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KAI RAUSALU

# Alphaviral nsP2 protease: From requirements for functionality to inhibition

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# Alphaviral nsP2 protease: From requirements for functionality to inhibition

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# TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS .................................................... 7

ABBREVIATIONS........................................................................ 8

1. INTRODUCTION........................................................................ 11

2. REVIEW OF LITERATURE ................................................................ 13
2.1. Alphaviruses ........................................................................ 13
2.2. Geographical distribution of alphaviruses ........................................ 14
2.3. Genome organization and replication of alphavirus RNAs ............... 14
2.4. Structures and functions of alphavirus-encoded proteins .................. 17
2.4.1. nsP1 .......................................................................... 17
2.4.2. nsP2 .......................................................................... 20
2.4.3. nsP3 .......................................................................... 25
2.4.4. nsP4 .......................................................................... 29
2.4.5. Capsid protein.......................................................... 33
2.4.6. Envelope proteins ................................................... 35
2.5. Inhibitors of CHIKV infection.......................................................... 40

3. AIMS OF THE STUDY........................................................................ 45

4. MATERIALS AND METHODS............................................................ 46

5. RESULTS AND DISCUSSION ............................................................ 55
5.1. Analysis of the requirements for CHIKV nsP2 protease activity and the impact of mutations of protease active site residues on CHIKV infectivity, RNA synthesis and ns polyprotein processing (I) ............ 55
5.1.1. Requirements of CHIKV nsP2 for its protease activity............ 56
5.1.2. Effects of the C478A, S482A and W479A substitutions on the protease activity of nsP2 of CHIKV, the activity of the CHIKV RNA replicase and virus rescue ........................... 60
5.2. Importance of the timeliness in alphavirus ns polyprotein processing (II) ........................................................................ 64
5.2.1. Optimization of the sequence of the 1/2 site is detrimental for SFV ........................................................................ 65
5.2.2. Deoptimization of the 1/2 site increases the infectivity of SINV harboring a hyperprocessive protease mutation ......... 69
5.2.3. Accelerated processing of P123 reduces RNA synthesis of SFV and SINV.................................................................. 70
5.2.4. The N-terminal region of SFV nsP2 is involved in the processing of the 2/3 site ........................................................ 73
5.3. Compounds targeting CHIKV nsP2 inhibit its protease activity and virus replication in cell culture (III) ............................................ 74

6. CONCLUSIONS........................................................................ 78

SUMMARY IN ESTONIAN ................................................................ 81

REFERENCES ... 84
ACKNOWLEDGMENTS ... 107
PUBLICATIONS ... 109
CURRICULUM VITAE ... 166
ELULOOKIRJELDUS ... 169

# LIST OF ORIGINAL PUBLICATIONS

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

I. **Rausalu, Kai**, Age Utt, Tania Quirin, Finny S. Varghese, Eva Žusinaite, Pratyush Kumar Das, Tero Ahola, and Andres Merits. 2016. 'Chikungunya Virus Infectivity, RNA Replication and Non-Structural Polyprotein Processing Depend on the NsP2 Protease's Active Site Cysteine Residue'. *Scientific Reports* 6 (November). https://doi.org/10.1038/srep37124.

II. Lulla, Valeria, Liis Karo-Astover, **Kai Rausalu**, Sirle Saul, Andres Merits, and Aleksei Lulla. 2018. 'Timeliness of Proteolytic Events Is Prerequisite for Efficient Functioning of the Alphaviral Replicase'. *Journal of Virology* 92 (14). https://doi.org/10.1128/JVI.00151-18.

III. Ivanova, Larisa, **Kai Rausalu**, Maksim Ošeka, Dzmitry G. Kananovich, Eva Žusinaite, Jaana Tammiku-Taul, Margus Lopp, Andres Merits, and Mati Karelson. 2021. 'Novel Analogues of the Chikungunya Virus Protease Inhibitor: Molecular Design, Synthesis, and Biological Evaluation'. *ACS Omega* 6 (16): 10884–96. https://doi.org/10.1021/acsomega.1c00625.

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This thesis also contains some unpublished data.

Author contributions:

I. I purified the wild-type and mutant recombinant CHIKV nsP2 proteins, participated in the experimental design, performed the circular dichroism spectroscopic analysis and protease assays, analyzed the data, prepared the figures and participated in the writing of the manuscript.

II. I performed the *in vitro* translation and immunoprecipitation experiments and analyzed the data. I also participated in analyzing the RNA infectivity of the constructs, in plaque purification of the viruses and in the analysis of the sequencing data of plaque-isolated viruses. I participated in the writing of the manuscript.

III. I designed and participated in performing the protease assays and western blot analysis, analyzed the data from these experiments and participated in the writing of the manuscript.

# ABBREVIATIONS

| | |
|---|---|
| aa | amino acid (residue) |
| ASALV | Agua Salud alphavirus |
| AUD | alphavirus unique domain |
| CD | circular dichroism |
| CD2AP | CD2 associated protein |
| cDNA | complementary DNA |
| CHIKV | Chikungunya virus |
| CP | capsid protein |
| CPE | cytopathic effect |
| DABCYL | 4-[[4-(dimethylamino)phenyl]-azo] benzoic acid |
| DdRp | DNA-dependent RNA polymerase |
| DRM | detergent resistant membrane microdomain |
| dsRNA | double-stranded RNA |
| $EC_{50}$ | 50% effective concentration |
| EEEV | Eastern equine encephalitis virus |
| EILV | Eilat virus |
| EMCV | encephalomyocarditis virus |
| ER | endoplasmic reticulum |
| FDA | (USA) Food and Drug Administration |
| FFLuc | firefly luciferase |
| FHL1 | four-and-a-half LIM domain protein |
| FRET | fluorescence resonance energy transfer |
| G3BP | Ras-GAP SH3 domain binding protein |
| GLuc | *Gaussia* luciferase |
| GTase | guanylyltransferease |
| HCV | hepatitis C virus |
| HDV | hepatitis delta virus |
| HIV | human immunodeficiency virus |
| hpi | hours post-infection |
| hpt | hours posttransfection |
| $IC_{50}$ | 50% inhibitory concentration |
| ICA | infectious center assay |
| icDNA | infectious cDNA |
| IP | immunoprecipitation |
| IRES | internal ribosome entry site |
| ISV | insect specific (alpha)virus |
| MAYV | Mayaro virus |
| MOI | multiplicity of infection |
| MTase | methyltransferase |
| MTL | methyltransferase-like |
| MWAV | Mwinilunga alphavirus |
| NES | nuclear export signal |

| | |
|---|---|
| NGS | next generation sequencing |
| NHC | β-d-$N^4$-hydroxycytidine |
| NLS | nuclear localization signal |
| ns | nonstructural |
| nsP | nonstructural protein |
| NTD | N-terminal domain (of nsP2 or nsP4) |
| NTPase | nucleoside triphosphatase |
| ONNV | o’nyong’nyong virus |
| ORF | open reading frame |
| PCR | polymerase chain reaction |
| p.t. | post-transfection |
| PVDF | polyvinylidene fluoride |
| RC | replication complex |
| RdRp | RNA-dependent RNA polymerase |
| RLuc | *Renilla* luciferase |
| RRV | Ross River virus |
| RTPase | RNA triphosphatase |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| SDV | sleeping disease virus |
| SESV | southern elephant seal virus |
| SFV | Semliki Forest virus |
| SG | subgenomic |
| SINV | Sindbis virus |
| SPDV | salmon pancreas disease virus (also known as salmonid alphavirus, SAV) |
| TALV | Taï Forest alphavirus |
| TF | transframe |
| Trx | thioredoxin |
| UTR | untranslated region |
| VEEV | Venezuelan equine encephalitis virus |
| WEEV | Western equine encephalitis virus |
| wt | wild-type |

# 1. INTRODUCTION

Viruses are noncellular infectious agents, and their particles (virions) come in a variety of sizes and shapes. Virus particles consist of genetic material protected by the protein capsid and, sometimes, also by a membranous envelope originating from the host cell. The viral genetic material can be either DNA or RNA, single or double stranded; the genome can be composed of one molecule or several segments. The variation in size – of both virions and genomes – is larger than was thought a few decades ago. Parvoviruses can be taken as an example of the smallest viruses, with a capsid of ~20 nm in diameter and a DNA genome of ~5 kb in length, while the largest viruses (e.g., pandoraviruses) have a genome (DNA) size of ~2.5 Mbp, and their oval-shaped virions, which measure ~1x0.5 µm, are easily visible under a light microscope. However, regardless of the size, viruses have no independent metabolism, encode no protein synthesis machinery and have to infect host cells to reproduce themselves.

Alphaviruses have a positive-strand RNA genome that is ~12 kb in size. Their virions are ~70 nm in diameter and consist of icosahedral nucleocapsids and envelope membranes. Alphaviruses are widely spread throughout the world and can be found on all continents, except Antarctica. These viruses are mostly arboviruses, as they are spread by arthropod vectors to vertebrate hosts, including mammalian hosts such as humans. Some alphaviruses cause severe diseases, with either encephalitic (e.g., Eastern equine encephalitis virus, EEEV) or arthritic (e.g., chikungunya virus, CHIKV) symptoms. CHIKV is very widespread in warmer climates. Its infection in humans is almost always symptomatic, and although the acute phase of CHIKV disease passes in a couple of weeks, the debilitating joint pain associated with CHIKV infection persists, sometimes for months and even years afterward, in nearly half of patients. Currently, there is no approved drug for the treatment of CHIKV infection. The situation with vaccines to prevent CHIKV infection is somewhat better, with at least one vaccine candidate waiting for Food and Drug Administration (FDA) approval.

Alphaviruses encode four nonstructural proteins (nsPs; nsP1-nsP4), which all perform different functions during viral replication. The first protein, nsP1, is a capping enzyme that anchors viral replication complexes (RCs) to cellular membranes. The third protein, nsP3, has ADP-ribose 1′′-phosphate phosphatase activity and facilitates interactions with host factors during viral infection. The fourth protein, nsP4, is a viral RNA-dependent RNA polymerase. The second protein, nsP2, is a multifunctional protein with many different roles in the viral life cycle. Its N-terminal part is the viral RNA helicase/NTPase, and its C-terminal part is the viral protease, which cleaves the viral nonstructural (ns) polyprotein into individual nsPs. This is a key event in the alphavirus replication cycle; if the virus is unable to carry out the cleavage of the ns polyprotein, it fails to replicate. Furthermore, if the processing is disturbed, i.e., either the order or timing of processing events is not correct, the replication will fail and/or will be detected and suppressed by the host. In part, this is because nsP2 also participates in the shutoff

of cellular transcription and translation and interferes with cellular antiviral responses. Considering all these multiple functions, nsP2 plays a central role in the alphavirus life cycle.

The aim of this study was to further our understanding of the functioning of alphaviral nsP2, with a focus on its protease activity. Our results clarified, without a doubt, that nsP2 of CHIKV was a cysteine protease. We acquired information about the importance of temporally regulated cleavage between the nsP1 and nsP2 parts of the ns polyprotein for the functioning of the alphaviral RNA replicase. The sequence of the nsP1/nsP2 cleavage site in alphaviruses was found to be suboptimal for efficient cleavage. It is not that this site cannot be cleaved more efficiently, as it certainly can be cleaved faster. However, the premature cleavage of this site was found to lead to negative consequences for the virus, including its inability to properly initiate, perform or complete negative-strand RNA synthesis and RC (also called spherule) formation during early stages of virus replication; as a result, the virus loses (partly or completely, depending on the defect) its infectivity. The key role of nsP2 in alphavirus replication also strongly suggests that compounds interfering with any of its activities have the potential to act as antivirals. However, numerous studies have shown that targeting nsP2 is not easy. Herein, we studied a set of novel CHIKV nsP2 protease inhibitors, which were rationally designed by optimizing the structure of a previously known CHIKV inhibitor. Among these, we identified a compound that had approximately ten times better antiviral activity against CHIKV infection in cell culture than the original hit compound.

# 2. REVIEW OF LITERATURE

## 2.1. Alphaviruses

Alphaviruses (genus *Alphavirus*) belong to the family *Togaviridae*. The genus *Alphavirus* contains more than 30 recognized virus species. Historically, the family *Togaviridae* also contained the genus *Rubivirus*, but this genus was transferred to the family *Matonaviridae* in 2019 (Walker et al. 2019). Alphaviruses have a positive-strand RNA genome of 10–12 kb in length. The alphavirus virions are approximately 70 nm in diameter and have a membrane that is derived from the cellular plasma membrane or membrane of the endosomal compartment. The latter property is reflected in the name of the virus family (from Latin *toga*).

The majority of currently known alphaviruses are arboviruses, i.e., they are transmitted to vertebrates by arthropod vectors (mostly mosquitoes but also some other hematophagous insects) (R. Chen et al. 2018). The vertebrate hosts of alphaviruses include mammals, birds, rodents, amphibians, reptiles and fishes (J. H. Strauss and Strauss 1994). Some alphaviruses are restricted to only mosquitoes and do not infect vertebrates; therefore, they are also termed insect-specific viruses (ISVs). To date, three such viruses have been found in the Old World, namely, Eilat virus (EILV), Taï Forest alphavirus (TALV) and Mwinilunga alphavirus (MWAV). The first insect-restricted alphavirus, EILV, was isolated from a pool of *Anopheles coustani* mosquitoes in Israel (Nasar et al. 2012). Nasar *et al.* also found EILV insect-only host restriction to be present at both the cell entry and RNA replication levels (Nasar et al. 2014). A few years later, TALV was discovered in *Culex decens* mosquitoes collected in the Ivory Coast, and phylogenetic analysis placed TALV, together with EILV basal, to the Western equine encephalitis virus (WEEV) complex (Hermanns et al. 2017). MWAV was found in *Culex quinquefasciatus* mosquitoes collected in Mwinilunga in Zambia (Torii et al. 2018). Recently, another ISV, named Agua Salud alphavirus (ASALV), was found in the New World in *Culex declarator* mosquitoes in Panama (Hermanns et al. 2020). Given the abundance of mosquitoes and the low number of studies performed to characterize viruses infecting them, it is logical to assume that ISVs are numerous and possibly represent the largest group of alphaviruses. Two alphavirus species, namely, southern elephant seal virus (SESV) (Linn et al. 2001) and salmon pancreas disease virus (SPDV) (J. H. Weston et al. 1999), also known as salmonid alphavirus (SAV), have aquatic hosts. Additionally, rainbow trout sleeping disease virus (SDV) was partly characterized, and the sequences of its structural proteins were shown to be similar to those of alphaviruses, indicating that SDV is probably also an alphavirus (Villoing et al. 2000). However, the high sequence similarity between SDV and SPDV indicates that these viruses might be isolates of the same virus (J. Weston et al. 2002). SESV was isolated from the louse, *Lepidophthirus macrorhini,* collected from southern elephant seals, *Mirounga leonina*, on Macquarie Island in Australia (Linn et al. 2001). Therefore, it is often assumed that aquatic alphavirus species are also transmitted

by arthropod vectors. As of now, there is no clear proof that this is indeed the case; however, it has been demonstrated that SPDV can replicate in mosquito cells (Hikke et al. 2014).

## 2.2. Geographical distribution of alphaviruses

Alphaviruses can be found worldwide. Arbovirus members of the genus are often divided into Old World and New World alphaviruses based on their spread; however, this is not always correct, as several Old World alphaviruses, such as Mayaro virus (MAYV) (Tesh et al. 1999), are spread in the Americas. The division is nonetheless useful, as it also reflects the type of illnesses these viruses typically cause (Jose, Snyder, and Kuhn 2009; Powers et al. 2001). Examples of New World alphaviruses are WEEV, EEEV and Venezuelan equine encephalitis virus (VEEV), and as the names suggest, they are encephalitic. Old World alphaviruses include chikungunya virus (CHIKV), Sindbis virus (SINV) and Semliki Forest virus (SFV). Many of these alphaviruses are medically important pathogens, and the diseases they cause are associated, depending on the virus species, with fever, rash and arthritis. However, in experimental hosts, some Old World alphaviruses (for example SFV and SINV) can also be encephalitic. CHIKV is a medically important pathogen because of its tendency to cause massive outbreaks of disease called chikungunya fever. In addition, debilitating arthralgia frequently (up to 60% of cases, depending on the virus genotype) caused by CHIKV infection can last for months and even years thereafter (Suhrbier, Jaffar-Bandjee, and Gasque 2012; Schwartz and Albert 2010). Within the last two decades, CHIKV has caused multiple epidemics. In 2006, a massive outbreak occurred on La Réunion Island (Renault et al. 2007), and shortly after, epidemics occurred in other countries of the Indian Ocean region and Asia (Sissoko et al. 2008; Lahariya and Pradhan 2006; Yoosuf et al. 2009; Beesoon et al. 2008). There have also been endemic cases of CHIKV disease in northern Italy and France (Rezza et al. 2007; Gould et al. 2010). In 2013, CHIKV spread to the Americas (Leparc-Goffart et al. 2014) and has since become endemic there (Gutierrez-Saravia and Gutierrez 2015).

## 2.3. Genome organization and replication of alphavirus RNAs

The alphavirus genome is a positive-strand RNA of approximately 12 kb in length. The alphavirus genome organization is depicted in Figure 1. The genome has a 5' cap0 structure and a 3' poly(A) tail. There are three untranslated regions (UTRs): a relatively short 5' UTR, a longer 3' UTR and a short intergenic sequence located between the open reading frames (ORFs). The genome contains two ORFs that cover most of its length. The first ORF encodes the precursor of four viral nonstructural proteins (nsPs), nsP1, nsP2, nsP3 and nsP4. The second ORF, located downstream of the subgenomic (SG) promoter and expressed from SG

RNA, encodes precursors of viral structural proteins, the capsid protein (CP), viral glycoproteins (E1, E2 and E3), and two additional small proteins, 6K and transframe (TF); the latter is the frameshift product of the 6K protein-encoding region (J. H. Strauss and Strauss 1994; Firth et al. 2008).

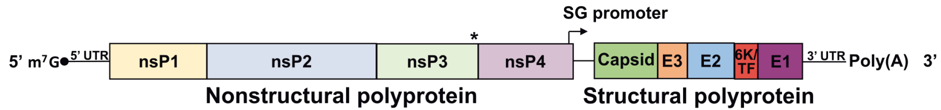



**Figure 1.** Alphavirus genome organization. m$^7$G – 7-methylguanosine (forms the cap0 structure), UTR – untranslated region, nsP – nonstructural protein, SG – subgenomic, TF – transframe protein; * represents the opal stop codon that most alphaviruses have at the end of the nsP3-encoding region.

Alphavirus replication complexes (RCs) are formed on the plasma membrane of infected cells, where nsP1 preferentially binds to anionic phospholipids. Viral nsPs induce the formation of membrane invaginations (termed spherules), which represent structures where synthesis of viral RNAs (starting from the synthesis of negative-strand RNA) occurs. For many alphaviruses, such as SFV, the assembled RCs are subsequently internalized by the endosomal pathway. For these viruses, spherules can be found in cytopathic vacuoles, which represent modified endo- and lysosomes (Kujala et al. 2001; Froshauer, Kartenbeck, and Helenius 1988; E. I. Frolova et al. 2010).

nsP1 is the only alphaviral nsP that interacts with cellular membranes and is therefore crucial for the formation of spherules and anchoring other components of the viral RC to the membranes (Lampio et al. 2000; Johan Peränen et al. 1995). Twelve copies of nsP1 form a dodecameric ring structure that is located at the neck of a spherule (K. Zhang et al. 2021). According to a recent study by Tan *et al.*, a single copy of nsP4 is enclosed in the central pore of the nsP1 ring, more toward the spherule interior. There are 10 different contact surfaces between nsP1 subunits and nsP4; these contacts are likely essential for the proper folding of nsP4. Interestingly, no contacts are observed between nsP4 and two nsP1 molecules of the ring structure; instead, these molecules form a channel-like structure that is most likely used for viral RNA. A single molecule of nsP2 found in an RC extends toward the cytoplasm from the disk structure of the nsP1/nsP4 complex. nsP2 is placed on top of the nsP4 structure, and the contact surface is formed by its N-terminal region (for details of nsP2 organization, see 2.4.2 below). Active RCs also have another ring structure located on the cytoplasmic side of the nsP1 ring. This structure probably consists of nsP3 and, possibly, host proteins needed for viral RNA replication. The outer (cytosolic) ring appears amorphous in cryo-EM studies and is probably not required for viral RNA synthesis *per se,* as, at least in the test tube, the RNA synthesis reaction can be performed by the replicase core structure (12 × nsP1+nsP4+nsP2 complex). Nonetheless, this structure is essential for virus replication to occur, as it can be found only in active RCs located in infected cells (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022).

Alphavirus nsPs are first synthesized in the form of a single polyprotein (P1234) precursor. The majority of alphaviruses (for example, SINV and most CHIKV strains) have an opal termination codon in the region encoding the C-terminus of nsP3; for these viruses, the major ns polyprotein synthesized is P123. The synthesis of P1234, which is less abundant (approximately 10% of the amount of P123), occurs via translational readthrough (G Li and Rice 1993; J. H. Strauss and Strauss 1994). Ns polyproteins have several enzymatic activities but cannot perform viral RNA replication. To activate the replicase activity, step-by-step proteolysis of P1234 is needed; this is performed by protease activity residing in nsP2 (and the ns polyprotein region corresponding to nsP2). nsP4 is the first ns protein to be released from P1234, and as a result, the so-called early replicase, consisting of P123 and nsP4, is formed. The early replicase synthesizes new viral negative-strand RNAs. The synthesis is associated with spherule formation and is most likely a single event (i.e., each positive-strand RNA can be used as a template for the synthesis of one negative-strand RNA) that results in the formation of a double-stranded (ds) RNA molecule located inside the formed spherule. Subsequent cleavage of P123 separates nsP1 and is quickly followed by the final cleavage between nsP2 and nsP3. This completes the formation of the late replicase, consisting of the four individual nsPs. The late replicase uses the negative strand in double-stranded RNAs (dsRNAs) as a template to synthesize new positive-strand RNA genomes and SG RNAs for the production of structural proteins (Lemm et al. 1994). The translation of SG RNAs also results in the synthesis of polyproteins, which are subsequently cleaved into individual structural proteins. The capsid protein (CP), which contains a C-terminal protease domain, is cleaved off first and is used, together with the newly synthesized genomic RNA, to form new nucleocapsids in the cytoplasm of infected cells. Autocatalytic removal of the CP exposes the membrane localization signal in the translation product, and as a result, the rest of the structural polyprotein is synthesized on endoplasmic reticulum membranes. Its processing is performed by cellular enzymes and is associated with its transport via the Golgi complex to the plasma membrane (in insect cells, to internal membranes). When new viral particles bud from the cell, they acquire a membrane embedded with the viral glycoproteins (reviewed in (Jose, Snyder, and Kuhn 2009)). The life cycle of alphaviruses is depicted in Figure 2.

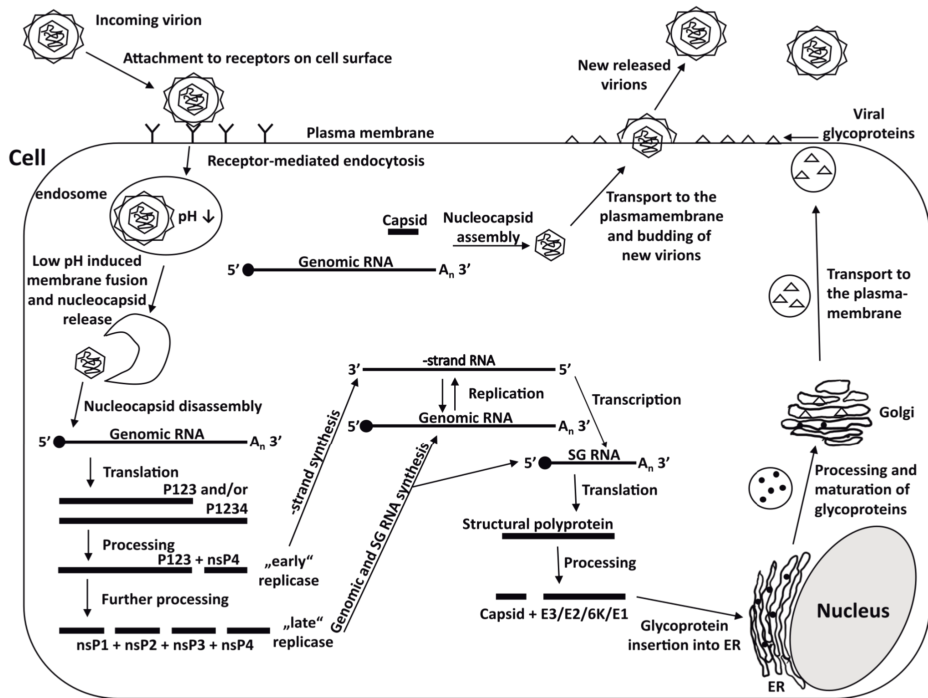



**Figure 2.** Life cycle of alphaviruses. nsP – nonstructural protein, ER – endoplasmic reticulum, SG – subgenomic.

## 2.4. Structures and functions of alphavirus-encoded proteins

### 2.4.1. nsP1

nsP1 corresponds to the N-terminal part of the alphavirus-encoded P123/P1234 polyproteins. nsP1 of CHIKV is 535 amino acid (aa) residues in length (537 aa in SFV) and has a molecular mass of approximately 60 kDa. nsP1 is responsible for anchoring the viral RC to the cell membrane (Johan Peränen et al. 1995; Bakhache et al. 2020) and for capping viral positive-strand RNAs (T Ahola and Kääriäinen 1995; C. Li et al. 2015; K. Zhang et al. 2022). The scheme of the CHIKV nsP1 organization and its 3D structure are depicted in Figure 3.

nsP1 has N7-guanine methyltransferase (MTase) and guanylyltransferase (GTase) activities, which are needed for the synthesis of viral cap0 structures. The methyl group is derived from S-adenosyl-methionine and transferred to GTP. Then, nsP1 covalently binds $m^7$GMP (releasing pyrophosphate), and as a final step, the guanylate residue is transferred to viral RNA to form the 5’ cap0 structure (T Ahola and Kääriäinen 1995; C. Li et al. 2015; T. Ahola et al. 1999; Laakkonen, Ahola, and Kääriäinen 1996).

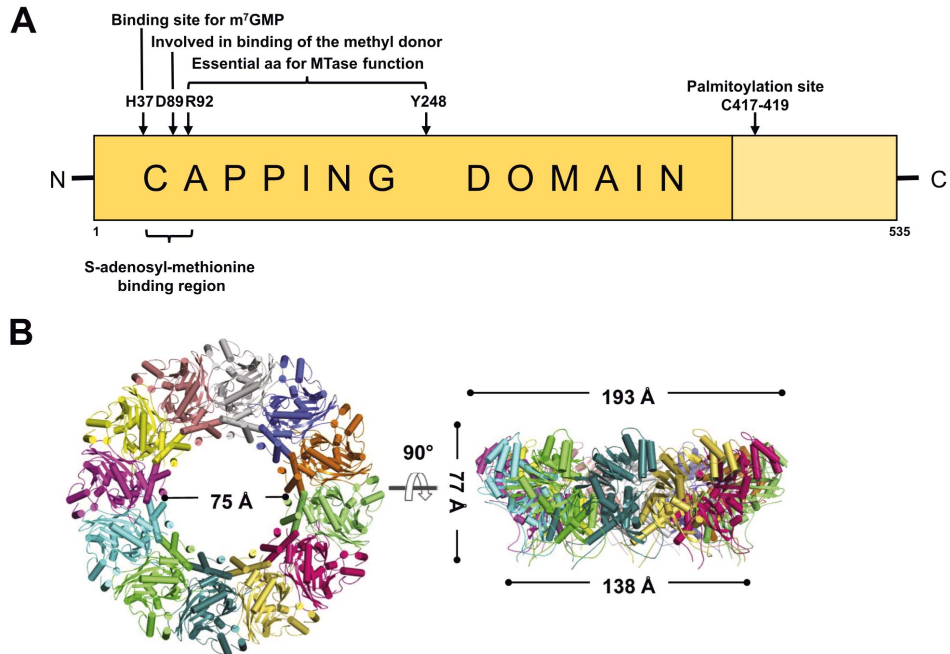



**Figure 3.** CHIKV nsP1. A) Schematic overview of nsP1. The amino acids needed to carry out nsP1 enzymatic functions are indicated by arrows. m$^7$GMP – guanosine monophosphate methylated at position 7, MTase – methyltransferase. B) "Top view" (left) and "side view" (right) of the nsP1 ring structure. The twelve nsP1 monomers are marked by different colors. The image is reproduced from (K. Zhang et al. 2021).

