

AILAR OMLER

From Barmah Forest virus to host
RNA-derived PAMPs: divergent replicase
functions and conserved innate immune
activation by alphavirus replicases



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Institute of Bioengineering, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which are referred to in the text by Roman numerals:

- I. **Omler, Ailar***, Margit Mutso*, Mihkel Vaher, Joseph R. Freitas, Adam Taylor, Cassandra T. David, Gregory W. Moseley, Xiang Liu, Andres Merits, and Suresh Mahalingam. 2024. 'Exploring Barmah Forest Virus Pathogenesis: Molecular Tools to Investigate Non-Structural Protein 3 Nuclear Localization and Viral Genomic Determinants of Replication'. *mBio* 15(8):e0099324. doi:10.1128/mbio.00993-24.
- II. Lello, Laura Sandra, Agneta Miilimäe, Liubov Cherkashchenko, **Ailar Omler**, Rachel Skilton, Rachel Ireland, David Ulaeto, and Andres Merits. 2023. 'Activity, Template Preference, and Compatibility of Components of RNA Replicase of Eastern Equine Encephalitis Virus'. *Journal of Virology* 97(1):e0136822. doi:10.1128/jvi.01368-22.
- III. **Omler, Ailar**, Anna Rutmane, Suresh Mahalingam, and Andres Merits. 2026. 'The Ability of Alphavirus Replicases to Synthesize Non-Viral Type I Interferon-Inducing RNAs Correlates with Viral RNA Synthesis and Has a Diverse Impact on Virus Replication and Pathogenicity'. *Journal of Virology* 100(2):e0216225. doi:10.1128/jvi.02162-25.

*These authors contributed equally.

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This thesis also contains unpublished data.

Author contributions:

- I. Performed cell culture infection and transfection experiments, confocal microscopy experiments, *trans*-replicase construct cloning, *trans*-replicase assays, *in vitro* translation experiments, *in vivo* mouse infection, scoring, and tissue harvesting and titration, data analysis, figure preparation, and participated in writing of the manuscript.
- II. Experimental setup and data acquisition for the template RNA biosensor microscopy analysis with replicases and Old World alphavirus infection, data and figure preparation for publication.
- III. Conducted most of the experiments, excluding those with mosquito cells, participated in experimental design, data analysis, figure preparation and writing of the manuscript.

ABBREVIATIONS

AlbPolI	RNA polymerase I promoter of <i>Aedes albopictus</i>
ApoER2	apolipoprotein E receptor 2
AUD	alphavirus unique domain
AURAV	Aura virus
BEBV	Bebaru virus
BFV	Barmah Forest virus
CARD	caspase activation and recruitment domain
CD2AP	CD2-associated protein
cGAMP	cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthase
CHIKV	chikungunya virus
CMV	human cytomegalovirus
CP	capsid protein
Cryo-EM	cryo-electron microscopy
DC-SIGN	dendritic cell-specific ICAM-3-grabbing non-integrin
dsRNA	double-stranded RNA
EEEV	Eastern equine encephalitis virus
EGFP	enhanced green fluorescent protein
eIF	eukaryotic initiation factor
EILV	Eilat virus
ER	endoplasmic reticulum
FHL1	four-and-a-half LIM domain protein 1
Fluc	firefly luciferase
FXR	fragile X mental retardation protein
G3BP	Ras-GTPase-activation protein (SH3-domain)-binding protein
GETV	Getah virus
Gluc	<i>Gaussia</i> luciferase
GTase	guanylyltransferase
hpt	hours post transfection
HSPoII	RNA polymerase I promoter of <i>Homo sapiens</i>
HSP-90	heat shock protein 90
HVD	hypervariable domain
icDNA	infectious cDNA
IFIT	interferon-induced proteins with tetratricopeptide repeats
IFN	interferon
IFNAR	interferon α/β receptor
IRF	interferon regulatory factor

ISAV	insect-specific alphavirus
ISG	interferon-stimulated gene
ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulated response element
JAK	Janus activated kinase
LDLR	low-density lipoprotein receptor
LDLRAD3	low-density lipoprotein receptor class A domain-containing 3
L-SIGN	liver-specific ICAM-3-grabbing non-integrin
MAVS	mitochondrial antiviral signalling protein
MAYV	Mayaro virus
MDA5	melanoma differentiation-associated protein 5
MEF	mouse embryonic fibroblast
MIDV	Middelburg virus
MTase	methyltransferase
MTL	methyltransferase-like
Mxra8	matrix remodelling-associated protein 8
NB	northern blot
NLS	nuclear localization signal
NRAMP2	natural resistance-associated macrophage protein 2
ns	non-structural
nsP	non-structural protein
nsP2h	helicase region of non-structural protein 2
nsP2p	protease region of non-structural protein 2
NTD	N-terminal domain
NTPase	nucleoside triphosphatase
NW	New World
OAS	2'-5' oligoadenylate synthase
ONNV	o'nyong'nyong virus
ORF	open reading frame
OW	Old World
p.i.	post infection
PAMP	pathogen-associated molecular pattern
PARP	poly-ADP-ribose polymerase
PERK	PKR-like ER kinase
PFU	plaque forming unit
PHB1	prohibitin-1
PI3K	phosphatidylinositol 3-kinase
PI3K/Akt/mTOR	phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin
PRR	pattern recognition receptor

RC	replication complex
RdRp	RNA-dependent RNA polymerase
RIG-I	retinoic acid-inducible gene 1
RLR	RIG-I-like receptor
RLU	relative light unit
rPAMP	replicase-only PAMP RNA
Rpbl	catalytic subunit of RNA polymerase II
RRV	Ross River virus
RTPase	RNA triphosphatase
saRNA	self-amplifying RNA (replicon)
SAV	salmonid alphavirus
SAGV	Sagiyama virus
SESV	Southern elephant seal virus
SFV	Semliki Forest virus
sg	subgenomic
SG	stress granule
sgRNA	subgenomic RNA
SH3KBP1	SH3-domain kinase-binding protein 1
SIE	superinfection exclusion
SINV	Sindbis virus
SPDV	Salmon pancreas disease virus
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
STING	stimulator of IFN genes
TALV	Tai Forest alphavirus
taRNA	trans-amplifying RNA
TF	Transframe
TIM-1/4	T cell immunoglobulin mucin domain 1/4
TLR	Toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TYK2	tyrosine kinase 2
Ubi	<i>Aedes aegypti</i> polyubiquitin promoter
UPR	unfolded protein response
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus
WHATV	Whataroa virus
WT	wild-type

INTRODUCTION

Alphaviruses are a genus of enveloped, positive-sense RNA viruses comprising the family *Togaviridae*. They are widely distributed, being present on all continents except Antarctica. As arboviruses, they are primarily transmitted by mosquito vectors, which infect vertebrate hosts, including humans, during feeding. Consequently, several alphaviruses pose significant public health risks, causing illnesses that range from mild febrile symptoms to severe conditions such as lethal encephalitis or chronic arthritis. Large outbreaks or epidemics typically arise when ecological conditions, such as rainfall and temperature, favouring vector proliferation, coincide with the establishment of substantial viral reservoirs in susceptible vertebrate hosts, and ultimately lead to spillover into human populations given overlaps in habitats.

Over the past century, alphaviruses have spread to new geographical regions, and this expansion is expected to continue. Several medically important alphaviruses have demonstrated the capacity to emerge in previously unaffected regions, highlighting their growing public health significance. Among these, chikungunya virus (*Alphavirus chikungunya*, CHIKV) provides the clearest cautionary example of the global spread potential of highly pathogenic alphaviruses. It has caused multiple epidemics of chikungunya fever in densely populated urban areas where competent vector species thrive, facilitating high infection rates and sustaining urban transmission cycles. Originally endemic to sub-Saharan Africa, it has expanded over the past century to South and Southeast Asia, Indian Ocean islands, and Central and South America, where it has established new endemic regions. Additionally, warmer parts of Europe are considered at similar risk, with several localized outbreaks reported since 2007. The continued emergence of alphaviruses in previously unaffected regions depends largely on the availability of suitable mosquito vectors, which have themselves expanded northward due to continued climate change.

In recent years, two alphavirus vaccines, both against CHIKV, have been introduced: IXCHIQ, based on an attenuated virus platform, and Vimkunya, a virus-like particle vaccine. However, IXCHIQ has faced considerable setbacks, including use restrictions following reports of adverse effects, sometimes with lethal outcome, in older recipients, while Vimkunya has yet to achieve widespread application. Thus, the development of effective vaccines and targeted therapeutics against alphavirus infection remains an ongoing priority.

Currently, there are no approved vaccines available for general use against other alphaviruses of significant public health concern. These include the encephalitic Eastern equine encephalitis virus (*Alphavirus eastern*, EEEV) and several arthritogenic alphaviruses, such as o'nyong'nyong virus (*Alphavirus onyong*, ONNV), Mayaro virus (*Alphavirus Mayaro*, MAYV), Sindbis virus (*Alphavirus sindbis*, SINV), Ross River virus (*Alphavirus rossriver*, RRV) and Barmah Forest virus (*Alphavirus barmah*, BFV). BFV is the second most prevalent arbovirus causing human disease in Australia. It is genetically distinct within the genus and

represents its own antigenic complex, with no closely related viruses currently identified. Infection is typically characterized by rash, fever, muscle and joint pain, fatigue, and headaches. BFV disease is nationally notifiable in Australia, and its epidemiology is actively monitored. Despite this, molecular biology of BFV remains relatively poorly understood.

To address this gap, the work presented here focuses in part on developing molecular tools to better characterize BFV. Specifically, we constructed an infectious cDNA (icDNA) clone of BFV to enable genetically defined studies of viral infection. During this process, we identified and corrected an error in the publicly available sequence of the BFV prototype isolate, which led to the discovery of a functional link between the replicase proteins nsP2 and nsP4 during troubleshooting. The corrected icDNA also facilitated the establishment of a BFV *trans*-replicase system, allowing targeted investigation of mutations towards replicase activity. Using the infectious clone, we also observed nuclear localization of BFV nsP3, the least understood replicase protein of alphaviruses, during infection – a rare feature within the genus.

Furthermore, our tools enabled investigation of the BFV replicase in its ability to induce a type I interferon (IFN) response in the absence of viral RNA templates. This finding shows that BFV replicase can utilize host RNAs as templates to generate RNA species that activate intracellular immune sensors. While this phenomenon had previously been described for Semliki Forest virus (*Alphavirus semliki*, SFV), RRV, and SINV, this additional finding prompted a broader analysis across a more diverse range of alphavirus replicases. Results obtained using replicases harbouring different mutations indicated that the efficiency to utilize host-derived RNA is virus species-specific and may influence outcomes of infection.

Overall, these findings expand our understanding of the diversity of alphavirus infection strategies. Although recent progress in CHIKV vaccine development marks a significant advance, similar breakthroughs have not yet been achieved for other medically important alphaviruses. As further demonstrated by our findings with BFV, individual alphaviruses may exhibit unique and previously unrecognized features, suggesting that therapeutic and vaccine development will likely require virus-specific approaches. A detailed understanding of viral mechanisms and host interactions remains essential for discovering and designing effective countermeasures. Moreover, as alphavirus replicase- and replicon-based technologies – such as self-amplifying RNA (saRNA) and *trans*-amplifying RNA (taRNA) platforms – continue to be developed and improved, our findings on innate immune activation in the absence of viral RNA templates may have broader implications for vaccine design and immunogenicity.

1. LITERATURE REVIEW

1.1. Alphaviruses

The genus *Alphavirus* currently includes over 30 species of arboviruses that belong to the family *Togaviridae* (1). Alphaviruses are found worldwide, and many serve as human and veterinary pathogens. Based on geographic distribution, they have traditionally been classified as New World (NW) and Old World (OW) alphaviruses, distinguishing those found in the Americas from those found elsewhere. This division also reflects differences in the pathology caused by NW and OW alphaviruses (2). The NW alphaviruses (Fig. 1) include Venezuelan equine encephalitis virus (*Alphavirus venezuelan*, VEEV), EEEV and Western equine encephalitis virus (*Alphavirus western*, WEEV), which can cause disease in humans, ranging from febrile illness to encephalitis (3). While EEEV is endemic in North America and human cases are relatively rare, encephalitis caused by EEEV has a mortality rate exceeding 30%, making it a significant public health concern (4). Additionally, EEEV is capable of infection via aerosolized particles and is therefore classified as a potential bioweapon (5, 6).

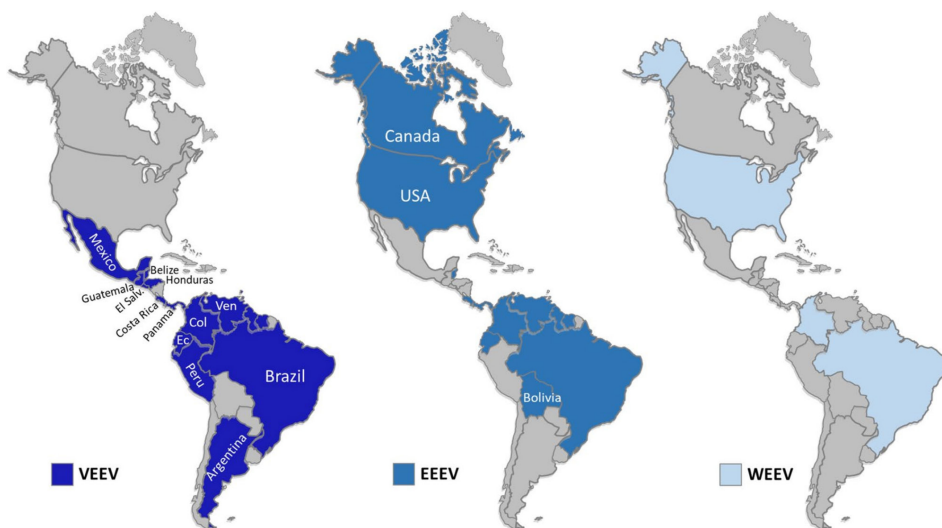


Figure 1. Presence of encephalitic alphaviruses in the Americas by country. VEEV – Venezuelan equine encephalitis virus, EEEV – Eastern equine encephalitis virus, WEEV – Western equine encephalitis virus. Adapted from (7).

OW alphaviruses (Fig. 2), including CHIKV, ONNV, SINV, RRV, SFV, and BFV, are typically arthritogenic and cause illness characterized by fever, myalgia, rash, and arthritis. For some of these viruses (most prominently CHIKV), the infection often leads to chronic illness (8). However, the classification based on symptoms is not absolute; for instance, arthritogenic alphaviruses such as SFV and SINV have widely been used to study alphavirus-induced encephalitis in animal models, and even for CHIKV, cases of neurological disease have been reported (9–11). Conversely, MAYV, which is endemic to Central and South America (Fig. 2),

primarily causes febrile illness, with more severe cases presenting arthritogenic symptoms similar to those caused by OW alphaviruses (12); thus, despite its origin, it is usually grouped together with OW alphaviruses.

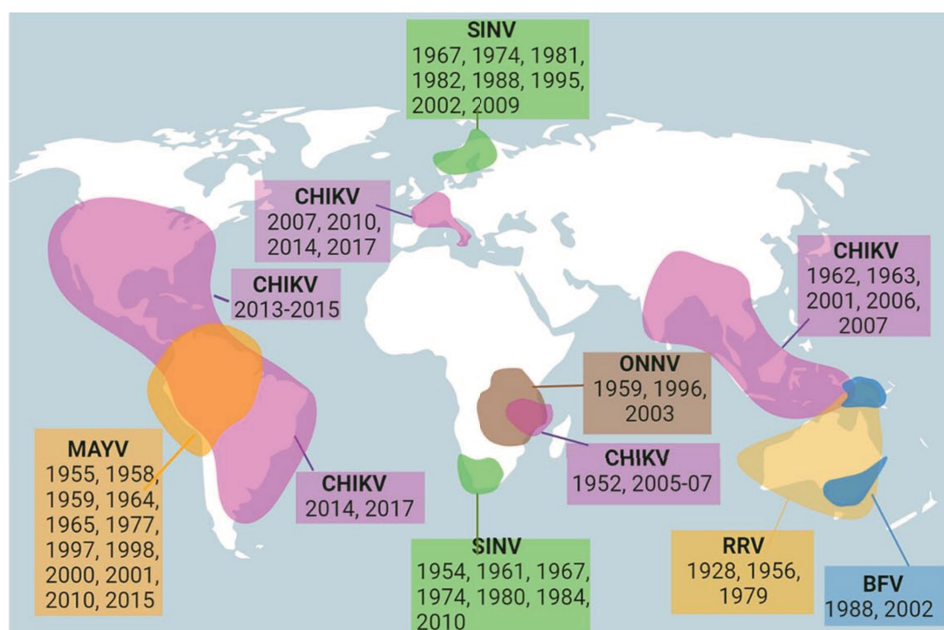


Figure 2. Geographic distribution of arthritogenic alphaviruses. Notable outbreaks indicated by year. CHIKV – chikungunya virus, ONNV – o’nyong’nyong virus, SINV – Sindbis virus, RRV – Ross River virus, MAYV – Mayaro virus, BFV – Barmah Forest virus. Figure adapted from (13).

Among alphaviruses, CHIKV represents the most significant public health concern due to its ability to cause severe illness, including high fever, rash, headache, myalgia and polyarthrititis, which can persist for months or even years after infection (14). Historically, CHIKV was endemic to sub-Saharan Africa and Southeast Asia, but early in the 21st century, it has spread globally. In 2004–2006, CHIKV caused a massive outbreak across several Indian Ocean islands, with over 250 000 cases in La Réunion alone, before reaching mainland India, where over 1.3 million cases were reported (15, 16). Infected travellers from highly affected regions have since introduced CHIKV to Europe, causing localized outbreaks in Italy (2007, 2017) and France (2010, 2014, 2017) (17–19). Since 2013, CHIKV has become endemic in the Americas, with nearly 3.7 million reported cases between 2013 and 2023 (20). From November 2024 to October 2025, 458 840 CHIKV cases and 146 deaths were reported (21). The global expansion of CHIKV has been driven by both travellers and the local presence of competent mosquito vectors. In its ancestral regions, CHIKV is maintained through enzootic cycles, where non-human primates serve as amplification hosts and *Aedes aegypti* mosquitoes act as transmission vectors (22). Human infection in enzootic regions typically represent spillover events. Large CHIKV outbreaks occur when human-amplified virus spreads through urban epidemic cycles involving mosquitoes in

densely populated areas. Urban transmission of CHIKV is facilitated by the presence of *Aedes aegypti* and *Aedes albopictus*, which may or may not have overlapping habitats (23). *Aedes aegypti* is commonly found in cities (often being called “urban mosquito”) and is well adapted to tropical and subtropical climates; it acts as the key vector in Africa, Southeast Asia, and the Americas (20). *Aedes albopictus*, while largely sharing the same habitat, is more common in rural and peri-urban areas and can also survive in more temperate climates, playing a crucial role in European outbreaks (18, 24). Currently, *Aedes albopictus* has been reported in 16 European countries, with its northernmost established populations found in Belgium and Germany (25). Climate change is predicted to expand its range as far north as Great Britain and southern Scandinavia by 2050 (26). Similar concerns have been raised regarding potential MAYV expansion to Europe. In endemic regions of South America, *Haemagogus janthinomys* is the primary MAYV vector; however, under laboratory conditions, *Aedes albopictus* has also been shown to be a competent vector (27, 28). Thus, there is growing concern that highly pathogenic alphaviruses may continue to expand into previously unaffected areas in the coming decades.

Alphaviruses are also classified into seven distinct antigenic complexes: Western equine encephalitis complex, Eastern equine encephalitis complex, Venezuelan equine encephalitis complex, Middelburg complex, Ndumu complex, Barmah Forest complex, and Semliki Forest complex (1). Members of these complexes are not confined to specific hemispheres, as exemplified by SINV, which belongs to the Western equine encephalitis complex, and MAYV, which is classified in the Semliki Forest complex (1). The Semliki Forest complex is particularly notable for including clinically significant arthritogenic viruses, such as CHIKV, RRV, ONNV and MAYV (29). At the same time, BFV, Middelburg, and Ndumu viruses are the sole representatives of their respective eponymous complexes (1).

The genus *Alphavirus* also includes several viruses with host ranges restricted to mosquitoes, collectively termed insect-specific alphaviruses (ISAVs). Known species include Eilat virus (*Alphavirus eilat*, EILV), Tai Forest alphavirus (TALV), Agua Salud virus, and Yada Yada virus and, most likely, their numbers will grow. While not officially designated, these viruses have been proposed to form a distinct clade within the genus (30–34). Furthermore, in addition to the recognized antigenic complexes, there are so-called aquatic alphaviruses, including Southern elephant seal virus (*Alphavirus seal*, SESV) and Salmon pancreatic disease virus (*Alphavirus salmon*, SPDV) (29). SESV was isolated from the blood-feeding louse *Lepidophthirus macrorhini* and has been associated with infections in southern elephant seals (35). SPDV, also known as salmonid alphavirus (SAV) is a serious concern in aquaculture as it causes disease in salmon and trout, negatively affecting product quality. Although SPDV/SAV has been isolated from the salmon louse (*Lepeophtheirus salmonis*), its role as a definitive vector species has not been conclusively established (36). These highly distinct alphavirus species have been instrumental in elucidating alphavirus phylogenetics, and the marine origin of the *Alphavirus* genus remains a valid hypothesis for its evolutionary ancestry (29).

1.1.1. Barmah Forest virus

BFV was first isolated in 1974 from a pool of *Culex annulirostris* mosquitoes in south-eastern Australia. The virus was named after the nearby Barmah Forest, the mosquito collection site within the Murray Valley region of the state Victoria (37). Due to its lack of cross-reactivity with known alphaviruses, the initial serological classification efforts mistakenly associated BFV with bunyaviruses (37). However, subsequent characterization of virion morphology, structural components, viral RNA, and envelope protein amino acid sequences correctly placed BFV within the *Alphavirus* genus (38). Since then, multiple complete BFV genomes have been sequenced, and cryo-electron microscopy (cryo-EM) studies have revealed the virion structure, further confirming its classification (39–41).

Human infection with BFV was first reported in 1986 when the virus was found in the serum samples collected in New South Wales; in 1988, the virus was associated with disease (42, 43). Awareness of the virus increased following several documented outbreaks in the 1990s, including outbreaks in the Northern Territory (1992), Western Australia (1992, 1993, and 1994), and New South Wales (1994–1995) (44–46). The state of Queensland has recorded the highest number of BFV infections in Australia, accounting for over half of all confirmed cases (47, 48). The first outbreak of BFV in Tasmania was reported in 2019 (49).

As of now, BFV is the second most prevalent arbovirus causing human disease in Australia, following RRV. Both viruses cause similar illnesses characterized by fever, fatigue, myalgia, arthritis and arthralgia. However, BFV disease is considered milder in terms of joint pain but is more frequently associated with a prominent maculopapular rash, covering up to 75% of the body surface (50). There has been one reported case of BFV infection associated with Guillain-Barré syndrome (51). Over the past decade, approximately 400 cases of BFV infection have been reported annually in Australia, and the virus is considered a public health concern (48). Since there are no vaccines or approved antiviral treatments for BFV, public health authorities urge preventive measures to minimize mosquito exposure (52).

