Realia et naturalia

DISSERTATIONES
BIOLOGICAE
UNIVERSITATIS
TARTUENSIS
281

PRATYUSH KUMAR DAS

Biochemical perspective on alphaviral nonstructural protein 2: a tale from multiple domains to enzymatic profiling

TARTU ÜLIKOOL · UNIVERSITAS TARTUENSIS · 1632

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
281

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
281

PRATYUSH KUMAR DAS

Biochemical perspective on alphaviral nonstructural protein 2: a tale from multiple domains to enzymatic profiling

UNIVERSITY OF TARTU
PRESS
1632

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

This dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Gene Technology on 1st of July, 2015 by the Scientific Council of the Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu, Estonia.

Supervisor: Andres Merits, PhD, Professor of Applied Virology, Institute of Technology, University of Tartu, Estonia

Co-supervisor: Aleksei Lulla, PhD, Research Scientist, Institute of Technology, University of Tartu, Estonia

Reviewer: Juhan Sedman, PhD, Professor and Head, Chair of General and Microbial Biochemistry, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Opponent: Bruno Canard, PhD, CNRS Directeur de Recherche and Principal Investigator Viral Replication: Structure, Mechanisms and Drug Design, Architecture and Functions of Biological Macromolecules Laboratory, Aix-Marseille Université, Marseille, France

Visiting Scientist, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA

Commencement: Estonian Biocentre auditorium, Riia 23B-105, Tartu on 9th of October 2015 at 12.15.

This research is supported by European Social Fund's Doctoral Studies and Internationalisation Programme DoRa, which is carried out by Foundation Archimedes.

The publication of this dissertation is granted by the Institute of Technology, Faculty of Science and Technology, University of Tartu and by the Graduate School in Biomedicine and Biotechnology created under the auspices of European Social Fund.

ISSN 1024-6479
ISBN 978-9949-32-914-4 (print)
ISBN 978-9949-32-915-1 (pdf)

University of Tartu Press,
www.tyk.ee

Dedicated to my beloved mother and late father;
Sanjukta Das and Pratap Kumar Das

“To invent, you need a good imagination and a pile of junk.”

Thomas A. Edison

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications, referred to in the text by their associated Roman numeral.

I. Rathore, A.P.S., Haystead, T., **Das, P.K.**, Merits, A., Ng, M.L., and Vasudevan, S.G. (2013). Chikungunya virus nsP3 & nsP4 interacts with HSP-90 to promote virus replication: HSP-90 inhibitors reduce CHIKV infection and inflammation in vivo. Antiviral Res. 103C, 7–16.

II. **Das, P.K**., Merits, A., and Lulla, A. (2014). Functional Crosstalk between Distant Domains of Chikungunya Virus Non-Structural Protein 2 Is Decisive For Its RNA-Modulating Activity. J. Biol. Chem. jbc.M113.503433.

III. Utt, A., **Das, P.K.**, Varjak, M., Lulla, V., Lulla, A., and Merits, A. (2015). Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2. J. Virol. 89, 3145–3162.

*Some unpublished data have also been presented or referred to in this thesis.

The articles listed above have been printed with the permission of the copyright owners.

My personal contribution to the above mentioned articles is detailed as follows:

I. I purified the recombinant CHIKV RdRp (nsP4) that was used to raise rabbit anti-nsP4 polyclonal serum.
II. I purified all the recombinant proteins, designed and performed all the experiments, analyzed the data and wrote the manuscript.
III. I purified the wild type and mutant recombinant nsP2s, carried out their CD spectroscopic analysis, designed and performed all the biochemical experiments, analyzed the data and participated in the preparation and writing of the manuscript.

LIST OF ABBREVIATIONS

aa	amino acid (residue)
ATPase	Adenosine triphosphatase
BFV	Barmah Forest virus
BHK	Baby Hamster Kidney
C	Capsid
CD	Circular Dichroism
CHIKV	Chikungunya virus
CoV	Coronavirus
CPV	cytopathic vacuole
CSE	conserved sequence element
DENV	Dengue virus
ds	double-stranded
E	Envelope protein
EAV	Equine arteritis virus
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
GTPase	Guanosine triphosphatase
HCV	Hepatitis C virus
HEV	Hepatitis E virus
HIV-1	Human immunodeficiency virus type 1
MCM	Mini-chromosome maintenance
MD	Macro domain
MTL	Methyltransferase-like
NA	nucleic acid
NCT	noncytotoxic
NLS	Nuclear localization signal
ns (nsP)	nonstructural (protein)
NTPase	Nucleoside triphosphatase
NTR	Non-translated region
nts	nucleotide (s)
p.i.	post-infection
PDB	Protein data bank
RdRp	RNA dependent RNA polymerase
RRV	Ross River virus
RTPase	RNA triphosphatase
SARS	Severe acute respiratory syndrome
SF	Superfamily
SFV	Semliki Forest virus
sg	Subgenomic
SINV	Sindbis virus
SL	stem-loop
ss	single-stranded
TATase	Terminal adenylyltransferase

TEV	Tobacco Etch Virus
TF	Transframe
ToMV	Tomato mosaic virus
VEEV	Venezuelan equine encephalitis virus
VV	Vaccinia Virus
WEEV	Western equine encephalitis virus
WNV	West Nile virus
wt	wild-type
YFV	Yellow Fever virus
ZBD	Zinc binding domain

1. PREFACE

1.1. Viruses

In 1892, Dmitri Iosifovich Ivanovsky, by employing Chamberland filter which had pores smaller than the size of bacteria, was able to demonstrate the existence of non-bacterial infectious agents, which in the later time came to be known as 'viruses'. However, the word 'virus,' which was apparently derived from Sanskrit, has an overall meaning of negativity comparable to 'wiesel' in German, 'weasel' in English or 'vison' in French, all of which refer to an animal that sprays a strong fluid when threatened. Whatever might be the origin of this important word since its foundation, the science now called 'virology' has been expanded and has travelled a great distance (1–3). In the simplest terms, viruses can be described as small obligate parasites with an RNA or DNA genome surrounded by a protective coat (4, 5).

These tiny sub-microscopic entities are found throughout the ecosystem, including under extreme conditions of temperature and solvent medium (6). For example, it has been proposed that the ocean contains approximately 10^{31} virus particles; these particles contribute close to 20% of oceanic biomass production per day. If these number of viruses could be placed side by side, it could easily cross nearest 60 galaxies to the planet Earth (7). As viruses can invade any form of life starting from archaea to prokarya to eukarya, they represent a very significant repository and combination of genetic diversity (2, 6).

1.2. Classification of viruses

The rules set by the International Committee on Taxonomy of Viruses (ICTV) along with the Baltimore system are primarily used to classify viruses at the present time (8, 9). The ICTV system guides the classification starting from order, family, subfamily, genus to species level, while the Baltimore classification system is based on the basic viral strategy to produce mRNA from the genetic material of the virus (8, 9). According to the Baltimore classification, viruses are classified into seven different groups as follows (Fig. 1).

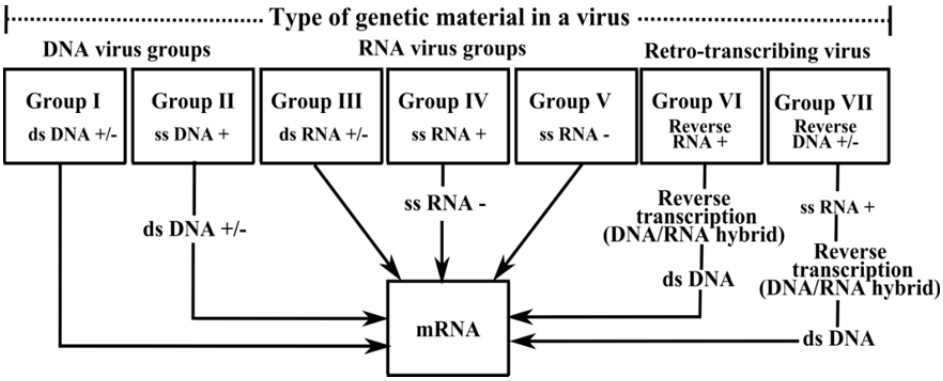


Figure 1. Baltimore classification of viruses according to their genetic material; adapted from *http://viralzone.expasy.org* (8).

1.3. Size of viruses

Viruses have evolved with amazing diversity in their size and shape. They range from as small as 20 nm to as large as 1000 nm in size, while their shapes are equally astonishing. For example, there are filamentous viruses such as Ebola virus, helical viruses such as Tobacco Mosaic Virus, icosahedral viruses such as rhinovirus and complex shapes such as T7 bacteriophage could be put forth. However, due to the focused nature of the current thesis, I have tried to very briefly outline the average sizes of some of the most common viruses to compare their relatively miniature natures (Table 1).

Sr No.		**Resolution of**		**Average Size (nm)**
1		Human eye		>100,000
2		Light microscope		200
3		Electron microscope		2
Ref.	http://www.biology.arizona.edu/cell_bio/tutorials/cells/cells2.html			
Sr No.		**Size of**		**Average Size (nm)**
4		Plant cell		100,000
5		Animal Cell		10,000
6		Bacterial cell		1000
7		Length of an aa		0.34 (10)
Ref.	http://www.biology.arizona.edu/cell_bio/tutorials/cells/cells2.html			
Sr No.	**Family**	**Representative member**	**Associated Disease**	**Average Size (nm)**
8	Pandoravirida	Pandoravirus	None yet	1000 (11)
9	Mimiviridae	Mimivirus	None yet	800 (12)
10	Poxviridae	Variola virus	Smallpox	200–300
11	Paramyxoviridae	Measles virus	Measles	80–250
12	Herpesviridae	Herpesvirus	Herpes	150–160
13	Rhabdoviridae	Rabies virus	Rabies	70–180
14	Coronaviridae	CoV	SARS	80–160
15	Adenoviridae	Adenovirus	Common cold	70–80
16	Reoviridae	Rotavirus	Diarrhea	60–80 (13)
17	Togaviridae	Rubella virus	Rubella	60–80
17	Papillomaviridae	Human papillomavirus	Cervical cancer	40–60 (14)
18	Hepadnaviridae	Hepatitis B virus	Hepatitis B	42
19	Picornaviridae	Poliovirus	Polio	20–30
20	Parvoviridae	Parvovirus	Fifth disease	18–22 (15)
Ref.	http://www.elu.sgul.ac.uk/rehash/guest/scorm/343/package/content/size.htm			

Table 1. Viruses show amazing diversity in their sizes.

2. INTRODUCTION

Chikungunya Virus (CHIKV), a representative member of the Alphavirus genus, is a relatively small RNA virus which is primarily transmitted to humans by mosquitos and causes high fever, skin rash, headache, muscle pain and polyarthralgia. Currently there is no approved therapeutics or vaccines against this pathogen. Furthermore, the current level of understanding regarding the mechanisms of infection and hijacking of the host cell machinery is broadly drawn from studies of Old World alphaviruses such as SINV, SFV, which are similar but certainly not identical to CHIKV.

The nonstructural proteins (nsPs), which are the prime gears of CHIKV, are the main components that empower the virus to ensure the establishment of RNA replication machinery inside the host cell and channel or modulate host cell resources to support viral replication to complete a successful cycle of infection. However, the roles of individual ns proteins are still very incompletely understood; in consequence, our understanding of the overall replication of this highly medically important virus is clearly inadequate. Moreover, it is apparent that the isolated individual ns proteins could behave differently than their counterparts inside finely calibrated and highly sophisticated replication complexes. Characterizing the biochemical and biological functions of the nsPs in these two entirely different settings is essential to understand and analyze the regulation of their enzymatic activities. Along with biophysical and structural approaches, these strategies could lead towards the ultimate goal of cultivating an understanding the molecular alterations occurring in these greatly organized macromolecular productive assemblies, and characterization of which is inevitably required to understand the overall molecular biology underlying virus replication. In this thesis, I summarize several studies that aimed to biochemically and biophysically address some of these aspects of viral enzymatic proteins in a relatively comprehensive and fine-tuned manner.

Out of the four ns proteins, nsP2 is exceedingly multitalented. It not only harbors a number of vital enzymatic functionalities for assisting the viral RNA replication but also represents quite possible determinant of the cytopathic properties of the virus. Thus, the aim of these studies was to biochemically analyze the properties of this protein in relation to RNA modulation, one of the leading functions of this comparatively large viral protein, in the context of a biochemically purified recombinant protein. Furthermore, an attempt was made to determine how the individual domains of nsP2 (in the context of a multidomain protein arrangement) contribute towards the RNA-associated functionalities of nsP2. The information gathered in such studies can be used towards the ultimate objective of proposing and standardizing a biochemical platform that would be essential for the screening of small molecule inhibitors capable of constraining CHIKV infection. In addition, the biochemical expertise developed during this study has been further used to develop alphavirus research tools such as antibodies, which could enhance our understanding of virus replication and virus-host interactions. These antibodies were not only confined to our lab

in Tartu, Estonia but were also used for global collaborative studies; by our best estimation, these antibodies represent the most efficient (and in some cases unique) tools for the detection of CHIKV proteins. As of March 2015, over 20 laboratories on all continents (with the obvious exception of Antarctic) have requested and obtained these tools. Accordingly, these antibodies have been used for localizing alphaviral individual ns proteins inside the host cell, to determine their cellular interacting partners, to analyze the dynamics of viral protein synthesis, to study the effect of antiviral compounds on viral replication etc. Similarly, expression constructs created during these studies are in use by at least ten different research teams. This highlights what is the last, but not the least, aim of these studies: to contribute to international collaboration and facilitate studies of important emerging pathogen.

3. REVIEW OF LITERATURE

3.1. Alphavirus

3.1.1. An overview of Alphaviruses

Alphavirus like CHIKV belongs to the family *Togaviridae*. CHIKV was first reported during the 1952-53 epidemic in Makonde plateau in Tanzania, where it was isolated from the serum of a febrile patient (16). The name "Chikungunya" was derived from a word spoken by a population in the Mozambique region that means "bends up," referring to the arthritic symptoms frequently caused by this virus (17). Recently, in 2005, CHIKV, which was known for its wide-spread geographical spectrum, re-emerged in East Africa and was transmitted to La Reunion Island located in the Indian Ocean, where it was reported to cause over 300,000 cases. Subsequently, the virus was transmitted to India with an estimation of six million infected cases, while European countries such as Italy and France as well as commonwealth countries such as Australia and also regions of southeast Asia were severely affected by this virus (18–27). The overlap between symptoms of CHIKV infection, which is characterized by high fever, head ache, nausea/vomiting, persistent myalgia/arthralgia and maculo papular rash, and those of DENV often leads to mis-attribution of the causative agent, instigating a severe socio-economic burden (28, 29). As DENV is more common than CHIKV, the number of actual CHIKV cases may be severely underestimated. The virus is also a great traveler: in November 2013 it emerged in the Caribbean islands and has by now (as of March 2015) affected more than 30 countries in the region (including the Americas), with more than 1,300,000 confirmed and suspected cases (http://www.paho.org). Currently, there are no therapeutics available against this virus, which demonstrates that we still lack an essential understanding of the mechanism employed by this virus to cause disease (20, 30). A recent review summarizes it all to a very simple expression: "CHIKV no longer simply threatens; it has arrived as a significant, global pathogen" (Fig. 2) (31).

Based on serological cross-reactivity, the genus Alphavirus contains seven groups of viruses (called serogroups). Out of these, the VEEV, SFV and SINV groups are most prominent and accordingly are the best-studied. CHIKV belongs to the SFV serogroup of alphaviruses, which also comprises ONNV (the closest relative of CHIKV) and RRV (which has been well studied by different research groups as it is endemic and causes regular outbreaks in Australia and Oceania) (25). There are also members of the serogroup that have received little if any attention. Before 2004, CHIKV used to be one of these; however, this has been considerably improved. Despite efforts in the last 10 years to study CHIKV, still it remains relatively inadequately investigated, and many aspects of the molecular biology of this highly infective virus (such as the basis of chronic infection) are still completely enigmatic.

