UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Technology

Melis Nur Konno

Generation of Flaviviral Replicon Expressing Stable Cell Lines

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Supervisor(s):
MD, DSc Eva Zusinaite

MSc Sandra Koit

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Abstract:

Flaviviruses are a group of arboviruses that are considered re-emerging pathogens which can cause severe illnesses in vulnerable populations. Although a significant issue for the healthcare system, there are no effective treatments available, and flavivirus countermeasures rely on preventative actions like the control of vector spread and vaccination. The majority of flaviviruses are biosafety level 3 (BSL3) agents, which necessitate special precautions when working with infectious viruses. This complicates the development of antivirals and vaccines. To address this issue, when investigating the antiviral mechanism of inhibitors at lower biosafety levels, subgenomic replicons can be used to study viral replication events without virus entry or assembly. In this work, we generated stable cell lines based on flavivirus replicon RNAs, including reporter marker genes and the puromycin N-acetyltransferase (PAC) gene. The resulting puromycin-resistant stable cell lines persistently expressed non-structural proteins and fluorescent or luminescent reporter proteins. These stable cell lines can be used for high-throughput screening of potential replication inhibitors for the development of novel antiviral therapies against flaviviruses.

Keywords:

flaviviruses, TBEV, KUNV, replicon, stable cell line, non-structural protein, puromycin N-acetyltransferase

CERCS: T490 Biotechnology

Flaviviiruste replikone ekspresseerivate stabiilsete rakuliinide genereerimine

Lühikokkuvõte:

Flaviviirused on rühm arboviiruseid, mis põhjustavad haiguspuhanguid maailma erinevates piirkondades ning võivad just riskigruppides tekitada väga tõsiste tagajärgedega nakatumisi. Tervishoiusüsteemile võib see põhjustada omakorda väga tõsiseid probleeme, kuna efektiivseid ravimeid pole saadaval ning flaviviiruste-vastased meetmed põhinevad enamasti ennetavatel tegevustel - näiteks kandjate/nakatunute jälgimisel ja vaktsineerimisel. Enamus flaviviirustest on 3. bioohutuse taseme (BSL3) haigustekitajad, st nende viirustega töötamisel on vaja järgida erakordselt rangeid ettevaatusabinõusid, mis teeb keerulisemaks viirusevastase ravimite ja vaktsiinide väljatöötamise. Just nimelt selle probleemi lahendamiseks on antud uurimistöös kasutatud subgenoomseid replikone, mis võimaldavad uurida viiruse replikatsiooni madalama ohuklassi laboris ja ilma viirust rakkudes vabastamata. Käesolevas töös disainiti vastavad flaviviiruste replikonid ja genereeriti nendel põhinevad stabiilsed rakuliinid, mis sisaldavad reporter-markergeene ja puromütsiini N-atsetüültransferaasi (PAC) geeni. Antud stabiilsed rakuliinid on võimelised konstantselt ekspresseerima mittestruktuurilisi valke ja fluorestseeruvaid/luminestseeruvaid reportervalke. Neid saab kasutada replikatsiooni inhibiitorite testimisel, mis on suureks abiks flaviviirusevastaste ravimeetodite välja töötamisel.

Võtmesõnad:

flaviviirused, TBEV, KUNV, replikon, stabiilse rakuliin, mittestruktuurse valgu, puromütsiini N-atsetüültransferaas

CERCS: T490 Biotehnoloogia

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ABBREVIATIONS

BSL	biosafety level
C protein	capsid protein
cDNA	complementary DNA
dsRNA	double-stranded RNA
DPBS	Dulbecco's modified PBS
E protein	envelope protein
ER	endoplasmic reticulum
FCS	fetal calf serum
FMDV	Foot-and-mouth disease virus
GMEM	Glasgow's modified essential medium
IF	immunofluorescence
IgG	immunoglobulin G
JEV	Japanese encephalitis virus
Kb	kilobase
KUNV	Kunjin virus
LB	lysogeny broth
M	membrane protein
NanoLuc	nanoluciferase
NC	nucleocapsid
NS protein	Non-Structural protein
ORF	open reading frame
PAC	puromycin N-acetyltransferase
PBS	phosphate buffered saline
PFA	paraformaldehyde
prM protein	pre-membrane protein

RC	replication complex
RdRp	RNA dependent RNA polymerase
RLU	relative light unit
Rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TBEV	Tick-borne encephalitis virus
UTR	untranslated region
WB	western blot
WNV	West Nile virus

INTRODUCTION

Viruses are small infectious agents that are on the cusp of being regarded as living organisms. Viruses are composed of DNA or RNA as their genetic material, which is encased in a coat of capsid protein despite their diverse shapes. Additionally, some viruses have an outer envelope lipid layer that facilitates their entry and exit from host cells. Viruses are obligate intracellular parasites, which means they lack the metabolic machinery to produce energy or protein on their own and rely on a host cell to perform these essential tasks for their survival and reproduction. Infecting microorganisms, animals, and plants, their host range is extraordinarily varied. Certain viruses that can cause severe illnesses and outbreaks are transmitted by arthropod vectors and can replicate in both the vector and the vertebrate host. The genus *Flavivirus* is majorly composed of such arboviruses and uses mostly mosquitoes and ticks as their vectors.

Flaviviruses are positive-sense, single-strand RNA viruses that can cause severe diseases and mortality in humans. Flaviviruses are regarded as re-emerging threats due to their widespread global distribution, which has expanded in recent decades due to population growth, increased travel, and vector spread due to climate change. There are no effective treatments for flavivirus infections, and there are only a handful of licensed vaccines. Vector control is the primary method for combating flaviviruses, and the lack of countermeasures highlights the need for new approaches and tools to develop new treatment and prevention methods. As most flaviviruses are regarded as biosafety level 3 (BSL3) agents, handling them requires special precautions, which hinders the development of new antivirals and vaccines. A suggested approach is to use replicons, which can replicate in host cells but cannot produce infectious particles. Lack of infectious particles can allow the analysis of viral replication events without virus entry or assembly when studying the antiviral mechanism of inhibitors, bypassing the need for BSL3 conditions.

In this study, we generated flavivirus replicon-carrying stable cell lines that could be handled in BSL2 conditions. To this end, tick-borne encephalitis virus (TBEV) strain 93/738 was chosen because it is a highly neurovirulent and pathogenic strain that is relevant to Estonia and other European countries. Furthermore, the Kunjin virus (KUNV) was chosen because of its well-characterized replicon systems, which can be used as a good control while working with other flavivirus replicon systems. These stable cell lines can be used for high-throughput screening of potential replication inhibitors in order to develop new antiviral therapies against flavivirus infections.

1 LITERATURE REVIEW

1.1 Flaviviruses

1.1.1 General Overview of Flaviviruses

The genus *Flavivirus* (*Orthoflavivirus* since 2022), which contains 53 species consisting of more than 75 viruses, is one of the four genera belonging to the positive-sense RNA virus family *Flaviviridae* (Burrell et al., 2017; International Committee on Taxonomy of Viruses, 2022). Although there are several ungrouped viruses, most members of the genus are grouped into 15 groups based on the similarities of nucleotide sequences and shared neutralization epitopes. Most flaviviruses are arthropod-borne viruses, commonly known as arboviruses, and are spread through arthropod vectors. About half of the known flaviviruses, such as West Nile virus (WNV), Zika virus (ZIKV), yellow fever virus (YFV), Dengue virus (DENV), and Japanese encephalitis virus (JEV), are transmitted via mosquitoes of either *Aedes* or *Culex* spp. (Burrell et al., 2017; Rosen, 1988). Some others, such as tick-borne encephalitis virus (TBEV) and Powassan virus (POWV), are transmitted via a variety of ticks such as *Haemaphysalis, Ixodes,* and *Dermacentor* spp., while there are also flaviviruses that do not use an arthropod vector (Johnson et al., 2012; Labuda & Nuttall, 2004; Ličková et al., 2020). Many of the flaviviruses are naturally maintained in a viremic transmission cycle of blood-feeding arthropod vectors to vertebrate hosts, including humans (Burrell et al., 2017). However, for some flaviviruses, the transmission cycle is not identified, and some flaviviruses can also be transmitted between vectors via processes like non-viremic, transovarial, and transstadial transmission (Burrell et al., 2017; Kurnia et al., 2022; Simmonds et al., 2017).

Flaviviruses are being considered re-emerging threats as they have an extensive worldwide distribution, which has increased over the last few decades due to reasons including population growth, increased travel, and vector spread due to climate change (Robert et al., 2020; Tabachnick, 2016). They are associated with many human infections, and the most well-studied flaviviruses are important pathogens with a significant burden on human health globally (Bhatt et al., 2013; Burrell et al., 2017; Roehrig, 2013). The diseases caused by these viruses can vary from mild fever to life-threatening severe syndromes like hemorrhagic fever, encephalitis, paralysis, and congenital defects (Burrell et al., 2017; Carteaux et al., 2016; Flint et al., 2015). In addition to humans, flaviviruses like JEV, WNV, and Louping ill virus (a tick-borne flavivirus) can also be pathogenic to a wide range of animals, including

domesticated animals like livestock, and can pose a threat to the global economy (Ilkal et al., 1994; Jeffries et al., 2014; Katayama et al., 2013). Despite flaviviruses posing a continuous threat to health and the economy, preventative measures like vector control remain the main tool to control flaviviruses as there are no specific antiviral drugs against flaviviruses and there are only a few licensed vaccines, which require frequent boosting for immunity, for YFV, JEV, and TBEV (Bhatt et al., 2013; Burrell et al., 2017; Pierson & Diamond, 2020). The lack of countermeasures against flaviviruses highlights the need for different approaches and tools for developing new treatment and prevention methods in addition to studying flaviviruses.

1.1.2 Viruses Included in This Work

TBEV belongs to a tick-borne virus complex that comprises nine genetically and antigenically related viruses. TBEV causes about 10,000 human cases of tick-borne encephalitis annually throughout Eurasia and is considered one of the most important causative agents of arbovirus infections in Europe. There are three main subtypes of TBEV: European, Far Eastern, and Siberian (Burrell et al., 2017; Lindqvist et al., 2020). The infection is usually manifested as a two-phase disease where patients develop unspecific symptoms like fever, fatigue, and headache in the first phase, and then symptoms of inflammation in the central nervous system in the second phase. The disease can result in long-term neurological conditions that can manifest as paralysis, atrophy of neck muscles, weakness of voluntary movement in the neck and lower extremities, poor memory, and difficulties with coordination and speech (Burrell et al., 2017; European Centre for Disease Prevention and Control, 2023; Lindqvist et al., 2020). The European subtype usually is related to the least severe disease in comparison to the other two main subtypes. The virus is maintained in nature through a complex cycle between *Ixodid* ticks and feral mammals, where ticks can serve as reservoir hosts as well. The virus can be passed on from one tick to another through transstadial or transovarial transmission. Human infection is usually caused by the bite of an adult tick but there are also cases of outbreaks where the infection occurred due to the consumption of unpasteurized milk products from infected animals (Burrell et al., 2017; Lindqvist et al., 2020). The genome of TBEV is approximately 10 kilobases (kb) long and the features associated with the other tick-borne flavivirus RNA are also found in the TBEV RNA sequence. The variation in the amino acid sequence of the polyprotein is not high between the TBEV subtypes but the diversity of subtypes correlates with the pathogenicity (Kutschera & Wolfinger, 2022; Zakotnik et al., 2022; Zhang M. et al., 2022).

KUNV is a mosquito-borne zoonotic flavivirus of the Japanese encephalitis complex that was isolated in 1960 for the first time. It is a subtype of the West Nile virus (WNV) that is endemic to Australia (Westaway et al., 2003). Like WNV, KUNV is maintained in a natural transmission cycle including mosquito vectors and avian reservoir hosts with horses and humans being incidental hosts (Burrell et al., 2017; Hall et al., 2002; Scherret et al., 2002; Westaway et al., 2002). The infection by KUNV usually causes much milder disease compared to WNV infection and rarely causes encephalitis (Burrell et al., 2017; Hall et al., 2002). The genome of KUNV is approximately 11 kb long and the features associated with the other mosquito-borne flavivirus RNA are found in the KUNV RNA sequence (Westaway et al., 2002).

1.1.3 Morphology of Flavivirus Virion

Flavivirus virions are approximately 50 nm in diameter, roughly spherical in shape, and surrounded by a lipid envelope that surrounds a nucleocapsid (NC) core (Figure 1). They are assembled in the secretory pathway of the host cell using capsid (C), premembrane (prM), and envelope (E) structural proteins, the viral genomic RNA, and the host-derived lipid bilayer (Burrell et al., 2017; Flint et al., 2015; Lindenbach et al., 2013). The outer shell of the virion is arranged in an icosahedral symmetry anchored to the lipid membrane, surrounding the NC, which has no distinguishable symmetry (Lindenbach et al., 2013).

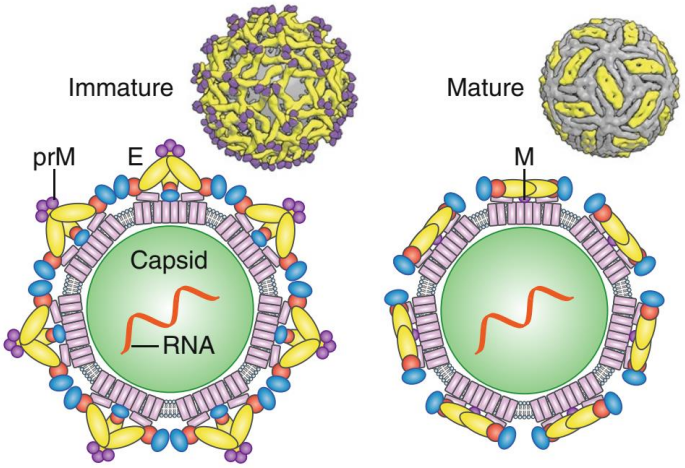

Figure 1. Arrangement of immature and mature virions of flaviviruses. E proteins of flaviviruses are arranged distinctively depending on the form of the virion. Immature (left) virion displays prominent spikes due to the heterotrimeric prM-E formation. Mature (right) virion displays a smooth outer layer as the dimeric E glycoproteins are arranged in a herringbone pattern with M protein. Both virion structures enclose a nucleocapsid that consists of capsid protein that tightly packs the viral genomic RNA (Pierson & Diamond, 2020).

The virion can exist in two forms: mature and immature. The mature virion has an outer shell composed of E glycoprotein and membrane (M) protein. The E glycoproteins are arranged in a herringbone pattern, with 180 copies of E and M, tightly packed, providing a smooth surface over the lipid bilayer (Lindenbach et al., 2013; Simmonds et al., 2017; Zhang, W. et al., 2003). In contrast, the immature virions contain prM instead of M protein, and they are larger, measuring 60 nm in diameter. The outer shell of immature virions is not smooth like that of mature virions but rather displays 60 prominent spikes on the surface due to the incorporation of prM into the envelope as heterotrimeric prM-E (Figure 1). Normally, the shape of the immature particles undergoes drastic changes as the glycoproteins are rearranged in the trans-Golgi network and prM is cleaved by the host furin enzymes to M, resulting in the formation of mature virions. However, sometimes this process is not efficient, and the host cell may release significant quantities of immature virions along with the mature virions (Burrell et al., 2017; Hardy et al., 2021; Lindenbach et al., 2013; Pierson & Diamond, 2020). This generalization of the flavivirus virion structures holds true except for one isolate of TBEV. One study showed that the reconstruction of TBEV virion structure from the TBEV Kuutsalo-14 isolate had the presence of an M-M protein interaction at the interheterotetramer interface in comparison to the previous models, which suggested that the conformation might have a role in virion stabilization due to the interactions between M proteins as it is compensating for the weaker E protein interactions (Füzik et al., 2018; Pulkkinen et al., 2022).

1.1.4 Genome Organization

Flaviviruses have a positive-sense single-stranded RNA genome that is approximately 10-11 kb long (Figure 2). The 5’ end of the genome is capped with a type 1 cap, m^7GpppAmN, that stabilizes the viral genome, initiates translation, and disrupts innate antiviral defenses while the 3’ end lacks the polyadenylated tail (Lindenbach et al., 2013; Polacek et al., 2009).

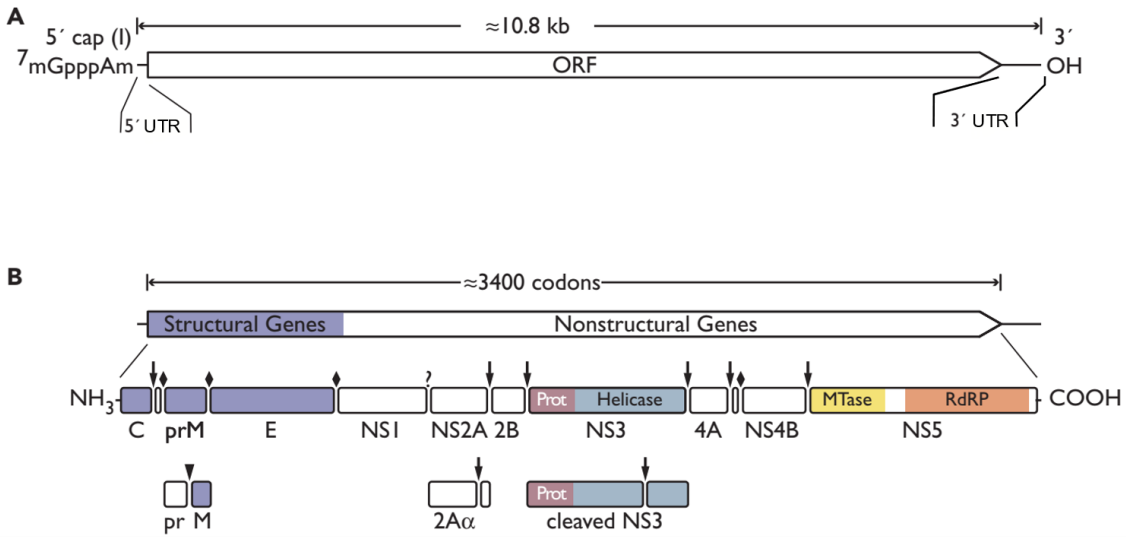

Figure 2. Genome structure and protein expression of flaviviruses. A. Genome structure. The flaviviral genome is shown with the open reading frame (ORF), the 5' cap, and 5' and 3' untranslated regions (UTR). The genome is approximately 11 kb long. **B.** Flavivirus polyprotein processing and products. Boxes below the polyprotein depiction show the precursor and mature proteins generated by the enzymatic processes. Structural proteins are colored purple, and the nonstructural (NS) proteins are colored white or shaded according to their enzyme subunits. ♦ indicates the cleavage sites for host signalase, ↓ indicates the sites for viral NS2B/3 serine protease, ▼ represents the site for furin, and ? shows the unknown proteases (Modified from (Lindenbach et al., 2013)).