In general, the enzymatic activities of nsP1 are related to its binding to cellular membranes (T. Ahola et al. 1999; Jones et al. 2020; K. Zhang et al. 2021). SFV nsP1 segment 245–264 was thought to form an amphipathic helix. This structure can indeed be observed in the corresponding synthetic peptide but cannot be located on the revealed 3D structure of full-length nsP1. The amphipathic region was thought to be important for the first step of nsP1 binding to the membrane. In light of recent structural data (K. Zhang et al. 2021; Jones et al. 2020), it is not clear whether this is indeed the case. However, without a doubt, the region is crucial for nsP1 function. Thus, mutational analysis of charged aa residues located in this region (K253, K254, and R257; aa numbers correspond to nsP1 of SFV) revealed their necessity for nsP1 MTase activity (for example, the K253E substitution completely abolished the MTase activity). It was also shown that in the case of synthetic peptides, the hydrophilic side of the α-helix interacted with polar head groups of phospholipids, and the hydrophobic side interacted with hydrophobic lipid chains in the cell membrane (T. Ahola et al. 1999). Although SFV nsP1 needs to be associated with lipids for enzymatic activities, membrane binding is not necessary for the MTase and GTase activities of SINV nsP1 (Tomar et al. 2011). The R252E substitution in nsP1 strongly reduces CHIKV (for CHIKV, aa 252 corresponds to aa 253 of SFV) and SFV replication and

disrupts membrane localization of SFV replicase proteins (T. Ahola et al. 1999; Spuul et al. 2007; Utt et al. 2019). The role of the W259 residue, which is supposed to work as a membrane anchor (Lampio et al. 2000), is also somewhat different for different alphaviruses; the W259A substitution is lethal for SFV, but an analogous mutation in nsP1 of CHIKV only results in a temperature-sensitive phenotype of the virus (Spuul et al. 2007; Utt et al. 2019).

Membrane binding of nsP1 is strengthened by palmitoylation of cysteine residues. The palmitoylation site is situated in the C-terminal part of the protein (C418-420 for SFV; C417-419 for CHIKV). Either or several of these cysteines may be palmitoylated, and palmitoylation is the main cause of the strong membrane association of nsP1. If nsP1 of SFV is not palmitoylated, the strength of its association with the membrane is reduced, but nsP1 remains functionally active (Laakkonen, Ahola, and Kääriäinen 1996). If these cysteines are replaced with alanines, the nsP1 localization to filopodia-like extensions, formed on the plasma membrane of nsP1-expressing cells, is reduced (Utt et al. 2019; N. Zhang, Zhao, and Zhang 2019). Similar to mutations in the 245–264 region, the impact of the replacement of palmitoylated cysteine residues with alanines depends on the virus. For SINV (having only a single palmitoylated Cys residue in nsP1), the effect is nearly undetectable; for SFV, the replacement has a devastating effect on virus replication and causes accumulation of second-site mutations; and for CHIKV, this mutation is unconditionally lethal (Žusinaite et al. 2007; Utt et al. 2019). These data are highly consistent with those from a recent study, which revealed that the presence of nsP1 in cholesterol-rich, detergent-resistant membrane microdomains (DRMs) of alphavirus-infected cells was absolutely necessary for CHIKV RNA replication but not for SINV RNA replication. Although, similar to CHIKV nsP1, SINV nsP1 is also targeted to DRMs, the latter is equally found in detergent-soluble membranes (Tero Ahola et al. 2000; Bakhache et al. 2020; Lampio et al. 2000; Spuul et al. 2007).

In the last couple of years, the cryo-EM structure of nsP1 of CHIKV has been revealed (K. Zhang et al. 2021; Jones et al. 2020). As shown in Figure 3B, twelve copies of the nsP1 molecule are organized into a dodecameric ring structure with dimensions of 19.3 × 7.5 × 7.7 nm (outer diameter, inner diameter and height of the ring). The upper part of the ring is formed by the MTase/GTase catalytic domains of nsP1, which add a cap0 structure to newly synthesized RNAs, and the lower part of the ring is formed by the membrane association and oligomerization domains of nsP1. The C-terminal tail of nsP1 after residue 474 remains disordered (K. Zhang et al. 2021).

Alphavirus infection causes the formation of structures called spherules in the cell. Spherules are sites where virus RNA replication takes place. nsP1 directly participates in the formation of spherules via its membrane association. It has been shown that the binding of nsP1 to lipid bilayers induces changes in the membrane curvature (Gottipati, Woodson, and Choi 2020). As the only strongly membrane-binding nsP of alphaviruses, nsP1 anchors the whole RC to the membrane (Johan Peränen et al. 1995; Bakhache et al. 2020; Spuul et al. 2007). In localization studies, individually expressed nsP1 has been found in the plasma membrane and filopodia-

like structures (Salonen et al. 2003; Laakkonen et al. 1998; Kujala et al. 2001). In infected cells, nsP1 also localizes in spherules. Spherules first arise at the plasma membrane, followed by their internalization in cytoplasmic vesicles, and finally localize in type I cytopathic vacuoles. However, this general scheme varies among alphaviruses. In the case of SINV and CHIKV infections, spherules predominantly remain at the site of their formation (plasma membrane). In contrast, in SFV-infected cells, spherules are subsequently internalized and can be found on endosomal membranes and in cytopathic vacuoles (Spuul et al. 2010; E. I. Frolova et al. 2010; Thaa et al. 2015). Both actin and phosphatidylinositol 3-kinase are involved in the internalization process, and later, microtubules are also involved in the trafficking of spherules to type I cytopathic vacuoles (Spuul et al. 2010).

For several decades, the architecture of alphavirus spherules has been a topic of speculation. It is clear that the size of spherules depends on the length of replicating RNA (Kallio et al. 2013). Furthermore, *in situ* proximity ligation experiments have revealed that nsP1 localizes inside the spherule and that spherules contain all nsPs at a very low concentration; the localization of nsPs on the cytosolic (neck) side of the spherule and their colocalization with viral RNA were also observed (E. I. Frolova et al. 2010). These findings are consistent with a model in which nsPs are located at the neck region of the spherule, which connects the interior of the spherule with the cytosol. This assumption was clearly confirmed by two recent cry-EM studies (Laurent et al. 2022; Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022). These studies revealed that the core of the alphavirus RNA replicase is formed by nsP1, nsP2 and nsP4, which have 12, 1 and 1 copies, respectively. nsP4 is located within the nsP1 dodecameric ring structure toward the spherule interior, and nsP2 is also located within the nsP1 ring structure but toward the cytosol (Figure 6C). The spherule interior contains one copy of viral dsRNA. nsP4 makes contact with 10 out of the 12 copies of nsP1 in the dodecameric nsP1 ring, thus leaving a channel on one side of the ring interior for the translocation of single-stranded RNAs. Also associated with the nsP1 ring structure is another, larger ring structure on the cytosolic side of the spherule, which is thought to consist of nsP3 and host factors involved in alphavirus replication (Laurent et al. 2022; Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022).

### 2.4.2. nsP2

Alphavirus nsP2 (798 aa in CHIKV), with a molecular mass of ~90 kDa, is the largest ns protein. In infected cells, approximately half of nsP2 localizes in the cytoplasm, and the other half localizes in the nucleus (J Peränen et al. 1990; Utt et al. 2015). nsP2 has four enzymatic functions: nucleoside triphosphatase (NTPase)/RNA triphosphatase (RTPase), RNA helicase and protease. Additionally, nsP2 has a large number of nonenzymatic activities. Unlike conserved enzymatic activities, these are different for different alphaviruses and their groups. nsP2 of Old World alphaviruses is, for example, involved in host cell transcriptional shutoff and plays a key role in the suppression of the host cell antiviral

response (reviewed in (Tero Ahola and Merits 2016; Rupp et al. 2015)). nsP2 consists of two basic regions (helicase and protease) and forms five domains, as depicted in Figure 4.

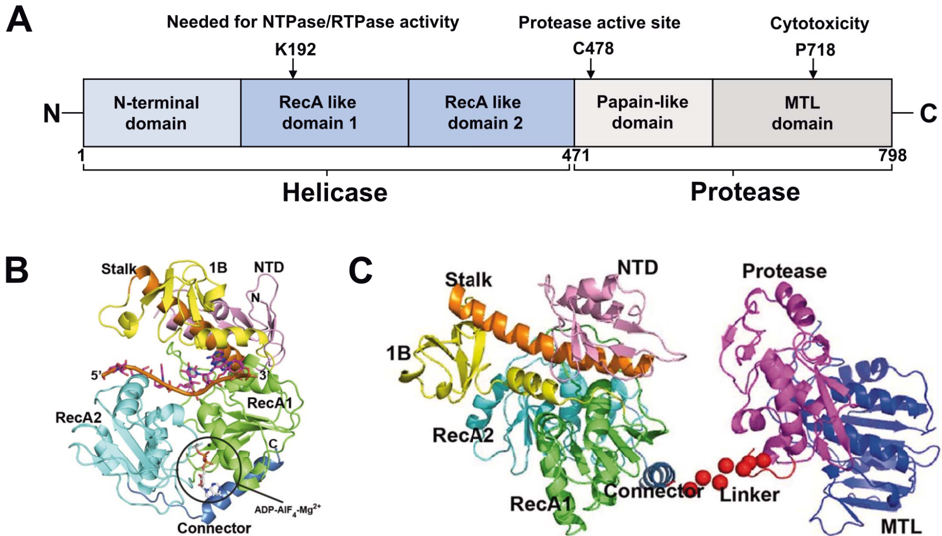



**Figure 4.** CHIKV nsP2. A) Schematic presentation of the organization of nsP2. Important amino acid residues for carrying out different functions of the protein are indicated by arrows. NTPase – nucleoside triphosphatase, RTPase – RNA triphosphatase, MTL – methyltransferase-like. B) Crystal structure of the nsP2 helicase region. The nsP2 helicase region is in the form of a complex with a 14-mer single-stranded RNA (orange line with magenta sticks) and a nonhydrolyzable transition-state nucleotide analog, ADP-AlF$_4$ (red and blue sticks). The image is reproduced from (Law et al. 2019). C) Structure of full-length nsP2. The image is reproduced from (Law et al. 2021). In panels B and C, the N-terminal domain (NTD) of nsP2 is in pink, the stalk domain is in orange, domain 1B is in yellow, the RecA1 domain is in green, the RecA2 domain is in cyan, the connector is in sky blue, the protease domain is in magenta, and the MTL domain is in blue; red dots represent seven linker residues missing in the resolved 3D structure.

The N-terminal part of nsP2 is the NTPase/RTPase and helicase. It consists of three domains: the N-terminal domain, which is unique to alphaviruses (Das, Merits, and Lulla 2014; Law et al. 2019), and two RecA-like domains. The C-terminal part of nsP2 is the viral protease and has two domains: a papain-like protease domain and a methyltransferase-like (MTL) domain (A. Russo, White, and Watowich 2006) (Figure 4B, C). The MTL domain is structurally similar to Ftsj-methyltransferase, but it lacks the residues needed for enzymatic activity (A. Russo, White, and Watowich 2006), and indeed, no MTase activity has been found for nsP2. Alphavirus nsP2 is a cysteine protease, and C478 is the catalytic cysteine residue of CHIKV nsP2 (Rausalu et al. 2016).

The NTPase and RTPase activities of nsP2 were first discovered for the enzymes encoded by SFV and SINV (M Rikkonen, Peränen, and Kääriäinen 1994; Vasiljeva et al. 2000). The RTPase activity removes γ-phosphate from the 5' end of new viral positive-strand RNAs, thus allowing these RNAs to be capped by nsP1. Additionally, SFV nsP2 was demonstrated to have weak RNA helicase activity (Gomez de Cedrón et al. 1999). NTPase and RTPase activities were also confirmed for CHIKV nsP2 (Karpe, Aher, and Lole 2011). Both functions are dependent on $Mg^{2+}$ ions. ATP is the most preferred substrate for the hydrolysis by NTPase, and both activities were shown to be reduced by mutations of either K192 (NTP binding) or D251 and E252 (involved in $Mg^{2+}$ binding) to alanine, indicating that these enzymatic activities share the functional domains of nsP2 (Karpe, Aher, and Lole 2011). Das *et al*. demonstrated that in addition to the N-terminal helicase part, CHIKV nsP2 needs its C-terminal part for RNA helicase activity (Das, Merits, and Lulla 2014). In these experiments, only the full-length CHIKV nsP2 showed 5'→3' RNA helicase and RNA rewinding activities, while nsP2 lacking the C-terminal part lacked these activities (Das, Merits, and Lulla 2014).

The most important aa residue in nsP2 for NTPase/RTPase and RNA helicase functions is the conserved lysine (K192) in the NTP-binding motif of the first RecA-like domain. If this lysine is replaced with asparagine, nsP2 loses its NTPase/RTPase and RNA helicase activities (M Rikkonen, Peränen, and Kääriäinen 1994; Vasiljeva et al. 2000; Gomez de Cedrón et al. 1999). This lysine is necessary for virus viability because transfection of cells with RNA of SFV carrying the K192N mutation was reported to give rise to revertant viruses with the wt lysine residue in this position (Marja Rikkonen 1996). It should be noted that these data have never been reproduced and may represent an experimental error due to imperfect methods used in this rather early study. It was later shown that CHIKV RNA harboring the K192A mutation was not infectious (Law et al. 2019). Importantly, the CHIKV replicase harboring the K192A or K192N substitution in nsP2 lacks any ability for RNA synthesis (Law et al. 2019; Bartholomeeusen et al. 2018), making rescue and reversion of the corresponding virus impossible. Several other residues of the helicase part of nsP2 have also been shown to be crucial for CHIKV infectivity. These residues include Y161 and F164, which are involved in stacking interactions with RNA bound to nsP2 (Law et al. 2019).

Alphavirus ns protease function was mapped to the C-terminal part of nsP2 quite a long time ago (Hardy and Strauss 1989). SINV nsP2 was suggested to be a papain-like cysteine protease with C481 and H558 residues comprising the protease catalytic dyad (E. G. Strauss et al. 1992). nsP2 is the only protease involved in alphavirus ns polyprotein processing (Merits et al. 2001). The processing that leads to the formation of functional RCs follows a strict order and timing. Thus, during processing, alphavirus P1234 is first cleaved between nsP3 and nsP4, resulting in P123 and nsP4 that form the early replicase. The next cleavage is *cis*-cleavage between nsP1 and nsP2, and only after that can *trans*-cleavage between nsP2 and nsP3 occur, yielding mature nsPs (nsP1-nsP4) that

form the late replicase (Vasiljeva et al. 2003) The early replicase synthesizes negative-strand RNAs, while the late replicase synthesizes new positive-strand RNAs, representing new genomes and SG RNAs. Thus, the processing of P123 represents a switch from negative- to positive-strand RNA synthesis (Y Shirako and Strauss 1994; Lemm et al. 1994). It has been shown that in the core of the mature RNA replicase complex, the nsP2 molecule interacts via its N-terminal part with the nsP4 molecule (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022) (Figure 6C). The complex interactions of nsP2 with other components of the RC (including viral RNA) are the likely reasons why processing of P1234 must occur in a strictly organized manner. Indeed, we have observed that the efficiency of the alphavirus RNA replicase prominently depends on the perfect timing of processing events (V. Lulla et al. 2018). In part, the fixed cleavage pattern and timing can be attributed to the cleavage site preference of nsP2; the aa sequence upstream of the nsP2 protease cleavage site determines the cleavage efficiency of the site. Thus, alphavirus nsP2 most efficiently cleaves a site located between nsP3 and nsP4 (3/4 site; hereafter, the other cleavage sites are referred to in a similar manner), followed by the 1/2 site, while the 2/3 site is cleaved with the lowest efficiency (A. Lulla et al. 2006), a pattern that concurs with the alphavirus ns polyprotein processing order. Earlier protease studies, performed using nsP2 of SFV, revealed that the protease needed quite a short sequence around the 1/2 and 3/4 cleavage sites for the cleavage to occur (A. Lulla et al. 2006; A. Lulla, Lulla, and Merits 2012). CHIKV nsP2 was also found to have similar requirements for its cleavage sites. The protease substrates representing the 1/2 or 3/4 cleavage site, containing 10 aa upstream and 5 aa downstream of the scissile bond, are cleaved efficiently (Utt et al. 2015). However, the short sequence of the cleavage site is not the only factor determining the cleavage order and efficiency; it was observed that the processing was also regulated by macromolecular assembly of the replicase precursor (A. Lulla, Lulla, and Merits 2012). Thus, the short substrate corresponding to the 2/3 site cannot be cleaved by nsP2 of SFV or CHIKV. To perform this cleavage, nsP2 requires a much longer sequence to be present downstream of the 2/3 cleavage site; a substrate with 10 aa upstream and 170 aa downstream of the scissile bond is cleaved with a high efficiency. In addition, the processing is regulated by the presentation of processing sites to the protease (V. Lulla, Karo-Astover, et al. 2013) and, likely, by other factors, such as the interaction of nsP2 with other replicase proteins, host factors and/or viral RNAs. These multiple mechanisms ensure the ability of the nsP2 protease to play a leading role in the regulation of the alphavirus infection cycle in the cell.

In addition to enzymatic functions, nsP2s of Old World alphaviruses play a role in the development of cytopathic effect (CPE) in vertebrate cells. Garmashova *et al*. have demonstrated that SINV nsP2 is responsible for the development of CPE and that CPE is at least partially caused by transcriptional shutoff. Additionally, CPE development requires free nsP2, as changes in the ns polyprotein processing can diminish/abolish the cytotoxicity of nsP2 (Garmashova et al. 2006). There are several mechanisms underlying the cytotoxicity of nsP2s of Old World alphaviruses. Possibly, the most important of them is related to

nsP2-induced degradation of the catalytic subunit of cellular RNA polymerase II (I Akhrymuk, Kulemzin, and Frolova 2012). Mutations in nsP2 that eliminate or reduce this ability also reduce the cytotoxicity of Old World alphavirus infection (Ivan Akhrymuk, Frolov, and Frolova 2018; Ivan Akhrymuk et al. 2019). In SINV-infected cells, nsP2 also has the ability to affect cellular translation. However, this effect is independent of the ability of nsP2 to induce the shutdown of transcription (Gorchakov, Frolova, and Frolov 2005). Furthermore, it has been shown that nsP3, not nsP2, has a leading role in the SINV-induced shutdown of translation (Ivan Akhrymuk, Frolov, and Frolova 2018). Interestingly, unlike those of Old World alphaviruses, nsP2s of New World alphaviruses do not induce CPE in vertebrate cells. Instead, in the case of New World alphaviruses, the development of CPE is mostly induced by their capsid proteins (Aguilar, Weaver, and Basler 2007; Garmashova, Gorchakov, et al. 2007).

In SINV-infected cells, the suppression of cellular transcription is also the principal strategy to avoid the cellular antiviral response, especially the activation of interferon-inducible genes (Gorchakov, Frolova, and Frolov 2005). Therefore, it is not surprising that nsP2s of different Old World alphaviruses have been shown to play a role in suppressing the cellular interferon response (Breakwell et al. 2007; E. I. Frolova et al. 2002). Similar to the shutdown of cellular transcription, this function is attributed to a mature (individual) nsP2 because SINV mutants that are unable to process P123 or P23 polyproteins induce a much higher interferon response in infected cells than wt SINV. In cells with a functional interferon response, cleavage-deficient SINV mutants are unable to induce complete transcriptional and translational shutoff. Consequently, such mutants can only effectively replicate in cells with defective interferon production and signaling (Gorchakov et al. 2008). However, it is also clear that the inhibition of host cell transcription is not the only mechanism by which nsP2 affects cellular antiviral responses. Thus, it has been shown that CHIKV nsP2 inhibits the interferon response by inhibiting JAK/STAT signaling. Upon infection with CHIKV or transfection with a plasmid encoding CHIKV nsP2, the nuclear translocation of phosphorylated STAT is suppressed, and as a result, the activation of interferon-stimulated genes is inhibited (Fros et al. 2010). In SINV, mutation of P726 disrupts the viral RNA synthesis (Sawicki et al. 2006), CPE development and virus replication (Frolov et al. 1999). Mutation of the corresponding residue (P718) in nsP2 of CHIKV renders the virus unable to prevent the nuclear translocation of phosphorylated STAT and thus reduces the ability of the virus to suppress JAK/STAT signaling (Fros et al. 2010). In addition, CHIKV infection inhibits the unfolded protein response in cells, and nsP2 is one of the proteins that are responsible for this inhibition (Fros et al. 2015). Remarkably, the substitution of the same P718 residue with glycine renders CHIKV noncytopathic and blocks its ability to inhibit the unfolded protein response (Fros et al. 2015).

The nonenzymatic functions of nsP2 are not limited to the suppression of cellular antiviral responses. nsP2 has also been shown to participate in the formation of alphavirus virions. It has been shown that nsP2 of VEEV regulates the packaging of the viral genome (Kim et al. 2013). It is possible that this

mechanism is universal for alphaviruses, as we have observed a genetic link between sequences of the capsid protein and N-terminal residues of nsP2 of SFV, an Old World alphavirus (V. Lulla et al. 2018). Finally, it has been shown that unlike other nsPs, nsP2 of SINV is present in virions (Schuchman et al. 2018). Thus, nsP2 is important for alphavirus virions (and/or their formation), although its role(s) is currently poorly understood.

In the alphavirus replicase complex, nsP2 interacts with nsP4 (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022). It is also possible that it interacts with other nsPs, as pulldown of nsP2 from SINV-infected cells results in coprecipitation of a set of cellular proteins similar to those coprecipitated by pulldown of nsP3 or nsP4 (Atasheva et al. 2007; Cristea et al. 2006; 2010). This makes the pulldown method (at least using alphavirus-infected cells) poorly suitable for studies of interactions between nsP2 and cellular proteins. To overcome this limitation, a study was performed using yeast two-hybrid methods. It identified 22 cellular proteins that interacted with CHIKV nsP2 and nsP4. The identified proteins included proteins involved in protein degradation and/or autophagy and in the control of gene expression. However, siRNA-mediated knockdown of the expression of the majority of the identified proteins had little or no effect on CHIKV replication. Only knockdown of heterogeneous nuclear ribonucleoprotein K (nuclear protein) and ubiquilin 4 (involved in ubiquitin pathways) reduced CHIKV replication; a tetratricopeptide repeat protein 7B was shown to be involved in nsP2-induced host cell shutoff (Bouraï et al. 2012).

### 2.4.3. nsP3

CHIKV nsP3 (531 aa residues) has a molecular mass of approximately 60 kDa. nsP3 has three distinct domains: the macro domain, alphavirus unique domain (AUD) and hypervariable domain (HVD). The length and sequence of the HVD domain are, as the name suggests, variable among different alphaviruses. The scheme of CHIKV nsP3 is depicted in Figure 5A.

The N-terminal macro domain of nsP3 is ~160 aa in length. Malet *et al.* determined the crystal structure of the CHIKV and VEEV macro domains (Malet et al. 2009). The alphavirus macro domain has a globular structure, containing a six-stranded β-sheet in the middle, three α-helices on one side and one α-helix on the other side (Figure 5B). The CHIKV macro domain can bind mono- and poly-ADP-ribose and RNA, and the D10 residue is needed for these functions. If D10 is replaced with alanine, the CHIKV macro domain loses its ability to bind ADP-ribose. The CHIKV nsP3 macro domain also has ADP-ribose 1′′-phosphate phosphatase activity. Important aa residues for this activity are N24 and Y114; the N24A and Y114A substitutions of these residues abolish the ADP-ribose 1′′-phosphate phosphatase activity of the macro domain. For this reaction, the D10 residue is less critical, as its substitution with alanine only decreases (but does not eliminate) the ADP-ribose 1′′-phosphate phosphatase activity (Malet et al. 2009). Studies of the SFV macro domain have shown that in contrast to the CHIKV macro domain, it does not bind mono-ADP-ribose; however, its ability

to bind poly-ADP-ribose and RNA is similar to that of its counterpart from CHIKV. The SFV macro domain also has a weak ADP-ribose 1´´-phosphate phosphatase activity (Neuvonen and Ahola 2009; Egloff et al. 2006). These contrasting results indicate that the macro domains of different alphaviruses may have somewhat different functions during virus infection. The CHIKV nsP3 macro domain also has mono-ADP-ribose hydrolase activity, which is necessary for CHIKV replication in cell culture and for virulence in mice (McPherson et al. 2017). The ADP-ribose hydrolase activity of the macro domain also participates in stress granule disassembly during CHIKV infection (Jayabalan et al. 2021). As nsP3 is part of the viral RC, the RNA-binding activity of the macro domain may help provide RNA templates for the viral replicase. Alphavirus infection can induce the synthesis of poly-ADP-ribose, which contributes to cell death (Nargi-Aizenman et al. 2002); therefore, nsP3 poly-ADP-ribose-binding activity may help counteract the antiviral response in infected cells.

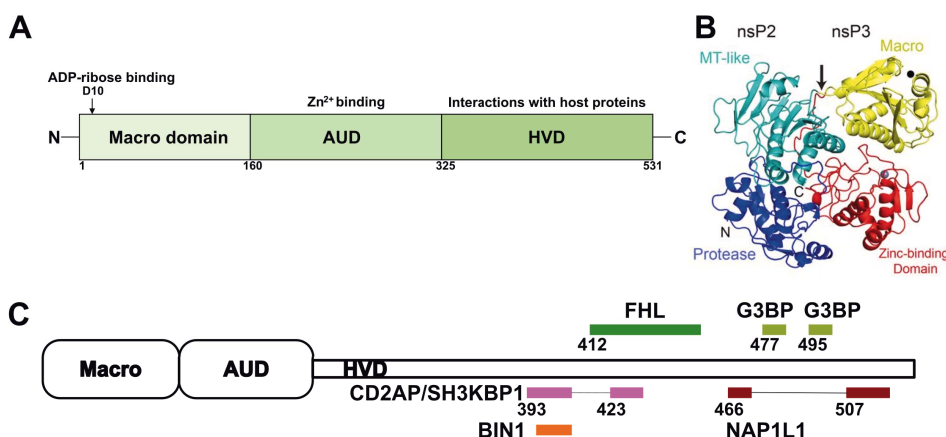



**Figure 5.** Alphavirus nsP3. A) Schematic presentation of the domain organization of CHIKV nsP3. D10 in the macro domain is involved in ADP-ribose binding. The alphavirus unique domain (AUD) is involved in $Zn^{2+}$ binding. The hypervariable domain (HVD) is involved in interactions with host proteins. B) 3D structures of the macro and AUD domains of SINV. The structure is derived from the SINV P23 precursor protein, covering the nsP2 protease and MTL domains and the nsP3 macro and AUD (zinc-binding) domains (aa residues 1011–1675 of SINV P1234; from nsP2 aa 471 to nsP3 aa 328). The arrow indicates the cleavage site between nsP2 and nsP3, the gray sphere indicates the zinc ion, and the black filled circle indicates the ADP-ribose-binding site. The protease domain is in blue, the MTL domain is in teal, the macro domain is in yellow, and the AUD (zinc-binding) domain is in red. The image is reproduced from (Shin et al. 2012). C) Localization of sites for interaction with host proteins in the CHIKV HVD. FHL – four-and-a-half LIM domain protein, G3BP – Ras-GAP SH3 domain binding protein, CD2AP – CD2 associated protein, SH3KBP1 – SH3-domain containing kinase-binding protein, BIN1 – amphiphysin-2, NAP1L1 – protein from nucleosome assembly protein 1 family.

The nsP3 macro domain is followed by the AUD, a domain thus far found only in alphaviruses. The AUD has a globular shape and is made up of an α-helical bundle and two β-strands. The AUD also contains a zinc-binding site (Figure 5B) wherein $Zn^{2+}$ is coordinated by four conserved cysteines (in the SINV nsP3 AUD, these are C263, C265, C288 and C306). Replacement of any of these cysteines with alanine is lethal for the virus (Shin et al. 2012), indicating that the binding of $Zn^{2+}$ is important; however, its role is not exactly known. Other mutations in the AUD have been shown to affect negative-strand RNA and SG RNA synthesis, ns polyprotein processing and neurovirulence of the viruses (Dé et al. 2003; Lastarza, Grakoui, and Rice 1994; Gao et al. 2019; M. Tuittila and Hinkkanen 2003).