CHIKV is an arbovirus that is transmitted by the *Aedes* species of mosquitos, primarily by *A. aegypti* (more common in urban areas) and *A. albopictus* (more common in rural areas) (32). *A. albopictus* has, however, expanded its distribution and is currently present in metropolitan areas of Europe and the United States of America (17). It has been suggested that CHIKV had been maintained in a sylvatic cycle between wild primates and *Aedes* mosquito species in Africa, from where it spread to Asia (33). Whatever may be the cause, CHIKV is capable of establishing an urban cycle (human-mosquito-human) of transmission. Due to this ability, it has (since its discovery) caused more than ten million patient cases worldwide.

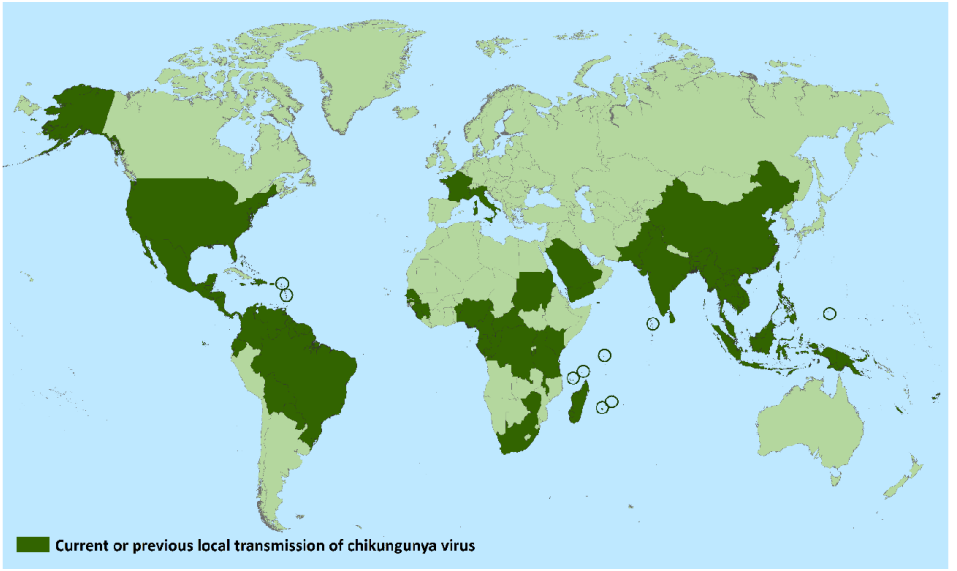


Figure 2. Global distribution of CHIKV as of March 10, 2015; www.cdc.gov/chikungunya/geo/index.html

CHIKV clearly originates from Africa. It has three different genotypes. Currently, most of the available data concerning the functions of the ns proteins originate from studies of proteins encoded by viruses belonging to the East/Central/South African (ECSA) genotype (typically from the Indian Ocean outbreak of 2005-2007). Two other genotypes of CHIKV, West African (WA) and Asian, have been studied to much lesser extent. It should be noted that the ns polyproteins encoded by viruses belonging to different genotypes demonstrate 95% (WA compared to the other two genotypes) to 97% (ECSA compared to the Asian genotype) identity. While the basic functions of ns proteins from viruses belonging to different genotypes are likely similar (possibly identical), a 3-5% difference in the aa sequence certainly suggests the possibility

that some of the non-enzymatic functions of nsPs encoded by different CHIKV genotypes may be substantially different.

3.1.2. Virion

Like other members of the genus Alphavirus, CHIKV is relatively small (~70 nm), spherical, and produces enveloped virions (25, 34–37). The structure of the whole CHIKV virion has been studied by electron microscopy, while the individual structural proteins have been investigated using X-ray crystallography methods. Such studies have revealed important insights into the overall organization of the whole virion as well as details about the structures of its surface proteins (38, 39). The cryo-electron microscopic structure of the CHIKV virion-like particle (VLP) reveals that its overall structural organization is quite similar to virions of other alphaviruses (Fig. 3) (40, 41). The alphavirus virion has a molecular mass of approximately 5.2 x 10^6 Da and carries a protein-NA complex called the "nucleocapsid" which is a firm assembly of a positive strand viral genomic RNA with an approximate size of 11.5 kb and 240 copies of capsid protein arranged in icosahedral symmetry with a diameter of ~40 nm and triangulation number of four (T=4) geometry (25, 42, 43). The 110 N-terminus aa of the capsid protein, which is assumed to lack defined structure, is positively charged, corroborating its interaction with viral genomic RNA (44). The nucleocapsid is further enclosed by a close-fitting, host-derived lipid bilayer that is also covered by an arrangement of almost continuous layer of envelope glycoproteins such as E1 and E2 and an extrinsic membrane protein, E3, in an icosahedral lattice with T=4. A small amount of the membrane protein 6K is also reported to be unstoichiometrically associated with the viral particle (42, 45–47). The glycoproteins E1 and E2 together form 240 copies of rigid heterodimers and are arranged as 80 spikes protruding out from the lipid membrane of the viral envelope (Fig. 3A) (41, 48, 49). These spikes are representative arrangements of trimers of heterodimers (80 x [3 x E1-E2]) in the case of CHIKV (Fig. 3A) or trimers of heterotrimers (80 X [3 X E1-E2-E3]) as reported for SFV and VEEV (41, 50). These trimeric spikes constitute three distinct parts: first, a hollow, three-lobed extended projection of a 4.5 nm ectodomain from the surface for optimal interaction; second, a thin plate called a 'skirt' which covers the lipid bilayer; and finally, the transmembrane helix of E1 and E2, which both efficiently transverse the lipid bilayer in a pair-wise manner to establish direct physical contact with one monomer of capsid protein (44, 50, 51). The envelope glycoprotein E2 and capsid protein are involved in an interaction that has been proposed to be critical for alphavirus budding (52). The lipid bilayer membrane between the nucleocapsid and the envelope layer is also associated with cholesterol and sphingolipids, which govern both virus fusion and budding (Fig. 3B) (53–56).

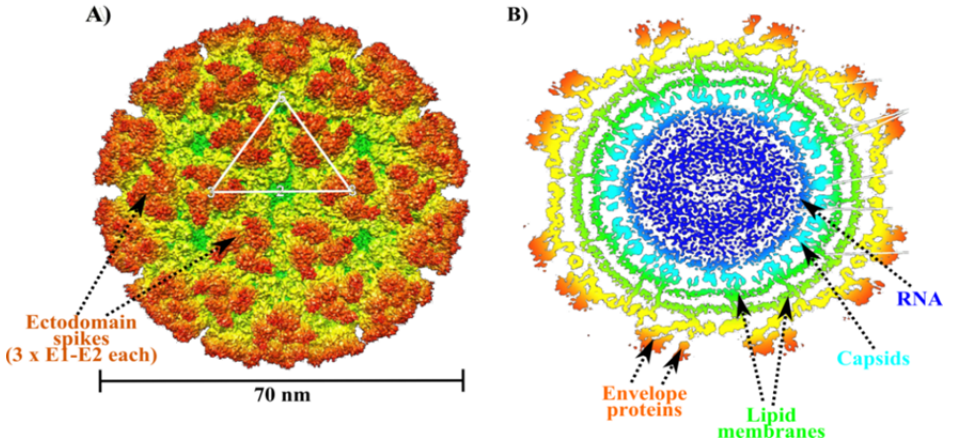


Figure 3. Structure of a CHIKV-like particle (CHIK-VLP) showing the arrangement of surface proteins, adapted from (41). A) Cryo-electron microscopy analysis of CHIK-VLP ectodomain spikes, each of which is a trimer of heterodimers (E1-E2). The VLP is approximately 70 nm in diameter. B) Transectional arrangements of different layers towards the interior, starting from the envelope proteins, lipid membranes, capsids and RNA, which are color coded. Please refer to the text for more detail.

3.1.3. Genome organization

Alphaviruses have positive strand RNA genomes approximately 11,600 nt in length (SINV: 11,700, SFV: 11,500 and CHIKV: 11,800-12,000) that are capped and polyadenylated at the 5' and 3' ends, respectively (Fig. 4) (57, 58). After introduction into the host cell, this genome directly acts as an mRNA that is immediately ready to be translated. The genome contains two ORFs. The first ORF, corresponding to nearly two thirds of the genome, codes for the ns polyprotein or polyproteins (nsP1234 or nsP123 and nsP1234) depending on the presence or absence of a termination codon near the end of the nsP3 coding region (Fig. 4) (59). These polyproteins are precursors of ns proteins that form the virus-encoded part of the replicase and are fundamental towards viral RNA replication. The third of the genome at the 3' end contains a smaller ORF that codes for the viral structural proteins: C, E1 and E2 and also certain small proteins such as E3, 6K or TF, which broadly contribute to the encapsidation of the viral genome. Like ns proteins, the structural proteins are also produced in the form of polyprotein precursors.

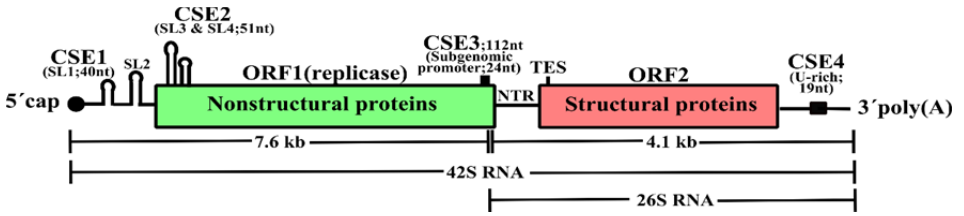


Figure 4. Alphavirus genome structure, showing the conserved sequence element (CSE) (25, 60). The translational enhancer sequence (TES) is characteristic for some viruses (SFV) but absent from the genomes of other (CHIKV) alphaviruses. Note also the genomic RNA stretch (42S RNA) and the sg RNA stretch (26S RNA) (25); refer to text for more details.

The ns polyprotein is directly translated from the viral genome, which is also designated 42S RNA in case of SFV or 49S in case of SINV. In contrast, the structural portion is translated from a sg RNA which is often designated 26S RNA (Fig. 4). Both the genomic and sg RNA have a 5' terminal cap and 3' polyadenylation similar to eukaryotic mRNA (25). During RNA replication a negative polarity RNA complement that carries an unpaired G residue at the 3' end but lacks poly(U), which is complementary to the poly(A) tail of genomic RNA, is generated based on the template of the positive polarity genomic RNA. Negative strand RNA (most likely in duplex with genomic RNA) acts as a template for subsequent transcription of both genomic and sg RNA. The negative strand RNA lacks a cap structure (61, 62). The viral RNA genome also contains three NTRs that are present at both ends of the genome and at the intersection of ns and the structural ORF. These regions are essential for the controlled synthesis of replicative RNA intermediates and also for immune evasion (Fig. 4) (63). The three NTR regions include three CSEs: one towards the 5' end, one near the 3' end and one present at the intersection of ns and the structural ORF (Fig. 4). The final CSE (CSE2) is located in the coding region of the ns polyprotein. CSE1 towards the 5' terminus has demonstrated considerable sequence variability however, the SL1 comprising approximately the first 40 nt of this region are structurally conserved among alphaviruses. CSE2, which comprises 51 conserved nt starting approximately 150 nt from the 5' terminus, is located in the nsP1 coding region and forms two stable hairpins (SL 3 and 4) (Fig. 4) (64). The complements of CSE 1 and 2 at the 3' end of the negative strand were shown to affect both negative and positive strand RNA synthesis (60, 65). As indicated above, the replication intermediate, which represents dsRNA comprising both the positive and negative RNA strands, acts as a template for successive genomic and sg RNA synthesis (58, 66, 67). For genomic RNA synthesis, the complement of SL1 in CSE1 at the 3' end of the negative strand RNA acts as a core promoter (68), while adjacent elements of this region improve the efficacy of synthesis of genomic RNA. CSE2, including SL 3 and 4, is important for both positive and negative RNA synthesis (Fig. 4) in a host-specific manner: it is indispensable for genomic RNA synthesis in insect cells but not in

vertebrate cells while deletion of SL2 leads to enhancement of RNA replication in vertebrate cells (60, 64). This leads to the hypothesis that these elements bind to cell-type specific interacting partners (69). CSE3, which overlaps with the region encoding for the C-terminus of nsP4 and extends up to the non-coding region upstream of the structural region, was identified as a sg promoter. Indeed, sequence complementary to this sequence on the negative strand RNA acts as a promoter for the synthesis of sg RNA (Fig. 4). The minimal length of this region capable of supporting sg RNA synthesis could be as short as 24 nt (-19/transcription start site/+5), while for efficient sg RNA synthesis this region should be long, up to 112 nt (-98/transcription start site/+14) in length (70–72). To note, these data are implied for SINV, although the lengths of the minimal (and/or maximal) sg promoters of other alphaviruses could be different. CSE4, comprising 19 nt immediately preceding the poly(A) tail of the genomic RNA, acts as a part of the promoter required for the synthesis of negative strand RNA (Fig. 4) (60, 73, 74). Mutational studies in these regions have shown that initiation of negative strand RNA occurs at the last nucleotide before the poly(A) tail. The start site of the negative strand could be shifted using certain mutations; however, this type of non-wt initiation compromises the productivity of elongation (75).

In a late stage of infection, the 5' portion of the capsid gene of SINV, encoding for the first 34 aa of the capsid protein, acts as translational enhancer and facilitates efficient translation of structural proteins in infected cells (76, 77). This region, located close to the 5' region of sg RNA, also carries several stable SL structures that assist in cap-independent translation, compensating for the viral-induced shutdown of host cell translation (76–79). This function, however, is not conserved. Capsid enhancer is found in the corresponding region of the SFV genome but lacks in the genomes of other alphaviruses, including VEEV and importantly also for CHIKV. The region encoding for the 6K protein has been shown to carry a -1 ribosomal frame shift signal, recognition of which gives rise to a small structural protein called the TF (80). The alphaviral genome also carries a packaging signal sequence, but the position of this signal and its sequence vary considerably between different alphavirus species. In the case of SINV, nt 945-1076 of the nsP1 region are important for packaging of the genome. In the case of SFV, the packaging signal is located in the region between nt 2767-2990 (nsP2 encoding region), and for RRV several probable packaging signals have been mapped (81, 82).

3.1.4. Alphavirus replication cycle

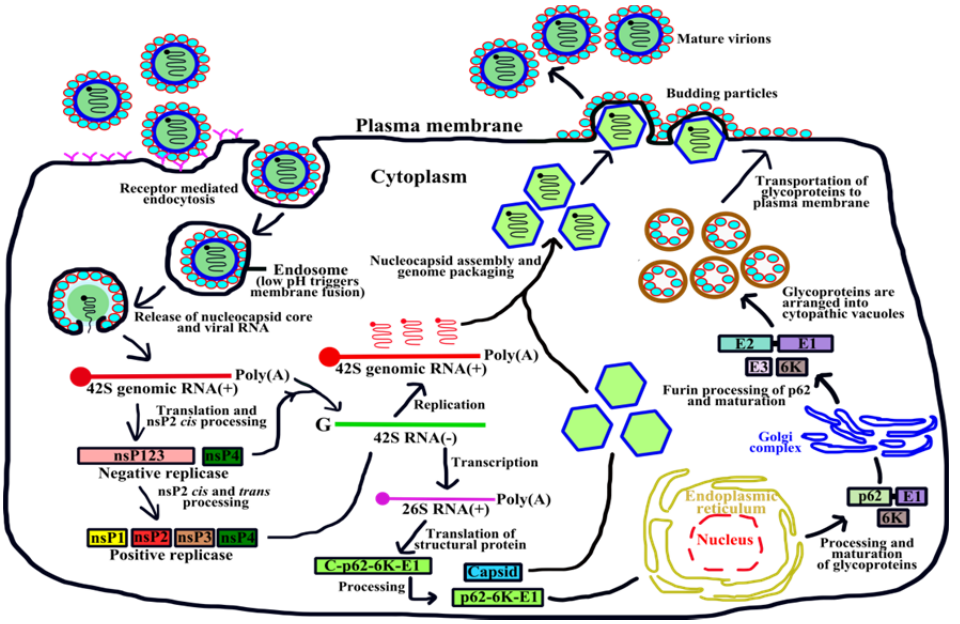


Figure 5. Replication cycle of alphaviruses: from virus entry to budding and escape. Note that for the sake of simplicity, all cell organelles have not been shown (83, 84).

