

Characterizing important flavivirus-host interactions

*I dedicate this thesis
to my family*

Örebro Studies in Medicine 255



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**Characterizing important flavivirus-host interactions:
Replication, assembly, restriction factors,
and vaccine development**

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Title: Characterizing important flavivirus-host interactions:
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Publisher: Örebro University 2022

www.oru.se/publikationer

Print: Örebro University, Repro 02/2022

ISSN 1652-4063

ISBN 978-91-7529-426-1

Abstract

Pham Tue Hung Tran (2022): Characterizing important flavivirus-host interactions: replication, assembly, restriction factors, and vaccine development. *Örebro Studies in Medicine* 255.

The genus *Flavivirus* (family *Flaviviridae*) consists of important zoonotic viruses that cause morbidity and mortality worldwide. These viruses are enveloped and have a positive-sense single-stranded RNA genome encoding a polyprotein. Cleavages of the polyprotein by host and viral proteases result in individual viral proteins, including the structural capsid (C), pre-membrane (prM), envelope (E) proteins, and seven nonstructural proteins. Removal of the C-prM-E genes in the flavivirus genome results in replicons that can replicate in transfected cells but do not generate infectious virus particles. The replicon can be co-expressed with the C-prM-E genes in trans, resulting in packaging of the replicon and generation of replicon virus-like particles (RVPs).

During cellular infection, various host proteins are employed, supporting multiple stages of the virus life cycle. In this thesis, we identified and characterized functions of the host lunapark protein and two members of the Endosomal Sorting Complexes Required for Transport Machinery – ALIX and CHMP4A. We also revealed how the host proteins were recruited by virus proteins during infection.

To counteract the virus infection, virus-infected cells can express antiviral proteins. We demonstrated the antiviral mechanism of interferon-stimulated gene (ISG) 15 and the E3 ligase for ISG15 conjugation HERC5, which degrades ALIX and CHMP4A, indirectly targets virus infection. Furthermore, using proteomic screening, we identified tripartite motif-containing proteins (TRIM) – TRIM21 and TRIM14 – as restriction factors to Langkat virus and Zika virus.

We also established and characterized an RVP production system based on the West Nile virus (WNV) Kunjin strain. The system was used as a vector to express antigens from Ebola virus (EBOV), which can potentially be developed as a vaccine platform against WNV and EBOV.

Keywords: Flaviviruses, virus-host cell interaction, lunapark, ESCRT, ALIX, CHMP4A, ISG15, TRIMs, replicon virus-like particles, vaccine

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List of papers

This thesis is based on the following studies, which are referred to by their Roman numerical I o IV

- I. **Tran P-T-H**, Asghar N, Johansson M, Melik W. Roles of the Endogenous Lunapark Protein during Flavivirus Replication. 2021;13(7):1198
- II. **Tran P-T-H**, Chiramel AI, Johansson M, Melik W. Roles of ESCRT Proteins ALIX and CHMP4A and Their Interplay with Interferon-Stimulated Gene 15 during Tick-Borne Flavivirus Infection. 2022. 96(3): p. e01624-21.
- III. **Tran P-T-H**, Karlsson R, Karlsson A, Asghar N, Melik W, Johansson M. Proteomic Screening Identifies TRIM21 and TRIM14 as Antiviral Proteins against Langkat Virus and Zika Virus, manuscript.
- IV. **Tran P-T-H**, Asghar N, Höglund U, Larsson O, Haag L, Mirazimi A, Melik W, Johansson M. Development of a Multivalent Kunjin Virus Reporter Virus-Like Particle System Inducing Seroconversion for Ebola and West Nile Virus Proteins in Mice. 2020;8(12):1890

List of abbreviations

aa	Amino acids
ADE	Antibody-dependent enhancement
ALIX	ALG-2-interacting protein X
ATLs	Atlastins
BHK-21	Baby hamster kidney cell line
BSA	Bovine serum albumin
C	Capsid
cDNA	Complementary DNA
CHMP4	Charged multivesicular body protein 4
CMV	Cytomegalovirus
CoIP	Coimmunoprecipitation
DB	Dumbbells
DENV	Dengue virus
ds	Double-stranded
E	Envelope
EBOV	Ebola virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FMDV2A	Foot-and-mouth disease virus protease 2A
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOIs	Genes of interest
HA	Haemagglutinin
HDV _r	Hepatitis delta virus ribozyme
HIV	Human immunodeficiency virus
IFN	Interferon
IRES	Internal ribosome entry site
IRF	IFN regulatory factors
ISGs	Interferon-stimulated genes
JEV	Japanese encephalitis virus
KFDV	Kyasanur forest disease virus
L	Late

LC	Liquid chromatography
LDS	Lithium dodecyl sulphate
LGTV	Langat virus
LNP	Lunapark
Luc	Luciferase
MBFVs	Mosquito-borne flaviviruses
mRNA	Messenger RNA
MS	Mass spectrometry
Neo/KanR	Neomycin/kanamycin resistance
NHPs	Non-human primates
NS	Nonstructural
ORF	Open reading frame
pA	Poly A
PABP	Poly-A-binding protein
PCR	Polymerase chain reaction
prM	Precursor membrane
PRRs	Pattern recognition receptors
PTGS	Post-transcriptional gene silencing
PTMs	Post translation modifications
qPCR	Quantitative real-time PCR
RC	Replication complex
RTNs	Reticulons
RVPs	Replicon virus-like particles
sHP	Small hairpin element
siRNA	Small interfering RNA
SL	Stem-loop
SPCS1	Signal peptidase complex subunit 1
STAT	Signal transducer and activator of transcription
TBEV	Tick-borne encephalitis virus
TBFVs	Tick-borne flaviviruses
TBK1	TANK-binding kinase 1
TEM	Transmission electron microscopy
TGN	Trans-Golgi network

TLR	Toll-like receptor
TM	Transmembrane
TRIMs	Tripartite motif-containing proteins
UTRs	Untranslated regions
WB	Western blotting
Ve	Replication vesicle
VLPs	Virus-like particles
WNV	West Nile virus
WNV _{KUN}	Kunjin West Nile virus
vRNA	Viral genome RNA
YFV	Yellow fever virus
ZIKV	Zika virus

Introduction

Flavivirus, infection outcomes, and epidemiology

The genus *Flavivirus* (family *Flaviviridae*) consists of 53 species (1), which infect at least 400 million people annually (2, 3). Most members of the genus are arboviruses, transmitting to humans via mosquito or tick bites. These infections can lead to severe diseases including neurotropic disease (encephalitis, cognitive impairment, and flaccid paralysis), vesical disease (hemorrhagic fever, hepatitis, and shock syndrome), and congenital disease (microcephaly and placental insufficiency) (3). Within the genus, infections of notable mosquito-borne flaviviruses (MBFVs) – e.g., dengue viruses (DENV), Zika virus (ZIKV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) burden healthcare systems. In addition to MBFVs, tick-borne flaviviruses (TBFVs) form a serocomplex consisting of severe pathogenic viruses such as tick-borne encephalitis virus (TBEV), Powassan virus, and Kyasanur forest disease virus (KFDV). In this group, Langat virus (LGTV), which is naturally attenuated (4), is often used as a virus model to study TBFVs.

DENV, YFV, and ZIKV can lead to infections that cause vesical disease in humans and non-human primates (NHPs). DENV infects approximately 390 million people per year worldwide, causing 100 million clinical cases (5). The virus contains four serotypes and exists in more than 100 tropical and temperate countries in Asia, Africa, America, and Australia. The epidemiology of dengue disease relies on the distribution of the mosquito vector *Aedes aegypti*. The severity of dengue disease varies between primary and consecutive infections. During the secondary infection by DENV, the produced antibodies may not neutralize the virus but enhance infection to the monocyte via antibody-dependent enhancement (ADE) (6). Regarding YFV, the virus originated in Africa and spread to America and Europe during 18th and 19th centuries. Despite an effective vaccine, the virus still causes 130,000 severe cases leading to around 78,000 deaths in Africa each year (7). The virus also has established and circulated in the zoonotic cycle of NHPs - the mosquito - humans in South America, causing many outbreaks (8). About ZIKV, before 2013, the virus had caused only mild vesical disease in Africa. However, the virus triggered epidemics in Brazil and parts of America in late 2013, when millions of infections occurred, and congenital disease was one of the outcomes of infection

during the epidemics. Molecular virology studies have shown that genetic changes in the epidemic strains of ZIKV, including A188V (9) and S139N (10), enhance the infectivity in the vector mosquito and neuroprogenitor cells, respectively. In addition, mutations at the 3' untranslated region (UTR) of the viral genome RNA (vRNA) allows it to hijack the host musashi-1 protein, which functions to differentiate neuronal progenitor cells (11).

WNV, JEV, and members of the TBEV serocomplex can cause neurotropic disease. Both WNV and JEV infect humans via *Culex* mosquitos. The zoonotic cycle of WNV consists of *Culex* mosquitos, birds, and humans. In addition to humans, some mammals such as equine species are dead-end hosts. Infection of WNV can result in febrile and mild neurotropic disease in parts of Africa, the Middle East, Asia, and Australia. However, since the outbreak of WNV in New York, 2,381 deaths due to WNV infection have been recorded in the United States (12). JEV is prevalent in Asia and has a case fatality rate reaching 30% (13). In addition to mosquitos, water birds and swine are amplifying hosts in the zoonotic virus cycle. Despite effective vaccines, JEV is still the major cause of encephalitis worldwide and 50% of the world population is living in the endemic regions of JEV (14). TBEV is another important neurotropic virus, infecting humans mainly through *Ixodes* ticks. The virus causes thousands of infection cases annually in Northern China, Russia, and Central and Eastern Europe (15). Strains of the virus are divided into three groups: European, Siberia, and Far Eastern. The fatality rates of infection are 1–2%, 6–8%, and 30%, respectively (15, 16). In Sweden, European TBEV is the only flavivirus that exists, and its incidents of infection have been increasing (17, 18).