The AUD is followed by the intrinsically disordered HVD. A degradation signal is located at the C-terminal end of the HVDs of nsP3s of SFV and SINV. The removal of the last 6–10 aa of SFV nsP3 and the last 36 aa of SINV nsP3 increased the half-lives of the nsP3s compared with those of the full-length nsP3s, and conversely, the addition of these aa significantly reduced the half-life of the luciferase reporter (Varjak, Žusinaite, and Merits 2010). Similar to the disordered domains found in replicase proteins of other viruses, the HVDs of alphaviruses contain multiple short linear interaction motifs and are involved in interactions with a large number of cellular proteins, some of which are shown in Figure 5C. The HVDs of Old World alphaviruses interact with Ras-GAP SH3 domain binding proteins (G3BPs), and through this interaction, inhibit stress granule formation in infected cells (Panas, Ahola, and McInerney 2014; Fros et al. 2012). However, the main role of this interaction is related to viral RNA replication; if nsP3 lacks motifs for the interaction with G3BPs or genes encoding G3BPs have been knocked out, the replication of Old World alphaviruses is reduced (SINV) or completely blocked (CHIKV) (Kim et al. 2016). The defect is due to the inability of the mutant virus (or knockout cells) to support negative-strand RNA synthesis (Götte et al. 2020). Interestingly, replication of New World alphaviruses is independent of the interaction with G3BPs; instead, it depends on the interaction of the HVD with cellular Frx-proteins (Kim et al. 2016). To date, G3BPs (or Frx-proteins) remain the only host proteins absolutely required for alphavirus RNA replication. It has been reported that four-and-a-half LIM domain protein 1 (FHL1) is also absolutely required for the replication of CHIKV and o'nyong'nyong virus (ONNV) (Meertens et al. 2019); however, this has been challenged by another study that revealed that FHL1 was dispensable for CHIKV replication (Lukash et al. 2020). However, it would be incorrect to assume that the interaction of nsP3 with other cellular proteins is not important. In fact, it has been shown that to perform RNA replication, the HVD must interact with G3BP (or Frx) and at least one other cellular protein (Meshram et al. 2018). The list of these interacting proteins is long and growing. For example, the HVDs of many alphaviruses contain a conserved Src homology 3 (SH3) domain-binding motif (PxxPxR). The same proline-rich sequence motif binds the cellular proteins amphiphysin-1 and amphiphysin-2. SFV, SINV and CHIKV bind to amphiphysins in an nsP3 SH3-domain-dependent manner, and this binding affects the replication and virulence of SFV in mice (Neuvonen et al. 2011). Another protein

that binds to similar motifs in the HVD of nsP3 of CHIKV is CD2 associated protein (CD2AP) (Mutso et al. 2018; Agback et al. 2019); its binding also supports alphavirus replication.

Alphavirus nsP3 is a phosphoprotein (Guangpu Li et al. 1990; Teppor, Žusinaite, and Merits 2021; Johan Peränen et al. 1988). The HVD is phosphorylated on multiple serine and threonine residues. In nsP3 of SFV, the phosphorylated residues are located at the beginning of the HVD (Vihinen and Saarinen 2000), and their phosphorylation has been shown to be important for SFV virulence in mice (Vihinen et al. 2001). Recently, nsP3s of CHIKV and ONNV have been shown to be phosphoproteins. In contrast to nsP3 of SFV, phosphorylated serine and threonine residues can be found throughout the HVD of nsP3 of CHIKV. Substituting all potentially phosphorylated residues with alanine residues is lethal for CHIKV (Teppor, Žusinaite, and Merits 2021). A possible reason for this may be the interactions of CHIKV nsP3 with the host NAP1L1 and NAP1L4 proteins. These proteins are essential for CHIKV replication, and their binding sites in the HVD overlap with phosphorylation sites. It has been demonstrated that the interactions with NAP1L1 and NAP1L4 require phosphorylation of their binding sites (Dominguez et al. 2021).

Given the presence of a large number of interaction motifs, it is not surprising that in cells, nsP3 has been shown to colocalize and/or interact with multiple cellular proteins, including G3BPs, cytosceletal proteins, eIF1A, chaperones, hnRNPs, ribosomal proteins, and 14-3-3 proteins (E. Frolova et al. 2006; Cristea et al. 2006; Scholte et al. 2015; Schulte et al. 2016). Altogether, 92 interaction partners have been described for nsP3s of Old World alphaviruses (Götte, Liu, and McInerney 2018). Multiple interactions with host proteins suggest localization of nsP3 in the cytosolic part of the alphavirus RC; only in this case would it be accessible for host proteins. Indeed, a recent study has revealed that nsP3 is not a part of the core of the alphavirus RNA replicase. Instead, it is thought to be a part of another mandatory component of an active RC, the cytosolic ring structure (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022).

Given the abilities of nsP3 to be modified in infected cells and to interact with host components, it is hardly surprising that this protein is involved in the modulation of cellular processes. nsP3 regulates the internalization of alphavirus RCs by modulating Akt-mTor signaling (Thaa et al. 2015). The same signaling pathway is used to activate proviral metabolic changes in infected cells (Mazzon et al. 2018). At the same time, nsP3 is also a key determinant of the shutdown of translation in alphavirus-infected cells (Ivan Akhrymuk, Frolov, and Frolova 2018). These functions, as well as multiple interactions with host components, make nsP3 a likely candidate for a factor underlying the different host and/or vector specificities of alphaviruses. Indeed, nsP3 participates in the determination of the alphavirus vector specificity. The insect vector for ONNV is *Anopheles gambiae*, while CHIKV uses *Aedes aegypti* and *Aedes albopictus* mosquitoes. The substitution of CHIKV nsP3 with ONNV nsP3 grants the chimeric virus the ability to infect *A. gambiae* mosquitoes (Saxton-Shaw et al. 2013). It is possible that this is due to the ability of nsP3 to interact with different cellular proteins, possibly including the mosquito homolog of G3BPs, called Rin (or Rasputin). Similarly,

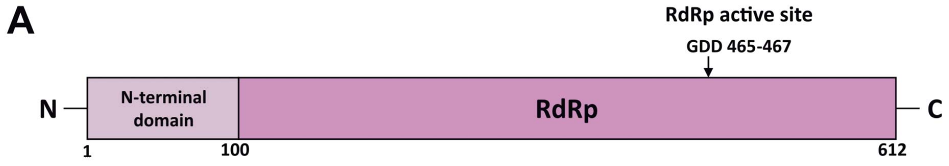
one can expect that nsP3 might play a role in host cell/tissue specificity and, therefore, also have a role as a determinant of alphavirus virulence. This has been directly demonstrated for SFV, in which nsP3 is involved in the determination of neurovirulence in mice. Analysis by swapping nsP3 regions between neurovirulent and nonvirulent SFV strains has shown that nsP3 determines the outcome of the infection, that is, whether the mice die or survive (M. T. Tuittila et al. 2000; Saul et al. 2015).

### 2.4.4. nsP4

nsP4 is alphaviral RNA-dependent RNA polymerase (RdRp). Although all ns proteins are needed to form a functional RC, nsP4 specifically performs the synthesis of new viral RNAs. nsP4 has a molecular mass of approximately 70 kDa and, in the case of CHIKV, consists of 612 aa residues. Approximately 100 N-terminal amino acids of nsP4 are unique to alphaviruses and form the N-terminal domain (NTD), while the rest form the RdRp region, which is organized similarly to other viral RdRps (Rubach et al. 2009; Tomar et al. 2006). The scheme of CHIKV nsP4 is depicted in Figure 6A.

The amount of nsP4 in infected cells is lower than that of other nsPs. There are two main reasons for this. First, the N-terminal aa residue of nsP4 is tyrosine. According to the N-end rule, it is a destabilizing residue that facilitates nsP4 degradation in proteasomes (Groot et al. 1991). Second, in many alphaviruses, the synthesis of nsP4 is downregulated by a readthrough stop codon at the end of the nsP3 region. Owing to this opal stop codon, the amount of nsP4 synthesized is approximately 10-fold lower than that of other nsPs (J. H. Strauss and Strauss 1994; G Li and Rice 1993). Combined, these mechanisms can result in infected cells with a molar ratio of nsP4 to nsP1 (or other nsPs) as low as 1:100.

The 3D structures of the RdRp domains of Ross River virus (RRV) and SINV nsP4s were recently determined using X-ray crystallography (Figure 6B). The proteins were found to have a “right-hand fold” typical for viral RdRps. Compared with other viral RdRps, the alphavirus nsP4 structure is richer in α-helices. RRV RdRp forms an encircled ring structure, with finger domains reaching toward the thumb. The palm, thumb, middle and ring fingers form a protected, buried structural core of RdRp, while the pinky and index fingers are more exposed to the surrounding solution. RdRps of alphaviruses have dynamic structures with a number of unfolded (not visible on the crystal structure) regions. The NTD also seems to have a dynamic structure, and no strong interaction was observed between the NTD and RdRp parts of nsP4 (Tan, Lello, Liu, Law, Kang, Lescar, Zheng, Merits, et al. 2022). However, once nsP4 forms a complex with the dodecameric ring structure of nsP1, its folding significantly changes. In this complex, most regions of nsP4, including the NTD and C-terminal tail of the protein, are well folded (Figure 6C). As described above, there are 10 nonidentical contact surfaces of nsP4 and nsP1 subunits, as well as channel structure formed between nsP4 and the remaining two nsP1 molecules. The cytosolic side of nsP4 forms a contact surface with the N-terminal part of the nsP2 molecule (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022).


A
RdRp active site
GDD 465-467
N
N-terminal domain
RdRp
C
1
100
612

B
NTD
INDEX
PINKY
RING
MIDDLE
PALM
THUMB
RRV
SINV
flex
135-184
SER3
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109
611
91
610
G
F
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D
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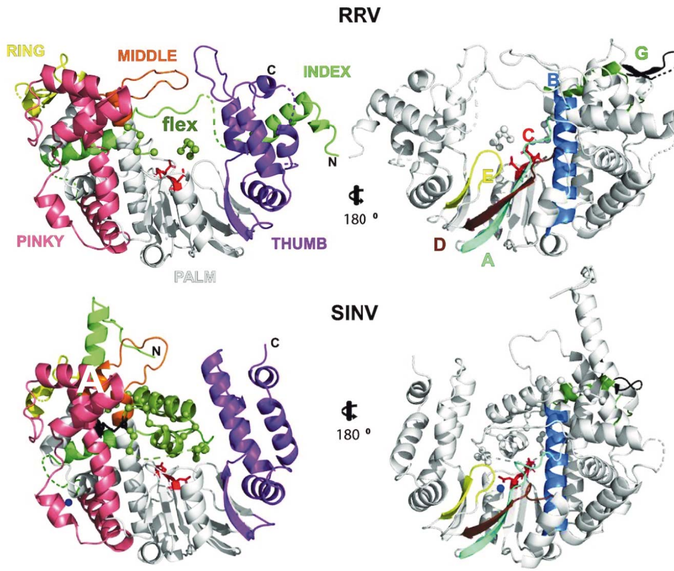
motif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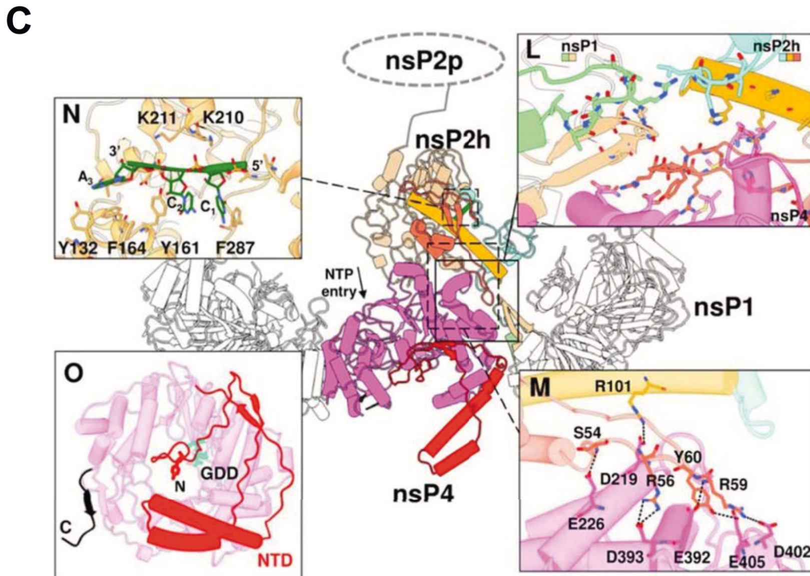
C
nsP2p
nsP2h
nsP1
nsP4
NTP entry
N
K211
K210
Y132
F164
Y161
F287
L
nsP1
nsP2h
M
R101
S54
Y60
D219
R56
R59
E226
D393
E392
E405
D402
O
GDD
NTD

**Figure 6.** Alphavirus nsP4. A) Schematic structure of CHIKV nsP4. The active site of RNA-dependent RNA polymerase (RdRp) is indicated by an arrow. B) Schemes and crystal structures of the nsP4 RdRp domains of RRV and SINV. The image is reproduced from (Tan, Lello, Liu, Law, Kang, Lescar, Zheng, Merits, et al. 2022). C) Overall structure of the CHIKV RNA replicase core, comprising twelve molecules of nsP1, one molecule of nsP2 and one molecule of nsP4. The proper folding of nsP4 depends on its interaction with the nsP1 ring structure. The image is reproduced from (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022), and the names of the blown-up windows are from the original article. NTP – nucleotide triphosphate. L) Interactions between nsP1, nsP2 and nsP4 in the CHIKV RC. The helicase part of nsP2 (nsP2h, aa 1-465 of nsP2) is shown as follows: the N-terminal domain (NTD) is in orange, the stalk is in gold, 1B is in cyan, and RecA domains are in peru; the nsP1 chains located in proximity are in light green and tan. The structure of the nsP2 protease part (nsP2p, aa 466-798) was not resolved by this analysis, and this region was added to the image for better visualization. nsP4 is in magenta. M) Interactions between nsP2 and nsP4. Dotted lines – hydrogen bonds. N) Interactions between nsP2h and RNA (green); the involved aa residues of nsP2h are labeled. O) The "bottom" view of nsP4. The C-terminus (aa 600-611) is shown in black, the NTD (aa 1-105) is shown in red, and the GDD, nsP4 active site, is shown in cyan.

Individual recombinant nsP4 is a poorly soluble protein that has a strong tendency to aggregate; most likely, these properties originate from the dynamic structure of the protein. The poor solubility and low activity have hampered the characterization of the enzymatic activities of nsP4 in cell-free reactions. To some extent, the solubility of recombinant nsP4 depends on its source, with SINV nsP4 being the most soluble and thus easier to work with. Rubach *et al*. purified full-length nsP4 of SINV as a recombinant protein, demonstrated its ability to *de novo* synthesize RNA and observed its ability to add terminal adenosine residues to the 3' end of RNA (Rubach et al. 2009). The terminal adenylyltransferase activity was also demonstrated for recombinant nsP4 lacking 97 N-terminal aa residues; similar to the RdRp activity, it depended on the intactness of the catalytic site, as the GDD to GAA mutation rendered the enzyme inactive (Tomar et al. 2011). RNA synthesis and terminal adenylyltransferase activities have also been demonstrated for the RdRp core domain of CHIKV nsP4 (M. W. Chen et al. 2017). Likewise, nsP4s of RRV and SINV were demonstrated to have low RdRp activity; the addition of up to 7 residues to a provided RNA template required the incubation for several hours (Tan, Lello, Liu, Law, Kang, Lescar, Zheng, Merits, et al. 2022). The formation of a complex with nsP1 drastically improved the polymerase activity of nsP4, and the addition of nsP2 resulted in increased terminal adenylyltransferase activity. Thus, although some activities can be observed for individual nsP4, they are fully displayed only in the 12 (nsP1):1 (nsP2):1 (nsP4) complex, which was therefore termed the core alphavirus RNA replicase (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022).

Mutations in the NTD of nsP4 impair RNA synthesis and host cell translation shutoff. The N-terminal tyrosine residue that causes instability of an individual

nsP4 protein has been shown to be critical for RNA replicase activity; in an active replicase, it can only be replaced with another aromatic residue or histidine. Coupled with recent structural information, this finding suggests that the N-terminal residue of nsP4 may be required for priming RNA synthesis: the aromatic ring of tyrosine likely forms a stacking interaction with the nucleobase of GTP, the nucleotide used to initiate the synthesis of negative-strand RNA. A substitution of a nonaromatic residue for the N-terminal tyrosine residue does not completely inactivate the virus; the compromised RNA replicase activity can be restored by compensatory mutations at the 5' end of the virus genome and/or in other nsPs; these findings indicate that the N-terminal part of nsP4 interacts with these components (Rupp, Jundt, and Hardy 2011). The R183 residue in the RdRp domain of nsP4 has been shown to be essential for the efficient negative-strand RNA synthesis (Fata, Sawicki, and Sawicki 2002b). Studies have demonstrated the importance of interactions between nsP4 and nsP1 for negative-strand RNA synthesis during alphavirus replication (Fata, Sawicki, and Sawicki 2002a; Yukio Shirako, Strauss, and Strauss 2000), a finding that is clearly supported by the existence of multiple contacts between nsP1 and nsP4 within the RNA replicase core of the alphavirus (Figure 6C).

The data reviewed above indicate that nsP4 plays a role in alphavirus RNA recognition. Using a *trans*-replicase assay and analysis of different combinations of replication proteins/transcription templates, it was demonstrated that for alphaviruses of the SFV complex, nsP4 determines the recognition and use of the SG promoter and, presumably, the genomic promoter (Lello et al. 2021). Interestingly, biochemical evidence suggests that these RNA elements are recognized by different motifs in nsP4. Thus, the critical amino acid residues in nsP4 for the recognition of the SG promoter in SINV are R331 and R332. Replacement of these residues with alanines renders nsP4 unable to bind the SG promoter; in the context of the virus genome, this results in lethal defects (M.-L. Li and Stollar 2004). At the same time, the genomic promoter interacts with the sequence LGKPLPAD, which corresponds to aa 531-538 in nsP4 (M.-L. Li and Stollar 2007).

There is not much information about the interactions of nsP4 with host proteins. This is understandable for nsP4 that is included in the RNA replicase core as well as for nsP4 present in the form of inactive complexes; in both cases, nsP4 forms extensive contacts with nsP1 and nsP2 and is mostly not accessible for interaction with cellular proteins. The only "free" surface of the protein is facing the spherule interior in functional RCs or the plasma membrane in inactive complexes (Tan, Chmielewski, Law, Zhang, He, Chen, Jin, and Luo 2022). The only form available for interactions with host proteins is free nsP4, which is present in very low amounts due to its instability. Nevertheless, some interactions of this protein with host components have been revealed. Thus, the cellular protein HSP-90 has been shown to interact with nsP4 of CHIKV, an interaction that is beneficial for virus replication. Inhibitors of HSP-90 reduced CHIKV infection in cell culture and inflammation caused by *in vivo* infection (Rathore et al. 2014). There is also a report describing numerous host cell proteins pulled down

via nsP4 of SINV (Cristea et al. 2010). However, the data presented in the latter study are almost certainly an artifact caused by the flawed design of the experiment. It has been demonstrated decades before that free (individual) nsP4 is formed only at early stages of SINV infection; at late stages, nsP4 formation ceases, and P34 accumulates (de Groot et al. 1990). Thus, Cristea and coauthors mostly, if not exclusively, precipitated proteins bound to the P34 polyprotein, which includes a region corresponding to nsP3, the main ns protein of alphaviruses involved in interactions with host proteins (see 2.4.3 above). Therefore, it is not surprising that the list of presumed interactors of nsP4 that the authors obtained is extremely similar to the ones that they and others obtained using nsP3 pulldown (Cristea et al. 2006; E. Frolova et al. 2006).

### 2.4.5. Capsid protein

Similar to nsPs, the structural proteins of alphaviruses are synthesized in the form of polyprotein precursors. The capsid protein (CP; approximately 30 kDa) is the first structural protein encoded by the second ORF of alphaviruses. The CP is involved in the packaging of viral genomes into nucleocapsid cores, virus budding and virion assembly. The viral nucleocapsid contains 240 copies of the CP (J. H. Strauss and Strauss 1994; K E Owen and Kuhn 1996; Henrik Garoff, Sjöberg, and Cheng 2004). The scheme of the CHIKV CP is depicted in Figure 7.

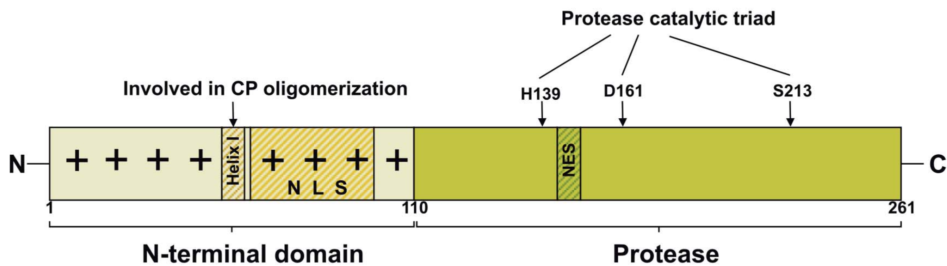


**Figure 7.** Schematic presentation of the CP of CHIKV. The helix I region involved in CP oligomerization and the aa residues forming the catalytic triad of the protease domain are indicated by arrows. The approximate locations of the nuclear export signal (NES) and nuclear localization signal (NLS) are also shown. "+" indicates the general positive charge of the N-terminal domain of the CP.

The CP consists of two domains: a positively charged N-terminal domain (NTD) (Lundberg, Carey, and Kehn-Hall 2017; H Garoff et al. 1980) and a C-terminal domain, which is a chymotrypsin-like serine protease (Hahn, Strauss, and Strauss 1985; H Garoff et al. 1980). The protease domain of the CHIKV CP consists of two β-barrel subdomains connected via the linker region. The protease catalytic triad (H139, D161 and S213; Figure 7) is located in the cleft between the subdomains. Following its synthesis, the CP is autoproteolytically cleaved from the rest of the structural polyprotein. Studies of the CP of SINV showed that the protease is used only to release the CP after its translation (Choi et al. 1991). After

release of the CP from the remaining structural polyprotein, the C-terminal W261 residue of the CP is left in the protease substrate-binding pocket, thus inactivating the protease (Sharma et al. 2018). The protease domain of the CP also contains a hydrophobic pocket that interacts with the cytoplasmic domain of the E2 glycoprotein, and this interaction plays a role in virus budding (Henrik Garoff, Sjöberg, and Cheng 2004; Jose et al. 2012; Sharma et al. 2018; Tang et al. 2011; Katherine E. Owen and Kuhn 1997).

The NTD of the CP is approximately 110 aa long, and in the crystal structure, it appears to be disordered (Choi et al. 1991). The NTD contains many positively charged aa residues that mediate the interactions with negatively charged RNA (Lundberg, Carey, and Kehn-Hall 2017; K E Owen and Kuhn 1996). The NTD can be divided into two regions. Region I (aa 1-81) is involved in viral RNA packaging (K E Owen and Kuhn 1996; V. Lulla, Kim, et al. 2013) and CP dimerization (Hong, Perera, and Kuhn 2006; Perera et al. 2001). Region II (aa 81-114) is involved in the interaction with the RNA packaging signal (Weiss, Geigenmüller-Gnirke, and Schlesinger 1994; K E Owen and Kuhn 1996). Interactions with RNA enable the packaging of viral genomes into nucleocapsid cores. In SINV, the region responsible for RNA binding lies between aa 75-116 (Geigenmüller-Gnirke, Nitschko, and Schlesinger 1993). Aa 38-55 in SINV form a leucine zipper motif, termed helix I, which is associated with CP dimerization stabilization (Perera et al. 2001). Thus, the NTD of the CP is involved in interactions between the capsomeres in the nucleocapsid (Cheng et al. 1995).

A study by Kim *et al.* revealed that the alphavirus CP recognizes a packaging signal located in the region encoding nsP1. The packaging signal is formed by multiple stem–loops carrying the GGG motif at the stem tips. SFV complex viruses are exceptions to this rule, as they contain packaging signals with a similar structure in their nsP2-encoding region (Kim et al. 2011). Whether SFV complex viruses require these signals remains unclear, as recent mutagenesis studies have shown that previously described packaging signals are not required for the formation of infectious virions of SFV and CHIKV. Instead, multiple CP-binding sites were identified that are enriched in genomic RNA-specific regions of the viral genome (i.e., absent in the region corresponding to SG RNA) and promote RNA packaging and infectious virion formation (Brown et al. 2020).

The alphavirus CP also contains nuclear localization signals (NLSs) and nuclear export signals (NESs) (Favre, Studer, and Michel 1994; Thomas et al. 2013; Atasheva et al. 2010). Their significance is better understood for New World alphaviruses. The CPs of these viruses are involved in host cell transcriptional shutoff by blocking nucleocytoplasmic traffic by the formation of complexes located in nuclear pores (Garmashova, Atasheva, et al. 2007). This is less clear for Old World alphaviruses; however, it is clearly not accidental, as mutations introduced in the NES or NLS affect the properties of these viruses. The localization of the CP of CHIKV to the nucleolus has also been shown to be important for the virulence of the virus. Mice infected with a CHIKV mutant lacking nucleolar localization of the CP did not develop typical footpad swelling and showed reduced viremia upon infection (Taylor et al. 2017).

### 2.4.6. Envelope proteins

Alphaviruses encode five envelope proteins: the major glycoproteins E1 and E2, which are always present in mature virions, and smaller peptides, called E3, 6K and TF proteins. The order of these proteins in the structural polyprotein is E3-E2-6K/TF-E1.

**E3**. E3 consists of approximately 65 aa residues. E3 is an α/β-protein, with its N-terminal part forming a β-hairpin, followed by three α-helices, in a horseshoe shape. It is absolutely necessary for virion assembly (Liljeström and Garoff 1991; Lobigs, Zhao, and Garoff 1990) but is not always present in mature virions. E3 is required for membrane attachment of the structural polyprotein; the release of the CP from the translated polyprotein exposes the N-terminal region of E3, which contains a signal sequence that directs the synthesis of the remaining structural polyprotein into the endoplasmic reticulum (ER) membrane (H Garoff et al. 1990). The scheme of the orientation of the structural polyprotein in the ER membrane is depicted in Figure 8. E3 is also needed for the assembly of spikes present in alphavirus virions. The E2 glycoprotein and E3 form a relatively stable polyprotein, known as p62 (and also as PE2). To initiate the assembly of spikes, p62 and E1 form heterodimers in the ER (J. H. Strauss and Strauss 1994), which are transported through the Golgi complex. These immature heterodimers are protected from premature fusion with cellular membranes, and E3 has a crucial role in this process. It is thought to form a clamp that stabilizes the E2-E1 heterodimer during spike assembly (L. Li et al. 2010; Voss et al. 2010), preventing premature changes in the conformation during the transport of p62-E1 complexes in acidic compartments of the cell. The CHIKV p62-E1 heterodimer has a twisted plate shape, with E3 protruding on one side. In the heterodimer, E3 interacts with E2 and does not make contacts with E1. E3 forms a brace that keeps E2 in such a conformation that it forms a groove for the E1 fusion loop (Voss et al. 2010).

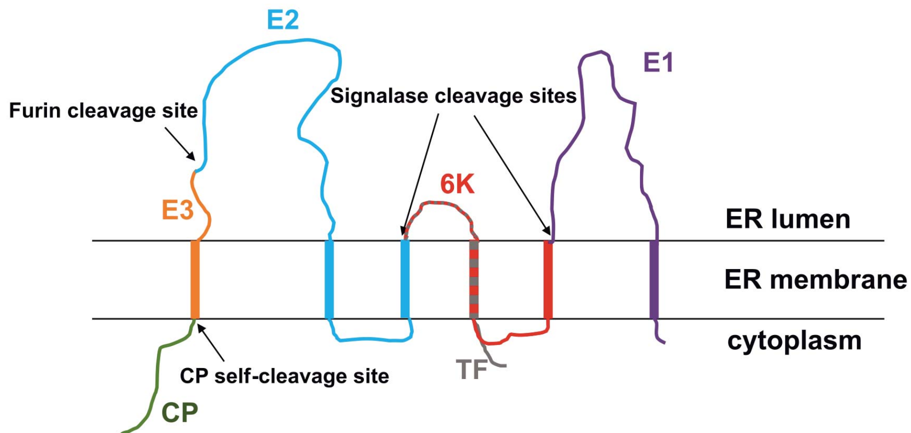


**Figure 8.** Topology of alphavirus glycoproteins in the ER membrane. The cleavage sites between the glycoproteins are indicated by arrows. If the alternative form of the 6K protein, the TF protein (result of a frameshift), is produced, the E1 glycoprotein, which follows 6K in the polyprotein, is not produced. CP – capsid protein, ER – endoplasmic reticulum, TF – transframe protein.