3.1.4.1. Alphavirus entry

Alphavirus entry is primarily initiated by the binding of E2 glycoprotein with one or multiple cell surface receptors (Fig. 5) (85, 86). However, as alphaviruses target very diverse cell types, from vertebrates to invertebrates, which are very different in their surface architecture, an omnipresent receptor is highly unlikely to facilitate the binding of virions to the cell surface. Thus, use of altered or multiple receptors on different cell types is more likely to contribute to this event. It has been demonstrated that SINV employs natural resistance-associated macrophage protein (NRAMP), a divalent metal ion transporter that enables its entry into both mammalian and insect cells. RRV, which belongs to the SFV serological complex, does not exploit the same receptor. Thus, generalization of the attachment process is difficult (87). It has also been shown that SINV uses the laminin receptor to assist its entry into mammalian and insect cells, as an antibody directed towards the C-terminal domain of laminin partially blocked viral entry into mosquito cells (88). In a another study, SEC61A and valosin-containing proteins were reported to regulate SINV entry into insects and mammalian cells (89). The entry of RRV into mosquito cells has also been reported to be supported by the binding of E2 glycoprotein to laminin (90). DC-SIGN and L-SIGN, proteins belonging to C-type lectin family, which preferentially binds to mannose-rich glycoproteins, are also broadly proposed to act as

receptors for alphavirus entry (91). In addition, heparan sulfate has been demonstrated to be the binding receptor for SINV entry (92, 93). Using anti-idiotypic rabbit polyclonal sera, it has also been shown that on chicken cells an anonymous 63 kDa protein could act as a receptor for SINV entry (94), while in the case of SFV, class I major histocompatibility antigens were found to mediate infection of human and murine cells (95).

Receptor-mediated endocytosis has been extensively investigated and is supported by solid data (44, 96–99). Recently, it was reported from genome-wide RNAi screening that a homologue of Fuzzy, a protein involved in planar cell polarity and cilia biogenesis, supports clathrin-dependent alphavirus internalization (100). Thus, in general, cumulative evidence supports the receptor mediated endocytosis mode of alphavirus entry. Still, there have been reports of other modes of initiation for virus infection in which the virus comes in direct contact with the plasma membrane, bypassing the stage of classical endocytosis. According to this alternate hypothesis, a pore complex derived from viral and host proteins is formed, which connects the interior of the virus to the susceptible cell cytoplasm (101).

According to a more widely accepted model, the binding of virions to cell surface receptors is followed by swallowing the particle, which is later found in endocytic vesicles. Acidification of endosomes leads to the fusion of viral and endocytic membranes, and the nucleocapsid is released into the cytoplasm (Fig. 5) (102). This fusion is also reported to be driven by the presence of cholesterol and sphingolipid in the target membrane (53, 55). Few detailed studies suggest that the low pH of the endocytic vesicle destabilizes the E1-E2 heterodimer to expose the fusion loop of E1, which otherwise (at neutral pH) would have remained hidden within the E1-E2 complex (103, 104). The fusion loop is inserted into the target membrane, where it trimerizes. This is associated with melting of the viral and endocytic membranes and leads to the creation of a pore through which the nucleocapsid exits into the cytoplasm. In the case of several alphaviruses, including SINV, SFV and CHIKV (but not in the case of structurally related DENV, a member of the *Flaviviridae* family), it has been further described that a tetraspanin membrane protein, TSPAN9, has a critical role in this type of low pH-activated membrane fusion (100, 105–108). The changes in the viral envelope architecture are followed by structural changes in the nucleocapsid, which is the viral RNA-protein complex (102, 109, 110). Once the nucleocapsid is in cytoplasm their disassembly happens very quickly within 1 to 2 minutes by the recruitment of ribosomes which interact with the capsid proteins permitting the release of viral genomic RNA which becomes available for translation (96, 106).

3.1.4.2. Overview of RNA replication

The alphaviral genome directly serves as mRNA for the production of viral ns polyprotein cleavage products, which form the virus-encoded portion of the

replication complex. The regulated synthesis of different types of viral RNAs (negative strand, new genomes and sg RNAs) is associated with the meticulous processing of the viral ns polyprotein (111). In alphaviruses such as SINV, VEEV and some strains of SFV, an opal termination codon (UGA) is present towards the end of the nsP3 coding region such that in these cases the viral genome serves as a template for the synthesis of a dominant nsP123 and a small proportion of full length nsP1234 polyproteins. The latter is possible due to the ribosomal read-through that occurs in 10-20% of cases (59, 112). In a complete nsP1234, the nsP3 portion also carries an additional 6 aa in its C-terminus. These originate from a region located downstream of the stop codon and forms part of the cleavage site between nsP3 and nsP4. Thus, in cells infected with these viruses, two forms of alphaviral nsP3 exist (25, 113, 114). It has also been shown that the presence of an opal termination codon between nsP3 and nsP4 enhances ONNV virus fitness during mosquito infectivity and could also alter virulence (115, 116). Conversely, a number of alphaviruses only produce nsP1234 due to the absence of an in-frame opal termination codon. Examples include several SFV isolates such as SFV4 and SFV L10 and CHIKV isolates such as LR2006OPY1 (cloned variant, clinical isolate has terminator), SGP011 and IND91. It is very likely that natural isolates of alphaviruses contain genomes with and without a terminator.

The peptidase activity residing in the C-terminal portion of nsP2 is solely responsible for the cleavage of nsP1234 to liberate nsP1, nsP2, nsP3 and nsP4 in a precisely regulated manner that is tightly connected to the synthesis of viral related RNA of different polarities (Fig. 5) (66). In the first 6 hours p.i., the viral replicase protein nsP4, which possesses RdRp enzymatic activity, and nsP123 (still uncleaved) direct the synthesis of the genome-length negative strand RNA, which later acts as a template for the synthesis of genomic and sg RNA of a positive polarity (117). It is likely that each positive strand can be used only for the synthesis of one negative strand, which forms dsRNA with its template. The replicase proteins for negative RNA synthesis are therefore constantly reintroduced from 4–6 hour p.i., after which no new negative strand synthesis complexes are made (118, 119). Thus, maximal amounts of negative strand RNA are reached approximately 6 hours p.i., and these amounts remain at the same levels thereafter. This could, however, be altered by certain mutations in nsP2: for corresponding viruses, incessant minus strand synthesis is observed beyond the mentioned time frame (120–122). In contrast to minus strand synthesis, positive strand synthesis is stable and continues for several hours, even tolerating the addition of protein synthesis inhibitors (57). Due to the presence of a relatively stronger promoter for sg RNA synthesis (compared with promoters used for genomic RNA synthesis), sg RNA synthesis proceeds more proficiently than genomic RNA in later stages of infection (60). Quantitation of minus strand RNA copies per BHK-21 cell gave a number of approximately 27,000 copies per cell; in chicken embryo fibroblasts, this amount was considerably less (likely due to the smaller sizes of these cells). In vertebrate

cells, the positive strands are clearly more abundant with copy numbers of 200,000 and above and genomic to sg RNA ratios of 1:2 to 1:5 (120, 123, 124).

3.1.4.3. Replication machinery

Although the alphaviral genome codes for approximately 10 different proteins, only 4 ns proteins and their precursors are absolutely required for RNA replication. The proteins contribute to the formation of highly organized and fine-tuned replication complexes inside the host cell, the modulation of which is primarily governed by polyprotein processing by a peptidase residing in the nsP2 portion of the polyprotein (Fig. 5) (125–128). In infected cells, along with other host factors, the viral portion of the replicase complex involves different cleavage products of nsP1234. As infection proceeds, their processing as well conformational alterations lead to the formation of early, intermediate and late replication complexes; this determines which polarity of viral RNA will be synthesized:

- At an early stage of infection, during translation, nsP4 is *cis* cleaved out from nsP1234 and together with the nsP123 polyprotein; it functions in the production of genome-length negative polarity RNA. Thus, nsP4 + nsP123 are referred to as the early replicase complex (or negative strand replicase) (Fig. 6).
- Another *cis* cleavage, which occurs between the nsP1 and nsP2 regions of nsP123, leads to the formation of a complex consisting of nsP1, nsP23 and nsP4. This entity is known as the intermediate replication complex and is capable of synthesizing viral RNAs of both polarities. The complex is very short lived, at least in the case of SFV and SINV.
- The final cleavage, which occurs in *trans*, functions to separate nsP2 and nsP3 and leads to the formation of a stable complex consisting of individual ns proteins. This is termed as the late replicase complex or positive strand replicase complex, as it produces both genomic and sg RNA of a positive polarity, a process that lasts until the death of the infected cells (Fig. 5 and 6) (117, 126, 129–131). The formation of the late replication complex as a result of the *trans* cleavage of nsP2 and nsP3 is the final commitment of the replicase to exclusively synthesize positive polarity genomic and sg RNA from dsRNA intermediates, which results in a point of no return according to current dogma (Fig. 6) (122, 130–134).

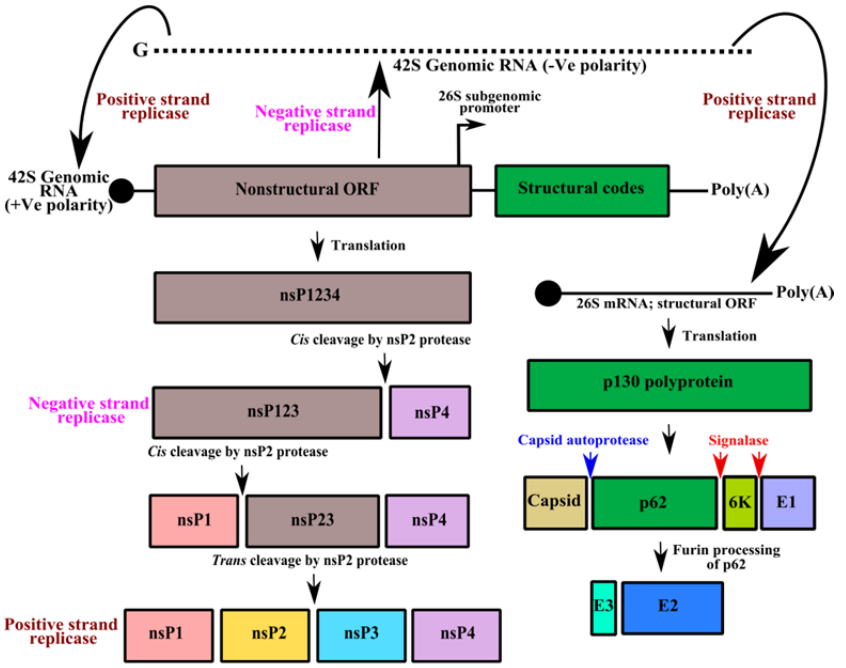


Figure 6. Sequential polyprotein processing in alphavirus. Note how the processing supports the replication of RNA with different polarities (positive and negative). The proteases responsible for the cleavage-processing of ns and structural proteins have also been shown (25) and http://viralzone.expasy.org/all_by_protein/3.html.

At a later stage of infection (6 h p.i. and later) when the concentration of individual nsP2 builds up in the cytoplasm, the newly synthesized nsP1234 is processed in a different order: cleavage of the 2/3 site occurs first, leading to the formation of the nsP12 and nsP34 polyproteins. The cleavage occurs co-translationally or just after the synthesis of the new nsP1234 (135). These molecules or their cleavage products cannot form replicase complexes and are used to produce individual nsPs, which are not included in replicase complexes. Nonetheless, they are important for virus infection and the functionality of each is described in later sections.

3.1.4.4. Site of RNA replication

The replication of positive strand RNA virus is a highly regulated process. The replication machinery is not dispersed throughout the host cell; instead it is concentrated and located in compartments, often co-opting the membrane-bound infrastructure in such a way that the confined settings not only provide the required microenvironment for the process, and only the molecules related

to or supporting viral RNA replication can be transported in and out, but also optimal usage of the available resources is ensured.

In infected cells, alphaviral RNA replication occurs inside a compartment (sometimes called the "virus replication organelle") derived from modified endosome and lysosome called "CPV I or CPV" with a size ranging from 600 to 2,000 nm (136). Inside these CPVs, multiple small, regular, light bulb-shaped membrane invaginations called "spherules2 (with a diameter of approximately 50 nm; visible by electron microscopy) are located. These "spherules" are true replication complexes and contain all alphavirus replication machinery. Formation of spherules is coupled with the synthesis of negative strand RNA; no spherule is formed without RNA replication (137, 138). Electron microscopy images reveal that the size of a spherule is determined by the length of the RNA packed into these structures (139–143). It has also been found that all four replicase proteins are associated with the spherule, and anchorage of the replicase complex to the spherule membrane is governed by nsP1. For material exchange (transport of nucleotides and RNA molecules), the spherules are connected to the cytoplasm by a neck-like structure with an inner diameter of 8 nm (139, 142, 144, 145).

Live cell imaging techniques along with electron microscopy suggest that the spherules are first formed and accumulate along the plasma membrane. Their formation is followed by internalization via an endocytosis-like process that is driven by the phosphatidylinositol 3-kinase and actin-myosin network. The process occurs efficiently in the case of SFV, and vesicles, containing spherules, are later fused to acidic endosomes and displaced in multiple directions using microtubules, leading to the formation of CPVs, which localize around the nucleus (146). CPVs carry different late endosomal and lysosomal marker proteins, while the ns proteins are identified on the cytoplasmic face of the limiting membrane of the CPV (139, 142). These processes are tightly reinforced by ns proteins, especially nsP1 and nsP3. NsP1 anchors to the plasma membrane, while nsP3 is essentially needed for transportation to the lysosome (147–149). The internalization of spherules is not an absolute requirement, and inhibitors of the process do not diminish viral RNA replication. Furthermore, for several alphaviruses, including CHIKV, the internalization of replicase complexes is rather inefficient. In cells infected with CHIKV, replicase complexes stay near the plasma membrane, and prominent CPVs are not formed. The molecular bases of these different behaviors are unknown.

3.1.5. Structural proteins and their processing

Alphavirus sg RNA acts as an mRNA for the translation of the structural proteins. These are produced as polyprotein precursors, and their processing and post-translational modifications are important and demand specific elaboration (25). The order of mature proteins in the alphavirus structural polyprotein is C-E3-E2-6K-E1. The E3-E2 precursor is commonly called p62 based on its

molecular mass (Fig. 6) and has the longest half-life among the precursors of the structural proteins (125, 127, 150, 151). For wt SFV, a vertebrate cell-specific translational enhancer involving a region encoding the first 102 aa of the capsid protein has also been reported in sg RNA (77, 152). The capsid protein is autocatalytically *cis* cleaved out from the structural polyprotein. This cleavage liberates the N-terminus of p62, which carries the ER membrane translocation signal. The translocation is essential as the membrane-bound portion of the polyprotein undergoes further extensive modifications and is cleaved by cellular proteases to individualize the p62, 6K and E1 proteins followed by palmitoylation and glycosylation (Fig. 6) (151, 153, 154). As alphaviruses shuttle between vertebrate and invertebrate hosts, it is tempting to postulate that post-translational modification could be different based on the host species. Along this line of thought, it has been shown recently in case of SFV by ion mobility mass-spec and collision-induced dissociation analysis that E1 and E2 could be glycosylated to a different degree. The composition of glycans associated with these proteins varies according to the structure of the individual surface proteins and host-specific enzymes (155). Binding to the membrane is essential for proper folding, modification and processing of these proteins.