The flaviviral genome encodes one open reading frame (ORF), which is about 3400 codons long, that is flanked by untranslated regions (UTR) with loop-like structures. 5' UTR is generally about 100 nucleotides long, while 3' UTR length can range from 400 to 800 nucleotides (Lindenbach et al., 2013; Ramos-Lorente et al., 2021). The 5' UTR sequence varies among flaviviruses, but the overall RNA structure is mostly conserved and contains common secondary structures. The 5' UTR consists of *cis*-elements, bifurcating stem-loops, and a complementary sequence that interacts with the 3' UTR to create a panhandle, which is responsible for the RNA genome cyclization (Chambers et al., 1990; Lindenbach & Rice, 2003; Wallner et al., 1995). The 3' UTR sequence contains three domains: a highly conserved region that carries stem-loop structures and is preserved across mosquito and tick-borne flaviviruses; a partially conserved region that contains hairpin motifs; and a variable region that is not conserved between flaviviruses (Asghar et al., 2016; Lindenbach & Rice, 2003; Ramos-Lorente et al., 2021; Villordo et al., 2015).

Translation of the large ORF results in one polyprotein that is cleaved by the host cell and viral proteases. The cleavage results in ten functional proteins including three structural proteins (C, prM, E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3,

NS4A, NS4B, NS5) (Figure 2). Apart from the cleavage of prM into pr and M by the host cell's furin enzyme, the majority of the cleavages within the structural protein region and the cleavage near the C terminus of NS4A is carried out by the host cell signalase (Barrett & Weaver, 2012; Lindenbach et al., 2013; Lücke et al., 2022). The virus-encoded serine protease (NS2B/3) cleaves at the junctions of NS proteins, but the cleavage process at the NS1-2A junction is carried out by an unknown signalase pathway (Falgout & Markoff, 1995; Flint et al., 2015; Lindenbach et al., 2013). While structural proteins are majorly involved in particle formation and cell entry, NS proteins are responsible for viral replication in addition to hindering the host antiviral responses (Chambers et al., 1990).

1.1.5 Structural Proteins

Capsid (C) protein is the primary structural component of the viral nucleocapsid measuring about 30 nm in diameter and is responsible for protecting the viral genome from external factors and ensuring the virion's structural stability. Flavivirus C protein is a highly basic, dimeric alpha-helical protein of 11 kDa that is about 114 amino acids long. The N-terminus of the protein is unstructured and the charged residues at the C-terminus of the protein are likely to be involved with RNA binding (Lindenbach et al., 2013; Ma et al., 2004; Sampath & Padmanabhan, 2009). Nascent C protein carries a hydrophobic tail at the C-terminus, which functions as a signal for prM endoplasmic reticulum (ER) translocation. The anchor is cleaved in two steps; first by the viral NS2B/3, then by the host signal peptidase (Chambers et al., 1990; Lindenbach et al., 2013; Sampath & Padmanabhan, 2009). The flaviviral C protein is associated with high functional flexibility as it can tolerate large deletions (Lindenbach et al., 2013). For example, TBEV can tolerate deletions of up to 16 amino acids from the core hydrophobic helix, but this increases the number of empty particles produced (Kofler et al., 2002). The C protein of YFV can package RNA even after deleting 40 residues from the N-terminus or 27 residues from the C-terminus. However, deletions in the internal hydrophobic area are not tolerated (Patkar et al., 2007). It was also established that at least the first 20 codons of C protein are essential for RNA replication as this region contains *cis*-acting elements (Corver et al., 2003; Khromykh & Westaway, 1997).

Precursor Membrane (prM) is the 26 kDa glycoprotein precursor of the M protein (approximately 8 kDa). The signal produced by the hydrophobic tail at the C-terminus of the C protein translocates prM into the ER (Lindenbach et al., 2013). The signal recruits host signalase to cleave the prM from the polyprotein at the N-terminus of C-prM and the C-terminus of prM-E sites. During flavivirus virion assembly, prM and E are arranged in a

heterodimeric structure, resulting in the formation of an immature virion that transits through the Golgi compartment. In the low pH environment of the Golgi compartment, reversible conformational changes occur in the E protein, but once the precursor is cleaved to form structural protein M via host furin in the trans-Golgi network, the conformational change in E becomes irreversible. The cleaved prM (pr) is not released until the mature virion is secreted out into the neutral pH environment of the extracellular space, which serves to safeguard the E protein from premature fusion (Li, L. et al., 2008; Lindenbach et al., 2013; Yu et al., 2008).

The envelope (E) protein has a molecular weight of 53 kDa and is the major component of the flavivirus virion surface (Chambers et al., 1990). It is found as a trimer in the immature virion, a dimer in the mature virion, and a trimer again prior to fusing with the host cell membrane due to the conformational changes induced by pH changes (Bressanelli et al., 2004; Modis et al., 2004). The mature virion E protein can bind, fuse, and enter host cells (Li, L. et al., 2008; Sampath & Padmanabhan, 2009). E protein domains DI, DII, and DIII form an antiparallel dimer at the mature virion surface. The highly conserved fusion loop at the DII tip initiates membrane fusion and cell entrance (Bressanelli et al., 2004). DIII has an integrin-binding motif and may be important in receptor binding and cell recognition (Mlera et al., 2014; van der Most et al., 1999). The crystal structures of mosquito and tick-borne flavivirus E proteins share domain composition and amino acid sequences (Chambers et al., 1990; Wu et al., 2003; Zhang, Y. et al., 2004).

1.1.6 Non-Structural Proteins

NS proteins are responsible for flavivirus replication and virion assembly. Additionally, NS proteins interact with the host cells and interfere with cellular functions by activating or inhibiting the host immune signaling pathways. Most of the NS proteins are associated with the replicase complex that catalyzes RNA accumulation (García et al., 2017; Lindenbach et al., 2013; Youn et al., 2012).

NS1 is a multifunctional glycoprotein of 46-55 kDa, depending on the degree of glycosylation. The glycosylation of NS1 is highly variable as it depends on the flavivirus. The process is important for the maturation of this protein and is crucial for flaviviral virulence, replication, and secretion (Falgout & Markoff, 1995; Lindenbach et al., 2013; Rathore et al., 2011). NS1 of flaviviruses shares a certain degree of homology and is also a major selection target in flavivirus evolution. This protein is translocated via the signal sequence that is located at the last 24 amino acids of E to the ER where it is cleaved from

the polyprotein at the NS1/2A and E/NS1 by the host signalase (Falgout & Markoff, 1995; Lindenbach et al., 2013; Sironi et al., 2016). NS1 can be found at different locations in the host cell, including the vesicular compartments that are associated with the cell surface and membrane in addition to extracellular space as a secreted lipid-rich non-virion species (Lindenbach et al., 2013; Mason, 1989; Winkler et al., 1988). In early replication, intracellular NS1 associates with NS4A and NS4B for the formation of the replicase complex. Also, intracellular NS1 co-localizes with dsRNA, which suggests that NS1 plays an important role in viral replication (Carpio & Barrett, 2021; Lindenbach & Rice, 1999; Tan et al., 2020).

NS2A is a hydrophobic transmembrane protein of 22-25 kDa that is processed in the ER lumen by an unknown host protease and NS2B/NS3 protease at the N-terminus and C-terminus, respectively (Chambers et al., 1990; Lindenbach et al., 2013). The protein does not have an enzymatic function but it is necessary for viral RNA replication and capsid assembly as it can interact with and bind to the 3' UTR, NS3, and NS5. NS2A is believed to play an important role in the production of infectious viruses as it seems to be associated with the incorporation of genomic RNA into the budding virion (Kümmerer & Rice, 2002; Leung et al., 2008; Lindenbach et al., 2013; Liu et al., 2003).

NS2B is a small membrane-associated protein of 14 kDa and is composed of three hydrophobic domains. Two of the hydrophobic domains are transmembrane segments that lie at N- and C-terminus. The NS2B hydrophobic domain at the central region, which is 47 amino acids long, acts as a co-factor of NS2B/3 protease. NS2B also binds to the NS3 protein in a stable complex at the cellular membrane to anchor the complex (Lindenbach et al., 2013; Sampath & Padmanabhan, 2009).

NS3 is a multifunctional protein of approximately 70 kDa which has two functional domains: an N-terminal protease with a chymotrypsin-like domain that is responsible for most of the cleavages of the polyprotein, and a C-terminal helicase. The helicase activity is needed for genome replication and viral RNA synthesis as it is believed necessary for unwinding the double-stranded RNA (dsRNA) replication intermediate (Burrell et al., 2017; Davidson et al., 2020; Li, K. et al., 2014; Lindenbach et al., 2013; Luo et al., 2015). The protease activity is gained after NS3 binds with its cofactor NS2B, forming the NS2B/3 viral serine protease which is responsible for cleaving the C-terminus of the mature capsid protein in addition to recognizing and cleaving several polyprotein junctions (Lindenbach et al., 2013).

NS4A and **NS4B** are small hydrophobic proteins of 16 and 27 kDa, respectively. Neither of the proteins shows an identified enzymatic activity but both have roles in viral replication. NS4A binds to other NS proteins and participates in RNA replication by assembling replicase complexes at the ER membrane (Cedillo-Barrón et al., 2018; Lindenbach et al., 2013). NS4B co-localizes with NS3 at sites of RNA replication and is believed to have roles in regulating virus replication through interactions with NS1, NS3, and NS4A (Cedillo-Barrón et al., 2018; Youn et al., 2012). DENV and WNV NS4B also are reported to have roles in inhibiting the interferon (IFN) response (Lindenbach et al., 2013).

NS5 is a highly conserved, large multifunctional protein of 103 kDa which is the biggest protein encoded by flaviviruses (Burrell et al., 2017). NS5 performs two enzymatic activities structurally separated by the interdomain region. The N-terminal domain contains the S-adenosylmethionine methyltransferase (MTase) activity which is responsible for the formation of the viral 5' cap structure. The C-terminal domain contains the RNA-dependent RNA polymerase (RdRP) which is shown to initiate RNA synthesis through a *de novo* mechanism (Chambers et al., 1990; Lindenbach et al., 2013; Park et al., 2007). Additionally, flaviviral NS5 is shown to disrupt innate immune signaling such as by suppressing the Jak-STAT pathway of IFN signaling and inducing interleukin-8 (IL-8) expression to enhance viral spread (Best et al., 2005; Cedillo-Barrón et al., 2018; Lindenbach et al., 2013; Park et al., 2007).

1.2 *Flavivirus* Replication Cycle

1.2.1 Binding and Entry

Flaviviruses follow a conserved replication cycle that starts with host cell entry (Figure 3). Virion E glycoprotein interacts with and attaches to host receptors on the surface of the cell plasma membrane. The virion diffuses along the plasma membrane until it comes across an entry receptor localized at a clathrin-coated pit (Flint et al., 2015; Lindenbach et al., 2013; van der Schaar et al., 2008). The clathrin-coated pit invaginates and forms a clathrin-coated vesicle that mediates the entry of the virion into the cell via endocytosis. After this particle is internalized, it is delivered to early endosomes, which mature into late endosomes. The maturation of endosomes creates an acidic environment, which triggers the viral membrane fusion with the endosomal membrane. The fusion process releases the NC into the cytoplasm, which results in NC degradation that releases the viral positive-sense single-strand RNA genome into the cytoplasm. Once the viral genome is released into the

cytoplasm, it can be further processed for protein translation and genome replication (Flint et al., 2015; Lindenbach et al., 2013; Smit et al., 2011).

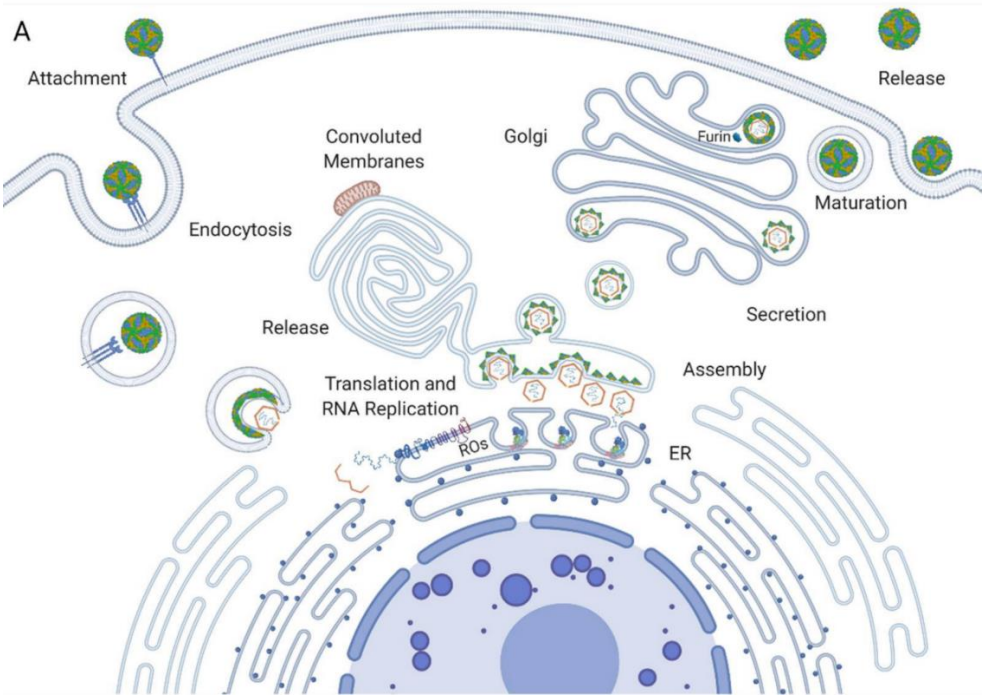

Figure 3. Replication cycle of flaviviruses. Flavivirus infection starts with receptor-mediated binding to the host cell and entry via clathrin-mediated endocytosis. A decrease in endosome pH triggers virion and envelope membrane fusion, releasing the viral RNA genome into the host cell cytoplasm. The viral genome is translated into a polyprotein that is processed by the host and viral proteases into ten proteins. Non-structural viral proteins form replication complexes in the ER, whereas structural viral proteins are assembled and loaded with viral genetic material. Furin protease cleavage of prM transforms immature virions into mature, infectious virions. These virions leave the cell through exocytosis and continue the replication cycle by infecting other host cells (van den Elsen et al., 2021).

1.2.2 Translation and Replication

Flavivirus replication begins once the viral RNA genome is released into the host cytoplasm and the host cell machinery initiates the cap-dependent translation of the polyprotein at the ER. Ribosomes on the rough ER translate viral RNA into a single polyprotein that is anchored to the ER membrane. The ER-bound polyprotein is processed by the host and viral proteases into three structural and seven NS proteins. The NS proteins form replication complexes that mediate the replication of viral RNA genome in invaginated ER compartments, which have a pore that connects them to the cytoplasm. The NS proteins recruit the positive-sense RNA genome to the replication complexes where NS5 RdRP uses it as a template to generate the antisense RNA, producing the dsRNA intermediate. The

antisense RNA is used as a template for viral RNA synthesis (Lindenbach et al., 2013; Reid et al., 2018; van den Elsen et al., 2021).

Flaviviral RNA synthesis yields approximately 10-fold more positive strands compared to antisense strands. All NS proteins are essential in the RNA replication process. NS5 serves as a methyltransferase that is involved in the capping of the viral genomic RNA, in addition to its RdRP function. NS3, with NS2B as its co-factor, acts as a protease to correctly process the viral polyprotein. NS3 also acts as a helicase that unwinds the dsRNA intermediate during the replication (Lindenbach et al., 2013; Luo et al., 2015). The roles of NS1, NS4A, and NS4B transmembrane proteins are not clear but they are associated with the formation of the replication complexes. NS4B is known to colocalize with NS3 and dsRNA in the replication complexes, indicating a role in RNA replication. Also, in DENV, NS4B was shown to support the dissociation of NS3 from single-strand RNA to stimulate the helicase recruitment to the dsRNA, thus promoting the unwinding of the intermediate product (Lindenbach et al., 2013; Mackenzie et al., 1998; Miller et al., 2006).

The progeny positive-strand RNA exits the ER compartment through the pore and can be used as mRNA for a new translation cycle, as a template for antisense RNA, or as the genetic material that is packed into the virions (Lindenbach et al., 2013; Reid et al., 2018; van den Elsen et al., 2021).

1.2.3 Assembly and Release of Virions

In the ER lumen, structural proteins are assembled with genetic material into immature virions. The C protein binds to the synthesized viral RNA and this complex is packaged in the ER-derived lipid bilayer containing the E and prM proteins. The premature fusion of the viral particle during its transport through the trans-Golgi network is prevented by the prM. The immature virions are processed by host furin protease to cleave off prM to produce mature virions in the trans-Golgi network. Mature virions exit the cell via exocytosis and continue the same cycle with the other host cells (Apte-Sengupta et al., 2014; Fishburn et al., 2022; Lindenbach et al., 2013).

1.3 Replicons and Replicon Harboring Stable Cell Lines

Reverse genetics has made it possible to store and manipulate complete viral genomes hosted/encoded on DNA-based plasmids. As manipulations can be introduced on the DNA level, RNA viruses, such as flaviviruses, need to be first reverse transcribed to a complementary cDNA and cloned into a plasmid vector under the control of a bacteriophage

RNA polymerase promoter. The bacteriophage RNA polymerase promoter is upstream of the sequence of interest and allows template-directed RNA synthesis from cDNA *in vitro* using the respective RNA polymerase. As flaviviruses are positive-strand RNA viruses, *in vitro* transcribed RNA from a cDNA clone acts as a messenger RNA and can start the viral life cycle once it is transfected into a susceptible host cell (Kümmerer, 2018; Westaway et al., 2003).