Flavivirus genome and polyprotein processing

Flaviviruses are enveloped positive-sense single-stranded RNA viruses, whose genomes are approximately 10–11 kb. Their genomes encode an open reading frame generating a transmembrane polyprotein spanning the endoplasmic reticulum (ER) membrane. Cleavages of the polyprotein by host and viral proteases result in the functional proteins necessary for the virus life cycle: capsid (C), precursor membrane (prM), and envelope (E) proteins for RNA genome encapsidation and envelopment. On the polyprotein, the C and the prM proteins are connected by an anchored transmembrane domain. To release the two proteins, a viral protease is initially

required to cleave the C protein at its C-terminus (19-21); this is followed by the protease activity of the signal peptidase complex subunit 1 (SPCS1), which cleaves at the N-terminus of the prM (22) (Figure 1). The cleavage at the junction between the C-terminus of prM and E relies on an unknown signal peptidase (22) (Figure 1). The polyprotein also encodes for seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These proteins are cleaved from the polyprotein by the viral NS2B-NS3 protease (23), except the cleavage of the NS1-NS2A junction, which is cleaved by an unknown host peptidase (24), and the 2K-NS4B junction, which is cleaved by SPCS1 (22) (Figure 1). Similar to the C-prM junction, the NS4A and NS4B are bridged by a transmembrane domain 2K. NS4A-NS4B is initially cleaved by the NS2B-NS3 protease at the C-terminus of NS4A, which is followed by the activity of SPCS1 at the N-terminus of NS4B (22, 25) (Figure 1). At the ER membrane, the NS proteins assemble into a replication complex (RC), which invaginates the membrane into vesicles (Ve) and supports vRNA replication (26-30) (Figure 1). Table 1 provides a summary of the multiple functions of the NS proteins. TBEV Toro strain was used as the reference for protein sizes.

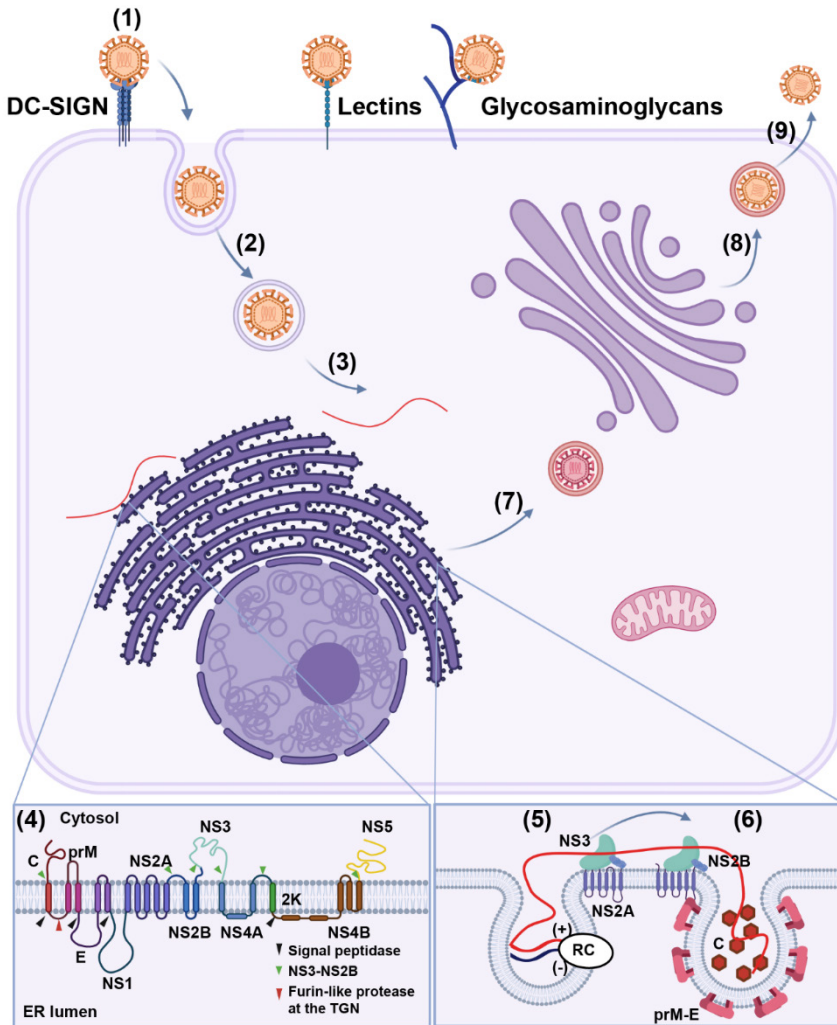


Figure 1. Schematic representation of the flavivirus life cycle. The virus particles initially attach to cellular receptors on the plasma membrane (1), which are either the C-type lectins (31), glycosaminoglycans (32), or DC-SIGN (33). The virus then enters the cell by endocytosis (2). Fusing of the membrane of the virus particles with the endocytosis vesicle membrane and uncoating the capsid (C) proteins release the positive-sense RNA virus genome (3). The initial translation generates a polyprotein, located at the endoplasmic reticulum (ER) membrane and cleaved by the host and the virus proteases (4) into functional proteins, including C, precursor membrane (prM), envelope (E), and nonstructural (NS) proteins (NS1–NS5).

The ER is invaginated by the NS4A/NS4B from the replication complex (RC) of NS proteins to form vesicles for virus replication (5). The genome RNA transits to assembly sites via the NS2A-NS3-NS2B complex (6) for encapsidation and envelopment. The assembled particles (in red color) bud through the ER and egress to the Golgi (7). At the trans-Golgi network (TGN), the prM protein on the virus surface is cleaved by the Golgi protease into the M protein, generating mature particles (in orange color) (8). They are finally released from the infected cells by exocytosis (9). The scheme was generated using the webtool Biorender.com.

Table 1. Characterized functions of non-structural proteins

Protein	Size (aa)	Functions
NS1	352	NS1 can be secreted into the circulatory system (34). DENV NS1 can bind to toll-like receptor (TLR) 4 on the plasma membrane resulting in pro-inflammatory cytokine production and therefore increased endothelial permeability and vascular leak (35, 36). The protein can recruit the host receptor for activated C kinase 1 protein, which supports virus replication (37). Furthermore, the protein can evade the interferon I (IFN-I) immune response by inhibiting TANK-binding kinase 1 (TBK1) phosphorylation (38).
NS2A	218	NS2A is one of the transmembrane proteins scaffolding the RC at the ER membrane. The protein can interact with prM-E, NS3, and the vRNA, supporting viral packaging (39, 60). The protein can suppress the IFN-I response by induced degradations of the signal transducer and activator of transcription (STAT) 1 and STAT2 (39).
NS2B	130	Like NS2A, NS2B is a transmembrane protein of the RC. The protein is the cofactor of NS3, which supports the protease activity of NS3 (40).
NS3	618	NS3 and NS2B function as a protease for polyprotein cleavage (23, 25, 41). Furthermore, the C-terminal domain of NS3 possesses an ATPase activity with a role for capping of the vRNA and a helicase activity for separation of the intermediate double-strand (ds) RNA formed during virus replication (42). In addition to these enzymatic roles, NS3 also regulates virus assembly (43) by interacting with prM-E, NS2A, and the vRNA (44, 45).
NS4A	286	NS4A plays a role in formation of the Ve (46, 47). The protein also acts as an indirect stabilizer of the intermediate viral ds RNA

		during flaviviral replication through its interaction with the host polypyrimidine tract binding protein (48).
NS4B	112	Like NS4A, NS4B is an important protein to generate Ve (26). Furthermore, NS4B acts as a suppressor of IFN-I signaling by inhibiting STAT1 (49).
NS5	900	NS5 is the largest and the most conserved protein. The protein consists of two domains: the N-terminal methyltransferase, which methylates the viral RNA cap, and the C-terminal RNA-dependent RNA polymerase, which is necessary for viral RNA replication (50-52). Furthermore, similar to NS4B, the protein suppresses IFN-I signaling by inhibiting STAT1 and STAT2 (53-57).

The flavivirus life cycle

The flavivirus infection begins when virus particles attach to host cells, which results from several interactions, including interactions between sugars on viral structural proteins and host C-type lectins (31), interactions between charged areas of the viral E protein and host glycosaminoglycans (32), or interactions between viral lipid envelope and host receptor proteins (33). Upon attachment, flaviviruses enter cells that depend on clathrin-mediated endocytosis (58), generating small endosomes containing viral particles inside infected cells. Under low pH conditions of endosomes, the E protein undergoes conformational modifications, fusing the viral membrane to the host endosome membrane (59). Next, uncoating of the C protein releases the vRNA into the cytosol, followed by vRNA translation to generate the transmembrane polyprotein at the ER. Then, NS proteins and host proteins including – reticulons (RTNs) (60) and alastins (ATLs) (61, 62) – remodel the ER membrane, which results in the formation of the Ve for the vRNA replication. The vRNA transits to assembly sites via the NS2A-NS3-NS2B complex (43, 44, 63, 64) for encapsidation with the C protein and envelopment with membranes containing the prM and the E proteins at the ER (65). These newly assembled particles are transported through the Golgi apparatus and matured by cleavages of the prM to the M protein by the host furin-like protease at the trans-Golgi network (65, 66). Finally, an exocytosis releases the newly synthesized matured viral particles to infect other cells, completing the single virus life cycle (65) (Figure 1).