Later, during transport to the plasma membrane, p62 is cleaved in the *trans*-Golgi network by furin protease, resulting in individual E3 and E2 glycoproteins (X. Zhang et al. 2003; Jain, DeCandido, and Kielian 1991; Salminen et al. 1992). Mature viral spikes are formed by E2 and E1 heterodimers. E2-E1 heterodimers or E3-E2-E1 heterotrimers are transported to the plasma membrane (Ziemiecki and Garoff 1978). Even after p62 is cleaved, E3 remains in association with E2-E1 heterodimers in a pH-dependent manner. Under acidic conditions in the late secretory pathway, E3 continues to protect spikes from premature fusion with cellular membranes, acting as a clamp to keep E2 in place. Only under neutral pH outside the cell (i.e., after budding), E3 dissociates (for many, but not all, alphaviruses), and the spike complex becomes ready to initiate the fusion during a new round of infection (Sjöberg, Lindqvist, and Garoff 2011; Wahlberg, Boere, and Garoff 1989). For some alphaviruses, such as SFV, E3 remains associated with mature virions.

A study using E3 chimeric viruses showed that viruses that had E3 from a virus belonging to a different clade than the parental virus had reduced infectivity and abnormal particle morphology and composition compared with those of wild-type viruses and chimeras in which E3 was derived from a virus belonging to the same clade as the parental virus. These data suggest that a mismatched E3 is probably unable to stabilize the p62-E1 heterodimer during transport through the Golgi complex and allows the formation of abnormal spikes (A. J. Snyder and Mukhopadhyay 2012).

**E2**. E2 is the C-terminal part of p62. Cellular signalase cleaves the structural polyprotein in the ER between E2 and 6K/TF (Figure 8). p62 forms heterodimers with E1, which occurs cotranslationally in the ER (Voss et al. 2010). E2/E1 heterodimers form trimers making up the viral spikes. In total, alphavirus virions have 240 E1/E2 heterodimers, which form 80 spikes. E2 is a transmembrane protein whose function is the recognition of viral receptors on the cell surface (reviewed in (Jose, Snyder, and Kuhn 2009)). The distal part of the E2 cytoplasmic tail functions as a signal for the membrane translocation of 6K, which is the next protein following E2 in the structural polyprotein (Liljeström and Garoff 1991). The p62/E1 heterodimer has a twisted leaf shape and is approximately 150 Å long, 50 Å wide and 25 Å thick. The crystal structure of E2 reveals that it is a β protein that has three immunoglobulin-like domains, named A, B and C. The C domain is situated the closest to the membrane, the B domain is the most distant from the membrane, and the A domain is located between the C and B domains. A β-ribbon motif connects the domains to each other. Domain B covers the viral fusion loop in domain II of the E1 glycoprotein. E2 and E1 make contact with each other along E1 domain II (Voss et al. 2010; Rui Zhang et al. 2011). In addition to domains A, B and C, E2 contains subdomain D, also referred to as the stem region, which is located close to the membrane. Subdomain D contains aa residues important for SFV budding (Byrd and Kielian 2019). The transmembrane domains of E2 and E1 also interact with each other, and this interaction is needed for efficient budding (Sjöberg and Garoff 2003; Byrd and Kielian 2019).

The C-terminus of E2 interacts with the CP. The endoplasmic tail of E2 contains a Tyr-X-Leu motif that makes contacts with a hydrophobic pocket on the CP; this interaction is necessary for virus budding. Residue Y399 of E2 of SFV forms aromatic interactions with residues W251 and Y184 in the hydrophobic E2-binding cavity of the CP. If residue Y399 of E2 is removed, the viral budding activity is abolished (Skoging et al. 1996; Zhao et al. 1994; Lee et al. 1996; Katherine E. Owen and Kuhn 1997). The cytoplasmic endodomain of E2 is also palmitoylated. The palmitoylation of SINV E2 occurs after the exit of p62/E1 dimers from the ER (Bonatti, Migliaccio, and Simons 1989). The palmitoylation occurs at cysteine residues and is important for a virus, as substitutions of these cysteine residues result in defects in the budding of virions (L Ivanova and Schlesinger 1993).

The A and B domains of E2 of CHIKV have been shown to be involved in the recognition of Mxra8, one of the cellular receptors for CHIKV. Mxra8 also plays a role as a receptor for other arthritogenic alphaviruses, such as MAYV, ONNV and RRV, but it seems not to be important for SINV. However, Mxra8 is not the only cellular receptor for CHIKV, as some infection occurs in the absence of Mxra8, albeit at reduced levels (Rong Zhang et al. 2018), indicating that the interaction with other cellular components can (at least partly) substitute for the interaction with Mxra8. These cellular components include, for example, heparan sulfate receptors, which have been implicated in interactions with the E2 protein (Tanaka et al. 2017). SINV can use C-type lectins as attachment receptors, and the interaction probably occurs with E2 or E1 (Klimstra et al. 2003). Phosphatidylserine receptors are also involved in the attachment of several alphaviruses (CHIKV, SINV, RRV, and EEEV) (Jemielity et al. 2013). Several cellular proteins have been shown to be used as receptors for different alphaviruses; for example, the natural resistance-associated macrophage protein acts as the main receptor for SINV (Rose et al. 2011). The laminin receptor has also been proposed to interact with the E2 protein and act as a receptor for SINV (K. S. Wang et al. 1992) and VEEV (Malygin et al. 2009). Another protein, prohibitin 1, has been identified as a potential receptor for CHIKV (Wintachai et al. 2012).

Receptor binding triggers conformational changes in the alphaviral spike. Prior to the receptor binding, E2-E1 trimeric spikes have E2 in the center of the spike, while E1 is located in the spike periphery (Voss et al. 2010). In its fusion form, E1 is, however, a homotrimer. Both receptor binding by E2 and acidic pH in the endosome after endocytosis of virions are proposed to act as triggers for the dissociation of E2-E1 dimers (Voss et al. 2010; L. Li et al. 2010). According to one molecular docking study, heparan sulfate binds to a positively charged pocket on the E2-E1 trimer. Upon receptor binding, the C domain of E2 moves toward E1 and pushes E1 away from the spike. The B domain of E2 moves away from the A domain and reveals a previously hidden E1 fusion loop (Sahoo and Chowdary 2019).

**6K**. 6K is a small glycoprotein with a molecular mass of 6 kDa (which gives it its name). It is cotranslationally translocated into the ER lumen (Liljeström and Garoff 1991). Cellular signal peptidase cleaves 6K free from E2/p62. In 2008,

Firth *et al*. discovered that the 6K protein had two different variants, 6K and transframe (TF), the latter being produced as a result of a -1 frameshift during the translation of the protein. The frameshifting occurs with a ~10–18% frequency in the UUUUUUA motif in SG RNA. Thus, both proteins share an identical N-terminus but have different C-terminal sequences. SFV virions were analyzed for the presence of 6K and TF and were found to primarily contain TF and not the 6K protein (Firth et al. 2008). After synthesis, 6K is associated with p62/E1 complexes in the ER and is transported to the plasma membrane along with the complexes (Lusa, Garoff, and Liljeström 1991). 6K seems to interact with E2, as 6K mutations causing a budding defect can be compensated by mutations in the E2 ectodomain (Lidia Ivanova, Lustig, and Schlesinger 1995). Additionally, chimeric SINV harboring 6K of RRV is severely defective in virion formation (compared with wt SINV), again implying that interactions between 6K and alphavirus glycoproteins are necessary for proper virion production (Yao, Strauss, and Strauss 1996).

6K has two transmembrane domains. The first transmembrane domain of 6K is involved in ion-channel activity (i.e., functions as a viroporin), and the second transmembrane domain is the signal sequence for E1 (Liljeström and Garoff 1991). TF only has the first of these transmembrane domains and probably also functions as a viroporin (J. E. Snyder et al. 2013). The cation channel formed by 6K/TF probably functions in the ER, Golgi complex or plasma membrane. The 6K ion channels of RRV and BFV are more permeable to monovalent than to divalent cations. The permeability sequence of the 6K viroporin is $Na^+ > K^+ > Ca^{2+}$. In the Melton *et al*. study, the holding potential necessary to activate 6K ion-channel activity was similar to the membrane potential of the cells; therefore, the ion-channel activity of 6K/TF that reaches the plasma membrane would also be activated (Melton et al. 2002).

**TF**. The TF protein is also palmitoylated, and its palmitoylation is important for successful alphavirus infection. If cysteine residues in the palmitoylation sites of SINV TF are mutated, the resulting virus produces virions at lower yields. Furthermore, such virions are often morphologically changed and contain multiple cores, unlike wt virions (Gaedigk-Nitschko et al. 1990). Ramsey *et al*. showed that TF was palmitoylated at its N-terminal cysteine residues, located upstream of the residues encoded by the frameshift site. The same cysteines are also present in 6K but are not palmitoylated; this suggests that the signal for the palmitoylation of TF might be located in its C-terminus. The results also demonstrated that TF localization to the plasma membrane was dependent on the palmitoylation state of these cysteines. Localization to the plasma membrane would easily enable TF to participate in the budding process. Analysis of the protein composition of virions showed the presence of TF but not 6K (Ramsey et al. 2017).

If the region encoding 6K is deleted from the SFV genome, the virus production is reduced, although the produced virus particles seem to be similar to wt particles (Liljeström et al. 1991; Loewy et al. 1995). A SINV mutant with a deletion in 6K that also includes the later-discovered TF frameshift site (therefore, the mutant is also lacking TF in addition to lacking a proper 6K) shows

defects in glycoprotein processing and virus budding, indicating that 6K plays a role in the budding process (Sanz and Carrasco 2001). In the Sanz and Carrasco experiments, 6K provided in *trans* did not rescue the deficient phenotype, indicating that TF might also play a role in the affected processes. In a different study, mutations resulting in a deletion or mutations in SINV TF caused a reduced viral yield and lower mortality and morbidity in infected mice (J. E. Snyder et al. 2013). Taken together, it is assumed that 6K has a function in viral glycoprotein processing and that both 6K and TF probably function in the late stages of virus assembly and budding. However, these proteins are not vital for the virus either *in vitro* or *in vivo*, although their presence affects the phenotype of the virus.

**E1**. E1 is the C-terminal part of the main structural polyprotein. If a –1 frameshift occurs during 6K translation and TF is produced instead, no E1 protein, which otherwise follows 6K in the structural polyprotein, is synthesized. E1 is the viral fusion protein; it facilitates fusion of the viral and endosomal membranes after virus entry into the cell. Fusion is triggered by a low pH in the endosome (Margaret Kielian 1995). The E1 structure is made up of three β-barrels (domains I, II and III); the fusion loop lies at the tip of domain II, which is the most distant from the viral membrane. E1 also has a transmembrane domain that connects to domain III through a stem region.

Similar to E2, E1 is the major building block of the outer protein shell of virus particles (Lescar et al. 2001; Voss et al. 2010). Eighty E1/E2 trimer spikes interact with each other to form the protein shell of virions, and this shell covers nearly all of the viral membrane. The interactions between the spikes are facilitated only by the E1 protein. In the prefusion form of the spike protein, the B domain of the p62/E2 protein covers the fusion loop of E1, thus preventing premature fusion during glycoprotein transport, oligomerization and virion assembly. Only following budding, do mature E2/E1 heterodimers acquire the fusion-competent conformation (Roussel et al. 2006; Lescar et al. 2001; Voss et al. 2010). An SFV mutant in which the furin cleavage site in p62 is mutated (and, therefore, spikes consist of p62/E1 heterodimers) is basically noninfectious because the p62/E1 heterodimers are much less sensitive to low pH-induced dimer dissociation (Salminen et al. 1992). This contrasts with natural virions, in which during the fusion process, the viral spike structure changes, E2/E1 heterodimers dissociate (Wahlberg and Garoff 1992), and E1 homotrimers are formed (Wahlberg et al. 1992). E1 proteins rotate around their long axes, E2 molecules change the position from the inside of the spike to the outside of the E1 trimer, and E2/E1 heterodimers dissociate (Mukhopadhyay et al. 2006). After E2/E1 dimer dissociation, the E1 fusion loop is exposed and can be inserted into the target membrane. Further conformational changes in the E1 homotrimer facilitate membrane fusion. In the prefusion form, the E1 long axes are oriented tangentially to the viral membrane, but in E1 homotrimers, E1 is elongated and oriented perpendicularly to the membrane. In homotrimers, E1 proteins make contact with each other through domains I and II. Domain III moves toward the fusion loop, thus creating a hairpin structure. This movement forces the viral membrane toward the target membrane. Neighboring E1 trimers make contacts by fusion loops. Fusion loops

interact with lipid heads in the target membrane and induce its deformation toward the viral membrane; as a result of this process, the two membranes eventually merge. Fusion probably requires the involvement of several (likely five or six) E1 trimers (Gibbons et al. 2004; 2003). Alphaviruses also require the presence of specific lipids in the target membranes for fusion to occur. Depleting cell membranes of cholesterol inhibits the viral fusion process, while the presence of cholesterol and sphingolipids promotes fusion and infection (M. Kielian et al. 2000; Wilschut et al. 1995; Ahn, Gibbons, and Kielian 2002). Membrane fusion results in the release of viral nucleocapsids into the cytoplasm (Sánchez-San Martín, Sosa, and Kielian 2008; H. Garoff et al. 1994). E1 also creates ion-permeable pores in the cell membranes. These pores are permeable to $Na^+$, $K^+$ and $Ca^{2+}$ ions and enable endosomal protons to flow into the cytoplasm, thereby facilitating the unpacking of viral nucleocapsids in this region of the cell (Wengler et al. 2003).

## 2.5. Inhibitors of CHIKV infection

CHIKV caused massive epidemics in the first decades of the 21st century. It continues to cause outbreaks in Africa, Asia, southern Europe and the Americas, making it currently the most medically important pathogen among alphaviruses. There is no vaccine to prevent CHIKV infection, a situation that will likely change in the near future, as on August 18, 2022, Valneva SE filed an application to the FDA for the approval of the CHIKV vaccine VLA1553. VLA1553 is a single-shot vaccine based on live attenuated CHIKV; it has passed all phases of clinical trials and was found to be safe, well tolerated and effective. The vaccine itself represents CHIKV harboring a deletion in the HVD of nsP3, and it was originally designed, constructed and tested by a consortium of European institutions, including the University of Tartu (Hallengärd, Kakoulidou, et al. 2014; Hallengärd, Lum, et al. 2014; Roques et al. 2017). Various other vaccine candidates are at different stages of clinical trials. However, vaccines alone cannot solve problems associated with CHIKV and other alphavirus infections; their outbreaks are unpredictable and often extremely massive, with many people becoming infected in a short period of time. Therefore, vaccines should be complemented by antivirals, ideally suitable for the treatment of infections caused by different alphaviruses.

At the moment, there are no approved drugs to treat CHIKV or other alphavirus infections. Treatment of CHIKV disease consists of alleviating the symptoms, mainly with painkillers and nonsteroidal anti-inflammatory drugs (Kennedy Amaral Pereira and Schoen 2017). There has been much research into potential inhibitors of alphavirus replication (reviewed in (Battisti, Urban, and Langer 2021; Hucke and Bugert 2020)), and this direction remains active. In principle, all stages of the viral replication cycle could be targets for antiviral drugs, beginning from virus attachment to cell receptors, virus entry, all stages of virus RNA replication and translation, virion formation and ending with budding of new virions. Both virus-encoded proteins and the host factors involved in these

processes can be targeted; compounds targeting virus-encoded components are termed directly acting antivirals, and compounds targeting host components and processes are termed host-targeting antivirals. Many different methods have been used to identify anti-CHIKV compounds, from rational drug design to *in vivo* studies. Compounds with potential anti-alphavirus activity have been identified using virtual screening (*in silico*) as well as high-throughput screening. Several CHIKV-inhibiting compounds have been described in the literature. The mechanism of action of directly acting antivirals is more or less clear, while it is less understood for host-targeting compounds. The efficacy of the obtained compounds has been evaluated using computational methods, cell-free experiments using recombinant alphavirus enzymes, various cell-based experiments, and, albeit in relatively few cases, animal experiments to determine the efficacy of potential inhibitors at the organism level. This has resulted in a list of diverse compounds, some of which are described below. The existing progress does not, however, mean that CHIKV antivirals will become available any time soon. Most existing CHIKV inhibitors are still at early stages of development and, in general, tend to have rather low efficiencies and poorly studied side effects, and more often than not, their activity in infected organisms (i.e., *in vivo*) is not known. Below, I have summarized the data published on such inhibitors, with an emphasis on compounds known or suspected to act as inhibitors of the protease activity of nsP2.

Arbidol and suramin are compounds that inhibit early stages of CHIKV infection, interfering with virus attachment and entry. The anti-influenza drug amantadine also has some anti-CHIKV activity, being detrimental to the ion-channel activity of 6K. 5-iodotubercidine inhibits the MTase activity of nsP1. Virtual screening and molecular docking have identified several potential hits against nsP3 (NCI-61610 and NCI-25457 were among the top five hit compounds), but the ability of these compounds to inhibit CHIKV infection *in vitro* or *in vivo* has yet to be demonstrated (Nguyen, Yu, and Keller 2014). nsP4-inhibiting compounds are primarily nucleoside analogs, such as the anti-influenza drug favipiravir and the anti-hepatitis C virus (HCV) drug sofosbuvir. Picolinic acid interferes with the interaction between the viral CP and E2 glycoprotein and with the budding of new virions (reviewed in (Battisti, Urban, and Langer 2021)). CHIKV replicon cell lines developed in our laboratory were used by researchers from Emory University (USA) to screen a library of nucleoside analogs that inhibit CHIKV replication. Among nearly 1000 compounds, β-d-$N^4$-hydroxycytidine (NHC) was identified as the most potent inhibitor (Ehteshami et al. 2017). As a nucleoside analog, NHC targets nsP4 and RdRp of CHIKV. However, NHC is not a chain terminator; instead, it is a hypermutagenic drug, making the virus unable to produce accurate enough copies of its genome. NHC has a high activity against CHIKV and other alphaviruses and thus has clear potential to become a clinically used alphavirus inhibitor. In some twist of fate, it has already made to the clinic but for the treatment of infection by another virus; NHC is the active part of molnupiravir, a drug licensed for the treatment of COVID-19.

Potential antivirals targeting host factors include viral entry and fusion inhibitors (such as chloroquine), inhibitors of lipid biosynthesis pathways (such as the fatty acid synthase inhibitor orlistat), inhibitors of pyrimidine and purine syntheses (such as ribavirin, which inhibits inosine monophosphate dehydrogenase, an enzyme needed for *de novo* synthesis of guanosine), inhibitors of protein synthesis (such as harringtonine, which inhibits eukaryotic translation of mRNAs), inhibitors of cellular enzymes (such as the furin inhibitor decanoyl-RVKR-chloromethyl ketone, blocking p62 cleavage), inhibitors of cellular receptors (such as digoxin, a sodium-potassium ATPase inhibitor that interferes with the virus's ability to affect the ionic environment in the cell), immunomodulatory agents (such as tilorone, which helps to induce interferon, needed for the cellular antiviral response) and some compounds with unknown targets (such as quinolones, which have been suggested to affect early stages of infection, but their precise mode of action is unknown) (reviewed in (Battisti, Urban, and Langer 2021)). As these compounds target cellular proteins, viruses have difficulty developing resistance against them. On the other hand, universal downsides of host-targeting antivirals are the possibility of adverse side effects due to interference with necessary cellular mechanisms and their relatively low potency against viruses. Perhaps the best-known example of this kind of inhibitors is ivermectin, an antiparasitic drug that has been falsely claimed (but still widely used) as an inhibitor of SARS-CoV-2 replication. Interestingly, ivermectin is also effective against CHIKV, inhibiting CHIKV replicons and *trans*-replicase with an effective concentration 50 ($EC_{50}$) of ~0.5 μM (Varghese, Kaukinen, et al. 2016), i.e., at a considerably lower concentration than is required to inhibit SARS-CoV-2 replication ($EC_{50}$ of 1.9 μM in Vero E6 cells and no detectable inhibition of SARS-CoV-2 replication in human airway-derived cells) (Dinesh Kumar et al. 2022). However, the concentration is still considerably higher than those used for its intended purpose (to treat parasitic infections). According to Baraka *et al.,* after administering an ivermectin dose of 150 μg/kg, the maximum plasma concentration of the drug is 52 ng/ml (Baraka et al. 1996), which is equivalent to 0.059 μM. Therefore, ivermectin cannot be considered a valid drug to treat alphavirus (or SARS-CoV-2) infections.

Studies performed by our research group have also contributed to the list of host-targeting antivirals. We found obatoclax to be a highly effective inhibitor of alphaviruses; its mechanism of action is based on the prevention of acidification of endosomes and inhibition of fusion of viral and cellular membranes (Varghese et al. 2017). Posaconazole was found to inhibit multiple steps in alphavirus infection (Varghese et al. 2022), and the alkaloid berberine acts by reducing CHIKV-induced mitogen-activated protein kinase signaling (Varghese, Thaa, et al. 2016). Tomatidine reduces the formation of the CHIKV infectious progeny by altering virus protein synthesis (Troost-Kind et al. 2021); the same was observed for bortezomib, an inhibitor of cellular proteasomes (Kaur et al. 2020). Synthetic and natural compounds were found to block virus entry or possess a virucidal effect (Santos et al. 2021; de Oliveira et al. 2020), and silymarin was found to affect virus protein synthesis and virion formation/release (Lani et al. 2015).

The majority of known directly acting anti-CHIKV compounds target the protease activity of nsP2. There are several reasons for this. In general, protease inhibitors are the second most abundant group (after polymerase inhibitors) of antiviral compounds and are used to treat human immunodeficiency virus (HIV), HCV and SARS-CoV-2 infections. Furthermore, the structure of the alphavirus nsP2 protease was available in 2006, being the first among alphavirus ns proteins. This allowed the application of *in silico* design to obtain a list of compounds that should target the protease activity of nsP2. Several studies found potential nsP2 inhibitors using *in silico* methods, but the first study to evaluate the efficiency of identified compounds was performed by Bassetto *et al.* (Bassetto et al. 2013). The best identified hit compound (1) in their study had an $EC_{50}$ of ~5 μM. Using target-based modeling, Das *et al*. chose 12 compounds and identified several novel nsP2 inhibitors by analyzing these compounds first in cell-free nsP2 protease inhibition assays and then evaluating their effects on CHIKV replication in cell culture. The most potent inhibitor found in this study was compound 8, with an $EC_{50}$ of ~1.5 μM. Surprisingly, in Das *et al*. experiments, compound B1 (same as compound 1 in Bassetto's study), which was used as a base for predictions of their new compounds, did not inhibit the protease activity of nsP2; therefore, it was concluded that B1 must have affected some other function of nsP2 (Das et al. 2016). Our research group has also participated in subsequent projects that used molecular modeling methods to further improve compounds found in the Das *et al*. (2016) study and to obtain novel and more potent CHIKV nsP2 inhibitors (Larisa Ivanova, Rausalu, Ošeka, et al. 2021; Larisa Ivanova, Rausalu, Žusinaite, et al. 2021).

Singh *et al*. identified two small peptidomimetic compounds (PEP-I and PEP-II) using a fluorescence resonance energy transfer (FRET)-based assay. PEP-I and PEP-II inhibited nsP2 protease activity with inhibitory concentrations 50 ($IC_{50}$) of ~34 μM and ~42 μM, respectively (Singh et al. 2018). Another study identified five derivatives of 1,3-thiazolidin-4-one as inhibitors of CHIKV ($EC_{50}$ values between 0.42 and 40.1 μM), and based on molecular docking simulation, it was suggested that these compounds inhibited the nsP2 protease (Jadav et al. 2015). Drug repurposing is one of the ways to reduce the time and cost for the development of new treatments, as the analysis of safety has already been performed for drugs in clinical use. The downside is that the efficacy (at least for monotherapy) is generally low. Nevertheless, the search for new purposes for approved drugs is widely used, including for the inhibition of alphavirus infections. Tripathi *et al*. screened a library of ~3000 FDA-approved drugs for anti-CHIKV activity and identified novobiocin and telmisartan as CHIKV inhibitors. In *in vitro* protease assays, these compounds had $IC_{50}$ values of ~2 μM and ~5 μM, and in *in vitro* cell culture assays, the $EC_{50}$ values were ~20 μM and ~45 μM, respectively (Tripathi et al. 2020). Similarly, Bhakat *et al.* chose a panel of FDA-approved HIV/HCV inhibitors and studied their efficacies against CHIKV. They found that nelfinavir had an $EC_{50}$ of ~14 μM against CHIKV (Bhakat et al. 2015).

Protease activity is not the only function of nsP2 that can be targeted by antiviral compounds. To date, no inhibitor targeting the NTPase/RNA helicase activities of the enzyme has been developed, possibly because the structure of this part of nsP2 was only resolved a few years ago. nsP2 also has crucial nonenzymatic activities; as reviewed above, nsP2 of CHIKV is a viral protein that induces the shutoff of cellular transcription. Lucas-Hourani *et al*. developed a phenotypic screening assay to identify compounds that target the nsP2-mediated cellular shutoff and found a natural compound, ID1452-2, that had a moderate effect on CHIKV replication ($EC_{50}$ of 31 μM) (Lucas-Hourani et al. 2013). Thus, the availability of the structure of the nsP2 helicase part, the structure of nsP2 inside the replicase core of the RNA replicase and an increasing understanding of the nonenzymatic activities of nsP2 that are crucial for alphavirus infection provide a basis for the development of new types of nsP2 inhibitors.

# 3. AIMS OF THE STUDY

CHIKV remains a medically important pathogen against which there is no approved vaccine to prevent the infection nor any antiviral to cure/treat infected people. While the approval of vaccines for CHIKV is on the horizon, the development of antivirals is at early stages. CHIKV antivirals can be developed against host components required for CHIKV infection or against CHIKV-encoded proteins, especially nonstructural proteins that are essential for viral RNA replication. Of these proteins, nsP2 has the largest number of known enzymatic activities, making it both the best studied and the most important component of the alphavirus replication machinery. Furthermore, nsP2 is not only an integral part of the alphavirus replicase complex but also plays a major role in its formation; it is nsP2 that cleaves the CHIKV nonstructural polyprotein into individual nsPs, a step that is absolutely essential for successful replication of the virus. If nsP2 is unable to carry out its protease function, CHIKV is also unable to replicate and eventually produce new virions. Even partial inhibition of the protease activity of nsP2 will likely have a devastating impact on virus infection, altering its replication efficiency and resulting in excessive synthesis of agonists of the type-I interferon system. A better understanding of the functions of CHIKV nsP2 would therefore promote the discovery of ways to interfere with CHIKV infection; multiple crucial functions of nsP2 in virus infection will likely limit the ability of the virus to develop resistance against such treatments. Thus, nsP2 is a tempting target for the development of novel antivirals against CHIKV and/or other pathogenic alphaviruses.

The objectives of this thesis research were as follows:

I. To confirm that CHIKV nsP2 is a cysteine protease. Earlier literature indicates that nsP2 of the alphavirus is a cysteine protease (E. G. Strauss et al. 1992; A. Russo, White, and Watowich 2006), but this view has been challenged in a more recent publication claiming that nsP2 of CHIKV could be a serine protease (Saisawang, Saitornuang, et al. 2015).

II. To study the importance of the timing and speed of ns polyprotein processing for the success of alphavirus infection.

III. To participate in the development of efficient CHIKV inhibitors that block the protease activity of nsP2.

# 4. MATERIALS AND METHODS

The methods used in this study have been described in the Materials and Methods sections of publications **I**, **II** and **III** as well as in several other publications from our lab (Utt et al. 2015; 2016; Das, Merits, and Lulla 2014; A. Lulla et al. 2006; V. Lulla, Karo-Astover, et al. 2013). Therefore, only the principles of the main approaches as well as the reasons why these methods were selected are outlined and illustrated below. These key approaches were the use of highly active purified recombinant nsP2 of CHIKV, carefully controlled experiments with wt and mutant virus genomes and corresponding viruses, and highly sensitive *trans*-replication assays.

**Expression, purification and use of recombinant nsP2 of CHIKV.** Alphavirus nsP2 is seemingly easy to produce – if to do this in the wrong way. The mistakes made in nsP2 production have repeatedly resulted in doubtful data about the properties of nsP2 in transfected cells (Boussier et al. 2020; Rana et al. 2014). In the case of purified recombinant proteins, these mistakes have resulted in data that are not only doubtful but have been shown to be outright wrong (Saisawang, Saitornuang, et al. 2015). Many of these troubles originate from the failure of researchers to recognize that for many functions of nsP2, an authentic N-terminal region and a native N-terminal aa residue are strictly needed. No argument like "just a small tag that unlikely affects properties of the enzyme" or "for simplicity, the start codon in a strong context was added" is valid; nsP2 has literally zero tolerance for any alterations in the N-terminal region and does not tolerate the addition of even a single extra N-terminal aa residue, including methionine originating from an artificially added initiation codon. Predictably, all attempts to work with a flawed protein can only result in flawed data or experimental artifacts. Even worse, some functions of nsP2 are not (seriously) affected by modifications at the N-terminus, providing researchers false confidence that they are using acceptable tools. To avoid such mistakes, a significant effort was dedicated to obtaining nsP2 in its precise and completely functional form.