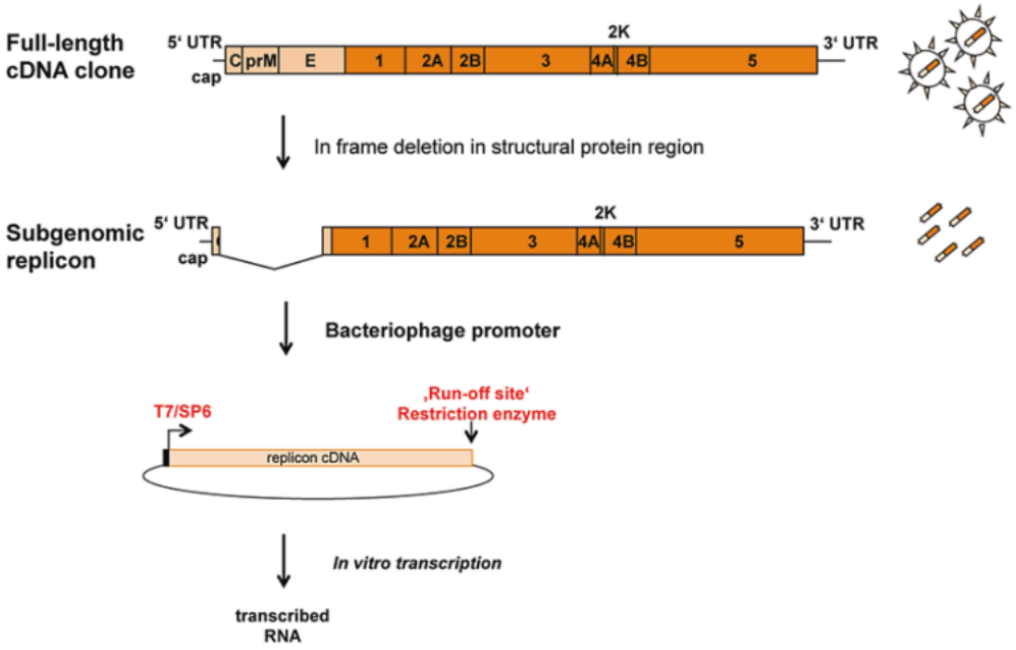

Figure 4. Schematic representation of flaviviral full-length cDNA clone and subgenomic replicon. Full-length cDNA clone encodes for genomic RNA allowing virion formation; the subgenomic replicon with the in-frame deletion in the structural protein region only mediates the replication of subgenomic RNA without virion formation. The subgenomic replicon sequences can be cloned in a plasmid under the control of a bacteriophage promoter. A unique restriction site is added downstream of the sequence for linearization and viral 3' end production as a result of *in vitro* transcription (Modified from (Kümmerer, 2018)).

Subgenomic replicons of flaviviruses, on the other hand, have all the genetic components needed for self-replication but lack the structural components needed to produce infectious virions. Generally, flavivirus replicons display a large in-frame deletion that surrounds the region of the genome that encodes the structural components (C-prM-E) of the virion (Figure 4). The deletion excludes at least the first 20 codons of the C protein, as the region contains *cis*-acting elements necessary for RNA replication (Corver et al., 2003; Khromykh & Westaway, 1997). Additionally, 24-30 codons from the C-terminus of the E protein are retained as they encode the signal sequence needed for NS1 protein translocation and cleavage. The flavivirus replicon sequence can be cloned into a plasmid under the control of

a bacteriophage-originated promoter like the SP6 or T7 RNA polymerase promoter to initiate the start of replicon RNA *in vitro* transcription. In addition, just downstream of the 3' end of the viral genome, a unique restriction site is added. This site is used to linearize the plasmid and obtain the authentic 3' end of the viral genome since, during *in vitro* transcription, the RNA polymerase of choice will fall off the DNA template at the linearized site, resulting in an *in vitro* transcribed replicon RNA ending with the authentic 3' end. *In vitro* transcribed RNA, then, can be used to transfect susceptible host cells, which results in the replication of subgenomic replicon RNA in the cells without virus production (Khromykh & Westaway, 1997; Kümmerer, 2018; Shi et al., 2002). Consequently, subgenomic replicons can make well-suited tools to study viral genome replication independently from the virion assembly.

Several flavivirus replicons have been developed to this day, including replicons for YFV, DENV, ZIKV, TBEV, and WNV, with KUNV being the most studied and best characterized (Hayasaka et al., 2004; Jones et al., 2005; Kümmerer, 2018; NG et al., 2007; Shi et al., 2002; Westaway et al., 2003; Xie et al., 2016). Replicons can be used for various applications such as testing inhibitory compounds, studying viral translation and replication, determining the expression of heterologous genes, or characterizing adaptive mutations (Khromykh & Westaway, 1997; NG et al., 2007). As subgenomic replicons are suited for manipulation, they can be modified to express reporter and selection genes in conjunction with all the NS proteins. The introduction of reporter protein genes can help with monitoring flavivirus replicon replication, as it has been shown that the level of reporter gene expression correlates with the level of genome replication (Varnavski & Khromykh, 1999). Reporter proteins commonly utilized for RNA replication detection include those that facilitate luminometric detection, such as *Renilla*, *Firefly*, or NanoLuc (NLuc) luciferase, as well as those that enable fluorescent detection, such as green fluorescent protein (GFP) or mCherry. Reporter protein genes are usually introduced in the section that covers the in-frame deletion in the structural region. These genes are often inserted in-frame with a foot-and-mouth disease virus (FMDV) 2A autoprotease at its C-terminus, which mediates the cleavage of the reporter protein from the remaining E protein sequence that is necessary for processing of the NS1 protein (Jones et al., 2005; Kümmerer, 2018; Li, S.-H. et al., 2013; Lücke et al., 2022; NG et al., 2007).

Although transient replication of flavivirus replicons in susceptible host cells can be used for testing antiviral compounds affecting RNA replication, replicon harboring stable cell lines could provide a tool for high-throughput antiviral screening. Stable cell lines can persistently replicate flaviviral replicon RNA in the host cell cytoplasm for a longer period

with low cytopathicity (Jones et al., 2005; Khromykh & Westaway, 1997; Kümmerer, 2018; Shi et al., 2002; Xie et al., 2016). To generate stable cell lines, flavivirus replicons with selection genes that provide resistance against their corresponding antibiotics have been described. Commonly used selection genes like puromycin N-acetyltransferase (PAC) or neomycin phosphotransferase II (NEO) help replicon-harboring cells sustain themselves under the selection of respective antibiotics. These genes also need FMDV 2A autoprotease to release the product of the gene mediating antibiotic resistance (Khromykh & Westaway, 1997; Kümmerer, 2018; Varnavski & Khromykh, 1999).

In conclusion, flavivirus replicon harboring stable cell lines can be particularly useful when studying the antiviral mechanisms of inhibitors of RNA replication. As the replicons lack structural genes, the replicon system cannot produce virions, which makes it non-infectious, thus reducing the biosafety concern while screening antiviral compound libraries. Additionally, stable cell lines can prove helpful while profiling escape mutants generated from resistance to antiviral compounds in drug discovery (Khromykh & Westaway, 1997; NG et al., 2007).

2 THE AIMS OF THE THESIS

Flaviviruses have caused epidemic outbreaks in the last few decades and are considered re-emerging pathogens that can cause severe diseases in susceptible populations. The medical treatment of flaviviral infections usually relies on symptomatic relief as there are no effective treatments available. Flavivirus countermeasures rely heavily on preventative actions like vector surveillance and control and vaccination, which is only available for a couple of flaviviruses. This highlights the significance of the need for antivirals and vaccines against flaviviruses. However significant it may be, the development of antivirals and vaccines is hampered by the fact that most flaviviruses are biosafety level 3 (BSL3) agents and require special handling when working with infectious viruses. The need for BSL3 laboratories can be avoided by the use of subgenomic replicons that allow the analysis of viral replication events without the virus entry or assembly, especially when studying the antiviral mechanism of inhibitors.

In this study, we aimed to generate stable cell lines harboring flavivirus replicons that can be handled in BSL2 conditions as tools for RNA replication studies and inhibition assays. The replicons are engineered to express fluorescent/luminescent marker genes (mCherry, oxGFP, or NanoLuc) and the Puromycin-N-acetyltransferase gene as a selection marker, which is the main driver for stable expression of the replicons in cell lines.

3 EXPERIMENTAL PART

3.1 Materials and Methods

3.1.1 Design of *Flavivirus* PAC Replicons

Six *Flavivirus* replicons with Puromycin N-acetyltransferase (PAC) gene were designed and constructed in this work. The PAC replicons were based on replicons that were constructed previously by colleagues. The cDNA sequences of KUNV and TBEV replicons, which were constructed by colleagues, are under the control of the SP6 RNA polymerase promoter that allows replicon RNAs to be synthesized *in vitro.* The replicon sequences encode for the replicon 5'UTR, C protein, marker sequence, FMDV 2A sequence, some part of the structural region, non-structural proteins' coding sequence (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5), and 3' UTR. Most of the structural regions in the replicon constructs were removed, except for the C protein and the last 30 codons (35 codons in TBEV) from the C-terminal region of the E protein. The C protein coding sequence is conserved as it is involved in RNA cyclization during replication (Tsetsarkin et al., 2016). The C-terminal region of the E protein is conserved in the replicon constructs as it is necessary for the correct translocation of the NS1 protein in ER membranes (Rastogi et al., 2016). The removed structural part in the replicons was replaced by marker genes encoding mCherry, oxGFP, or NanoLuc that are expressed during the replicon RNA replication in cells. mCherry is a red fluorescent protein, oxGFP is a green fluorescent protein and NanoLuc is a small and stable enzyme that reacts with its corresponding substrate, a coelenterazine derivative, to produce bioluminescence which can be measured from the cell lysates in the presence of the said substrate (England et al., 2016). The marker gene is followed by FMDV 2A autoprotease that cleaves itself from the C-terminal end and releases the marker protein from the flavivirus polyprotein. These replicons' encoded sequences were cloned into the pCCI vector which is a low-copy plasmid containing the Chloramphenicol resistance gene. This vector allows the production of flaviviral replicon cDNAs in low amounts to prevent bacterial cell death due to the toxicity caused by the viral protein expression (Ávila-Pérez et al., 2018).

Constructed replicons:

1. pCCI-KUNV-mCherry-PAC-Replicon
2. pCCI-KUNV-oxGFP-PAC-Replicon
3. pCCI-KUNV-NanoLuc-PAC-Replicon

4. pCCI-TBEV-mCherry-PAC-Replicon
5. pCCI-TBEV-oxGFP-PAC-Replicon
6. pCCI-TBEV-NanoLuc-PAC-Replicon

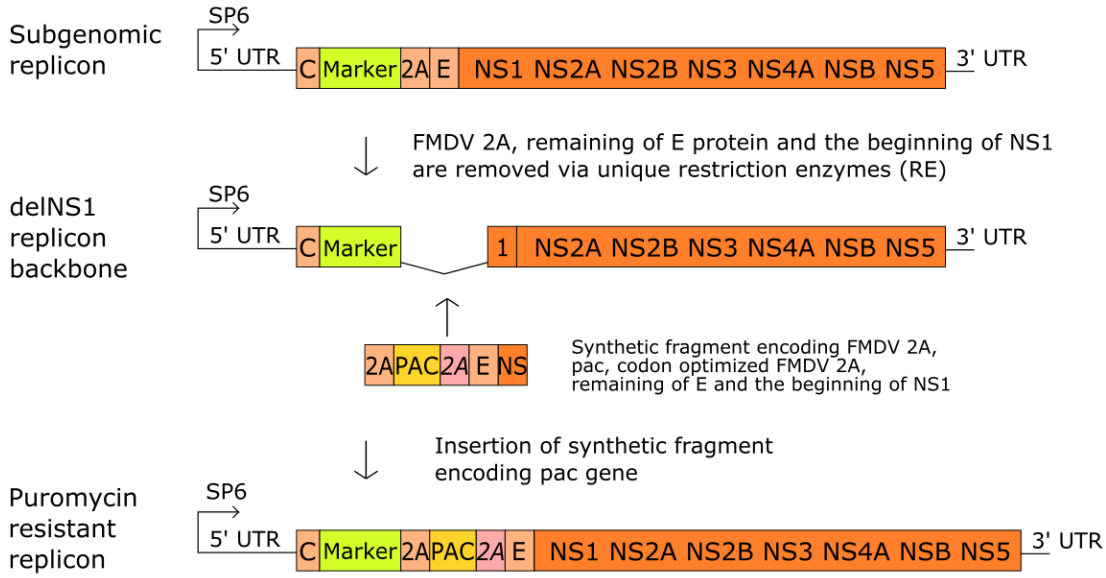

Figure 5. Scheme of marker gene insertion into the replicon sequences. Deletion of the region comprised of FMDV 2A, remaining of the E protein, and the beginning of NS1 sequences is introduced to the flaviviral subgenomic replicon via unique restriction enzymes. A synthetic fragment encoding the resistance marker PAC gene along with FMDV 2A, codon-optimized FMDV 2A, remaining codons of the E protein, and the beginning of NS1 is inserted into the removed region via ligation. The resulting replicon encodes the inserted puromycin resistance gene.

The PAC replicon sequences were designed to encode the PAC gene flanked by FMDV 2A autoprotease sequences (Figure 5). The FMDV 2A sequence downstream of the marker gene was intentionally codon optimized. The PAC gene with FMDV 2A autoprotease sequences was inserted between the fluorescent marker gene and the remaining section of E protein-encoded sequences. The aim was to express the replicons in mammalian cell lines, with the help of Puromycin, to create stable cell lines that harbor flaviviral replicons. Puromycin acts as an inhibitor of protein synthesis, causing the premature release of nascent polypeptide chains from the ribosome (De La Luna & Ortin, 1995). To achieve resistance against puromycin, the PAC gene from *Streptomyces alboniger*, which encodes for puromycin acetyltransferase, was introduced. PAC inactivates puromycin by acetylating the amino position of its tyrosinyl moiety (Lahoz et al., 1991). This resistance mechanism selects only the cells that carry the replicon RNA.

3.1.2 Molecular Cloning of *Flavivirus* PAC Replicons

All cloning was carried out using basic cloning techniques. The synthetic DNAs encoding FMDV 2A, PAC gene, optimized FMDV 2A, end of E protein, and the beginning of NS1 sequences (flanked with unique restriction sites) in pUC57/Kan high-copy plasmid were ordered from Twist Bioscience (USA). The restriction enzyme digest reactions were carried out using FastDigest enzymes and buffers (Thermo Fisher Scientific) to create vector and insert fragments, following the manufacturer's instructions, at 37˚C for 30 minutes. To prevent the self-ligation of the vector, the FastAP alkaline phosphatase (Thermo Fisher Scientific) was added to dephosphorylate vector DNA (1 µl enzyme per 1 µg DNA) for 30 minutes at 37˚C. The dephosphorylation treatment was heat inactivated via incubation at 75˚C for 5 minutes. Restriction products of the vector and insert constructs were analyzed via gel electrophoresis where the fragments were separated on the 0.8% TAE agarose gel. The correct fragments were cut out from the gel and purified using a ZymoClean Gel DNA Recovery kit (Zymo Research) following the manufacturer's instructions and eluted with 12 µl of nuclease-free water. Concentrations of the purified DNAs were quantified using a spectrophotometer (NanoDrop™ 2000C, Thermo Fisher Scientific). After purification, the vector and insert were ligated at a 1:3 molar ratio at 12˚C overnight using 1 µl T4 DNA Ligase and 4 µl 5× T4 DNA Ligase Buffer (Thermo Fisher Scientific) in a 20 µl reaction mixture. The ligated DNAs were then precipitated with ethanol prior to bacterial transformation to remove the salt coming from the ligation mixture to prevent electrical arcing during electroporation. For 20 µl of ligation mix, 4 µl of 5 M ammonium acetate, 0.5 µl 20 mg/ml glycogen, and 100 µl 96% ethanol were added into the mixture and incubated at -20˚C for 30 minutes. Then the mixture was centrifuged at 15000× g for 10 minutes, and the pellet was washed with 200 µl 70% ethanol. The wash step was repeated, and the pellet was air dried, then dissolved in 2 µl nuclease-free water.

The plasmid DNAs resulting from ligated DNAs were transformed via electroporation into the competent *Escherichia coli* EPI300 strain cells. For each transformation, 80 µl of thawed competent cells were mixed with the purified ligation mixture, then transferred to a 2 mm electroporation cuvette and electroporated with 2500 V in a BioRad Gene Pulser Xcell unit. The electroporated cells were immediately rescued with 800 µl of SOC media (SOB media supplemented with 20 mM glucose) and incubated at 37˚C for 45 minutes on a shaker (220 rpm). The cells were then plated onto lysogeny broth (LB) agar plates supplemented with chloramphenicol at a final concentration of 12.5 µg/ml and were incubated at 37˚C for 16

hours. Following the transformation, the bacterial colonies were picked for propagation in 2.5 ml of tryptic soy broth (TSB) medium supplemented with chloramphenicol at a final concentration of 12.5 µg/ml and incubated at 37˚C for 16 hours on a shaker (220 rpm). The plasmid DNAs were isolated by the FavorPrep Plasmid DNA Extraction Mini Kit (Favorgen Biotech Corp.) following the manufacturer's instructions and eluted in 20 µl nuclease-free water. To verify that the isolated plasmid DNAs were correct, restriction analysis was carried out with *Aat*II FastDigest enzyme for KUNV-PAC replicon DNAs and *Sgs*I+*Nhe*I for TBEV-PAC replicon DNAs (as described in 3.1.2) using all the material (20 µl) obtained from the mini preparations. The restriction mixtures were analyzed with gel electrophoresis on 0.8% TAE agarose gel. Correct clones were chosen based on the results of the restriction analysis and were propagated in larger volumes to obtain larger amounts of plasmid DNA. The cells were grown in 50 ml of TSB media supplemented with chloramphenicol at a final concentration of 12.5 µg/ml and were incubated at 220 rpm, 37˚C for 16 hours on a shaker. Then the media volume was adjusted to 200 ml, and the amplification of low-copy plasmid was induced with the addition of 0.1% L-arabinose at 37˚C for 5 hours on a shaker (220 rpm).