Multiple roles of vRNA

The UTRs

The vRNA is flanked by two UTRs at their terminuses. The UTRs have several conserved secondary structures, including stem-loop (SL), dumbbells (DB), and small hairpin (sHP) (Figure 2 and 3). These structures have important roles in replication (67, 68), translation (69), encapsidation (70), regulation of the innate immune response (71), virulence (11, 72), and arthropod vector adaptation (73). At the 5'UTR, there is a type I cap structure containing methyl groups (51) and a conserved Y shape SL (73), termed SLA. The less conserved 3'UTR is divided into a variable region, a moderately conserved region, and a highly conserved region with respect to other flaviviruses (72, 74-76) (Figure 3). Mutations and deletions at the variable region do not significantly hinder vRNA replicability (77). Furthermore, during infection, the vRNA can be digested by the host 5'-3' exoribonuclease 1. The process is terminated at the SL2 of the 3'UTR of MBFVs (78), generating the subgenomic flavivirus RNA (sfRNA).

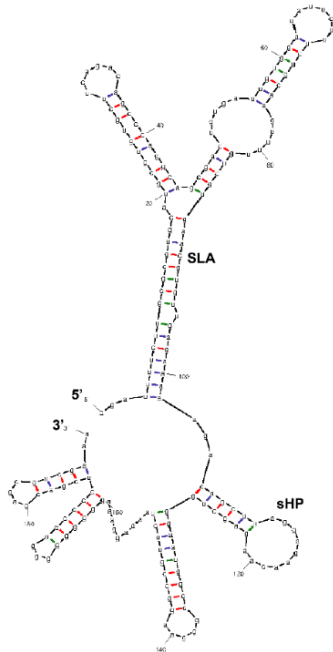


Figure 2. Secondary structure of the 5' untranslated region (UTR) and the first 20 codons of the capsid protein of the Langkat virus (LGTV) TP21 strain (Accession number: AF253419.1), illustrated using the web tool <http://www.unafold.org/>. The region contains a conserved stem-loop A (SLA) and several small hairpin (HP) structures.

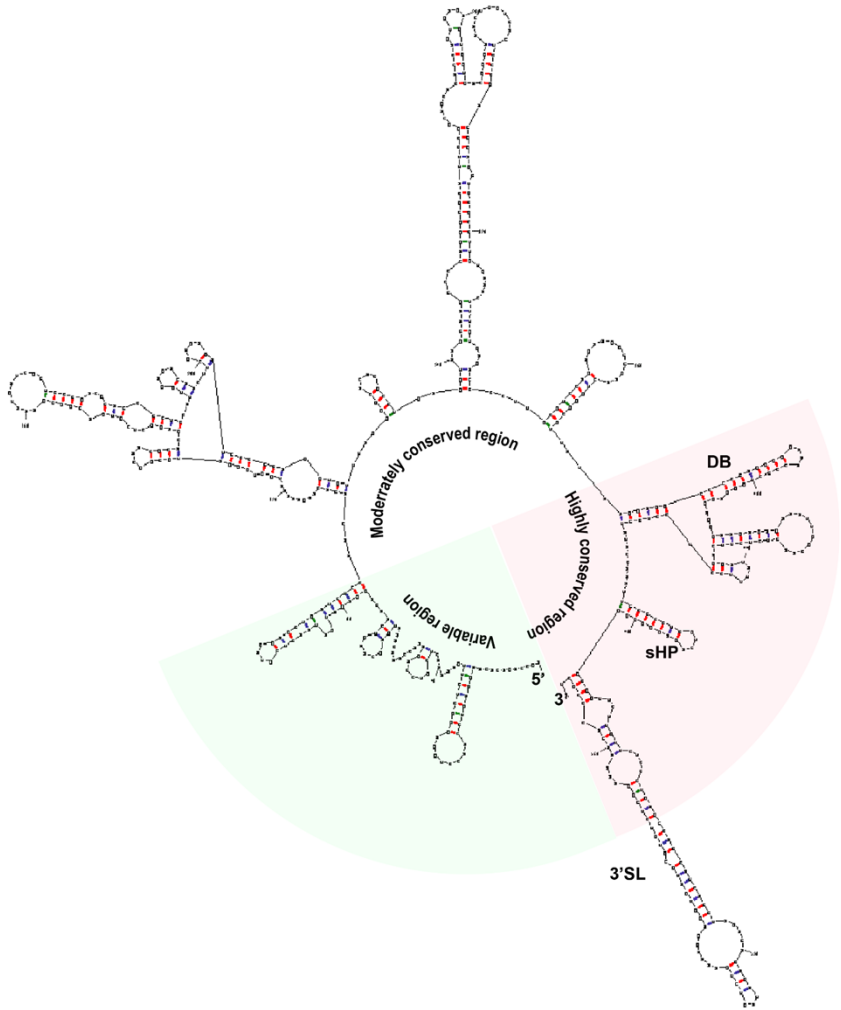


Figure 3. Secondary structure of the 3'UTR of the LGTV TP21 strain (Accession number: AF253419.1), illustrated using the web tool <http://www.unafold.org/>. The 3'UTR consists of a variable region, a moderately conserved region, and a highly conserved region (the last 190 nucleotides) (72, 76) with secondary structures such as 3' stem-loop (3'SL), small hairpin (sHP), and dumbbell (DB).

vRNA translation

Replication of the vRNA relies on the RNA-dependent RNA polymerase activity of NS5 and the helicase activity of NS3. However, these proteins do not exist in infectious virus particles. Thus, the initial translation of vRNA is essential for vRNA replication. Like messenger RNA (mRNA), initiation of vRNA translation is cap-dependent. The capping of a newly synthesized vRNA is mediated by the methyltransferase activity of NS5 coupled with the nucleotide triphosphatase activity of NS3 (50-52). According to a study on DENV, although the vRNA does not possess a pA tail, vRNA contains A-rich sequences flanking the DB structure before the terminal 3'SL motif at the 3'UTR, which can interact with the host cell poly-A-binding protein (PABP) (79). During translation, PABP binds with the cap-binding complex of eukaryotic initiation factor 4F, which stabilizes and mediates the circularization of the translating RNA (80). However, this mimic of pA tail role in translation is unclear in the context of TBFVs. Furthermore, translation of vRNA also relies on other cis-acting elements on the C-coding region (81).

vRNA replication

vRNA replication relies on the RNA-dependent RNA polymerase (RdRp) activity of NS5, which complementary synthesizes the negative-strand intermediate RNA in the 5'-3' direction based on the 3'-5' positive-strand vRNA template. The process initiates by binding of NS5 to SLA at the 5'UTR, while vRNA is circularized by interactions between the 5' and 3'UTRs. This conformation allows NS5 to move from the 5'UTR to the 3'SL at 3'UTR using a pppAG dinucleotide as a primer for the negative strand synthesis (82). During this process, the intermediate dsRNA, which is the hybrid between vRNA and the negative strand RNA, is generated and unwound by the helicase activity of NS3. New copies of vRNA are then generated from the negative strand RNA by the similar mechanism of RNA circulation. During replication, the vRNA SLA-bound NS5 can directly transfer to the 3'SL of the negative strand rather than that of the vRNA (52). Thus, a lower amount of negative strand is required, favoring a high amount of positive strand synthesis during the replication (Figure 4).

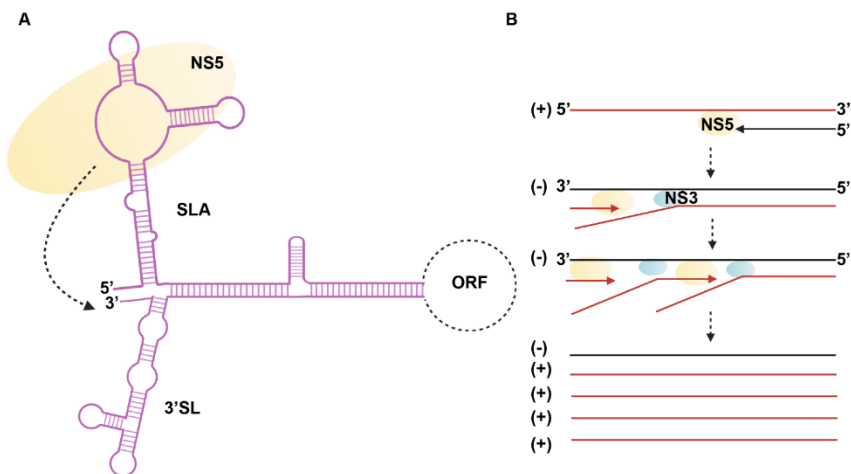


Figure 4. (A) Schematic illustration of the viral genome circulation by the interactions between the 5'UTR and the 3'UTR for virus replication. By virtue of the circularized vRNA, NS5 can egress from the 5'UTR to the 3'UTR, initializing the complementary synthesis of the RNA negative strand (-) from the positive strand (+) vRNA as in (B). In addition to the RNA-dependent RNA polymerase activity from NS5, the NS3's helicase activity unwinds the intermediate double strand RNA. During replication, preferred binding of the NS5 to the (-) strand results in a polarity to the (+) strand synthesis. ORF: open reading frame. The scheme was generated using the webtool Biorender.com.

