global suppression of JAK1-dependent cytokine signaling (Valmas et al., 2010). Interestingly, a similar MOA has been shown also for MLAV VP40 (Williams et al., 2020), however, it remains unclear for now if this MOA substitutes for the VP24 dependent inhibition of importin-α5 mediated STAT import (Bodmer et al., 2025).

It has been noted in earlier studies that EBOV and MARV VP24 proteins suppress both type I (IFN-β) and type III (IFN-λ1) interferon pathways, which is concordant with current studies, although through distinct mechanistic basis (Reid et al., 2006; Valmas et al., 2010; He et al., 2017). Following previous research foundations, the current research reveals consistent results of type I/III interferon pathway inhibition, indicating a broader (i.e., spanning across filoviral strains) involvement in inhibiting antiviral transcriptional responses. According to these

results, VP24 proteins interfere with the transcriptional activation of ISGs at a downstream node of IFN signalling, and after IRF3 activation step. The suppression of IFN-promoters activity following RIG-I stimulation suggests that VP24 interferes with signaling upstream or at the level of IRF3 activation, in addition to its established role in inhibiting STAT1 nuclear import.

MARV VP24, however, did not suppress karyopherin-mediated STAT1 nuclear import, even at high expression levels which reflected a functional divergence from other members of Filoviridae family. MARV VP24 lacks the importin-binding nuclear localisation signal (NLS) needed for interfering with STAT1 nuclear localisation. This feature of MARV prevented it from repressing IFN-α-mediated transcription and separated it from members of the genus *Orthoebolavirus* through functional divergence, as supported by the outcomes of current analysis.

Cluster 3, with positively charged residues like Lys142 and Arg137, establishes a hydrophobic and electrostatic interaction with importin α5, enabling VP24 to outcompete STAT1 (Reid et al., 2006). The lack of inhibitory potential in NLS-mutated VP24 constructs in EBOV, SUDV, BOMV and RESTV established that functional integrity of cluster 3 is essential for IFN-suppression in the VP24 NLS, aligning with the existing findings where VP24 and phosphorylated STAT1 compete for importin α5 binding via overlapping docking surfaces (Mateo et al., 2010; Schwarz et al., 2017).

The study data further showed that sequence alterations in the NLS region (at cluster 3) affect IFN suppression efficacy across *Orthoebolavirus* species (NLS mutated EBOV, BOMV, RESTV, and SUDV), highlighting that this region is a functionally constrained determinant of host innate immune antagonism. These conservative changes (leucine to glutamine or valine) can markedly affect importin-binding affinity and resultant nuclear localisation. The variability associated with these changes are supported by previous observations that small VP24 alterations can affect virulence and host specificity despite substantial conservation (Mateo et al., 2010; Schwarz et al., 2017).

6.4 VP35 Supresses RIG pathway activation

The results have shown that VP35 proteins from all tested *Orthoebolavirus* species including EBOV, SUDV, RESTV, TAFV, BOMV, BDBV, *Orthomarburgvirus* species (MARV, MLAV) and *Cuevavirus* species (LLOV) suppress RIG-I-dependent IFN signalling, but do not impact IFN-induced promoter activation. VP35 is a multifunctional viral protein that inhibits innate antiviral responses in the cytoplasm (Leung et al. 2011).

Across mammalian-infecting filoviruses, the viral protein VP35 is a conserved and central antagonist of IFN-I production. VP35-mediated inhibition of IFN

induction has been experimentally demonstrated for EBOV, RESTV, MARV, LLOV, and MLAV, and is considered conserved across Orthoebolaviruses, Orthomarburgviruses, Cuevaviruses, and Dianloviruses (Bodmer et al., 2025). It has also been reported that EBOV VP35 inhibits RIG-I and MDA5 activation by binding to dsRNA and preventing cytosolic PRR from recognising it. VP35 antagonizes type-I interferon production through four coordinated mechanisms: it binds and shields viral dsRNA to prevent recognition by RIG-I and MDA5 (Reid et al., 2005; Kimberlin et al., 2010); it inhibits PACT-mediated activation of RIG-I (Luthra et al., 2013); it blocks phosphorylation and nuclear translocation of IRF3 and IRF7 by interfering with TBK-1 and IKKε (Basler et al., 2003; Prins et al., 2009); and it sequesters IRF3 into viral inclusion bodies (Zhu et al., 2024), further preventing IFN-α/β gene transcription. This subsequently inhibits IFN-induction at an early cytoplasmic stage.

Amongst the different filoviral species, mechanistic evidence for EBOV in the literature is more prevalent as compared to MARV, but VP35 proteins of MARV have also been well-established in earlier studies (Ramanan et al., 2016; Edwards et al., 2016; Williams et al., 2020). In addition, similar MOA of LLOV VP35 protein to EBOV VP35 has also been reported (Feagins & Basler, 2015).