For many years, BFV was thought to be endemic exclusively to Australia. However, in 2014, a locally acquired case was detected in a child in Papua New Guinea with no history of travel to Australia. Phylogenetic analysis revealed that the local BFV strain was genetically distinct from Australian strains, suggesting that BFV may be endemic in Papua New Guinea as well (53). A serological study conducted among Papua New Guinea military personnel further supported the endemicity hypothesis, showing evidence of prior BFV infection in the local population (54).

BFV has been isolated from multiple mosquito species in Australia, including *Aedes*, *Culex*, and *Anopheles* mosquitoes. In the coastal areas, common vectors are *Aedes camptorhynchus* (predominantly in southern Australia), and *Aedes vigilax* (primarily in northern and eastern Australia); the latter has been experimentally confirmed as an important transmission vector (55). In freshwater and inland areas, vectors are *Aedes notoscriptus* and *Culex annulirostris*; both species have been confirmed to be susceptible to BFV infection in laboratory experiments

(56, 57). The definitive vertebrate reservoir hosts for BFV remain unknown. However, serological studies have detected natural BFV infection in marsupials such as eastern gray kangaroos, quokkas, common brushtail possums and koalas. BFV can also infect non-marsupial mammals, including domestic cats, dogs, cattle, and horses (58–61). However, due to the genetic stability of BFV isolates across geographically distant areas, it has been hypothesized that avian species may serve as the primary BFV reservoir (62).

BFV is also capable of infecting laboratory animals. To better understand BFV pathogenesis, disease progression has been studied in a mouse model. The historic BFV isolate from 1974, BH2193, was found to be the most pathogenic for mice and was used to evaluate disease severity in a C57BL/6 mouse model, which was originally established to study RRV infection. While blood of infected mice exhibited high BFV titres, the virus was found to be significantly less pathogenic than RRV, likely due to less prominent infection of joint tissue and even lesser infection of muscle tissue (63). This aligns with clinical observations in humans, where BFV disease tends to present with less severe joint pain than RRV infection (50). The BFV mouse model is also employed in ongoing research, including Publication I of this thesis, to further elucidate BFV disease mechanisms.

1.1.2. Alphavirus virion structure and genome organization

The alphavirus virion (Fig. 3) is an enveloped icosahedral particle of approximately 70 nm in diameter (41). The outer shell consists of 240 heterodimers of the envelope proteins E1 and E2, which are organized into 80 trimeric spikes on the virion surface (64). E1 and E2 are anchored to the host-derived lipid bilayer by their C-terminal transmembrane helices, while the C-terminal tails of E2 extend beyond the bilayer to interact with the 240 monomers of capsid protein (CP) (65). CPs encapsulate a single copy of the viral genome, forming the nucleocapsid (64).

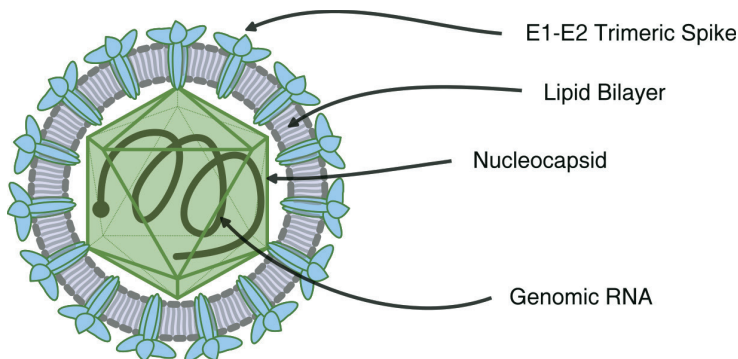


Figure 3. Schematic representation of the alphavirus virion. The outermost layer of the approximately 70 nm virion is comprised of E1-E2 trimeric spikes (blue) embedded into the host-derived lipid bilayer (gray). 240 copies of CP comprise the capsid shell (light green) encapsulating the viral genome (dark green), together forming the icosahedral nucleocapsid.

The alphavirus genome is a positive-sense RNA molecule, approximately 11–12 kb in length, with a 5'-cap and a 3'-polyA tail (Fig. 4). It contains two open reading frames (ORFs). The first ORF (approximately two-thirds of the genome) encodes the non-structural (ns) polyprotein, a precursor that is later processed into the non-structural proteins (nsP1-4). The second ORF encodes the structural polyprotein precursor, which is cleaved into CP, envelope proteins E3, E2, and E1 and the small transframe (TF)/6K proteins (2, 66).

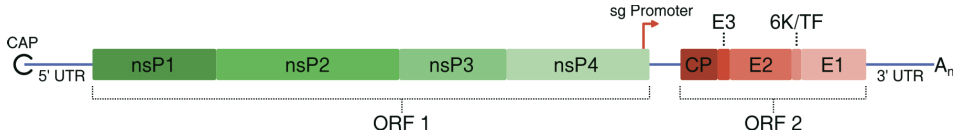


Figure 4. Organization of the alphavirus genome. The alphavirus genome mimics mRNA by including a 5'-cap (CAP) and a 3'-polyA tail (A_n). The two ORFs encode the non-structural polyprotein (ORF 1) and the structural polyprotein (ORF 2). 5' UTR, 3' UTR – untranslated regions, sg Promoter – subgenomic promoter.

The untranslated regions (UTRs) in the 5'-end, 3'-end, and intergenic region contain critical sequence and structural elements involved in RNA replication and translation regulation (67). The 5' UTR contains at its 5'-end a highly conserved non-base-paired AU dinucleotide, essential for replication by replicase proteins, for synthesis of the negative-strand RNA, initiating positive-strand RNA synthesis, and for effective RNA capping (68–70). It houses a core promoter which contains several stem-loop structures; its copy at the 3'-end of the negative-strand transcript regulates genomic RNA synthesis (67). The *cis*-acting elements required for RNA replication are also present at the beginning of ORF1; most notable of these is a 51-nucleotide conserved sequence element that functions as an enhancer of the core promoter (71). The intergenic region contains a part of the subgenomic (sg) RNA (sgRNA) promoter; however, most of this element overlaps with the end of nsP4 coding sequence. The optimal sg promoter (i.e. the sequence sufficient and essential for high levels of sgRNA synthesis) spans positions –98 to +14 (+1 being the first base incorporated into sgRNA), while the minimal active sg promoter is much shorter, extending from –19 to +5. Most of the sequence of the minimal sg promoter (–19 to +2) is highly conserved across alphaviruses (72, 73). The length of the 3' UTR varies significantly between alphaviruses: fish-infecting alphaviruses have 3' UTR-s of ~90 bases in length while CHIKV strains have 3' UTRs ranging from 510 to 930 nucleotides (74, 75). The variation in CHIKV 3' UTR length is due to differing numbers of direct repeat elements, which are associated with increased CHIKV fitness in mosquitoes (76). Most of the 3' UTR is dispensable for RNA replication, however, its very end, that includes a highly conserved 19-nucleotide element along with a polyA-tail, which must be at least 11 bases long, is crucial for efficient replication (77, 78).

1.2. Alphavirus infection cycle

1.2.1. Attachment and entry

Alphaviruses infect a wide range of hosts and exhibit diverse tissue tropism. Their virions interact with host cell surface factors via the trimeric spike formed by E1 and E2 envelope proteins. These proteins compose an antireceptor complex, with the receptor binding mediated by epitopes on both E1 and E2. A number of host surface factors have been identified that either mediate virion attachment or serve as actual entry receptors.

Several ubiquitous cell surface components have been identified as attachment factors for alphaviruses. The most prominent among them is heparan sulphate, a negatively charged glycosaminoglycan that facilitates virion attachment via positively charged lysine and arginine residues on E2 (79, 80). Heparan sulphate has been identified as an attachment factor for EEEV, RRV, CHIKV, SINV, and VEEV (81–85) and likely has a similar role for other alphaviruses. However, heparan sulphate binding effects vary significantly. CHIKV, SINV, and VEEV adaptations toward increased heparan sulphate increase infectivity *in vitro* but result in faster virus clearance in mice, leading to attenuation (86, 87). Therefore, for these viruses the increased binding of heparan sulphate is likely an adaptation for growth in cell culture. Conversely, however, EEEV heparan sulphate dependence has been linked to increased neurovirulence, influencing tissue tropism, and similar effects towards neurovirulence have been observed for SFV (88, 89).

In addition to heparan sulphate, alphaviruses such as SINV and Aura virus bind C-type lectins dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) and liver-specific ICAM-3-grabbing non-integrin (L-SIGN) (90–92). It has been shown that DC-SIGN/L-SIGN expression in non-permissive cells enabled SINV infection and replication, and that mosquito cell derived SINV has higher infectivity in DC-SIGN/L-SIGN expressing cells than mammalian cell derived virus, likely due to host-specific glycosylation differences (90). Other cellular components associated with infection of SINV, CHIKV, RRV, and EEEV are phosphatidylserine receptors T cell immunoglobulin mucin domain 1 (TIM-1), TIM-4, and CD300a (93–96). These recognize phosphatidylserine lipids in the host-derived viral envelope rather than the viral E1/E2 proteins (97).

Until recently, the identity of actual cellular receptors crucial for alphavirus entry remained enigmatic. Few proteins were shown indirectly to participate in alphavirus entry including the 67-kDa high-affinity laminin receptor for SINV and VEEV (98, 99), prohibitin-1 (PHB1) and ATP synthase β for CHIKV (100, 101), and CD147 protein complex for SINV, CHIKV, RRV, WEEV, and EEEV (102). Subsequently, natural resistance-associated macrophage protein 2 (NRAMP2) was identified in a *Drosophila*-based study as an entry receptor for insect and murine cells (103).

The recent advances in alphavirus receptor research are due to application of different screening technologies. Unlike the case of earlier receptor candidates, the role of these receptors has typically unequivocally been confirmed by follow-

up cryo-EM analyses. The first receptor that was identified is matrix remodelling-associated protein 8 (Mxra8), that was confirmed as an entry receptor for CHIKV, SFV, ONNV, RRV, and MAYV (104–106). Subsequently, it was shown that Mxra8 from avian species serves as a receptor for SINV and WEEV, alphaviruses that naturally infect birds rather than mammals (107). Numerous alphaviruses were shown to exploit proteins from the low-density lipoprotein receptor family. Low-density lipoprotein receptor class A domain-containing 3 (LDLRAD3) was identified as an entry receptor for VEEV (108, 109), very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) as receptors for EEEV, and SFV (110) and low-density lipoprotein receptor (LDLR) as an entry receptor for Getah virus (*Alphavirus getah*, GETV) (111). For EEEV, WEEV, and SFV, LDLR has low binding affinity, and it was suggested that it may function as an entry cofactor (112).

Following receptor binding, alphaviruses primarily enter host cells via clathrin-mediated endocytosis (113). Clathrin-coated vesicles transport virions to endosomes, where acidification triggers membrane fusion (114). The fusion pH threshold varies by alphavirus species and even by strains (~pH 6.2 for SFV, ~pH 5.6 for SINV) (115–117). Viral genome release is known to rely largely on disassembly activity presented by 60S ribosomal subunits, which specifically interact with the incoming nucleocapsid, although a complete mechanism has yet to be elucidated (118, 119).

1.2.2. Replicase complex formation

Alphavirus positive-sense genomic RNA mimics cellular mRNA by including a 5'-cap and a 3'-polyA tail. Genome release is immediately followed by the translation of ORF1, resulting in the production of two different non-structural polyprotein precursors: one that includes all four nsPs (P1234) and the shorter P123. This is due to a UGA termination codon, also known as the opal stop codon, typically present at the end of the nsP3 coding region. This codon is described as “leaky” because ribosomal readthrough results in P1234 production at approximately 10% efficiency (120). All four nsPs are required for active replication complex (RC) assembly. The full-length P1234, however, is not capable of RNA synthesis, and the proteolytic activity in nsP2 is required to release nsP4 from the P1234 precursor (121, 122). The resulting P123+nsP4 is termed the early replicase and is primarily active in synthesizing the viral negative-strand RNA complement, although it can also produce positive-strand genomic RNA and sgRNA at lower efficiency (122, 123). Next, nsP1 is cleaved from P123, forming a short-lived nsP1+P23+nsP4 replicase capable of synthesizing RNA of either polarity. However, this intermediate is short-lived and likely contributes little to RNA production; instead it serves as a transitional step needed to shift from the early replicase to the late replicase (124). Cleavage at the nsP2-nsP3 junction irreversibly produces the late replicase (nsP1+nsP2+nsP3+nsP4), which is now responsible for positive-sense genomic RNA and sgRNA synthesis (122, 124). A schematic summary of the infection cycle is depicted below in figure 5.

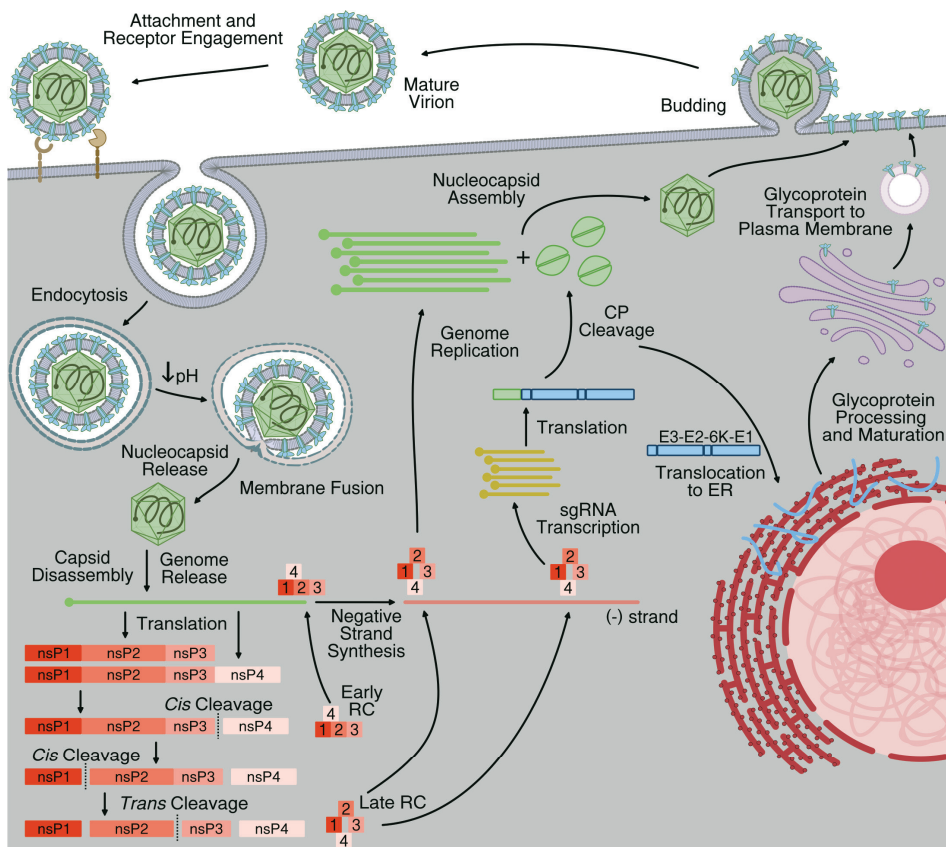


Figure 5. A general overview of the alphavirus infection cycle. Detailed explanations are provided in main text.

Thus, for alphaviruses to successfully initiate viral genome replication, the sequential processing of the P1234 precursor is crucial. The first cleavage, releasing nsP4 from P1234, occurs *in cis* and is a rapid process. Studies have shown that early replicase functionality is lost if P123 is processed too quickly (125, 126). Therefore, the subsequent *in cis* cleavage between nsP1 and nsP2 is slower, possibly as an adaptation to allow the early replicase to form and complete minus-strand RNA synthesis (126, 127). This cleavage also exposes the N-terminus of nsP2, which can now process the remaining P23 into nsP2 and nsP3; this cleavage occurs *in trans* (126) as *in cis* cleavage of this site is impossible due to steric constraints (128). As infection progresses, more individual nsPs accumulate, increasing the *trans*-activity of nsP2 protease. This results in a shift in P1234 processing pattern: P1234 is instead converted to P12 and P34. These intermediates are subsequently cleaved *in cis* and *in trans*, respectively (129). P12, P34 and mature nsPs are unable to trigger RC formation. Thus, the accumulation of RC-free nsP2 determines the timeframe for RC formation (typically 4–6 hours from the beginning of infection); after this, new RCs are not formed and synthesis of negative-strand RNAs stops (129).

Alphavirus RCs are membrane-bound, a function attributed to nsP1. RNA replication itself occurs in replicase-induced organelles observable as membrane invaginations called spherules (Fig. 6) (130). Spherule formation always begins at the plasma membrane and is initiated by the components of the early replicase; it has also been demonstrated that the RNA synthesis activity is a crucial determinant of spherule formation (131). For SFV, spherules are subsequently internalized and localize in modified endosomes and lysosomes termed cytopathic vacuoles (132). Internalization has been linked to nsP3 interactions with host factors that lead to increased phosphatidylinositol 3-kinase (PI3K) activity and occurs more frequently during SFV infection compared to CHIKV or SINV (131, 133, 134). For CHIKV, the majority of RCs remain associated with plasma membrane and display a poorly understood phenomenon – their gradual increase in size as infection progresses (135).

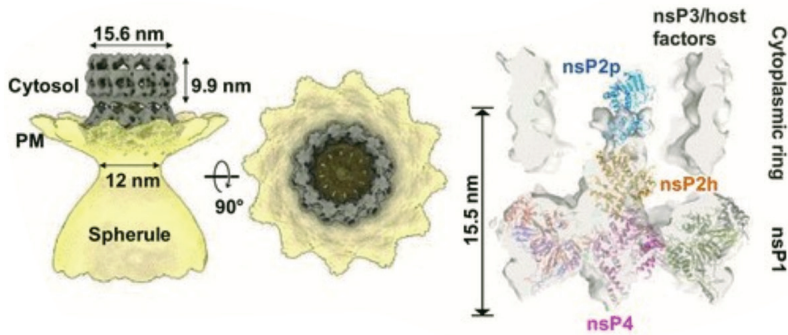


Figure 6. 3D-structure of the CHIKV spherule neck region and RC. (Left) A cryo-EM subtomogram depicting the side and top view of the CHIKV spherule. (Right) The RC core complex (CHIKV nsP1 + CHIKV nsP2 + ONNV nsP4) structure as fitted to the spherule model. Figure adapted from (136).

The spherule itself is bulb-like in appearance, with an approximate diameter of 50–60 nm and features a narrow neck area connecting the compartment to the cytoplasmic environment. For SFV, the spherule size correlates with the template length currently under replication (137). Recent advancements in elucidating the alphavirus RC and spherule structures have supported earlier hypotheses that the RC sits within the narrow spherule neck and that viral negative-strand RNA is retained within the spherule, most likely as a double-stranded RNA (dsRNA) duplex formed with positive-sense genomic RNA (138). In fact, structural studies of the late RC have revealed that nsP1 forms a ring-like scaffold consisting of 12 monomers, which anchor the ring structure, and thus the RC, into the membrane via two membrane-associating loops present in each copy of nsP1 (139). This dodecameric ring positions nsP4 within its central pore, where it interacts with nsP2 via the N-terminal helicase domain, presumably leaving the nsP2 protease domain facing outward from the spherule neck (136). The area surrounding the estimated position of the protease domain has been reported to

be sheathed by a symmetrical crown complex, which is suggested to be composed of nsP3 and unknown host factors (136, 138). The latter was confirmed by a more recent study which revealed that mature CHIKV nsP3 forms oligomeric tubular structures of helical symmetry. Superpositioning of the nsP3 helical structures onto the previously reported replication pore complex supports the hypothesis of nsP3 involvement in the crown complex (140). Despite these remarkable achievements, a complete structural model of the RC remains unknown, likely due to involvement of multiple host factors. Most importantly, the structure of the early RC has yet to be determined.

1.2.3. Structural protein expression, virion formation and release

sgRNA is transcribed from the negative-strand RNA using the internal sg promoter. Like the viral genome, sgRNAs are also capped and polyadenylated, allowing for the expression of ORF2 (2). The viral structural polyprotein precursor is translated in two variants: either as CP-E3-E2-6K-E1 or as CP-E3-E2-TF, the latter resulting from a -1 ribosomal frameshift during translation of the 6K region (66).

CP is autoproteolytically released from the nascent polyprotein by the serine protease activity in its C-terminal domain, after which its proteolytic capability is exhausted (141). CP is then involved in genomic RNA packaging, mediated by specific sites in its N-terminal domain. Genome recognition must be targeted toward unique features found only in genomic RNA, distinguishing it from sgRNA or any other genetic material abundant in the cytoplasm (142). This process is proposed to function through packaging signals, which are not conserved across the genus. For example, an important packaging signal for Semliki Forest complex viruses resides in the nsP2 coding region. Interestingly, CHIKV CP can also recognize packaging signals in the nsP1 region of other alphaviruses, as demonstrated by CHIKV CP-mediated packaging of VEEV genomes (143). Recent SFV-based studies suggest that CP recognizes multiple sites on a single genome using multiple copies of itself, as several critical recognition sites have been identified within the first two-thirds of the genome. At least one stem-loop structure has been experimentally shown to be essential for CP binding (144, 145).

The release of CP results in the formation of a shorter polyprotein, E3-E2-6K-E1, with a newly exposed signal peptide at the N-terminus of E3. This facilitates polyprotein translocation to the endoplasmic reticulum (ER) (146). Host proteases further process the membrane-bound polyprotein to yield p62 (E3-E2, still uncleaved), 6K, and E1. The p62 and E1 proteins interact to form heterodimers (147). The processing delay of p62 is crucial for prolonging E3-mediated protection of E1 from exposure to low-pH environments, which would otherwise prematurely activate the E1 fusion peptide during translocation. The p62-E1 heterodimer remains more stable than the mature E2-E1 heterodimer (148). Subsequent trafficking of p62-E1 complexes through the *trans*-Golgi secretory pathway leads to post-translational modification of glycoproteins and final furin-mediated cleavage of p62, yielding separate E2 and E3 proteins. This results in

the formation of mature E2-E1 heterodimers, which form trimers and are transported to the plasma membrane (149). CPs in assembled nucleocapsids interact with the C-terminal region of the E2 protein on the cytosolic side of the plasma membrane, initiating the budding process (150). A recent cryo-EM study investigating CHIKV budding suggests that the process is driven by pre-formed E2-E1 trimeric spikes located on the plasma membrane; the icosahedral symmetry is formed during budding (151). Successful budding results in the acquisition of the viral envelope (152).

There is also evidence that the small 6K protein or its frameshift variant, TF, are incorporated into viral envelopes. TF has been shown to be palmitoylated at N-terminal cysteine residues, whereas 6K, despite having an identical N-terminal domain, is not (153). Palmitoylation promotes TF localization to the plasma membrane and its incorporation into virions. While a TF palmitoylation-defective SINRV mutant is still able to grow to high titres, it exhibits altered virion morphology. Furthermore, palmitoylated TF, rather than 6K, is preferentially incorporated into virions (153). The complete absence of the 6K coding sequence is tolerated by SFV but results in significantly lower virus production. Thus, 6K is dispensable for virion assembly, but likely acts as an accessory factor that enhances virion budding efficiency (154).