The innate immune system

On sensing viral RNA, cells produce IFN, where type I and III IFN responses are rapidly induced and the expression of interferon-stimulated genes (ISGs) are upregulated, establishing an antiviral state (83). These two IFN responses are initiated when cells recognize invading viruses by their pattern recognition receptors, including cytoplasmic retinoic acid-inducible gene I, melanoma differentiation-associated protein 5, endosomal TLR3, and TLR7. This recognition activates TBK1, I κ B kinase- ϵ , phosphorylating IFN regulatory factors (IRF) 3, and IRF7, which translocate to the nucleus to elicit production of type I IFNs such as IFN α/β . These released cytokines then bind to IFN α/β receptors on the plasma membrane of adjacent cells, leading to activation of the Janus kinase and STAT pathway, finally inducing ISG expression (84, 85). NS proteins

(Table 1) and sfRNA has been shown to function in antagonism of the IFN-I response (76, 78).

Engineering of vRNA to generate replicons and replicon virus-like particles (RVPs)

Since emerging of the genetic engineering, studies on flaviviruses have been facilitated by generation of recombinant viruses using reverse genetics technology, which allows the introduction of mutations or deletions into vRNA. Here, vRNA can be purified, followed by reverse transcription to generate complementary DNA (cDNA), and cloned into plasmids under control of T7 or SP6 promoters from bacteriophages. At the DNA level, the viral genome can be modified simply by PCR reactions. The DNA products are *in vitro* transcribed by T7 or SP6 RNA polymerase, generating vRNA. Transfection of vRNA into cells can initiate the virus life cycle.

In-frame removal of the C-prM-E genes in the vRNA results in replicons that can replicate in transfected cells but do not produce infectious virus particles (86-89). Here, parts of the N-terminus of the C protein and parts of the C-terminus of E protein are retained as it contains the cis-acting elements for efficient replication and the signal peptide to maintain the correct topology of the viral polyprotein, respectively (77, 90). Kunjin WNV (WNV_{KUN}) was one of the first flavivirus replicon systems constructed (77). Since then, replicons of other flaviviruses with other modifications have been developed. DNA constructs expressing replicons in transfected cells directly without *in vitro* transcription can be generated. The construct is driven by a eukaryotic promoter such as the cytomegalovirus (CMV) promoter and the pA tail. Cleavage of the pA tail is mediated by a hepatitis delta virus ribozyme (HDVr) inserted downstream of the 3'UTR. To monitor expression of replicons, genes that code for reporter proteins such as luciferase or fluorescent proteins can be cloned in-frame into the replicons at the locus of the removed C-prM-E genes, followed by the foot-and-mouth disease virus autoprotease 2A (FMDV2A) sequence to release the reporter proteins during the polyprotein processing. Furthermore, the replicons can be co-expressed with the corresponding structural genes in trans, resulting in the packaging of the replicon and generation of RVPs (63, 86, 88, 91, 92). Both replicons and RVPs are essential for virus replication and assembly studies, respectively. The systems also allow for functional studies of host proteins during these stages of the virus life cycle. Finally, as RVPs do not have genes coding for the packaging proteins, they

infect cells only in a single cycle and are therefore safe as a putative vaccine platform. Several studies have demonstrated the potential of flavivirus RVPs as a vaccine platform (93-96).

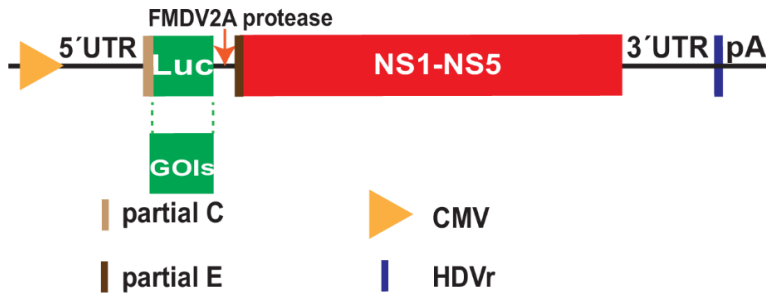


Figure 5. Schematic illustration of the Kunjin West Nile virus (WNV_{KUN}) DNA replicon construct used in the studies. The replicon is driven by a cytomegalovirus (CMV) promoter, followed by the replicon genome, and a poly A (pA) tail. Inside the replicon genome, the C-prM-E genes is substituted with luciferase (Luc) gene, which retain 27 codons of C and 28 codons of E. A foot-and-mouth disease virus protease 2A (FMDV2A) gene was inserted after the Luc to release the reporter protein from the polyprotein during expression. During expression, the hepatitis delta virus ribozyme (HDVr) gene inserted after the 3'UTR removes the pA, generating the RNA replicon ending at 3'UTR. The replicons can express genes of interest (GOIs) by substitutions of the Luc gene with GOIs.

Important virus-host interacting proteins characterized in the papers

Lunapark (LNP) protein

In eukaryotic cells, the ER is a constant-remodeling organelle with three major morphologies: the nuclear envelope, the peripheral ER cisternae, and the interconnected tubular network (97). To induce and maintain the highly curved tubular structure, host machinery is employed such as the RTNs family, DP1/YOP1 protein (98, 99), the ATLS family (100, 101), and lunapark (LNP) protein (102, 103). At the ER junctions, LNP stabilizes the ER junction (103), whose depletion leads to a dense sheet-like ER (102, 104, 105). The protein is constituted by an N-terminal cytoplasmic myristoylation motif, two transmembrane domains, and a C-terminal cytoplasmic domain (106). Localization of LNP at the three-way junctions relies on the protein dimerization at a zinc finger motif within the C-

terminal domain of the protein (107). However, during mitosis, when the ER sheet form is preferred, phosphorylation of LNP diminishes the dimerization and remodeling of the tubular ER network into sheets (105). Furthermore, the protein can also be ubiquitinated at the N-terminus, supporting its localization and recruitment of the rapamycin complex-1 from the lysosomes to the ER three-way junction (108). Ubiquitination of LNP is regulated by the cullin-associated and neddylation-dissociated 1 protein (109). Inhibition of the LNP ubiquitination can elicit neurodevelopmental defects (108).

Endosomal sorting complex required for transport (ESCRT) machinery

In eukaryotic cells, the endosomal sorting complex required for transport (ESCRT) machinery is formed by conserved membrane remodeling proteins. The complex has various roles in cellular life (110). For enveloped viruses, the roles of ESCRT proteins during budding of retroviruses, influenza viruses, and filoviruses are well characterized (111-114). The generation of ESCRT complex is initiated by recruiting early proteins – i.e., ALG-2-interacting protein X (ALIX), ESCRT-0, ESCRT-I (TSG101), and ESCRT-II proteins – to ubiquitinated proteins or to the late (L) domain motif (PPXY, PT/SAP, and YXXL) containing proteins. This recruitment is followed by the attachment of ESCRT-III proteins, which the charged multivesicular body protein 4 (CHMP4) is the most essential for forming a collar of protein polymer at the membrane necks anchoring viral buds to the plasma membrane. Constraint of protein collars by changes in their monomer conformation coupled with ESCRT III disassembly by the ATPase activity of the vacuolar protein sorting 4 homolog A results in membrane fission and releases viral buds from the plasma membrane (110, 115-117). In the context of MBFVs, TSG101 and CHMP4 have been shown to have roles in virus assembly at the ER (118).

Interferon-stimulated gene 15 (ISG15)

On sensing viral RNA, expressions of interferon-stimulated genes (ISGs) are induced, establishing an antiviral state (83). ISG15, which is a ubiquitin-like protein, can be conjugated to a variety of host proteins in a process called ISGylation. This process requires three-enzymatic steps involving E1 activating enzyme (UBE1L) (119), E2 conjugating enzyme (UBCH8) (120, 121), and E3 ligase (HERC5) (122, 123). Since HERC5 is predominantly in contact with polyribosomes, the ISGylation process tar-

gets newly synthesized proteins (124). By virtue of induced ISGylation, replications of an array of DNA/RNA viruses are inhibited (125). ISGylation of the host cell TSG101 protein inhibits budding of the human immunodeficiency virus (HIV) (115) and post-Golgi transportation of the influenza haemagglutinin (HA) protein (112).

Tripartite motif-containing proteins (TRIMs)

Among ISGs, tripartite motif-containing proteins (TRIMs) are endogenous proteins that can restrict virus infection. Most TRIM proteins have three conserved N-terminal domains – RING, B-box, and coiled-coil; however, the C-terminal is less conserved, containing either PRY domain and/or SPRY domain (126). The RING domain contains a zinc finger motif possessing E3 ubiquitin ligase activity, whereas the PRY-SPRY domain is often employed as a protein-protein interaction site (126). The antiviral activities of TRIM proteins are due to multiple mechanisms, including degradation of antibody-opsonized non-enveloped viruses (TRIM21), proteasome-dependent protein degradation of virus proteins (TRIM5 α , TRIM22), and formation of nuclear bodies to enhance production of antiviral molecules (TRIM19) (127). For flaviviruses, TRIM5 α and TRIM79 α inhibit TBFVs by inducing proteasomal degradation of NS3 and NS5, respectively (128, 129).