The recombinant CHIKV nsP2 and all of its mutant forms used in the studies included in the thesis were produced in *E. coli;* to eliminate premature termination of translation due to rare codons, the sequence encoding nsP2 was codon optimized for expression. To obtain a protein with an authentic N-terminus, the expression construct was designed to include a sequence encoding the peptide corresponding to the C-terminus of nsP1, i.e., the expressed recombinant protein started with the 1/2 cleavage site. This design allows nsP2 to process its own N-terminus, resulting in an authentic N-terminal glycine residue and, also likely, in a correct conformation that the protein acquires during its release (also via cleavage at its termini) from P1234 in virus-infected cells. Originally, we also used a hexa-histidine tag added to the C-terminus of nsP2, but in the final constructs, it was omitted because we observed that the recombinant nsP2 had a strong natural affinity to Ni-TED resin. Expression was performed at a low temperature using an autoinduction protocol. The obtained recombinant protein

was purified to homogeneity using a three-step protocol: Ni-affinity, cation exchange and finally, size exclusion chromatography. The purified proteins were analyzed by mass spectrometry to verify the authenticity of the N-terminal peptide generated by the protease activity of the recombinant protein and the authenticity of the inserted mutations. Circular dichroism (CD) spectroscopy was used to analyze the intactness of the protein secondary structure. The nsP2 purification and analysis scheme is outlined in Figure 9. Only protein samples that passed these quality controls were used in protease assays.

Another frequent flaw found in studies dedicated to the analysis of the protease activity of nsP2 and in studies of inhibitors of the protease activity of nsP2 is the use of an incorrect substrate. nsP2 is intrinsically unable to cleave short peptide substrates corresponding to the 2/3 site. Instead, a rather large recombinant protein of 180–190 aa residues (10–170' or 20–170' substrate) is needed to represent the 2/3 site (A. Lulla, Lulla, and Merits 2012; Utt et al. 2015). Thus, any study using a short peptide corresponding to the 2/3 site as a substrate for nsP2 cannot produce anything, except experimental artifacts. Shorter peptide substrates representing the 1/2 or 3/4 site can be cleaved by nsP2, but even then, an important restriction applies: the substrates should not be too short. The cleavage of such peptides is efficient if they include at least 10 residues from the P-side and several from the P'-side of the scissile bond (A. Lulla et al. 2006). Using shorter peptides is tempting because they are cheaper and easy to obtain at high quality, and peptides of similar length do work well for proteases encoded by some other RNA viruses.

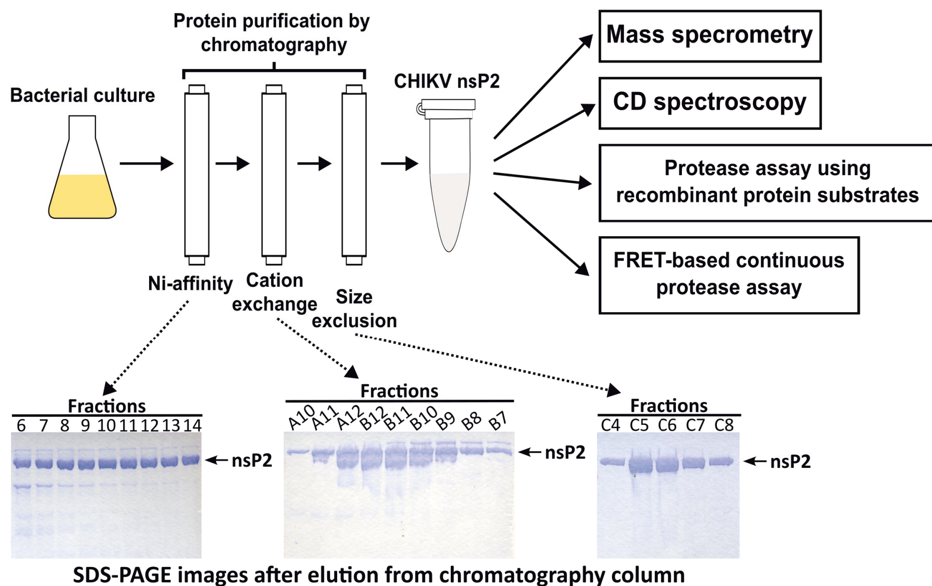


**Figure 9.** Scheme of purification of recombinant nsP2 of CHIKV and a scheme of analysis used in publication **I**. Examples of images of Coomassie-stained SDS-PAGE gels after each purification step are also shown. CD – circular dichroism, FRET – fluorescence resonance energy transfer, SDS-PAGE – SDS-polyacrylamide gel electrophoresis.

For alphaviruses, the use of such substrates is less than ideal; it does not result in false data, but it does not allow revealing the full activity of nsP2 (resulting in a drastic underestimation of different parameters of the enzyme). Herein, two types of substrates, each designed considering all these limitations, were used to analyze the protease activity of the purified recombinant nsP2. The first assay was based on the use of recombinant proteins comprising different nsP2 cleavage sites as substrates for nsP2; the cleavage products were detected using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The second assay was based on continuous measurement of fluorescence. The substrate for this assay was a peptide containing a 3/4 cleavage site (in an optimal substrate, it corresponded to residues P10 to P'5, i.e., the substrate had a length of 15 aa residues) between 4-[[4-(dimethylamino)phenyl]-azo]benzoic acid (DABCYL, a quencher) and EDANS (fluorophore) at the amino and carboxy termini, respectively. These methods are described in greater detail in publication **I**.

**Construction, rescue and analysis of recombinant viruses.** The constructs used for the rescue of recombinant viruses were obtained using previously constructed and functionally verified infectious cDNA (icDNA) clones of CHIKV, SFV and SINV and standard molecular cloning methods, including polymerase chain reaction (PCR)-based mutagenesis and DNA restriction and ligation. icDNAs were transcribed *in vitro,* and the obtained capped transcripts were used to transfect BHK-21 cells to obtain mutant viruses or to verify the impact of the introduced mutations. Importantly, RNA infectivity was always determined using an infectious center assay (ICA) (Figure 10A). This is a crucial step of analysis, omission of which can result is serious mistakes. The reason for this is simple: RNA viruses harboring unfavorable (but not lethal) mutations do revert, pseudo-revert or acquire compensatory changes. In addition, the replication of a virus is rapid, and the infection does spread from transfected cells to cells in which infection was not originally initiated. Consequently, harvesting virus at the time of CPE development does not provide information on how the infection started in transfected cell cultures, in a large number of cells (as is the case for cells transfected with transcripts obtained using wt icDNA) or in single/few cells (as is often the case for genomes harboring mutations that reduce RNA replication). The ICA reveals these differences and allows us to distinguish between genomes with wt-like infectivity and those that require reversions, pseudoreversions or compensatory changes to produce an infectious progeny. This information is crucial for understanding the true impact of the introduced mutations and for planning subsequent experiments.

Secondary virus stocks were created by infecting cells at a low to moderate multiplicity of infection (MOI) and collecting the resultant virus stocks. Viral titers were determined by the plaque assay. Plaque purification and sequencing of genomes of rescued viruses were performed if the ICA results indicated that viruses may have acquired reversions, pseudoreversions and/or second-site compensatory mutations during the infection process. Viral RNAs were purified from stocks of plaque-purified viruses and reverse transcribed into complementary DNAs (cDNAs), which were amplified by PCR and sequenced (Figure 10B).

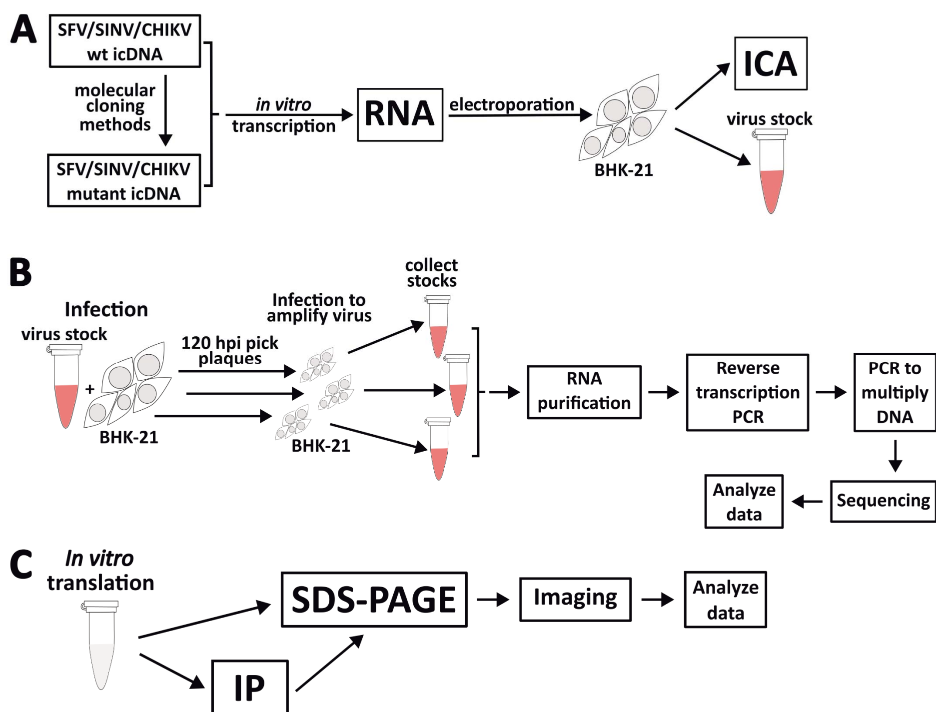


**Figure 10.** Experimental schemes used in publications **I** and **II**. A) Scheme for the determination of RNA infectivity and production of virus stocks. B) Scheme for the plaque purification assay. C) Scheme for the *in vitro* translation and immunoprecipitation assay. ICA – infectious center assay, PCR – polymerase chain reaction, SDS-PAGE – SDS-polyacrylamide gel electrophoresis, IP – immunoprecipitation, icDNA – infectious complementary DNA, hpi – hours post-infection.

The use of plaque-purified viruses was needed because in general, alphaviruses have many options to compensate for introduced nonlethal defects. Therefore, the rescued virus stock typically consists of a pool of viruses, each having one or more compensatory changes; however, often none of these viruses is dominant. Thus, Sanger sequencing of the cDNA corresponding to such a pool of viruses will show no difference from the original sequence. Certainly, the use of next-generation sequencing (NGS) could reveal compensatory changes in positions where two or more types of sequence variants coexisted and would show some degree of polymorphism. However, the intensity of the “second signal” is often low, and it is difficult to distinguish between adaptive changes and the natural polymorphism, which is characteristic for all RNA viruses. Furthermore, at the time when these experiments were performed, the only NGS system available to us was from Illumina, which generates rather short (at best, a few hundred nucleotides in length) reads that typically do not allow the detection of whether potential compensatory changes are linked to each other or are present in different genomes. Thus, the “old-fashioned” plaque purification and sequencing approach

was more reliable. It was inevitable that using this approach, some compensatory changes remained undetected, but on the other hand, the more limited data were easier to verify. In our case, we performed the verification of the functional impact of all mutations revealed in sequenced genomes (and there is a limit on how many different changes can be experimentally analyzed). Thus, our approach was sufficient to reveal a general picture, although there certainly was room for additional in-depth analysis.

The effects of different mutations introduced by us (and sometimes of those detected in rescued virus genomes) on the cleavage of the ns polyprotein was studied using *in vitro* translation and immunoprecipitation assays (Figure 10C). Again, there was a clear reason for the choice of this approach. The processing of the ns polyprotein can also be analyzed using virus-infected cells, and in many ways, this is biologically more relevant. However, for obvious reasons, such an analysis cannot be applied to viral RNAs that contain lethal mutations. Furthermore, it cannot be used for viral RNAs that undergo reversions, pseudoreversions or secondary changes in infected cells, as the data will not show the properties of originally constructed genomes. Hence, *in vitro* translation represented the best option because it does not require the viral genome to replicate and eliminates the impact of potential reversions, pseudoreversions or secondary changes (none of which occur in test-tube reactions).

***Trans*-replication assay.** *Trans*-replication assays enable the analysis of viral RNA replication and transcription outside of the context of the whole viral infection cycle. Using these assays enables the analysis of the effects of mutations that affect the functions of different nsPs or RNA sequences. For example, mutations in nsPs may potentially be lethal to the virus, and their impact on the virus RNA replicase is therefore impossible to study in the context of viral infection. The lack of need to use the whole virus also makes *trans*-replication assays safer to use because there is no risk of infection with a possibly pathogenic virus (e.g., CHIKV) to the researcher.

In the *trans*-replication assay, the expression of viral replicase proteins and viral RNA synthesis are uncoupled. For alphaviruses, this is achieved by the use of two different expression constructs. The first plasmid encodes all four viral nsPs (in the form of the P1234 precursor) that are needed for the formation of the viral replicase. The second construct is a plasmid that encodes an alphavirus minigenome containing reporter genes (e.g., firefly and *Gaussia* luciferases) under control of viral elements needed for the viral replicase to recognize the construct as its template and amplify/transcribe it with a high efficiency. Our lab has developed many versions of *trans*-replicases; the main differences are the promoters used for the transcription of plasmid constructs producing mRNA for replicase proteins and a minigenome (template RNA) and the mode of expression of replicase proteins (as P1234 or P123 and nsP4). The version of the *trans*-replication system using the bacteriophage T7 RNA promoter and the corresponding polymerase (Figure 11A) was used in my study, as this system has a high sensitivity and is easy to use in mammalian cells expressing T7 RNA polymerase.

Both replicase and template constructs contain all the elements needed for the propagation of the plasmid in bacteria (not shown) and for the expression of the gene of interest. For the latter the replicase plasmid contains the following elements: i) the promoter for bacteriophage T7 DNA-dependent RNA polymerase (DdRp); ii) the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) to allow translation from uncapped transcripts made by T7 DdRp; iii) the region encoding for the precursor of viral nonstructural proteins (nsP1-4), which are the components of the viral replicase; iv) an untranslated region, followed by 60 adenine residues (required because T7 DdRp does not polyadenylate transcripts); v) the antisense-strand ribozyme of hepatitis delta virus (HDV) to remove extra nucleotides from the 3' end of the transcript; and vi) a terminator for T7 RNA polymerase. Replication-negative control constructs are similar, with the only difference being the GDD → GAA mutation in the active site of the nsP4 RNA polymerase (inactivates the enzyme). The plasmid for the expression of the template RNA containing reporter genes consists of i) the T7 promoter in its minimal version to avoid extra nonviral residues at the 5' end of the transcript; ii) the viral 5' UTR; iii) a region encoding 77 N-terminal aa from viral nsP1 (in the case of CHIKV; the number of aa would be 74 for SFV and 66 for SINV), which, along with the 5' UTR, forms the part of the viral genomic promoter that drives replication of the RNA transcript; iv) a sequence encoding firefly luciferase (FFLuc) cloned in frame with a fragment of nsP1; v) the viral SG promoter, which drives the synthesis of SG RNAs used for the translation of the second marker present in the construct; vi) a sequence encoding for *Gaussia* luciferase (GLuc); vii) the viral 3' UTR, followed by 60 adenine residues, as another conserved sequence element necessary for the viral replicase to function; viii) the antisense-strand ribozyme of HDV; and ix) a T7 terminator.

The principle of the *trans*-replication assay is depicted in Figure 11B. BSR T7/5 is a cell line (derived from BHK-21 cells, which are one of the most efficient mammalian cells used for studies of alphavirus RNA replication) that constitutively expresses bacteriophage T7 DdRp (Buchholz, Finke, and Conzelmann 1999). T7 DdRp-encoding mRNA is produced in the cell nucleus by cellular RNA polymerase II, then transported into the cytoplasm and translated into the T7 DdRp protein. Thus, T7 DdRp is readily available in the cell cytoplasm. This makes the system relevant for alphavirus replication studies, as viral RNA synthesis also occurs in the cytoplasm. This may also have a positive impact on the efficiency, as in this system, *trans*-replicase plasmids do not need to enter the nucleus to be transcribed. Instead, T7 RNA polymerase present in the cytoplasm recognizes the T7 promoter in these plasmids when they are transfected into the cell and drives the production of replicase mRNA and template RNA. The EMCV IRES enables ribosomes to translate the replicase mRNA to the replicase polyprotein. In contrast, the template RNA produced by T7 DdRp is not translated, as it does not have a cap structure at its 5' end nor does it contain an IRES. Instead, the template RNA is recognized by the newly produced viral replicase, as its 5' and 3' sequences contain *cis*-sequences required for the alphavirus RNA replication.

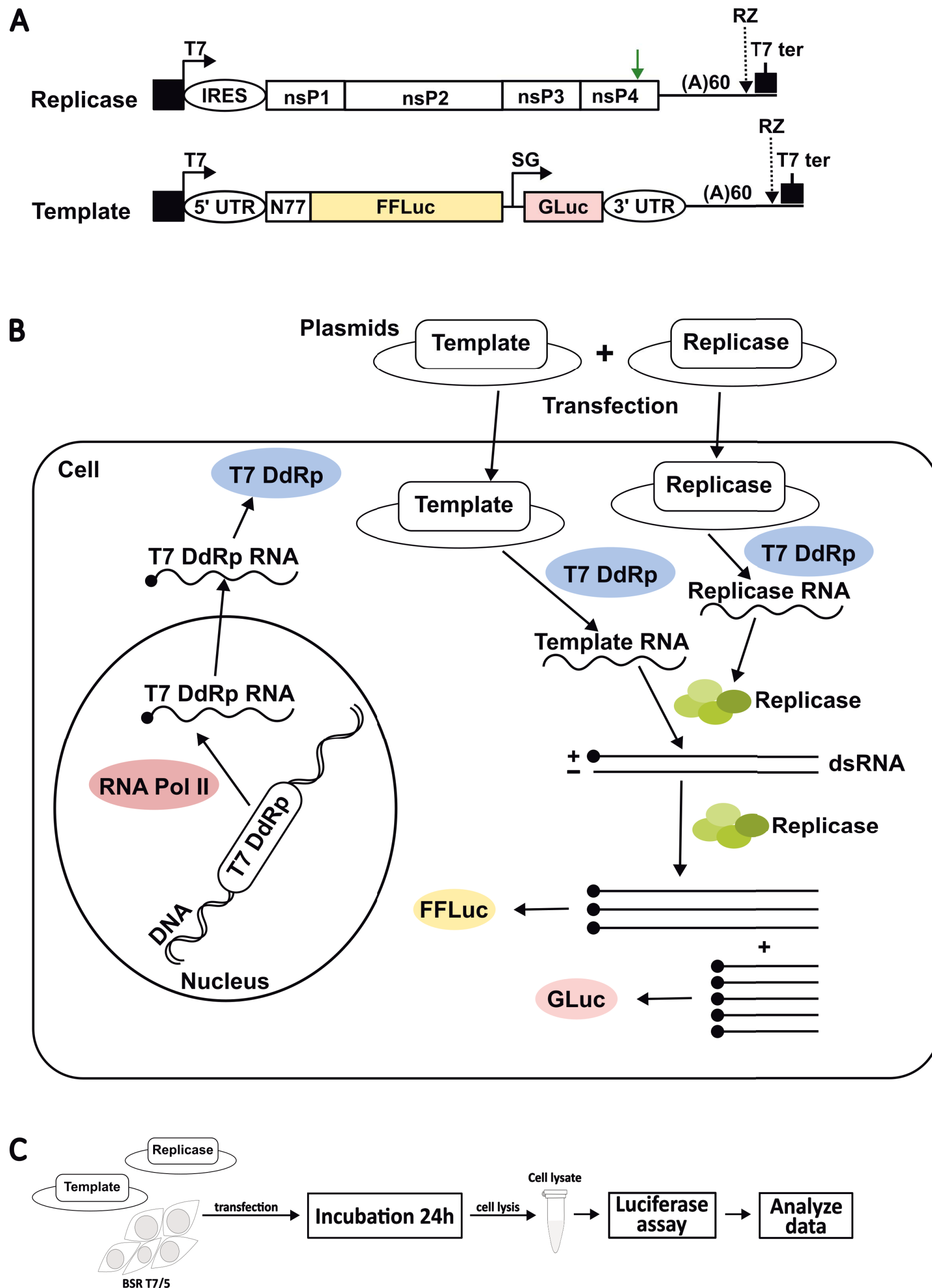


**Figure 11.** Alphavirus T7 *trans*-replication assay. A) Scheme of alphavirus T7 *trans*-replication assay constructs. T7 – T7 promoter, IRES – EMCV IRES, A(60) – 60 adenine residues, RZ – HDV ribozyme, T7 ter – T7 terminator. The green arrow indicates the location of the replicase-inactivating GAA mutation in the nsP4 RNA polymerase active site in the control constructs. N77 – sequence encoding 77 N-terminal aa of nsP1, FFLuc – firefly luciferase, SG – viral subgenomic promoter, GLuc – *Gaussia* luciferase. B) The principle of the *trans*-replication assay. dsRNA – double-stranded RNA. The black filled circle at the end of some RNAs represents the 5' cap structure. C) Scheme of the *trans*-replication assay.

The replicase produces a dsRNA, and then its negative strand is used as a template to produce capped mRNAs of the marker proteins. In the context of the *trans*-replication assay, the expression of the first marker (FFLuc) reflects viral replication (synthesis of analogs of new viral genomes), and the expression of the second marker (GLuc, under control of the viral SG promoter) reflects viral transcription (synthesis of mRNA for the viral structural polyprotein). The amount of the luciferase mRNA produced translates into the amounts of the luciferases, luminescence of which can be easily measured.

The experimental scheme of the *trans*-replication assay is depicted in Figure 11C. BSR T7/5 cells are transfected with the template RNA and replicase expression plasmids using the Lipofectamine 2000 reagent. At 24 h posttransfection (hpt), the cells are lysed in Passive Lysis Buffer (Promega), and FFLuc and GLuc activities are measured using the Dual-Luciferase Reporter Assay System (Promega). In data analysis, the luciferase activities are normalized against the negative control for which the activities are measured for cells treated exactly the same way, except using the polymerase-dead GAA mutant instead of the active replicase. This control provides data about the background levels of marker activities and allows the estimation of "boosts", i.e., an increase in reporter activities due to the presence of the functional replicase. In experiments in which the activities of wt and mutant replicases are compared, the activity of the latter can be expressed as a percentage of the activity of the wt replicase (considered 100%).

**Protease inhibition assay.** A protease inhibition assay was carried out similarly to the protease activity assays using a recombinant protein containing the 1/2 cleavage site as a substrate, recombinant nsP2 with verified quality as an enzyme and varying concentrations of different nsP2 inhibitors. The test-tube assay (Figure 12A) is the most certain way to confirm that the analyzed compounds indeed target the protease activity of nsP2. However, on its own, this assay does not provide information about the potential of the analyzed compound to be a practically applicable inhibitor of CHIKV. For example, the test-tube assay does not consider the potential toxicity of the compound and its ability/inability to enter cells. Furthermore, the test-tube assay may underestimate the potency of an inhibitor, as it is based on the use of relatively large amounts of the enzyme (compared with that synthesized in infected cells), measures inhibition of *trans*-cleavages (in alphavirus-infected cells, two cleavage sites in the ns polyprotein, 1/2 and 3/4 sites, are cleaved in *cis*) and does not take into account the possibility that blocking CHIKV infection does not necessarily require complete inhibition of the nsP2 protease activity; even a slight disturbance of the finely regulated processing pathway may reduce or even block virus infection. Therefore, we also analyzed the inhibitory potential of compounds in CHIKV-infected cells (Figure 12B, C). Two approaches were used. First, cells were infected with CHIKV-NanoLuc (virus containing a nanoluciferase gene under control of a duplicated SG promoter) at a very low MOI of 0.001. Under these conditions, virus infection in cell culture is a multistep process, including the maturation and release of virions in primarily infected cells and a second wave of infection of cells that originally

remained uninfected. The multicycle nature of infection makes this assay highly sensitive; the use of the reporter virus simplifies the analysis of the efficiency of virus infection (Figure 12C). In another setting, a high MOI was used to ensure synchronous infection of cells (Figure 12B). In this case, we performed western blot analysis of CHIKV-encoded proteins, as in general, their amounts correlate with the viral genome copy number, which in turn depends on the activity of the CHIKV replicase. Thus, this approach allows the detection of the impact of an inhibitor on the events occurring at early stages of infection when ns polyprotein synthesis, processing and RC formation take place. It should be mentioned that neither of these cell-based assays can directly confirm that virus infection was reduced because of the suppression of the protease activity of nsP2; it remains possible that compounds affect other CHIKV-encoded proteins or cellular protein(s)/ process(es) important for early stages of CHIKV infection. Only combining the results of cell-based assays with those from the cell-free protease activity inhibition assay provides a confirmation that the observed reduction in virus replication was most likely due to the inhibition of the protease activity of nsP2.

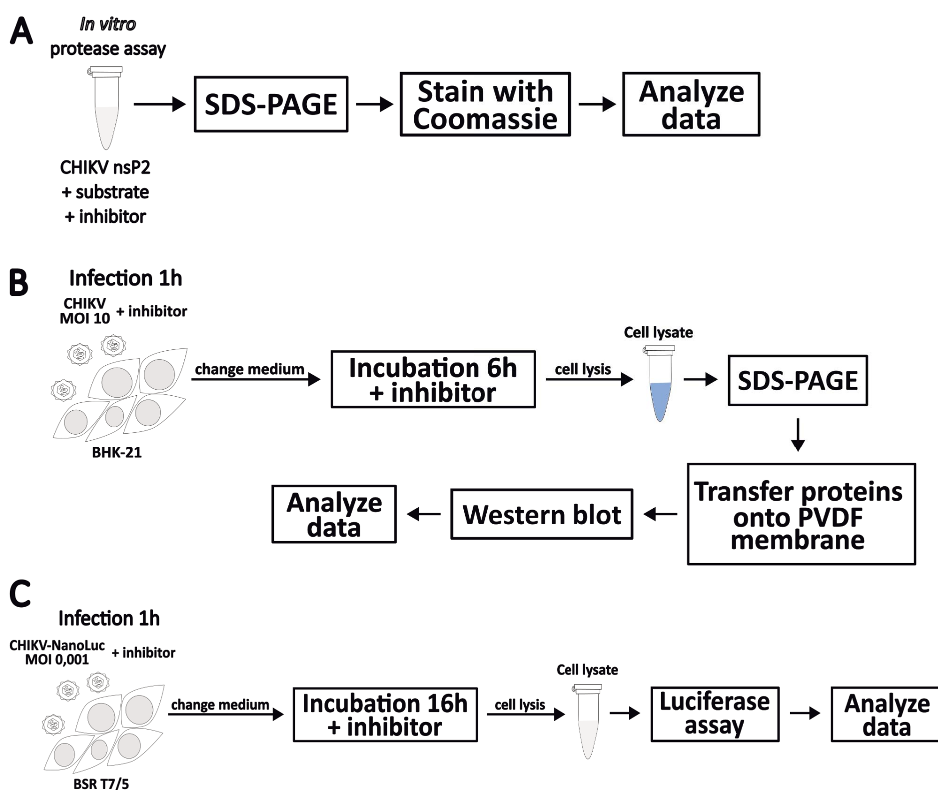


**Figure 12.** Experimental schemes from publication **III**. A) Scheme of the protease inhibition assay. B) Scheme of the virus inhibition assay used to analyze the amounts of viral proteins in infected cells. C) Scheme of the virus inhibition assay to analyze the efficiency of virus infection. SDS-PAGE – SDS-polyacrylamide gel electrophoresis, MOI – multiplicity of infection, PVDF – polyvinylidene fluoride.