As the induced bacteria was expected to be in large quantities, the plasmid DNA was isolated using a double purification method to reduce the amount of bacterial material within the samples. First, the bacterial culture was centrifuged at 4000× g for 10 minutes at 4˚C to obtain the bacterial cell pellet. The supernatant was removed, and the cells were resuspended in 8 ml of home-made SOL I resuspension solution (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA, pH 8.0). Then, 10 ml of home-made lysis solution SOL II (0.2 M NaOH, 1% SDS (sodium dodecyl sulphate)) was added to the resuspended mixture, gently mixed, and incubated at room temperature for 5 minutes. Finally, 10 ml of SOL III solution (3 M potassium acetate pH 4.5) was added to the mixture to neutralize the lysis solution, mixed, and centrifuged at 15000× g at 4˚C for 10 minutes. The supernatant was separated from the precipitate with an organza fabric filter, 0.7 volumes (20 ml) of room temperature isopropanol were added to the solution and the mixture was centrifuged at 15000× g at 4˚C for 30 minutes. The resulting precipitate was dissolved in 8 ml of RES buffer from the commercial purification kit NucleoBond® Xtra Midi EF (Machery-Nagel) and then the plasmid DNAs were purified using the kit following the manufacturer's instructions. The DNAs were eluted in 120 µl of endotoxin-free water; the concentration of the DNAs was quantified using NanoDrop™ 2000C spectrophotometer, and the resulting plasmid DNAs were verified by restriction analysis and Sanger sequencing.

3.1.3 *In Vitro* Transcription

Linearization of the *Flavivirus* PAC replicon cDNAs was performed with FastDigest *Bsh*T1 (Thermo Scientific™). 5 µg of plasmid DNA was added to the restriction mixture, and the linearized DNA was purified from the restriction mixture with DNA Clean and Concentrator kit (Zymo Research) following the manufacturer's instructions and eluted in 15 µl of nuclease-free water. 1 µg of linearized plasmid was transcribed *in vitro* using the mMESSAGE mMACHINE™ SP6 Transcription Kit (Invitrogen™, Thermo Fisher Scientific) according to the manufacturer's instructions. The reaction mixture was incubated at 37˚C for 2 hours. The integrity and yield of the *in vitro* transcribed RNA were assessed by gel electrophoresis in 0.8% agarose gels.

3.1.4 Cell Lines and Media

Baby hamster kidney cells (BHK-21**)** were maintained in Glasgow's modified essential medium (GMEM, Gibco) supplemented with 10% inactivated fetal calf serum (FCS, PAN Biotech), 2% tryptose phosphate broth, 10 mM HEPES pH 7.2, and 100 IU/ml Penicillin/1 µg/ml Streptomycin (Pen-Strep, Gibco). The BHK-21 cells harboring flaviviral replicons expressing the PAC gene were maintained in the same medium supplemented with 5 µg/ml puromycin (Gibco). All cells were maintained at 5% CO_2 and 37˚C.

3.1.5 Transfection of BHK-21 Cells with Replicon RNAs and Generation of Stable Cell Lines

BHK-21 cells were grown on a 100 mm diameter cell culture dish until 90-100% confluency, and then PAC gene expressing flaviviral replicons' *in vitro* transcribed RNAs were transfected into the cells to generate stable cell lines. The cells were resuspended in ice-cold phosphate-buffered saline (PBS, Gibco) and mixed with 19 µl of *in vitro* transcribed RNA mixture. Electroporation procedures were performed in 4-mm cuvettes (Bio-Rad) with two pulses at 850 V and 25 µF using a Bio-Rad Gene Pulser Xcell unit. The electroporated cells were then incubated for 24 h in a 100 mm cell culture dish containing fresh GMEM at 37˚C, 5% CO_2. After the incubation, the media was replaced with GMEM containing 5 µg/ml puromycin to select for puromycin-resistant cells, and the fresh puromycin-containing medium was replaced every 3-4 days. Puromycin-resistant colonies appeared in 4-5 weeks, which were picked and transferred into the 24-well plates to confirm their ability to keep their puromycin-resistant phenotype. The cell colonies were subsequently expanded to cell lines in 100 mm culture dishes under continuous puromycin selection which were stored

frozen in liquid nitrogen (three cell lines per replicon). Cell lines with mCherry and oxGFP markers were chosen based on their positive fluorescence signal observed by EVOS M5000 Imaging System (Thermo Fisher Scientific).

3.1.6 Verification of Nanoluciferase Stable Cell Lines

To confirm the expression of TBEV-NanoLuc-PAC replicon in stable BHK-21 cell lines, NanoLuc activity was measured from the cell lysates. In 24-well plates, an equal amount of TBEV-NanoLuc-PAC-Replicon stable cell suspension and BHK-21 cell suspension (1×10^5 cells/well) as control were added to each well and incubated at 37˚C, 5% CO_2 for 48 h. Following incubation, the cells were washed with PBS and lysed with 100 µl of 1× *Renilla* Luciferase lysis buffer (Promega), which was subsequently frozen at -80˚C and thawed at room temperature for 20 minutes with constant shaking at 200 rpm. The cell lysates were collected in 1.5 ml reaction tubes, and the coelenterazine substrate (TBD Pharmatech) was mixed with assay buffer (home-made, 50 mM Tris pH 7.5, 0.5 mM EDTA pH 8.0, 25 mM NaCl) to prepare the luciferase assay reagent. Next, 20 µl of cell lysate was mixed with 20 µl NanoLuc reagent, and relative light units (RLU) were measured with Glomax SIS (Promega) to determine NanoLuc activity. The results were assessed by GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California USA).

3.1.7 Immunofluorescence Assay of mCherry and oxGFP Stable Cell Lines

In order to visualize the KUNV and TBEV non-structural proteins of interest using microscopy, an immunofluorescence (IF) assay was carried out. Stable cell lines expressing respective flaviviral replicons (KUNV or TBEV) were seeded at an equal amount (5×10^5 cells/well) on glass coverslips in 6-well plates and incubated for 24 hours at 37˚C, 5% CO_2. Cells were washed with Dulbecco's PBS (DPBS), fixed with 4% paraformaldehyde (PFA) in DPBS at room temperature for 15 minutes, and permeabilized with 100% methanol at -20°C for 10 minutes. Cells were washed with DPBS and blocked with 5% FCS in DPBS overnight at 4˚C. KUNV- and TBEV- replicons bearing stable cells were stained overnight at 4˚C with rabbit anti-WNV NS5 (GTX131961, GeneTex) and chicken anti-TBEV NS3 (kindly provided by Dr. Anna Överby Wernstedt, Umeå University, Sweden) primary antibodies, respectively. Then, the cells were incubated at room temperature for 1 hour in the dark with secondary antibodies. Goat anti-rabbit Alexa Fluor™ 568, goat anti-rabbit Alexa Fluor™ 488, goat anti-chicken Alexa Fluor™ 647, and goat anti-chicken Alexa Fluor™ 488 (Invitrogen, Thermo Fisher Scientific) conjugated antibodies were used as

secondary antibodies for KUNV oxGFP, KUNV mCherry, TBEV oxGFP, and TBEV mCherry replicon cell lines, respectively. Nuclei were counterstained using SlowFade Gold reagent (Roche) containing 4',6'-diamidino-2-phenylindole (DAPI). Immunofluorescence images were obtained and analyzed using EVOS M5000 Imaging System (Thermo Fisher Scientific).

3.1.8 Verification of Non-Structural Protein Expression with Fluorescent Western Blot Analysis

To confirm the expression of TBEV NS3 and KUNV NS5 nonstructural proteins in stable BHK-21 cell lines harboring respective flaviviral replicons, a western blot (WB) analysis was carried out using the respective cell lysates. The replicon-harboring cell lines and an equal amount of BHK-21 (mock) cells (1.5×10^5 cells/well) as control were seeded to the 24-well plate and incubated at 37˚C, 5% CO_2 for 48 h. Cells were washed with PBS and lysed with 1× loading buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 10% glycerol, bromophenol blue) and heated for 10 min at 95°C. The proteins were separated on a 10% SDS polyacrylamide gel in 1× SDS running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) and transferred to the methanol-activated PVDF (polyvinylidene difluoride) transfer membrane (Thermo Fisher Scientific) with a constant voltage of 15 V using a trans-blot semi-dry (Bio-Rad) machine. The membranes were washed with semi-dry transfer buffer (48 mM Tris base, 39 mM glycine, 0.037% SDS, 20% methanol) and dried at room temperature for an hour. Prior to blocking with Intercept blocking buffer (LI-COR Biosciences) for an hour at room temperature, the membrane was activated with methanol, then washed with dH_2O and 1× TBS (20 mM Tris base, 150 mM NaCl). Membrane blocking was followed by incubation with the primary antibody mouse anti-β-actin (Santa Cruz Biotechnology) at 1:5000 dilution, and rabbit anti-WNV NS5 (GTX131961, GeneTex) or chicken anti-TBEV NS3 (kindly provided by Dr. Anna Överby Wernstedt, Umeå University, Sweden) at 1:1000 in Intercept blocking buffer containing 0.2% Tween20 overnight at 4°C on a shaker for KUNV and TBEV samples, respectively. The membranes were washed with 1× TBS/0.1% Tween20, then the secondary antibodies IRDye® 800CW goat anti-rabbit IgG (LI-COR Biosciences) to KUNV samples, and IRDye® 800CW donkey anti-chicken IgG (LI-COR Biosciences) to TBEV samples were applied at 1:20000 dilution in Intercept blocking buffer containing 0.2% Tween20 and 0.01% SDS for 1 hour at room temperature on a shaker. IRDye® 680RD goat anti-mouse IgG secondary antibody was used to develop the β-actin protein. For detection, the LI-COR

Odyssey FC 2800 Western Blot Imaging system (LI-COR Biosciences) at 2- and 10-minute acquisition times were used according to the manufacturer's protocol.

3.1.9 Comparative Nanoluciferase Assay of KUNV and TBEV Replicon RNA in Transfected BHK-21 Cells

To investigate if KUNV-NanoLuc-PAC replicon RNA transfected BHK-21 cells can express nanoluciferase protein or not, a nanoluciferase assay was carried out with TBEV-NanoLuc-PAC replicon RNA transfected BHK-21 cells as a positive control. The replicon cDNAs were linearized and *in vitro* transcribed to RNAs as described in 3.1.3. BHK-21 cells were grown on 100 mm cell culture dishes at 90-100% confluency prior to the transfection. BHK-21 cells were transfected with the replicon RNAs as described in 3.1.5, and then transfected cells were seeded on 6-well plates at an equal amount (7×10^5 cells/well). The cells were maintained in fresh GMEM at 37˚C, 5% CO_2. After the transfection, time points were taken at 24 h intervals for 6 days, and cell lysates were collected in 1.5 ml tubes. The NanoLuc reagent was prepared as described in 3.1.6, 20 µl of cell lysate was mixed with 20 µl NanoLuc substrate (3.1.6), and relative light units (RLU) was measured with Glomax SIS (Promega) to determine NanoLuc activity. The results were assessed by GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California USA).

3.2 RESULTS and DISCUSSION

3.2.1 Selection of Puromycin Resistant Colonies in BHK-21 Cells

The primary goal of this study was to create flavivirus replicon-carrying stable cell lines that could be handled in BSL2 conditions. The flaviviruses chosen for this goal were TBEV strain 93/738 (European subtype) and KUNV. The strain 93/738 of TBEV is a highly neurovirulent and pathogenic strain in comparison to strains from the other subtypes in addition to being a relevant virus to Estonia and other European countries (Lindqvist et al., 2020; Zhang, M. et al., 2022). On the other hand, KUNV replicon systems have been established and characterized well enough that KUNV replicons can be used as a good alternative as a control while working with other flavivirus replicon systems (Pijlman et al., 2006; Westaway et al., 2003). To create stable cell lines, we developed PAC gene-expressing replicons of TBEV and KUNV (referred to as PAC replicons). This gene has previously been used to successfully establish positive-sense RNA virus replicon harboring stable cell lines and demonstrated that it can provide the necessary expression of resistance in the selection process in BHK-21 cells (Kümmerer, 2018; Lücke et al., 2022; Utt et al., 2015; Varnavski et al., 2000). The PAC replicons constructed during the current study were based on the subgenomic replicons previously constructed by colleagues: the KUNV and TBEV subgenomic replicons were designed and constructed to carry a marker gene introduced to the in-frame deletion in the structural region of the flaviviral genome (Figure 6). Three versions of subgenomic replicons were created for KUNV and TBEV replicons, two of which encoding for the fluorescent proteins mCherry and oxGFP and one encoding a bioluminescent protein nanoluciferase. The PAC replicons were constructed for each version of the KUNV and TBEV replicons, for a total of six PAC replicons (see section 3.1.1). The PAC gene was inserted into the subgenomic replicons using basic cloning techniques based on restriction and ligation enzymes (see section 3.1.2). The synthetic fragments encoding for FMDV 2A autoprotease, PAC gene, codon-optimized FMDV 2A, the last 30 codons of the E protein (35 codons for TBEV), and the start of NS1 protein sequence. The subgenomic replicon-coding sequences carried unique restriction enzyme recognition sites located at the beginning of the FMDV 2A sequence and following the beginning of the NS1 protein coding sequence. These sites were utilized to remove a segment from the downstream of the marker gene to the end of approximately 200 codons of the NS1. This excised segment was replaced by the synthetic fragment carrying those removed segments in addition to the PAC and codon-optimized FMDV 2A genes. The sequences encoding the replicons were placed under

the control of the bacteriophage SP6 RNA polymerase promoter, which was used for *in vitro* transcription of the replicon RNA for cell transfection (Lücke et al., 2022). Given that both the marker and the PAC genes have an FMDV 2A autoprotease downstream, it was expected that both genes would be translated and cleaved from the polyprotein due to the FMDV 2A autocleavage mechanism based on ribosome skipping, providing detectable marker expression as well as resistance to puromycin that allows cells to persistently survive and grow under continuous selection (Minskaia & Ryan, 2013).

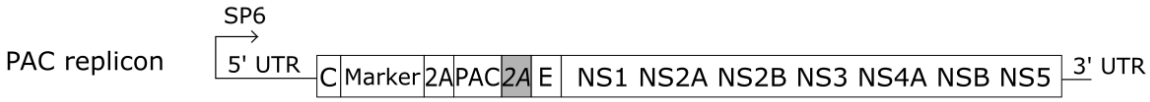

Figure 6. The basic scheme of flaviviral replicons bearing markers and puromycin resistance gene. Flaviviral PAC replicons have an in-frame deletion in the structural region that prevents them from producing virions. To visualize and analyze the expression of the replicon, a marker sequence with an FMDV 2A at its downstream is inserted into the deleted segment in the structural region. To establish stable cell lines, the PAC gene and a codon-optimized FMDV 2A are introduced to express puromycin resistance for the selection of replicon-positive cells.

After the PAC replicons were sequenced and verified to be aligning with the designed sequences, the replicons were *in vitro* transcribed to replicon RNAs (see section 3.1.3). *In vitro* transcribed RNAs were used to transfect BHK-21 cells via electroporation, and the continuous selection with puromycin (5 µg/ml) started on the next day (see section 3.1.5) (Frolov et al., 1999). The selection process was observed using a fluorescent microscope. Most of the cells died within a week after the start of the selection process, and it was possible to observe the surviving cells with detectable expression of the fluorescent markers (Figure 7A, Figure 8A). The appearance of KUNV replicon-transfected cells was similar to those of parental BHK-21 cells (Figure 7).

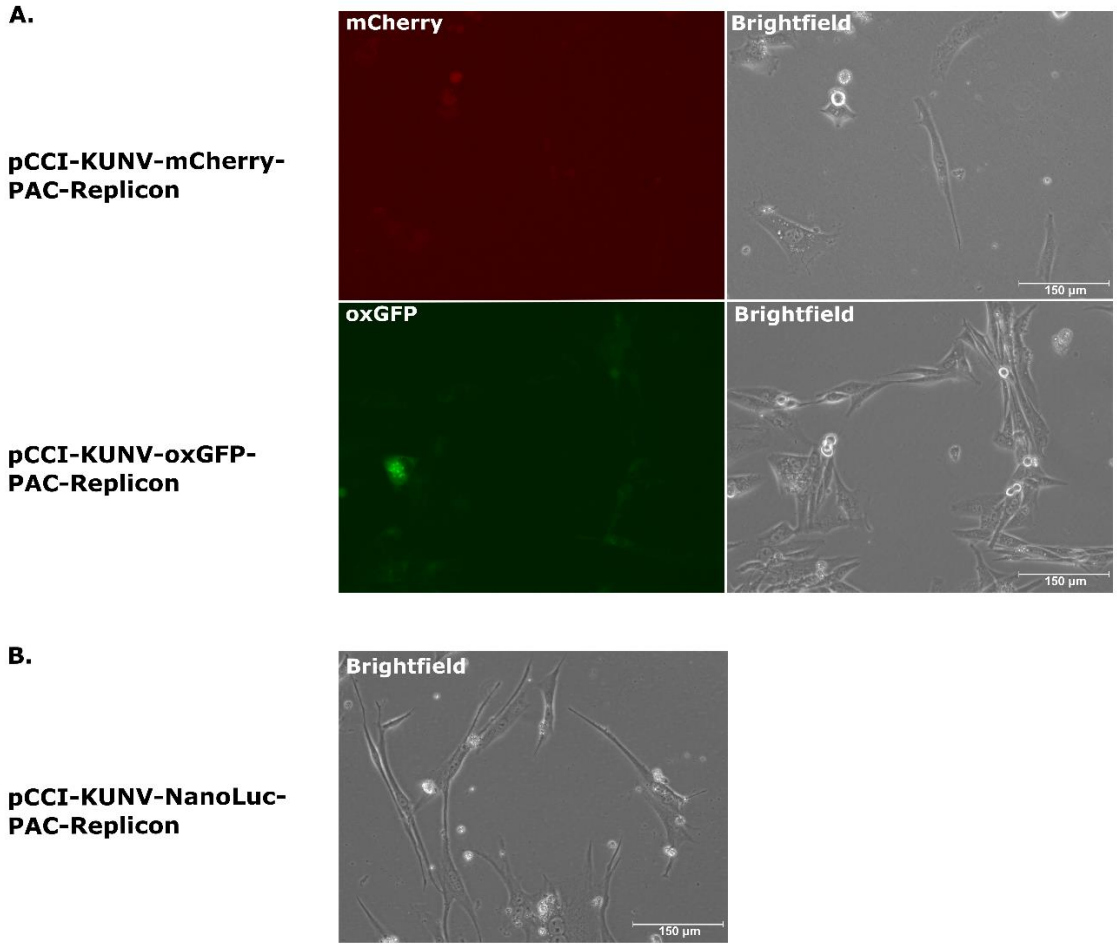

Figure 7. Selection of KUNV PAC replicon RNA transfected BHK-21 cells. BHK-21 cells were transfected with *in vitro* transcribed KUNV PAC replicon RNA via electroporation. Continuous puromycin selection started 24 hours after the electroporation. Transfected cells were imaged seven days later. **A.** The fluorescent markers mCherry and oxGFP were detectable via fluorescent microscopy. **B.** Brightfield image of the KUNV-NanoLuc-PAC replicon RNA transfected cells. The photographs were made using an EVOS M5000 microscope. The scale bar is 150 µm.