Vaccines against flaviviruses

Flaviviruses cause dangerous infectious diseases. However, because there is no approved anti-viral drug, vaccination is the only way to protect from infections or severe disease outcomes. Among vaccines, there are only TBEV, JEV, KFDV, YFV, and DENV vaccines available for human uses. All licensed TBEV and KFDV vaccines are based on inactivated whole virus and have a high protection level. However, TBEV vaccines are less effective in older individuals: more than 70% of vaccine failure cases are in individuals aged over 50 years (130, 131). YFV is based on the live attenuated 17D strain generated by passaging in mouse and chicken embryo tissues. Compared to the wild type YFV, the capacity to induce IFN-I response of the 17D strain is reduced (132). JEV vaccines are based on either live attenuated or inactivated virus (133). DENV is a chimeric vaccine based on substitutions of the prM and E proteins of YFV-17D with those of the 4 DENV serotypes (134). Administration of DENV vaccine to individuals who are naïve to any DENV serotypes elicits sensitivity to

dengue disease later due to ADE (135, 136). Moreover, the vaccine increases the hospitalization rate in children 2–5 years old (136, 137). Interestingly, DENV infection does not enhance ZIKV infection later, but the primary infection of ZIKV can enhance subsequent DENV infection via ADE (138), suggesting that ZIKV vaccine development should carefully consider avoiding ADE (139). Furthermore, as WNV vaccines are currently only approved for horses, a safe and effective vaccine platform against the virus is needed for humans.

Ebola virus

Ebolaviruses are enveloped, single-stranded negative-sense RNA virus with a genome of approximately 19 kb. The genus *Ebolavirus* consists of six distinct species: *Zaire ebolavirus*, *Bombali ebolavirus*, *Bundibugyo ebolavirus*, *Sudan ebolavirus*, *Tail Forest ebolavirus*, and *Reston ebolavirus* (140). In the genus, *Zaire ebolavirus*, commonly called Ebola virus (EBOV), is one of the most dangerous viruses with fatality rates between 25 and 90% (141). Currently, the World Health Organization has prequalified two EBOV vaccines: the recombinant vesicular stomatitis virus-based vector vaccine and the recombinant adenovirus-based vector combined with the vaccinia virus-based vector vaccine. As the virus is highly dangerous, we explored the potential of developing other vaccine platforms against the virus based on the available flavivirus vectors.

Aims of the thesis

This doctoral thesis aims to provide insights into important interactions of flaviviruses and host cells at multiple stages of the flavivirus life cycle. Furthermore, we also aim to identify and characterize the elicited host antiviral proteins during virus infection. The knowledge about these essential interacting checkpoints could be used to enhance the efficacy of flavivirus genome-based vaccines in the future. Thus, we attempt to establish and characterize a vaccine platform based on the modified WNV_{KUN} genome.

The specific aims of each paper:

- I. To characterize roles of the membrane-remodeling protein LNP during flavivirus infection and to determine a virus domain interacting with LNP.
- II. To characterize roles of ESCRT proteins during TBFV infection and interactions between the ESCRT proteins and the virus proteins. To identify the host cell response to inhibit the ESCRT proteins during the virus infection.
- III. To identify novel proteins that are essential for virus replication or assembly at the ER, and proteins that are induced to counteract these stages of the flavivirus life cycle.
- IV. To establish a vaccine platform based on the WNV_{KUN} RVPs, which can be used as a vector to transduce cells expressing EBOV antigens. To characterize seroconversion to antibodies against WNV_{KUN} and EBOV proteins after administration of the RVPs into mice.

Methods and materials

Molecular cloning

Molecular cloning generates DNA-based tools for molecular, biochemical, and imaging methodology. The technology emerged in the early 1970s after the discovery of restriction endonuclease enzymes. Molecular cloning refers to isolation of DNA sequences, which are often genes of interest (GOIs), insertion of the genes into a DNA vector for amplification, and expression inside host cells (either prokaryote or eukaryote). The inserted DNA can be obtained by using restriction enzymes to digest the DNA from plasmids, or specifically amplified by polymerase chain reactions (PCR). The isolated DNA is then ligated into a linearized vector plasmid, which is a self-replicative circular dsDNA inside *Escherichia coli*. The plasmid is then transformed into *E. coli* for propagation, and the transformed bacteria are selected by antibiotics (either ampicillin or kanamycin in the studies) by virtue of the antibiotic resistant gene cassette existing on the plasmid. For gene expression, the isolated gene is designed to be inserted in the plasmid forming an in-frame gene construct having a promoter upstream of the inserted gene and a pA tail at the downstream. Furthermore, the inserted gene can be fused in-frame with a reporter gene like luciferase or fluorescent protein-coding genes on the plasmid to monitor expression of the GOIs inside cells.

Cell culture

In our studies, we used established commercial mammalian cell lines under controlled conditions. The cells were cultivated in Dulbecco's Modified Eagle's Medium containing 1 g/L glucose supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin-streptomycin at 37 °C in 5% CO₂. To characterize functions of host cell proteins, we used the adenocarcinoma human alveolar basal epithelial cell line (A549) because it is a human cell line with high transfection efficiency and susceptibility to flavivirus infections (142). The baby hamster kidney (BHK-21) cell line was used to retain replicons as it has been traditionally used for flavivirus adaptation (143, 144). Vero cells, a kidney epithelial cell line from an African green monkey, was used for plaque assay and virus production as the cells are susceptible to flaviviruses, defects in the IFN-I response (145),

and have not lost their contact inhibition, generating a monolayer ideal for plaque assays (146, 147).

Generation of cell lines

Exogenous proteins can be transiently expressed by transfecting the gene construct cloned into plasmid vectors. However, the expressions rely on the transfection efficiency and decline gradually. To overcome these challenges, stable cell lines expressing foreign genes can be generated by either the lentivirus system or by transfection of a plasmid, which has an antibiotic resistance gene cassette for cell selection. In the first case, produced lentivirus particles packaging the GOIs can infect cells and transduce these cells by integrating the GOIs into the host cell genome. In our studies, we generated cell lines expressing GOIs by a lentivirus-independent system. The GOIs were fused with another gene cassette expressing an antibiotic resistance gene. Transfected cells with the gene construct are selected by the corresponding antibiotic.

In **papers I and II**, we generated cell lines expressing the WNV_{KUN} or LGTV replicons. Here, the replicon constructs were cloned into vector plasmids under control by a T7 promoter. An internal ribosome entry site (IRES) followed by a neomycin/kanamycin resistance (Neo/KanR) gene was inserted into the variable region of the 3'UTR, which confers kanamycin or G418 antibiotic resistance. The linearized DNA plasmid was *in vitro* transcribed by the T7 RNA polymerase and capped to generate a mimic RNA replicon product before transfecting into BHK-21 cells. Inside the cells, the RNAs were cleaved by HDVr at the terminus of the 3'UTR, generating the RNA replicons (Figure 6). Transfected cells expressing the RNA replicons were selected by supplementing the media with the G418 antibiotic. In **paper III**, we generated cell lines expressing TRIM proteins. Commercial vector plasmids expressing both TRIM proteins and the Neo/KanR gene were transfected into A549 cells, selected by the addition of G418. In **paper IV**, we generated a cell line expressing the WNV_{KUN} C-prM-E. Here, the gene expression was under the control of the CMV promoter, and an IRES followed by a Neo/KanR gene were added downstream the C-prM-E gene. The plasmid was linearized before transfecting into BHK-21 cells. Stable transformants were established by cultivating the cells in G418-supplemented medium.

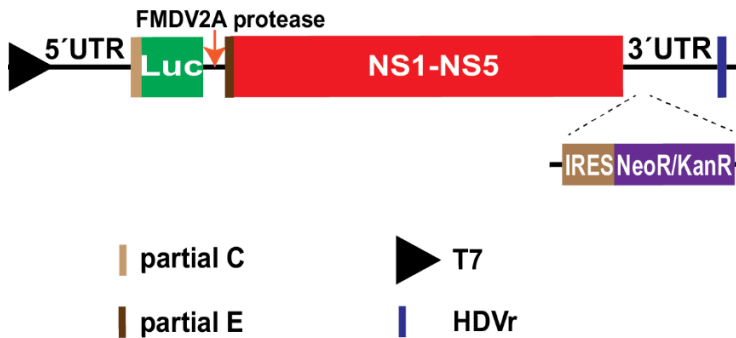


Figure 6. Schema illustration of the construct expressing the WNV_{KUN} RNA replicon used in the study. The construct is driven by a T7 promoter for *in vitro* expression, followed by the replicon genome and the HDVr, a process similar to the one used to create the DNA replicon construct described in Figure 5. A gene cassette expressing neomycin and kanamycin resistant (NeoR/KanR) gene driven by an internal ribosome entry site (IRES) was inserted inside the variable region of the 3'UTR.

Gene silencing using small interfering RNAs (siRNAs)

Post-transcriptional gene silencing (PTGS) was first observed in transgenic petunia plants, where attempts to overexpress endogenous genes resulted in blocking of the gene expressions (148). The process is initiated by cleavage of dsRNA by a dicer endonuclease into duplexes of siRNAs. Incorporation and processing of the siRNA duplex by the RNA-induced silencing complex and the argonaute protein generate a single strand siRNA-protein complex. The complex binds complementary to mRNA and cleaves the mRNA, downregulating expression of targeting genes (149).

In papers I–III, we used commercially modified ds siRNAs with enhanced specificity, characterized by microarray. Furthermore, each set of siRNAs contained a pool of four different algorithms-designed siRNAs, which increased the potency of siRNA treatment (150). The siRNAs were transfected into cells using a RNAiMAX lipofectamine specialized for siRNA transfection, which increases the transfection efficiency and reduces cytotoxicity (151). PTGS was characterized by either western blotting (papers I and II) or qPCR (paper III).