The current studies have further contributed to this existing understanding by demonstrating that all nine VP35 variants inhibit IFN-λ1 promoter activation, suggesting a conserved mechanism. Co-expression of VP35 with wild-type RIG-I or its agonists (PolyI:C and Sendai virus) significantly reduced promoter activity while overexpression of downstream effectors (ΔRIG-I, MAVS, or IRF3-5D) eliminated the inhibitory effect, reflecting that VP35 operates before adaptor molecule activation. Although previous studies have shown that VP35 can interfere with TBK1/IKKε-mediated phosphorylation of IRF3, our data indicate that overexpression of MAVS or constitutively active IRF3-5D bypasses VP35-mediated inhibition. This suggests that, in our experimental system, VP35 predominantly acts upstream of adaptor activation, likely at the level of viral RNA sensing.

The observation around the co-expression aligns with previous findings which established that the C-terminal interferon inhibitory domain (IID) of VP35 binds dsRNA via a patch and central β-sheet interface while protecting it from RIG-I helicase identification (Kimberlin et al. 2010). The important dsRNA-binding motif is conserved across all nine filovirus species, as displayed by sequence alignments and crystallographic investigations which explains uniform suppression pattern.

Immunoblotting analyses reveals no degradation or changed expression of signalling proteins, indicating that VP35-mediated suppression is caused by RNA sensing interference rather than protein destabilisation. The key mechanistic specificity complements structural studies that VP35 hides PAMPs, inhibiting RIG-I CARD domain activation and downstream signalling (Leung et al., 2011).

Moreover, the experiments have found that VP35-mediated suppression is not stimulus-specific, as polyI:C and Sendai virus as synthetic and natural RIG-I activators, respectively, displayed similar potential; which support the previous claim that VP35 broadly inhibits RIG-I signalling (Basler & Amarasinghe, 2009; Yamaoka & Ebihara, 2021). The studies conducted hereby have further established that VP35 prevents IRF3's phosphorylation and nuclear translocation which initiates interferon gene expression.

The fact that strongly expressed VP35 proteins do not impede interferon-induced promoter activation of ISGs like MxA and IFITM3 is significant to the current state of science because this finding defines antiviral suppression and validates mechanistic specificity of VP35. VP35 effectively inhibits IFN induction via RIG-I pathway but does not affect the JAK-STAT signalling cascade initiated by exogenous IFN-α. Unlike VP35, VP24 targets importin α and STAT1 nuclear import during the nuclear phase of interferon signalling (Ramanathan et al., 2023).

The inability of VP35 to reduce ISG promoter activation after IFN-stimulation supports its upstream mode of action, as it does not inhibit ISRE-driven transcriptional responses; the equivalency of MxA and IFITM3 expression levels in VP35-expressing and control cells highlighted that VP35 innate immune suppression is upstream of the JAK/STAT axis. The result that all nine VP35 proteins reduce RIG-I-dependent interferon induction, regardless of species, supports evolutionary conservation and establishes VP35 as a universal cytosolic antiviral signalling suppressor. Thus, functional homogeneity and inability to alter IFN-induced ISG transcription makes VP35 as a precise yet early-stage immune antagonist that disables interferon gene activation.

6.5 Implications and Future Directions

The current studies have established that Filoviridae evade host innate immune defences via the coordinated effects of VP24 and VP35 proteins. Despite their unique modes of inhibitory action, these proteins constitute an integrated and synergistic evasion network at the cytoplasmic and nuclear levels of the antiviral response. Most prior studies have examined individual filoviral proteins separately, often using distinct experimental systems, cell types, stimuli, and assay conditions, which complicates direct comparisons of antagonistic potency between species.

In contrast, studies for this doctoral thesis have looked at VP24 and VP35 proteins of nine filoviruses representing all four mammalian-infecting genera. The proteins were analysed side-by-side using identical expression systems, stimulation conditions, and readouts. By testing all selected filovirus VP24 and VP35 proteins in parallel, this approach minimised experimental variability and enabled a

standardised comparison of their individual effects on RIG-I and IFN-induced pathway.

Verifying these inhibitory mechanisms in primary human immune cells including macrophages, dendritic cells and endothelial cells is a key next step to build upon the current results. These cells are main targets of filovirus and control the systemic antiviral response, as it has been found earlier that in-vivo interactions between VP35 and cellular cofactors, such as PACT or PKR, influence antiviral signalling in ways that cannot be duplicated in simpler cell systems (Fang et al. 2017, Xu 2024).

The interaction of VP24/VP35 with host co-factors should be studied in physiologically relevant organoid and animal models while the comparative studies of recently developed or less deadly filoviruses like BOMV, LLOV and MLAV would also reveal the mechanistic evolutionary basis for these lineages to become less pathogenic in humans as compared to type species like EBOV and MARV.