# 5. RESULTS AND DISCUSSION

## 5.1. Analysis of the requirements for CHIKV nsP2 protease activity and the impact of mutations of protease active site residues on CHIKV infectivity, RNA synthesis and ns polyprotein processing (I)

Alphavirus nsP2 has historically been considered to be a papain-like protease. Earlier studies demonstrated the importance of cysteine residues in the catalytic site of nsP2 for several different alphaviruses, including SINV, SFV and VEEV (E. G. Strauss et al. 1992; A. Russo, White, and Watowich 2006; Merits et al. 2001; Golubtsov, Kääriäinen, and Caldentey 2006). In sharp contrast to these findings, one more recently published article claimed that the cysteine residue in the active site of the nsP2 protease of CHIKV was not strictly required for enzymatic activity, as its role can be carried out by a nearby serine residue (Saisawang, Saitornuang, et al. 2015), a claim based on computer modeling that seemed unlikely to reflect reality. Furthermore, the paper suffered from multiple technical flaws (see below); however, it did not directly contradict any published data. In particular, the alphavirus community has assumed that CHIKV nsP2 should have properties similar to those of nsP2s of other alphaviruses and therefore has not performed corresponding analysis. Or, they may have performed it only to confirm that the abovementioned assumption was correct and decided that the publication on protease activities of CHIKV nsP2 was therefore of little importance. This was indeed the case for our laboratory, where protease activities of nsP2 of CHIKV were analyzed several years ago, but nothing truly exciting/novel was found (Triin Lillesaar, 2009, MSc thesis). In hindsight, the reluctance of researchers (including our lab) to publish solid but not-too-exciting data opened the possibility for others to publish a paper that was exciting but deeply flawed. Based on our unpublished data, we immediately knew that the conclusions presented in this publication were wrong. As the flawed publication caused confusion in the field and could potentially have a negative impact on the development of protease inhibitors for CHIKV, we decided to “clear the field” and conclusively demonstrate the importance of the active site cysteine residue for nsP2 of CHIKV. However, it is much easier to publish flawed data (assuming an editor and reviewers do not spot mistakes) than to demonstrate that published data are incorrect. To this end, our preexisting unpublished data were not sufficient, and we decided to more thoroughly investigate the requirements for CHIKV nsP2 protease activity as well as its impact on CHIKV RNA replication and viability. Only a set of data from such experiments could, beyond any doubt, demonstrate the true importance of the protease active site cysteine and/or the nearby serine or tryptophan on nsP2 of CHIKV and on virus infection.

### 5.1.1. Requirements of CHIKV nsP2 for its protease activity

The protease activity of nsP2 is perhaps the most important enzymatic function revealed for alphaviral nsPs. This is the first and absolutely mandatory function that the alphavirus replicase precursor must have because without cleaving the ns polyprotein into P123 and nsP4, viral RNA replication cannot occur. More specifically, uncleaved P1234 is unable to produce negative-strand RNAs (Kallio et al. 2016). To activate this essential step, nsP4 must be present in free (individual) form. In an experimental system, this can be achieved by coexpression of P123 and nsP4 (Lello et al., 2021). In alphavirus-infected cells, however, the only option is the cleavage of P1234 by the nsP2 protease at the junction between nsP3 and nsP4, resulting in the release of nsP4 and the formation of the “early” (P123+nsP4) replicase that produces negative-strand RNA. Further processing of P123 into individual nsPs results in the formation of the “late” replicase that produces new positive-strand RNAs. This step is also important for alphavirus infection but is not strictly needed, as the replicase consisting of unprocessed P123 and nsP4 is, in principle, also capable of positive-strand RNA synthesis (Gorchakov et al. 2008; Lemm et al. 1994).

As highlighted in the Materials and Methods section, some researchers, especially those new to the alphavirus field, have a tendency to ignore crucial information regarding the nsP2 protease and its substrate requirements. One important piece of information is that a previous study by our group revealed that the nsP2 protease of SFV did not tolerate insertions or deletions at its N-terminus; even one or two missing (or extra) amino acids have serious adverse effects on its ability to cleave the junction between nsP2 and nsP3 (2/3 cleavage site). Equally important, SFV nsP2 also requires at least 165 amino acids from the nsP3 side in its substrate for the cleavage of the 2/3 site to occur (A. Lulla, Lulla, and Merits 2012). At the same time, substrates representing the 1/2 and 3/4 sites can be much shorter and can be processed by a truncated nsP2, including a recombinant protein corresponding to the protease region of nsP2. Although we had already had evidence that these rules also applied to nsP2 of CHIKV, we decided to demonstrate this in the most convincing manner.

First, the activity of the nsP2 protease of CHIKV was analyzed using recombinant protein substrates. These substrates consisted of GFP and thioredoxin (Trx), separated by sequences representing the 1/2, 2/3 or 3/4 cleavage site of CHIKV. Short substrates (GFP-10:5-Trx) were designed such that the linker sequence consisted of 10 and 5 amino acid residues derived from the P- and P’-sides of the cleavage sites. For the 2/3 cleavage site, a sequence representing the region from P10 to the P’170 residue was also used, resulting in a long substrate, GFP-10:170-Trx. GFP and Trx were used in these substrates because these recombinant proteins are large enough to be detected using SDS-PAGE. The benefit of this approach is simple, as one can directly visualize the substrate and its cleavage products. In contrast, peptide substrates and their cleavage products can only be detected using mass spectrometry or indirectly, for example, using a FRET-based approach. Certainly, both methods are valid and useful. However, there is a catch:

since these products are not visualized directly, there is a chance of mistaking a noise (always present in whatever assay) for a signal. With substrates and cleavage products detectable as bands in a Coomassie blue-stained gel, such a mistake is virtually impossible to make; there is either a cleavage or no cleavage and little-to-no room for doubts.

As expected (and similar to nsP2 of SFV), CHIKV nsP2 efficiently cleaved short GFP-10:5-Trx substrates containing 1/2 and 3/4 cleavage sites, while the cleavage of a short GFP-10:5-Trx substrate containing a 2/3 cleavage site could not be observed (**I**, Fig. 1A). However, similar to nsP2 of SFV, nsP2 of CHIKV was able to cleave a GFP-10:170-Trx (long) substrate containing a longer 2/3 cleavage site (**I**, Fig. 1A). Finally, CHIKV nsP2 did not tolerate deletions at its N-terminus; an enzyme missing just two N-terminal residues lost its ability to cleave the 2/3 long substrate, as has also been described for nsP2 of SFV. The only notable difference observed between nsP2s of SFV and CHIKV was that the latter tolerated the addition of two amino acids at its N-terminus (**I**, Fig. 1A), while the former did not. Whether this is due to differences in the protease or substrate remains unclear. However, the latter option is somewhat more likely, as the 2/3 site of CHIKV has a favorable P4 Arg residue (the 2/3 site of SFV has a P4 Thr residue), which may make the site cleavable by an enzyme containing some imperfections. Altogether, these data convincingly demonstrated that CHIKV nsP2 was functionally very similar to its counterpart from SFV. In addition, these experiments established a solid background for subsequent analysis of the impact of mutations in the nsP2 protease.

Despite providing clear-cut data, the assay using recombinant protein substrates and SDS-PAGE has numerous limitations; it requires large amounts of purified recombinant proteins and is rather time consuming and hard to scale up. These factors make it difficult (though not impossible) to use this assay for the analysis of processing kinetics. For this analysis, an assay allowing continuous measurements offers multiple benefits. Therefore, we also used a continuous protease assay based on a FRET-based approach. Substrates used in this assay consisted of a peptide containing a 3/4 cleavage site between DABCYL (quencher) and EDANS (fluorophore). The design of the substrate was based on a previous assay, which, beyond doubt, indicated that the sequences representing the 2/3 site could not be used in such a substrate (except as the negative control). The choice between peptides representing 1/2 and 3/4 sites was based on preexisting data on substrate preferences of the SFV protease. More specifically, the 1/2 site in P1234 of SFV is cleaved almost exclusively in *cis,* and cleavage of the corresponding substrate in *trans* is very inefficient (as shown in publication **II**, cleavage of the 1/2 site of SFV is slow because of the P4 His residue). Owing to this, there were no data about the processing kinetics for this site of SFV. For the 3/4 site, such data exist, making it a reasonable choice for a CHIKV protease study. For our purpose, it was also essential to determine the optimal length of the 3/4 cleavage site for the FRET-based protease assay. Based on several studies that were in progress in our lab, we did know that using substrates that have fewer than 6 P-side residues is unreasonable, as both P6 and P5 residues are clearly involved

in the regulation of cleavage. Hence, the first peptide substrate was designed to correspond to amino acid residues P6-P2' from the 3/4 cleavage site. It was observed that this substrate was cleaved by CHIKV nsP2, but the reaction velocity could not be saturated (**I**, Fig. 1B), indicating that this substrate was suboptimal. To overcome this problem, a longer peptide substrate, comprising P10-P5' residues of the 3/4 site, was designed and shown to be more efficiently cleaved (**I**, Fig. 1C vs. B). Importantly, for this substrate, the reaction velocity reached a plateau (**I**, Fig. 1D), thus indicating that a longer substrate is preferred.

The validated enzyme and substrates were used to analyze the efficiency of the CHIKV nsP2 protease. Based on the results of the protease assay performed using GFP-10:5-Trx substrates, CHIKV nsP2 is able to process, within 3 minutes, ~1.8-fold molar excess of the 1/2 substrate and ~14.5-fold molar excess of the 3/4 substrate (**I**, Fig. 1E), thus showing that it has a preference for the 3/4 cleavage site; however, compared to SFV nsP2, the difference in cleavage efficiencies of such substrates is relatively minor. The cleavage rate for the 3/4 substrate was ~0.08 $s^{-1}$ or 4.8 $min^{-1}$. We also determined $k_{cat}$ and $K_m$ values using a 3/4 long peptide substrate in a continuous protease assay and found $K_m$ to be 2.5 ± 0.1 μM and $k_{cat}$ to be ~2.5 $min^{-1}$. Thus, the $k_{cat}$ values obtained using the two different methods were quite similar. Furthermore, the $k_{cat}$ value of CHIKV nsP2 is very similar to that (~3.1 $min^{-1}$) revealed for the VEEV nsP2 protease domain using similar substrates (Hu et al. 2016). However, it was clearly lower than that reported for the protease domain of SFV, which is able to cleave a 200-fold molar excess of the 3/4 protein substrate in 5 minutes (Vasiljeva et al. 2001), i.e., has a $k_{cat}$ of ~40 $min^{-1}$. Importantly, the $k_{cat}$ value for CHIKV nsP2 determined in our experiments was ~50-fold higher than that (0.096 $min^{-1}$) reported by Saisawang *et al.* (Saisawang, Saitornuang, et al. 2015). The $k_{cat}$ determined by Saisawang *et al.* is almost certainly incorrect, as it is well outside the range observed for nsP2s (or the nsP2 protease region) of other alphaviruses. The $k_{cat}$ this low is not realistic from the point of view of virus infection either; if it were correct, then the enzyme would need ~10 minutes to cleave one substrate molecule. It is difficult to imagine a virus with such an ineffective protease (and, in the case of alphaviruses, ineffective activation of the viral RNA replicase) being viable; nor is the Saisawang *et al.* $k_{cat}$ value believable, considering the speed of CHIKV infection in cell culture, in which 8 hours post-infection, cellular protein synthesis has stopped and only that of viral proteins can be observed using radioactive pulse labeling.

Why is there such a large (~50-fold) difference between the $k_{cat}$ values obtained by our group and by Saisawang *et al.*? The obvious reason is the flawed experimental design used by our colleagues. The recombinant nsP2 used by Saisawang *et al.* was obtained as a fusion protein with an N-terminal maltose-binding protein-His tag, which was cleaved off with PreScission protease (Saisawang, Sillapee, et al. 2015). PreScission protease cleaves between glutamine and glycine residues in its recognition sequence (LEVLFQ↓GP), thus leaving 2 extra amino acids (GP) at the N-terminus of the recombinant nsP2. In general, alphavirus nsP2 prefers to have its native N-terminus for uncompromised enzymatic functions. In our study, a two-amino acid insertion (GA) at the CHIKV nsP2

N-terminus was tolerated and did not negatively affect the cleavage of the 2/3 site in the long GFP-10:170-Trx substrate. While the first added amino acid, glycine, was the same in our and Saisawang *et al.*'s enzymes, the second residue was different. Alanine is a small hydrophobic amino acid that should have only a minor effect on protein conformation, while proline has a rigid structure that can affect the conformation of the protein. Thus, it is conceivable that proline at the N-terminus of nsP2 may change the conformation of nsP2 and have a negative effect on its protease activity. Furthermore, because of the lack of full information about the sequence of the construct used by Saisawang *et al.,* we cannot exclude the possibility that additional amino acid residues were present between the cleavage site of PreScission protease and the N-terminal residue of nsP2, possibly resulting in an additional negative impact. However, the intactness of the N-terminal sequence of nsP2 mostly impacts its ability to cleave the 2/3 site and has a small (or no) impact on the cleavage of other substrates. Thus, although the design of the enzyme was far from ideal, it was unlikely to be the main problem. If the problem was an enzyme, then it originated from a less-than-perfect method of purification. If such problems existed, they remained unnoticed, as the quality of the enzyme was not verified using any method (such as CD). What seems to be certain is that the design of substrates was deeply flawed and was possibly the main source of flawed data. More specifically, the substrates that Saisawang *et al.* used in their protease assays included the P4-P5' regions of cleavage sites (Saisawang, Saitornuang, et al. 2015). For researchers familiar with alphavirus proteases, this choice is strange, as these substrates have a P-side that is too short for efficient recognition and cleavage. For example, the P5 position is important for the efficient cleavage of the 1/2 site in SFV; if the P5 tyrosine in the SFV 1/2 site is replaced with alanine, the majority of P12 remains uncleaved during the processing of the *in vitro* translated polyprotein (V. Lulla, Karo-Astover, et al. 2013). Additionally, comparison of the cleavage of short (P6-P2') and long (P10-P5') 3/4 site peptide substrates in a continuous protease assay by CHIKV nsP2 demonstrated that a longer substrate was optimal for CHIKV nsP2 (**I**, Fig. 1B, C). On the other hand, the length of the P'-side in the substrates used by Saisawang *et al.* is likely excessive. It is not harmful (as observed for the P10-P5' substrate used in our study) but is not strictly needed, as an alphavirus protease can cleave 3/4 substrates even if there are no native P' residues (as long as the P1' position is not a Pro) (A. Lulla et al. 2006). Most importantly, the design of the substrate corresponding to the 2/3 cleavage site in the experiments by Saisawang *et al.* (Saisawang, Saitornuang, et al. 2015) makes no sense whatsoever. The authors used a "substrate" that had the same length (P4-P5') as the other substrates that they used (containing 1/2 and 3/4 cleavage sites). It was shown many years earlier that the SFV protease was unable to cleave protein substrates containing short 2/3 cleavage sites (Vasiljeva et al. 2001) and that SFV nsP2 required at least the first 165 aa from the nsP3 macrodomain in the 2/3 substrate (A. Lulla, Lulla, and Merits 2012). Our experiments confirmed that CHIKV nsP2, similar to SFV nsP2, efficiently cleaved the longer GFP-10:170-Trx substrate but not the short GFP-10:5-Trx 2/3 substrate (**I**, Fig. 1A); therefore, the findings for nsP2 of SFV

are clearly also valid for CHIKV nsP2. Thus, at best, the P4-P5' substrate representing the 2/3 site can be used as a negative control. In fact, had Saisawang *et al*. used it for that purpose, they would have noticed that something was seriously off with their assays, as the "cleavage efficiency" of this virtually uncleavable substrate was very similar to that of 3/4 (and 1/2) substrates that should have been easily cleavable (if correctly designed). This should have been a "stop-and-go-back" sign, as experiments performed using a test system that does not work properly (or not at all) cannot possibly produce meaningful data.

### 5.1.2. Effects of the C478A, S482A and W479A substitutions on the protease activity of nsP2 of CHIKV, the activity of the CHIKV RNA replicase and virus rescue

Saisawang *et al*. made a surprising claim that the catalytic cysteine (C478) residue of the CHIKV nsP2 protease was functionally interchangeable with a nearby serine (S482) (Saisawang, Saitornuang, et al. 2015). To verify the validity of this claim, we replaced these amino acid residues (separately and together) or a tryptophan residue located next to the catalytic cysteine with alanines. These four mutations (C478A, S482A, C478A+S482A and W479A) were introduced into the CHIKV nsP2 expression construct, and the proteins were expressed in bacteria and purified using a three-step purification protocol (Figure 9). We used two types of nsP2 proteins in our experiments. First, for improved binding to the Ni-affinity column, nsP2 and its mutant forms were expressed with a 6xHis-tag at their N-termini (His-nsP2, His-nsP2$^{C478A}$, His-nsP2$^{S482A}$, His-nsP2$^{C478A+S482A}$, and His-nsP2$^{W479A}$). Different tags are often used in protein purification because they may simplify the purification procedure; however, tags can also be difficult to remove from the purified protein, and they may influence the protein properties and/or activities. Earlier work has demonstrated the importance of the native N-terminus for the ability of SFV nsP2 to cleave between nsP2 and nsP3 (A. Lulla, Lulla, and Merits 2012) and for the enzymatic activities of CHIKV nsP2 (Das, Merits, and Lulla 2014). Herein, we used a set of tagged proteins not only to simplify purification but also to obtain an identical set of recombinant proteins (with the exception of the introduced mutations). Our self-cleavage expression/purification protocol was unsuitable for this purpose because it relies on the protease activity of nsP2, and proteins harboring mutations inactivating this activity (we expected nsP2$^{C478A}$, nsP2$^{C478A+S482A}$ and nsP2$^{W479A}$ to be inactive) would have uncleaved tags at their N-termini. For this reason, the self-cleavage protocol, allowing us to obtain proteins in the form most relevant for virus infection, was applied only for the purification of wild-type nsP2 and nsP2$^{S482A}$, which we expected to be active as proteases. These two proteins were used to verify whether the N-terminal His-tag had any effect on the protease activity of CHIKV nsP2 in the used assays.

The results of the enzymatic assays confirmed, beyond any doubt, that C478 was essential for the protease activity of CHIKV nsP2 (**I**, Fig. 3). The assays using GFP-10:5-Trx substrates demonstrated that His-nsP2$^{C478A}$, His-nsP2$^{C478A+S482A}$ and His-nsP2$^{W479A}$ were unable to cleave substrates corresponding to 1/2 and 3/4 sites; only His-nsP2$^{S482A}$ was able to cleave these substrates, similar to His-nsP2. As expected, none of the His-nsP2 variants were able to cleave the short (GFP-10:5-Trx) 2/3 substrate, while the long (GFP-10:170-Trx) 2/3 substrate was cleaved by His-nsP2 and His-nsP2$^{S482A}$ but not by His-nsP2$^{W479A}$. Surprisingly, both His-nsP2$^{C478A}$ and His-nsP2$^{C478A+S482A}$ showed strongly reduced but still detectable cleavage of the long 2/3 substrate, an unexpected result, possibly indicating that the binding of nsP2 to such a substrate can cause its hydrolysis in a protease activity-independent manner (**I**, Fig. 3A). The molecular mechanism underlying this effect remains unknown, but it can be speculated that it may be related to the macromolecular assembly-dependent cleavage of this substrate. It is conceivable that interactions between nsP2 (including its N-terminal region) and the macrodomain of nsP3 in the substrate make a scissile bond available not only for protease-dependent but also (albeit to a lesser extent) for protease-independent hydrolysis.

We found that in the continuous protease assay, the variants of nsP2 behaved in the same manner as in the assay using recombinant protein substrates; only His-nsP2 and His-nsP2$^{S482A}$ demonstrated the ability to cleave the peptide substrates used (**I**, Fig. 3B). In the assays based on the use of recombinant proteins as substrates, we could not observe a significant difference in protease activities between wt nsP2 and His-nsP2 or wt nsP2 and nsP2$^{S482A}$ (**I**, Fig. 3C, D). However, in the continuous protease assay, both His-nsP2 and nsP2$^{S482A}$ had slightly lower (~2-fold) protease activities than that of wt nsP2 (**I**, Fig. 3E), indicating a very mild negative impact of the presence of the 6xHis-tag at the N-terminus of the enzyme or the presence of the S482A substitution near the active site on the protease activity of CHIKV nsP2. Combined, the results of our protease assays unequivocally prove that CHIKV nsP2 requires C478 for its protease activity. Thus, the data reported by Saisawang *et al*. (Saisawang, Saitornuang, et al. 2015) are flawed. At the same time, our data are consistent with those reported for other alphaviruses, including SFV, SINV or VEEV (E. G. Strauss et al. 1992; Merits et al. 2001; Golubtsov, Kääriäinen, and Caldentey 2006; A. Russo, White, and Watowich 2006).

The assays performed using purified nsP2 provided very clear results about the importance of C478 for the protease activity of CHIKV nsP2. However, during alphavirus infection, the nsP2 protease works in the context of the ns polyprotein. As alphavirus replicase proteins and their precursors interact with each other, it is possible that in the natural context, the impacts of mutations in/near the active site of nsP2 may be different. Therefore, we carried out a cell-free *in vitro* transcription/translation assay, coupled with immunoprecipitation of synthesized polyproteins and their cleavage products using antibodies against different nsPs (method described in Figure 10C). The results of this assay perfectly agreed with the data from the assays based on the use of purified nsP2.

Again, in the case of polyproteins harboring the C478A, C478A+S482A or W479A mutation in nsP2, the processing of P1234 did not occur, and only P1234 could be observed in the image obtained from an SDS-PAGE gel. The S482A mutation did not block P1234 processing, and the obtained image was similar to that obtained for wt P1234. A full-length P1234 could not be detected; instead, all the individual nsPs (1–4) and P1234 processing intermediates (P12 and P34) were revealed (**I**, Fig. 4).

The importance of the findings obtained using cell-free systems was also verified using cell-based assays, as this provides an indisputable proof of the impacts (or lack thereof) of introduced mutations on virus replicase activity/virus infectivity. First, we used a CHIKV *trans*-replicase assay (method described in Figure 11) to evaluate how the studied mutations affected the functionality of the CHIKV RNA replicase. BSR cells were transfected with plasmids expressing wt (T7-P1234) or mutant (T7-P12$^{CA}$34, T7-P12$^{SA}$34, T7-P12$^{CA+SA}$34, or T7-P12$^{WA}$34) CHIKV replicases; T7-P1234$^{GAA}$ was used as a replication-negative control and a replication-competent template RNA harboring the FFLuc (replication) and GLuc (transcription) markers (**I**, Fig. 5A). Lysates of transfected cells were analyzed by western blotting for P1234 expression and processing or by the luciferase assay for RNA replicase activity. Western blot analysis demonstrated the presence of individual nsPs in samples from cells transfected with the T7-P1234, T7-P12$^{SA}$34 and T7-P1234$^{GAA}$ plasmids, while in samples from T7-P12$^{CA}$34-, T7-P12$^{CA+SA}$34- and T7-P12$^{WA}$34-transfected cells, uncleaved P1234 could be observed, while individual nsPs could not be detected (**I**, Fig. 5B). The expression of the FFLuc marker from the template's first (5') ORF showed a more than 300-fold boost in samples from T7-P1234- and T7-P12$^{SA}$34-transfected cells, while FFLuc expression was not boosted in samples from cells transfected with the other mutant replicases. Similarly, the expression of GLuc from the second ORF (expression of GLuc occurs from SG RNA produced by the RNA replicase) was boosted ~100,000-fold by the replicases expressed from T7-P1234 and T7-P12$^{SA}$34. In contrast, only a very small (~10-fold) boost in GLuc expression was detected in cells expressing replicases containing the C479A, C479A+S482A or W479A substitution (**I**, Fig. 5C). Where may this minimal activity come from? Most likely, it originates from an RNA replicase generated by spontaneous degradation of the P1234 precursor. P1234 lacking protease activity is rather unstable and is slowly degraded, forming a large array of smaller proteins (**I**, Figs. 4, 5). Most likely, these degradation events occur at random positions of P1234, and if so, the odds of such an event occurring at the boundary of nsP3/nsP4 (given the size of P1234) are approximately 1:2500. A functional replicase consisting of P123 and nsP4 and formed as a result of such an event would be present at a very low amount, and because of the lack of P123 processing, its RNA replicase activity would be lower than that of the wt replicase (Lello et al. 2021). Combined, these effects may result in a 10,000-fold reduced (but still detectable) boost of GLuc expression. Regardless of its exact origin, the presence of this minimal RNA replicase activity has no impact on the overall conclusion based on the data from *trans*-replicase experiments; that is, the C478A

and W479A substitutions in CHIKV nsP2 prevent (and the S482A mutation does not prevent) P1234 processing in transfected cells, which, in turn, prevents the formation of a fully functional RNA replication complex in these cells. Additional northern blot analysis confirmed this directly, with the synthesis of negative- and positive-strand template RNAs above the background level observed only in cells transfected with the T7-P1234 and T7-P12$^{SA}$34 plasmids. The C478A and W479A mutations abolished RNA synthesis by the CHIKV replicase; the same was observed for the corresponding mutations introduced into the SFV replicase (**I**, Fig. 6).

Finally, the impacts of the set of these protease active site mutations were tested in the context of transcripts obtained from plasmids harboring wt and mutant cDNAs of CHIKV. It was observed that the C478A and W479A mutations blocked the rescue of the virus from corresponding transcripts; in the ICA, no plaques were detected, and there was no CPE in transfected cells even 144 hpt. In contrast, strong CPE could be observed in cells transfected with the transcripts from the wt CHIKV clone or from the CHIKV$^{S482A}$ clone as early as 18 hpt. The ICA analysis also revealed that the virus rescue efficiencies for wt CHIKV and CHIKV$^{S482A}$ were similar (**I**, Fig. 7A). Lysates of transfected cells were also analyzed by western blotting for the presence of the CHIKV CP, which serves as an indicator that active viral replication occurs (CP is translated not from *in vitro* made transcripts but from the SG RNA made by the viral replicase). The CP could be detected only in cells transfected with wt CHIKV or CHIKV$^{S482A}$ RNA but not in cells transfected with CHIKV RNA harboring the C478A or W479A mutation (**I**, Fig. 7B). Thus, the C478A or W479A substitution is clearly lethal for CHIKV, while the S482A substitution is not. To analyze its impact on P1234 processing in CHIKV-infected cells, we infected BHK-21 cells with wt CHIKV or CHIKV$^{S482A}$ and performed analysis of ns polyprotein processing using the pulse-chase method. No significant difference between these two viruses was observed (**I**, Fig. 7C). There were no detectable differences in electron microscopy images of spherules, cytopathic vacuoles or progeny virus formation in cells infected by these viruses (**I**, Fig. 8), confirming that the S482A substitution had no significant impact on the CHIKV life cycle.

Our results clearly demonstrate that C478 is crucial for the protease activity of CHIKV nsP2. When the C478A mutation was in the context of the whole ns polyprotein, this mutation also "killed" RNA replicase activity and was lethal for the virus. S482 was incapable of rescuing protease activity/ns polyprotein processing if the C478A substitution was present; accordingly, it cannot functionally substitute the C478 residue. Most likely, S482 is not involved in ns polyprotein processing at all, as the S482A mutation did not have a significant adverse effect on CHIKV nsP2 protease activity nor did it prevent the formation of a functional RNA replicase. The amino acid composition of the CHIKV nsP2 protease active site (476-NVCWAKS-482) is very similar to that of the VEEV nsP2 protease active site (475-NVCWAKA-481), in which S482 is naturally substituted with an alanine (A481). In addition, in VEEV, K480 interacts with P1' tyrosine in the 3/4 cleavage site, which probably contributes to the preference of the nsP2 protease

toward the 3/4 cleavage site (A. T. Russo et al. 2010). The same explanation probably applies to the CHIKV nsP2 protease's preference toward the 3/4 cleavage site because in CHIKV, there is also a lysine in the corresponding position (position 481) in nsP2, and the P1' residue in the 3/4 cleavage site is also a tyrosine.

Finally, what did we achieve in this study? Due to the controversial information about the organization of the protease active site of CHIKV nsP2, it was important to verify which residues were actually involved in protease function. After our work, there should be no more ambiguity regarding that CHIKV nsP2 is a cysteine protease; in all important aspects, it is very similar to nsP2s of other alphaviruses. Accurate knowledge about amino acid residues critical for the enzymatic function of a viral protein is essential for a better understanding of the viral life cycle and for the development of potential antivirals. The fact that CHIKV is a medically important pathogen only underlines the necessity for correct information about this virus. Thus, our study has cleared the field for the development of protease inhibitors for CHIKV. Such studies have been active in our lab (Das et al. 2016; Larisa Ivanova, Rausalu, Žusinaite, et al. 2021; Larisa Ivanova, Rausalu, Ošeka, et al. 2021), and the most recent of them is the final part of this thesis (publication **III**). A better understanding of the function of nsP2 of CHIKV is also important for the analysis of the structure and function of this protein (Law et al. 2021). It is important for studies of the role of nsP2 in superinfection exclusion (Cherkashchenko et al. 2022), which has opened novel and prospective directions of research and development, namely, the generation of transgenic mosquitoes with a limited capacity for pathogenic alphavirus transmission (Basu et al. unpublished data).