1.3. Alphavirus replicase proteins

1.3.1. nsP1

The two major functions of nsP1 are to act as the viral RNA capping enzyme and as a membrane anchor for the RC. Recent cryo-EM works have revealed that twelve nsP1 copies form a ring structure with a distinct base “skirt” that anchors the complex to the membranes, a central “waist” where contacts for oligomerization are established, and an upper, or cytosolic-side, “crown” that includes motifs needed for the capping activity (Fig. 7) (139, 155). Furthermore, the structural elucidation of a core RC showed that a 7–7.5 nm pore in the centre of the nsP1 dodecamer houses a single copy of nsP4 that interacts with 10 nsP1 subunits. The two missing contact surfaces form a molecular channel that is suggested to support the trafficking of nucleotides and RNA to and from the spherule, with the anchoring nsP1 ring placed at the neck region (136). It was also observed that nsP2 interacts with nsP4 on the cytosolic side of the spherule neck complex, proposing a model in which nascent genomic and sgRNA exiting through the molecular channel are exposed to the capping machinery consisting of nsP2 and nsP1 (136). Namely, nsP2 removes the γ -phosphate from the nascent RNA, yielding a 5'-diphosphate terminus (156); the rest of the activities required for cap synthesis reside in nsP1. The N7-methyltransferase (MTase) and guanylyltransferase (GTase) activities reside in the N-terminal half of nsP1. The MTase utilizes S-adenosyl methionine (SAM) as a methyl group donor to convert guanosine triphosphate (GTP) to m⁷GTP (139). During the guanylyl transfer, a covalent

nsP1-m⁷GMP complex is formed via a conserved histidine residue of nsP1 (H37 in CHIKV and VEEV). The GTase completes the cap synthesis, yielding a cap-0 structure (m⁷GpppAn) on viral positive-sense RNAs (155, 157, 158). However, the capping efficiency is not absolute, and a large proportion of uncapped genomes are produced during early SINV infection (159). This phenomenon may be linked to increased allosteric activation resulting from oligomerization of nsP1. In other words, the early replicase, which is essentially a tool for negative-sense RNA synthesis (which are non-capped) but is still capable of positive-strand RNA synthesis, may possess a conformation of nsP1 that is less optimal for capping activity. Supporting this, experiments have shown that viral RNA capping activity is not necessary for spherule formation and negative-strand RNA synthesis (160). Interestingly, nsP1 can also remove cap structures, at least *in vitro*, from capped mRNA substrates and transfer them to 5'-diphosphate RNAs (69, 139). The 5'-terminal conserved AU dinucleotide present in both genomic and sgRNA is required for effective capping *in vitro*; these nucleotides were observed to form base-specific interactions with the capping domain (69).

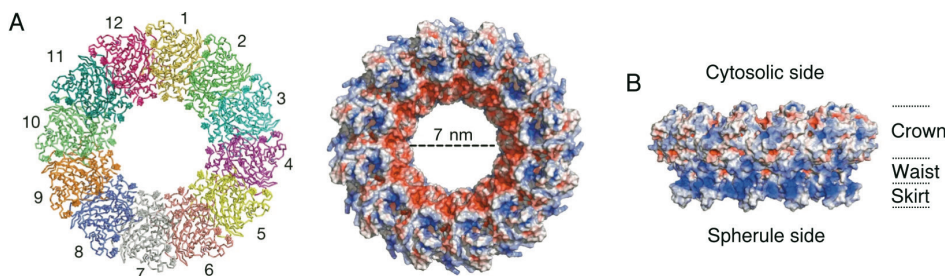


Figure 7. Spherule-pore complex cryo-EM structure of CHIKV nsP1. (A) Cytosolic top view of the dodecameric nsP1 pore symmetry and surface representation. (B) Side view of the nsP1 pore complex. Adapted from (155).

Membrane association of nsP1 has long been known to be essential for its enzymatic activity and, by extension, for alphavirus replication as a whole (161, 162). Membrane binding has been attributed to an amphipathic peptide covering amino acid residues 245 to 264 in SFV nsP1 (161, 163). It has been shown that substitutions R253E and W259E within this peptide render nsP1 localization cytoplasmic, and such a complete loss of membrane binding was shown to be lethal for SFV (163). The corresponding R252E substitution in CHIKV is also not tolerated; interestingly, however, the corresponding substitution of the tryptophan residue (W258A) in CHIKV nsP1 results in a temperature sensitive phenotype: the mutant virus exhibited severely attenuated replication at 37 °C and 39 °C, but replicated well at 28 °C (164, 165).

What is clear is that nsP1 membrane association is further enhanced by palmitoylation at cysteine residues 418–420 for SFV, 417–419 for CHIKV, and C420 for SINV (166, 167). The absence of this modification influences alphaviruses differently: corresponding cysteine-to-alanine mutations render CHIKV

non-infectious/non-replicative while the SFV and SINV mutants remain viable, with the SFV mutant acquiring second-site mutations within nsP1 upon passaging (164, 167, 168). This also led to the discovery that palmitoylation-deficient SFV (presumably harbouring one or more second-site compensatory mutations in nsP1 region) is non-neurovirulent in mice, which contrasts to the wild-type (WT) strain that produces high titres in the murine brain (167). The high impact of the palmitoylation of CHIKV nsP1 has been linked to a strong positive correlation with the location of cholesterol-rich micro-domains in the plasma membrane – the sites for the formation of CHIKV RCs. Interestingly, membrane association of SINV nsP1 appears to be less dependent on cholesterol which correlates with the smaller number of palmitoylated residues and minor impact of the C420A substitution (169).

Recent cryo-EM studies on the CHIKV nsP1 structure have helped contextualize these important function-related studies. Firstly, membrane association is directly mediated by two intertwining membrane association loops at the bottom of the “skirt” region; loop 1 spans amino acid residues 220–233 and loop 2 spans residues 407–427, with the latter containing three palmitoylated cysteines (139, 155). Secondly, the amphipathic helix was not observed to directly associate with membranes. Instead, it has been proposed to be important for proper protein folding, a finding that correlates with a study showing that when introduced as a synthetic peptide corresponding to the CHIKV nsP1 amphipathic helix, the R252E and W258A substitutions do not hinder binding to phospholipids (170).

Studies with temperature-sensitive mutants have implied an important role for nsP1 in negative-strand RNA synthesis. For example, a T349K substitution in nsP1 was observed to compensate for the replacement of the native tyrosine residue in the nsP4 N-terminus with a non-aromatic residue, a change that is otherwise lethal. The resulting mutants were permissive for negative-strand synthesis at 30 °C but defective at 40 °C, and when nsP1-T349K was introduced alone, virus growth was similarly temperature sensitive (171). The adjacent position was shown to be similarly important, as the A348T substitution in SINV nsP1 causes growth defects and impairment of negative-strand RNA at the nonpermissive temperature, highlighting that residues 348–349 in SINV nsP1 are likely critical for interactions between nsP1 and nsP4 N-terminus in the early replicase (172). In fact, as revealed by the cryo-EM analysis, the regions of the CHIKV nsP1 dodecameric ring that associate with nsP4 inside the central pore indeed map to residues 335–380 (136).

1.3.2. nsP2

The largest alphavirus ns protein is nsP2, which hosts the enzymatic activities necessary for RNA replication, modification and polyprotein processing. nsP2 consists of two major regions: the N-terminal helicase region (nsP2h) and the C-terminal protease region (nsP2p), which are interconnected by a short flexible linker (173). The larger nsP2h region includes an N-terminal domain (NTD), a helical stalk domain, a superfamily 1 accessory domain B (1B), and two RecA-

like domains, RecA1 and RecA2, which compose the helicase core structure, as well as an additional C-terminal α -helix termed the connector (Fig. 8) (174). The nsP2p region consists of the papain-like cysteine protease domain and a methyltransferase-like (MTL) domain, although the latter lacks methyltransferase activity (173).

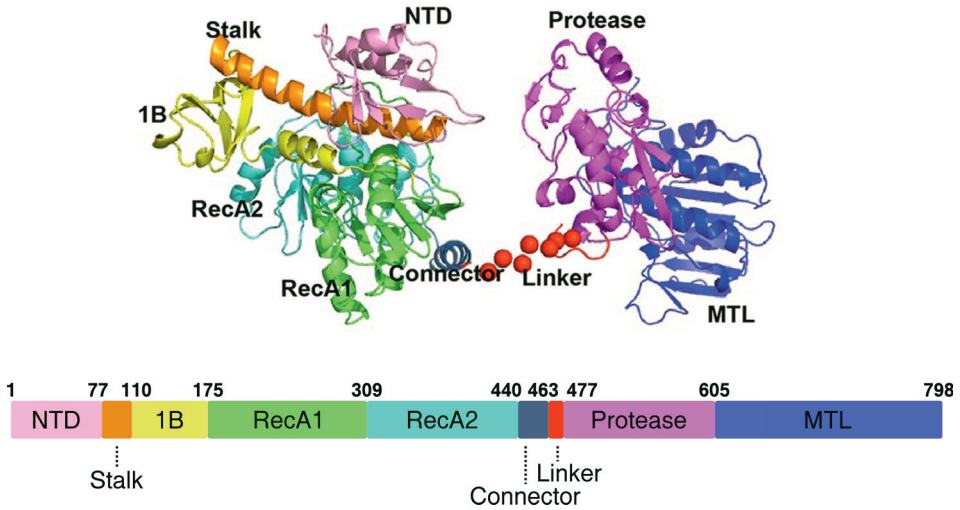


Figure 8. Overview of CHIKV nsP2 structure. NTD – N-terminal domain, 1B – superfamily 1 accessory domain B, MTL – methyltransferase-like domain. Figure adapted from (173).

nsP2h exhibits nucleoside triphosphatase (NTPase) and RNA triphosphatase (RTPase) activities (175–177). The NTPase activity drives the helicase unwinding process during RNA replication; ATP is the preferred substrate for hydrolysis (156). A conserved lysine at position 192 is instrumental for this activity as both K192R and K192A substitutions abolish NTPase activity of nsP2 (156, 176). Further evidence of the importance of this residue comes from experiments showing that a mutated N192 residue in SFV nsP2 reverts to the original lysine when corresponding infectious RNA is directly transfected into cells (177). Experiments with truncated CHIKV nsP2 lacking much of the C-terminal protease domain, along with K192A, D251A, and E252A substitutions in the nsP2h region, demonstrated that these positions are also necessary for RTPase activity, which specifically cleaves the γ - β triphosphate bond to generate the RNA 5'-diphosphate end required for subsequent capping steps (156). Interestingly, nsP2h contains all motifs required for RNA helicase activity yet is unable to function as a helicase on its own; thus, the C-terminal nsP2p is required to unwind RNA strands. It was shown that the nsP2p binds RNA more effectively, and, in order to unwind dsRNA substrates, the linker between nsP2h and nsP2p is important. Thus, both major regions, their proper positioning, and the need for high RNA binding affinity are required for RNA helicase activity (173).

nsP2 is the only protein that cleaves alphavirus ns proteins. Since full-length P1234 is not active as a replicase, the protease activity of nsP2p is essential for establishing viral infection (178, 179). nsP2 is a cysteine protease with a catalytic dyad of cysteine and histidine corresponding to residues 478 and 548 for SFV and CHIKV, or 481 and 558 for SINV nsP2 (180–182). The importance of these residues is clearly demonstrated by SINV mutants with substitutions C481S/G/R or H558A/Q/Y, which lack nsP2 proteolytic activity and are non-infectious (181); similar observations have been made for the C478A CHIKV nsP2 mutant (183) and SFV nsP2 with substitutions C478A and H548A (182).

P1234 is processed at three cleavage sites: the 1/2 site (between nsP1 and nsP2), the 2/3 site, and the 3/4 site. The sequences of these sites are not highly conserved, except for a conserved glycine residue in the P2 position (the second residue upstream of the scissile bond) (184). The processing follows a sequential pattern, and each cleavage is properly timed; as described above, this is essential for the formation of the early replicase and its subsequent conversion to the late replicase. Briefly, upon P1234 synthesis, nsP4 is rapidly released by *cis* cleavage, and the early replicase, P123+nsP4, is formed. 1/2 and 2/3 site cleavage-deficient SINV can establish a complete infection in cell culture, albeit the replication is inefficient and requires a second-site mutation to be present in nsP4 (185). The subsequent 1/2 cleavage also occurs *in cis* but compared to the 3/4 site cleavage, is less effective and somewhat delayed. The 1/2 site cleavage is a pre-requisite for the 2/3 site processing, as the liberated N-terminus of nsP2 within P23 is essential for the final *trans* cleavage to take place (126). It has been demonstrated that the processing rate can determine the *in vivo* phenotype of an alphavirus. Substitution H534R (the P4 position of the 1/2 site), along with mutation V515E in nsP2 (associated with reduced protease activity) significantly reduce SFV6 neurovirulence (186). These substitutions increase ns polyprotein processing rates for SFV6. Reciprocal changes alter the phenotype of the A774 strain from avirulent to neurovirulent (186). Additionally, a hyperprocessive nsP2 protease in SINV with an N614D mutation greatly reduces infectivity of the virus (187). Thus, there appears to be selective pressure to maintain the 1/2 site sequence so that protease recognition and cleavage occur at an optimal rate. The protease activity of nsP2 of OW alphaviruses has also been implicated in superinfection exclusion (SIE), a process in which the initially infecting virus prevents subsequent infections. Most likely it is due to the ability of free nsP2 to cleave P1234 of the superinfecting virus at the 2/3 site and thus prevent RC formation (188–190).

As evident from the stoichiometry of the replicase core, only a fraction of nsP2 directly participates in viral RNA synthesis. RC-free nsP2 is found both in the cytoplasm and nuclei of alphavirus infected vertebrate cells (191). The nuclear localization signal (NLS) of SFV nsP2 lies in the C-terminal MTL domain. Nuclear localization of nsP2 of OW alphaviruses is important for host antagonism, as disruption of the NLS of SFV abolishes neurovirulence in mice and results in an elevated type I IFN response in cultured mouse embryonic fibroblasts (MEF) (192–194). Moreover, SINV nsP2 alone has been shown to be cytotoxic for vertebrate cells and to inhibit cellular transcription (191). The

principal mechanism by which OW alphaviruses inhibit host transcription is by inducing the degradation of the catalytic subunit of RNA polymerase II (RpbI), a process mediated by nsP2 (195). Interestingly, this activity is not attributable to the nsP2 protease function, but rather to its general ability to bind DNA, and likely the RNA polymerase II complex, resulting in transcriptional stalling and subsequent RpbI degradation by host mechanisms (195). Independently of the transcriptional shutoff, nsP2 of VEEV, SINV, and CHIKV inhibits the activation of interferon-stimulated genes (ISGs) by downregulating the Janus activated kinase (JAK) and signal transducer and activator of transcription (STAT) signalling pathway (196, 197). In this pathway, STAT1 and STAT2 are phosphorylated upon activation and form heterodimers that function in the nucleus as transcription factors; the presence of CHIKV nsP2 has been shown to prevent nuclear localization of these STAT dimers. Although the nuclear localization of nsP2 is essential for this activity, the native NLS is not directly responsible. Instead, the MTL domain is essential for promoting the nuclear export of STAT1 (198).

1.3.3. nsP3

nsP3 is composed of three domains: the N-terminal macro domain, the middle alphavirus unique domain (AUD), and the C-terminal hypervariable domain (HVD) (Fig. 9A). Historically, nsP3 has been the most elusive of the alphavirus replicase proteins, as it has no enzymatic function directly related to RNA replication.

The conserved macro domain of nsP3 is not unique to alphaviruses; similar domains are found in proteins of other RNA viruses such as coronaviruses and in many cellular organisms (199). Alphavirus macro domains are known to bind ADP-ribose and, moreover, act as ADP-ribosylases capable of removing this modification from proteins; it is the only enzymatic activity currently associated with nsP3 (200–202). ADP-ribosylation is a post-translational protein modification important for DNA damage response, cell growth, transcription, metabolism, cellular stress response, and gene expression, among others, and is catalysed by poly-ADP-ribose polymerases (PARPs) (203). The impact of ADP-ribose binding and hydrolase activity on CHIKV infection is significant, as D10A, G112E, and G32A substitutions in nsP3 that diminish ADP-ribose binding and enzymatic activity render the viral replicase inactive and the virus nonviable (204). In addition, similar mutations in SINV nsP3 resulted in significantly lesser neurovirulence in a mouse model (205). Recently, PARP10 was characterized as a potential CHIKV nsP2 antagonist, as it was found to modify the protein directly and downregulate its protease activity. In turn, the nsP3 macro domain was shown to counteract the effect of PARP10 by removing the mono-ADP-ribose modification from nsP2, helping restore its protease activity (206). More recently, ADP-ribosylation of nsP3 and nsP4 of SFV by PARP7 and PARP12 was demonstrated. Again, nsP3 ADP-hydrolase activity was associated with the removal of the modification (207). On another note, the presence of the macro domain is also important in the

trans cleavage of the 2/3 site. This finding lead to the proposed model in which the 2/3 site cleavage is not necessarily driven by the specific sequence at the scissile site, but rather by macromolecular assembly of the polyprotein and its cleavage substrates (208).

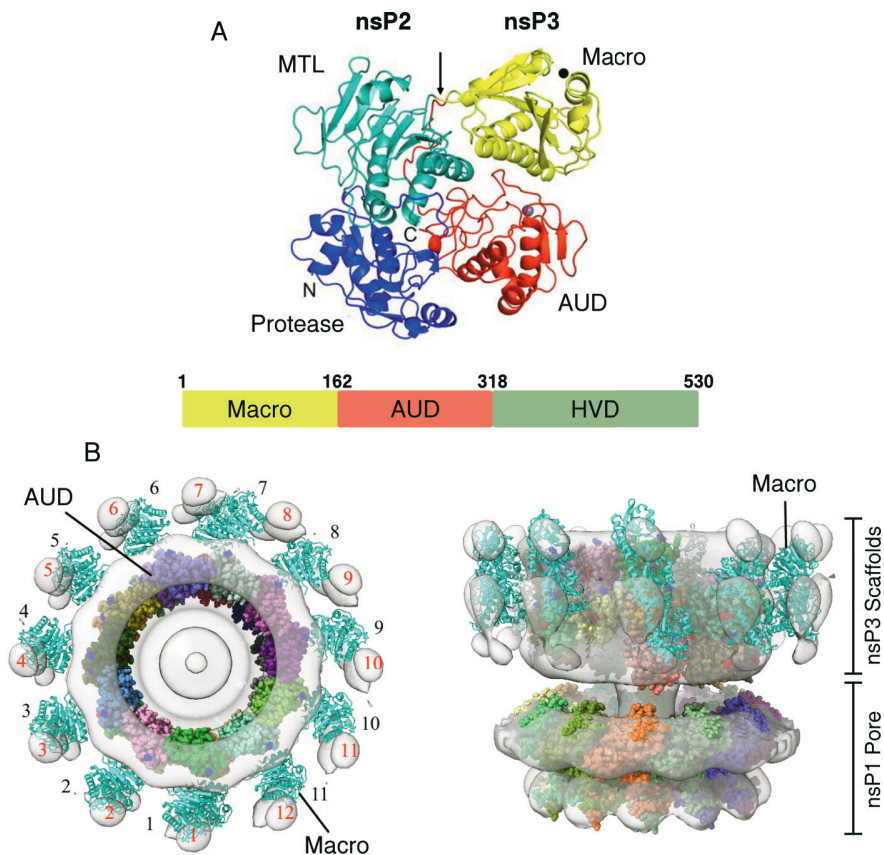


Figure 9. Structural overview of nsP3. (A) Ribbon representation of SINV nsP3^{Pro-AUD} and linear organization of CHIKV nsP3; arrow depicts the 2/3 cleavage site. MTL – methyltransferase-like domain, AUD – alphavirus unique domain, HVD – hypervariable domain. Figure adapted from (128). (B) Superposition of CHIKV nsP3 helical oligomer structures onto the CHIKV core RC tomogram. Figure is adapted from (140).

The central AUD, also called the zinc-binding domain, coordinates a single zinc ion via four conserved cysteine residues (128). Substitution of these cysteines with alanine abolishes CHIKV replication in mammalian and insect cells, suggesting that these cysteines and the associated ion play a role in RNA binding (209). A more recent study found that alphavirus nsP3 forms oligomers through interactions mediated by the AUD and organises into tubular helical scaffolds during CHIKV infection (Fig. 9B). Upon individual protein expression, the phenomenon was also observed for EEEV, ONNV, SINV, and MAYV nsP3 (140). Interestingly, when CHIKV AUD is expressed alone or together with the HVD, very long fibrous structures form (140, 210). The oligomerization was shown to be

blocked by the presence of nsP2 and cannot be observed until nsP3 is released from maturing RCs, presumably this regulates the formation of the nsP3 helical crown utilized as part of the mature RC. In later stages of infection, RC-independent nsP3 organizes into cytoplasmic granular clusters. Recent evidence suggests that this clustering likely also occurs through AUD oligomerization, and in some cases, these clusters have been observed to include viral genomic RNA and CP, suggesting a role in virion assembly (140, 211).

The C-terminal HVD is highly variable in length and sequence and accounts for the size differences of alphavirus nsP3s. For example, BFV nsP3 is 469 amino acid residues long, whereas ONNV nsP3 is 570 residues in length (212). The HVD is intrinsically disordered (128, 140). Despite this, a conserved feature of the HVD is its modification by phosphorylation on serine and threonine residues, which has been observed in nsP3 of CHIKV, ONNV, SINV, SFV, and VEEV (213–216). The effects of nsP3 phosphorylation vary between alphaviruses: SINV mutants lacking these modifications exhibit moderately attenuated infection in vertebrate cells but are severely attenuated in mosquito cells; similar effects were observed with VEEV nsP3 phosphorylation-deficient mutants (217, 218). Additionally, reduced phosphorylation of SINV nsP3 correlated with diminished negative-strand RNA synthesis (219). Deletion of 50 residues within the HVD of SFV that contained phosphorylation sites resulted in moderate attenuation in cultured mammalian cells and avirulence in mice (220). In contrast to these viruses, CHIKV lacking HVD phosphorylation is not viable (215). Collectively, these observations indicate that conserved phosphorylation modifications influence alphavirus species differently and that the effects can be host specific, suggesting that phosphorylated residues might be involved in interactions with host cell factor(s).