Concentrating virus particles and isolating the ER membrane by ultracentrifugation

Flavivirus particles can be concentrated under ultra-high-speed centrifugation, which is larger than $100,000 \times g$ (152). To achieve such a high speed, a vacuum environment is required as it reduces the friction between the rotor and the air inside the centrifuge chamber. In our studies, infectious virus particles and RVPs were concentrated and separated from organelles and microsomes by allowing particles to sediment through a 25% sucrose cushion under $150,000 \times g$ speed for 2.5 h at 4°C (153). To purify ER membrane (**paper III**), the precleared cell lysates were loaded on a gradient of sucrose from 1.5 M to 1.3 M to 1 M (51%, 44.5%, and 34%, respectively) and centrifuged at $150,000 \times g$ for 70 min at 4°C . By virtue of the density and sedimentation coefficient of the ER, the membrane was concentrated as a single band on the gradient of sucrose after centrifugation. The ER band were collected and subsequently concentrated by centrifugation at $126,000 \times g$ for 45 min at 4°C (140).

Plaque assay

Plaque assay is a standard assay to determine concentration of lytic infectious virus particles. Here, a monolayer of Vero cells (**papers I–III**) were inoculated for 1 h with a serial dilution of virus with unknown concentration. The media were removed and replaced with an overlay of fresh media containing 1.2 % Avicel, which indiscriminately restricts viral infection after the virus inoculation. Viruses from infected cells will continue spreading to surrounding cells, inducing cell lysis, and eventually result in increasing distinct zones of cell death (plaques) after 2–5 days, depending on the infectious kinetics of viruses. After incubation, the overlay was removed, and the cell monolayer was fixed and counterstained with crystal violet 1%, which reveals distinct plaques. Based on the number of plaques at a specific dilution and a known volume used to inoculate cells, concentration of infectious particles can be determined, assuming that one plaque was generated by one infectious particle. In some cases, viruses do not generate distinct lytic plaques in a short time range of a cell passage. In these cases, virus proteins are visualized by immunostaining. In **papers I–III**, an antibody against anti-TBEV E protein was used to immuno-stain and ultimately visualize foci of LGTV-infected cells.

Quantitative real-time PCR (qPCR)

qPCR quantifies gene expression at the RNA level. Here, the amount of an expressed gene in cells can be estimated by calculating its relative ratio to the amount of constitutively expressed genes (housekeeping genes) or “absolutely” by extrapolation from external standard curves of Ct values versus known gene copy numbers. To detect and quantify gene expression from small amounts of RNA in cell lysates, amplification of the gene transcript is necessary. RNA is reverse transcribed into cDNA, which is used as templates for amplification by PCR. To monitor the DNA amplifications in real time, two established techniques are often used. In the first one, a dsDNA dye like SYBR Green is added to the qPCR assay, which stains the amplified PCR products. Thus, omitted fluorescent signals increase by each PCR cycle. The accuracy of this assay is reduced as putative primer dimers in the PCR assay also may be stained by the dye. In our studies, the TaqMan assay was used to monitor the real time PCRs. Rather than using a dye, we used a fluorescent reporter DNA-based probe complementary binding to a specific DNA sequence within the PCR amplicon. At the two terminuses of the probe, there is a fluorescent reporter and a quencher of fluorescence, which prevents the release of fluorescent signal. During the elongation step of PCR, the 5' to 3' exonuclease activity of the Tag DNA polymerase degrades the probe, releasing the reporter from the quencher which fluoresce when excited with a laser. Thus, as PCR product increases with each PCR cycle, generating a proportional increase of the fluorescent signal.

In qPCR, numbers of PCR cycles (Ct) required for the fluorescent signal to cross a threshold level determine amount of input nucleotides. When there is higher amount of RNA in the samples, fewer cycles are required to reach the threshold of the fluorescent signal. In our studies, genome copy numbers of viruses or replicons from the materials were determined by extrapolation from the standard curves of Ct values from known genome copy number solutions. The genome copy numbers of the solutions for constructing the standard curve were determined by multiplying the moles of DNA replicon constructs with the Avogadro constant. In **paper III**, expressions of TRIM genes were normalized by expression of the house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Luciferase assay

As the firefly (*Photinus pyralis*) luciferase gene was used as a reporter gene of our replicons, a luciferase assay was used to monitor the expression. In the replicon constructs, the luciferase gene was fused in-frame after the first 20–27 codons of the C protein depending on the replicons, which was followed by the FMDV2A gene coding an autoprotease to release the luciferase enzyme during the polyprotein expression. The level of polyprotein/luciferase expression can be monitored by the intensity of luminescence measured by a luminometer, supplementing the substrates of the enzyme: luciferin and ATP. To reduce experimental variability resulting from differences in cell viability or transfection efficiency, a second plasmid expressing renilla (*Renilla reniformis*) luciferase was co-transfected with the replicons and used as an internal control in **papers II and III**. The firefly luciferase normalized by renilla luciferase can be measured from transfected cell lysates using the Dual-Luciferase® Reporter Assay System. In this system, the firefly luciferase reporter is initially measured after adding Luciferase Assay Reagent II containing substrates of this enzyme. The signal is then quenched, and the second type of luminescence produced by renilla luciferase are released by adding The Stop & Glo® Reagent.

Coimmunoprecipitation (CoIP)

Coimmunoprecipitation (CoIP) is used to study protein-protein interaction, and the proteins can be either endogenously expressed or overexpressed. Furthermore, the technique can be used to isolate the proteins of interest by pre-knowing the interactions. In **papers I and II**, gene constructs expressing virus proteins tagged with mCherry (a red fluorescent protein) or human influenza HA were transfected into cells. Inside the cells, these proteins are expressed and may interact with host protein. Cell lysates were incubated with a mix of antibodies binding to the tags and nanobeads. Next, the mixture was exposed to a magnetic field, which retains the complexes of bead-antibody-virus proteins and host proteins. The host proteins were released from the complexes by dissolving them with surfactants such as lithium dodecyl sulphate (LDS) before western blot analysis (**papers I and II**) or with acidic solutions such as formic acid (0.5%) before mass spectrometry analysis (**paper III**).

Western blotting (WB)

Western blotting (WB) is a standard technique to analyze proteins qualitatively and semi-quantitatively. WB separates proteins from lysates by gel electrophoresis. The input proteins are often treated with strong surfactant such as LDS to break the proteins' non-covalent bonds, to degrade the protein tertiary structure, and to mask the intrinsic charges on the proteins. Thus, the protein separation by the electrophoresis relies solely on protein mass, not charge and size. Separated proteins on gels are then transferred to membranes, which are made from either nitrocellulose (**papers I, II, and IV**) or methanol-activated polyvinylidene difluoride. To detect the proteins on the membranes, the proteins are stained with specific antibodies. Here, the membranes are initially blocked by milk proteins or bovine serum albumin (BSA) to reduce non-specific bindings of the antibodies to the membranes. The membranes are then incubated with primary antibodies, which can bind specifically to the target proteins. Secondary antibodies labeled with probes such as fluorescence proteins, biotin, or enzyme conjugates (e.g., alkaline phosphatase or horseradish peroxidase) (in **paper I, II, and IV**) are used to bind to the primary antibodies. In the case of using enzyme-conjugated antibodies, substrates of the enzymes are added to the membrane, which produces detectable chemiluminescent signals that are captured by a charge-coupled device camera. To semi-quantify protein expression, the intensity signals obtained from WB of targeting proteins were normalized to expression level of house-keeping proteins such as GAPDH in **paper II**. Furthermore, modifications of proteins such as phosphorylation, glycosylation, and covalently polymerization of proteins during PTMs can be studied by comparing non-treatment and treatments of protein lysates to remove the modifications before gel electrophoresis.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a plate-based technique for detecting and quantifying protein substances such as peptides, cytokines, antibodies, and hormones. As with WB, ELISA is based on the binding of antigens and probe-labeled antibodies. In **paper IV**, antigens were coated on the plates before blocking the plate with BSA to cover all uncoated sites. Diluted sera from immunized mice were added to each well for antigen-antibody binding. An anti-mouse secondary antibody labeled with horseradish peroxidase was then added before the addition of the

3,3',5,5'-tetramethyl-benzidine substrate for colorimetric development. The concentrations of antibodies in the sera were extrapolated from a standard curve of antibody concentration-predetermined solutions supplied in the ELISA kit versus their absorbance values.

Laser scanning confocal microscopy

Since Anton van Leeuwenhoek used a microscope to observe bacteria, spermatozoa, and red blood cells in 17th century, many generations of microscopes have been developed, including the laser scanning confocal microscope. In conventional widefield optical epi-fluorescence microscopes, all emitted fluorescent signals are collected from the large illuminated areas on specimens, which hinders resolutions of the signals at the objective focal plane. However, confocal microscopes use lasers as a light source and these lasers excite specific fluorophores at an objective focal plane, removing the background fluorescence of the whole specimen. In addition to the laser beam, the out-of-focus emitted light can also be re-filled from the detector by virtue of a pin hole in the system, which allows only the desired focus emitted light to reach the detector. This system improved resolutions of the obtained images in **papers I–IV**.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) uses electrons rather than photons as the excitation beam and a detector collecting the emitted electrons from the specimen. Thus, TEM functions like an optical microscopy system, which electrons and magnetic lenses are employed rather than photons and glass lenses. One advantage of the TEM is the super high-resolution image produced, compared to other optical microscopy system. In microscopy, resolution is defined as the ability to distinguish two distinct points on images, which is proportional to the wavelength of the light source and inversely proportional with the numerical aperture of the lens. As electrons have smaller wavelengths compared to photons, the TEM system overcomes the spatial resolution limits at around 200 nm of the optical microscopy. Thus, TEM can capture reliable images with high resolutions at high magnifications, such as the RVPs in **paper IV** (flavivirus particles are approximately 50 nm in diameter).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Liquid chromatography-tandem mass spectrometry (LC-MS/MS), an advanced technique for analyzing proteins, was used in **paper III**. Here, the proteins are cleaved into small peptides before injected into the LC system for separation. The separated peptides are then ionized at the interface between the LC and the MS system before entering the MS system to detect the mass-to-charge (m/z) ratio of ionized peptides. In analytical chemistry, these m/z ratios reveal the detected peptides and are used to identify the proteins. A second MS system can be employed, tandem mass spectrometry (MS/MS), to enhance the detection resolution. Ionized peptides of a particular m/z from the first MS are obtained and then split into smaller fragments by high collision-induced dissociation before entering the second MS for separation and detection based on their m/z ratio. This second fragmentation step separates ions that have similar m/z ratios in the first MS.