## 5.2. Importance of the timeliness in alphavirus ns polyprotein processing (II)

As previously mentioned, ns polyprotein processing is a central process during the alphavirus infection cycle. The first cleavage in P1234 occurs at the 3/4 site and creates the P123+nsP4 complex that starts the synthesis of negative-strand RNA. This is also the only absolutely mandatory processing event; the absence of 3/4 site cleavage means no replication. However, although not strictly required for the replication to occur, subsequent processing events are also important for successful infection. While there is no clear evidence that 3/4 site processing in P1234 is regulated (it seems more likely that the rule "the faster the better" applies), it has been known for a long time that the processing of remaining P123 is regulated. One of the reasons for this is that the lifetime of the "early" replicase (or its precursor) must be sufficient to allow transport to the plasma membrane, attachment to membranes, formation of the oligomeric replicase core and synthesis of negative-strand RNA to ensure success of the following steps of the replication cycle. The presence of P123 and/or P23 is clearly necessary for the formation of spherules, the alphavirus RCs in the infected cells (Hellström et al. 2017). It is clear that the cleavage of the 1/2 site functions as a molecular switch, after which

no more negative-strand RNA can be produced. The final cleavage, that of the 2/3 site, occurs quickly after the cleavage of the 1/2 site, and thus, the "late" replicase, consisting of individual nsPs, is created. Several studies have shown that changes in the cleavage patterns and/or a change in the protease activity of nsP2 affect the fitness of alphaviruses; for both SINV and SFV, the accelerated processing of P123 leads to decreased virulence (Heise, Simpson, and Johnston 2000; Saul et al. 2015). However, there are also many controversies regarding the importance and regulation of P123 processing, which may be due to several factors. Corresponding studies were performed using different alphaviruses and/or different types of host cells, as well as assays with different sensitivities. Therefore, it is not easy to decide which data one believes or to make solid and broad conclusions. To generate a broad picture and put together the existing fragments of knowledge, several studies were carried out in our lab in parallel. In this study, we investigated the consequences of interfering with the timing of cleavages in the ns polyprotein for SFV and SINV; in a parallel study published as an accompanying paper, the same was conducted using CHIKV (Bartholomeeusen et al. 2018). The picture that emerged from these studies is highly coherent and provides a good context for the explanation of data from earlier studies.

### 5.2.1. Optimization of the sequence of the 1/2 site is detrimental for SFV

Cleavage site sequences in alphaviruses are evolutionarily conserved but, with the exception of the 3/4 site, are clearly suboptimal for cleavage. This indicates that these sequences are important to the virus, and there must be a reason (or reasons) for the selection. The 3/4 site is the first to be cleaved. It has been shown for SFV that the sequence of the 3/4 site is optimal (or nearly optimal) for the nsP2 protease, and consequently, it is the most efficiently recognized and cleaved (Vasiljeva et al. 2003); herein, the same was shown to be true for CHIKV (publication **I**). The P6-P1 regions of the cleavage sites in the SFV4 ns polyprotein are represented by the sequences EYHAGA, MHTAGC and LGRAGA for the 1/2, 2/3 and 3/4 sites, respectively. To study the effect of accelerating the cleavage of the 1/2 and/or 2/3 sites, we replaced the P6-P4 residues in these sites with the LGR sequence present in the 3/4 site (**II**, Fig. 1A).

The first approach to study the effects of these replacements was to determine the ability of the corresponding *in vitro* transcribed viral RNAs to initiate infection in transfected cells (for a lack of a better term, hereafter referred to as "RNA infectivity"). If RNAs were infectious, it was also possible to measure the plaque sizes and to analyze the emergence of reversions, pseudoreversions and/or second-site mutations in resultant virus stocks (methods described in the Materials and Methods section in Figure 10A, B). The presence of the LGR sequence in the 2/3 site (SFV4-2LGR) had no detectable effect on RNA infectivity, and the plaques formed by the mutant virus were of the same size as those formed by wt SFV. Consistent with the wt-like phenotype, we did not find any pseudoreversions or second-site mutations in the genomes of the obtained stock of SFV4-LGR (**II**,

Fig. 1B). These findings are in line with previous observations that the cleavage of the 2/3 site mostly depends on its presentation and not on its sequence (A. Lulla, Lulla, and Merits 2012; V. Lulla, Karo-Astover, et al. 2013). In contrast, the optimization of the sequence of the 1/2 site (i.e., accelerating its processing) had a dramatic negative effect on RNA infectivity. The infectivity of RNA corresponding to SFV4-1LGR was 1000-fold lower and that of RNA corresponding to SFV4-1+2LGR (a construct also optimized at the 2/3 site) 5000-fold lower than the RNA infectivity of wt SFV4. The plaques formed by these mutant viruses were also smaller (1–2 mm) than those formed by wt SFV (2–3 mm; **II**, Fig. 1B). This demonstrated that the LGR sequence in positions P6-P4 of the 1/2 site was not tolerated, although it was not lethal either. We have repeatedly observed that such a drastic drop in RNA infectivity is a strong indicator that the infectivity of the original mutant RNA was increased by some compensatory changes generated during virus rescue and propagation (Žusinaite et al. 2007; Thaa et al. 2015; Teppor, Žusinaite, and Merits 2021; Teppor et al. 2021; V. Lulla, Karo-Astover, et al. 2013). Analysis of 30 clones from the progenies of SFV4-1LGR and SFV4-1+2LGR indeed revealed that the LGR sequence in the 1/2 site was altered, with the exception of one clone from the SFV4-1+2LGR progeny. The substitutions occurred in the P4 position of the cleavage site in which the Arg residue was replaced by His (original P4 residue in wt SFV4) or by Cys, Ser or Leu (LG**R**AGA → LG**H**AGA/LG**C**AGA/LG**S**AGA/LG**L**AGA). Two of these replacements were introduced into SFV4-1LGR, and the obtained RNAs (SFV4-1LGH and SFV4-1LGL) were analyzed similarly to the original construct. This analysis confirmed that either His or Leu in the P4 position in the 1/2 site restored both the RNA infectivity and plaque size of the mutant virus to the levels of wt SFV4 (**II**, Fig. 1B).

As stated above, one clone isolated from the SFV4-1+2LGR progeny retained Arg in the P4 position in the 1/2 site. However, it had acquired two different substitutions; the P1 Ala was replaced by Val (LGRAG**A** → LGRAG**V**), and a second-site mutation, V515M, occurred in nsP2 (**II**, Fig. 1B). Hence, this mutant was named SFV4-1LGRAGV-2LGR-V515M. Next, we studied how these changes (LGRAGV in the 1/2 site and V515M in nsP2), as well as the introduction of Arg in the P4 position in the 1/2 site, affected SFV4 RNA infectivity. The latter substitution was made because there is a natural nonvirulent SFV isolate, A7(74), which has Arg in the P4 position in the 1/2 site (EY**R**AGA), and the residue at position 515 in nsP2 is also different from that in SFV4, namely, Glu in SFV A7(74) and Val in SFV4. Interestingly, a very similar combination is also found in CHIKV, which has Arg as the P4 residue in the 1/2 site and Glu as residue 515 of nsP2. The substitution of His for P4 Arg or Val for E515 was found to have negative consequences for CHIKV replication, while the combination of both mutations (i.e., same as the combination present in wt SFV4) was well tolerated (Bartholomeeusen et al. 2018). It was found that the Val residue introduced into the P1 position of the 1/2 site (SFV4-1LGRAGV) completely restored the RNA infectivity of SFV4-1LGR. If the LGR sequence was present not only in the 1/2 site but also in the 2/3 site (SFV4-1LGRAGV-2LGR), the P1 Val residue also resulted in a large improvement in RNA infectivity (which increased ~1000-fold),

although it still remained somewhat (2.7-fold) lower than the RNA infectivity of wt SFV4. We assumed that the remaining small difference was due to the lack of the V515M substitution in nsP2; however, additional experiments revealed that this was not the case, and the infectivity of RNA harboring both of these changes (SFV4-1LGRAGV-2LGR-V515M) remained ~3.5-fold lower than that of wt SFV4 RNA (**II**, Fig. 1C). The Arg residue in the P4 position of the 1/2 site (SFV4-1R$^{P4}$) had a less prominent negative effect than the LGR substitution; however, it clearly reduced the RNA infectivity (~10-fold compared with that of wt SFV4). These data demonstrate that the P5 and P6 positions in the 1/2 site are also important determinants of infectivity, most likely by contributing to the processing efficiency of the site. The V515M or V515E mutation alone (SFV4-V515M or SFV4-V515E) also slightly reduced the RNA infectivity compared with that of SFV4 (~1.5- and ~1.3-fold, respectively). However, in combination with the P4 Arg residue (SFV4-1R$^{P4}$-V515M and SFV4-1R$^{P4}$-V515E), either of these mutations was sufficient to restore the RNA infectivity to a level similar to that of wt SFV4 (**II**, Fig. 1C). The observation that Arg in the P4 position in the 1/2 site and E515 in nsP2 represent a highly efficient combination was also evident from a parallel study performed using CHIKV mutants; the same can be concluded based on the analysis of naturally occurring combinations of these residues, which are also found in several other alphaviruses, including RRV and ONNV.

Our data clearly indicated that the Arg residue in the P4 position of the 1/2 site of SFV had a major impact on the virus. As Arg is the preferred P4 residue of the alphavirus protease (A. Lulla et al. 2006), its impact is almost certainly linked to premature (too fast) processing of the 1/2 site, which reduces the half-life of P123 and that of the virus early replicase. Thus, it was logical to hypothesize that an array of compensatory mutations detected in our experiments had a common denominator: they all slow down 1/2 site processing and increase the half-life of P123. By far, the most common strategy used by the mutant virus to achieve this goal is the deoptimization of the 1/2 site. Most likely, the V515M mutation serves the same purpose. Indeed, in VEEV, E513 in nsP2 (corresponding to V515 in SFV nsP2) has been predicted to be involved in the recognition of the P4 residue in the 1/2 site (A. T. Russo et al. 2010); thus, it is likely that the V515M substitution acts by reducing the activity of the nsP2 protease toward this cleavage site. Several assays were performed to experimentally confirm this hypothesis. In the context of an infectious virus, this can be demonstrated only indirectly; the use of SFV4-1LGR (presumably having accelerated processing of the 1/2 site) is impossible, as this virus has a too low activity and immediately acquires various compensatory changes. Therefore, we first analyzed ns polyprotein processing in the four SFV isolates originating from the progeny of SFV4-1LGR; all these viruses were plaque purified and named (according to the compensatory changes they harbored) SFV4-1LGR(R/H), SFV4-1LGR(R/C), SFV4-1LGR(R/S), and SFV4-1LGR(R/L). Plaque-purified SFV4-1LGRAGV-2LGR-V515M from the progeny of SFV4-1+2LGR was also included in this analysis. A pulse-chase experiment revealed that the ns polyprotein processing of SFV4-1LGR(R/H), SFV4-1LGR(R/S) and SFV4-1LGR(R/L) was very similar to that of wt SFV4;

the amounts of P1234 and its processing intermediates were only slightly lower than those of wt SFV4, indicating that despite the compensatory changes in the P4 position, the 1/2 site cleavage rates remained slightly higher than that in wt SFV4, an effect almost certainly caused by the P5 Leu and P6 Gly residues in the altered 1/2 site. In contrast to the viruses harboring other compensatory changes in the P4 position, SFV4-1LGR(R/C) demonstrated a slightly delayed processing of P123 compared to that of wt SFV4. The ns polyprotein processing of SFV4-1LGRAGV-2LGR-V515M differed from that of wt SFV4 to the largest extent; there was a clear delay in the processing of P123 and P12, and as a result, a delay in the accumulation of mature nsP2 was also observed (**II**, Fig. 2). Nevertheless, these changes were all rather minor, allowing us to conclude that the processing patterns of P1234 in these mutant viruses were quite similar to that in wt SFV4. Hence, we can assume that the restoration of disturbed (artificially accelerated) processing by the compensatory changes allowed the RNA infectivity to increase to the wt level (or to the level close to it).

Taken together, this part of our data leads to a simple conclusion that the alphavirus does not care (at least not much) about the optimal structure of the 1/2 cleavage site or a high activity of the nsP2 protease; instead, an actual requirement is the proper balance between the two. If the 1/2 site is optimal for processing, the virus needs to have nsP2 with a suboptimal activity toward this target. Indeed, this situation naturally occurs, for example, in SFV A7(74), CHIKV, RRV and ONNV. However, if the 1/2 cleavage site is suboptimal, the virus can have, and likely has, a highly efficient protease, as is the case with SFV4. It is not fully clear what the consequences of an improper balance are. It is possible that if the cleavage of the 1/2 site is accelerated, the virus does not have enough time to complete the negative-strand RNA synthesis. However, it may also be that if P123 is not stable enough, there is not enough time for its transport to the plasma membrane and/or to participate in successful spherule formation. Given the importance of the proper timing of P123 processing, it is highly likely that this process is regulated on more than one level. The fact that SFV4-1LGR could produce a viable progeny (albeit thanks to compensatory changes) is in line with this hypothesis. One option is that the virus can use its RNA genome to regulate (slow down) 1/2 site processing. There is also a clear functional link between the presence of Arg as the P4 residue in the 1/2 site and the sensitivity of an alphavirus to the depletion of an important host factor, the G3BP protein (Götte et al. 2020). The proper balance between the cleavability of the 1/2 site and the activity of nsP2 also impacts the *in vivo* properties of alphaviruses. In cell culture, both SFV4 and SFV A7(74) replicate well; *in vivo,* however, the latter is avirulent. Analysis performed using a panel of SFV mutants and chimeras clearly showed that the ability of SFV to cause encephalitis depended on the stability of P123, with isolates with more stable P123 being highly neurovirulent (Saul et al. 2015). The same seems to be the case for SINV or at least some isolates of this virus (Heise et al. 2003). Interestingly, the opposite was observed for RRV (Liu et al. 2018) and CHIKV (Chan et al. 2019), as mutants of these viruses with a reduced speed of 1/2 site processing were avirulent. Revealing the molecular basis of

these effects and the reasons why *in vivo* properties of different alphaviruses are affected differently is clearly both important and interesting; however, such a study requires in-depth analysis of *in vivo* infection, including complex interactions between the virus and the host antiviral responses.

### 5.2.2. Deoptimization of the 1/2 site increases the infectivity of SINV harboring a hyperprocessive protease mutation

The processing of P1234 can be accelerated not only by changing the sequences of cleavage sites; certain substitutions in the nsP2 protease may increase its activity and consequently accelerate polyprotein processing. For example, nsP2 of SFV with the V515 residue is more active than the enzyme with M515 or E515 residue. However, the best known – and widely used – substitution in the nsP2 protease, resulting in a hyperactive enzyme, has been described for SINV. This N614D substitution increases the speed of SINV ns polyprotein processing in cell-free assays (E. G. Strauss et al. 1992). It has also been reported to be lethal for SINV, as attempts to rescue the virus harboring this mutation were unsuccessful. However, the latter has never been confirmed using modern RNA transcription/transfection methods. Furthermore, the basis of this phenotype has not been demonstrated. The lethal phenotype is unlikely to be due to enhanced cleavage of the 3/4 site; if anything, accelerated formation of the P123+nsP4 replicase should activate virus replication, not suppress it. Based on the results obtained for SFV and CHIKV, it seemed highly likely that the real reason might be accelerated processing of the 1/2 site.

Our attempt to copy the N614D mutation into the SFV backbone was not successful, as the obtained virus was temperature sensitive and genetically unstable. This outcome is not surprising, as SINV is only distantly related to SFV. In another study, it was found that SFV chimeras in which nsP2 or its protease part was replaced by the corresponding sequence of SINV, were not viable (Teppor et al. 2021), suggesting that there are some differences between the processing of P1234 between SINV and SFV. Therefore, we performed subsequent analysis using an icDNA clone of SINV. First, we analyzed the RNA infectivity of wt SINV and SINV-N614D. As expected, RNA of wt SINV was highly infectious. Somewhat surprisingly, in our hands, SINV-N614D RNA was also infectious. However, it had ~2500-fold reduced infectivity compared with that of its counterpart from wt SINV (**II**, Fig. 3). Thus, in contrast to a previous report (E. G. Strauss et al. 1992), the N614D mutation is actually not lethal to SINV. The most likely explanation for this discrepancy is that the transfection methods used by us were much more effective than those available to researchers in the early 1990s; because of the lower efficiency of virus rescue, the 2500-fold drop in the infectivity prevented virus rescue, and the mutation was (mistakenly) considered lethal. Next, we added two more viruses for comparison. These viruses harbored mutations in the 1/2 site that were assumed to result in a slower cleavage. In the SINV 1/2 site, the P6-P1 sequence is QADIGA. SINV-N614D-1G$^{P4}$ had a single substitution in the 1/2 cleavage site, Asp→Gly in the P4 position; thus, the sequence

of the mutant 1/2 site for this virus was QA**G**IGA. In the genome of SINV-N614D-1RDG, the P6-P4 residues of the 1/2 site were replaced with those from the 2/3 site (the least efficiently processed cleavage site, with the sequence RDGVGA); the resulting sequence of the mutant 1/2 site was therefore **RDG**IGA. Notably, the actual cleavage efficiencies of the corresponding mutant polyproteins (see 5.2.3) confirmed that the introduced mutations had the expected effects. SINV-N614D-1G$^{P4}$ RNA had a 2.5-fold higher infectivity than that of SINV-N614D, while the infectivity of SINV-N614D-1RDG, harboring more extensive deoptimization in the 1/2 site, was increased 110-fold (**II**, Fig. 3). As deoptimizing the 1/2 site clearly improves the infectivity of RNA of SINV harboring hyperprocessive nsP2, this clearly indicates that a too fast cleavage of the 1/2 site might be the main reason for the low infectivity of SINV-N614D.

Taken together, our results imply that similar to SFV (and CHIKV), accelerated processing of the 1/2 site is detrimental for SINV. The defects caused by accelerated cleavage at the 1/2 site, due to hyperactive nsP2 of SINV, can be compensated for by changes in the viral genome that decelerate P1234 processing. Thus, similar to other analyzed alphaviruses, SINV replicase formation depends on the proper balance between the nsP2 protease activity and cleavage efficiency of the sequence present in the 1/2 site.

### 5.2.3. Accelerated processing of P123 reduces RNA synthesis of SFV and SINV

The ICA rescue results presented thus far serve only as indirect evidence that the mutations that we introduced in the cleavage sites and/or into nsP2 affect the processing of P1234. Unfortunately, the processing of P1234 in virus-infected cells could only be analyzed for mutant viruses that replicated well and were genetically stable. To also include mutants that were not viable without pseudo-reversions/second-site mutations, we used an *in vitro* transcription/translation/processing assay. Products of these reactions were separated by SDS-PAGE, nsP2 and P123 band intensities were quantified, and nsP2/P123 ratios were calculated and normalized to either SFV or SINV wt P1234 values. It was observed for SFV (**II**, Fig. 4) that the processing of the P1234-1LGR and P1234-1+2LGR polyproteins was indeed accelerated in contrast to that of P1234-2LGR, which was similar to the processing of wt P1234. Compared with that of P1234-1LGR, both P1234-1LGH and P1234-1LGL displayed reduced processing speeds; nevertheless, the processing of these polyproteins remained faster than that of wt P1234. P1234-1LGRAGV and P1234-1LGRAGV-2LGR were processed similarly to P1234-1LGL or P1234-1LGH. Compared with that of parental P1234-1LGR, the processing speed was reduced but still remained higher than in the case of wt P1234. The V515M substitution in P1234-1LGRAGV-2LGR-V515M further reduced the processing speed, which became similar to that of wt P1234. P1234-V515M and P1234-V515E both had reduced polyprotein processing speeds compared with that of wt P1234, confirming that these substitutions reduced the protease activity of nsP2. Similar to P1234-1LGR, P1234-1R$^{P4}$ had accelerated

polyprotein processing, which was reduced by the addition of the V515M or V515E substitution but still remained higher than the processing speed of wt P1234.

In the case of SINV constructs, the N614D mutation indeed led to increased P1234 processing, and deoptimization of the 1/2 site sequence in P1234-N614D-1G$^{P4}$ or in P1234-N614D-1RDG reduced the ns polyprotein processing speed to a level below that observed for wt P1234 (**II**, Fig. 4). Combined with the data obtained in virus rescue experiments, these results indicate that the increased speed of ns polyprotein processing, whether caused by changes in the cleavage site sequence (e.g., studied SFV mutations) or by mutations in the nsP2 protease (e.g., the N614D substitution in SINV), is unfavorable for a virus and is compensated for by pseudoreversions in the cleavage site and/or by additional second-site mutations. The results of the *in vitro* processing assay confirmed that the N614D mutation in nsP2 of SINV indeed enhanced the P1234 processing speed and revealed that deoptimizing the 1/2 site sequence decelerated its processing.

The logical question to ask was: How does the accelerated processing of P1234 at the 1/2 site reduce the infectivity of the corresponding mutant RNA genome? As processing of P1234 plays a central role in the regulation of alphaviral RNA synthesis, it is logical to assume that accelerated processing of the 1/2 site has a negative impact on alphavirus RNA synthesis. To confirm this directly, we used *trans*-replication (method described in Figure 11), as this assay allowed the inclusion of constructs corresponding to genetically unstable viruses. The design of the replication template was such that the boost in the FFLuc signal reflected the synthesis of viral genomic RNAs and the boost in the *Renilla* luciferase (RLuc) signal reflected the production of SG RNAs. In this specific setup, the cotransfection of the template plasmid with a plasmid expressing wt P1234 of SFV activated the expression of the FFLuc and RLuc markers ~200- and ~12,000-fold, respectively; in the case of the template and P1234 of SINV, the boosts were ~80- and ~20,000-fold, respectively (**II**, Fig. 4). As is typical for alphavirus *trans*-replicases, the amplification of the SG marker signal (in this case, RLuc) was much larger; as RLuc is encoded by the second ORF in the template construct, its background expression is very low, and the translation almost exclusively occurs from replicase-generated SG RNA. Therefore, the conclusions about the effects of the studied mutations on the activity of the RNA replicase were based on the observed boost in the RLuc signal. Similar to the CHIKV *trans*-replicase (publication **I**), it was observed that the P12$^{CA}$34 replicase of SFV, which contains the Cys→Ala mutation in the active site of the nsP2 protease, was unable to boost the expression of either marker; therefore, the construct was used as a negative control. The inability of the P12$^{CA}$34 replicase to perform any detectable RNA synthesis also confirmed the importance of the cysteine residue in the active site of the nsP2 protease. The lack of replicase activity was clearly due to the lack of cleavage of the 3/4 site, as another control, SFV P1^2^34 (^ indicates that the cleavage between these regions is blocked by mutations in the corresponding cleavage sites), which was able to perform only 3/4 site cleavage, clearly boosted FFLuc and RLuc expression (12- and ~870-fold, respectively). In the *trans*-replication assay, all SFV polyproteins harboring mutations that significantly

accelerated 1/2 site processing had strongly reduced abilities to activate the expression of the luciferase markers. The effects were most prominent for SFV P1234-1LGR and SFV P1234-1+2LGR, but the ability of P1234-1R$^{P4}$ to perform RNA replication was also severely reduced. Both 1LGH and 1LGL pseudo-reversions restored the ability of the mutant replicase to activate marker expression, a result that was consistent with the data from the P1234 processing assay. Apparently, the restoration of the processing speed due to these pseudo-reversions was also sufficient to achieve near-wt levels of RNA infectivity. The V515M and V515E mutations, which slowed down the processing of P1234, boosted RLuc expression more efficiently than wt P1234, thus indicating that slower ns polyprotein processing increases the replication and transcription of the template RNA expressed from the reporter plasmid. When combined with the 1R$^{P4}$ mutation, the V515M and V515E substitutions increased, to an extent, the ability of the mutant replicase to activate RLuc expression. Again, these results are in accordance with earlier predictions about the importance of the amino acid residue in this position for the binding of the P4 residue of the 1/2 site (A. T. Russo et al. 2010) and align with previous findings that revealed that the V515E substitution reduces the activity of SFV nsP2 in virus-infected cells (Saul et al. 2015). SINV P1234-N614D almost completely lacked the ability to activate the expression of markers encoded by the template RNA (**II**, Fig. 4), a finding that aligns well with the severely reduced RNA infectivity of the corresponding virus. Both of these effects may be due to premature cleavage at the 1/2 site, and the processing of P1234 harboring the N614D substitution is accelerated. Interestingly, the 1G$^{P4}$ or 1RDG mutation did not increase FFLuc expression by SINV P1234 harboring the N614D substitution, and only a modest, ~3-fold increase in RLuc expression was observed. Although the change seems small, in the case of the 1RDG mutation, it was associated with an ~100-fold increase in RNA infectivity (**II**, Fig. 3).

The *trans*-replicase assay is very sensitive and thus can reveal minor defects that cannot be observed using infectious viruses. These effects may be further enhanced by the obligatory *trans*-binding of the replicase precursor to the replication template (in the case of a virus, binding can occur in *cis,* i.e., the replicase typically binds the viral RNA genome that was used as mRNA for its translation). Such a requirement likely increases the impact of altered P1234 processing on RNA replicase activity, as in the case of *trans*-binding, additional time is needed for the replicase precursor to find and bind its template. Thus, *trans*-replicase assays can provide some unexpected results that do not *per se* contradict those obtained using infectious RNAs or viruses. In the case of SINV P1234 with the N614D substitution, the 1RDG mutation had an unexpectedly small positive impact on RNA replicase activity. This may indicate that the N614D mutation also accelerates cleavage of the 2/3 site (which remained unmodified in the P1234 expression construct and could be targeted by abnormally active nsP2). Alternatively, or in addition, this may indicate that the requirements for the processing of the 1/2 site in SINV P1234 are somewhat different from those for the 1/2 site of SFV. One possible explanation is that the processing of the 1/2 site of SINV is

negatively regulated by genomic RNA of the virus; such regulation would explain why in the context of the virus genome, mutations in this site have a more prominent impact than in the case of the *trans*-replicase assay. Several such effects were also observed for SFV polyproteins. For SFV P1234, the LGRAGC sequence in the 2/3 cleavage site affected the ns polyprotein ability to activate template RNA replication/transcription. Compared to wt P1234, P1234-2LGR had a strongly reduced ability to activate the expression of the luciferase markers (**II**, Fig. 4). The same applied to P1234-1GRAGV and P1234-1GRAGV-2LGR-V515M. P1234-1GRAGV-2LGR had an even more diminished ability to activate the expression of the markers. Thus, the conclusion from these experiments is that accelerated cleavage of the 2/3 site also has a negative effect on template RNA replication. This finding likely implies that premature cleavage at the 2/3 site is also harmful for the virus, although not to the same degree as premature cleavage of the 1/2 site, and is difficult to detect in standard virus rescue/propagation experiments.

### 5.2.4. The N-terminal region of SFV nsP2 is involved in the processing of the 2/3 site

The optimization/deoptimization of the 1/2 site revealed the importance of the temporally regulated cleavage of this site. This cleavage not only releases nsP1 from the polyprotein but also generates a native N-terminus of nsP2; the sequence of this region is quite conserved among alphaviruses (**II**, Fig. 5A). The release of the N-terminus of nsP2 is also significant, as it is important for the NTPase and RNA helicase activities of nsP2 (Das, Merits, and Lulla 2014). Herein, we wanted to analyze whether the N-terminal residues of nsP2 are involved in the regulation of the cleavage of the 1/2 site (as these residues form the P' side of the 1/2 site) and/or are important for the cleavage of the 2/3 site, as previously described (A. Lulla, Lulla, and Merits 2012). We replaced single residues 3–7 in the nsP2 N-terminus of SFV with alanines (the sequence of this region is VETPR; hence, the mutants were designated V3A, E4A, T5A, P6A and R7A, respectively) and studied the impact of these mutations on the processing of P123 of SFV. An *in vitro* transcription/translation/processing assay showed that all these substitutions resulted in slowed processing of P123 (**II**, Fig. 5B), and the effects were the strongest for the V3A and R7A mutations. The R7A mutation resulted in reduced amounts of individual nsP2 and nsP3 and in an increased amount of uncleaved processing intermediate P23, while the amount of nsP1 remained the same as for wt P123. Therefore, we concluded that the R7 residue is involved in the recognition of the 2/3 site and/or sequences in nsP3 that are needed for the cleavage of the 2/3 site to occur. The V3A mutation had a different effect on P123 processing, resulting in the accumulation of P123 instead of P23. Thus, it seems that the V3 residue (P3' residue) is important for the cleavage of the 1/2 site, and its substitution with Ala suppresses cleavage of the 1/2 site.