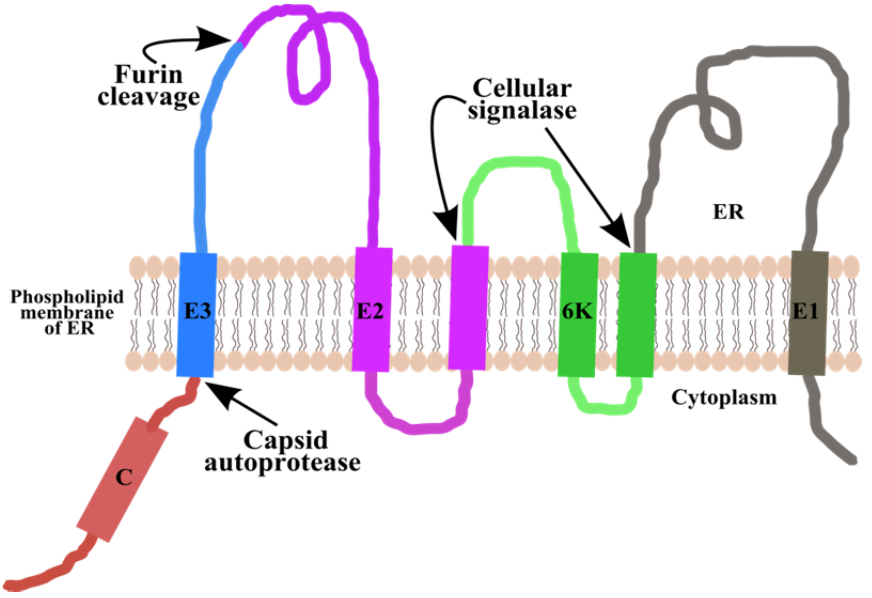


Figure 7. Alphavirus structural protein arrangement on the membrane of the endoplasmic reticulum showing capsid, E1, E2, E3 and the protease responsible for the cleavage of individual protein (156).

P62 and E1 form a heterodimer in the ER that is transported to the Golgi; during this time, p62 is processed by furin to liberate E3 and E2 (Fig. 6 and 7). This

leads to E2-E1 heterodimer formation, and these complexes are transferred to the plasma membrane where they participate in virion formation. In the case of SFV and VEEV, E3 is also incorporated into the virion; however, in the case of SINV (and CHIKV), E3 is absent or may not contribute during this process. The 6K protein is present in virions in very small amounts and may influence the interactions between E2 and E1 (25, 41, 44, 50, 84, 84, 157). If a ribosomal frame shift occurs during 6K synthesis, a TF protein is synthesized. The existence of this protein has been confirmed using mass spectrometry; however, its functions are largely unknown except that it is somehow important for alphavirus virion assembly (80, 158).

3.1.6. Individual structural proteins

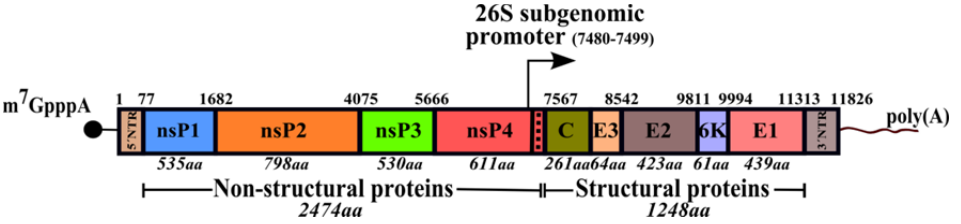


Figure 8. CHIKV genomic RNA with 5' cap, 3' poly(A) and NTR regions at both ends of the genome. Sizes of mature ns and structural proteins and start positions of corresponding coding regions are shown. Note that sg RNA codes for the structural proteins ((35, 159, 160) and UniProt entry Q8JUX5).

3.1.6.1. Capsid protein

Capsid protein (33 kDa) is an enzymatically active protein. The C-terminal domain of capsid protein exhibits sequence conservation towards a serine protease (161, 162). So this protein has protease activity that is used for *cis* cleavage at its C-terminus (Fig. 8) (163). After this processing event, protease activity of the capsid protein is inhibited as the catalytic site is occluded due to a conformational change (25). The N-terminal variable region comprising 96 aa (SINV), which is rich in positively charged aa residues, has no defined 3D structure and binds viral RNA. This interaction is crucial for the formation of nucleocapsid. The C-terminal conserved portion of this protein binds to the E2 glycoprotein (25).

Capsid protein also has functions that are not related to virion formation. This is especially evident for New World alphaviruses. It has been shown that one of the N-terminal sub-domains of VEEV capsid protein is not necessary for viral particle assembly; instead, this region regulates viral RNA encapsidation. Mutation in the capsid protein has been reported to be compensated for by mutation in nsP2 (164) and vice versa (unpublished data from our lab), indicating some involvement in RNA synthesis and/or selection. For New World

alphaviruses, the capsid protein is the main determinant for the shutdown of host cell transcription and antiviral responses. The protein has both nuclear export and nuclear import signals and, by interacting with corresponding cellular proteins, it blocks nucleo-cytoplasmic transport (165–167). In the case of CHIKV, it has also been demonstrated that the capsid protein interacts with karyopherin α to translocate into the nucleus, whereas interaction with the export receptor CRM1 results in displacement of this viral protein from the nucleus (168). Thus, trafficking in and out of nucleus is also characteristic of capsid proteins of Old World alphaviruses; however, the biological significance of this process is not yet known.

3.1.6.2. Envelope proteins 3 and 2

E3 (11 kDa) is a glycoprotein resulting from the cleavage of p62; this cleavage is performed by furin in the trans-Golgi compartment (Fig. 6, 7 and 8). E3 facilitates proper folding of p62; p62 itself interacts with E1 to influence spike function. E3 is also believed to influence E2-E1 heterodimeric complex formation and allows its transportation to the site of budding via acidic compartments of the cell. E3 interacts with E2 by Tyr47, which protects E1 from low pH (169). The cleavage of E3 from the spike complex activates the fusion ability of the E1 protein. Thus, E3 is important for viral particle assembly and indirectly also for viral entry (44, 170, 171).

The 51 kDa **E2** protein, another product of the furin cleavage of p62, is a transmembrane protein. It is glycosylated and palmitoylated; both of these modifications are essential for virion budding (Fig. 6, 7 and 8) (172, 173). The 260 aa from the N-terminus of E2 form an ectodomain, while the next 100 aa form a stem-like region and the remaining 30 aa form a transmembrane region (171, 174). The C-terminus of the E2 protein also interacts with the nucleocapsid. As mentioned above, E2 eventually forms E1-E2 heteroduplex complexes. In these complexes, E2 is responsible for binding to the cell surface receptor and subsequent endocytosis (25, 44). It has also been described that an anti-idiotypic neutralizing antibody binds to an epitope in the E2 protein. A monoclonal antibody against this glycoprotein is reported to protect mice against CHIKV disease; a number of studies have also shown that this structural protein is a major viral antigen and thus represents a possible therapeutic target (94, 175–177). Recently, it has been observed that E2-64 and E2-208 mutations lower the dissemination of the E1-226V CHIKV variant in *Aedes aegypti* but not in *Aedes albopictus* mosquitos. This is apparently due to an aberrant interaction between CHIKV and mutant E2:E1-226V spikes and cellular receptor in the *A. aegypti* midgut (178, 179).

3.1.6.3. 6K and Transframe proteins

6K is a relatively small 60 aa long structural protein. Translation of this coding frame can also lead to formation of another protein with a C-terminal extension known as TF. **TF** is an 8 kDa protein, and its synthesis requires a ribosomal frameshift. It has been shown that this frameshift occurs with an approximate frequency of 10-18% (Fig. 8). In the SFV virion, the TF protein is present to a considerable extent. In contrast, 6K is present in much smaller amounts. 6K facilitates virion formation but is not absolutely required (which is true for TF as well). These two proteins both undergo acylation; the TF protein also undergoes palmitoylation, whereas 6K undergoes glycosylation (46, 80, 157). Manipulation or mutations in the 6K region affect infectious virus production; however, even complete deletion of this region is not fatal. It has been shown that mutations and deletions in 6K can be compensated for by mutation in E2, providing evidence that these two proteins interact (46, 150, 180, 181). The 6K protein is also involved in the modification of membranes in cells of mammalian origin, and it likely represents a cation-specific viroporin (182–186).

3.1.6.4. Envelope protein 1

E1 is a 50 kDa transmembrane glycoprotein that is also modified by the addition of fatty acids and resembles flavivirus E protein (172, 187). In virions, it forms a continuous icosahedral shell covering the lipid bilayer. E1 is palmitoylated and this modification contributes to the stability of budding virions (Fig. 8) (188). The E1 protein has been reported to be responsible for mediating viral membrane fusion with the host endosomal membrane during virus entry (189). Indeed, at low pH the E1 becomes capable of fusion. A specific histidine residue at the third position of the E1 protein assists in low pH-dependent refolding during the course of fusion (44, 190).

The E1 protein is also reported to play a crucial role in viral adaptation and evolution. It has been clearly shown that it was the E1-A226V mutation that resulted in added fitness of CHIKV for the *A. albopictus* mosquito vector. This mutation greatly facilitated the spread of the virus via *A. albopictus* mosquitos and was clearly among the major factors contributing to the 2005-2006 Indian Ocean epidemics (23, 179, 191). In a study carried out to identify post-2005/2006 epidemic mutations, two mutations, V80I and A129V, were mapped to the E1 region when mosquitos were infected with the E1-A226V CHIKV strain (192). This mutation also induces the virus to acquire multiple second-site mutations such as E2-L210Q, which is proposed to contribute towards “super adaptive CHIKV” with enhanced transmission efficiency (193). Interestingly, however, the E1-A226V mutation does not facilitate transmission of CHIKV of Asian genotypes. This is due to the T98 residue in the E1 protein of this CHIKV genotype (194)

3.1.7. Nonstructural proteins

As described earlier, the alphavirus genome encodes for four ns proteins. As these proteins along with other host factors form viral replicase complex and also have additional essential functions, they are immensely important for the viral replication inside the infected host cell. The ns proteins also interact with host factors; these interactions are important both for replicase function and for influencing cellular functions to support infection. In this line of thought, it is plausible to imagine that almost all the ns proteins individually or as a part of the replication complex multitask to a large extent. Clearly, these proteins are able to carry out versatile activities to complement the other ns proteins in this process; thus, detailed descriptions of the individual ns proteins follow.

3.1.7.1. Nonstructural protein 1

NsP1 (60 kDa; 535 aa in SFV and CHIKV) determines the localization of the replication complex inside infected cells because it has an inherent ability to bind to the plasma membrane. This is facilitated by the presence of a positively charged-hydrophobic peptide of 19 aa between residues 245 and 263 (in case of SFV, the sequence of the peptide is GSTLYTESRKLLRSWHLPS). This peptide can form an amphipathic helix and is crucial for viral replication. Though the initial interaction with membrane occurs via the amphipathic helix, the final membrane anchoring of the protein is mediated by palmitoylation of cysteine residues between aa 418–420 (Fig. 9). The amphipathic-like helix is arranged in plasma membrane in such a way that the hydrophobic residues can interact with the lipid bilayer while the positively charged residues interact with the negatively charged group of phospholipids. Comparable peptides can be found in many viruses related to alphavirus (144, 147–149, 195, 196). In the case of SFV, mutations in this conserved peptide not only affect the membrane binding properties of the protein but also hamper the enzymatic activities of nsP1 (see below). However, this is not universal as nsP1 of SINV, which is devoid of membrane binding, is still partly biochemically active. In the case of SFV, it was shown in a competition experiment that a putative membrane binding peptide could compete with nsP1 to bind liposomes containing phosphatidylserine (147, 196, 197). The specific affinity of nsP1 for the negatively charged phospholipids and the lipid bilayer leads to the formation of tunnel-like projections of a nano-scale size that resemble filopodia-like structures via an unknown mechanism (195, 198). Palmitoylation is an important requirement for nsP1 to induce these types of structure (148, 195). These structures, which are common to many RNA viruses and retroviruses, have also been reported to be associated with membrane proteins such as ezrin and glycoprotein receptors such as CD44. However, it remains unknown to what extent these structures could be used by alphaviruses to spread infection from infected cells to adjacent cells (198–201). Additionally, it has been revealed that if nsP123 or nsP1234 are expressed as an

uncleavable polyprotein, the nsP1 portion of these molecules can still undergo palmitoylation and guide the polyprotein to cytoplasmic membranes (145). Mutations preventing the palmitoylation of nsP1 reduce virus infectivity. This effect is most likely not due to weaker membrane attachment but due to disturbed interactions between nsP1 and nsP4. It has been found that multiple compensatory mutations can restore these interactions. Interestingly, these mutations also restore the formation of filopodia-like structures, at least to some extent (202). The direct interaction between nsP1 and nsP4 has also been reinforced by studies involving co-immunoprecipitation techniques. One of these study reported that the N-terminus of nsP4 interacts with nsP1 during the initiation of minus strand synthesis, while another study reported that modification of the N374 residue in nsP1 of SINV influences the ability of nsP4 to synthesize minus strands (203, 204). From these pieces of evidence, it could be broadly perceived that nsP1 plays a direct or an indirect role in the synthesis of negative strand RNA by interacting with nsP4.

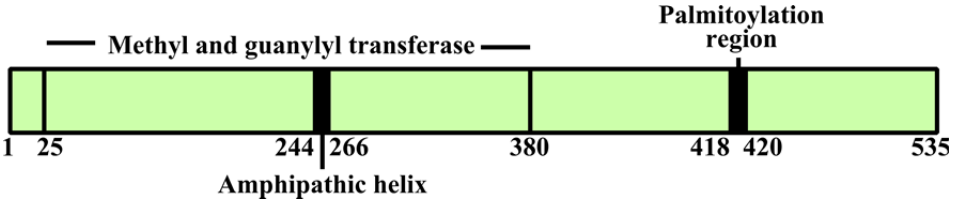


Figure 9. Schematic representation of alphaviral nonstructural protein 1. Regions crucial for enzymatic (methyl- and guanylyl transferase) activity, the amphipathic helix and the palmitoylation region are shown. Amino acid residues are numbered based on nsP1 of SFV (35, 149, 195).

NsP1 also harbors enzymatic activities that are essential for cap synthesis. This is pivotal for making viral genomic and sg RNA essentially non-distinguishable from host mRNA (205) (Fig. 9). These actions of nsP1 contribute to the formation of a cap-0 structure that supplements and complements the RdRp activity of nsP4, which synthesizes new RNA of a positive polarity and the RTPase activity of nsP2, respectively (206–208). Briefly, nsP4 synthesizes new positive strand RNA that carries three phosphates at its 5' end. Subsequently, the gamma phosphate is removed by the RTPase activity of nsP2 to give rise to an RNA molecule with two phosphates at the 5' end. Afterwards, a GTP molecule is methylated at the 7' position of the guanine (m7GTP) by the guanine-7-methyltransferase (MTase) activity of nsP1, which uses S-adenosyl-L-methionine (Ado-Met) as a methyl group donor. Later the guanylyltransferase (GTase) activity of nsP1 catalyzes the release of two terminal phosphates which allows the formation of methylated-GMP covalently bound to nsP1 (m7GMP-nsP1) transfer complex. Finally, the methylated GMP (m7GMP) is transferred to the 5' terminus of the RNA to conclude the formation of a 5'-5' covalent bond between

RNA di-phosphate and the methylated GMP moiety (205, 206, 209–211). Recently it has been reported_that S-Adenosyl-L-homocysteine (Ado-Hcy), a byproduct of the preceding MTase reaction is essential for the following GTase reaction (211). Mutations changing key residues in the "MTase motif" (H39, R91, N94, and Y249 residues; numbered based on nsP1 of SINV) to alanine residues abolishes MTase activity and viral infectivity (212). As indicated above, MTase and GTase activities of nsP1 of SFV are dependent on the membrane binding of nsP1. Thus, washing out the phospholipid by detergent abolishes the enzymatic activity of nsP1, while supplementing these settings with phosphatidylserine restores the activity. In contrast, SINV nsP1 did not demonstrate such dependence; instead, a nonionic detergent abolished nsP1 GTase activities without affecting the MTase activity (147, 197).