Interestingly, we observed some morphological differences in TBEV (mCherry and oxGFP) replicon-transfected BHK-21 cells, which did not resemble the parental cells (Figure 8A) reminding epithelial cells' morphology. Generally, cell morphology is rather dynamic and highly dependent on the status of the cells as well as on external factors. One of the reasons for the observed morphological change could be due to cells getting stressed and cells responding via a cell senescence program that involves the remodeling of the morphology (Ben-Porath & Weinberg, 2004; Fulda et al., 2010). During the electroporation and selection process, the BHK-21 cells undergo different events that are unnatural in comparison to the "normal" cell culture conditions. Electroporation compromises the cell membrane while puromycin causes premature peptide chain termination during translation in ribosomes, thus

affecting protein synthesis (Aviner, 2020). Additionally, the morphological changes could be due to the foreign RNA introduced to the cells. *In vitro* synthesized flavivirus replicon RNA is readily available for translation in the cells, and it expresses NS proteins that remodel the ER to create an environment that maximizes the efficiency of replicon RNA replication (Fishburn et al., 2022). It is known that disturbances in the ER functions lead to a conserved cell stress response called unfolded protein response (UPR) which restores ER protein homeostasis. Previously, it was shown for JEV-infected BHK-21 cells that the JEV infection acts as an ER stress inducer and a trigger of a UPR, as infected cells undergo apoptosis once the stress is persistent (Su et al., 2002). Additionally, UPR is mediated by inositol-requiring protein 1α (IRE1α) which is also responsible for the regulation of cytoskeleton remodeling by binding to filamin A (Gurel et al., 2014; Ren et al., 2021). Thus, it can be suggested that the TBEV PAC replicon RNA transfected cells might have had a morphological change due to the stress caused by external factors such as puromycin and internal factors like ER stress. This morphological change was not detected later in the stable cell lines, as the cells resembled parental BHK-21 cells (Fig 11).

Selection of nanoluciferase expressing replicon cell lines was performed “blindly”. Cell morphology and colony formation were observed without detecting the marker expression prior to the generation of stable cell lines. The nanoluciferase activity was evaluated when the culture of stable cell lines reached subconfluency on the 100 mm dishes. The KUNV-NanoLuc-PAC-Replicon transfected BHK-21 cells did not survive the continuous puromycin selection, as after three weeks post-transfection no viable cells were found on the 100 mm culture dish. This was unexpected because the other KUNV PAC replicon RNA transfected BHK-21 cells produced colonies successfully and the PAC replicons' sequences were confirmed to be correct. One theory was that the concentration of the puromycin used was too high for the selection of those cells. We assume that further optimization is required for KUNV-NanoLuc-PAC replicon harboring cell selection using puromycin since the puromycin concentration was chosen based on multiple experiments (not reflected in this master thesis) and was sufficient for selecting replicon harboring BHK-21 cells for the other five cell lines and on the previous publications (Liu et al., 2004; Lücke et al., 2022; Utt et al., 2015). Further analysis was also carried out to assess the functionality of the KUNV-NanoLuc-PAC replicon RNA in BHK-21 cells after electroporation, which will be discussed later (see section 3.2.5).

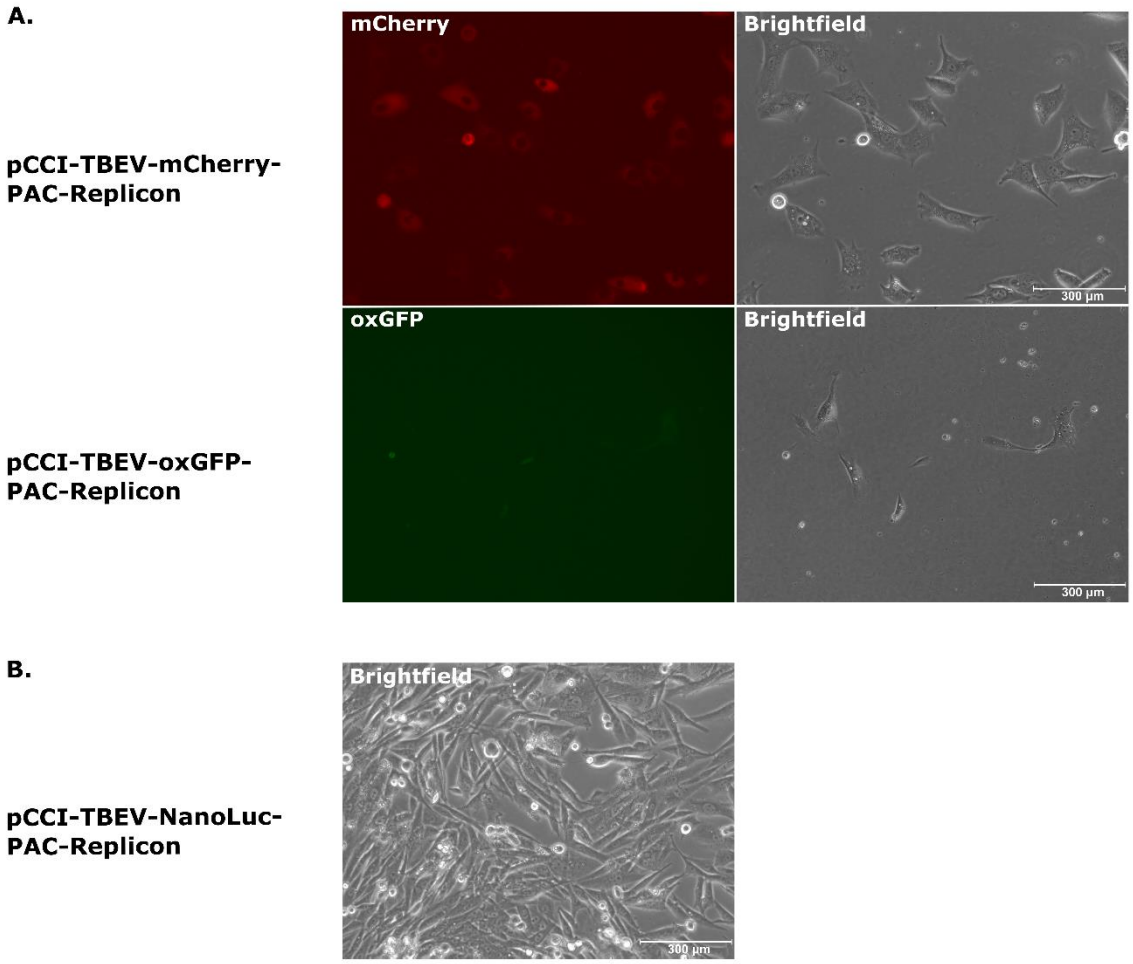

Figure 8. Selection of TBEV PAC replicon RNA transfected BHK-21 cells. BHK-21 cells were transfected with *in vitro* transcribed TBEV PAC replicon RNA via electroporation. Continuous puromycin selection started 24 hours after the electroporation. Most of the cells died within a week and on day seven, transfected cells were imaged. The scale bar is 300 µm. **A.** The fluorescent markers mCherry and oxGFP expressions were detectable via fluorescent microscopy. **B.** Brightfield image of the TBEV-NanoLuc-PAC replicon RNA transfected cells was taken as the bioluminescent marker is not microscopically detectable. The photographs were made using an EVOS M5000 microscope.

At approximately four weeks after the transfection, colonies of puromycin-resistant cells were formed in the selective media, except for KUNV-NanoLuc-PAC-Replicon transfected BHK-21 cells. For each cell line, excluding KUNV-NanoLuc-PAC-Replicon transfected cells, three single colonies were picked and transferred to 24-well plates. These cells were allowed to reach 90% confluency and transferred to 100 mm cell culture dishes, which were then subsequently expanded to stable cell lines under continuous puromycin selection. At three or four passages, stable cell lines were frozen to keep a low passage number for future use. Stable cell lines were further analyzed to detect the expression of KUNV/TBEV NS proteins and fluorescent/bioluminescent reporters.

3.2.2 Detection of Nanoluciferase Activity in TBEV-NanoLuc-PAC-Replicon Harboring Stable Cell Line

Passaging surviving cells continuously in a medium containing 5 µg/ml puromycin resulted in the generation of stable cell lines that persistently expressed the replicon RNAs. The detection of fluorescent signals microscopically provided the first evidence for the expression of replicon RNAs with fluorescent markers. However, for the TBEV-NanoLuc-PAC replicon harboring stable cells, detection of the visual microscopical signal was not possible, as the marker in this replicon was a bioluminescent reporter. Nanoluciferase reporter requires furimazine and molecular oxygen as substrates to produce bioluminescence that could be detected from cell lysate by a luminometer (England et al., 2016). Thus, to verify that the cells harboring TBEV-NanoLuc-PAC replicon were expressing a marker protein, we employed a nanoluciferase assay which is simple and rapid while being very sensitive.

TBEV-NanoLuc-PAC replicon harboring stable cells and BHK-21 cells as negative control (mock) were seeded 48 h prior to the assay. The nanoluciferase activity of the cells was measured from cell lysates using the appropriate substrate and a luminometer (see section 3.1.6). The results confirmed that the stable cell line harboring the TBEV-NanoLuc-PAC replicon could express the marker protein, indicating that the puromycin-based continuous selection was successful and the replicon RNA was functioning as expected (Figure 9).

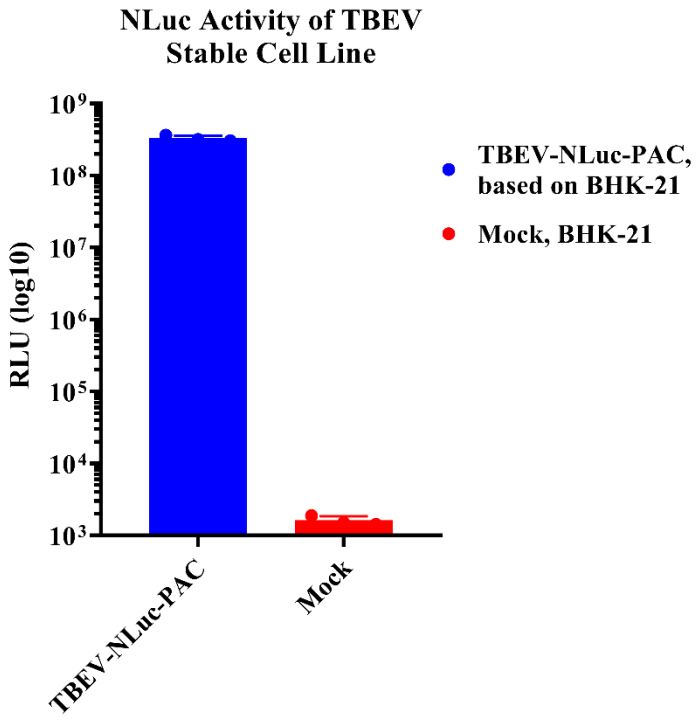

Figure 9. Detection of nanoluciferase activity in TBEV-NanoLuc-PAC replicon harboring stable cell line. Nanoluciferase activity was measured at 48 h after cell seeding from the collected cell lysates of TBEV-NanoLuc-PAC replicon harboring stable BHK-21 cells and mock (parental BHK-21 cells as the negative control). The measured bioluminescence data were normalized against cell count and expressed as mean±SE (*n*=3). The luminescence was read using a Glomax SIS luminometer and the data was processed using GraphPad Prism v. 8.0.1.

3.2.3 Detection of Marker and NS Proteins with Immunofluorescence Assay

The initial evidence for the established stable cell lines with the reporter markers was the detection of the fluorescent signal microscopically and the nanoluciferase assay. Although the initial evidence gave an idea for the functionality of the replicon RNA in BHK-21 cells, it was important to visualize the expression and localization of NS proteins in the stable cell lines to prove that the flaviviral replicon polyprotein processing was taking place as it is a crucial part of the replicon RNA replication. Thus, further evidence was sought for the replication of flaviviral replicon RNA via IF assay with antibodies against KUNV NS5 and TBEV NS3 proteins.

IF analysis with primary anti-NS5 antibody was used to detect the NS5 protein in KUNV PAC replicon harboring stable cell lines. The secondary antibodies were selected based on the marker that the respective stable cell line was expressing so that both the NS and the marker proteins could be visualized in the separate wavelength channels (see section 3.1.7).

The nuclei were counterstained with a DNA stain DAPI. Assaying of KUNV PAC replicon harboring stable cell lines by IF showed that the fluorescent marker was expressed in the cells (Figure 5). Similarly to the results in section 3.2.2, it can be suggested that both the reporter and the PAC proteins are functional. The cell shape and size were similar to those of parental BHK-21 cells. Moreover, we observed that the NS5 protein was localized in the cell cytoplasm of the KUNV PAC replicon harboring stable cells (Figure 10). According to an earlier study, KUNV NS5 is confined only to the cytoplasm where it acts as an RdRp for the synthesis of new genomic RNAs during the replication (Mackenzie et al., 2001, 2007). This indirectly proves that replicon RNA replication takes place in stable cells with the presence of NS5. The detected KUNV NS5 protein in the stable cell line can also indicate the presence of NS2B/3 protease as it is necessary for the cleavage of NS5 (to be functional) from the polyprotein. Thus, the IF assay provides evidence that the KUNV PAC replicon harboring stable cell lines can persistently express reporter markers along with the NS proteins.

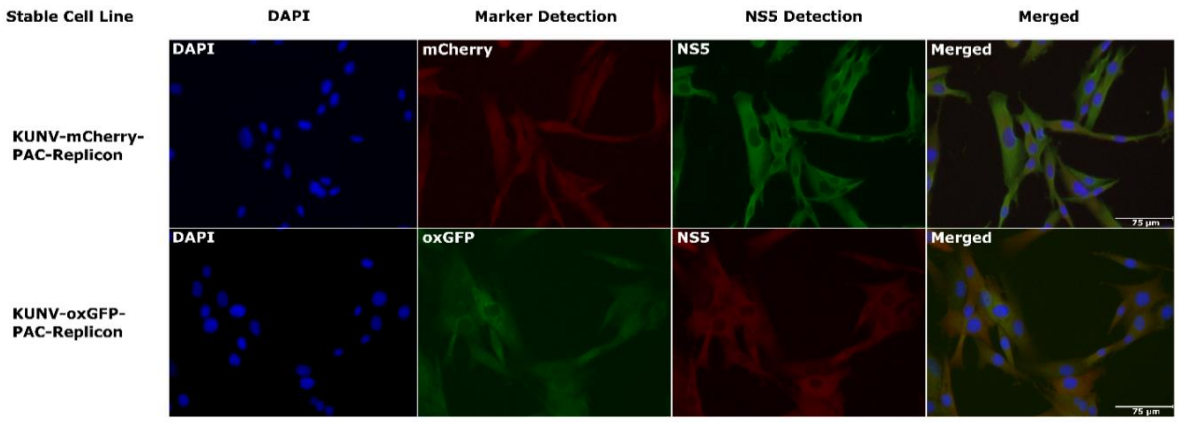

Figure 10. Detection of NS5 and fluorescent markers in KUNV PAC replicon harboring stable cell lines with IF assay. The cells were fixed with 4% PFA 24 h after cell seeding and stained with primary rabbit anti-WNV NS5 antibody overnight at 4˚C before the hour-long staining with secondary antibodies at room temperature in the dark. Goat anti-rabbit Alexa Fluor 488 (green) was used for KUNV-mCherry-PAC replicon harboring stable cell line, whereas goat anti-rabbit Alexa Fluor 568 (red) was used for KUNV-oxGFP-PAC replicon harboring cell line. The nuclei were counterstained with DAPI included in the cover slips' mounting medium. "Marker detection" represents the reporter marker expressed by the replicon RNA in the stable cell lines. The imaging was carried out using EVOS M5000. The scale bar represents 75 µm.