The HVD of nsP3 serves as a hub for interactions with host proteins (212). The disordered HVD structure remained unresolved in the cryo-EM analysis of the nsP3 helical scaffolds, but these residues were shown to be exposed on the surface of the oligomeric structures, making the domain accessible for the numerous interactions associated with nsP3 (140). The list of proteins shown to interact with nsP3 HVD is long and continuously growing. While most of the individual proteins interacting with the HVD have a relatively mild impact on alphavirus infection, there are some that stand out. For instance, Ras-GTPase-activation protein (SH3-domain)-binding proteins (G3BP1 and 2) are known interactors of OW alphavirus nsP3 (221, 222). G3BPs play a role in the assembly of stress granules (SGs), cytoplasmic RNA-protein complexes that form as a stress response, including during viral infections, to regulate translation stalling (223). Alphavirus antagonism of SG formation is beneficial, as these viruses depend on cellular translation factors to establish successful infections (224). nsP3 of OW alphaviruses contain FGDF motifs that directly bind G3BPs; substituting the first residue of each motif with alanine in SFV HVD results in virus attenuation and enhanced SG formation (221, 225). These effects are much more pronounced for CHIKV infectivity which is completely dependent on G3BP availability (226). Several other OW alphaviruses, including ONNV, RRV, MAYV, BFV, and the

avirulent SFV strain A774, have also been shown to be sensitive to G3BP deletion, although to varying degrees (227). The same study demonstrated that slower processing of the 1/2 site reduces G3BP dependency for CHIKV. The prominent impact on CHIKV replication and link with one of the key elements for RC formation strongly suggests the involvement of G3BP in RNA synthesis. Interestingly, the FGDF motifs are also crucial for nsP3 interaction with the mosquito ortholog of G3BP, Rasputin (or Rin); mutagenesis of these motifs inactivates CHIKV replication in the *Aedes aegypti* cell line Aag2 (228). NW alphaviruses VEEV and WEEV lack FGDF motifs in their HVDs and consequently, their nsP3 does not bind G3BPs. Instead, their nsP3 can bind another SG-related protein, fragile X mental retardation protein (FXR), as demonstrated for VEEV nsP3 (229, 230). In the absence of FXR, VEEV remains viable, although viral replication is significantly less efficient. As OW alphaviruses are similarly sensitive to G3BP depletion, it was concluded that these SG-related proteins are important for viral replication, possibly having similar functions in RNA replication (230). To support this assumption, Kim et al. confirmed that FXR and G3BPs are indeed recruited to RCs at the plasma membrane, and their absence likely disrupts complex formation, resulting in aberrant RNA production (230). Notably, the HVD of EEEV contains binding sites for both FXR and G3BPs, and significant effects on viral replication occur only when interactions with both factors are blocked (231). At later stages of infection, as free nsP3 accumulates, cytoplasmic aggregates of FXR/G3BP become more prominent. These aggregates sometimes include viral genomic RNA, which is consistent with a role beyond SG formation inhibition (230). The sequestration of essential host proteins by the HVD of nsP3 has even been suggested to contribute to SIE during mammalian cell infection with VEEV (232).

Another conserved feature of alphavirus nsP3 is the presence of proline-rich regions in the HVD. These motifs mediate interactions with another group of host factors – amphiphysins, proteins associated with membrane trafficking and endocytosis (233, 234). Phage library based screening identified amphiphysins as binding partners for CHIKV, SFV, and SINV nsP3; mutating the proline-rich region impairs SINV and SFV RNA replication and significantly attenuates their neurovirulence in mice (234). In addition to amphiphysins, these motifs bind multiple SH3 domain proteins, such as CD2-associated protein (CD2AP) and SH3-domain kinase-binding protein 1 (SH3KBP1) (235). Individually, each of these proteins has limited impact on alphavirus replication, however, to achieve a detectable level of virus replication, the HVD needs to bind members of at least one more protein family in addition to G3BPs (236, 237). One of these proteins is four-and-a-half LIM domain protein 1 (FHL1). CHIKV infection is also highly dependent on this factor, as demonstrated in FHL1 knockout cell lines and mouse models (238, 239). The effect on virus infectivity appears species and strain specific; most CHIKV and ONNV isolates are sensitive to FHL1 absence, whereas a West African CHIKV strain (37997) is less so, and other tested alphaviruses (MAYV, RRV, SINV, SFV, EEEV, WEEV, and VEEV) are insensitive (238). The binding region of FHL1 within CHIKV nsP3 HVD has been mapped to be

upstream of the G3BP binding motifs; mutating this site results in strong CHIKV attenuation in some cell lines (238) but not in others (240).

As an integral part of RCs, a fraction of nsP3 localizes near the plasma membrane. In the case of some alphavirus infections, RCs internalize into cytopathic vesicles, and nsP3 can be observed to colocalize with dsRNA at both sites (132, 241). nsP3 of some alphaviruses, via its HVD, has also been shown to (hyper-)activate the phosphatidylinositol-3-kinase-Akt-mammalian target of rapamycin (PI3K/Akt/mTOR) signalling pathway (133, 242). This is most exemplified by SFV, in which a YXXM motif in the HVD is essential for the hyperactivation. The interaction with PI3K activates the pathway and directs the host toward a pro-survival state, leading to higher activation of glycolysis and the pentose phosphate pathway, ultimately resulting in increased virion production. This suggests that regulation of the PI3K/Akt/mTOR pathway is especially important for the later stages of infection (242). The pathway has also been associated with the internalization of RCs from the plasma membrane to cytopathic vesicles, a property that distinguishes SFV and CHIKV. Although both viruses activate the PI3K/Akt/mTOR pathway, SFV has been described to hyperactivate it (133, 134). nsP3 of RRV also includes a YXXM motif, altering the motif to FXXM results in attenuated infection in a mouse model. Conversely, SINV inhibits the pathway in HEK293T cells but activates it in insect cells, potentially supporting persistent infection in arthropod hosts (243, 244).

Since HVD sequences vary considerably within the genus, this region of nsP3 tolerates large insertions. Attaching fluorescent tags to the C-terminal region of nsP3 has facilitated studies of its subcellular localization (245, 246). Enhanced green fluorescent protein (EGFP) tagged nsP3s from Bebaru virus (*Alphavirus bebaru*, BEBV), ONNV, SINV, Whataroa virus (*Alphavirus whataroa*, WHATV), TALV, and BFV have been observed to partially localize in cell nuclei (246); utilizing HVD-tagging, BFV nsP3 nuclear localization is also investigated in the current work as part of Publication I.

nsP3 is an important determinant of alphavirus virulence as seen in experiments performed using virulent (SFV6 or SFV4) and avirulent (A774) strains of SFV (186, 247). Swapping nsP3 between these strains results in changes in virulence but the impact is incomplete and not reciprocal. Comparing these swap mutants led to the discovery of additional determinants of SFV neurovirulence within the ns polypeptide. Specifically, the avirulent A774 exhibits more rapid ns polypeptide processing. The effect of swapping nsP3 from SFV6 to A774 dominated over these differences, whereas swapping nsP3 from A774 to SFV6 did not. When these mutations, together with nsP3 swapping, were introduced into SFV6, the virus became significantly less lethal in mice (186). Furthermore, nsP3 has a significant impact on alphavirus vector preference. Such an effect is best observed for CHIKV and ONNV, that are closely related but have a major difference in their vector usage; CHIKV is transmitted by *Aedes* mosquitoes and is unable to infect the primary ONNV vector, *Anopheles gambiae*. However, it was found that chimeric CHIKV encoding the nsP3 of ONNV is infectious in these mosquitoes, indicating that nsP3 is a major determinant of ONNV vector specificity (248).

1.3.4. nsP4

In infected cells, the levels of nsP4 are much lower than those of other ns proteins. This indicates that alphaviruses actively suppress nsP4 levels. A strategy apparent to many, but not all alphaviruses, is the use of the “leaky” opal stop codon (UGA) at the region encoding for the C-terminus of nsP3 (2, 249). Exceptions from this rule include the fish-infecting alphaviruses that thrive in colder environments; these viruses encode glutamine instead (249). The neurovirulent SFV4 and SFV6 strains also produce solely P1234 precursors by encoding an arginine at the end of nsP3, whereas the avirulent A774 includes the opal stop codon, indicating that both codons occur naturally (247). ONNV isolates also possess either the opal or arginine codon (250, 251). The presence of the leaky stop codon has been reported to enhance ONNV infectivity in the mosquito *Aedes albopictus* cell line C6/36 and the golden hamster originating BHK21 cells, and importantly, in its natural host *Anopheles gambiae* (252). Conversely, serial passaging of ONNV in Vero cells results in positive selection for the arginine codon via a single base mutation (UGA to CGA), suggesting that during natural circulation either codon may emerge depending on the host used for replication (252). The arginine codon has also been found in some CHIKV strains, and its occurrence has been linked to severely attenuated phenotype in a mouse model (253, 254). The more universal strategy to reduce nsP4 levels is by targeting it for degradation. As nsP4 is a cleavage product of P1234, it lacks an N-terminal methionine residue, instead the N-terminal residue is tyrosine. This is a destabilizing residue, and when exposed, it leads to nsP4 degradation via the N-end rule pathway (255). However, as part of the RC, nsP4 is stabilized.

nsP4 is an RNA-dependent RNA polymerase (RdRp) and is composed of two domains: the N-terminal domain (NTD), which is unique to alphaviruses, and the C-terminal RdRp domain (Fig. 10) (256). The resolution of the crystal structures of SINV and RRV nsP4 confirmed the characteristic viral RdRp fingers-palm-thumb structure of the C-terminal domain, which contains the highly conserved GDD amino acid sequence in the catalytic active site (257). Manipulation of the GDD motif, such as substituting it with GAA, eliminates the enzymatic activity of nsP4; such mutants have proven useful as RdRp activity-absent negative controls in alphavirus *trans*-replication systems (258, 259).

The NTD, as well as the C-terminus of nsP4, cannot be visualized using X-ray crystallography. The cryo-EM structure of a mature RC helped resolve the structures of these regions, indicating that these are not unfolded, but rather their folding depends on interactions with other RC subunits, most prominently with nsP1. Furthermore, the *in vitro* RNA synthesis activity of nsP4 subunits was greatly enhanced when being part of the complex (136, 257). nsP4 was observed to be stably positioned at the centre of the dodecameric nsP1 pore, where it established contacts with 10 nsP1 subunits via its NTD and the fingers-palm-thumb subdomains of the RdRp domain. It was further stabilized through interactions with nsP2 (136). Within the RC, the conserved N-terminal tyrosine residue of nsP4 was shown to be tucked into the internal RNA-binding groove (136).

Importantly, in SINV nsP4, the tyrosine residue can be substituted with other aromatic residues or histidine (260). Non-aromatic residues are only tolerated when additional AU, AUA, or AUU sequences are added to the 5'-end of the genomic RNA, suggesting that nsP4 likely interacts with the 5'-end of the genome (68). Combining this functional data with that from structural analysis suggests the following model: the tyrosine residue is positioned within the RNA binding pocket of the RC, and its aromatic ring is required for the optimal RNA positioning via stacking interactions (136).

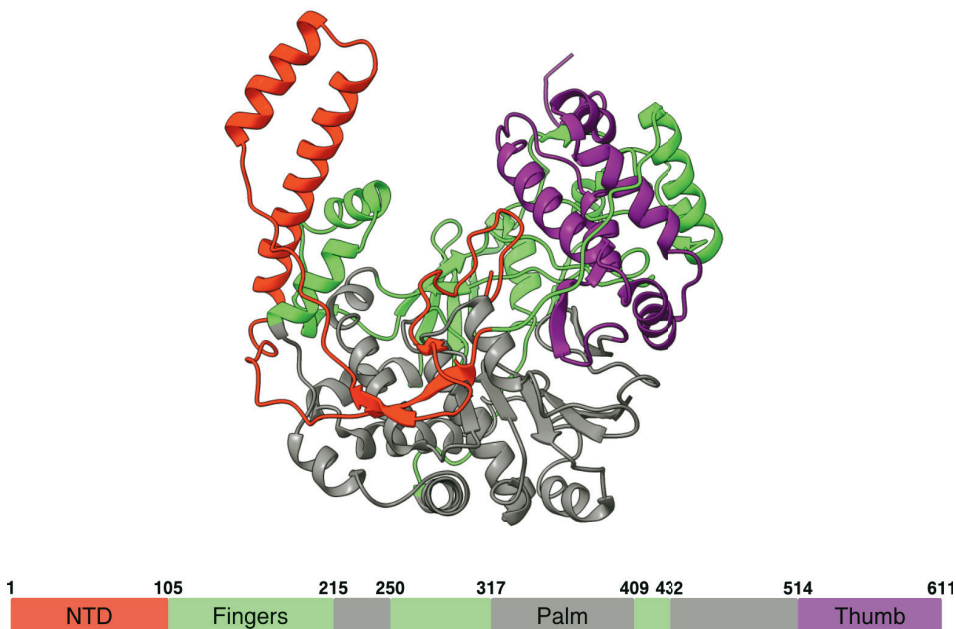


Figure 10. Structural overview of ONNV nsP4. Full length nsP4 structure inside of the core of mature RC (PDB ID: 7Y38). Linear organization schematic is adapted from (136).

As an RdRp, nsP4 must, directly or indirectly, interact with promoter elements in the viral genome and negative-strand RNA. One of these elements is a conserved sequence located immediately upstream of the polyA tail, and it has been shown that the minimal polyA length required at the 3'-end of alphavirus genomic RNA to efficiently support negative-strand RNA synthesis is 11 bases (78). This seemingly peculiar requirement is due to the initiation of negative-strand RNA synthesis that does not occur at the end of the polyA tail, but instead on the last non-A residue of the 3' UTR; as a result, the negative-strand RNAs do not contain polyU sequences. The synthesis of the polyA tail is still a property of nsP4, but it is additionally synthesized for the ends of positive-strand viral RNAs rather than being copied (261, 262). This is possible as nsP4 exhibits terminal adenylyltransferase activity that relies on its core RdRp function; the GDD-to-GAA mutation in the catalytic site also prevents polyA tail addition to RNA templates (262).

Despite the mechanisms described above that lead to significantly reduced nsP4 abundancy during infection, the protein has been suggested to function as an unfolded protein response (UPR) antagonist (263). Specifically, CHIKV infection and standalone nsP4 expression were shown to suppress Ser-51 phosphorylation of eukaryotic initiation factor 2 α (eIF2 α), which is usually performed by activated PKR-like ER kinases (PERK) under stress conditions and serve to halt translation (263). Furthermore, CHIKV nsP4 was detected interacting directly with the UPR effector chaperone protein heat shock protein 90 (HSP-90) in co-immunoprecipitation experiments (264). Targeting HSP-90 production via genetic knockdown or using chemical inhibitors impaired CHIKV replication and significantly reduced CHIKV-induced inflammation in mice, suggesting that HSP-90 may be utilized by CHIKV to stabilize nsP4 (264).

1.4. Alphaviruses and the type I IFN response

The host innate immune response is the initial and rapid defence against pathogen activity. Alphavirus replication occurs solely in the cytoplasm of infected cells and relies on host factors present in this compartment to propagate its genomes. In response, the host actively works to counteract viral replication by detecting pathogen-associated molecular patterns (PAMPs), which, in the case of alphaviruses, consist primarily of various RNA replication products. Thus, alphaviruses must manipulate host cell metabolism to gain an advantage, antagonizing innate immune mechanisms while maintaining a cellular environment conducive to viral RNA transcription and translation. This section describes the interaction of alphaviruses with the vertebrate innate immune system, focusing on type I IFN-related mechanisms that detect nucleic acids generated during infection. A schematic summary of alphavirus-related PAMP sensing is provided below in figure 11.

A major early host response to alphaviruses is the type I IFN pathway, which is triggered when viral RNAs, that differ from normal RNAs in the cell, are detected by intracellular pattern recognition receptors (PRRs). This leads to the production of IFN- α and IFN- β , which mediate autocrine and paracrine signalling to establish an antiviral state in both infected and neighbouring cells. Important PRRs involved in alphavirus detection include endosomal toll-like receptors (TLRs): TLR3, which recognizes dsRNA, and TLR7/8, which detect viral single-stranded RNA (ssRNA) (265–267). Upon recognizing PAMPs, TLR3 recruits the adaptor TIR-domain-containing adaptor protein inducing IFN- β (TRIF), while TLR7/8 recruit myeloid differentiation primary response 88 (MyD88). These signalling cascades activate interferon regulatory factor 3 (IRF3) and IRF7, which translocate to the nucleus to upregulate type I IFN production (265, 268). IFN- α and IFN- β act as extracellular signalling molecules that bind to the heterodimeric IFN- α/β receptor (IFNAR), composed of IFNAR1 and IFNAR2 subunits (269). Upon ligand binding, IFNAR activates JAK1 and tyrosine kinase 2 (TYK2) on the cytosolic side, leading to phosphorylation of STAT1 and STAT2

(270). These phosphorylated proteins form a heterodimer that associates with IRF9, creating the IFN-stimulated gene factor 3 (ISGF3) complex, which translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) to activate the transcription of ISGs (270).

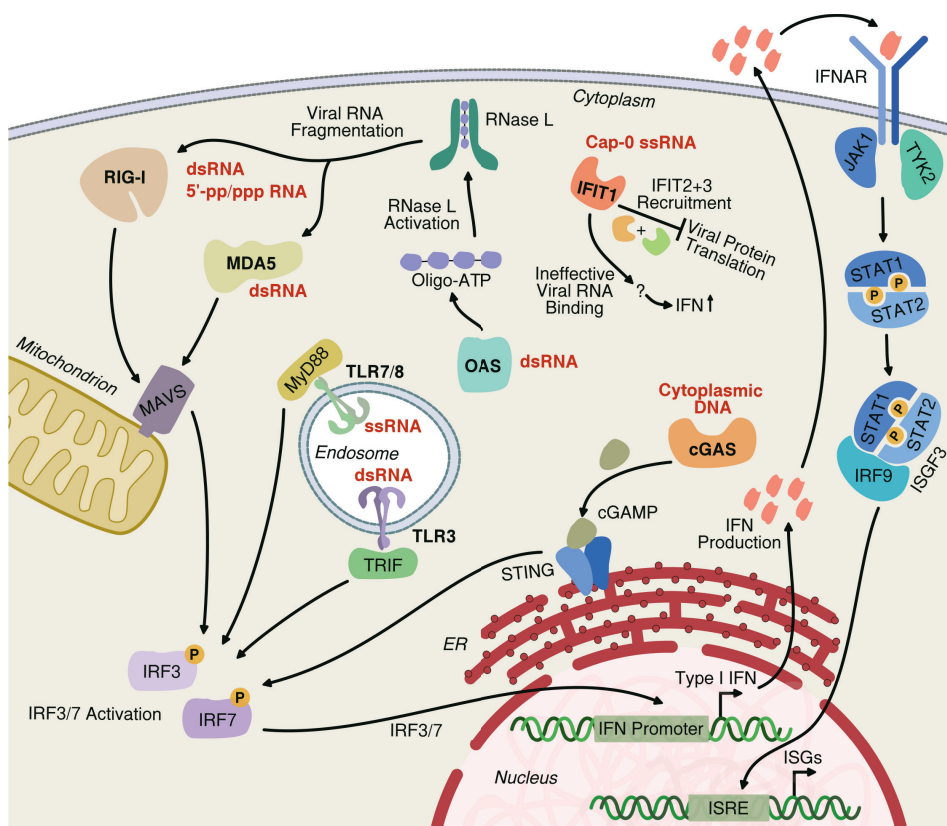


Figure 11. Overview of type I IFN induction related to alphavirus sensing. The principal sensors detecting nucleic acid PAMPs are highlighted in bold black text, and related PAMPs highlighted in bold red text.

The critical role of type I IFN in controlling alphavirus infection is well established. IFNAR-deficient mice exhibit high mortality following infection with both arthritogenic and encephalitic alphaviruses (271–274). Furthermore, mouse model experiments demonstrate that type I IFN is particularly crucial during the early stages of infection, as IFN treatment is ineffective against already established CHIKV infection (275). Additionally, the importance of TLR3 and TRIF in CHIKV infection control has been demonstrated in gene knockout murine and human cell lines, where their absence significantly enhanced viral replication. TLR3 knockout (TLR3^{-/-}) mice infected with CHIKV exhibited more severe disease progression, higher tissue viral loads, extensive dissemination, and delayed clearance (276). Similarly, RRV infection in TLR7^{-/-} and Myd88-deficient

mice resulted in more severe disease and prolonged viral presence in infected tissues compared to WT mice, highlighting the role of these PRRs in viral clearance (266). IRF3 and IRF7 signalling also plays a crucial role in limiting CHIKV pathogenesis. IRF3/7^{-/-} double knockout mice do not survive CHIKV infection, similar to IFNAR-deficient mice, further demonstrating the essential role of type I IFN in restricting viral replication (272, 277). IFNAR1-deficient mice are also highly susceptible to VEEV infection, and IRF7 (but not IRF3) has been identified as the key factor necessary for effective IFN α/β induction and secretion (278). IRF3-mediated immune responses are critical for neuronal survival during WEEV infection, and neurovirulent SINV disease is significantly more severe in IRF7^{-/-} mice, emphasizing the role of type I IFN-associated PRRs in controlling encephalitic alphaviruses (279, 280).

The cyclic GMP-AMP synthase (cGAS) and stimulator of IFN genes (STING) pathway has been also implicated in type I IFN signalling during CHIKV infection (281). This pathway primarily detects DNA rather than RNA and activates type I IFN responses when cytoplasmic DNA is detected (282). Upon DNA binding, cGAS synthesizes cyclic GMP-AMP (cGAMP), which acts as a signalling ligand for the ER membrane-bound STING, triggering downstream signalling that activates IRF3 and induces type I IFN production (283). However, alphavirus-derived PAMP RNA is not a direct target for cGAS, instead CHIKV infection has been shown to induce the re-localization of cellular DNA into the cytoplasm, where it activates cGAS (281). Interestingly, CHIKV antagonizes this pathway by promoting cGAS degradation through its interaction with viral CP, suggesting that the pathway plays a role in CHIKV infection control and that endogenous DNA release into the cytoplasm is a consequence of infection that must be counteracted for optimal viral replication (281). The study did not determine the exact source of the endogenous DNA found in the cytoplasm during CHIKV infection. However, another positive-sense RNA arbovirus, Dengue virus (family *Flaviviridae*), has been shown to trigger the release of mitochondrial DNA for cGAS recognition (281, 284). STING deficiency has also been associated with more severe arthritis in CHIKV infected mice (285).

Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are soluble cytosolic receptors belonging to the RIG-I-like receptor (RLR) family, which are crucial for detecting RNA virus activity (286). Both of these PRRs recognize dsRNA. In addition, RIG-I recognizes 5'-triphosphate (5'-ppp) or diphosphate (5'-pp) RNA that forms short duplexes, where the terminal nucleotide must also participate in base-pairing, and lack ribose 2'-O-methylation (287–290). Additionally, RIG-I can be activated by binding RNA with a cap-0 structure that forms a short 5'-terminal hairpin (290). While the specific ligands for MDA5 are less well defined, it has been shown to oligomerize into filaments when binding dsRNA and is activated when mitochondrial dsRNA is introduced to the cytoplasm (286, 291, 292). Both RIG-I and MDA5 contain two tandem caspase activation and recruitment domains (CARDs), which, upon ligand binding, interact with mitochondrial antiviral signalling protein (MAVS) to initiate a cascade leading to IRF3 and IRF7 activation and subsequent type I

IFN production (286). Both RIG-I and MDA5 act as sensors of SINV and VEEV replication in the murine NIH-3T3 cell line; however, type I IFN induction is far more impaired in cells lacking both receptors (293). Consistently, CHIKV infection in RIG-I^{-/-} or MDA5^{-/-} mice is only slightly attenuated compared with WT animals, whereas MAVS deficiency results in more severe disease progression (294). Together with the SINV and VEEV findings, this indicates that RLRs function cooperatively in alphavirus detection. In addition, RIG-I expression has been shown to be upregulated in RRV infected murine cells, indicating the importance of this RLR in sensing RRV infection (271). During SFV infection, RIG-I- and MDA5-dependent PAMP detection has been demonstrated in COP5 murine fibroblasts, and, furthermore, strong IFN- β induction occurs in these cells upon expression of SFV replicase alone. The study further demonstrated that RLR-recognized PAMP RNAs are produced by SFV replicase utilizing host cell RNA templates (295). The ability of several alphavirus replicases in producing these replicase-only PAMP RNAs (rPAMPs) in the absence of viral template RNAs is investigated in the current work as part of Publications I and III.

IFNAR stimulation also activates 2'-5'oligoadenylate synthases (OASs) during several RNA virus infections, including SINV (296). OASs are activated by dsRNA and convert ATP substrates to specific adenosine oligomers that serve as signalling molecules for the activation of RNase L, that subsequently degrades ssRNA and dsRNA substrates (297). Although they are prominently upregulated during induction of ISGs, OAS proteins are also constitutively expressed and can function as PRRs, to subsequently activate RNase L, which can then supply RIG-I and MDA5 with ligands to activate type I IFN signalling (297–299). More specifically, OAS3 has been associated with RNase L activation during SINV infection, and SINV replicates to significantly higher titres in OAS3-deficient cells (296). Furthermore, OAS3 activity has been shown to restrict CHIKV and SFV replication; however, this effect appears to be RNase L-independent, suggesting additional mechanisms for OAS3-mediated alphavirus inhibition (300).

The interferon-induced proteins with tetratricopeptide repeats (IFIT) family of ISGs includes two members, IFIT1 and IFIT3, that have been associated with inhibition of alphavirus protein translation (301, 302). IFIT1 is a cytosolic protein that detects capped RNA lacking 2'-O-methylation, i.e., alphavirus positive-strand RNAs containing a 5'-cap-0 structure (303). There is also evidence that IFIT1 recognizes uncapped 5'-ppp RNA and recruits IFIT2 and IFIT3 to form a translation-inhibitory complex during RNA virus replication (304). IFIT complexes function to inhibit translation initiation through interactions with eIF3, thereby excluding it from translation initiation complexes (303). IFIT1 has been shown to negatively impact translation of alphavirus proteins, and this effect is significantly enhanced when cells are primed by IFN treatment (302). Altering the third G nucleotide to A in VEEV genome (a key attenuation in VEEV vaccine strain named TC-83) renders the virus more susceptible to IFIT1 recognition, resulting in attenuation both *in vivo* and *in vitro*; furthermore, IFIT1^{-/-} mice succumb to VEEV infection regardless of the nucleotide in this position. These

results suggest that the potential resistance to IFIT1 recognition is mediated by differences in the secondary structures within the 5' UTR (305). Additionally, another report showed that the 5' UTRs of SINV, VEEV, SFV, and CHIKV are less susceptible to IFIT1 targeting, although to varying efficiency, due to natural differences in the structure and presence of stem loops in this region, suggested to inhibit IFIT1 interaction with the 5'-cap-0 structure. Interestingly, elevated levels of IFIT1 make these IFIT1-resistant alphaviruses more potent IFN inducers, suggesting that in such cases, IFIT1 may act as an innate immune response inducer, rather than a direct antiviral effector (302). These findings provide evidence that certain alphaviruses have evolved to evade IFIT1-mediated translation inhibition by the inclusion of such stem-loops in the 5' UTR, however, this may come at a certain cost towards further type I IFN induction.

To conclude, there is no shortage of evidence demonstrating the critical role of the type I IFN response in controlling alphavirus infection, as has been repeatedly evidenced directly by the high mortality in IFNAR-deficient mice. However, alphaviruses do not elicit uniform immune responses, suggesting that the PAMP profile varies between different alphavirus species and/or they exhibit different strategies for antagonizing host IFN responses.

2. AIMS OF THE STUDY

In general, the study focused on a less investigated alphavirus, as well as on less explored aspects of alphavirus molecular biology, with the aim of advancing the knowledge of alphavirus-host interactions and the unique strategies that some members of the genus employ during infection. A large portion of the work was focused on BFV, a medically important alphavirus in Australia. BFV is not closely related to other known alphaviruses, and its molecular virology remains poorly understood, partly because of the lack of specific molecular tools. However, understanding the mechanisms of pathogenesis and molecular characteristics of each virus is prerequisite for the successful development of therapeutic and vaccine strategies. Therefore, part of this study focused on developing molecular tools that enable investigation of BFV infection using a reverse genetics platform. The investigation also addressed the ability of BFV to produce PAMP RNA in the absence of viral template RNAs. The latter part of the dissertation examined in greater detail this less investigated phenomenon accompanying alphavirus replication. The overall aim was to uncover the characteristics of alphavirus replicases in utilizing cellular RNAs, resulting in activating the innate immune response.

The following objectives were pursued in the thesis:

1. To construct a correct BFV icDNA clone and to validate the *in vitro* and *in vivo* properties of the clone-derived virus in comparison with the parental natural isolate, in order to use the viral clone in future studies.
2. To investigate the significance of a replication defect caused by an erroneous database sequence-derived mutation in the nsP4 region, and of a spontaneous compensatory mutation in nsP2.
3. To study the importance and impact of the unique nuclear localization of BFV nsP3 in cultured mammalian and mosquito cell lines, and in a mouse model.
4. To characterize the capacity of alphavirus replicases to synthesize PAMP RNAs in the absence of viral RNA templates in mammalian and mosquito cell lines.
5. To determine possible reasons for variation in replicase activity towards utilizing host-cell RNA as templates.
6. To identify the cellular RNA template(s) utilized by alphavirus replicases by NGS.

3. MATERIALS AND METHODS

The materials and methods used in this study are described at the usual level of detail in the corresponding sections of Publications I, II, and III. Select methods are, however, described further below to facilitate reading the following sections of the dissertation.

***Trans*-replicase assay (Publication I, II, and III)**

Experiments in all three publications included in this study employed the *trans*-replication system. This system takes advantage of the inherent *trans*-activity of alphavirus replicases. Several versions have been developed over the years in our group, leading to the currently used iteration. In simple terms, it employs a two-plasmid platform in which replicase proteins and recombinant viral template RNA are supplied from two separate plasmid constructs and delivered to cells by co-transfection (Fig. 12).

The expression of replicase proteins in this system is designed to rely on cellular machinery. In the mammalian cell version of *trans*-replicase, the complete ns polyprotein ORF is placed under the control of the human cytomegalovirus immediate early promoter (CMV promoter) to produce 5'-capped and polyA-tailed mRNA. The template RNA represents a minigenome designed to express marker proteins in a replicase activity dependent manner. The initial template RNA is produced by the host cell using *Homo sapiens* RNA polymerase I promoter (HSPolI), generating uncapped transcripts to minimize background translation. The template RNA retains all necessary *cis*-acting elements to ensure its utilization by the replicase as a genuine viral genome: the complete viral 5' UTR followed by the first 231–330 nucleotides of the nsP1 region (length depends on the specific viral genome), the full sg promoter, the final 110 nucleotides of the 3' UTR, and a 60-nucleotide-long polyA-tail. However, it is devoid of viral genes as ORF1 and ORF2 in the minigenome encode for firefly luciferase (Fluc) and *Gaussia* luciferase (Gluc), respectively. To adapt the system for use in mosquito cells, the replicase expression construct contains the *Aedes aegypti* polyubiquitin promoter (Ubi) instead of the CMV promoter, while template RNA transcription is placed under the control of the RNA polymerase I promoter of *Aedes albopictus* (AlbPolI) (Fig. 12).

Overall, the architecture of this system is designed to uncouple the production of replicases from their activity in replicating template RNAs. This provides a means to study alphavirus replication outside the context of viral infection, making also the system suitable for use in low-biosafety conditions. Importantly, the system is genetically stable, which facilitates determination of the significance of distinct ns protein elements specifically for RNA replication, without the risk of reversions or compensatory mutations that could otherwise rescue a detrimental phenotype.

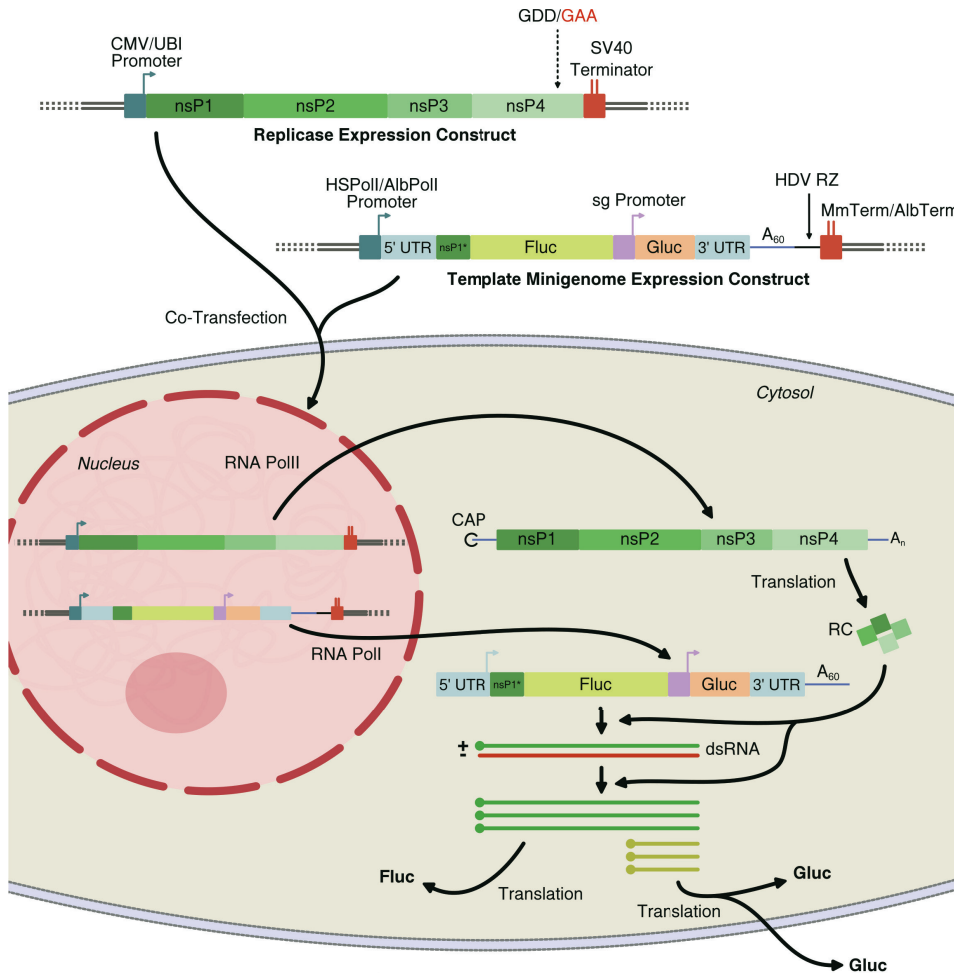


Figure 12. Components and principle of the *trans*-replication assay. The replicase expression constructs contain the following elements: CMV Promoter – cytomegalovirus immediate early promoter (for use in mammalian cell lines) or UBI Promoter – *Aedes aegypti* ubiquitin promoter (for use in mosquito cell lines), GDD/GAA – indicates either active (GDD) or inactive (GAA) viral RdRp catalytic motif, SV40 Terminator – late polyadenylation sequence of simian virus 40. The template minigenome expression constructs consisting of the following: HSPolI Promoter – *Homo sapiens* RNA polymerase I promoter (for use in mammalian cell lines) or AlbPolI Promoter – *Aedes albopictus* RNA polymerase I promoter (for use in mosquito cell lines), 5' UTR – complete 5' UTR of a specific alphavirus, nsP1* – the first 231 to 330 nucleotides of the nsP1 encoding region (length depending on viral species), sg Promoter – subgenomic promoter of a specific alphavirus, 3' UTR – the final 110 nucleotides of the 3' UTR of a specific alphavirus, A₆₀ – 60-nucleotide-long polyA-tail sequence, HDV RZ – hepatitis delta virus ribozyme, MmTer – *Mus musculus* terminator for RNA polymerase I (for use in mammalian cell lines) or AlbTer – *Aedes albopictus* terminator for RNA polymerase I (for use in mosquito cell lines). In the assay, cells are co-transfected with the replicase and template RNA expression plasmids. RNA polymerase II transcribes the replicase expression mRNAs from CMV or Ubi promoters, which are subsequently transported to the cytoplasm for

translation. The uncapped template minigenome is provided by the activity of RNA polymerase I. The minigenome is targeted by the viral replicase to produce negative-strand copies which in turn act as templates to produce full-length capped copies of the minigenome and shorter capped sgRNAs. Firefly luciferase (Fluc) is translated from the full-length minigenome copies and *Gaussia* luciferase (Gluc) from the sgRNA.

In the experiments presented here, murine or human cells were transfected with CMV promoter-based replicase expression constructs designated, for example, CMV-P1234-BFV, together with template RNA constructs HSPolI-FG-BFV (FG indicating Fluc and Gluc). For experiments in mosquito cells, the corresponding constructs were Ubi-P1234-BFV and AlbPolI-FG-BFV.

Replicase activity follows the principles of alphavirus RNA amplification. First, the host-transcribed uncapped template RNA is converted into negative-strand RNA through recognition of the 5' and 3' UTR sequences. Subsequently, full-length 5'-capped complementary RNAs are produced through recognition of the sequence complementary to the 5' UTR, while shorter transcripts are synthesized from the sg promoter. To distinguish promoter usage in this system, 5' UTR-dependent RNA synthesis was designated viral RNA replication, representing genomic RNA synthesis, whereas sg promoter utilization was designated viral RNA transcription, representing sgRNA synthesis. In this context, viral RNA replication results in the expression of the Fluc marker, whereas viral RNA transcription results in the expression of the Gluc marker (Fig. 12). The activity of either can be assessed from transfected cell lysates by luciferase assay.

To distinguish replicase-amplified signal from background activity, negative control samples used HSPolI-FG or AlbPolI-FG constructs together with replicase expression constructs containing an inactive nsP4, generated by a GDD-to-GAA mutation in the RdRp catalytic motif. This allows analysis of raw luciferase activity by comparing direct luminescence signals of different *trans*-replicases, recorded as relative light units (RLU), or by expressing activities as a “boosted” signal ratio over the RdRp-negative controls; that is, the normalized fold change of CMV-P1234-BFV + HSPolI-FG-BFV luciferase activity relative to CMV-P1234-BFV^{GAA} + HSPolI-FG-BFV activity (or other combinations of WT and GAA replicases). Because Gluc is also secreted from cells after translation, cell culture medium samples can also be collected easily at different time points to generate kinetics curves for *trans*-replication.

rPAMP production and detection assay (Publications I and III)

Previous work in our group showed that the *trans*-activity of alphavirus replicases can extend beyond the use of viral RNA templates. Initially, expression of CMV-P1234-SFV alone was used as a negative control when assessing type I IFN response activation in *trans*-replication experiments. However, transfection of the replicase expression construct in the absence of a viral template RNA-producing construct also resulted in significant IFN- β induction. This observation led to further studies demonstrating that alphavirus replicases can utilize host RNAs as templates to produce PAMP RNAs that are sensed by cytosolic PRRs

(295). To distinguish these molecules from classical PAMP RNAs associated with positive-sense RNA virus infection, these replicase-generated PAMP RNAs were termed rPAMPs in Publication III and in the present study.

Previously, rPAMP synthesis had been assessed by measuring IFN- β secreted directly from COP5 cells expressing P1234. For pilot experiments and assay optimization, we also tested direct P1234 expression in COP5 cells, different time points for rPAMP production, different time points for IFN- β sample collection, and compared a column-based RNA purification method with TRIzol reagent-based RNA isolation. Overall, the time point of 48 hours post transfection (hpt) for rPAMP production and for type I IFN induction were selected, as these conditions produced the strongest signals and provided confidence in detecting rPAMP signals even from replicases with lower activity. However, obtained results indicated a possible bias arising from the overall activity of replicases in COP5 cells, as well as differential antagonism of host transcription mediated by ns proteins of some alphaviruses but not others.

To overcome these issues, we developed a novel two-step approach. First, one cell line was transfected with plasmids encoding alphavirus ns polyproteins (e.g., CMV-P1234-SFV), thereby serving as the rPAMP production system. Total RNA was then isolated and subsequently transfected into type I IFN response-competent cells. These recipient cells functioned as rPAMP sensors, providing biological evidence of rPAMP presence and potency through measurement of secreted IFN- β in the cell culture medium by ELISA (Fig. 13). This strategy effectively decouples the generation and confirmation of immunostimulatory RNA from any potential host antagonism mediated by alphavirus ns proteins, which may vary between virus species. It therefore allows to assess whether the type I IFN response is induced specifically by RNA generated in the initially transfected cells. For robust evaluation of replicase activity in rPAMP production, HEK293T were selected as the human cell model, as our *trans*-replication experiments had shown relatively high and comparable activity across most alphavirus systems tested in this cell line. COP5 cells were used as the type I IFN-competent sensor cell line. However, the system would permit the use of essentially any cell line suitable for *trans*-replication assays as the rPAMP production platform. Accordingly, Ubi promoter-based replicase expression constructs were also used to assess the breadth of rPAMP production in the mosquito-derived *Aedes albopictus* C6/36 cell line.

In addition, we evaluated different methods for rPAMP purification. Column-based RNA purification rendered most replicases apparently rPAMP-negative, producing lower IFN induction signals than their RdRp-inactive GAA counterparts. This suggested that column purification may result in the loss of certain RNA species. In contrast, TRIzol purification provided a more complete recovery of total RNA and was therefore used in all subsequent experiments.

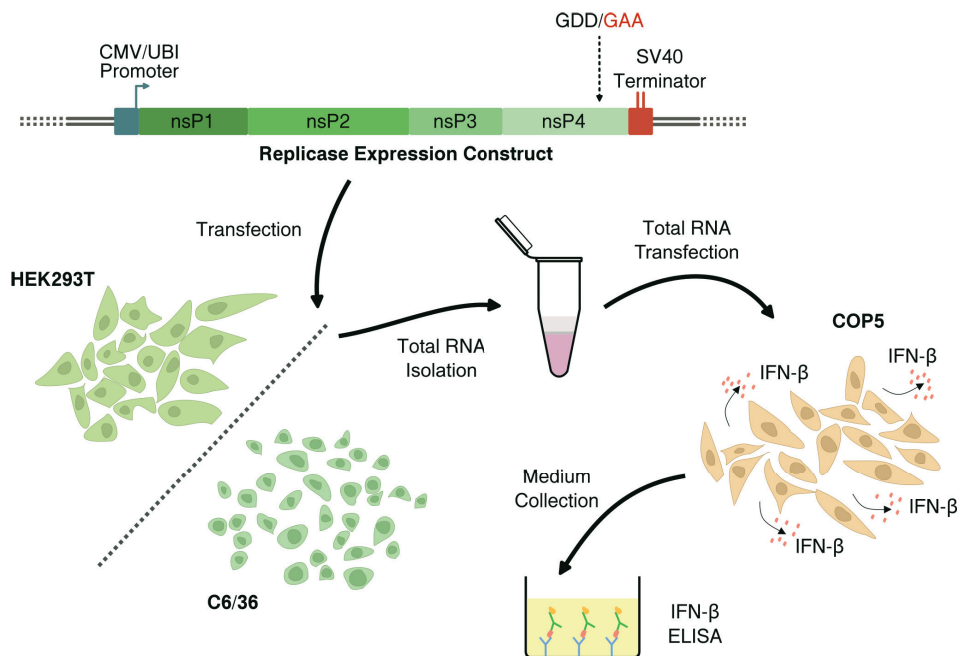


Figure 13. Experimental workflow for the two-step rPAMP production and detection assay. Replicase expression construct elements are as indicated for Fig. 12. HEK293T or C6/36 cells were transfected either with the CMV promoter or Ubi promoter driven replicase expression plasmids, respectively. After 48 hpt, the cells were lysed for RNA isolation. The total RNA fraction was then used to transfect COP5 cells and after 48 hpt, the cell culture medium was collected and analysed for the presence of mouse IFN- β by ELISA.

Mouse BFV infection disease scoring (Publication I)

The *in vivo* BFV infection mouse model used in this study was previously developed (63), and was based on the earlier established C57BL/6 model for RRV (306). It follows the same subcutaneous route of administration and uses the same parameters for visual assessment of disease symptoms and assignment of clinical scores, which are presented below in Table 1.

Table 1. Clinical score evaluation chart for BFV infection in C57BL/6 mice.

CLINICAL SCORE	DESCRIPTION OF SIGNS	INTERVENTION STEPS
0	no disease signs	None
1	ruffled fur	None
2	very mild hind limb weakness, ruffled fur, some lethargy	None
3	mild hind limb weakness, starting to show signs of delicate walking, increase in lethargy	None
4	moderate hind limb weakness and mild dragging of hind limbs, shows limited gripping ability	Wet food and gel pack added
5	severe hind limb weakness/dragging, limited movement & stride of hind legs when walking, moderately lethargic, $\leq 15\%$ weight loss (monitored closely for weight loss).	Wet food and gel pack added; twice daily monitoring
6	Immediate humane end point (euthanasia).	Humane end point (euthanasia)

4. RESULTS AND DISCUSSION

4.1. Construction and explorative use of BFV molecular tools to further characterize a genetically distinct alphavirus (Publication I)

Along with illnesses caused by two other alphaviruses, RRV and CHIKV, BFV disease and infection are listed as nationally notifiable in its native Australia, indicating significant prevalence and clinical importance in the region. Consequently, most research on BFV has been focused on virus epidemiology and clinical disease, often in comparison with RRV. Earlier laboratory studies determined the genomic sequence of the prototype strain field isolate BH2193 (hereafter referred to as BFV2193-FI, corresponding to the nomenclature used in Publication I) and described BFV as phylogenetically distinct within the genus *Alphavirus* (1, 40). Another Australian research group led by Suresh Mahalingam developed a mouse model to study BFV pathology *in vivo*, testing several natural isolates and determining that the BFV2193-FI prototype exhibited the highest virulence (63). In contrast, the molecular virology of BFV has remained relatively poorly studied. Relying on intragenomic similarities with other arthritogenic alphaviruses to draw firm conclusions regarding viral replication and host interaction landscapes carries the risk of overlooking unique properties of this genetically distinct member of the genus. In part, this has been due to the lack of molecular tools, such as a BFV icDNA clone, a gap that our research group sought to address. The project saw us collaborating with Suresh Mahalingam and his team, which also enabled me to briefly join their laboratory for further *in vivo* experimentation with BFV. Importantly, this collaboration provided access to the BFV2193-FI natural isolate for comparative cell culture and animal experiments and, in addition, made it possible to determine the precise genomic sequence of the prototype strain in order to generate a correct BFV icDNA clone for future studies.