3.1.7.2. Nonstructural protein 2

NsP2 (90 kDa; 799 aa in SFV and 798 in CHIKV) is the largest multi-tasking and the most multifaceted protein among the ns proteins of alphaviruses. NsP2 possesses four enzymatic functions: RTPase, NTPase, protease and helicase activities; additionally, a number of important non-enzymatic activities such as causing cytotoxic effects related to the shutdown of cellular transcription and translation machineries have been associated with this protein (Fig. 10). Thus, it is very plausible to anticipate that a number of extensive studies have been carried out to characterize these features of nsP2 (166, 209, 210, 213–220). Bioinformatical analysis and structural data have assigned this protein five different domains: N-terminal domain (NTD) aa 1–167, 1st RecA-like domain aa 168–311 (1A), 2nd RecA-like domain aa 312-470 (2A), protease domain aa 471–605 and MTL domain aa 606-798 (aa residues numbered based on nsP2 of CHIKV; Fig. 10). The functional significance of these domains has been discussed in different contexts throughout this thesis. Furthermore, the subcellular localization of nsP2 inside infected cells is also intriguing. In the case of SFV, 25% of nsP2 is associated with virus replicase organelles, another 25% is diffusely present throughout the cytoplasm and a large fraction of approximately 50% is present inside the nucleus, especially the nucleolus. These distributions are believed to assist in hijacking or manipulating host cell machineries to support viral RNA replication (142, 221).

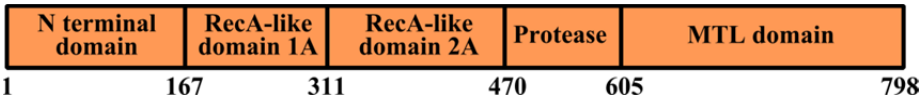


Figure 10. Schematic representation of the domain organization of nsP2 of CHIKV. The C-terminal region starting at 470 is historically considered to be a protease domain, while the N-terminal region is responsible for the NTPase and RTPase activity (210, 215, 220, 222). Please follow the text for more details.

The N-terminal aa 1–470, in the case of nsP2 from SFV, and fragments spanning from aa 1–470 or aa 166-630, in the case of nsP2 from CHIKV, have been expressed and purified as recombinant proteins. Biochemical analysis has revealed that these fragments possess NTPase and RTPase activities. Hydrolysis of NTPs or dNTPs and RTPase are divalent cation-dependent (especially Mg^{+2} or Mn^{+2}) (209, 210, 220). There are a number of signature motifs that have been mapped to the N-terminus of the proteins associated with these activities. These are located in a region between aa 167–470, which, according to bioinformatics predictions, is reported to contain two RecA-like domains indispensable for NTPase and RTPase activity (Fig. 10) (215, 223, 224). These RecA-like domains contain seven classical motifs essential for NTP hydrolysis, of which Walker A (motif I) is marked by the signature sequence $GSGK^{192}S$, Walker B (motif II) is distinguished by the landmark sequence $D^{252}E^{253}$, and motif VI, denoted by R^{416} known as the "arginine-finger," represents the indispensable motifs that pivotally drive NTPase and RTPase reactions by interacting with different skeletal portions of the NTP molecule; therefore, these motifs are commonly called the "core" motif of NTPase (215, 225). In the case of recombinant proteins, replacement of the highlighted aa residues individually (or in combinations) indeed completely abolished NTPase and RTPase activities (209, 210, 215, 226). Interestingly, however, the infectious clones of SFV harboring a K192N mutation in nsP2 were reported to be infectious and capable of reverting this mutation, which has been reported to produce infectious virus progeny (Rikkonen, 1996). This finding is puzzling as experiments in our laboratory have shown that the corresponding mutation completely inactivates the replicase of CHIKV and the corresponding clone lacks infectivity (our unpublished data). A biochemical study reported that a recombinant protein corresponding to aa 1–470 of SFV could carry out an RTPase reaction with a substrate affinity (K_m) of 3 μM and catalytic rate (K_{cat}) of 5.5/min and an NTPase reaction with K_m of 90 μM and K_{cat} of 230/min. These data suggest that the affinity of the enzyme for the RNA substrate is approximately 30 times higher than that of the NTP molecules; in contrast, the catalysis rate of the NTP molecule is approximately 42 times higher than that of the RNA triphosphates. The RTPase activity of nsP2 is essential for making precursor RNA for nsP1 to carry out final capping reactions (210). The role of the NTPase activity of nsP2 in virus infection, however, is not clear.

Sequence alignment categorized the alphaviral helicase as SF1 (227). The helicase activity of nsP2 is imperatively associated with its NTPase activity to power the RNA unwinding: NTP hydrolysis must drive the helicase activity. In case of SFV or CHIKV, full-length nsP2 has clearly been demonstrated to unwind dsRNA in a 5'-3'directionally biased manner. Interestingly, truncated forms of nsP2 (fragments ranging from aa 1–470 or aa 166-630) lack this activity completely (209, 215, 217). The NTPase activity of the protein corresponding to aa 166-630 of nsP2 was also insensitive to the presence of oligonucleotides. The C-terminal region of nsP2 (aa 471–798) contains two distinct domains; the first is the functionally active protease domain and the second is

the MTL domain. The latter cannot be active as an enzyme because it lacks classical structural elements required for methyltransferase activity; instead, it functions as part of a non-structural protease. To note, the lack of methyltransferase activity could be an excellent proxy for performing ancillary but important functionalities. Surprisingly, this most distant domain is crucial for RNA helicase activity. First, the pI of this domain is >10, which means that at approximately neutral pH it is heavily protonated, rendering it capable of binding the RNA. Second, there is indirect evidence that the MTL domain folds towards the NTD domain (pI < 6); this interaction could form a binding pocket for the RNA (Fig. 11). As this topic is central for the current thesis, it will be discussed in great detail in later sections (209, 215, 222).

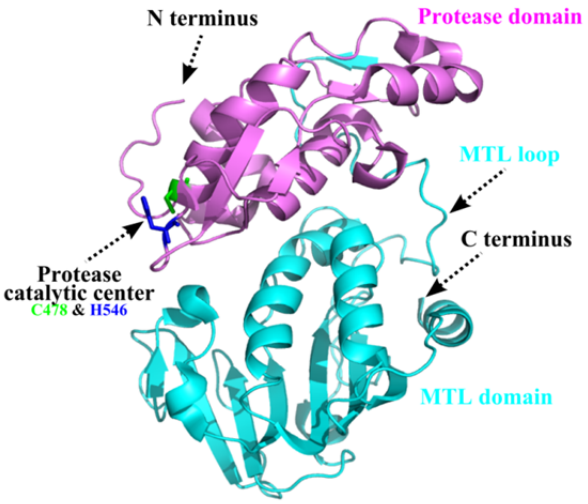


Figure 11. X-ray crystallographic structure of the protease domain of CHIKV adapted from the PDB ID 3TRK, showing the protease domain and the C-terminal MTL domain (222, 228). The protease catalytic center is also highlighted. Note that the MTL domain possesses a long loop connecting the protease and MTL domains. All the structures from the PDB throughout this thesis are modified using PyMOL (229).

The peptidase activity of nsP2 is associated with its C-terminal region (aa 471–798 of nsP2; Fig. 11 and 12). A study involving X-ray crystallography revealed that this region contains a papain-like protease domain and MTL domain featuring an FtsJ like fold towards the extreme C-terminus of this protein. In the case of SFV nsP2, the C478 and H548 form the catalytically active center of the protease (Fig. 11). In corresponding studies, the protease domain has always been analyzed together with the MTL domain (222); indeed, the papain-like protease domain is apparently not active. This may be attributable to two rea-

sons. First, the MTL domain can provide necessary structural support for the protease domain to be active. Second, it is also possible that expressing or purifying only the protease domain could expose hydrophobic patch that otherwise would have been buried under or by the MTL domain, and this leads to the aggregation and inactivation of the recombinant protein. The latter hypothesis is supported by experimental evidence that suggests that nsP2 lacking only the MTL domain shows signs of aggregation in gel filtration chromatography profile and CD spectroscopy (215). Although the MTL domain lacks any professional enzymatic activity, it is important not only for accommodating RNA (see above) but also for the regulation of protease activity. Furthermore, in the case of SINV, a genetic study suggested that positively charged conserved aa residues of this domain have broad functional implications towards minus strand RNA synthesis. Mutation 615A in the loop region of MTL also affected positive polarity viral RNA synthesis and resulted in viruses that could not inhibit host cell translation. Intriguingly, however, this mutation had minimal effects on cellular transcription or on viral polyprotein processing and minus strand synthesis. This suggests that this residue could likely contribute towards the establishment of a functional replicase complex (230). Taken together, it could be concluded that the MTL domain has evolved to be more participatory rather than a direct role in any enzymatic or biological activity involving nsP2 (Fig. 12) however, clearly our understanding of this domain is far from adequate to draw any bold conclusions regarding its purpose in the course of alphavirus infection.

The protease activity of nsP2 is an important and essential requisite for the regulation of replication, which is associated with the processing of nsP1234 (128, 131, 219). It has also been demonstrated from evidence of biochemical assay that the protease activity of nsP2 could be inhibited by divalent cations such as Zn^{+2} and Cu^{+2}. However, Co^{+2} and Ni^{+2} partially block this activity, whereas Ca^{+2}, Mg^{+2} and Mn^{+2} had no effect on the protease activity (231). The site between nsP123 and nsP4 (3/4 site, similar designations are used for other sites hereafter) is readily processed through an *in cis* cleavage during or just immediately after translation to release active nsP4. This is also the only processing event that is absolutely required for virus infectivity and leads to the formation of negative strand replicase (nsP123 + nsP4) (117, 129, 130, 232, 233). A second cleavage, again *in cis,* takes place at the 1/2 site. This cleavage regulates replication complex formation and affects cellular anti-viral responses and the ability of virus to counteract these cellular activities (unpublished data from our lab). This cleavage apparently leads to structural reconfiguration of the replicase complex and permits the involvement of additional transcriptional cofactors to provisionally switch synthesis towards positive strand viral RNA (117, 129). Finally, the 2/3 site is processed.

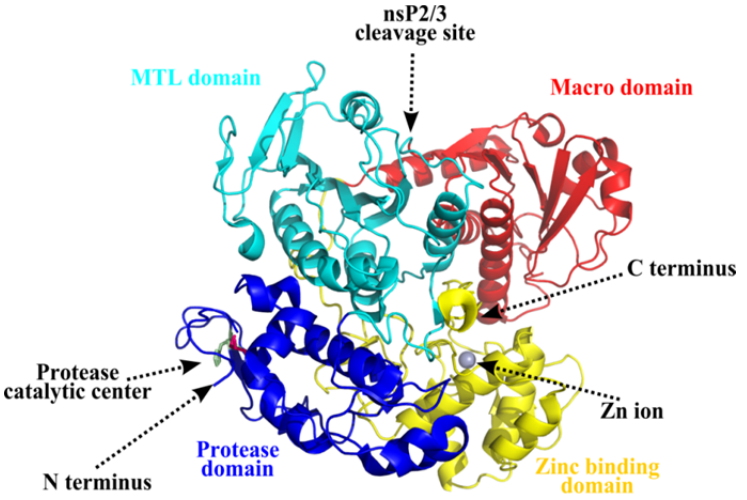


Figure 12. This picture has been adapted from (234), with an associated PDB ID 4GUA, showing the pre-cleavage conformation of the nsP2/3 site of SINV. Different domains are color-coded; the protease and MTL domains belong to nsP2 while the macro domain and ZBD belong to nsP3. The scissile bond (cleavage site), the protease active center and a zinc cation are highlighted. Note that the protease active center is far away from the cleavage site, which supports the *trans* mode of cleavage of this particular site in alphaviruses.

Both biochemical data and the 3D structure of the nsP2 protease domain indicate that this can occur exclusively *in trans* (Fig. 12). Final cleavage leads to the liberation of all individual nsPs to commit their activities for viral replication; in many ways, it is a point of no return, which means that negative polarity viral RNA would no longer be synthesized (Fig. 12) (232). It has been shown that for processing of the 2/3 site, short peptides representing cleavage site is not enough; a minimal substrate should contain a few aa upstream of the cleavage site and a large fragment ~170 aa from the N-terminus of nsP3. Unlike other cleavages, processing of the 2/3 site requires a full-length nsP2 protease with an authentic N-terminus; even minor changes at the N-terminus of enzyme are poorly (or not at all) tolerated (Fig. 23) (132). It should be noted that alphavirus mutants, which are unable to process the 2/3 site, are generally viable (although often have a temperature-sensitive phenotype). Commonly these mutants have an inability to shut down negative strand synthesis. This phenotype highlights another, possibly the most important, significant aspect of 2/3 site processing. Later in infection, when the nsP2 concentration in infected cells exceeds a certain threshold, the free protease starts to cleave nsP1234 between the 2/3 sites. As indicated in the sections above, it abolishes the formation of new replicase complexes and prevents negative strand RNA synthesis, leaving only the synthesis of positive strand RNA active (135).

NsP2 of Old World alphaviruses such as SINV and SFV is also associated with cellular transcription and translation shutdown to induce cytotoxicity. The cessation of transcription and translation are independent events, as mutations such as P726 in SINV and P718 in SFV selectively relieve cellular transcription (65, 216, 235–237). Recently it has been shown that nsP2 of SFV, SINV and CHIKV inhibits cellular transcription by causing the rapid degradation of Rpb1, a catalytic subunit of the RNA polymerase II complex. This ability of nsP2 is specific for vertebrate cells and is blocked by mutations inhibiting NTPase or helicase activities of nsP2 but not by mutations blocking the proteinase activity of nsP2 (213). The SFV and SINV also take control of protein synthesizing machineries in the cell: after just a few hours of infection, all the translating machinery as well as related systems are directed towards the synthesis of viral proteins (mostly structural proteins). This is aided by employing ribosomes at the site of viral replication and by establishing interactions between nsP2 and ribosomal protein elements (78, 79, 237–240). Additionally, the nsP2 proteins of Old World alphaviruses can enter the nuclei of infected cells; this is required to cause Rpb1 degradation and also for blocking of antiviral interferon signaling. It is, however, not clear how nuclear transport works. NsP2 of SFV is reported to carry NLS (PRR649RV sequence). Indeed, mutations in this element block nsP2 translocation to the nucleus and abolish the ability of nsP2 to interfere with interferon signaling. However, this effect is temperature-dependent (237), and corresponding regions of nsP2 of CHIKV (241) and SINV (242) do not contain functional NLS elements. Recently, it has been reported that nsP2 of CHIKV inhibits the unfolded protein response, a cellular defense mechanism against high concentrations of misfolded protein, in the host cell. Thus, nsP2 interacts with many cellular processes (sometimes in a virus- or host-specific manner) and regulates viral RNA replication (166, 214, 216, 237, 242–251).

3.1.7.3. Nonstructural protein 3

NsP3 (53 kDa; 482 aa in SFV and 58 kDa; 530 aa in CHIKV) is the least understood of the alphaviral ns proteins. However, recent revelations about the versatility of its activities have necessitated focused investigations. NsP3 contains three different regions. The N-terminal 160 aa domain forms the macro domain (MD), while another 160 aa domain following this region is conserved among alphaviruses and has been recently classified as ZBD. The C-terminal-most region of nsP3 is known as the hypervariable region (Fig. 12, 13 and 14) (25, 234, 252). NsP3, when expressed as a single protein, localizes to the cytoplasm, where it forms inclusions of random sizes. However, if nsP3 is expressed as a part of the nsP123 polyprotein, it localizes to the endosomal and lysosomal membranes. These structures, which are devoid of spherules, are still comparable to the CPVs (145).

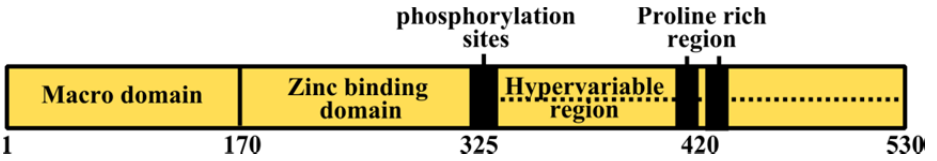


Figure 13. Schema of alphavirus nsP3 showing the macro domain, zinc binding domain and hypervariable region. Phosphorylation sites and the proline-rich region are highlighted (234, 253, 254).