To quantify the proteins in the samples, digested peptides can be chemically labeled by isobaric mass tags (TMT) before entering the LC-MS/MS system. The TMT is a set of molecules with the same mass but generate unique “reporter ions” after fragmentation by MS. At the end of the LC-MS/MS, intensities from each variant of reporter ions were detected to determine the abundances of the peptides.

Animal experiments

In **paper IV**, six-week-old female BALB/cN mice were used. These mice are albino and were inbred in 1923 by McDowell. As the mice polarly induce helper T cell type 2 response, supporting generation of antibodies after immunization (154), they are highly suitable for vaccine studies. Mice were subcutaneously administrated with RVPs or phosphate-buffered saline three times before euthanization. All animal experiments were conducted following ethics approval (4570-2019) by the Regional Animal Experimental Ethics Committee in Stockholm (North), Sweden.

Statistics

Statistical differences between the means were determined using Student's t-test (2 groups comparison) or one-way ANOVA (multiple group comparisons) followed by the Bonferroni post hoc test. $p < 0.05$ was considered to be a statistically significant difference in the comparison of groups. GraphPad Prism version 5 (**papers I and IV**) or version 9 (**papers**

II and III) were used for all statistical analyses. The values are presented as the mean \pm the standard error of the mean.

Results and discussion

LNP is essential for virus replication

As LNP functions in the maintenance of the three-way tubular junctions within the ER (102, 103), which are highly curved structures similar to the Ve, we postulated that LNP may play a role in virus replication and generation of the Ve. In **paper I**, we found that the depletion of LNP mediated by siRNA revealed that the protein plays an important role in virus production. To specifically study the role of LNP during the replication stage of the virus life cycle, we generated cell lines expressing the WNV_{KUN} or LGTV replicons, which represent MBFVs and TBFVs, respectively. By measuring the expression of the reporter gene luciferase from the replicon and replicon genome copy numbers during LNP depletion, we demonstrated that LNP plays essential roles in virus replication. Using TEM imaging, we showed that LNP is important for generation of the Ve. More imaging techniques could be conducted such as immune gold staining with antibodies targeting the Ve and measuring the exhausted area, which indicates the Ve.

LNP is recruited by the virus NS4B protein

As NS4A and NS4B are two virus proteins important in the generation of the Ve (26-28), we hypothesized that the LNP's roles in formation of the Ve are due to the interaction between LNP and either of the two virus proteins. Immunofluorescent staining and CoIP revealed that LNP interacts with NS4B, and the C-terminus containing the transmembrane (TM) domain 4–5 of NS4B is crucial for the interaction.

The roles of LNP during flavivirus replication are similar to other ER three-way junction inducers and stabilizers such as RTNs (155, 156) and ATLS (100, 101). Indeed, RTN3 and ATL2 have been shown to play roles in the generation of the Ve by interacting with NS4A of WNV_{KUN}/DENV-2/ZIKV (60) or NS3 of DENV-2/ZIKV (62), respectively.

The two ESCRT proteins – ALIX and CHMP4A – are required for TBFV life cycle

In **paper II**, we characterized the roles of three important ESCRT proteins – TSG101, ALIX, and CHMP4A – during TBFV infection. During siRNA treatments, we showed that TSG101 is redundant, while ALIX and

CHMP4A are important for virus production. Using cell line expressing replicons and the RVP platform, we found that ALIX has roles in virus replication and CHMP4A has roles in virus assembly, respectively.

ALIX and CHMP4A are recruited by the TBFV NS3 and E proteins

As ALIX and CHMP4A proteins are essential for the virus life cycle, we investigated the mechanism underlying their functions. Using immunofluorescent staining, we showed that ALIX is recruited to the Ve and the NS3 protein. Using CoIP and direct mutagenesis, we showed that the L domain motif LYXLA within NS3 interacts with ALIX and CHMP4A, whereas the E protein interacts only with CHMP4A. The identified motif is conserved in the TBFVs with the polar YT/S residues flanked by non-polar residues. However, we found that the equivalent ISTRV motifs within NS3 of the MBFVs differ to the putative TBFV L domain motif (Figure 7). Therefore, MBFVs may have other L motifs within NS3. Indeed, another putative L domain motif within the YFV NS3 protein supporting ALIX employment has been previously proposed (157).



Figure 7. Alignment of the conserved late (L) domain motif LYTLA starting at the 1795th codon of the polyprotein, using TBEV_{Toro} as the reference virus.

ISG15 protein can restrict ALIX and CHMP4A during TBFV infection

To counteract the employment of ESCRT proteins by viruses, the host cells may induce defense mechanisms. We illustrated that expression of ISG15 or HERC5 reduced virus replication by eliciting degradation of ALIX and CHMP4A. However, we did not eliminate the roles of ISG15 in targeting other host proteins and other stages of the flavivirus life cycle (124, 125). ISG15, indeed, has been shown to restrict ZIKV entry (158) and DENV release (159). Although ISGylation has been shown during DENV infection (160), we could not identify any signs of ISGylation dur-

ing LGTV infection within A549 cells. Thus, in this paper, we illustrated that the unconjugated ISG15 and HERC5 can inhibit LGTV replication by triggering ESCRT protein degradation (Figure 8). Indeed, ISG15 or HERC5 can, independent of ISGylation, restrict infections of chikungunya virus (161) and HIV-1 (162).

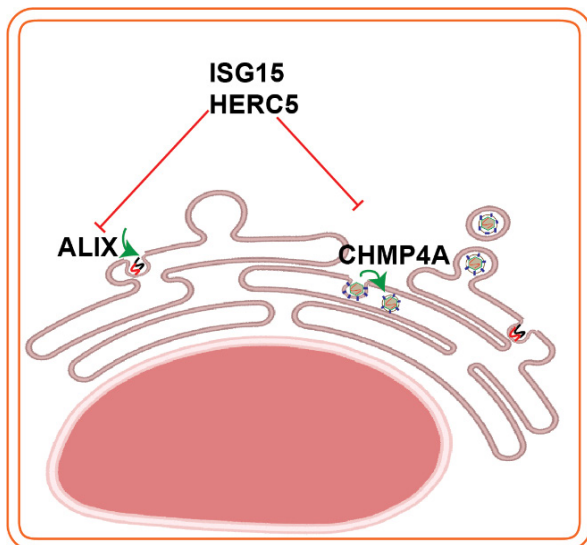


Figure 8. Schema illustrates roles of the ESCRT proteins and their interplay with ISG15/HERC5. ALIX is important for virus replication and CHMP4A is important for virus assembly. To counteract the employment of ALIX and CHMP4A during the virus life cycle, infected cells may induce ISG15/HERC5 to degrade the ESCRT proteins. The figure was generated using the webtool Biorender.com.

Identification of antiviral proteins – TRIM21 and TRIM14 – restricting LGTV and ZIKV infection

In paper III, by screening for enriched host proteins located at ER and pulled down by the NS1 and NS4A antibodies during WNV_{KUN}, LGTV, ZIKV infection, we identified three TRIM proteins – TRIM38, TRIM21, and TRIM14. Using cell lines expressing these proteins or by siRNA treatments, we illustrated the essential antiviral roles of TRIM21 and TRIM14 against LGTV and ZIKV infection. TRIM38 can also help inhibit ZIKV. Using the DNA replicon platforms, we demonstrated that TRIM21 and TRIM14 restrict replication of LGTV. As ubiquitinating

proteins, TRIM21 and TRIM14 can target virus proteins or host proteins supporting the virus via proteasome-dependent protein degradation (127). Indeed, in the context of influenza virus infection, ubiquitination of the virus nucleocapsid protein by TRIM14 induces degradation of the protein (163). Furthermore, TRIM21 can ubiquitinate the mitochondrial adaptor molecule, enhancing the innate immune response to Newcastle disease virus and Sendai virus infection (164). In the context of flaviviruses, up-regulation of TRIM21 has been reported during JEV infection (165), but the antiviral mechanisms of both TRIM14 and TRIM21 remain unclear. Therefore, to further elucidate the antiviral mechanisms of TRIM14 and TRIM21, future studies should identify the flavivirus proteins targeted by these proteins.

The WNV_{KUN} RVPs infect cells in a single cycle and can be used as a delivery vector for vaccine development

In paper IV, we established a platform to generate the WNV_{KUN} RVPs and characterized their potentials as a vector to transduce cells, expressing EBOV antigens. We initially enhanced the production of RVPs by generating a cell line expressing C-prM-E as the packaging system for the WNV_{KUN} replicons. We transfected cells with the WNV_{KUN} construct C-prM-E-IRES-NeoR/KanR and selected for transformants. This strategy is safer than using a lentivirus system as we eliminated the risk of unexpected recombination of lentivirus with WNV_{KUN} packaging genes. Using qPCR and TEM imaging, we characterized the generation of RVPs that could infect cells in a single cycle.