4.1.1. BFV icDNA construction and phenotype validation

When construction of the BFV icDNA clone began, only one nearly complete BFV2193-FI genome sequence was publicly available (GenBank: U73745.1). This sequence required correction of a single missing residue in the isolate's 3' UTR (correct sequence was obtained from GenBank: AF339488.1). Assembly of the icDNA clone is summarized in Fig. 1A of Publication I, in which five synthetic DNA fragments were joined to generate the construct. However, the infectivity of RNA transcribed from the assembled clone was very low (~100 plaque forming units/μg, PFU/μg). Although an initial stock was eventually obtained, rescue of the P0 virus took unexpectedly long. Sequencing of the P1 stock revealed a Thr-to-Pro substitution at position 792 of nsP2 (corresponding to residue 1325 in

P1234; hereafter referred to as T1325P). Introduction of this substitution into the original icDNA clone increased RNA infectivity nearly 100-fold.

Interestingly, the T1325P substitution lies at the P7 position of the 2/3 cleavage site, a region previously described as a hotspot for compensatory mutations arising in response to defects in ns proteins (307). This suggested that the substitution represented an adaptation compensating for suboptimal replicase activity in virus rescued from the original clone. We therefore re-sequenced BFV2193-FI and identified three non-synonymous differences relative to the GenBank sequence: nsP4-V1911D, E2-H383N, and E1-A826G (a full list of differences, including synonymous changes, is provided in Table 1, Publication I). Because the nsP4 substitution was the most likely cause of the replication defect, it was reverted in the icDNA clone. This increased transcript infectivity to $\sim 3 \times 10^4$ PFU/ μ g, which is comparable with that of transcripts generated from icDNAs of other alpha-viruses. This indicated that the database error was indeed responsible for the low rescue efficiency and the selection of a potential compensatory mutation. This corrected clone was designated as pSP6-BFV-IC, the original clone as pSP6-BFV^{V1911D}, and the adapted clone harbouring the compensatory mutation as pSP6-BFV^{T1325P+V1911D} (Fig. 1B, Publication I). In conclusion, although the assembly of icDNA clones from synthetic DNA has become increasingly accessible, success still depends on the accuracy of reference genome sequences.

Taking advantage of our access to the original field isolate, we compared the phenotype of the virus derived from the corrected clone (BFV-IC) with that of BFV2193-FI. In Vero cells, BFV2193-FI showed a slight growth advantage (Fig. 2A, Publication I), and a similar trend was observed at early time points in IFNAR^{-/-} MEFs (Fig. 2B, Publication I). Importantly, the growth curves of BFV2193-FI and BFV-IC were nearly identical in type I IFN-competent WT MEFs (Fig. 2C, Publication I). Furthermore, growth kinetics differed markedly between the two MEF cell types: peak titres were reached by 12 h post infection (p.i.) in IFNAR^{-/-} MEFs but only by 24 h p.i. in WT MEFs, indicating a restrictive effect of the type I IFN response on BFV replication.

Overall, BFV2193-FI and BFV-IC displayed nearly identical growth phenotypes in cultured cells. Using the established BFV mouse model (63), we also observed highly similar disease scores for both viruses. Peak disease occurred at 10 days p.i., and symptoms resolved by day 14 (Fig. 3A, Publication I). No effect on weight gain was observed in the juvenile mice in any experimental group (Fig. 3B, Publication I). Serum and tissue titres (Fig. 3C–G, Publication I), as well as muscle tissue pathology (Fig. 3F, Publication I), were likewise comparable between BFV2193-FI and BFV-IC. Together, these results confirm successful construction of a WT BFV icDNA clone that faithfully reproduces the phenotype of the natural isolate.

4.1.2. Functional impact of the sequence error in nsP4 is rescued by a second-site mutation and results in attenuated phenotype

RNA transcripts from the original pSP6-BFV^{V1911D} clone exhibited extremely low infectivity, and rescue attempts instead yielded BFV^{T1325P+V1911D}. This suggested that the spontaneously arising nsP2-T1325P substitution acted as a second-site compensatory mutation for the nsP4-V1911D defect. To investigate this hypothesis, a third clone, pSP6-BFV^{T1325P} was generated and included in the subsequent analyses.

Because the nsP2-T1325P substitution lies near the 2/3 cleavage site, we hypothesized that it modifies the ns polyprotein processing rate. *In vitro* translation assays revealed that the mutation slows processing: elevated levels of the P1234 precursor were detected after a 30-min chase period in the case of BFV^{T1325P} and BFV^{T1325P+V1911D} (Fig. 9C, Publication I). As mutations within this region have been previously associated with increased sensitivity to type I IFN and reduced viral replication (185, 308), we compared the growth kinetics of BFV^{T1325P} and BFV^{T1325P+V1911D} with those of WT virus. In IFNAR^{-/-} MEFs, both mutants replicated more slowly than BFV-IC at early time points up to 12 h p.i.; thereafter, BFV^{T1325P} titres exceeded those of the double mutant (Fig. 4A, Publication I). In WT MEFs, BFV^{T1325P} was mildly attenuated at 6 and 9 h p.i., whereas BFV^{T1325P+V1911D} exhibited significantly reduced growth from 6 h p.i. onward compared with BFV-IC (Fig. 4B, Publication I). Thus, the nsP2-T1325P substitution alone had only a modest effect on BFV replication in murine cells, whereas the combination of T1325P+V1911D markedly increases sensitivity to the type I IFN response. Although the adaptation arose in mammalian cells (BHK-21), we also tested whether these substitutions affect the viral phenotype in mosquito cells. In *Aedes aegypti* derived AF319 cells, BFV^{T1325P+V1911D} replicated similarly to BFV-IC, whereas the nsP2-T1325P mutation alone caused attenuation throughout infection (Fig. 4C, Publication I). In *Aedes albopictus* derived C6/36 cells, both mutants replicated more slowly than BFV-IC: titres of BFV^{T1325P+V1911D} were significantly lower from 12 h p.i. onward, while BFV^{T1325P} showed reduced replication throughout the experiment (Fig. 4D, Publication I). Thus, nsP2-T1325P appears better tolerated in mosquito cells when compensating for nsP4-V1911D than when present alone.

The corrected icDNA clone also enabled construction of a BFV *trans*-replicase platform, that was used in our group to study alphavirus RNA replication (259, 309). Here, the *trans*-replicase assay was used to compare RNA synthesis by BFV-IC-Rep, BFV^{T1325P}-Rep, BFV^{V1911D}-Rep, and BFV^{T1325P+V1911D}-Rep. The *trans*-replicases were also analysed by northern blot (NB) to further characterize viral RNA synthesis profiles. As expected, the nsP4-V1911D mutation alone drastically reduced replicase activity (Fig. 9A, 9B, Publication I), and the impaired production of viral RNAs was confirmed by NB analysis (Fig. 14). In contrast, BFV^{T1325P+V1911D}-Rep performed similarly to BFV-IC-Rep in the *trans*-replicase assay and even produced slightly higher Fluc signals (Fig. 9A, 9B, Publication I). NB results mirrored this observation, showing slightly increased

levels of both viral positive- and negative-sense RNA (Fig. 14). Interestingly, the replicase containing only the nsP2-T1325P mutation was significantly more active than both BFV-IC-Rep and BFV^{T1325P+V1911D}-Rep (Fig. 9A, 9B, Publication I; Fig. 14). These results indicate that the replicase encoded by BFV^{V1911D} was capable of viral RNA amplification at a critical threshold sufficient to allow emergence of adaptative mutations, and that the combination of the two substitutions effectively cancelled out each-other, producing a replicase with similar activity to that produced by WT virus. Together, these findings strongly support the hypothesis that nsP2-T1325P arose as a compensatory mutation for the nsP4-V1911D defect.

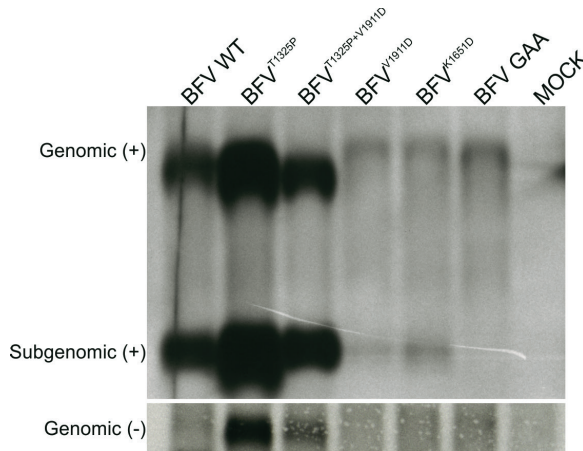


Figure 14. Viral RNA synthesis profiles of BFV replicases. HEK293T cells were co-transfected with HSPoll-FG-BFV and either with BFV-WT-Rep, BFV^{T1325P}-Rep, BFV^{T1325P+V1911D}-Rep, BFV^{V1911D}, BFV-GAA-Rep expression constructs, or mock transfected. At 18 hpt, total RNA was isolated and assayed by NB using a probe complementary to the Fluc reporter gene to detect negative strands (Genomic [-]) or a probe complementary to the Gluc reporter gene to detect positive strands (Genomic [+], Subgenomic [+]).

In conclusion, the accidental nsP4-V1911D substitution proved informative for studying alphavirus replication. The affected residue lies between the 97 amino acid alphavirus-specific nsP4 NTD and the conserved RdRp core, which begins near residue 150 (262, 310). To our knowledge, the function of this intervening region has not been previously characterized in any alphavirus and came to attention here by chance. Using the genetically stable *trans*-replicase system, we confirmed that nsP4-V1911D severely reduces replicase activity. The *trans*-replication results further demonstrated that nsP2-T1325P compensates for the nsP4 defect and, when present alone, increases replicase activity beyond WT levels. This suggests a functional link between residue 792 in nsP2 (T1325P) and residue 110 in nsP4 (V1911D) of BFV.

Functional interactions between nsP2 and nsP4 have been reported previously. For example, repeated passaging of CHIKV containing a mutation in the nsP4 RdRp catalytic domain (C483Y) in the presence of mutagens resulted in the

emergence of a companion nsP2 mutation (G641D) that further increased replicase fidelity (311). In another study, impaired sgRNA synthesis caused by an nsP4 NTD mutation (D41A) in SINV was restored by a second-site compensation in the nsP2 helicase domain (M282L) (312).

Further studies will be required to clarify the functional role of this nsP4 region in replicase assembly. However, the severe defect caused by the V1911D substitution indicates that this position likely requires Val or another residue with similar physicochemical properties. Indeed, sequence analysis shows that this position is occupied by a hydrophobic residue in most alphavirus nsP4s (Fig. 15A). Consistent with this, our corrective NGS revealed that BFV2193-FI encodes Val at this position, whereas sequences of more recent isolates available in databases encode Ile instead (Data S2, Publication I). In the available cryo-EM structure of the mature alphavirus core RC (136), the corresponding residue in ONNV nsP4 (Ile111) does not directly interact with nsP2, but instead forms hydrophobic contacts with one of the nsP1 monomers (Fig. 15B and C). Several hydrophobic patches are present between nsP4 and the nsP1 dodecameric ring in the core RC structure, and a substantial portion of these lies opposite the molecular channel, including the Ile111 region (Fig. 15B and C). Substitution with the hydrophilic Asp would likely disrupt these interactions and destabilize the RC. This suggests that nsP4-V1911D substitution interferes with optimal replicase assembly. Although functional early RCs can still form, as indicated by the *trans*-replicase NB data (Fig. 14), this likely occurs with very low efficiency, allowing for the emergence of viruses harbouring compensatory mutations that rapidly out-compete the original (nsP4-V1911D) variant. As most alphaviruses encode Val, Ile, or Leu at this nsP4 position, it would be valuable to examine whether the requirement for a hydrophobic residue at this site is broadly conserved and whether this region could represent a potential antiviral target for disrupting replicase assembly.

Compensation for the reduced RNA synthesis caused by nsP4-V1911D appears to occur through modulation of ns polyprotein processing, as the suppressor mutation changes the P7 residue near the 2/3 cleavage site from Thr to the rigid amino acid Pro, thereby slowing the cleavage of P1234 (Fig. 9C, Publication I). This delay may allow greater accumulation of P1234 precursor molecules, increasing the probability of successful early RC assembly. Such a mechanism may also help achieve an appropriate stoichiometry of nsP4, the availability of which is already limited by the opal stop codon at the end of BFV nsP3 region (40). In addition, failure to incorporate nsP4 into the RC would result in its degradation (255). Alternatively, or in parallel, the Pro substitution near the 2/3 cleavage site may delay maturation of the RC and prolong the early replicase stage capable of negative-strand RNA synthesis. This would provide additional time for negative-strand synthesis, given the hypothesized hindrance of the V1911D mutation towards achieving proper conformational integrity. Slower precursor processing would also delay the release of free nsP2 capable of antagonizing host transcription and translation, potentially contributing to the additional attenuation observed for BFV^{T1325P} and BFV^{T1325P+V1911D} in WT MEFs

(Fig. 4B, Publication I). Because the initial rescue of the adapted virus occurred in BHK-21 cells, it would be interesting to determine whether alternative strategies would be employed if the original pSP6-BFV^{V1911D} were rescued in cells with a complete type I IFN response.

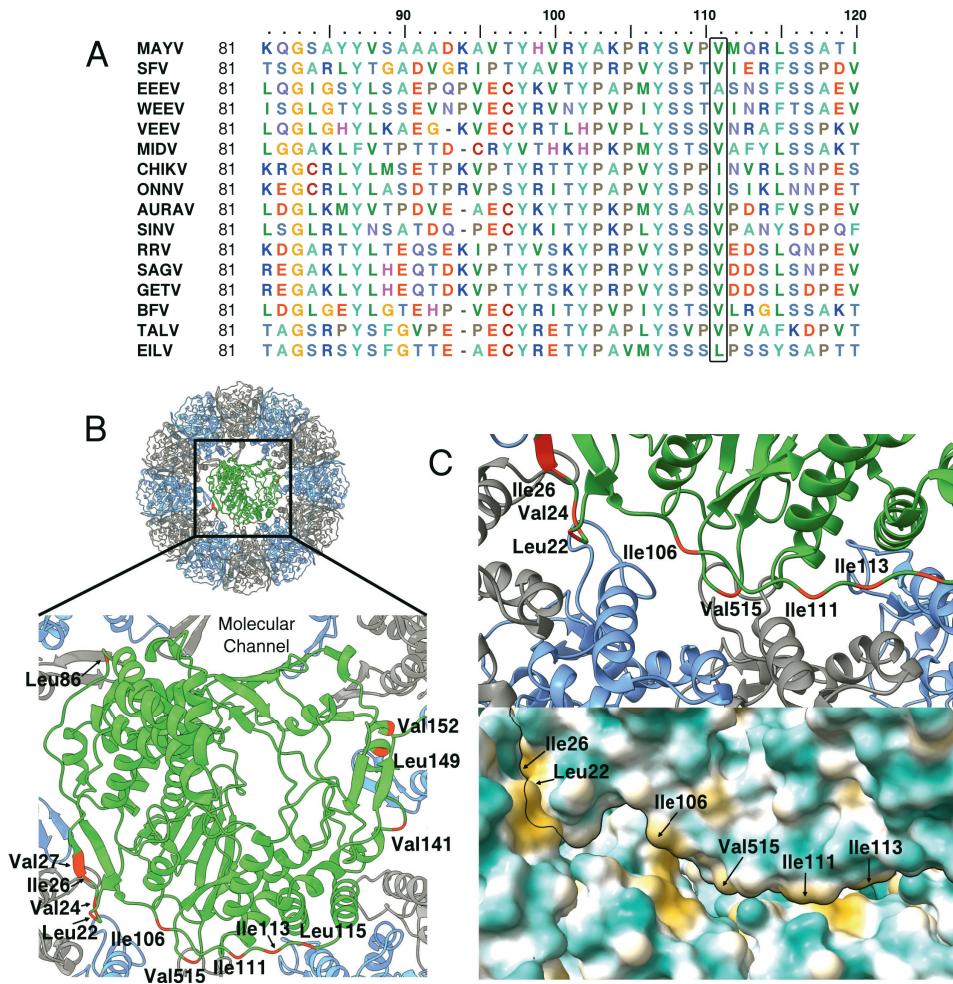


Figure 15. Hydrophobic interaction of Ile111 of ONNV nsP4 with nsP1 in the mature RC core complex. (A) Sequence alignment of several alphavirus nsP4s; MIDV – Middelburg virus, *Alphavirus middelburg*, AURAV – Aura virus, *Alphavirus aura*, SAGV – Sagiya virus. The position of the BFV nsP4-V1911D substitution is outlined and corresponds to Ile111 of ONNV nsP4. (B) Ribbon representation of the core RC complex; nsP1 monomers are in blue and gray, nsP4 in green. Closeup showing hydrophobic residues of nsP4 (in red) observed to form hydrophobic interfaces with nsP1 copies. (C) (Top) A stretch of nsP4 residues (including Ile111) seen to form hydrophobic interfaces with nsP1 monomers. (Bottom) A hydrophobic surface representation of the same region as top, nsP4 residues indicated; hydrophilic-to-hydrophobic range depicted from cyan-white-golden. Figure was compiled using PDB ID: 7Y38 as published by (136).

4.1.3. BFV nsP3 contains a functional nuclear localization signal

The subcellular localization of individual BFV nsPs was first analysed roughly a decade ago as part of my BSc project. Expression constructs were generated to produce nsP1-nsP4 fused to N-terminal EGFP in mammalian cells (Fig. 6B, Publication I). Confocal microscopy revealed one unexpected observation: BFV EGFP-nsP3 localized to both the cytoplasm and the nuclei of U2OS cells (Fig. 6B, Publication I). At the time, nuclear localization of alphavirus nsP3 had not been reported. Only some years later, Nowee et al. demonstrated that nsP3 proteins from several alphaviruses, such as ONNV, BEBV, SINV, TALV, and WHATV, also exhibit nuclear localization when expressed as EGFP fusions (246). However, the mechanism behind, and the importance of, nuclear localization of nsP3 had not been examined during *bona fide* viral infection.

Alphavirus nsP3 tolerates substantial insertions within the HVD without severely affecting viral fitness, and marker protein insertions within this region do not alter subcellular localization (229, 245). To examine BFV nsP3 localization during infection, the mCherry coding sequence was inserted into the HVD region, generating pSP6-BFV-P3mCh and the corresponding virus BFV-P3mCh (Fig. 5A, Publication I). It was observed that, like its counterparts from other alphaviruses, the HVD of BFV nsP3 tolerates large insertions without severely compromising viral replication, thereby enabling straightforward visualization of nsP3 in infected cells. In BHK-21 cells infected with BFV-P3mCh, nsP3-mCherry formed cytoplasmic foci and was also prominently detected in the nucleus (Fig. 5B, Publication I) confirming the nuclear presence of this protein in mammalian cells in the context of authentic viral infection. Similarly, nuclear nsP3 was observed in Vero cells, where nsP3 formed more concentrated structures (Fig. 5D, Publication I), frequently displaying a rod-like morphology (Movie S1, Publication I). Nuclear localization was also confirmed in WT MEFs, where higher-resolution imaging revealed rod-like or fibrous nsP3-mCherry structures (Fig. S1, Publication I). In contrast, nsP3 remained cytoplasmic in infected mosquito cells (Fig. 5C, Publication I), suggesting that BFV nsP3 localization and functions may differ between mammalian and insect cells. Host-specific differences in nuclear trafficking are consistent with observations made for other alphavirus proteins. In mammalian cells, nuclear translocation of nsP2 and CP contributes to antagonism of host transcription and translation, thereby promoting productive infection and often leading to cell death. In contrast, these proteins typically remain cytoplasmic during infection of mosquito cells. For example, CP of CHIKV (313), nsP2 of SINV (314), and CP of VEEV (315) have been reported to not enter mosquito cell nuclei. These observations suggest that the nuclear functions of these proteins may be specifically adapted to vertebrate host factors, possibly to avoid interfering with the establishment of persistent infection in mosquito vector hosts.

Alphavirus nsP3 is present in different cytoplasmic complexes during infection, not all of which are associated with viral RNA replication (185, 316). Thus, we examined the spatial relationship between nsP3-mCherry and dsRNA. In Vero

cells, dsRNA foci were primarily located at cell periphery (Fig. 5D, white arrows, Publication I), where nsP3-mCherry did not co-localize. Some internal cytoplasmic nsP3 foci appeared near dsRNA signals, but many lacked detectable dsRNA (Fig. 5D, yellow arrow). These observations resemble previously reported CHIKV nsP3 localization patterns (316) and support the idea that nsP3 participates in multiple functional complexes during infection. In contrast, nsP3-mCherry co-localized with dsRNA in large cytoplasmic vesicles in C6/36 cells (Fig. 5D, magenta arrow, Publication I), alongside dsRNA-negative nsP3 foci (Fig. 5D, cyan arrow, Publication I). Again, such distribution is consistent with previous observations for CHIKV infection in mosquito cells (317), and together suggest that the cytoplasmic nsP3 complexes differ between vertebrate and insect hosts.

In silico analysis identified a putative NLS at the beginning of the HVD of BFV nsP3. Because Lys and Arg residues are essential for nuclear import (318), Lys1651 was mutated to Asp (K1651D) to generate pSP6-BFV^{K1651D} and pSP6-BFV^{K1651D}-P3mCh (Fig. 7A and 6A, Publication I). This substitution abolished the nuclear localization of nsP3-mCherry in infected Vero and WT MEF cells (Fig. 6A, Fig. S1, Movie S2, Publication I). Similarly, EGFP-nsP3^{K1651D} expressed in the absence of viral infection remained cytoplasmic (Fig. 6B, Publication I), confirming that Lys1651 is essential for BFV nsP3 nuclear import.

Nuclear trafficking of alphavirus proteins has previously been described for nsP2 and CP, both of which contribute to suppression of host antiviral responses, including type I IFN signalling (319, 320). Mutations that disrupt nuclear localization of these proteins attenuate viral replication when type I IFN responses are intact (194, 313, 321). To evaluate the significance of BFV nsP3 nuclear localization, we compared the replication of BFV^{K1651D} and BFV-IC.