MDs are not only confined to viruses but have been found in many proteins from archaea, bacteria and also eukaryotes (255). MDs with an approximate representative size of 160 aa are also encoded by several other positive strand RNA viruses such as HEV, CoVs (including the SARS-CoV) and rubella virus (256). The crystallographic structure of MD from CHIKV and VEEV has been resolved. The proteins have been reported to be active as adenosine di-phosphoribose 1″-phosphate phosphatases, and a single aspartic acid residue is reportedly responsible for binding to the adenine base. This study also reports that MD engages the exposed positively charged patches, which are present distantly from the enzymatic active center, to interact with DNA, RNA and poly(ADP-ribose). The specificity of these interactions still needs to be validated; however, a general conclusion is that MD is involved in the binding of RNA during viral replication (257). Interestingly, isothermal titration calorimetry analysis suggested that MD of SFV could bind to poly(ADP-ribose) with considerable affinity, while it could not bind ADP-ribose; in contrast, the MD of CHIKV and VEEV binds ADP-ribose with low micromolar affinity (Fig. 14) (255–257). This finding is surprising because SFV is an Old World alphavirus and one of the closest relatives of CHIKV. Thus, it remains to be elucidated whether this finding could be attributed to the true and unique properties of SFV MD or is a result of the instability of purified recombinant protein (256). The MD, along with a few aa downstream of its C-terminus, has been shown to be very essential for the processing of the 2/3 site (132).

The ZBD has a size comparable to MD (Fig. 12 and 13). Recent X-ray crystallography data along with virological evidence has considerably elevated our understanding of the functions of this region. ZBD consists of an antiparallel alpha helical bundle, two parallel beta strands and a cysteine-rich zinc coordination site, positioned adjacent to the protease domain of nsP2 (Fig. 12). A mutation in the conserved zinc coordinating site results in less productive virus infection. Along with MD, this domain offers a long platform for RNA binding. ZBD was also proposed to play a critical role in switching template preference towards the synthesis of positive strand RNA after 2/3 site cleavage (Fig. 12) (232, 234).

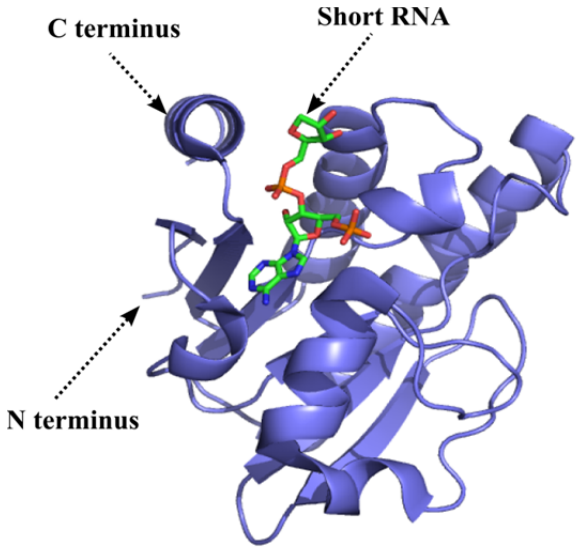


Figure 14. The structure of the macro domain of CHIKV nsP3 (1-160 aa) complexed with short RNA (PDB ID 3GPQ). It has also been proposed that the same binding pocket can bind other negatively charged polymers such as poly(ADP-ribose) or DNA, as the binding is relatively unspecific (occurs due to the presence of a positively charged patch) (257).

The C-terminal-most region of nsP3 is highly variable, not only in terms of sequence but also in its length; hence, it is called the "hypervariable region" (Fig. 13) (25). However, upon closer scrutiny, this region demonstrates some special arrangements. It contains repetitive short sequence motifs that are believed to interact with numerous host factors that are important for virus replication. In SINV-infected BHK-21 cells, nsP3 is found in different complexes, some of which are associated with the plasma membrane and endosome-like vesicles while others are also associated with nuclei (258). In the same study, it was shown that nsP3 of SINV (but not nsP3 of VEEV) binds to proteins involved in stress granule formation such as G3BP1 and G3BP2 or to their homolog in invertebrate cells (Rasputin); these observations have been confirmed by several subsequent studies (258–260). Furthermore, it has also been shown that the proline-rich motif in the nsP3 of SFV, SINV and CHIKV interacts with amphiphysin; mutations changing these proline residues or silencing of amphiphysin expression affects viral RNA replication in HeLa cells (254). The proline-rich region of the hypervariable domain is not essential for G3BP1 binding (261); instead, the interaction with G3BPs is mediated by repeated motifs located at the extreme C-terminus of nsP3. This region clearly possesses several other functions as well: it overlaps with the 3/4 processing site and is shown to carry a degradation signal for nsP3. Thus, removal of 10 aa from the C-terminus of nsP3 increased the half-life of nsP3 from 1 hr to 8 hr; more ex-

tensive (40 aa) deletion in the same region also reduces the synthesis of sg RNA (262). It has also been demonstrated that nsP3 networks with vimentin, a filamentous protein found in non-epithelial cells, which surround and support the replication complex of CHIKV in infected cells (263).

A stretch of serine and threonine residues is located between the ZBD and hypervariable region (~aa 320-350 for nsP3 of SFV). It has been shown that these residues are phosphorylated in host cells (Fig. 13) (264, 265). In the case of VEEV, phosphorylation of this region is reported to be dispensable in vertebrate cells but not in insect cells. The entire hypervariable region of nsP3 is actually dispensable in BHK-21 cells: viruses harboring deletions in this region can recover just by acquiring one or two compensatory mutations. This has permitted the use of this region for the insertion of marker proteins. Furthermore, all hypervariable regions of nsP3 can be replaced with a heterologous protein, at least for VEEV. In the case of SINV, in which the phosphorylation of nsP3 is especially prominent, it has been shown that reduction in the levels of nsP3 phosphorylation is directly proportional to reduced levels of negative strand RNA synthesis (266, 267).

3.1.7.4. Nonstructural protein 4

NsP4 (~68 kDa; 614 aa in SFV and 611 aa in CHIKV) always has an aromatic Tyr residue at its N-terminus and possesses an unstructured N-terminal region (approximately 100 aa, unique for alphaviruses) and a C-terminal region that harbors RdRp activity (Fig. 15) (268–270). Mutational analysis of the N-terminal region suggests that this region acts as an adaptor domain and is involved in interactions with other ns proteins. Curiously, it was also found that viruses containing mutations in this region are also unable to affect host cell translation (269). The C-terminal region of nsP4 contains a conserved signature motif with the signature sequence GDD which is strongly related to RdRps (Fig. 15). Based on sequence analysis, the alphavirus RdRp belongs to supergroup III, which also includes obamovirus, tobravirus, hordeivirus, tricornaviruses, beet yellows virus, rubivirus, furoviruses, HEV, potexvirus, carlavirus, tymoviruses, and apple chlorotic leaf spot virus (271). The crystal structure of the alphavirus RdRp has yet to be determined; however, it is very likely that this enzyme may have a "fingers-palm-thumb" structure characteristic of typical RdRps (272, 273).

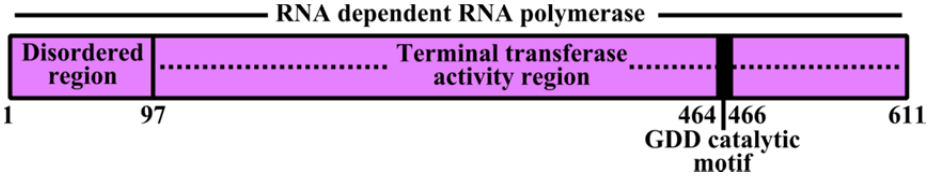


Figure 15. Schema of alphavirus nsP4. N-terminal disordered region, region required for terminal transferase activity and GDD catalytic motif for RdRp are shown (268, 274).

The alphavirus nsP4 is an unstable protein and this property is at least partially due to the presence of an N-terminal Tyr residue that leads to the rapid degradation of free nsP4 by the proteasome (275, 276). The N-terminus itself is, however, absolutely required for the formation of functional replicases, as unprocessed nsP1234 polyproteins form a nonfunctional replicase (128, 130, 277). Therefore, it is likely that rapid degradation of free nsP4 is used to maintain tight control over the production of nsP4. In addition, most alphaviruses down regulate nsP4 expression with an opal termination codon upstream of the region encoding nsP4. The read through of the terminator occurs with approximate 5–20% efficiency (275, 278, 279). The N-terminal Tyr is not only needed to promote nsP4 degradation; it is also needed for nsP4 activity. Thus, Tyr can be replaced by any other aromatic aa or H or to some extent, M (270), while other residues at this position suppress the viability of the virus. The viruses harboring such mutations can, however, recover by acquiring compensatory mutations in nsP1 or in nsP4. These data again strongly suggests that the N-terminus of nsP4 is involved in a productive interaction with nsP1 during the initiation of minus strand synthesis. Interestingly, a follow up study described one more possibility to compensate for a non-aromatic residue at the N-terminus of nsP4. In this case, compensation is achieved by including an extra 3'-UA-5'-rich region into a putative promoter site for the initiation of positive polarity RNA synthesis. This finding corroborates the notion that the N-terminus of nsP4 is also involved in the initiation of genomic RNA synthesis (204, 280).

The alphaviral nsP4 has also terminal TATase activity; the enzyme preferentially adds adenine nts to the 3' end of RNA. This activity is likely necessary for synthesis, maintenance and repairs of the poly(A) tail, which cannot be synthesized in a template-dependent fashion (Fig. 15). This activity was first demonstrated for purified SINV nsP4 devoid of 97 aa at the N-terminus (nsP4del97). Mg^{+2} or Mn^{+2} -dependent TATase activity was abolished by a GDD to GAA mutation in the catalytic center of nsP4 (Fig. 15) (274). It has also been shown that in the case of SINV, nsP4 could add poly(A) or AU-rich regions to the ends of defective or damaged viral genomes (281).

It was later shown that the N-terminal region, which was missing in the original recombinant protein, holds some importance as its presence considerably increased TATase activity. This sequence was, however, strictly required for the RdRp activity of nsP4: in the presence of nsP123, the purified nsP4 with the correct N-terminus (but not nsP4del97) was able to synthesize negative polarity RNA. This synthesis was abolished by mutations in the 3' CSE and poly(A) region. Combined, these data indicate that the unstructured N-terminal region of nsP4 is involved in efficient RNA recognition. It has also been shown that individual nsP4 (thus, in the absence of other viral ns proteins) is still capable of transcribing positive and negative polarity viral RNA but not sg RNA. Therefore, it can be suggested that the N-terminal region could be involved in a decisive, but regulated, interaction with nsP123 that may lead to an altered conformation preferring specific promoter recognition and subsequent synthesis of a particular polarity of RNA over others (268, 269, 282).

RNA-protein crosslinking experiments have shed some light on promoter recognition by alphaviral nsP4. It has been shown that sequences of the nsP4 molecule, used for the recognition of promoters for sg and genomic RNA synthesis, are different. In the case of SINV, the sg promoter is recognized and bound by peptide 329LVRRLT334, while peptide 531LGKPLPAD538 is used for recognition of the genomic promoter. Importantly, data from crosslinking experiments were clearly supported by reverse genetic experiments. Thus, changing the R residues at positions 331 and 332 affected only the synthesis of sg, not the genomic RNA (283–285).

In the course of alphavirus infection, the synthesis of negative or positive polarity viral RNA is regulated by ns polyprotein processing. When nsP4 and unprocessed nsP123 form the early replication complex, mostly negative polarity viral RNAs are synthesized. This indicates that the complex recognizes promoter elements for negative strand synthesis in the positive polarity genomic RNA. In contrast, the late replicase complex consisting of mature nsPs does not normally recognize the promoter for the synthesis of negative strand RNA but rather only the promoters for positive polarity RNA (129, 130, 232, 233, 277). These findings strongly suggest that not only the existence of differential recognition elements on the same nsP4 molecule is important for alterations in promoter specificity but this is also affected by fine-tuned conformational changes. These changes may be triggered by protease cleavage to ensure the synthesis of viral RNA of the appropriate polarity. However, it is more likely that conformational changes (or parts of them) actually occur prior to proteolytic cleavages, which are used to make these changes essentially irreversible. If so, these proteases are tools to recognize the changes, not tools to make them happen. This hypothesis is in line with the observation that SINV, harboring non-cleavable nsP123 and one compensatory change in nsP4, is capable of synthesizing RNAs of both polarities (247). Similarly, some temperature-sensitive mutants of SINV are capable of re-activating negative strand synthesis even in the presence of inhibitors of protein synthesis (286). Thus, the regulation of viral replicase complex specificity is not rigid, and the same end results could be achieved using different approaches.

3.1.8. Alphavirus-based vectors

Alphavirus-based vectors represent promising tools in the field of gene therapy, cancer treatment and vaccine development (287). These vectors are widely studied and used. As these constructs are based on RNA, regulatory elements, which almost exclusively originate from the "DNA world," cannot be used for these vectors. Nevertheless, there are multiple options to control and regulate gene expression with these vectors. For example, alphavirus gene expression (at least for SFV and SINV) could be regulated using different strategies such as using a capsid enhancer sequence (77, 152, 288).

The advantage of alphavirus vectors over other viruses derives from its very basic characteristics, such as the production of high-titer virus stock in a relatively short period of time, a broad host range, a high rate of RNA replication and transient gene expression (289–291). In anti-tumor and anti-microbial treatments, the drawbacks, which are intrinsically related to alphavirus infection (such as the shutdown of host cellular machineries, induction of programmed cell death and short term mode of expression) could actually be advantageous for such therapies (292). Mutations in the viral ns proteins could be used to limit RNA replication and to develop non-cytopathic replicons (the term replicon is used to indicate self-replicating RNA that cannot form infectious virions); such RNAs are capable of persistent replication in mammalian cells (293). Such mutations are most often mapped to nsP2; however, several manipulations in the nsP3 or nsP1 region can also reduce the cytotoxicity of alphaviruses and their replicons (195, 265, 294–297). Alphavirus vectors can also be used for basic studies. For example, such vectors have an added advantage in neural research as these constructs can be used specifically for the infection of neural cells. Such an approach may result in a high percentage (> 90%) of GFP-positive neuronal cells, while glial cells may remain uninfected (287, 298).

As briefly mentioned above, alphavirus-derived replicon vectors are constructed by replacing the structural portion of the genome with multiple cloning sites. Thus, these vectors contain a natural ns protein-encoding region and all CSEs (including the sg promoter), which are essential for replication and transcription. The viral replicon particles, which are similar to alphavirus virions (except that they contain replicon RNA instead of the viral genome), could be produced by co-transfecting cells with *in vitro* transcribed replicon RNA and helper RNAs used for the synthesis of the structural proteins (290, 299). The expression of foreign genes could be performed by transfecting these engineered RNAs directly into the cytoplasm or infecting cells with viral replicon particles carrying corresponding RNA. In many cases, the fact that replicons are limited to the infected (transfected) cells and cannot escape to infect nearby cells is beneficial. To ensure this, the packaging signals were purposefully deleted from helper-RNAs making replicons of single-cycle vectors. As many alphaviruses are pathogenic for humans, the safety of alphavirus replicons is an important concern. Indeed, as replicons and helper-RNAs contain identical sequences, there is risk that these two RNAs may recombine, resulting in wt virus. Multiple approaches can be used to reduce such risk. The most straightforward approach is to split the coding region for structural proteins into capsid and glycoprotein portions resulting in two helper vectors. This manipulation greatly reduces the probability of successful recombination (300–303).