Furthermore, we generated WNV_{KUN} RVPs that deliver the EBOV envelope GP and the matrix VP40 genes into cells by substitution of the Luc gene of the replicon with the EBOV genes. As there is no EBOV capsid protein in the system, these envelope and matrix proteins cannot function as an EBOV packaging system, eliminating the risk of forming chimeric WNV_{KUN}-EBOV. In fact, we demonstrated that the RVPs carrying these genes infected cells in a single cycle. Administrated mice with these RVPs survived and showed no differences in weights and health status, compared to the control groups. Finally, we showed that RVPs elicited seroconversions to produce the antibodies against the NS1 and E proteins of WNV in all immunized mice and EBOV proteins in half of the vaccinated mice. Further optimizations can be conducted in the future to enhance the potential of WNV_{KUN} RVPs as a vaccine platform.

Conclusion

In summary, this thesis sheds new light into flavivirus-host cell interactions and host response to virus infection, using molecular, biochemical, and imaging methodology. The insights from the first three studies can be used to enhance the potential of the WNV_{KUN} RVP platform as a vaccine strategy established in the last paper. The significant findings of each paper are summarized below.

Paper I: The ER membrane-remodeling protein LNP acts during flavivirus infections. Using the established replicon-expressing cell lines, we highlighted the important roles of LNP for virus replication and the generations of the Ve. During the virus infection, LNP can be employed by the C-terminus of NS4B.

Paper II: During TBFV infection, two important members of ESCRT protein, ALIX and CHMP4A, are present. ALIX plays a role in virus replication and CHMP4A functions in virus assembly. The ALIX-CHMP4A complex is recruited to NS3 through the putative L domain motif within NS3 and CHMP4A interacts with the E protein. Furthermore, ISG15 and HERC5 can induce degradations of ALIX and CHMP4A, indirectly hindering the virus infection.

Paper III: By screening for enriched host proteins targeting flavivirus replication at the ER, we identified TRIM21 and TRIM14 as antiviral proteins restricting replication of LGTV and ZIKV.

Paper IV: In this study, we established a platform to enhance WNV_{KUN} RVP production by transfecting the replicon into a cell line expressing C-prM-E. Generated WNV_{KUN} RVPs were modified to transduce cells to express EBOV GP and VP40 in a single round. The RVPs induced seroconversions to have antibodies against EBOV GP, EBOV VP40, WNV NS1, and WNV E proteins.

Future perspective

During infection, various host proteins are employed either to support or to restrict the virus life cycle. In this thesis, we identified and characterized interacting checkpoints between virus-host proteins, which may have therapeutic applications, including vaccine development. Indeed, the viral motifs targeted by ISGs can be modified on the RVP systems, allowing the replicons to bypass the antiviral effects of ISGs during cell transduction, increasing the potentials of RVPs as a vaccine strategy. Furthermore, the cell lines expressing the replicons and the RVPs established in the studies can be used as a platform to screen for antivirals targeting the replication and assembly stages. The characterized virus motifs required to recruit host proteins (LNP, ALIX, and CHMP4A) can be used as targets for antiviral development. In addition to the general future perspective for this thesis, we highlight the perspectives to further elaborate the findings in each paper more thoroughly in the future and translate them into applications.

We found that LNP functions in the *Ve* generation, and NS4B C-terminus is important for LNP recruitment. Future studies could focus on identifying motifs crucial for the NS4B-LNP interaction. Furthermore, the LNP protein can be ubiquitinated, which inhibitions of the activity elicits neurodevelopmental defects and enhanced sensitivity to epilepsy (108). These outcomes are similar to those of neurotropic flavivirus infection. We postulate that the LNP-NS4B interaction during flavivirus infection may restrict the LNP ubiquitination, resulting in neural cytopathic effects. In addition, flavivirus infections can induce cell cycle arrest and attenuate the mitosis rate (166-168). During mitosis, the role of LNP in the maintenance of the three-way junctions of ER tubules is inactivated by protein phosphorylation, resulting in more sheet-like ER (105). Thus, it will be important to elucidate whether flavivirus can antagonize the inactivation of LNP by phosphorylation to support the *Ve* maintenance.

We characterized the employments of ALIX and CHMP4A during TBFV infection at different stages of the virus life cycle. To restrict this recruitment, cells can express ISG15, inducing degradation of the ESCRT proteins. However, we have not elucidated the mode of protein degradation, which can depend on either proteasome or lysosome. In addition, although we have shown that NS3 can interact with the ESCRT proteins,

it remains unclear whether the virus protease can cleave ISG15 to antagonize its antiviral function.

We identified TRIM21 and TRIM14 as antivirals that target virus replication. In the future, we will characterize the mechanisms behind these antiviral effects by identifying the virus proteins or upsurged host proteins for virus replication that are targeted by the TRIM proteins. The identifying interaction motifs within the virus proteins can be used to develop replicon/RVP vaccine candidates that can elude the endogenous antivirals in infected cells, enhancing the expression of GOIs.

We developed a vaccine platform based on the WNV_{KUN} RVPs that can be used as a vector to express GP or VP40 EBOV in transduced cells. We showed that the RVPs induced seroconversion to EBOV GP, EBOV VP40, WNV E, and WNV NS1. WNV_{KUN} RVPs conveying EBOV VP40 to elicit immunological response were shown for the first time. In the future, we intend to co-express both GP and VP40 in the RVP platform, which may enhance seroconversion to VP40 as their co-expression can enhance VP40 release by 40-fold (169). The release of VP40 virus-like particles (VLPs) indeed relies on the ubiquitination of the PPxY motif within the VP40, followed by recruitments of the ESCRT proteins (170, 171). Thus, such an enhanced ubiquitinated VP40 with more L motifs can be generated to enhance the release of VP40 VLPs, inducing higher seroconversion. Furthermore, we want to translate our findings in virus-host cell interactions in **paper III** to generate the replicons that can bypass the antiviral effects of TRIM proteins. Such replicons can last longer in the transduced cells to express GOIs, improving the seroconversions by RVPs. The potential vaccine against EBOV and WNV should also be further characterized for T cell response, neutralizing antibodies, and animal survival after challenges with virus infections.

Acknowledgement

This thesis would not be achieved without invaluable helps and supports from my supervisors, colleagues, friends, and family. I would like to take this opportunity to express my sincere gratitude to those who has always by my side during the journey of becoming a Ph.D.

First of all, I would express my gratefulness to my supervisor **Prof. Magnus Johansson**, who has trusted in me and gave me the opportunity to become a Ph.D. student at Örebro University. You have been a good leader, encouraging a liberal and professional working environment within the group. I am thankful for your conscientious reviews and suggestions, which improved my manuscripts.

Assistant Prof. Wessam Melik, you are a great supervisor and friend. Your knowledge and experience in the molecular biology of flavivirus are extensive and impressive. I appreciate your initiations in many projects and your valuable comments during reviewing the manuscripts in this thesis. Thank you for your mentorship and supports during difficult times over last years. I did enjoy the fika times, discussing about science, life, politics, philosophy, and so on.

To my co-supervisor **Dr. Naveed Asghar**, I appreciate your supports during the Ph.D. student time, conferences, introductions in the first year, and meticulous reviews for the papers in the thesis.

I thank the co-supervisor **Prof. Sören Anderson** for your advice in many projects.

I appreciate previous colleagues in the group, also Wessam, Naveed, and **Dr. Sezin Gunaltay-Schenk**, who generated some molecular tools for the thesis. I thank Sezin for your advice and recommendations to recruit me for this Ph.D. position. I thank **collaborators and co-authors** for important inputs in this thesis. I thank **Dr. Anders Karlsson** and **Dr. Roger Karlsson** for supporting in proteomics experiments. I thank **Dr. Urban Hoglund** for supporting in animal experiments. I thank **Prof. Ali Mirazimi** for EBOV gene constructs. I thank also other scientists and organizations, who generously gave me other reagents and materials for the studies.

I appreciate the supports from the study administrators, **Susanne J, Julia K, and Sofia L.**

I thank for supports I got from the members of KFC staff, especially for introductions in using machines at the USO campus by **Anders, Zarah** and assisting in purchasing/delivery of reagents by **Lena.**

I thank external reviewers and opponents from the journals, conferences, and members of the half-time and final Ph.D. defense committee who gave and give me valuable comments and inquiries to amend the papers and the thesis.

I appreciate the supports, advice, scientific discussions, and encouragements from friend and colleagues at Örebro university, **Anna V, Amani, Amelie W, Assim, Alex P, Berhane, Frida F, Kaja, Kristine, Geena, Madelene, Mulugeta, Julia R, Julia S, Ole, Omer,** and more.

I am grateful to my **first biology teacher**, who inspired me by her excellent pedagogic skills. Every lesson with her was adventurous time and full of pleasure. A praise also for all **other teachers** who has enlightened my journey of educations.

I sincerely apologize if I forgot to acknowledge anyone for what they have done for me or taught me lessons within or beyond the schools during past years.

I dedicate my gratitude to my beloved family and other friends in life. My **father and mother**, you raised a soon-to-be Ph.D. I also thank **my aunts** and **my brother**. My academic achievement cannot be possible without your love, orientations, and sacrifices. I thank my greatest friends, **Thanh Thuy** and **Uy Vien**, for their patiently supports and encouragements during the toughest times in life and thus, deserve to share the glory with me. Thank **Hrishikesh** and **Pieter C** for your disinterested supports during the student time in Leuven. Thank **Dr. Luyen V** for scientific discussions and advices in life. Cheek kisses to **Levi** and **Stella**. Big embraces to all other friends in my life.

And the final words are for **myself**, for being strong to accept my mistakes, and for being consistent in pursuing dreams.

This thesis was supported by Örebro University and grants from the Swedish Knowledge Foundation.

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