For the TBEV PAC replicon harboring stable cell lines, the IF analysis with primary anti-NS3 antibody was used to detect the NS3 protein. The secondary antibodies were selected based on the marker that the respective stable cell line was expressing so that both the NS and the marker proteins could be visualized in the separate wavelength channels (see section

3.1.7). The nuclei were counterstained with DAPI. Assaying of TBEV PAC replicon harboring stable cell lines by fluorescent microscopy showed the presence of the fluorescent marker in the cells (Figure 11) which suggested that both the reporter markers and the PAC gene are functional. As expected, no fluorescent signal was detected in the TBEV-NanoLuc-PAC replicon harboring stable cell lines (Figure 11). The presence of the nanoluciferase in that stable cell line was previously verified (see section 3.2.2). The size and shape of the cells were comparable to parental BHK-21 cells. Although this contrasts the morphology of the cells during the selection process (Figure 8A), normal cell morphology was the expected result in stable cell lines, and it can be suggested that the changes were likely a result of the cellular stress from the transfection and selection process. Furthermore, the localization of the NS3 protein in the stable cell lines was confined to the cell cytoplasm (Figure 11). This was in line with the reported NS3 localization of TBEV in mammalian cells (Selinger et al., 2019). As NS3 acts both as a protease in polyprotein processing and helicase in RNA replication for the unwinding of dsRNA in the TBEV life cycle (Lindenbach et al., 2013), it can be suggested that TBEV PAC replicon harboring stable cell lines are capable of replication of the replicon RNA. Moreover, the IF assay indicates that the stable cell lines are capable of expressing their fluorescent markers persistently.

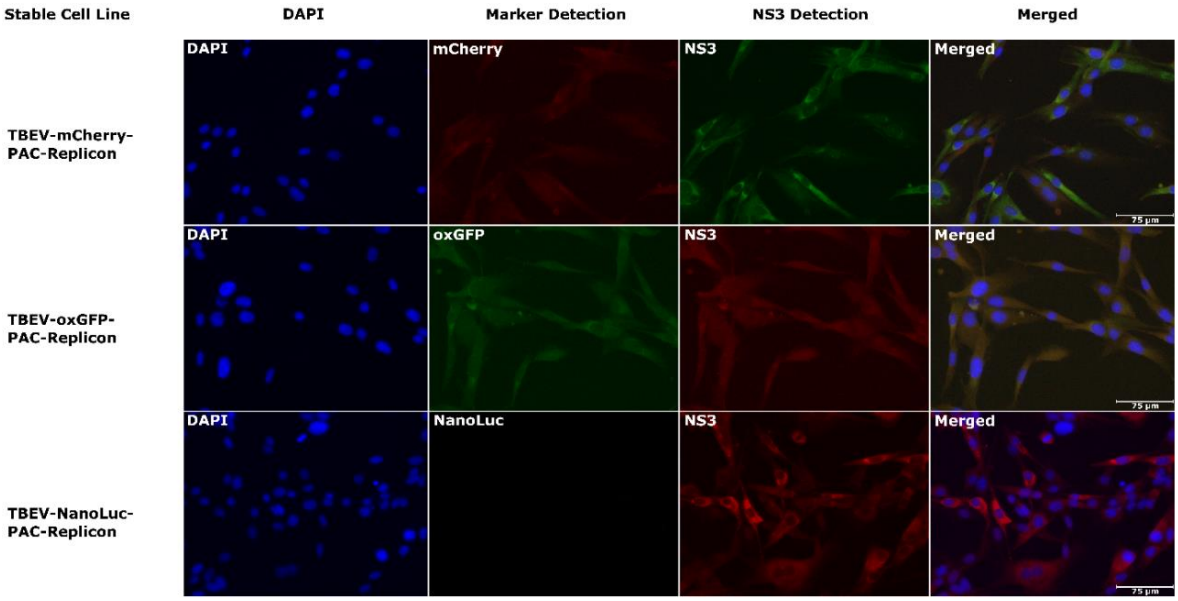

Figure 11. Detection of NS3 and fluorescent markers in TBEV PAC replicon harboring stable cell lines with IF assay. Stable cell lines were fixed with 4% PFA 24 h after seeding. The cells were stained with primary chicken anti-TBEV NS5 antibodies overnight at 4˚C before the hour-long staining with secondary antibodies at room temperature in the dark. Goat anti-chicken Alexa Fluor 488 (green) was used for TBEV-mCherry-PAC replicon harboring stable cells, whereas goat anti-chicken Alexa Fluor 647 (red) was used for TBEV-oxGFP and NanoLuc-PAC replicon harboring cells. The nuclei were counterstained with DAPI included in the mounting medium. "Marker detection" represents the marker protein expressed by the replicon RNA in the stable cell lines. The imaging was carried out using EVOS M5000. The scale bar represents 75 µm.

3.2.4 Detection of NS Proteins with Western Blot Assay

IF assay results gave the initial evidence that the chosen NS proteins were expressed in the cells of KUNV and TBEV replicon-bearing stable cell lines. To confirm the specificity of the IF assay results regarding the NS protein expression, the WB assay was performed with the stable cell lines as it can provide information about the size of the proteins and distinguish between proteins with similar molecular weight. β-actin was detected in the WB assays as an internal control to ensure even sample loading. This 42 kDa protein is one of the most conserved and abundant eukaryotic host cell proteins (Lee et al., 2016).

WB analysis with primary anti-NS5 antibody was used to detect the NS5 protein in KUNV PAC replicon harboring stable cell line lysates. Primary anti-β-actin antibody was used to detect the β-actin housekeeping protein in KUNV PAC replicon stable cell and mock (parental BHK-21 cells) lysates. The secondary antibodies were chosen to visualize NS5 and β-actin in separate wavelength channels (see section 3.1.8). β-actin can be detected both in stable and mock cells' lysates, and the band intensity is similar throughout samples which

indicates that the loaded lysates are similar in amount. In addition, for both stable cell lines (expressing mCherry and oxGFP markers) based on KUNV PAC replicons, WB assay shows that NS5 protein, which can be detected around the 100 kDa band as reported in earlier studies, is expressed in the cells, further verifying the IF detection of the protein expression in the cells (Figure 12A) (Mackenzie et al., 2007; Westaway et al., 2003). As mock sample lanes do not show any bands that would associate with the NS5 protein expression, it can be said that the expression of NS5 is specific to the stable cell lines of KUNV PAC replicons.

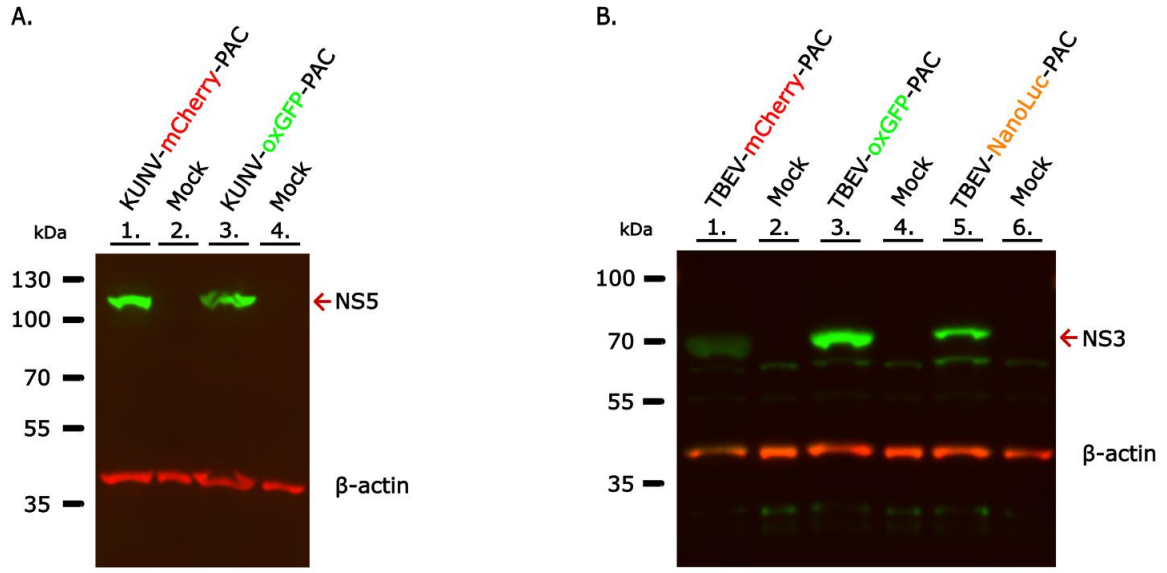

Figure 12. Detection of NS proteins in stable cell lines' lysates with WB assay. Stable cells and mock (parental BHK-21 cells as negative control) lysates were used to carry out the WB assay. The proteins were separated using a 10% SDS-polyacrylamide gel and transferred to a PVDF transfer membrane. WB assay images were done using LI-COR Odyssey FC 2800 Western Blot Imaging system at a 10-minute acquisition time. **A. Detection of KUNV NS5 protein.** After the protein transfer, the membrane was incubated with primary mouse anti-β-actin and rabbit anti-WNV NS5 antibodies overnight for the detection of β-actin and KUNV NS5 proteins. This incubation was followed by the secondary antibody incubation with IRDye 800CW goat anti-rabbit IgG (green) and IRDye 680RD goat anti-mouse IgG (red), which were visualized at 800 and 700 nm wavelengths, respectively. **B. Detection of TBEV NS3 protein.** After the protein transfer, the membrane was incubated with primary mouse anti-β-actin and chicken anti-TBEV NS3 antibodies overnight for the detection of β-actin and NS3 proteins. This incubation was followed by the secondary antibody incubation with IRDye 800CW donkey anti-chicken IgG (green) and IRDye 680RD goat anti-mouse IgG (red), which were visualized at 800 and 700 nm wavelengths, respectively.

Additionally, WB analysis with primary anti-NS3 antibody was used to detect the NS3 protein in TBEV PAC replicon harboring stable cell line lysates. Primary anti-β-actin antibody was used to detect the β-actin housekeeping protein in TBEV PAC replicon stable

and mock (parental BHK-21 cells) cells' lysates. The secondary antibodies were chosen to visualize NS3 and β-actin in separate wavelength channels (see section 3.1.8). β-actin can be detected both in stable cell and mock lysates, and the band intensity is similar throughout samples. Moreover, for all stable cell lines bearing TBEV PAC replicons, WB assay shows that NS3 protein (69 kDa), which can be detected around the 70 kDa band as reported in earlier studies, is expressed in the cells (Figure 12B) (Pugachev et al., 1993). As mock sample lanes do not show any bands similar to the NS3 protein size, it can be said that the expression of TBEV NS3 is specific to the stable cell lines of TBEV PAC replicons. One unexpected thing we detected was the presence of nonspecific bands in the WB assay image (Figure 12B). Nonspecific bands can be caused by a variety of reasons such as primary antibody concentration, sample amount, poor sample quality, or the way the transfer membrane was handled, like insufficient blocking or washing (Jensen, 2012; Yang & Mahmood, 2012). Although it is expected to have some variation between different WB assays, the assays were carried out in parallel, and all the conditions were the same, thus we think that the reason behind it is likely coming from the primary antibody. This can either be an issue due to the primary antibody concentration being too high, it is 5× higher than the primary antibody used to detect KUNV PAC replicon NS5, or the antibody specificity being poor. Nonspecific bands in the WB assay results could be resolved with further optimization, but in the scope of what was expected from the results of this assay, we can say that the WB assay verifies the IF detection of the NS3 protein expression in the cells.

In conclusion, based on the results that support each other, it can be said that the stable cell lines of TBEV PAC, KUNV-mCherry- and KUNV-oxGFP-PAC replicons were successfully generated. These cell lines can express the reporter markers, puromycin resistance, and NS proteins that were introduced in them within the replicon RNAs, indicating that replicon RNA replication takes place. These stable cell lines could further be used for inhibition assays to find potential compound candidates that can target the NS proteins that are responsible for the viral genome replication in nature, such as NS5 RdRp or NS3 helicase considering we have proved these proteins are present within the established stable cell lines.

3.2.5 Comparison of Nanoluciferase Activity in KUNV and TBEV-NanoLuc-PAC Replicon Transfected Cells

Stable cell lines for TBEV PAC were successfully established. As mentioned before (see section 3.2.1), KUNV-NanoLuc-PAC replicon transfected cells did not survive the puromycin selection and died out after three weeks, producing no colonies and therefore no

stable cell lines. One of the main reasons why this was an unexpected result for us was that the replicon sequence was confirmed to be correct like the other replicons that were used to generate stable cell lines. Additionally, compared to the other reporter marker genes we have used, the nanoluciferase coding sequence is a relatively short fragment and does not require post-translational modifications in mammalian cells, which may reduce the metabolic burden on the BHK-21 cells once the replicon RNA is introduced (England et al., 2016; Frei et al., 2020; Glick, 1995). Since we did not know what was causing the problem, we decided to test if BHK-21 cells transfected with this replicon RNA could express nanoluciferase protein transiently without puromycin selection. TBEV-NanoLuc-PAC replicon RNA was chosen as a control in this experiment, as it was already established that this replicon RNA is functional in BHK-21 cells.

To accomplish this, *in vitro* synthesized replicon RNAs of TBEV-NanoLuc-PAC and KUNV-NanoLuc-PAC were electroporated into BHK-21 cells, and timepoints were taken at 24 h intervals for six consecutive days to assess NanoLuc activity using the nanoluciferase assay (see section 3.1.9). During the experiment, the cells were kept in cell culture media without the addition of puromycin. Puromycin selection was not started because it was unclear whether the problem was caused by the selection or by the replicon RNA. The nanoluciferase assay indicated that the KUNV-NanoLuc-PAC replicon RNA transfected cells could express the marker protein. More importantly, the activity of the nanoluciferase in KUNV-NanoLuc-PAC replicon transfected cells followed a similar pattern to the TBEV-NanoLuc-PAC replicon transfected cells (Figure 13). The graph demonstrates that the KUNV-NanoLuc-PAC replicon RNA is readily translated into protein by the host cell as it can be deducted from the nanoluciferase activity from the first day. The steady increase in the nanoluciferase activity until the third day shows that the replicon RNA can replicate in the host cell and can be translated to produce more reporter protein. Then we see the activity drop, which is expected since there was no selection, and the replicon RNA eventually is degraded by the host cell RNA degradation pathways or diluted by cell division (Houseley & Tollervey, 2009).

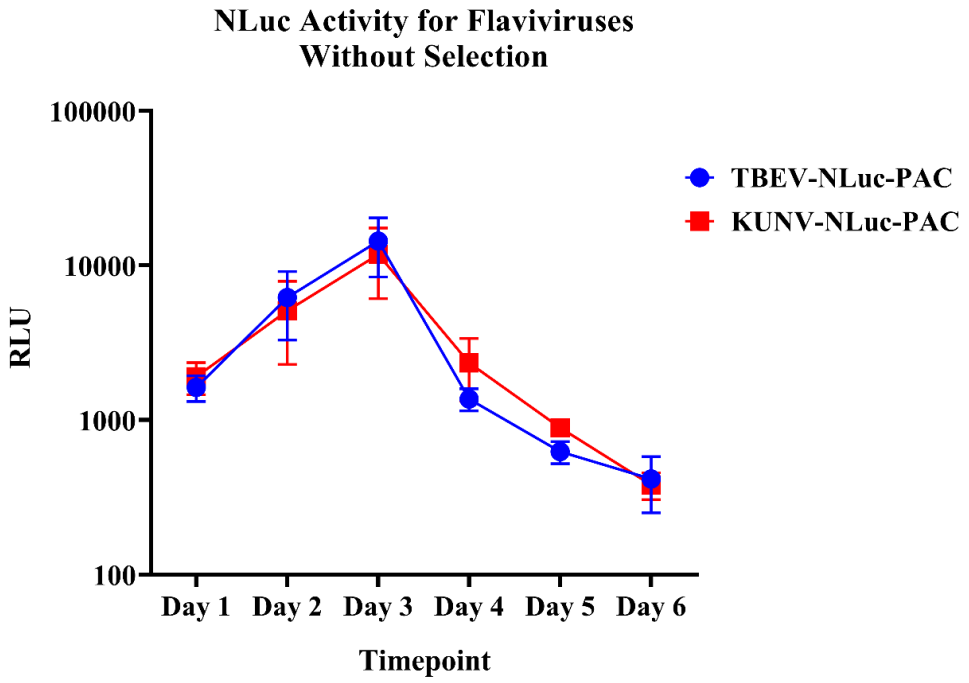

Figure 13. Nanoluciferase activity comparison between KUNV and TBEV-NanoLuc-PAC replicon transfected cells. Nanoluciferase activity was measured at 24 h intervals for six days after electroporation of BHK-21 cells with KUNV-NanoLuc-PAC and TBEV-NanoLuc-PAC replicon RNAs. Nanoluciferase activity was measured from cell lysates. The measured bioluminescence data were normalized against cell count and expressed as mean±SE (*n*=3). The luminescence was read using a Glomax SIS luminometer and the data was processed using GraphPad Prism v. 8.0.1.

It is likely that the KUNV-NanoLuc-PAC replicon is functional but cannot survive the selection process for some other reason, as the KUNV-NanoLuc-PAC replicon RNA transfected cells can express nanoluciferase (Figure 13) to the level comparable to the TBEV-NanoLuc-PAC replicon. To better understand the impact of puromycin selection on nanoluciferase expression, this experiment could be repeated with modified conditions, including the collection of data for a longer period and under puromycin selection. It is also possible to confirm the replicon's functionality by performing a WB assay on cell lysates to detect the NS5 protein. For cells transfected with KUNV-NanoLuc-PAC replicon RNA, the puromycin selection concentration may be too high; consequently, this concentration may be lowered and optimized to generate stable cell lines.

SUMMARY

Flaviviruses are positive-sense single-strand RNA viruses that belong to the family *Flaviviridae.* Most of them are arboviruses that can infect vertebrates through arthropod vectors such as mosquitoes and ticks. The most well-studied flaviviruses are important pathogens with a significant burden on global health as they can cause serious illnesses or even death. Despite the existence of a few vaccines, there are no specific antiviral drugs available, and vector control remains the primary strategy for managing flavivirus spread. The lack of countermeasures highlights the significance of vaccine and antiviral compound development. As most flaviviruses are BSL3 agents and require special handling, the development of vaccines and antivirals is hampered. A possible strategy to address this issue could be the utilization of replicon-based stable cell lines. Since replicons lack the majority of the structural genes and do not produce infectious particles, the biosafety concern is reduced while studying viral replication and inhibiting compounds against it. Thus, we aimed to generate KUNV and TBEV PAC replicon harboring stable cell lines that can be handled in BSL2 conditions as tools for RNA replication studies and inhibition assays.