Another type of alphavirus vector, called a “marker virus,” has been constructed and studied. In these cases, the foreign gene (most commonly a marker gene such as EGFP) is inserted into the structural or ns portion of the virus genome, or its expression is derived from an extra copy of the sg promoter (sometimes also by IRES from another virus). For example, one such vector based on the SINV genome has EGFP linked to “auto-cleaving” 2A sequence

from foot-and mouth disease virus, placed in between the capsid and E3 regions. This virus is claimed to show more stability than the analogous vectors containing a duplicated sg promoter (304). Comparable work has been carried out by different research groups by inserting a marker gene into the replicase portion of the SINV genome; the obtained viruses have been used to study the inhibition of alphavirus replication, nsP3-specific complex formation and functions related to nsP2 (305–307). It should be mentioned that the design of such recombinant genomes cannot always be used for different viruses. For example, an SFV construct similar to that described by Thomas *et al*. (2003) suffers from instability and is completely avirulent for mice (308). While studies were not able to construct a recombinant SINV with EGFP inserted into the nsP1 protein, such constructs were obtained for both CHIKV and SFV (unpublished data from our lab). Regardless, the number of approaches that could be used to improve alphavirus-based expression systems is nearly endless. Systems may be engineered to achieve high-level and long-lasting expression of heterologous proteins (309), to increase cell-type specificity of the virus (310) and other functions.

3.2. Helicases

3.2.1. Introduction to Helicases

Helicases are NTPase or dNTPase enzymes that are capable of unwinding ds NA into its component single strands. Helicases can also be viewed as subsets of "translocases": enzymes that are capable of hydrolyzing nts to directionally travel on nucleic acids with or without unwinding them. Thus, all helicases can be translocases but the reverse may not hold true (225, 311, 312). For example, RIG-I, EcoR124I, SWI/SNF, and ISW2 are all translocases but do not possess helicase activity (313). Helicases are processive enzymes that are capable of carrying out multiple cycles of catalysis before releasing the product. This is different from distributive enzymes such as simple NTPases that undergo one round of catalysis and release the product to bind a new substrate (225).

Helicases were discovered close to 40 years ago. The first report of an enzyme of such type appeared in 1976: an enzyme from the Gram-negative bacterium *E.coli* that unwound DNA in an ATP-dependent manner was described. The authors of this article state that "the enzyme was found to denature these duplexes in an ATP-dependent reaction, without detectably degrading" (314). First, eukaryotic helicase (from meiotic cells of *Lilium*) was reported in 1978; in this study, the authors termed this enzyme as "DNA unwinding protein" or "U-protein" (315). Since then, helicases have been discovered in all forms of life. Their functions are not only confined to the unwinding of nucleic acids; it has been understood that they play a much greater and complex role in the metabolism of NA including protein displacement from DNA and RNA, remodeling of chromatin, DNA replication and repair, transcription, translation, ribosome

biogenesis, maturation of RNA, splicing, and nuclear export processes (312, 316). Furthermore, the importance of these enzymes can be highlighted by considering the number of diseases associated with their malfunctioning (317–321). Helicase has also been proposed as a drug target to limit viral infection (322–324).

Helicases often exhibit polarity or directionality, which demonstrates the unidirectional manner in which the helicase may unwind and/or translocate on the NA. Given the structure of NA, the direction could be a 5'-3' or 3'-5'; there have also been reports of bidirectional or bipolar helicases that can unwind NA in both directions. Examples of such enzymes are eukaryotic translation initiation factors and RH70, which unwinds RNA in a bipolar manner (325–327). Common differences between DNA and RNA helicases concern their processivity:

- DNA helicases tend to be more processive: they often translocate or unwind multiple basepairs before dissociating.
- RNA helicases are relatively less processive and typically act as NTP-dependent chaperones by binding and remodeling RNA to form a more productive conformation (312, 328).

3.2.2. Classification of Helicases

Helicases can be classified mechanistically on the basis of some of the very general features such as:

- Substrate specificity: whether the helicase acts exclusively on RNA, DNA or both or on RNA:DNA hybrids
- Directionality: whether it moves and unwinds in a 3'-5' (class A) or 5'-3' (class B) direction
- Whether the helicase is a *bona fide* unwinding machine or a simple translocase
- Whether the helicase molecule forms a toroid or ring around NA

However, these classifications are very broad, which provides minimal information about the individual helicases; therefore, such groupings are very rarely used in the helicase research community (225, 311).

The most robust and withstanding classifications are based on the enzyme sequence and structural information. Based originally on the sequence alignment and presence of at least seven signature motifs (motif I, Ia, II, III, IV, V, VI), Gorbalenya and Koonin classified helicases into three Superfamilies (SFs) and two families of putative helicases (316). Since then, many new helicases have been reported. Furthermore, there are also several putative helicases; these enzymes have NTPase activity but lack genuine NA unwinding activity. Therefore, the original classification was again amended in 2007 by Singleton *et al.* to

accommodate these new helicases. Furthermore, the newer classification does not rely exclusively on the primary sequences of the enzymes; the available structural information with approximate 3D structures and additional newly discovered motifs (TxGx, Q-motif, motif4a, TRG) were also taken into account. Based on this, helicases are now classified into 6 SFs (SF1-6). SF1 and SF2 are monomeric helicases that contain two RecA-like folds on the same polypeptide chain. In contrast, SF3-6 are usually hexameric helicases that comprise six individual RecA-like folds (each subunit or polypeptide contributes one of these folds) (224, 225). The above mentioned sequence motifs have been shown to represent signature peptide sequences present in the domains with the RecA-like folds or in between RecA-like subunits. All of these motifs contribute to the binding and hydrolysis of NTP molecules (329). However, out of seven motifs, Motif I (Walker A) and Motif II (Walker B), both of which bind to NTP, and motif VI (arginine-finger; important for energy coupling) contribute pivotally to the "Core Domain" of the helicase. Accordingly, mutations in these three motifs commonly result in a NTPase-negative enzyme (330, 331). A brief description for each SF is provided below; however, special emphasis has made on SF1, as alphavirus nsP2 helicase belongs to this group. SF2, to which the other helicases of RNA viruses (such as NS3 of HCV and DENV) belong (227), is also reviewed in a more detailed manner.

3.2.2.1. Superfamily 1

Helicases from SF1 have been extensively studied. They unwind NA either in a 3'-5' or 5'-3' direction. This SF includes prokaryotic (bacterial) helicases such as Rep, UvrD, PcrA, RecD and Dda, which are generally well-studied. It also includes relatively less-studied eukaryotic helicases such as Upf1, Pif1, Rrm3 and viral helicases from alphaviruses (nsP2), CoV (ORF-1b), HEV (ORF-1), and Rubivirus (p220) (225, 332–337). Thus, SF1 could be further classified into class A, which includes 3'-5' directional helicases, and class B, which includes 5'-3' directional helicases.

3.2.2.1.1. SF1 of class A

Crystal structures of the 3'-5' directionally biased PcrA (Fig. 16) and Rep helicases have been determined. These structures revealed that these molecules consist of two domains, each of which can be further subdivided into two sub-domains. The NTP binding site is situated in a cleft at the bottom of two RecA-like domains classified as 1A and 2A in PcrA. This region contains conserved motifs for binding and hydrolysis of NTP molecules (Fig. 16) (335, 337). It has also been observed that the minimum structural requirement for NTP hydrolysis and thereby for fuelling helicase activity is present in just two RecA-like domains. However, the other sub-domains, which are distantly present from the core RecA-like domain (1B and 2B in the case of PcrA) and probably do not

contribute directly to NTP hydrolysis, may nonetheless be important in unwinding activity as they could interact with the NA (Fig. 16) (337). These NA-binding domains, which could also be termed as "accessory domains," act by coupling NTP hydrolysis with large conformational changes along the NA (335, 338). It has been shown further that deletion of the 2B domain, which acts as an auto-inhibitor of the full-length Rep monomer, activates the helicase activity of the Rep monomer (normally the Rep monomer is relatively less processive than the usual dimerized enzyme) (339). However, a recent study proposed that 2B domain play a regulative role in case of Rep helicase; locking this domain in a closed conformation made Rep a monomeric super helicase (340).

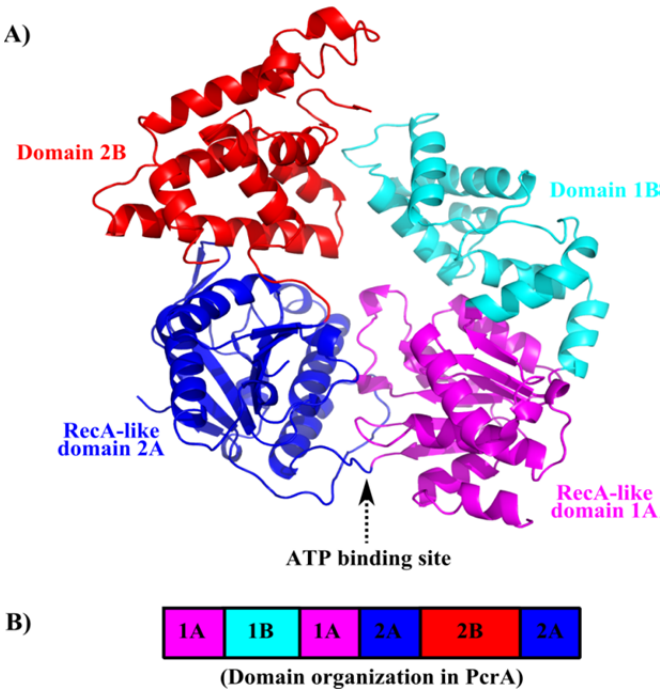


Figure 16. PcrA helicase from *Bacillus stearothermophilus* (337) and PDB ID 1PJR. A) The X-ray structure of the monomeric PcrA reveals omnipresent RecA-like domains and an ATP binding site. B) Schematic representation of the domain organization of PcrA; note that how distantly present sequences in the primary structure belong to the same or a single domain in the X-ray structure.

3.2.2.1.2. SF1 of class B

The structural and biochemical data for RecD from the RecBCD complex and biochemical evidence from studies of the Dda helicase contribute towards our understanding of SF1 helicases, which move in a 5'-3' directionally biased manner (Fig. 17) (225, 336, 341–344). The NTP binding and hydrolysis between

class A and B helicases are strikingly similar. The main differences derive from the mode of their interaction with the NA.

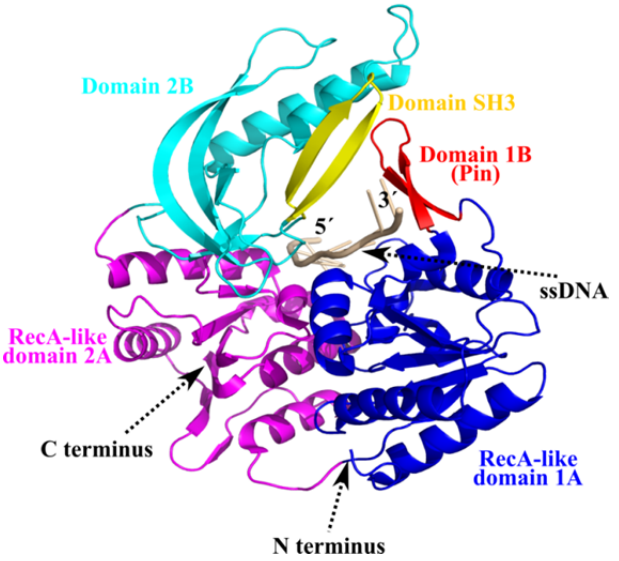


Figure 17. The 5'-3' SF1B Dda helicase (PDB ID 3UPU) from T4 phage co-crystallized with an ssDNA substrate. Domains 1A and 2A are the Rec-like domains, while the domain 1B, known as the 'Pin' domain, is required for the separation of dsDNA strands, which interact with the SH3 domain present primarily on domain 2B (342).

The well-studied RecD is part of the RecBCD heterotrimeric complex. Therefore, it is possible that the structural arrangement of this helicase may be evolved to complement the formation of the trimeric complex, and its structural conformation may not be fully comparable to those helicases that can act as monomers. This assumption is indeed supported by analysis of the crystal structure of RecD. The structure revealed the presence of an additional N-terminal domain (NTD; in heterotrimers, it is oriented toward the RecC subunit) that is not present in the Dda helicase (Fig. 17). Furthermore, RecD itself does not contact with the NA, as this interaction is mediated by the RecB subunit. Again, it is different for Dda. Thus, parallels between RecD and Dda outside the core RecA-like domains may or may not exist (225, 336, 343). Under single cycle conditions, it has been shown that the Dda helicase (Fig. 17) can unwind DNA as a monomer. However, irrespective of monomeric or dimeric structure, there is strong evidence that helicase molecules of SF1 can cooperatively unwind NA substrates; this results in enhanced processivity and productivity (225, 343).

3.2.2.2. Superfamily 2

SF2 is the largest SF of helicases and includes at least 10 subfamilies of helicases such as RecQ-like, RecG-like, Rad3/XPD, Ski2-like, type I restriction enzyme, RIG-I-like, NS3/NPH-II, DEAH/RHA, DEAD-box, and Swi/Snf families. SF1 and SF2 share the comparable core RecA-like domain for binding and hydrolyzing NTP molecules. However, distinct structural differences between these two families exist (225, 311). These are illustrated below with examples of helicases from the DEAD-box and NS3/NPH-II subfamilies (313).

3.2.2.2.1. DEAD box Helicase

The name of this group derived from the conserved peptide sequence representing motif II (DEAD stands for Asp-Glu-Ala-Asp). This is the largest group in SF2 and includes helicases from prokaryotes to higher eukaryotes (345). The helicases from this group are mostly involved in activities concerning RNA synthesis and functioning, including transcription, splicing, transport, ribosome biogenesis, translation, RNA/protein complex assembly, and degradation (313). Therefore, these enzymes are mostly ATP-dependent RNA chaperones that may not translocate or unwind ds NA processively. The landmark property of these enzymes is that their activity is enhanced upon binding to RNA. Generally, this binding occurs distantly from the unwinding active center of the enzyme (313, 346, 347). The protein from DEAD box family binds RNA in an ATP-dependent manner. However, as stated above, the protein does not translocate on the RNA but rather manipulates or remodels the structure of the RNA locally via protein-RNA interactions. This type of RNA remodeling involves strand rewinding and unwinding by prying of the individual strand. Thus, these enzymes can unwind only very short duplexes without any directionality (313, 348–351).

The DEAD box proteins are mainly comprised of two domains, both of which are primarily RecA-like domains. Certain fine-tuned features were discovered in these proteins, which specifically interact with nucleotides and RNA polymer (313). Motifs I-III belong to the N-terminal RecA-like domain, while motif IV-VI belong to the C-terminal Rec-A-like domain (348). A specific Q-motif, present in these proteins, interacts specifically with the adenine base of the ATP (352). The two Rec-A-like domains take an open conformation in the absence of ATP and RNA, while the domains close upon binding of these molecules (353, 354). In the case of DEAD proteins Ded1p, Mss116, and eIF4A, it has been shown that these proteins are capable of unwinding duplex RNA in the presence of non-hydrolysable ATP analogues such as ADP-BeF3 but not in the presence of ADP-AlF(4) or ADPNP. Thus, ATP binding but not its hydrolysis is necessary for strand separation. However, it has been shown that ATP hydrolysis leads to enzyme recycling, which could proceed through different degrees of affinity towards RNA (313, 349, 355–357).

3.2.2.2.2. NS3/NPH-II-like Helicase

This group includes a number of helicases from positive strand RNA viruses, mostly from the family *Flaviviridae:* the best studied examples are NS3 helicases of HCV (Fig. 18), DENV, WNV, YFV, and Japanese encephalitis virus.