None of these substitutions had a remarkable effect on the infectivity of the corresponding RNA (data not shown), possibly because none accelerated either 1/2 or 2/3 site processing. However, when the substitutions of two charged

residues were combined (E4A+R7A), a clear ~38-fold drop in RNA infectivity was observed (**II**, Fig. 5C). However, the sequencing of the progeny of this virus did not reveal any potential compensatory mutations. Most likely, this indicates that the infectivity of viruses harboring these mutations was too high for rapid emergence and selection of better replicating variants. To enhance the negative impact of substitutions in the N-terminus of nsP2 on SFV replication, we used charge-reversal mutations (E4R+R7E). RNA harboring these changes had a very low infectivity (~1600-fold lower than that of RNA corresponding to wt SFV). Furthermore, the rescued virus had a small-plaque phenotype and diminished CPE development. SFV containing a single charge-reversal mutation (R7E) also had a decreased RNA infectivity (~63-fold lower than that of wt). Sequencing of plaque-purified isolates obtained from the progenies of these viruses identified no reversions or second-site mutations in nsP2; instead, various substitutions were found in nsP1 (D119N and M241V in the MTase domain), nsP3 (F311I in the zinc-binding domain) and the CP (K100T). Although the effects of these second-site mutations were not separately analyzed, they likely represent adaptive mutations, as the same or similar mutations have been previously described in the literature. Thus, D119N in nsP1 in SFV is responsible for the temperature-sensitive phenotype (ts14) (V. Lulla et al. 2006; 2008); the corresponding virus has decreased P1234 processing and accumulates the P12 processing intermediate (V. Lulla et al. 2008). Residue F311 in SFV nsP3 corresponds to residue F312 in SINV nsP3, and the F312S substitution in SINV also causes a temperature-sensitive phenotype (ts7) and has a negative impact on negative-strand RNA synthesis (Y. F. Wang, Sawicki, and Sawicki 1994). K100T in the CP causes a charge change. For VEEV, it has been shown that charge-neutralized capsids are incapable of packaging viral RNA, and such a defect can be compensated for by adaptive changes in the N-terminus of nsP2 (V3A and T5A/I) (Kim et al. 2013). Taken together, as mutations in the N-terminus of nsP2 cause the accumulation of potential adaptive changes in different viral proteins, it can be assumed that there are multiple functional interactions between these proteins. This finding supports the idea of nsP2 as a central protein in the alphaviral life cycle, interacting with and affecting the functions of other viral proteins.

## 5.3. Compounds targeting CHIKV nsP2 inhibit its protease activity and virus replication in cell culture (III)

The studies presented above, as well as extensive data available in the literature, have revealed nsP2 as a protein with a central role in the alphaviral infection cycle. Its protease activity is necessary for viral ns polyprotein processing; thus, nsP2 is involved in the activation of RNA replication (3/4 site cleavage) as well as in the regulation of replication at subsequent stages of viral infection (other cleavages). nsP2 also has RNA helicase/NTPase/RNA triphosphatase activities and many nonenzymatic functions. For example, the nsP2 of Old World alphaviruses is also needed for the suppression of cellular antiviral responses and for the shutdown of

host cell transcription. Given its importance for the virus, nsP2 is an attractive target for the development of inhibitors. If nsP2 is stopped in carrying out one or several of its crucial functions, the virus cannot successfully complete the infection cycle, and in the case of pathogenic alphaviruses, the disease will be prevented. As stated above, nsP2 has many functions that can be targeted. However, in practical terms, its protease activity is the most promising target, as protease inhibitors are already in use against HIV-1, HCV and SARS-CoV-2. Furthermore, the structure of the alphavirus nsP2 protease was resolved 17 years ago, while that of the RNA helicase region was published only in 2019. Consequently, there has been much more time to use the rational structure-guided approach to develop inhibitors against the protease activity of nsP2 and rather little time for the development of inhibitors targeting other enzymatic activities. Similar to several other teams, our research group has also participated in the discovery and study of inhibitors targeting the protease activity of nsP2 of CHIKV (Das et al. 2016; Larisa Ivanova, Rausalu, Žusinaite, et al. 2021). In publication **III**, the structure of a previously identified CHIKV inhibitor was further optimized. *In silico* molecular modeling data were used to select compounds that should strongly bind to the CHIKV nsP2 protease. In total, 158 novel compounds were designed and evaluated using *in silico* molecular docking to the CHIKV nsP2 protease. Based on the obtained data, 11 structures were selected, the corresponding compounds were synthesized, and their activities against nsP2 protease and CHIKV infection were analyzed.

To avoid the possibility that the chosen compounds inhibit virus infection by damaging the host cell, we first evaluated their cytotoxicity. Nine out of the 11 compounds were nontoxic at a concentration of 100 μM. These compounds were used to inhibit the replication of CHIKV expressing a nanoluciferase marker (CHIKV-NanoLuc), and the obtained data were used to calculate the $EC_{50}$ values of the compounds (**III**, Table 1). Seven compounds were found to have antiviral activity at concentrations <100 μM, among which D160 was the most potent, with an $EC_{50}$ of 10.5 μM. D160 has four stereoisomers, D160a-d (**III**, Scheme 2), which were separated and used in the antiviral assay. It was found that under the conditions used, stereoisomer D160d was the most active, with an $EC_{50}$ of 4.8 μM. The analysis performed using the stereoisomers of D160 demonstrated that the antiviral activity of this compound strongly depended on its spatial configuration; while all four stereoisomers were active, they had rather different $EC_{50}$ values, from 4.8 μM to 70.7 μM (**III**, Table 1). In addition to D160, the three next best anti-CHIKV compounds were compounds D119, D127 and D161, with $EC_{50}$ values of 32 μM, 29.6 μM and 13.9 μM, respectively (**III**, Table 1). All the compounds with the lowest $EC_{50}$ values were subsequently evaluated using a cell-free nsP2 protease inhibition assay. This assay showed that all the tested compounds (at a concentration of 1 mM) inhibited the protease activity of nsP2; in their presence, the amount of the substrate that remained uncleaved was clearly higher than that in the control reaction with the vehicle control only (no inhibitor). Owing to the limited sensitivity of the experiment (we used Coomassie blue staining of polyacrylamide gels to detect the substrate and its cleavage products), it was difficult to document decreases in the amounts of cleavage products; the

exception was compound D160a, which clearly had the strongest inhibitory effect in this assay (**III**, Fig. 4). Interestingly, D160a was not the most effective inhibitor in the CHIKV-NanoLuc based assay (**III**, Table 1). Such a discrepancy between results obtained using different assays is rather common and can be due to various factors and their combinations. These may be of technical nature and related to different concentrations of the enzyme and substrate, different cleavage conditions or different reaction times. The list of potential biological factors is also long, including the ability of the compound to enter the cell and its stability in the cellular environment. Finally, the goals that inhibitors need to achieve are also different. In CHIKV-infected cells, the largest inhibitory effect originates from blocking the cleavage of the 3/4 site, as this cleavage is required to activate RNA replication. In infected cells, this cleavage mostly (or exclusively) occurs in *cis.* In contrast, the reaction inhibited in the cell-free assay is the *trans* cleavage of a recombinant substrate.

Compounds D160a and D160d were selected for molecular dynamics simulations. This analysis revealed that neither of them formed any contacts with the catalytic dyad residues C478 and H548 (referred to as Cys1013 and His1083 in publication **III**, in which the numbering of amino acid residues was based on CHIKV P1234, whereas in this thesis, the numbering is based on the individual nsP2, as in publication **I**). Instead, numerous other interactions with amino acid residues located in two potential binding sites were revealed (**III**, Fig. 6). Several of these interactions involve residues that have been shown (or predicted) to be important for the protease activity of nsP2. For example, D160a interacts with W549 (Trp1084) (**III**, Fig. 6A), a residue that has been shown to be necessary for the protease activity of nsP2 of SFV (Golubtsov, Kääriäinen, and Caldentey 2006). D160d also interacts with W549 as well as with N547 (**III**, Fig. 6B). According to Narwal *et al*., there is a flexible loop in nsP2, containing the H548, W549 and N547 residues (the latter two are involved in substrate binding). This loop blocks access to the protease active site and substrate binding cleft (Narwal et al. 2018). Interaction of a compound with W549 and/or N547 and additional binding of one or more residues in the flexible loop (L668, E669, and L670, i.e., Leu1203, Glu1204, and Leu1205), which is involved in conformational changes occurring upon substrate binding to nsP2, may be important for the activity of the inhibitor. It is therefore likely that D160d inhibits CHIKV nsP2 by interfering with substrate binding rather than by binding the catalytic dyad residues. However, to verify this experimentally, one needs to cocrystallize the nsP2 protease with the inhibitor compound; as of now, such an analysis has not been performed, and the true mode of interaction of D160d (or D160a) with nsP2 remains unknown.

Finally, the abilities of D160a, D160d and D161 to inhibit high-MOI CHIKV infection were also analyzed. In this case, the effect of an inhibitor was evaluated by a decrease in the expression of the viral replicase (nsP1 and nsP2) and structural (CP) proteins, as detected using western blotting. In this assay, D161 and D160a inhibited the expression of viral proteins at 50 μM with a dose-dependent increase in the inhibitory effect (**III**, Fig. 7A, B). This minimal inhibitory concentration observed in this assay is higher than the $EC_{50}$ values (13.9 μM and

26.8 μM, respectively; **III**, Table 1) found for these compounds using CHIKV-NanoLuc under low-MOI conditions. This is not unexpected, as different experimental conditions and different methods used in these assays can both affect $EC_{50}$ values. In general, inhibitors tend to have a lower effect on high-MOI infection, and western blotting used in the second virus-based assay is less sensitive than the measurement of nanoluciferase activity. More interestingly (and somewhat confusingly), D160d, which had the lowest $EC_{50}$ value (4.8 μM; **III**, Table 1) in the first virus-based assay, was a less potent inhibitor of CHIKV in the second assay, in which only a mild inhibitory effect on viral protein expression could be observed at the highest concentration (200 μM) of the compound (**III**, Fig. 7C). Why does MOI have such a profound effect on the activity of an inhibitor? In contrast to high-MOI infection experiments, low-MOI infections include the virion formation and release steps from initially infected cells and the repetition of the infection cycle in previously uninfected cells. It is conceivable that D160d may affect those additional steps of viral infection. Not far from the potential inhibitor-binding sites is the nsP2 linker region (nsP2 aa 463-468, located between the nsP2 N-terminal RNA helicase and C-terminal protease parts), which provides the flexibility to the nsP2 structure. If CHIKV nsP2 aa 463-468 are deleted, the viral replicase remains active (some decrease in activity is observed), while the infectivity of the corresponding RNA drops to zero, and no virus can be rescued (Law et al. 2021). It is conceivable that if an inhibitor binds near the linker region of nsP2, it may affect the flexibility of the nsP2 structure and thus have an adverse effect on virus production. This could explain why D160d inhibited CHIKV-NanoLuc at a low MOI, i.e., in the assay that involved multiple rounds of infection, more effectively than in the assay in which all cells were synchronously infected at a high amount of the virus.

As a result of optimizing the structure of a previously discovered CHIKV inhibitor 1c (Das et al. 2016), we managed to obtain compound D160d, which has an $EC_{50}$ value of 4.8 μM, i.e., approximately 10-fold lower than the $EC_{50}$ value of 1c (50 μM). The most likely mechanism of inhibition for compound D160 and its isomers seems to be the interference with conformational changes in nsP2 that are needed for protease substrate binding. It is also likely that the inhibitor affects some other process or processes that are crucial for the release of new virus particles and their spread in cell culture. As the development of inhibitory compounds has an ultimate goal to develop highly active antiviral drugs, it is good to ask how far from this goal we are. The honest answer is that our compounds are, at best, confirmed hit compounds. The possibility that D160 uses a unique mechanism of action (prevents binding of a substrate to the protease) and likely affects other nsP2-mediated activities in infected cells looks promising; targeting more than one function should increase the genetic barrier of resistance (i.e., make it difficult for a virus to develop resistance). However, optimizing a hit compound in such a way that all its antiviral activities would be increased is very challenging, and whether we like it or not, compounds with a single mechanism of action may be easier to optimize, and therefore, they may be more promising drug leads.

# 6. CONCLUSIONS

Alphavirus nsP2 has long been considered a papain-like cysteine protease. However, a recently published article claimed CHIKV nsP2 to be different from previously studied alphaviral proteases. As proteins of viruses belonging to the same genus may differ in how they carry out their functions, it seemed important to us to clarify this issue. Therefore, we considered it important to perform thorough research on the properties of CHIKV nsP2 protease activities.

Viral proteases do not cleave all available substrates, whenever and wherever possible, which would prevent the formation of replicase complexes. Thus, in addition to the ability of nsP2 to function as a protease, it is also important to understand how the protease activity of nsP2 is regulated. Processing of the viral nonstructural polyprotein P1234 determines the proceeding of the viral life cycle. Cleavage between nsP3 and nsP4 in P1234 activates the polymerase activity of nsP4 and initiates the synthesis of viral negative-strand RNA. The next cleavage, between nsP1 and nsP2, marks the completion of replicase complex formation and the switch from negative-strand to positive-strand genomic RNA and subgenomic RNA synthesis. This switch is made irreversible by the cleavage of the final bond, between nsP2 and nsP3, which marks a point of no return during alphaviral infection. Hence, we were interested in whether/how the amino acid sequences of the cleavage sites are involved in the regulation of the time of these cleavages.

As nsP2 protease function is essential for the viability of alphaviruses, it is an attractive target for the development of antivirals. The structure of a previously revealed CHIKV protease inhibitor was modified in several ways, and we analyzed how these new inhibitor compounds affected the activity of the CHIKV nsP2 protease and CHIKV replication in cell culture.

The data obtained in the studies included in this thesis allow a better understanding of the functioning of alphaviral (CHIKV/SFV/SINV) nsP2 proteases. The main conclusions that can be drawn from our research are as follows:

1. The requirements of CHIKV nsP2 for its protease activity are quite similar to those of previously studied alphavirus-encoded proteases. The 3/4 cleavage site has the sequence most favorable for nsP2-mediated cleavage and is easily accessible for the enzyme; therefore, in P1234, the protease cleaves it earlier and more efficiently than the other cleavage sites. The CHIKV nsP2 protease also cleaves a substrate containing a short (10:5) peptide 3/4 site and a similar substrate representing the 1/2 site. In contrast, the 10:5 substrate corresponding to the 2/3 site cannot be cleaved; to be processed, the 2/3 site substrate must contain a longer (10:170) peptide. This cleavage also requires full-length nsP2 with an intact N-terminus; CHIKV nsP2 tolerates only a very short insertion of two aa at the N-terminus without adverse effects, while deletion of two aa from the N-terminus abolishes the ability of the enzyme to cleave the 2/3 site.

2. Similar to proteases of other alphaviruses, CHIKV nsP2 is definitely a cysteine protease, and the active site residue C478 is absolutely required for the nsP2 protease activity. The function of this residue cannot be replaced by a nearby S482 residue. If C478 is replaced by Ala, either alone or in combination with a mutation of S482 (C478A and C478A+S482A mutations), the protease activity of nsP2 is lost. Likewise, the substitution of alanine for W479 abolishes CHIKV nsP2 protease activity. In contrast to the C478A or W479A mutation, the S482A substitution has no significant adverse effect on CHIKV nsP2 protease activity. The same principle applies not only to the activity observed using *in vitro* protease assays but also to other studied aspects of the viral life cycle. The C478A and W479A mutations abolish:
   a. the processing of CHIKV P1234 (both in a cell-free assay and in transfected cells)
   b. the activation of reporter expression in the CHIKV *trans*-replication assay
   c. viral RNA synthesis by the CHIKV replicase
   d. the rescue of infectious CHIKV from *in vitro* transcribed RNAs corresponding to the virus genome.

   In contrast, the S482A substitution has none of these negative effects. In $CHIKV^{S482A}$-infected cells, the processing of P1234 is unaffected, and the formation of spherules, cytopathic vesicles and virions is similar to that in cells infected with the wild-type virus.

3. Temporal regulation of cleavage between nsP1 and nsP2 is necessary for the correct formation and functioning of the alphaviral replicase. The timing of cleavage at the protease cleavage sites in P1234 is determined by several factors, including the sequences of the cleavage sites and the activity of nsP2. Mutations that cause accelerated/decelerated cleavage of the 1/2 and/or 2/3 sites, either due to changes in cleavage site sequences or in the activity of nsP2, have a major impact on corresponding viruses. Regardless of the reason (mutation in the cleavage site or in the protease), all mutations causing premature cleavage of the 1/2 cleavage site have detrimental effects on virus RNA infectivity. Analysis performed using the *trans*-replicase assay revealed that this was due to a major negative effect on the ability of a mutant replicase to carry out the synthesis of viral RNAs. Optimization/deoptimization of cleavage sites in P1234 of SFV and SINV revealed that residues occupying positions P6-P4 in the 1/2 and 2/3 cleavage sites were important for the determination of the efficiency of site cleavage by the nsP2 protease. Mutational analysis of the N-terminus of nsP2 of SFV (residues 3–7, also representing the P' side of the 1/2 cleavage site) revealed that alanine substitutions or charge-reversal mutations in this region might negatively affect P1234 processing and viral RNA infectivity. Some viruses harboring such mutations formed small plaques, had delayed development of cytopathic effect and accumulated second-site mutations in various other viral proteins.

4. Analysis of various novel compounds based on the structure of a previously analyzed inhibitor of the CHIKV nsP2 protease led to the identification of a compound (D160d) that had approximately ten times increased anti-CHIKV activity in cell culture.

Taken together, this research confirmed that the alphavirus P1234 polyprotein processing carried out by nsP2 is a well-orchestrated process that is crucial for virus infectivity and that affects multiple aspects of the virus infection cycle.

# SUMMARY IN ESTONIAN

## Alfaviiruste nsP2 proteaas: nõuetest funktsionaalsuseks inhibitsioonini

Alfaviirused kuuluvad perekonda *Alphavirus* ja sugukonda *Togaviridae*. Alfaviiruste genoom on positiivse polaarsusega RNA, 10–12 kb pikk ning virion on umbes 70 nm diameetriga. Viirused on ümbritsetud membraaniga, mis pärineb rakumembraanidest. Alfaviirused on arboviirused, mis tähendab, et viirus levib selgroogsetele putukvektorite vahendusel. Mitmed alfaviirused nakatavad ka inimesi ning võivad põhjustada tõsiseid haiguseid. Üks viirustest, mis on meditsiiniline probleem, on Chikungunya virus (CHIKV). CHIKV on levinud peamiselt troopilistes piirkondades ning põhjustab haigust, mille peamisteks sümptomiteks on lööve, palavik, peavalu ja liigesevalud. Kuigi esmane äge haigusefaas möödub umbes paari nädalaga, siis piinavad liigesevalud võivad kesta kuid või isegi aastaid peale nakatumist (Schwartz and Albert 2010; Suhrbier, Jaffar-Bandjee, and Gasque 2012). Hetkel on CHIKV nakkuse ravi vaid sümptomite leevendamine, pole heaks kiidetud CHIKV-vastast ravimit ega vaktsiini.

Alfaviiruste genoomis on kaks avatud lugemisraami, millest esimese pealt sünteesitakse mittestruktuurne liitvalk (P1234) ning teise pealt struktuurne liitvalk (sisaldab kapsiidivalku ja viiruse glükoproteiine). P1234 lõikamist individuaalseteks valkudeks (nsP1-nsP4) viib läbi viiruse proteaas, nsP2, ning see on oluline protsess viiruse elutsüklis. Kui proteaas pole funktsionaalne, siis pole viirus võimeline rakku edukalt nakatama. Proteaasi töö on rangelt reguleeritud, näiteks mittestruktuurse liitvalgu lõikamine nsP1 ja nsP2 vahel peab toimuma täpselt õigel ajal. Kui see lõikamine toimub liiga kiiresti, siis on viiruse nakatamisvõime tugevalt langenud. nsP2-l on ka teisi funktsioone lisaks proteaasi funktsioonile, see on ka viiruse RNA helikaas ning nsP2 osaleb nakatunud raku transkriptsiooni, translatsiooni ning viirusvastase reaktsiooni maha surumises.

Alfaviiruste nsP2 on pikalt peetud papaiin-sarnaseks tsüsteiin-proteaasiks. Samas, ühes mõne aasta eest ilmunud artiklis väideti, et CHIKV nsP2 on erinev varem-uuritud alfaviiruste proteaasidest. Ühte perekonda kuuluvate viiruste valgud võivad tõepoolest olla erinevad selles, kuidas need oma funktsiooni teostavad. CHIKV on meditsiiniliselt oluline patogeen ning kui CHIKV nsP2 tõesti erineks teistest alfaviiruste proteaasidest, siis tuleks seda arvesse võtta näiteks viirusvastaste ravimite välja töötamisel. Seetõttu pidasime oluliseks viia läbi põhjalikud uuringud CHIKV nsP2 proteaasi omaduste kohta.

Viiruste proteaasid ei lõika kõiki võimalikke substraate kusiganes ja miliganes võimalik – see takistaks replikatsioonikomplekside moodustumist. Lisaks nsP2 funktsionaalsusele proteaasina on seega oluline ka kuidas on reguleeritud nsP2 proteaasi aktiivsus. P1234 mittestruktuurse liitvalgu lõikamine määrab kuidas kulgeb viiruse elutsükkel. Lõikamine nsP3 ja nsP4 vahel aktiveerib nsP4 polümeraasi aktiivsuse ja algatab viraalse negatiivse polaarsusega RNA sünteesi. Järgmine lõikamine nsP1 ja nsP2 vahel viib lõpule replikatsioonikompleksi moodustumise ning sellega kaasneb ümberlülitumine positiivse polaarsusega

genoomsete ja subgenoomsete RNA-de sünteesile. Selle ümberlülituse muudab pöördumatuks viimane lõikamine nsP2 ja nsP3 vahel. Seetõttu olime huvitatud kas ja kuidas mõjutab proteaasi lõikesaitide aminohappeline järjestus aega, millal toimub lõikamine.

nsP2 proteaasi funktsioon on kesksel kohal alfaviiruste nakatamisvõime määramisel ning see muudab nsP2 atraktiivseks sihtmärgiks viirusvastaste ühendite arenduses. Seetõttu analüüsisime, kuidas ühe varasemalt kirjeldatud CHIKV proteaasiinhibiitori modifitseerimise tulemusena saadud uudsed ühendid mõjutavad CHIKV proteaasi aktiivsust *in vitro* ning CHIKV replikatsiooni rakukultuuris.

Selles väitekirjas sisalduvate uuringute tulemusena saadud teadmised aitavad paremini mõista kuidas funktsioneerib alfaviiruste (CHIKV/Semliki Forest viirus (SFV)/Sindbis viirus (SINV)) nsP2 proteaas. Peamised järeldused, mida meie uurimistöö põhjal saab teha on järgnevad:

1. CHIKV nsP2 proteaasi omadused on sarnased varem-uuritud alfaviiruste proteaasidele. nsP3 ja nsP4 vahelise lõikekoha (edaspidi 3/4 lõikekoht; ja teised lõikekohad samas stiilis) aminohappeline järjestus on nsP2 vahendatud lõikamise efektiivsuse vaatenurgast eelistatud järjestus ning ka ensüümile kergesti ligipääsetav. Seetõttu lõikab proteaas seda lõikekohta P1234 liitvalgus varem ja efektiivsemalt kui teisi lõikekohti. CHIKV nsP2 proteaas lõikab substraate, mis sisaldavad lühikest (10:5 aminohapet lõikekoha ümber) peptiidi 3/4 lõikekohaga sama hästi kui substraate 1/2 lõikekohaga. 2/3 lõikekohaga substraate, milles on lühike (10:5) lõikekoha peptiid CHIKV nsP2 ei lõika. Selle lõikamise toimumiseks peab lõikekoha peptiid olema pikem (10:170). See lõikamine vajab toimumiseks veel täispikka nsP2-e, millel on natiivne N-ots. CHIKV nsP2 talub ainult väga lühikesi insertsioone (kaks aminohapet) N-otsas ilma negatiivsete tagajärgedeta valgu aktiivsusele ning kahe aminohappe pikkune deletsioon nsP2 N-otsas hävitab ensüümi võime lõigata 2/3 lõikekohta.

2. Sarnaselt teiste alfaviiruste nsP2-dele on ka CHIKV nsP2 tsüsteiin-proteaas ning proteaasi aktiivsaidi aminohape C478 on vältimatult vajalik proteaasi aktiivsuseks. Lähedalasuv S482 pole võimeline proteolüüsil asendama C478-t. Kui C478 asendada alaniiniga, kas üksi või kombinatsioonis S482-ga (C478A ja C478A+S482A mutatsioonid), siis kaotab CHIKV nsP2 oma proteaasi aktiivsuse. Samuti hävitab CHIKV nsP2 proteaasi aktiivsuse W479 asendamine alaniiniga. Erinevalt C478A ja W479A mutatsioonidest, S482A asendusel pole märkimisväärset negatiivset mõju CHIKV nsP2 proteaasi aktiivsusele. Põhimõtteliselt sarnased tulemused saime lisaks *in vitro* proteaasikatsetele ka teisi viiruse elutsükli aspekte uurides. C478A ja W479A mutatsioonid hävitavad:
   a. CHIKV P1234 lõikamise individuaalseteks valkudeks (nii raku-vabades katsetes kui transfekteeritud rakkudes)

b. Reportevalkude ekspressiooni aktiveerimise *trans*-replikatsioonikatses
c. Viraalse RNA sünteesi CHIKV replikaasi poolt
d. Nakatamisvõimelise CHIKV kogumise rakkudest, mis on transfekteeritud viiruse genoomile vastava *in vitro* transkribeeritud RNA-ga

S482A mutatsioonil pole aga ühtegi neist negatiivsetest efektidest. $CHIKV^{S482A}$-ga nakatatud rakkudes on P1234 liitvalgu lõikamine ning sfäärulite, tsütopaatiliste vesiikulite ja virionide moodustumine sarnane metsiktüüpi CHIKV-ga nakatatud rakkudele.

3. nsP1 ja nsP2 vahelise lõikamise ajaline regulatsioon on vajalik alfaviiruse replikaasi korreksteks formeerumiseks ja funktsioneerimiseks. P1234 proteaasi lõikekohtade lõikamise aeg on määratud mitme erineva faktori koosmõjus, sealhulgas lõikekoha aminohappeline järjestus ning nsP2 proteaasi aktiivsus. Mutatsioonid 1/2 ja/või 2/3 lõikekohtade või proteaasi aminohappelises järjestuses, mis mõjutavad proteaasi aktiivsust nii, et liitvalgu lõikamine kiireneb/aeglustub, on viiruse jaoks suure mõjuga. Olenemata põhjusest, kõigil mutatsioonidel, mis põhjustavad nsP1 ja nsP2 vahelise lõikamise kiirenemise, on hävitav mõju viiruse RNA nakatamisvõimele. *Trans*-replikatsioonikatse näitas, et selle põhjuseks on tugev negatiivne efekt mutantse replikaasi võimele sünteesida viraalseid RNA-sid. SFV ja SINV P1234 lõikekohtade optimeerimine/deoptimeerimine demonstreeris, et 1/2 ning 2/3 lõikekohtade P6-P4 positsioonides olevad aminohapped on olulised määramisel kui efektiivselt nsP2 proteaas seda lõikekohta lõikab. SFV nsP2-e N-otsa mutatsioonanalüüs (aminohapped 3–7, P' pool 1/2 lõikekohas) näitas, et aminohapete asendamine alaniiniga või laengu ümberpööramismutatsioonid selles piirkonnas võivad mõjuda P1234 lõikamisele ja viraalse RNA nakatamisvõimele negatiivselt. Mõned viirused nende mutatsioonidega moodustasid väikseid lüüsilaike, olid hilinenud tsütopaatilise efektiga ning kogusid uusi mutatsioone erinevates viirusvalkudes.

4. Teadaoleva CHIKV nsP2 proteaasiinhibiitori struktuuri erinevate modifikatsioonidega saadud ühendite analüüsil leiti uus ühend (D160d), millel on rakukultuuris umbes kümme korda suurenenud anti-CHIKV aktiivsus võrreldes lähteühendiga.

Kokkuvõtteks, meie uurimistöö kinnitas, et alfaviiruste nsP2 poolt läbiviidav P1234 liitvalgu lõikamine on hästi-reguleeritud protsess, mis on ülioluline viiruse nakatamisvõime jaoks ning mõjutab mitmeid aspekte viiruse infektsioonitsüklis.

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# PUBLICATIONS

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**Publications**

Nefedova, Alexandra; **Rausalu, Kai**; Zusinaite, Eva; Vanetsev, Alexander; Rosenberg, Merilin; Koppel, Kairi; Lilla, Stevin; Visnapuu, Meeri; Smits, Krisjanis; Kisand, Vambola; Tätte, Tanel; Ivask, Angela (2022). Antiviral efficacy of cerium oxide nanoparticles. Scientific Reports, 12, 18746. DOI: 10.1038/s41598-022-23465-6.

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