Following their important role in viral pathology, previous research has recognised these viral proteins as master regulators of immune suppression (Basler & Amarasinghe, 2009), while current studies displayed that VP24 and VP35 act in a species-specific manner. This data on investigation of multiple filoviral species in concordant experimental settings and conditions fills the knowledge gap of prior studies. Hence, current results suggest that filoviruses adapt to host immunological barriers to improve replication fitness and pathogenicity. By demonstrating that VP35- and VP24-mediated antagonism of interferon production is both conserved and provides critical comparative insigths relevant to target validation, pan-filovirus therapeutic design, and host-directed antiviral strategies.

Small-molecule inhibitors or peptides that disrupt VP24-importin binding can restore STAT1 nuclear translocation and downstream ISG activation, restoring interferon-mediated antiviral activity in infected cells. The use of pharmacological strategies in viral-host protein interactions has been shown to reactivate innate immune signalling pathways compromised by viral protein components (Mateo et al., 2010; Schwarz et al., 2017). Targeting the C-terminal interferon inhibitory domain (IID) of VP35 to prevent dsRNA sequestration would restore RIG-I and MDA5 identification of viral RNA, resulting in the activation of the IRF3 and NF-kB pathways.

Currently, no therapies have been approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) for filoviruses other than EBOV. For Ebola virus disease (EVD), however, two monoclonal antibody–based therapies, Inmazeb (REGN-EB3) and Ebanga (mAb114), received FDA approval in 2020 for the treatment of adults and children (El Ayoubi et al., 2024). These approvals marked a transition from exclusively supportive care toward targeted therapeutic management of EBOV infection.

Recent advancements in high-throughput structural docking and fragment-based drug discovery allow screening for molecules that disrupt VP35-dsRNA binding surfaces (Leung et al., 2010; Kimberlin et al., 2010). Following the current data and recent advancements, a rational antiviral strategy would prospectively target VP24 and VP35 to restore interferon induction, and response mechanisms simultaneously which would better tailor therapeutic development for highly pathogenic filoviruses.

Additionally, four vaccines have received regulatory approval: Ervebo, Zabdeno/Mvabea, Ad5-EBOV, and GamEvac-Combi all stimulate immunity by targeting the EBOV glycoprotein (GP). Ervebo employs a recombinant vesicular stomatitis virus as its delivery vector (Saphire, 2020); Zabdeno/Mvabea and Ad5-EBOV are based on adenoviral vectors (Callendret et al., 2018; Wu et al., 2017); and GamEvac-Combi integrates both vesicular stomatitis virus and adenovirus platforms (Logunov et al., 2025). Of the vaccines currently licensed, Zabdeno/Mvabea is the only one designed to provide coverage against multiple species within the *Orthoebolavirus* genus, owing to its Mvabea component. The insights from current results further support vaccine developers in understanding effects of immune evasion mechanisms on antigenicity and protective immunity.

Recombinant Ebola viruses with mutations in VP35's IID domain generate greater IFN responses and provide protective immunity in animal models (Basler et al., 2019). VP24 NLS mutants have shown reduced pathogenicity but preserve replication competency, suggesting they could be live-attenuated vaccine backbones (Vogel et al., 2024). Thus, innovative vaccine platforms that demonstrate balanced innate and adaptive responses could solve the long-standing problem of lasting protection against various filovirus lineages by integrating these findings into rational vaccine design.

7 Conclusions

The current studies have elucidated host-filovirus interactions, particularly in the context of how VP24 and VP35 proteins facilitate innate immune evasion. The main objective of my thesis was to provide insights into mechanistic basis of host – filoviral interactions which can help in identifying key molecular targets of viral pathogenesis, that may lead to the development of novel antiviral therapeutics and vaccines.

Based on the results of this thesis, the following conclusions can be drawn:

1. Filovirus VP24 proteins function as conserved but pathway-specific innate immune antagonists. Functional divergence of VP24 reflects broader filovirus immune evasion strategies. Eight out of nine selected VP24 proteins inhibit IFN-induced pathway with varying degree of effectiveness. Species-specific exceptions (e.g., BOMV/MLAV in induction, MARV in signaling) demonstrate that VP24 function is conserved yet differentially deployed across virus species.

2. Filoviral VP24 proteins bind to importin-α5 and are localised in the nucleus, which shows their mode of action to be downstream of IRF3 phosphorylation during IFN induction. Disruption of NLS/importin interface consistently abrogates immune suppression, highlighting nuclear import as a central mechanistic requirement.

3. Selected filoviral VP35 proteins target the innate immune pathways which are upstream of the MAVS, which provide detailed information on their mode of action, that can help us in the development of novel antivirals

The growing structural and functional information on VP35 and VP24, and their complexes with host proteins substantially enhances the feasibility of rational, structure-guided antiviral drug design. The research presented here contributes to the global scientific effort to develop effective filovirus countermeasures by elucidating conserved mechanisms of host immune antagonism. Such advances have the potential to mitigate the severe pathogenic consequences of filovirus infection and to strengthen global preparedness for the emergence and spread of future outbreaks.

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