In this study, the PAC gene was inserted into KUNV and TBEV replicons that carry reporter markers. Transfection of BHK-21 cells with *in vitro* transcribed PAC replicon RNAs was followed by a continuous selection of replicon-harboring cells with puromycin. The surviving cells were monitored for colony formation and fluorescent marker gene expression. Single colonies were selected for the generation of stable cell lines. Nanoluciferase, IF, and WB assays were used to validate the stable cell lines. The results demonstrated that the cell lines could persistently express NS proteins, fluorescent or luminescent reporter proteins, and puromycin resistance, indicating RNA replication. The only stable cell line that was not successfully generated was based on KUNV-NanoLuc-PAC replicon RNAs. However, transient transfection with this replicon RNA resulted in nanoluciferase expression comparable to that of the TBEV-NanoLuc-PAC replicon, implying that the replicon RNA is functional but fails to survive the selection process.

In conclusion, the stable BHK-21 cell lines of TBEV-NanoLuc/mCherry/oxGFP-PAC, and KUNV-mCherry/oxGFP-PAC replicons have been successfully generated. High-throughput screening of potential inhibitors of KUNV and TBEV replication targeting NS proteins using these stable cell lines could lead to the development of novel antiviral therapies.

REFERENCES

Apte-Sengupta, S., Sirohi, D., & Kuhn, R. J. (2014). Coupling of replication and assembly in flaviviruses. *Current Opinion in Virology*, *9*, 134–142. https://doi.org/10.1016/j.coviro.2014.09.020

Asghar, N., Lee, Y.-P., Nilsson, E., Lindqvist, R., Melik, W., Kröger, A., Överby, A. K., & Johansson, M. (2016). The role of the poly(A) tract in the replication and virulence of tick-borne encephalitis virus. *Scientific Reports*, *6*(1), 39265. https://doi.org/10.1038/srep39265

Ávila-Pérez, G., Nogales, A., Martín, V., Almazán, F., & Martínez-Sobrido, L. (2018). Reverse Genetic Approaches for the Generation of Recombinant Zika Virus. *Viruses*, *10*(11), 597. https://doi.org/10.3390/v10110597

Aviner, R. (2020). The science of puromycin: From studies of ribosome function to applications in biotechnology. In *Computational and Structural Biotechnology Journal* (Vol. 18). https://doi.org/10.1016/j.csbj.2020.04.014

Barrett, A. D. T., & Weaver, S. C. (2012). Arboviruses: alphaviruses, flaviviruses and bunyaviruses. In *Medical Microbiology* (pp. 520–536). Elsevier. https://doi.org/10.1016/B978-0-7020-4089-4.00066-4

Ben-Porath, I., & Weinberg, R. A. (2004). When cells get stressed: an integrative view of cellular senescence. *Journal of Clinical Investigation*, *113*(1), 8–13. https://doi.org/10.1172/JCI200420663

Best, S. M., Morris, K. L., Shannon, J. G., Robertson, S. J., Mitzel, D. N., Park, G. S., Boer, E., Wolfinbarger, J. B., & Bloom, M. E. (2005). Inhibition of Interferon-Stimulated JAK-STAT Signaling by a Tick-Borne Flavivirus and Identification of NS5 as an Interferon Antagonist. *Journal of Virology*, *79*(20), 12828–12839. https://doi.org/10.1128/JVI.79.20.12828-12839.2005

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R. W., Simmons, C. P., Scott, T. W., Farrar, J. J., & Hay, S. I. (2013).

The global distribution and burden of dengue. *Nature*, *496*(7446), 504–507. https://doi.org/10.1038/nature12060

Bressanelli, S., Stiasny, K., Allison, S. L., Stura, E. A., Duquerroy, S., Lescar, J., Heinz, F. X., & Rey, F. A. (2004). Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. *The EMBO Journal*, *23*(4), 728–738. https://doi.org/10.1038/sj.emboj.7600064

Burrell, C. J., Howard, C. R., & Murphy, F. A. (2017). Flaviviruses. In *Fenner and White's Medical Virology* (pp. 493–518). Elsevier. https://doi.org/10.1016/B978-0-12-375156-0.00036-9

Carpio, K. L., & Barrett, A. D. T. (2021). Flavivirus NS1 and Its Potential in Vaccine Development. *Vaccines*, *9*(6), 622. https://doi.org/10.3390/vaccines9060622

Carteaux, G., Maquart, M., Bedet, A., Contou, D., Brugières, P., Fourati, S., Cleret de Langavant, L., de Broucker, T., Brun-Buisson, C., Leparc-Goffart, I., & Mekontso Dessap, A. (2016). Zika Virus Associated with Meningoencephalitis. *New England Journal of Medicine*, *374*(16), 1595–1596. https://doi.org/10.1056/NEJMc1602964

Cedillo-Barrón, L., García-Cordero, J., Shrivastava, G., Carrillo-Halfon, S., León-Juárez, M., Bustos Arriaga, J., León Valenzuela, P., & Gutiérrez Castañeda, B. (2018). *The Role of Flaviviral Proteins in the Induction of Innate Immunity* (pp. 407–442). https://doi.org/10.1007/978-981-10-8456-0_17

Chambers, T. J., Hahn, C. S., Galler, R., & Rice, C. M. (1990). FLAVIVIRUS GENOME ORGANIZATION, EXPRESSION, AND REPLICATION. *Annual Review of Microbiology*, *44*(1), 649–688. https://doi.org/10.1146/annurev.mi.44.100190.003245

Corver, J., Lenches, E., Smith, K., Robison, R. A., Sando, T., Strauss, E. G., & Strauss, J. H. (2003). Fine Mapping of a *cis* -Acting Sequence Element in Yellow Fever Virus RNA That Is Required for RNA Replication and Cyclization. *Journal of Virology*, *77*(3), 2265–2270. https://doi.org/10.1128/JVI.77.3.2265-2270.2003

Davidson, R. B., Hendrix, J., Geiss, B. J., & McCullagh, M. (2020). RNA-Dependent Structures of the RNA-Binding Loop in the Flavivirus NS3 Helicase. *The Journal of Physical Chemistry B*, *124*(12), 2371–2381. https://doi.org/10.1021/acs.jpcb.0c00457

De La Luna, S., & Ortin, J. (1995). pac Gene as Efficient Dominant Marker and Reporter Gene in Mammalian Cells. In *Recombinant DNA Methodology II* (pp. 129–138). Elsevier. https://doi.org/10.1016/B978-0-12-765561-1.50015-1

England, C. G., Ehlerding, E. B., & Cai, W. (2016). NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconjugate Chemistry*, *27*(5), 1175–1187. https://doi.org/10.1021/acs.bioconjchem.6b00112

European Centre for Disease Prevention and Control. (2023). *Factsheet about tick-borne encephalitis (TBE)*. Factsheet about Tick-Borne Encephalitis (TBE). https://www.ecdc.europa.eu/en/tick-borne-encephalitis/facts/factsheet#:~:text=Tick-borne%20encephalitis%20is%20caused%20by,subtype%2C%20transmitted%20mainly%20by%20I.

Falgout, B., & Markoff, L. (1995). Evidence that flavivirus NS1-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. *Journal of Virology*, *69*(11), 7232–7243. https://doi.org/10.1128/jvi.69.11.7232-7243.1995

Fishburn, A. T., Pham, O. H., Kenaston, M. W., Beesabathuni, N. S., & Shah, P. S. (2022). Let's Get Physical: Flavivirus-Host Protein–Protein Interactions in Replication and Pathogenesis. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.847588

Flint, S. J., Racaniello, V. R., Rall, G. F., & Skalka, A. M. (2015). *Principles of Virology* (4th ed.). ASM Press.

Frei, T., Cella, F., Tedeschi, F., Gutiérrez, J., Stan, G.-B., Khammash, M., & Siciliano, V. (2020). Characterization and mitigation of gene expression burden in mammalian cells. *Nature Communications*, *11*(1), 4641. https://doi.org/10.1038/s41467-020-18392-x

Frolov, I., Agapov, E., Hoffman, T. A., Prágai, B. M., Lippa, M., Schlesinger, S., & Rice, C. M. (1999). Selection of RNA Replicons Capable of Persistent Noncytopathic Replication in Mammalian Cells. *Journal of Virology*, *73*(5), 3854–3865. https://doi.org/10.1128/JVI.73.5.3854-3865.1999

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: Cell survival and cell death. In *International Journal of Cell Biology*. https://doi.org/10.1155/2010/214074

Füzik, T., Formanová, P., Růžek, D., Yoshii, K., Niedrig, M., & Plevka, P. (2018). Structure of tick-borne encephalitis virus and its neutralization by a monoclonal antibody. *Nature Communications*, *9*(1), 436. https://doi.org/10.1038/s41467-018-02882-0

García, L. L., Padilla, L., & Castaño, J. C. (2017). Inhibitors compounds of the flavivirus replication process. *Virology Journal*, *14*(1), 95. https://doi.org/10.1186/s12985-017-0761-1

Glick, B. R. (1995). Metabolic load and heterologous gene expression. *Biotechnology Advances*, *13*(2), 247–261. https://doi.org/10.1016/0734-9750(95)00004-A

Gurel, P. S., Hatch, A. L., & Higgs, H. N. (2014). Connecting the cytoskeleton to the endoplasmic reticulum and Golgi. In *Current Biology* (Vol. 24, Issue 14). https://doi.org/10.1016/j.cub.2014.05.033

Hall, R. A., Broom, A. K., Smith, D. W., & Mackenzie, J. S. (2002). *The Ecology and Epidemiology of Kunjin Virus* (pp. 253–269). https://doi.org/10.1007/978-3-642-59403-8_13

Hardy, J. M., Newton, N. D., Modhiran, N., Scott, C. A. P., Venugopal, H., Vet, L. J., Young, P. R., Hall, R. A., Hobson-Peters, J., Coulibaly, F., & Watterson, D. (2021). A unified route for flavivirus structures uncovers essential pocket factors conserved across pathogenic viruses. *Nature Communications*, *12*(1), 3266. https://doi.org/10.1038/s41467-021-22773-1

Hayasaka, D., Yoshii, K., Ueki, T., Iwasaki, T., & Takashima, I. (2004). Sub-genomic replicons of Tick-borne encephalitis virus. *Archives of Virology*, *149*(6), 1245–1256. https://doi.org/10.1007/s00705-003-0262-y

Houseley, J., & Tollervey, D. (2009). The Many Pathways of RNA Degradation. *Cell*, *136*(4), 763–776. https://doi.org/10.1016/j.cell.2009.01.019

Ilkal, M. A., Prasanna, Y., Jacob, P. G., Geevarghese, G., & Banerjee, K. (1994). Experimental studies on the susceptibility of domestic pigs to West Nile virus followed by Japanese encephalitis virus infection and vice versa. *Acta Virologica*, *38*(3), 157–161.

International Committee on Taxonomy of Viruses. (2022). *Genus: Orthoflavivirus*. Virus Taxonomy: 2022 Release. https://ictv.global/taxonomy/taxondetails?taxnode_id=202203070

Jeffries, C. L., Mansfield, K. L., Phipps, L. P., Wakeley, P. R., Mearns, R., Schock, A., Bell, S., Breed, A. C., Fooks, A. R., & Johnson, N. (2014). Louping ill virus: an endemic tick-borne disease of Great Britain. *The Journal of General Virology*, *95*(Pt 5), 1005–1014. https://doi.org/10.1099/vir.0.062356-0

Jensen, E. C. (2012). The Basics of Western Blotting. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, *295*(3), 369–371. https://doi.org/10.1002/ar.22424

Johnson, N., Voller, K., Phipps, L. P., Mansfield, K., & Fooks, A. R. (2012). Rapid Molecular Detection Methods for Arboviruses of Livestock of Importance to Northern Europe. *Journal of Biomedicine and Biotechnology*, *2012*, 1–18. https://doi.org/10.1155/2012/719402

Jones, C. T., Patkar, C. G., & Kuhn, R. J. (2005). Construction and applications of yellow fever virus replicons. *Virology*, *331*(2), 247–259. https://doi.org/10.1016/j.virol.2004.10.034

Katayama, T., Saito, S., Horiuchi, S., Maruta, T., Kato, T., Yanase, T., Yamakawa, M., & Shirafuji, H. (2013). Nonsuppurative Encephalomyelitis in a Calf in Japan and Isolation of Japanese Encephalitis Virus Genotype 1 from the Affected Calf. *Journal of Clinical Microbiology*, *51*(10), 3448–3453. https://doi.org/10.1128/JCM.00737-13

Khromykh, A. A., & Westaway, E. G. (1997). Subgenomic replicons of the flavivirus Kunjin: construction and applications. *Journal of Virology*, *71*(2), 1497–1505. https://doi.org/10.1128/jvi.71.2.1497-1505.1997

Kofler, R. M., Heinz, F. X., & Mandl, C. W. (2002). Capsid Protein C of Tick-Borne Encephalitis Virus Tolerates Large Internal Deletions and Is a Favorable Target for Attenuation of Virulence. *Journal of Virology*, *76*(7), 3534–3543. https://doi.org/10.1128/JVI.76.7.3534-3543.2002

Kümmerer, B. M. (2018). *Establishment and Application of Flavivirus Replicons* (pp. 165–173). https://doi.org/10.1007/978-981-10-8727-1_12

Kümmerer, B. M., & Rice, C. M. (2002). Mutations in the Yellow Fever Virus Nonstructural Protein NS2A Selectively Block Production of Infectious Particles. *Journal of Virology*, *76*(10), 4773–4784. https://doi.org/10.1128/JVI.76.10.4773-4784.2002

Kurnia, N., Kaitana, Y., Salaki, C. L., Mandey, L. C., Tuda, J. S. B., & Tallei, T. E. (2022). Study of Dengue Virus Transovarial Transmission in Aedes spp. in Ternate City Using Streptavidin-Biotin-Peroxidase Complex Immunohistochemistry. *Infectious Disease Reports*, *14*(5), 765–771. https://doi.org/10.3390/idr14050078

Kutschera, L. S., & Wolfinger, M. T. (2022). Evolutionary traits of Tick-borne encephalitis virus: Pervasive non-coding RNA structure conservation and molecular epidemiology. *Virus Evolution*, *8*(1). https://doi.org/10.1093/ve/veac051

Labuda, M., & Nuttall, P. A. (2004). Tick-borne viruses. *Parasitology*, *129*(S1), S221–S245. https://doi.org/10.1017/S0031182004005220

Lahoz, E. G., de Haro, M. A. L., & Esponda, P. (1991). Use of puromycin N-acetyltransferase (PAC) as a new reporter gene in transgenic animals. *Nucleic Acids Research*, *19*(12), 3465–3465. https://doi.org/10.1093/nar/19.12.3465

Lee, H.-G., Jo, J., Hong, H.-H., Kim, K. K., Park, J.-K., Cho, S.-J., & Park, C. (2016). State-of-the-art housekeeping proteins for quantitative western blotting: Revisiting the first draft of the human proteome. *PROTEOMICS*, *16*(13), 1863–1867. https://doi.org/10.1002/pmic.201500344

Leung, J. Y., Pijlman, G. P., Kondratieva, N., Hyde, J., Mackenzie, J. M., & Khromykh, A. A. (2008). Role of Nonstructural Protein NS2A in Flavivirus Assembly. *Journal of Virology*, *82*(10), 4731–4741. https://doi.org/10.1128/JVI.00002-08

Li, K., Phoo, W. W., & Luo, D. (2014). Functional interplay among the flavivirus NS3 protease, helicase, and cofactors. *Virologica Sinica*, *29*(2), 74–85. https://doi.org/10.1007/s12250-014-3438-6

Li, L., Lok, S.-M., Yu, I.-M., Zhang, Y., Kuhn, R. J., Chen, J., & Rossmann, M. G. (2008). The Flavivirus Precursor Membrane-Envelope Protein Complex: Structure and Maturation. *Science*, *319*(5871), 1830–1834. https://doi.org/10.1126/science.1153263

Li, S.-H., Li, X.-F., Zhao, H., Deng, Y.-Q., Yu, X.-D., Zhu, S.-Y., Jiang, T., Ye, Q., Qin, E.-D., & Qin, C.-F. (2013). Development and characterization of the replicon system of Japanese encephalitis live vaccine virus SA14-14-2. *Virology Journal*, *10*(1), 64. https://doi.org/10.1186/1743-422X-10-64

Ličková, M., Fumačová Havlíková, S., Sláviková, M., Slovák, M., Drexler, J. F., & Klempa, B. (2020). Dermacentor reticulatus is a vector of tick-borne encephalitis virus. *Ticks and Tick-Borne Diseases*, *11*(4), 101414. https://doi.org/10.1016/j.ttbdis.2020.101414

Lindenbach, B. D., Murray, C. L., Thiel, H.-J., & Rice, C. M. (2013). Flaviviridae: the viruses and their replication. In Knipe DM, Howley PM, Cohen JI, Griffin DE, Lamb RA, Martin MA, Racaniello VR, & Roizman B (Eds.), *Fields Virology* (6th ed., pp. 712–746). Lippincott Williams&Wilkins.