Replication kinetics of these viruses were near-identical in mosquito cell lines (Fig. 7B and C, Publication I), consistent with the absence of nsP3 nuclear localization in C6/36 and AF319 cells (Fig. 5C, Publication I). In Vero cells, which lack type I IFN signalling, BFV^{K1651D} also replicated to titres comparable to BFV-IC (Fig. 7D, Publication I). In murine fibroblasts, however, BFV^{K1651D} replication was reduced during early infection. Titres were approximately 7-fold lower in IFNAR^{-/-} MEFs at 12 h p.i. (Fig. 7E, Publication I). In WT MEFs, BFV^{K1651D} replication was further attenuated, with approximately 20- and 17-fold lower titres observed at 9 and 12 h p.i., respectively (Fig. 7F, Publication I). Despite this early effect, both viruses reached similar titres by 24 h p.i., indicating that nsP3 nuclear localization facilitates early BFV replication, but does not ultimately affect virion production. Consistent with the defect in early replication, the *trans*-replicase of BFV^{K1651D} was significantly less active than BFV-IC-Rep (Fig. 9A–B, Publication I), and the NB assay revealed that BFV^{K1651D}-Rep was severely impaired in viral RNA synthesis of all types (Fig. 14). As expected, ns polypeptide cleavage was not affected by the disruption of nsP3 NLS (Fig. 9C, Publication I). Furthermore, metabolic labelling revealed that the presence of nuclear nsP3 did not affect shutdown of host translation, whereas CP and viral glycoprotein synthesis signals were slightly weaker in BFV^{K1651D}-infected cells (Fig. 9D–E, Publication I).

Because disruption of nuclear localization of alphavirus proteins often results in virus attenuation *in vivo* (193, 313), and because BFV^{K1651D} replicated more slowly in murine cells, we examined the effect of the absence of nuclear BFV nsP3 in a mouse model. Both BFV-IC and BFV^{K1651D} produced similarly mild disease symptoms, with onset at day 4 p.i., peak disease at day 8, and recovery by day 13 (Fig. 8A, Publication I). Body weight changes were also comparable between all groups (Fig. 8B, Publication I). Despite this, BFV^{K1651D} showed reduced replication. Serum titres were significantly lower at day 1 p.i., and the virus was cleared earlier in some animals compared with BFV-IC (Fig. 8C, Publication I). Tissue titres in hind leg quadriceps and ankles were also reduced (Fig. 8D–E, Publication I), and inflammatory cell infiltration was lower in BFV^{K1651D}-infected mice (Fig. 8F–G, Publication I). Taken together, albeit both viruses caused similarly mild disease symptoms in mice, the absence of nuclear nsP3 is clearly disadvantageous for BFV replication *in vivo*.

Together, these findings suggest that nsP3 nuclear localization enhances BFV replication efficiency but is not strictly required for productive infection under the conditions tested. Importantly, the detectable reduction in viral titres demonstrates that the mouse model is sufficiently sensitive to assess attenuation of BFV variants, which may be valuable when evaluating potential vaccine candidates. The relatively mild disease phenotype observed in mice may also reflect the natural host range of BFV. Although definitive vertebrate hosts remain uncertain, serosurveys have suggested marsupials as potential BFV reservoirs. However, seroprevalence in these animals is generally lower than that observed for RRV (60), implying that additional hosts may contribute to virus circulation. Phylogenetic studies of Australian isolates have also revealed relatively high genomic stability of BFV compared to RRV, leading to the suggestion that avian hosts may play a role in its transmission cycle (62). While this remains to be investigated, examining BFV nsP3 nuclear localization in cell lines derived from macropod or avian hosts would therefore be of particular interest. In such models, nuclear trafficking of nsP3 might confer a more pronounced advantage, and the molecular tools developed here would facilitate such investigations.

The outstanding question remains as to the exact nature of the nuclear function of BFV nsP3 in mammalian cells. Alphavirus nsP3 acts as a hub for interactions with host proteins to support viral replication. Many of these interactions are virus species- or group-specific and are largely mediated by motifs within the HVD (230). Because nsP3 nuclear localization is rare within the genus, studies of nsP3 interactomes have only considered its cytoplasmic presence and functions (236, 237, 322, 323). However, many host proteins that interact with nsP3 or are present in virus replication organelles are nuclear proteins (324, 325), and there are examples of nuclear protein relocation to the cytoplasm acting inhibitory towards alphavirus replication (326). Thus, nsP3 might sequester inhibitory host factors in the nucleus or compete with their normal binding partners, thereby promoting viral replication. Nuclear localization of BFV nsP3 may also influence the redistribution of host proteins supportive towards RC functioning. The observation that BFV^{K1651D} replication was attenuated primarily during early stages of

infection, and that the corresponding replicase displayed very low activity in the *trans*-replicase assay, suggests that a cofactor required for efficient replicase activity may be unavailable when nsP3 cannot enter the nucleus. At present, the interactome of BFV nsP3 has not been characterized; the only host factors known to support BFV replication are G3BP proteins (227). It has been reported for some OW alphaviruses that nsP3 recruits G3BP complexes to facilitate efficient functioning of RCs (226, 327, 328). It is therefore plausible that preventing nuclear entry of nsP3 disrupts the recruitment or redistribution of additional host factors supporting replicase activity. Furthermore, the CPs of SINV, SFV, and CHIKV have occasionally been reported to participate in G3BP-nsP3 complexes (140, 211). Because the *trans*-replication system lacks viral structural proteins, the absence of CP or other structural components – combined with the inability of nsP3 to access the nucleus – may further limit replicase activity in this virus-free system by eliminating additional host-factor interactions. Characterizing the interactome of BFV nsP3 therefore represents an important direction for future work, as it may reveal additional mechanisms by which alphaviruses manipulate host cell biology during infection. It remains to be determined which host factors are targeted by nuclear-localized nsP3, and whether the ultimate effect is unique to BFV or whether other alphaviruses, despite lacking nuclear localization of nsP3, rely on different viral proteins to achieve similar outcomes.

BFV nsP3-mCherry formed clear rod-like or filamentous structures in the nuclei of mammalian cells. Initially, we suspected these structures to arise from interactions with host proteins. However, recent work has shown that mature nsP3 can assemble into oligomeric helical structures (140). Kril et al. demonstrated that the central AUD functions as the nsP3 oligomerization module and that substitution of key residues involved in subunit interactions renders CHIKV non-replicative (140). When expressed independently, or together with the HVD, CHIKV AUD forms highly elongated filaments (140, 210). Our unpublished results show that the AUD or AUD-HVD of BFV nsP3 expressed as EGFP fusions also form characteristic filaments, in the latter case both in the cytoplasm and nucleus (Fig. 16). This strongly suggests that the AUD of BFV likewise mediates oligomerization and that the filamentous localization of BFV nsP3-mCherry likely reflects intrinsic structural properties of the protein rather than interactions with host factors. Given the conservation of the residues involved in nsP3 oligomer formation across alphaviruses (140), this property is likely widespread, or even universal, within the genus and independent of the subcellular localization of the protein.

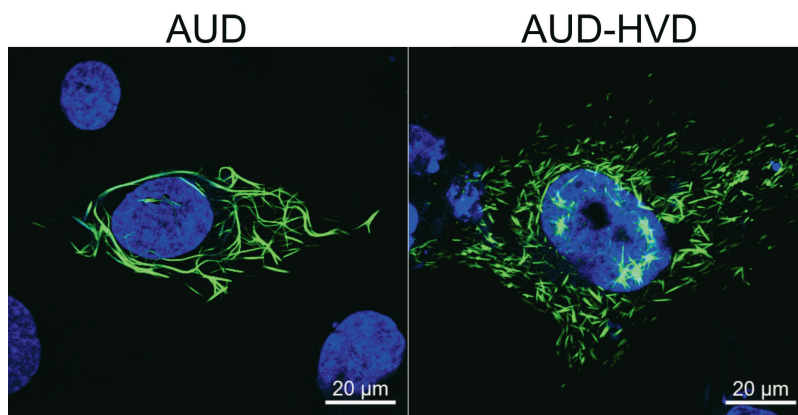


Figure 16. Filamentous localization of individually expressed BFV AUD and AUD-HVD. U2OS cells were transfected with plasmids encoding BFV EGFP-AUD or EGFP-AUD-HVD fusion proteins. Cells were fixed 24 hpt. Nuclei were counterstained with DAPI. Zeiss LSM710 confocal microscope was used for imaging.

4.1.4. BFV replicase uses host templates to produce rPAMPs

The *trans*-replicase system, which allows stand-alone expression of the viral replicase, provides a unique tool to specifically investigate type I IFN induction in the absence of viral RNA templates. Here, we found that the BFV replicase synthesizes rPAMPs in HEK293T cells (Fig. 10A, Publication I). Because the nsP2-T1325P compensatory mutation was shown to slow ns polyprotein processing, it became of particular interest in this context, as slower ns polyprotein processing has previously been linked to enhanced type I IFN response. For example, mutations that slow cleavage at the 1/2 site attenuate CHIKV replication in mice and increase type I IFN production (329). Similarly, delayed 1/2 site cleavage results in RRV attenuation in MEFs and in mice, accompanied by a stronger type I IFN induction. For the latter case, this has also been associated with increased rPAMP production (271). BFV^{T1325P}-Rep was indeed confirmed to be highly active in producing PAMP RNA in the absence of viral templates and, curiously, BFV^{T1325P+V1911D}-Rep was capable of rPAMP synthesis at levels close to WT replicase (Fig. 10A, Publication I). It is likely that the enhanced rPAMP production underlies the attenuation of BFV^{T1325P} in WT MEFs and suggests that increased replicase activity comes at the cost of stronger activation of innate immune responses.

The demonstration that the BFV replicase is capable of rPAMP synthesis increased the number of alphaviruses known to produce rPAMPs to four: SFV (295), RRV and SINV (271), and BFV (Fig. 10A, Publication I), raising the question of how widespread this property is across the alphavirus genus. Addressing this became the focus of the subsequent work presented in the latter part of this dissertation (Publication III).

4.2. Construction and validation of *trans*-replicase systems to expand the toolset for rPAMP synthesis investigation (Publications II and III)

An important advantage of *trans*-replicase systems is the reduced biosafety burden, as they can be employed using standard cell culture methods and plasmid DNA without the need to handle infectious agents. Furthermore, they provide a genetically stable platform for studying alphavirus RNA replication and virus-host interactions. For these purposes, our research group had previously constructed such systems for a number of OW alphaviruses (164, 309). However, this toolset did not fully cover the diversity of the genus, as among the three groups of NW alphaviruses only VEEV was represented, whereas other clinically important and highly pathogenic NW alphaviruses, EEEV and WEEV, were not included. Because both viruses require biosafety level 3 or higher containment, they remain comparatively understudied due to the strict and costly laboratory requirements.

Within this dissertation, Publications II and III represent important steps in expanding the toolkit of alphavirus *trans*-replicase systems, as these platforms were instrumental to the experimental approach used to assess the capability of different alphaviruses to produce rPAMPs. In this study, we assembled both a replicase expression construct and matching viral template RNA expression constructs for EEEV and WEEV for use in human and mosquito cells. The design follows that of our previously developed *trans*-replicase systems and is summarized in Fig. 1A of Publication II.

To enable use of the EEEV and WEEV replicases in rPAMP production experiments, it was necessary to assess their functionality in replicating viral RNA templates. In HEK293T cells, the EEEV *trans*-replicase proved highly active, producing Fluc and Gluc reporter signals at levels comparable to those observed with the more active *trans*-replicases in the panel, including RRV, SINV, MAYV, CHIKV, and SFV (Fig. 1B, Publication II). The EEEV replicase was also capable of utilizing template RNAs from other alphaviruses, showing highest compatibility with VEEV template, while being less efficient at replicating templates derived from members of the Semliki Forest complex (Fig. 2B, Publication II). Likewise, the WEEV *trans*-replicase proved to be highly active in mammalian cells (Fig. 1B–E, Publication III). Thus, the newly developed EEEV and WEEV *trans*-replication systems displayed high activity in human cells, a property relevant for assessing their potential to utilize host RNAs for rPAMP synthesis. Importantly, these systems provide low-biosafety-level tools for studying replicase properties of these highly pathogenic alphaviruses.

4.3. Investigating the breadth of alphavirus rPAMP synthesis and replicase properties associated with increased rPAMP production (Publication III)

Alphavirus RNA replication occurs in the cytoplasm and therefore carries a risk of activating cytosolic RNA-sensing receptors. Among the different viral RNA species produced by the alphavirus replicase, the most likely PAMP is uncapped negative-strand RNA. Although, free negative-strand RNAs have not been detected during alphavirus infection and are instead maintained in a dsRNA form (330), this dsRNA itself also represents a potent PAMP. Partly for this reason, these replication intermediates are concealed within membranous spherules, which provide a physical barrier against host sensors. In addition to dsRNA, alphaviruses have also been shown to synthesize large amounts of uncapped positive-strand RNAs (159) that are released into the cytosol, where they can act as PAMPs. Finally, our group's previous work has shown that expression of the replicase of certain alphaviruses alone is sufficient to stimulate the innate immune response via cytosolic RNA-sensing PRRs (271, 295).

Because it has remained unclear whether viral template RNA-independent replicase activity leading to PRR activation is a general feature across the alphavirus genus, we used our expanded toolkit of replicase expression constructs – typically employed in *trans*-replicase assays – to investigate type I IFN response-inducing rPAMP synthesis across 11 alphaviruses. These viruses represent all major phylogenetic groups of alphaviruses, including the Semliki Forest, Western equine encephalitis, Eastern equine encephalitis, Venezuelan equine encephalitis, and Barmah Forest complexes, as well as ISAVs.

4.3.1. Replicases of all tested alphaviruses produce rPAMPs in human cells and some also in mosquito cells

Previous data on rPAMP synthesis and the resulting IFN- β induction were obtained using P1234 expression in COP5 cells (271, 295). Because some alphaviruses (e.g. ONNV, CHIKV) replicate poorly in murine models, whereas others (e.g. SINV, SFV) replicate efficiently, we evaluated the suitability of the COP5 model for unbiased assessment of the overall activities of the different replicases using the *trans*-replicase assay. Gluc reporter kinetics revealed substantial differences in *trans*-replicase activity in these cells by 48 hpt: SFV *trans*-replicase activity exceeded that of ONNV by approximately 850-fold (Fig. 1B, Publication III). In HEK293T cells, however, absolute and normalized Fluc and Gluc activities differed (with the exception of EILV) by no more than approximately 50-fold (Fig. 1C–D, Publication III). This agrees with our previous reports (309; Fig 1B, Publication II) and further indicates that HEK293T cells are better suited for assessing robust alphavirus replicase activity.

However, HEK293T cells are unsuitable for direct measurement of type I IFN induction because the pathway is not fully functional (331). In addition, our

replicase panel includes alphaviruses that antagonize type I IFN responses via either nsP2 or CP. Thus, direct P1234 expression combined with robust replicase activity could introduce additional bias in type I IFN induction assays, as previously demonstrated with SINV, CHIKV, EEEV, and VEEV replicons (332). Indeed, in initial experiments in which we transfected COP5 cells directly with the replicase expression constructs, it was observed that SINV produced significantly lower amounts of IFN that would be predicted from Gluc reporter activities. EEEV, on the other hand, appeared to be among the strongest inducers of IFN- β production, whereas no IFN expression was detected for the replicases of CHIKV and ONNV (Fig. 17). These data suggest major bias arising from different modes of antagonizing IFN production: mediated either by nsP2 (present as part of the replicase) or by CP (absent in our system). Therefore, we adopted a novel two-step approach: HEK293T cells were used solely for rPAMP production (via P1234 expression), after which total RNA was isolated and transfected into COP5 cells to assess its ability to induce IFN- β production (Fig. 2A, Publication III).

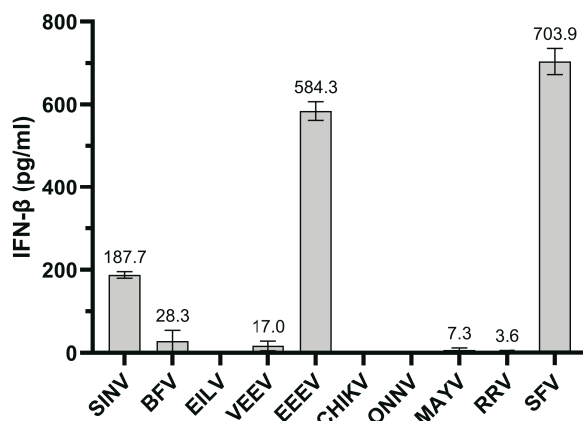


Figure 17. IFN- β induction in COP5 cells transfected with alphavirus replicase expression constructs. COP5 cells were transfected with P1234 expression constructs of indicated alphaviruses. Secreted IFN- β was measured from cell culture medium at 48 hpt. Data presented as amounts of measured IFN- β induced by WT replicases (CMV-P1234) with the corresponding polymerase-inactive replicase (CMV-P1234^{GAA}) induced amount value subtracted. Columns represent mean values (shown as numeric values) with SD from two independent experiments.

RNA extracted from HEK293T cells expressing SFV or SINV P1234 induced the strongest IFN- β levels (Fig. 2B, Publication III), consistent with earlier COP5-based observations (271, 295; Fig. 17). Moderate induction was observed for BFV, WEEV, CHIKV, and MAYV replicases, followed by EEEV, VEEV, RRV, ONNV, and EILV replicases (Fig. 2B, Publication III). Overall, rPAMP production broadly correlated with *trans*-replicase activities (309; Fig. 1C, Publication III),

with a few notable exceptions: RRV and EEEV replicases produced relatively low rPAMP levels despite high viral RNA replication activity. Significant variation in rPAMP abundance between replicases may reflect differences in template usage across cell types. Nevertheless, our data confirms that rPAMP synthesis is a general property of alphavirus replicases, including the insect-specific EILV, at least in permissive human cells such as HEK293T, here chosen for their suitability in enabling robust comparisons of replicase activity. However, alphaviruses replicate in a wide range of vertebrate cell types, including fibroblasts, dendritic cells, macrophages, muscle cells, and neurons. Future studies applying the two-step rPAMP detection assay across diverse cell types and a broader panel of replicases would provide additional valuable insights.

Next, we examined whether rPAMPs are produced in mosquito cells (Fig. 7C, Publication III). As mosquitoes do not have the type I IFN system, it was assumed that in these cells rPAMPs would not be produced. Unexpectedly, in mosquito cells, SFV replicase also produced high rPAMP levels, while CHIKV, MAYV, and WEEV produced lower but detectable levels (Fig. 7D, Publication III). Notably, the potency (amounts) of rPAMPs produced in mosquito cells did not correlate with *trans*-replicase activity in these cells, as even replicases with no detectable or weak rPAMP production typically generated strong Gluc signals (Fig. 7B and 7D, Publication III).

Interestingly, with the exception of WEEV, replicases of viruses capable of inducing rPAMP synthesis in mosquito cells were from viruses belonging to the Semliki Forest complex, whose members are particularly efficient at copying heterologous viral RNA templates (309). In contrast, in mosquito cells, SINV replicase did not produce detectable amounts of rPAMPs (Fig. 7D, Publication III), possibly reflecting its property to preferentially utilize matching template RNA in C6/36 cells, suggesting that stricter template requirements may limit the use of cellular RNA. Similar constraints may apply to other replicases, such as RRV and MAYV, which show limited rPAMP production despite efficient viral template RNA cross-utilization and high *trans*-replicase activity in HEK293T cells, including readily detectable negative-strand synthesis (309). However, not all potential non-self RNA templates – whether viral or cellular – are likely to be equally suitable across all members of the Semliki Forest complex.

4.3.2. Standard NGS library preparation methods are unsuitable for rPAMP sequencing

We initially attempted to sequence rPAMPs from total RNA isolated from SFV6-replicase-transfected or SFV-infected HEK293T cells. Sequencing using standard Illumina library preparation with rRNA depletion, however, failed to identify host-derived replicase products. Moreover, strand ratios of viral RNA were inconsistent with expected values: an approximately 1:300 ratio of negative- to positive-strand reads mapping to the SFV6 antigenome and genome was observed. Prior reports have indicated this ratio to be closer to 1:20, showing that for every 10,000 negative-strand complements, around 200,000 new genomic RNA copies

are produced (333). Additionally, an unexpected phenomenon was discovered: reads mapping to the antisense strand of the replicase ORF were abundant in samples from cells transfected with expression plasmids (Fig. 5B, Publication III). Most likely, these reads originated from cryptic internal transcription of the expression plasmid by host RNA polymerases, thereby creating noise and further complicating sequence analysis.

For the next attempt, we enriched the rPAMP pool in SFV6-replicase-transfected samples by co-immunoprecipitating RIG-I-associated RNAs. RNA co-immunoprecipitated from WT replicase transfected cells induced strong IFN- β levels (Fig. 6B, Publication III), confirming successful enrichment. However, once again, Illumina sequencing (this time omitting rRNA depletion) yielded low-complexity data dominated by PCR duplicates, and Oxford Nanopore Technologies platform sequencing produced very short (<200 nt), low-quality reads that failed to map reliably.

Current understanding, derived from SFV replicase studies, indicates that alphavirus rPAMPs are dsRNAs longer than 200 bp, lack polyadenylation, and are primarily recognized by RIG-I (295). What technical limitations may have prevented their identification? Most likely, standard NGS library preparation hindered effective dsRNA capture and cDNA synthesis. Similar challenges have been reported for sequencing the dsRNA genome of bacteriophage ϕ 6 using conventional Illumina protocols (334). These limitations likely arise from inefficient strand separation prior to cDNA synthesis. The inclusion of DMSO during heat denaturation has been shown to improve sequencing read yield for ϕ 6 by more than two orders of magnitude (335) and this approach has also proven useful in metagenomic discovery of novel dsRNA viruses (336). Future efforts to characterize rPAMP sequences should therefore prioritize methodological optimization, including improved dsRNA denaturation strategies.

Alternatively, rPAMPs could be metabolically labelled using cells treated with actinomycin D (which inhibits cellular DNA-dependent RNA polymerases but has no effect on alphavirus replicase activity), followed by pulldown and sequencing. There is also another intriguing, albeit unlikely, possibility: these RNAs are not generated using cellular RNA templates but are instead synthesized *de novo* by the replicases. Alphavirus infections can produce defective interfering RNAs containing tRNA fragments (337, 338), and replicases can add nucleotides in a template independent manner (78, 339). Consequently, rPAMPs may be highly heterogeneous and lack clear similarity to canonical host or viral RNAs, complicating their identification by NGS.

4.3.3. Replicases with slower ns polyprotein processing are more potent rPAMP producers

rPAMP production varied markedly between replicases – by up to approximately 30-fold even within the Semliki Forest complex (Fig. 2B, Publication III). Because the initial panel included replicases only from some prototypic strains, we next examined whether strain-specific variation contributes to these

differences. The SFV replicase used above corresponds to that of SFV6, an icDNA-derived copy of the virulent isolate L10 (89). Previous studies comparing this strain with the naturally occurring avirulent A7(74) strain identified key virulence determinants within the nsP3 region, and in the P4 residue of the 1/2 cleavage site (nsP1 position 534), together with the S4 subsite of the nsP2 protease (nsP2 position 515) (186). Swapping these determinants interconverts the virulence phenotypes. As replicases of these isolates and mutants were available (Fig. 3A, Publication III), the next analysis included two replicases from virulent (SFV6, A774-HV) and two from avirulent (A774, SFV6-74-RE) variants. Somewhat counterintuitively, RNA isolated from HEK293T cells expressing P1234 from the virulent strains induced significantly higher IFN- β levels in COP5 cells than RNA from cells expressing P1234 of the avirulent variants (Fig. 3D, Publication III). While all four replicases showed comparable overall activity in *trans*-replicase assays (Fig. 3B, Publication III), NB analysis revealed reduced positive- and negative-sense viral RNA synthesis for the avirulent A774 and SFV6-74-RE replicases (Fig. 3C, Publication III). The virulent SFV replicases exhibit slower 1/2 site cleavage (186), likely stabilizing early RCs and increasing negative-strand RNA synthesis, and by extension, resulting in greater positive-strand RNA production. This highlights an important link between viral RNA synthesis efficiency and rPAMP production, whereby relatively modest differences in replicase function can lead to substantial changes in the generation of type I IFN-inducing RNAs.