Lindenbach, B. D., & Rice, C. M. (1999). Genetic Interaction of Flavivirus Nonstructural Proteins NS1 and NS4A as a Determinant of Replicase Function. *Journal of Virology*, *73*(6), 4611–4621. https://doi.org/10.1128/JVI.73.6.4611-4621.1999

Lindenbach, B. D., & Rice, C. M. (2003). *Molecular biology of flaviviruses* (pp. 23–61). https://doi.org/10.1016/S0065-3527(03)59002-9

Lindqvist, R., Rosendal, E., Weber, E., Asghar, N., Schreier, S., Lenman, A., Johansson, M., Dobler, G., Bestehorn, M., Kröger, A., & Överby, A. K. (2020). The envelope protein of tick-borne encephalitis virus influences neuron entry, pathogenicity, and vaccine protection. *Journal of Neuroinflammation*, *17*(1), 284. https://doi.org/10.1186/s12974-020-01943-w

Liu, W. J., Chen, H. B., & Khromykh, A. A. (2003). Molecular and Functional Analyses of Kunjin Virus Infectious cDNA Clones Demonstrate the Essential Roles for NS2A in

Virus Assembly and for a Nonconservative Residue in NS3 in RNA Replication. *Journal of Virology*, *77*(14), 7804–7813. https://doi.org/10.1128/JVI.77.14.7804-7813.2003

Liu, W. J., Chen, H. B., Wang, X. J., Huang, H., & Khromykh, A. A. (2004). Analysis of Adaptive Mutations in Kunjin Virus Replicon RNA Reveals a Novel Role for the Flavivirus Nonstructural Protein NS2A in Inhibition of Beta Interferon Promoter-Driven Transcription. *Journal of Virology*, *78*(22). https://doi.org/10.1128/jvi.78.22.12225-12235.2004

Lücke, A.-C., vom Hemdt, A., Wieseler, J., Fischer, C., Feldmann, M., Rothenfusser, S., Drexler, J. F., & Kümmerer, B. M. (2022). High-Throughput Platform for Detection of Neutralizing Antibodies Using Flavivirus Reporter Replicon Particles. *Viruses*, *14*(2), 346. https://doi.org/10.3390/v14020346

Luo, D., Vasudevan, S. G., & Lescar, J. (2015). The flavivirus NS2B–NS3 protease–helicase as a target for antiviral drug development. *Antiviral Research*, *118*, 148–158. https://doi.org/10.1016/j.antiviral.2015.03.014

Ma, L., Jones, C. T., Groesch, T. D., Kuhn, R. J., & Post, C. B. (2004). Solution structure of dengue virus capsid protein reveals another fold. *Proceedings of the National Academy of Sciences*, *101*(10), 3414–3419. https://doi.org/10.1073/pnas.0305892101

Mackenzie, J. M., Kenney, M. T., & Westaway, E. G. (2007). West Nile virus strain Kunjin NS5 polymerase is a phosphoprotein localized at the cytoplasmic site of viral RNA synthesis. *Journal of General Virology*, *88*(4), 1163–1168. https://doi.org/10.1099/vir.0.82552-0

Mackenzie, J. M., Khromykh, A. A., Jones, M. K., & Westaway, E. G. (1998). Subcellular Localization and Some Biochemical Properties of the Flavivirus Kunjin Nonstructural Proteins NS2A and NS4A. *Virology*, *245*(2), 203–215. https://doi.org/10.1006/viro.1998.9156

Mackenzie, J. M., Khromykh, A. A., & Westaway, E. G. (2001). Stable Expression of Noncytopathic Kunjin Replicons Simulates Both Ultrastructural and Biochemical Characteristics Observed during Replication of Kunjin Virus. *Virology*, *279*(1), 161–172. https://doi.org/10.1006/viro.2000.0691

Mason, P. W. (1989). Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology*, *169*(2), 354–364. https://doi.org/10.1016/0042-6822(89)90161-X

Miller, S., Sparacio, S., & Bartenschlager, R. (2006). Subcellular Localization and Membrane Topology of the Dengue Virus Type 2 Non-structural Protein 4B. *Journal of Biological Chemistry*, *281*(13), 8854–8863. https://doi.org/10.1074/jbc.M512697200

Minskaia, E., & Ryan, M. D. (2013). Protein Coexpression Using FMDV 2A: Effect of "Linker" Residues. *BioMed Research International*, *2013*, 1–12. https://doi.org/10.1155/2013/291730

Mlera, L., Melik, W., & Bloom, M. E. (2014). The role of viral persistence in flavivirus biology. *Pathogens and Disease*, *71*(2), 137–163. https://doi.org/10.1111/2049-632X.12178

Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature*, *427*(6972), 313–319. https://doi.org/10.1038/nature02165

NG, C., GU, F., PHONG, W., CHEN, Y., LIM, S., DAVIDSON, A., & VASUDEVAN, S. (2007). Construction and characterization of a stable subgenomic dengue virus type 2 replicon system for antiviral compound and siRNA testing. *Antiviral Research*, *76*(3), 222–231. https://doi.org/10.1016/j.antiviral.2007.06.007

Park, G. S., Morris, K. L., Hallett, R. G., Bloom, M. E., & Best, S. M. (2007). Identification of Residues Critical for the Interferon Antagonist Function of Langat Virus NS5 Reveals a Role for the RNA-Dependent RNA Polymerase Domain. *Journal of Virology*, *81*(13), 6936–6946. https://doi.org/10.1128/JVI.02830-06

Patkar, C. G., Jones, C. T., Chang, Y., Warrier, R., & Kuhn, R. J. (2007). Functional Requirements of the Yellow Fever Virus Capsid Protein. *Journal of Virology*, *81*(12), 6471–6481. https://doi.org/10.1128/JVI.02120-06

Pierson, T. C., & Diamond, M. S. (2020). The continued threat of emerging flaviviruses. *Nature Microbiology*, *5*(6), 796–812. https://doi.org/10.1038/s41564-020-0714-0

Pijlman, G. P., Suhrbier, A., & Khromykh, A. A. (2006). Kunjin virus replicons: an RNA-based, non-cytopathic viral vector system for protein production, vaccine and gene therapy applications. *Expert Opinion on Biological Therapy*, *6*(2), 135–145. https://doi.org/10.1517/14712598.6.2.135

Polacek, C., Friebe, P., & Harris, E. (2009). Poly(A)-binding protein binds to the non-polyadenylated 3′ untranslated region of dengue virus and modulates translation efficiency. *Journal of General Virology*, *90*(3), 687–692. https://doi.org/10.1099/vir.0.007021-0

Pugachev, K. V., Nomokonova, N. Yu., Dobrikova, E. Yu., & Wolf, Y. I. (1993). Site-directed mutagenesis of the tick-borne encephalitis virus NS3 gene reveals the putative serine protease domain of the NS3 protein. *FEBS Letters*, *328*(1–2), 115–118. https://doi.org/10.1016/0014-5793(93)80977-3

Pulkkinen, L. I. A., Barrass, S. V, Domanska, A., Överby, A. K., Anastasina, M., & Butcher, S. J. (2022). Molecular Organisation of Tick-Borne Encephalitis Virus. *Viruses*, *14*(4). https://doi.org/10.3390/v14040792

Ramos-Lorente, S., Romero-López, C., & Berzal-Herranz, A. (2021). Information Encoded by the Flavivirus Genomes beyond the Nucleotide Sequence. *International Journal of Molecular Sciences*, *22*(7), 3738. https://doi.org/10.3390/ijms22073738

Rastogi, M., Sharma, N., & Singh, S. K. (2016). Flavivirus NS1: a multifaceted enigmatic viral protein. *Virology Journal*, *13*(1), 131. https://doi.org/10.1186/s12985-016-0590-7

Rathore, A. P. S., Paradkar, P. N., Watanabe, S., Tan, K. H., Sung, C., Connolly, J. E., Low, J., Ooi, E. E., & Vasudevan, S. G. (2011). Celgosivir treatment misfolds dengue virus NS1 protein, induces cellular pro-survival genes and protects against lethal challenge mouse model. *Antiviral Research*, *92*(3), 453–460. https://doi.org/10.1016/j.antiviral.2011.10.002

Reid, D. W., Campos, R. K., Child, J. R., Zheng, T., Chan, K. W. K., Bradrick, S. S., Vasudevan, S. G., Garcia-Blanco, M. A., & Nicchitta, C. V. (2018). Dengue Virus Selectively Annexes Endoplasmic Reticulum-Associated Translation Machinery as a Strategy for Co-opting Host Cell Protein Synthesis. *Journal of Virology*, *92*(7). https://doi.org/10.1128/JVI.01766-17

Ren, J., Bi, Y., Sowers, J. R., Hetz, C., & Zhang, Y. (2021). Endoplasmic reticulum stress and unfolded protein response in cardiovascular diseases. In *Nature Reviews Cardiology* (Vol. 18, Issue 7). https://doi.org/10.1038/s41569-021-00511-w

Robert, M. A., Stewart-Ibarra, A. M., & Estallo, E. L. (2020). Climate change and viral emergence: evidence from Aedes-borne arboviruses. *Current Opinion in Virology*, *40*, 41–47. https://doi.org/10.1016/j.coviro.2020.05.001

Roehrig, J. (2013). West Nile Virus in the United States — A Historical Perspective. *Viruses*, *5*(12), 3088–3108. https://doi.org/10.3390/v5123088

Rosen, L. (1988). Further Observations on the Mechanism of Vertical Transmission of Flaviviruses by Aedes Mosquitoes. *The American Journal of Tropical Medicine and Hygiene*, *39*(1), 123–126. https://doi.org/10.4269/ajtmh.1988.39.123

Sampath, A., & Padmanabhan, R. (2009). Molecular targets for flavivirus drug discovery. *Antiviral Research*, *81*(1), 6–15. https://doi.org/10.1016/j.antiviral.2008.08.004

Scherret, J. H., Mackenzie, J. S., Hall, R. A., Deubel, V., & Gould, E. A. (2002). *Phylogeny and Molecular Epidemiology of West Nile and Kunjin Viruses* (pp. 373–390). https://doi.org/10.1007/978-3-642-59403-8_18

Selinger, M., Tykalová, H., Štěrba, J., Věchtová, P., Vavrušková, Z., Lieskovská, J., Kohl, A., Schnettler, E., & Grubhoffer, L. (2019). Tick-borne encephalitis virus inhibits rRNA synthesis and host protein production in human cells of neural origin. *PLOS Neglected Tropical Diseases*, *13*(9), e0007745. https://doi.org/10.1371/journal.pntd.0007745

Shi, P.-Y., Tilgner, M., & Lo, M. K. (2002). Construction and Characterization of Subgenomic Replicons of New York Strain of West Nile Virus. *Virology*, *296*(2), 219–233. https://doi.org/10.1006/viro.2002.1453

Simmonds, P., Becher, P., Bukh, J., Gould, E. A., Meyers, G., Monath, T., Muerhoff, S., Pletnev, A., Rico-Hesse, R., Smith, D. B., & Stapleton, J. T. (2017). ICTV Virus Taxonomy Profile: Flaviviridae. *Journal of General Virology*, *98*(1), 2–3. https://doi.org/10.1099/jgv.0.000672

Sironi, M., Forni, D., Clerici, M., & Cagliani, R. (2016). Nonstructural Proteins Are Preferential Positive Selection Targets in Zika Virus and Related Flaviviruses. *PLOS Neglected Tropical Diseases*, *10*(9), e0004978. https://doi.org/10.1371/journal.pntd.0004978

Smit, J., Moesker, B., Rodenhuis-Zybert, I., & Wilschut, J. (2011). Flavivirus Cell Entry and Membrane Fusion. *Viruses*, *3*(2), 160–171. https://doi.org/10.3390/v3020160

Su, H.-L., Liao, C.-L., & Lin, Y.-L. (2002). Japanese Encephalitis Virus Infection Initiates Endoplasmic Reticulum Stress and an Unfolded Protein Response. *Journal of Virology*, *76*(9). https://doi.org/10.1128/jvi.76.9.4162-4171.2002

Tabachnick, W. J. (2016). Climate Change and the Arboviruses: Lessons from the Evolution of the Dengue and Yellow Fever Viruses. *Annual Review of Virology*, *3*(1), 125–145. https://doi.org/10.1146/annurev-virology-110615-035630

Tan, M. J. A., Brown, N. G., Chan, K. W. K., Jin, J. Y., Zu Kong, S. Y., & Vasudevan, S. G. (2020). Mutations in the cytoplasmic domain of dengue virus NS4A affect virus fitness and interactions with other non-structural proteins. *Journal of General Virology*, *101*(9), 941–953. https://doi.org/10.1099/jgv.0.001462

Tsetsarkin, K. A., Liu, G., Shen, K., & Pletnev, A. G. (2016). Kissing-loop interaction between 5’ and 3’ ends of tick-borne Langat virus genome “bridges the gap” between mosquito- and tick-borne flaviviruses in mechanisms of viral RNA cyclization: applications for virus attenuation and vaccine development. *Nucleic Acids Research*, *44*(7), 3330–3350. https://doi.org/10.1093/nar/gkw061

Utt, A., Das, P. K., Varjak, M., Lulla, V., Lulla, A., & Merits, A. (2015). Mutations Conferring a Noncytotoxic Phenotype on Chikungunya Virus Replicons Compromise Enzymatic Properties of Nonstructural Protein 2. *Journal of Virology*, *89*(6), 3145–3162. https://doi.org/10.1128/JVI.03213-14

van den Elsen, K., Quek, J. P., & Luo, D. (2021). Molecular Insights into the Flavivirus Replication Complex. *Viruses*, *13*(6), 956. https://doi.org/10.3390/v13060956

van der Most, R. G., Corver, J., & Strauss, J. H. (1999). Mutagenesis of the RGD Motif in the Yellow Fever Virus 17D Envelope Protein. *Virology*, *265*(1), 83–95. https://doi.org/10.1006/viro.1999.0026

van der Schaar, H. M., Rust, M. J., Chen, C., van der Ende-Metselaar, H., Wilschut, J., Zhuang, X., & Smit, J. M. (2008). Dissecting the Cell Entry Pathway of Dengue Virus by Single-Particle Tracking in Living Cells. *PLoS Pathogens*, *4*(12), e1000244. https://doi.org/10.1371/journal.ppat.1000244

Varnavski, A. N., & Khromykh, A. A. (1999). Noncytopathic Flavivirus Replicon RNA-Based System for Expression and Delivery of Heterologous Genes. *Virology*, *255*(2), 366–375. https://doi.org/10.1006/viro.1998.9564

Varnavski, A. N., Young, P. R., & Khromykh, A. A. (2000). Stable High-Level Expression of Heterologous Genes In Vitro and In Vivo by Noncytopathic DNA-Based Kunjin Virus Replicon Vectors. *Journal of Virology*, *74*(9), 4394–4403. https://doi.org/10.1128/JVI.74.9.4394-4403.2000

Villordo, S. M., Filomatori, C. V., Sánchez-Vargas, I., Blair, C. D., & Gamarnik, A. V. (2015). Dengue Virus RNA Structure Specialization Facilitates Host Adaptation. *PLOS Pathogens*, *11*(1), e1004604. https://doi.org/10.1371/journal.ppat.1004604

Wallner, G., Mandl, C. W., Kunz, C., & Heinz, F. X. (1995). The Flavivirus 3′-Noncoding Region: Extensive Size Heterogeneity Independent of Evolutionary Relationships among Strains of Tick-Borne Encephalitis Virus. *Virology*, *213*(1), 169–178. https://doi.org/10.1006/viro.1995.1557

Westaway, E. G., Mackenzie, J. M., & Khromykh, A. A. (2002). *Replication and Gene Function in Kunjin Virus* (pp. 323–351). https://doi.org/10.1007/978-3-642-59403-8_16

Westaway, E. G., Mackenzie, J. M., & Khromykh, A. A. (2003). *Kunjin RNA replication and applications of Kunjin replicons* (pp. 99–140). https://doi.org/10.1016/S0065-3527(03)59004-2

Winkler, G., Randolph, V. B., Cleaves, G. R., Ryan, T. E., & Stollar, V. (1988). Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology*, *162*(1), 187–196. https://doi.org/10.1016/0042-6822(88)90408-4

Wu, K.-P., Wu, C.-W., Tsao, Y.-P., Kuo, T.-W., Lou, Y.-C., Lin, C.-W., Wu, S.-C., & Cheng, J.-W. (2003). Structural Basis of a Flavivirus Recognized by Its Neutralizing Antibody. *Journal of Biological Chemistry*, *278*(46), 46007–46013. https://doi.org/10.1074/jbc.M307776200

Xie, X., Zou, J., Shan, C., Yang, Y., Kum, D. B., Dallmeier, K., Neyts, J., & Shi, P.-Y. (2016). Zika Virus Replicons for Drug Discovery. *EBioMedicine*, *12*, 156–160. https://doi.org/10.1016/j.ebiom.2016.09.013

Yang, P.-C., & Mahmood, T. (2012). Western blot: Technique, theory, and trouble shooting. *North American Journal of Medical Sciences*, *4*(9), 429. https://doi.org/10.4103/1947-2714.100998

Youn, S., Li, T., McCune, B. T., Edeling, M. A., Fremont, D. H., Cristea, I. M., & Diamond, M. S. (2012). Evidence for a Genetic and Physical Interaction between Nonstructural Proteins NS1 and NS4B That Modulates Replication of West Nile Virus. *Journal of Virology*, *86*(13), 7360–7371. https://doi.org/10.1128/JVI.00157-12

Yu, I.-M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., Chipman, P. R., Kuhn, R. J., Rossmann, M. G., & Chen, J. (2008). Structure of the Immature Dengue Virus at Low pH Primes Proteolytic Maturation. *Science*, *319*(5871), 1834–1837. https://doi.org/10.1126/science.1153264

Zakotnik, S., Knap, N., Bogovič, P., Zorec, T. M., Poljak, M., Strle, F., Avšič-Županc, T., & Korva, M. (2022). Complete Genome Sequencing of Tick-Borne Encephalitis Virus Directly from Clinical Samples: Comparison of Shotgun Metagenomic and Targeted Amplicon-Based Sequencing. *Viruses*, *14*(6), 1267. https://doi.org/10.3390/v14061267

Zhang, M., Tian, J., Li, H., & Cang, M. (2022). The comparative genomic analysis provides insights into the phylogeny and virulence of tick-borne encephalitis virus vaccine strain Senzhang. *PLOS ONE*, *17*(8), e0273565. https://doi.org/10.1371/journal.pone.0273565

Zhang, W., Chipman, P. R., Corver, J., Johnson, P. R., Zhang, Y., Mukhopadhyay, S., Baker, T. S., Strauss, J. H., Rossmann, M. G., & Kuhn, R. J. (2003). Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. *Nature Structural Biology*, *10*(11), 907–912. https://doi.org/10.1038/nsb990

Zhang, Y., Zhang, W., Ogata, S., Clements, D., Strauss, J. H., Baker, T. S., Kuhn, R. J., & Rossmann, M. G. (2004). Conformational Changes of the Flavivirus E Glycoprotein. *Structure*, *12*(9), 1607–1618. https://doi.org/10.1016/j.str.2004.06.019

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