Similar principles were observed for CHIKV. Mutations in nsP1 (R532H) and nsP2 (E515V), analogous to SFV virulence determinants, have previously been linked to enhanced type I IFN induction but, in contrast to SFV, result in CHIKV attenuation in mice (329). Here, CHIKV-H (R532H), CHIKV-V (E515V), and CHIKV-HV replicases (Fig. 4A, Publication III) showed that slowing the 1/2 site cleavage (replicases CHIKV-H and CHIKV-HV) increased rPAMP production, whereas for CHIKV-V, rPAMP synthesis levels were very low (Fig. 4D, Publication III). Interestingly, blocking P123 processing (replicase CHIKV-P1'2'34) had only a minor effect on rPAMP synthesis (Fig. 4D, Publication III). While *trans*-replicase activities were broadly similar for all of these replicases (Fig. 4B, Publication III), NB analysis revealed much higher RNA synthesis for CHIKV-H and CHIKV-HV replicases (Fig. 4C, Publication III), indicating, again, that mutations affecting ns polyprotein processing similarly influenced viral RNA and rPAMP synthesis.

The properties of BFV^{T1325P}-Rep (named BFV-P in Publication III) further illustrate this relationship. RNA from HEK293T cells transfected with BFV^{T1325P}-Rep also induced significantly stronger IFN- β production than RNA from WT BFV replicase transfected cells (Fig. 4D, Publication III) and was clearly more efficient in viral RNA synthesis (Fig. 4C, Publication III). Thus, slowing BFV ns polyprotein cleavage also yields a replicase that is more prone to utilizing host templates for RNA amplification. At the same time, when nsP2-T1325P compensates for the defect of nsP4-V1911D, rPAMPs are produced at levels similar to the WT BFV replicase (Fig. 10A, Publication I). While negative-strand viral RNA

synthesis is clearly enhanced by the presence of both mutations, positive-sense RNA amplification remains at a WT-like level (Fig. 14). The viral RNA synthesis profile of BFV^{T1325P+V1911D}-Rep more closely resembles that of the “locked” early RC replicase CHIKV-P1’2’34 (compare Fig. 14 and Fig. 4C, Publication III), together indicating that markedly increased rPAMP synthesis is not solely a consequence of elevated negative-strand RNA production.

4.3.4. What do we now know, what do we not know, and what may be the biological functions of rPAMPs?

Obviously, the greatest gap in current knowledge is that we still do not know exactly what rPAMPs are. In this regard, our study failed to provide major new insight, except to demonstrate that these dsRNAs bind to RIG-I and can be enriched by pulldown with the PRR (Fig. 6A–B, Publication III). What was established is that, for the most part, the ability of replicases to amplify their corresponding template RNA correlated with rPAMP synthesis. Because rPAMPs are dsRNAs, their production is likely to be largely mediated by the early RC. Our results support this interpretation, as mutations that stabilize the early RC further increased rPAMP production in the case of SFV, CHIKV, and BFV replicases (Fig. 3D and 4D, Publication III), together with viral negative-strand RNA synthesis (Fig. 3C and 4C, Publication III). In addition, slowed processing of RRV P123 has previously been shown to increase both rPAMP production and viral negative-strand synthesis in COP5 cells (271).

However, RNA isolated from HEK293T cells expressing replicase with uncleavable CHIKV P123 region did not induce significantly stronger IFN- β responses, despite clearly enhanced negative-strand viral RNA synthesis (Fig. 4C–D, Publication III). This indicates a crucial difference in rPAMP pool amplification between a “locked” early RC replicase (CHIKV-P1’2’34) and replicases that merely delay complex maturation (CHIKV-H, CHIKV-HV, SFV6, A774-HV, BFV^{T1325P}-Rep). Thus, it is plausible that the early P123+4 complex indeed represents the primary machinery responsible for rPAMP generation. In this model, the early RC initially captures suitable host RNA templates and converts them into dsRNA, which constitutes an rPAMP. As replication complexes mature, amplification shifts toward using the negative strand as a template, potentially leading to production of additional copies of the original host-derived template RNA. These copies may then serve as further templates for rPAMP generation, particularly when early RCs are more abundant or persist longer because of slower ns polyprotein processing. However, in the case of CHIKV-P1’2’34 replicase, such additional rPAMP template amplification would likely be far lower, and this may also apply to BFV^{T1325P+V1911D}-Rep. A likely explanation could be the lack of suitable *cis*-acting elements (those required for negative-strand synthesis are not the same as those recognized for positive-strand synthesis).

Furthermore, there is a strong possibility that, unlike true replication intermediates, rPAMPs are not incorporated into spherules and are therefore inacces-

sible to the positive-strand synthesis machinery. Despite similarities between viral RNA synthesis and rPAMP amplification in terms of general negative- and positive-strand RNA production, there is reason to consider that rPAMP-producing complexes differ from those responsible for viral RNA production. Spherule size is dependent on template RNA length (137), and very short templates would be expected to give rise to much smaller spherules. However, this has not been documented in infected cells. Moreover, rPAMP production is observed in the absence of viral template RNA by replicases formed from precursor polyproteins capable of undergoing the full processing cascade, a condition that does not result in detectable spherule formation (340). In this regard, it is interesting that even individual alphavirus ns proteins have been shown to assemble into complexes resembling mature replicase cores that are capable of limited extension of non-viral RNA templates. Such complexes are abundant in CHIKV-infected cells and are hypothesized to form in the absence of viral RNA templates (136). Therefore, it is plausible that these spherule-absent RCs, or their precursors, are the source of rPAMPs.

rPAMP synthesis appears to be a common property of alphavirus replicases, although it remains unclear whether rPAMPs are functionally important or merely by-products of highly efficient replicase activity. The observation that some replicases produce these molecules in mosquito cells supports the latter possibility. However, significant variation in rPAMP production among replicases from different mutants and naturally occurring strains with distinct phenotypes suggests that rPAMPs may have biological relevance depending on the viral species. In HEK293T cells, SFV and SINV replicases were the most active rPAMP producers (Fig. 2B, Publication III). We showed that SFV virulence determinants previously associated with P123 stabilization (186) result in elevated rPAMP levels (Fig. 3D, Publication III). Therefore, this does not appear to be detrimental to the virus, suggesting that, for SFV, the seemingly negative effects of increased type I IFN induction may be outweighed by enhanced replication efficiency. Although SINV replicases were not examined in the same detail here, similar trends have been observed: naturally occurring SINV strains with elevated rPAMP production (271) cause lethal encephalitis in mice (341). Because severe SINV encephalitis has been linked to neuronal apoptosis (342), and SFV replication can stimulate apoptotic pathways via MDA5 activation (343), rPAMPs may contribute to disease severity. Therefore, excessive type I IFN induction driven by rPAMPs may increase the susceptibility of central nervous system cells to SFV and SINV infection. In contrast, NW encephalitic alphaviruses exhibited low to moderate rPAMP production in HEK293T cells (Fig. 2B, Publication III). Virus-specific antagonism of host transcription, translation, and type I IFN responses is a key determinant of virulence and may obscure *in vitro* phenotypes. Given the diversity of virus-host interactions, these viruses may not rely on rPAMP-mediated immune stimulation to infect the central nervous system.

For arthritogenic alphaviruses, which lack naturally encephalitic strains (in mouse models) rPAMP synthesis appears to be under negative selection. Although mutations that slow ns polyprotein cleavage in CHIKV and BFV increase

replicase activity and rPAMP production – similar to earlier findings with RRV – these mutations also reduce CHIKV and RRV pathogenicity *in vivo* and attenuate BFV in cultured cells. This suggests that, unlike SFV and SINV, these viruses are not adapted to benefit from elevated rPAMP levels. Moreover, in contrast to the SFV and SINV examples, these processing mutations are artificial, as is the resulting increase in rPAMP synthesis, further supporting the idea that these viruses are not naturally adapted to exploit excessive rPAMP production.

Selective pressure to limit rPAMP production seems intuitive because of the need to evade host immune detection. Nevertheless, although alphaviruses possess well-characterized mechanisms to counteract type I IFN responses, activation of these pathways remains a hallmark of alphavirus infection. There is evidence, however, that this can also be beneficial for viral persistence. Notably, type I IFN responses can restrict arthritogenic alphavirus infection in dendritic cells, resulting in reduced antigen presentation and delayed CD8⁺ T-cell-mediated clearance (344). In this context, additional PRR activation via rPAMPs could instead benefit viral persistence by influencing tissue tropism, pathogenesis, and immune dissemination. On the other hand, rPAMP synthesis has been proposed to be an inevitable consequence of the high activity of viral replicases or RdRps, which hosts can exploit for virus detection (295). Consistent with this, there is evidence that other positive-strand RNA viruses also trigger type I IFN responses in the absence of viral template RNA via PRR activation. For example, expression of the RdRp of Hepatitis C virus (family *Flaviviridae*), norovirus (family *Caliciviridae*), and Theiler's murine encephalomyelitis virus (TMEV, family *Picornaviridae*) alone is sufficient to activate RIG-I and/or MDA5 (345–348). Moreover, transgenic mice stably expressing TMEV RdRp have been shown to effectively block incoming viral infection via the MDA5-MAVS pathway (349). Taken together, these findings indicate that both the infected vertebrate host and the infecting alphavirus may benefit from a robust innate immune response. Perhaps for alphaviruses, and many other positive-sense RNA viruses, rPAMP synthesis is unavoidable, but species-specific host antagonism, host-factor interactions and characteristic pathology lead to the differences observed in host RNA amplification.

Finally, these findings have potential implications for vaccine development. Alphavirus replicon- and replicase-based systems are actively being explored for saRNA and taRNA vaccines. Currently, one saRNA-based COVID-19 vaccine has been approved for use in Japan (350), the European Union (351), and the United Kingdom (352), and several others are in clinical trials. taRNA candidates have also shown promising pre-clinical results although, these have yet to enter clinical evaluation (353–355). mRNA vaccines are often described as self-adjuvanting because non-replicating mRNA backbones are subject to endosomal sensing (356). In contrast, saRNA and taRNA systems generate dsRNA, including rPAMPs, and are therefore expected to induce stronger innate immune responses. Demonstrating this potential indirectly, *in vitro*-transcribed RNA acting as a RIG-I agonist has been shown to be a potent adjuvant when immunizing mice against influenza (357). Alphavirus replicons derived from EEEV and VEEV

have been shown to strongly induce IFN- β production in both murine and human fibroblasts, whereas SINV replicons show weaker induction in human cells (332). Interestingly, stronger early type I IFN responses have been associated with reduced antigen production in saRNA-transfected mice, and delayed ns poly-protein processing can further decrease antigen expression while increasing IFN induction (358). As saRNA and taRNA platforms rely on the full alphavirus replication machinery, a deeper understanding of the mechanisms underlying innate immune activation is essential. Our data demonstrate that rPAMP synthesis varies significantly between viral species, adding another layer of complexity to vaccine design. Optimizing the balance between immune activation and antigen production may benefit from selecting replicases that not only generate rPAMPs but also exploit their effects to enhance vaccine efficacy.

5. CONCLUSIONS

The key results and findings of this thesis can be summarized as follows:

1. A BFV infectious clone was successfully assembled. The phenotype of the clone-derived recombinant virus closely corresponded to that of the original natural isolate.
2. A mutation in the nsP4 region between the NTD and the classical RdRp fold (V1911D) severely disrupted replication efficiency but could be compensated by a slowdown in ns polyprotein processing resulting from a mutation at the P7 position of the 2/3 cleavage site (T1325P) within the nsP2 coding region, providing evidence for a functional linkage between these positions in nsP2 and nsP4.
3. Unlike most nsP3 proteins of alphaviruses, BFV nsP3 also showed prominent nuclear localization owing to an NLS located at the beginning of the HVD of nsP3. Disruption of BFV nsP3 nuclear localization was tolerated but resulted in viral attenuation both *in vivo* and *in vitro*.
4. The utilization of host RNA templates by alphavirus replicases in human cells is likely a universal property within the genus, although the potency of rPAMP synthesis is highly variable between different alphavirus species.
5. Slower ns polyprotein processing resulted in greater rPAMP production, as demonstrated with CHIKV, SFV, and BFV replicase mutants. This is likely a consequence of longer-lived early RCs. Our results suggest that the early RC is the machinery responsible for host RNA recruitment and conversion into rPAMPs.
6. Greater rPAMP synthesis correlated with the previously reported virulent phenotype of SFV, and conversely, with the avirulent phenotype of CHIKV and attenuation of BFV replication in murine cells.
7. Replicases of some alphaviruses were capable of rPAMP production in mosquito cells.
8. Conventional NGS library preparation methods were unsuitable for determining the sequence or template origin of rPAMPs.

SUMMARY IN ESTONIAN

Barmah Forest viirusest peremeesraku RNA päritolu PAMP-ideni: alfaviiruste replikaaside eristuvatest funktsioonidest ning replikaaside konserveerunud omadusest aktiveerida kaasasündinud immuunvastust

Alfaviirused on positiivse polaarsusega RNA genoomiga viirused, mis on levinud ülemaailmselt. Alfaviiruseid levivad lüljalgsete vektorputukate, peamiselt sääskede kaudu, kes selgroogsete loomade verest toitumisel viirusnakkust edasi kannavad. Paljud alfaviirused põhjustavad seeläbi haigusi ka inimestel, mis tihti avalduvad kergemate sümptomitega, kuid võivad kujuneda ka surmavaks entsefaliidiks või krooniliseks artriidiks. Tulenevalt inimeste üha aktiivsemast reisimisest, uutes piirkondades leiduvatest sobilikest sääseliikidest ning kliimamuutustest tingitud vektorputukate levikualade laienemisest, on ka alfaviiruste levikuala laienemine jätkuvalt tõsiseks murekohaks. Rasket või ka kroonilist artriiti põhjustava chikungunya viiruse (CHIKV) praeguseks ulatuslikuks kujunenud levik on oluline näide alfaviiruste potentsiaalset jõuda uutele kaugetele aladele ning seal kanda kinnitada. Möödunud ning käesoleval sajandil on CHIKV levinud koduselt Aafrika mandrilt nii Aiasse kui Uude Maailma, aga põhjustanud kohalikke puhanguid ka Euroopa lõunapoolsemates riikides. Lähiaja läbimurdeks on olnud aga CHIKV vastaste vaktsiinide kasutuselevõtt. Paraku pole need osutunud täielikult ohutuks, seda eeskätt eakamate vanuserühmas, ning laialdane kasutus ei ole ka suurt hoogu kogunud. Ühtlasi pole kasutuslikku heakskiitu pälvinud teiste alfaviiruste vastased vaktsiinid või ravimid.

Meditiiniliselt oluliseks alfaviiruseks on ka Barmah Forest viirus (BFV), millega nakatumisel põevad inimesed lihas- ja liigesvalusid, palavikku, ulatuslikku nahalöövet ning peavalusid. BFV teadaolevaks levikualaks on Paapua Uus-Guinea ning kõige märkimisväärselt Austraalia, kus selle viirushaigusega nakatumisi aktiivselt ka jälgitakse. On varasemalt teada, et BFV on alfaviiruste perekonnas fülogeneetiliselt eristuv, esindades ainukesena omanimelist anti-geenset kompleksi, kuna lähedasi sugulasliike pole BFV-le avastatud. Samas pole aga BFV molekulaarbioloogiat palju uuritud, mis osaliselt tuleneb vastavate laboratoorsete molekulaarsete tööriistade puudumisest. Sellest lähtuvalt on väitekirja üheks osaks tulemused koostööprojektist Austraalia viroloogidega, mille käigus oli eesmärk luua laboratoorne BFV infektsiooniline kloon, et täpsemalt uurida selle unikaalse alfaviiruse infektsiooni kulgu ning omapärasid. Uurimuse tulemusel avastasime, et BFV infektsiooni käigus paigutub osa alfaviiruste kõige mõistatuslikumast replikaasi valgust, nsP3-st, imetajarakkude tuuma, mida pole aga ühegi teise alfaviiruse nakkuse korral varasemalt kirjeldatud. Katsetulemused rakukultuuride ning hiirte nakatamisest näitasid, et nsP3 lokaliseerimine raku tuuma pole BFV elumise tagamisel kriitiline, kuid andis ilmselge eelse viiruse replikatsioonil. Lisaks võimaldas pöördgeneetika ning BFV *trans*-replikaasi süsteemi rakendamine täheldada funktsionaalset seost BFV nsP2 ja nsP4 piirkondade vahel, kus viiruslike RNA-de sünteesidefekti põhjustanud mutatsioon

nsP4-s kompenseeriti teise mutatsiooni abil nsP2 valgus. Vastavat nsP4 mutatsiooni piirkonda pole samuti meile teadaolevalt varasemalt detailselt uuritud ning leidsime, et nsP4 mutatsiooni tõttu ilmnenud replikatsioonidefekt parendati mittestruktuurse liitvalgu lõikamisprotsesside aeglustamisega.

Üksnes BFV replikaasi ekspressioonil inimese rakuliinis näitasime, et tagajärjeks on I tüüpi interferoonide vastust aktiveerivate RNA-de süntees ka viirusliku RNA matriitsi puudumisel. Antud fenomeni, kus viiruse replikaas paljundab peremeesraku RNA-sid, et toota PAMP RNA molekule (käesolevas töös nimetatud kui rPAMP-id), mis on tuvastatavad raku ohureseptorite poolt, on eelnevalt kirjeldatud Semliki Forest viiruse (SFV), Ross River viiruse ning Sindbis viiruse (SINV) puhul. Lisanduv avastus BFV replikaasiga andis tõuke järgmisele projektile uurimaks, kui laialtlevik on rPAMP-ide sünteesimise omadus alfaviiiruste perekonnas.

Meie oluliselt täienenud replikaaside ekspressioonikonstruktid hulk võimaldas uurida rPAMP-ide tootmist 11 alfaviiruse replikaasi puhul, millega sai esindatud enamik alfaviiruste perekonna antigeensetest kompleksidest. Tulemused inimese rakuliinis HEK293T näitasid, et kõigi kasutatud alfaviiruste replikaasid sünteesisid rPAMP-e, sealhulgas ka üksnes putukarakke nakatava Eilat viiruse replikaas. Kõik uurimisalused replikaasid ei tootnud selliseid molekule võrdsel tasemel, kuid järeldasime, et võimekus paljundada peremeesraku päritolu RNA matriitse on alfaviiruste universaalne omadus, aga tõenäoliselt ei kasuta kõik replikaasid selliseid matriitse võrdse efektiivsusega. Üllatuslikult sünteesisid mõnede alfaviiruste replikaasid rPAMP-e ka putukarakkudes.

Järgnevalt võtsime luubi alla SFV replikaasid, mis olid pärit looduslikest virulentsetest (hiirtel surmavat entsefaliiti põhjustavatest) ning avirulentsetest (hiirtel oluliselt kergemat haiguskulgu põhjustavatest) variantidest ning nende põhistest kimääridest. Need võrdluskatsed näitasid, et virulentsete SFV variantide päritolu replikaasid on tõhusamad rPAMP-ide tootjad ning ühtlasi võimekamad paljundama viiruse negatiivse ja positiivse polaarsusega RNA-sid. Varasematest töödest oli ka teada, et antud virulentsed SFV variandid on aeglasemad protsessima viiruse mittestruktuurset liitvalku. Võrdluskatsed geneetiliselt muudetud BFV ja CHIKV replikaasidega näitasid, et seaduspära kehtib ka alfaviiruste puhul, mida seostatakse artriidi sümptomitega. Mutantsed BFV ja CHIKV replikaasid, mille mittestruktuursete liitvalkude lõikamine on samuti aeglasem, olid ka tõhusamad sünteesima viiruse RNA-sid ning tootsid ühtlasi suuremas hulgas rPAMP-e. Selliseid CHIKV mutante on aga varasemalt kirjeldatud kui oluliselt nõrgestatud viiruseid hiirte nakatamisel, ehk omadus toota suuremas hulgas rPAMP-e katus CHIKV puhul oluliselt kergemate sümptomitega, mis on vastupidine SFV tulemustele. Katsetulemustest järeldasime, et omadus sünteesida suuremas hulgas rPAMP-e on iseloomulik sellistele alfaviiruste replikaasidele, mida iseloomustab varajase replikatsioonikompleksi suurem stabiilsus, ehk selliste komplekside pikemaajalisem esinemine tänu aeglasemale mittestruktuurse liitvalgu lõikamisele. Samas järeldasime ka, et kõik alfaviirused pole üheselt kohastunud rPAMP-ide ülehulgale ning tõenäoliselt kasutavad nende paratamatut tootmist osad liigid (näiteks hiirtel entsefaliiti põhjustavad SFV ja SINV) edukalt

infektsiooni käigus ära, kuid näiteks artriiti põhjustavad CHIKV ja BFV selleks kohastunud pole.

Käesoleva töö eksperimentide tulemused avardasid üldisi teadmisi alfaviiruste replikaasi valkude funktsionaalsest mitmekesisusest. Kuigi lähiaastatel on alfaviiruste põhjustatud haiguste tõrjes tehtud märkimisväärsed arenguid CHIKV vaktsiinide näitel, siis pole teiste perekonna esindajate vastu veel vaktsiine ega inhibiitorravimeid saadaval. Meie leiud BFV-ga viitavad jätkuvalt sellele, et konkreetsed alfaviirused võivad olla piisavalt iseäralikud, et nende vastane tõrje- või ravistrateegia nõuab suure tõenäosusega spetsiifilisi lähenemisi ning seetõttu on tarvilik neid iseärasusi ka mõista. Teisalt võimaldasid meie katsed replikaasidega täheldada universaalset alfaviiruste omadust sünteesida rPAMP-e inimese rakuliinis, millel on potentsiaalne mõju ka väljaspool pelgalt alfaviiruste infektsiooni omapärade tundmist. Viimaste aastate jooksul on vaktsiiniarenduses hoogu kogunud alfaviiruste replikonide (saRNA) ning replikaaside (taRNA) põhiste platvormide arendus ning reaalne rakendamine, ehk vaktsiiniplatvormid, mille toime sõltub suuresti alfaviiruste replikatsioonikomplekside aktiivsusest. Sellistel juhtudel on kaasasündinud immuunvastuse ärgitamise mõistmine peremeesraku RNA matriitside paljundamise tagajärjel oluline element, mida võtta arvesse antud vaktsiiniplatvormide väljatöötamisel ning tõhustamisel.

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