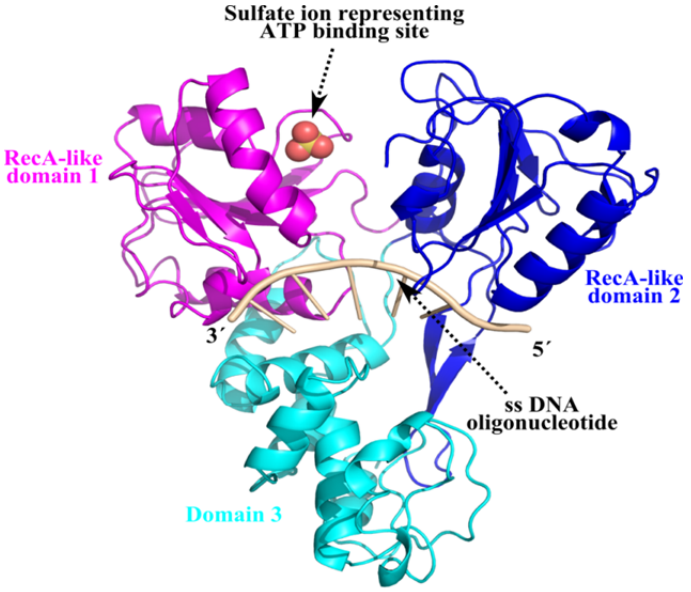


Figure 18. 3D structure of HCV NS3 helicase domain in complex with an ssDNA oligonucleotide. Domains 1 and 2 resemble classical RecA-like domains. A sulfate ion is bound to the ATP binding site. The ssDNA is bound in the channel between these two domains and the third (NA-binding) domain (358, 359) and PDB ID 1A1V.

The NS3 proteins from these viruses harbor helicase activity in their C-terminal region; the N-terminal region has peptidase activity. A few studies have suggested that communication exists between these two functionally different regions, which are important for helicase activity. Another extensively studied viral helicase from this group is NPH-II, which belongs to Vaccinia virus (VV) from the family *Poxviridae* (311, 313, 360–364). The NS3 helicase (Fig. 18) acts both on DNA, RNA and DNA:RNA hybrid substrates with a 3' ss region. In contrast, NPH-II prefers substrates with a RNA loading strand (313, 365–369). It has been shown that nicks in the loading strands pauses the enzyme when it is still engaged in the process of unwinding. Thus, these enzymes prefer to track on one strand while excluding the other strand (370, 371). The 3D structure of the NS3 helicase of HCV (Fig. 18) has been known for many years and has been intensively studied (359, 365, 369, 372, 373). This protein (and others similar to it) is likely to crystallize, a hypothesis that is supported by bioinformatical analysis of the NS3 primary sequence on the Xtal-pred server (374). All structural studies have shown that the NS3 helicase consists of three

domains: two RecA-like domains (named domain 1 and 2) and NA-binding domain 3 (Fig. 18). The NA is held in an electrostatic pocket formed between the two RecA-like motor domains and a 3rd domain (Fig. 18) (365).

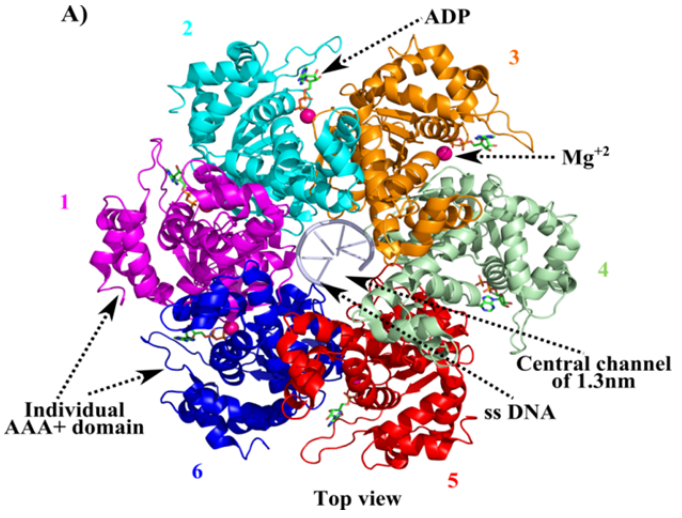
3.2.3. Hexameric Helicases

The SF1 and SF2 helicases can function as monomers, indicating that a single polypeptide can fold to form an NTP binding and hydrolysis domain as well as the NA binding domains. The ring-shaped (or toroid) helicases, which are classified as SF3, SF4 (DnaB-like), SF5 (Rho/V-F-ATPases), and SF6 (AAA^+: ATPases Associated with various cellular Activities), are considerably different both structurally as well as mechanistically from the first two SFs. The ring-shaped helicases are usually hexamers containing six identical or different subunits and are therefore called homohexamers (e.g., MCM complex from archea) or heterohexamers (e.g., MCM complex from eukaryotes), respectively (375–377). Each properly folded polypeptide, representing one subunit of hexamer, contributes the aa residues for the Walker A and B motifs, while the arginine finger motif comes from the neighboring subunit; thus, the NTP binding and catalysis centers are formed from elements belonging to different subunits (225, 378). The hexameric helicases are often associated with NA metabolism processes such as replication (e.g., T7, T4, and SPP1 helicases in bacteriophages), recombination (e.g., bacterial RuvB protein) and transcription (e.g., bacterial rho protein) (379). The topological contacts formed by these toroid enzymes with the DNA molecule empower these enzymes to be highly processive machines on the DNA. There are very well-controlled mechanisms that regulate how these oligomeric protein molecules load themselves onto the DNA molecule. These include controlled binding of NTP or Mg^{2+} (or both), which leads to the formation of hexamer. Binding of NTP and the hexameric form is necessary for DNA loading, which in turn stabilizes the complex further. Finally, DNA binding leads to the stimulation of NTPase activity of the enzyme, and, through an energy transduction process, NTP hydrolysis is coupled to the unwinding activity. Each of these steps contributes to additional pivotal regulation of the activities associated with these enzymes (379). These functional and regulatory events are clearly important. This is evidenced by a human genetic disorder known as Bloom's syndrome, which is concomitant with the disruption of one of the eukaryotic hexameric helicases, leading to chromosomal instability through an unknown mechanism (225, 379–383).

3.2.3.1. Superfamily 3

SF3 helicases were first reported in small DNA and RNA viruses in which these proteins not only contribute to the unwinding of NA but are also involved in the binding of replication origins and initiation or replication. These helicases form

hexamers or double hexamers and unwind NA in a 3'-5' (Type A) direction. SF3 includes helicases from Simian virus 40 (SV40), adeno-associated virus (AAV) type 2, and human papillomavirus type 18 (HPV18), which represent well-studied examples of enzymes of this type (225, 378, 384–388). Originally, three sequence motifs, known as A, B, and C, were identified in each subunit in a stretch of just ~100 aa. Later, a motif known as B' was identified between motifs B and C. Thus, in the case of SF3, just approximately 100 aa residues contain all four conserved motifs. The motifs A and B are equivalent to the classical Walker A and Walker B motifs of SF1 or SF2, while motif C (Asn residue placed downstream of a hydrophobic stretch) is SF3-specific. The crucial arginine finger is present after motif C (225, 385, 389). The arginine finger senses the presence of the γ phosphate of the NTP bound to the neighboring subunit via the Walker A and B motifs (223, 378). The core domain of the SF3 helicases has structural similarity to the AAA^+ class of proteins (broader group of ATPases and Walker type NTPases, which are mostly involved in protein folding complex disassembly, protein transport, and protein degradation) rather than to the RecA-like domains of SF1 and SF2 (378) (390, 391). The AAA^+ module contains two domains at the N- and C-termini. The N-terminal domain has alpha-beta-alpha folds and an nt binding pocket formed by the classical Walker A and B motifs, while the C-terminal domain is purely alpha helical. The N-terminal domain resembles a RecA-like fold, which carries parallel beta sheets flanked by alpha helices (224, 329). Walker A is present as a loop (P-loop) connecting beta strand 1 and alpha helix 2, while Walker B is present on beta strand 3 consequent of the P-loop (Fig. 19). The all-alpha C-terminal domain is less conserved across the AAA^+ class of NTPases and is proposed to stabilize the hexamer (225, 390–392). The conserved NTPase motif is preceded by an origin (*Ori*) DNA binding domain (225, 378).



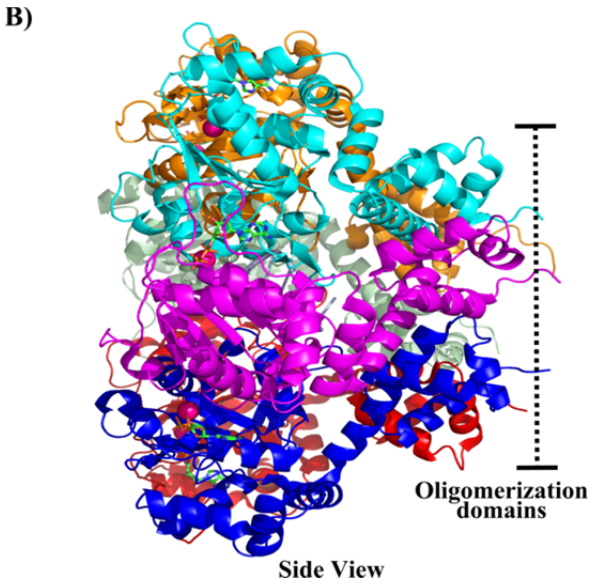


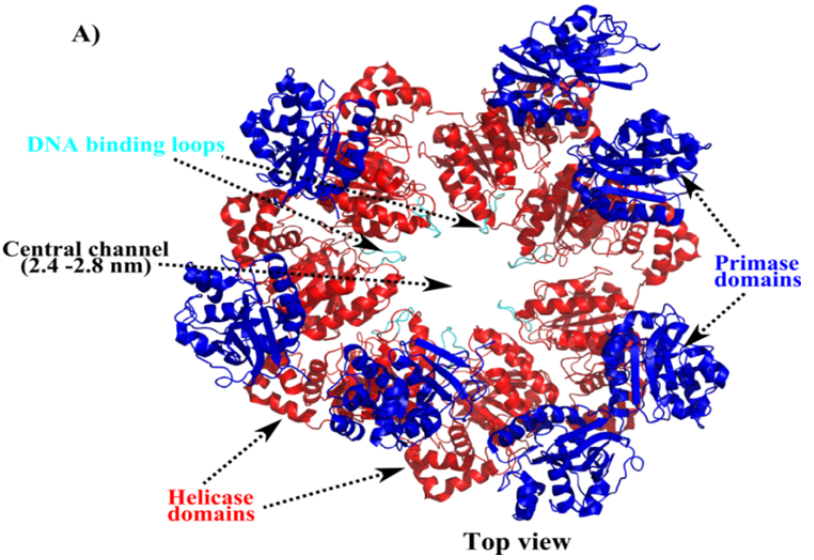
Figure 19. The hexameric Bovine papilloma virus (BPV) E1 helicase showing the arrangement of individual AAA+ subunits. A) Each subunit (1–6) is color-coded for representation. The ADP and Mg ions are bound to the interface between the subunits, while ssDNA is bound in the central channel. B) Lateral view of the BPV E1 helicase. The structure shows the oligomerization domains, which protrude out from the helicase (AAA+) domains and strongly interact with each other. This leads to the formation of a rigid collar which is proposed to contribute to the processivity of E1 helicase (393, 394) and PDB ID 2GXA. The author of the relevant article has reported two hexameric helicases bound to ssDNA; however, for the purpose of simplicity, only one has been presented here.

The papilloma Virus E1 hexameric helicase has been crystallized with a 13-nt-long ssDNA and ADP. The obtained structure revealed that oligomerization domain forms a sixfold symmetry collar while the AAA+ domains locate radially to the collar (Fig. 19). The central channel forms the ssDNA binding groove (Fig. 19), where a specific interaction between the B' motif and the phosphodiester backbone of DNA has been reported to exist. It has been proposed that the hexameric helicase has 6 ATP binding sites between subunits with three different conformations. These are interpreted to be moving around the ring at any point in time during the unwinding process: two places could be occupied by ATP (ATP-type conformation), while another two could carry ADP (ADP-type conformation) and last two may be empty (empty-type conformation). The position of the DNA binding loop reflects the class of the nt binding site. Thus, the ATP-type loop interacts towards the 5' end of the DNA while the ADP-type loop would interact with the DNA just below the ATP-type loop, and the empty-type loop places itself at the bottom of ADP-type loop on the DNA. The DNA is bound by multiple subunits: from one side there are

interactions with ATP-type loops, while from other side interactions with the empty-type loop occur. The loops move downwards with each catalysis of ATP, pulling the DNA with it; without ATP, it reverses itself to the top position to bind a new ATP molecule. The regulation and coordination of ATP hydrolysis between different subunits is supported by multiple interactions formed by the oligomerization domain, which safeguards the hexamer to continue to surround the ssDNA (Fig. 19). Recent evidence from single molecule experiments suggests that E1 unwinds DNA by a strand exclusion mechanism and that the N-terminal part of the helicase molecule surfaces towards the replication fork (225, 393, 395).

3.2.3.2. Superfamily 4

Helicases belonging to SF4 are primarily replicative helicases from bacteria and bacteriophages. In the bacterial system, the helicase is associated with primase activity. These functions reside in two separate polypeptides, *e.g.* DnaB and DnaG of *E. coli* or, as in the case of certain bacteriophage, in a single polypeptide. The helicase activity possesses a 5'-3' polarity, e.g., gp4 of bacteriophages T7 and T3 and alpha protein of bacteriophage P4. SF4 possesses five signature motifs designated H1, H1a, H2, H3 and H4 (396). Of these, the H1 and H2 are comparable to the classical Walker A and Walker B motifs, while other motifs have no comparable partners in other helicases (H3 often carries conserved Gln or in some cases His residues and has been proposed to be comparable to motif 3 of SF1) (225).



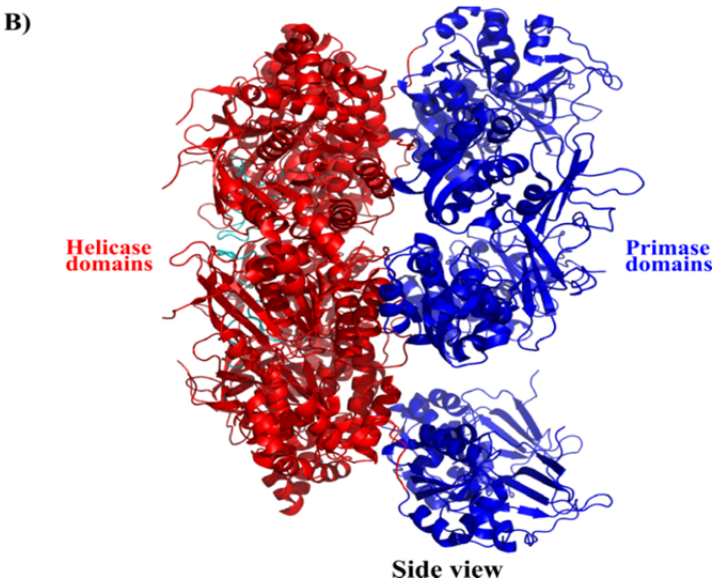


Figure 20. T7 gp4 is a heptameric primase-helicase. A) Top view showing primase domains (blue) and helicase domains (red). The DNA binding loop of the helicase domains are shown in cyan and are projected towards the central channel to bind dsDNA. The ring is essentially formed by the helicase domains with delicately associated primase domain. B) Side-view of gp4 shows crown-like shape of the complex (397) and PDB ID 1Q57.

The gp4 of phage T7 has been intensively studied, and the crystal structure of this enzyme has been obtained. It reveals the arrangement of subunits as hexamers (or heptamers) in which the primase domain is loosely staged over the helicase domain (Fig. 20) (382, 397). Interestingly, the polypeptide, which only supports NTPase activity, has also been crystallized; it is a helical filament with a RecA-like fold (398). The optimal arrangement of these subunits to couple the hydrolysis of NTP with translocation is still unclear; however, it could be suggested that cooperativity as well as regulatory control should exist between nt binding and DNA unwinding. Other SF4 helicases such as RSF1010 and *E. coli* DnaB have sixfold symmetry; in the case of the latter, threefold symmetry has also been reported (399, 400).

3.2.3.3. Superfamily 5

SF5 helicases exemplified by the Rho protein of bacteria closely resemble SF4 helicases; however, due to sequence differences these were assigned to a new SF. Rho is responsible for terminating RNA transcription in *E. coli.* It interacts with a signature sequence on the newly synthesized RNA strand and then unwinds the RNA:DNA hybrid resulting from the transcription process (Fig. 21) (225).

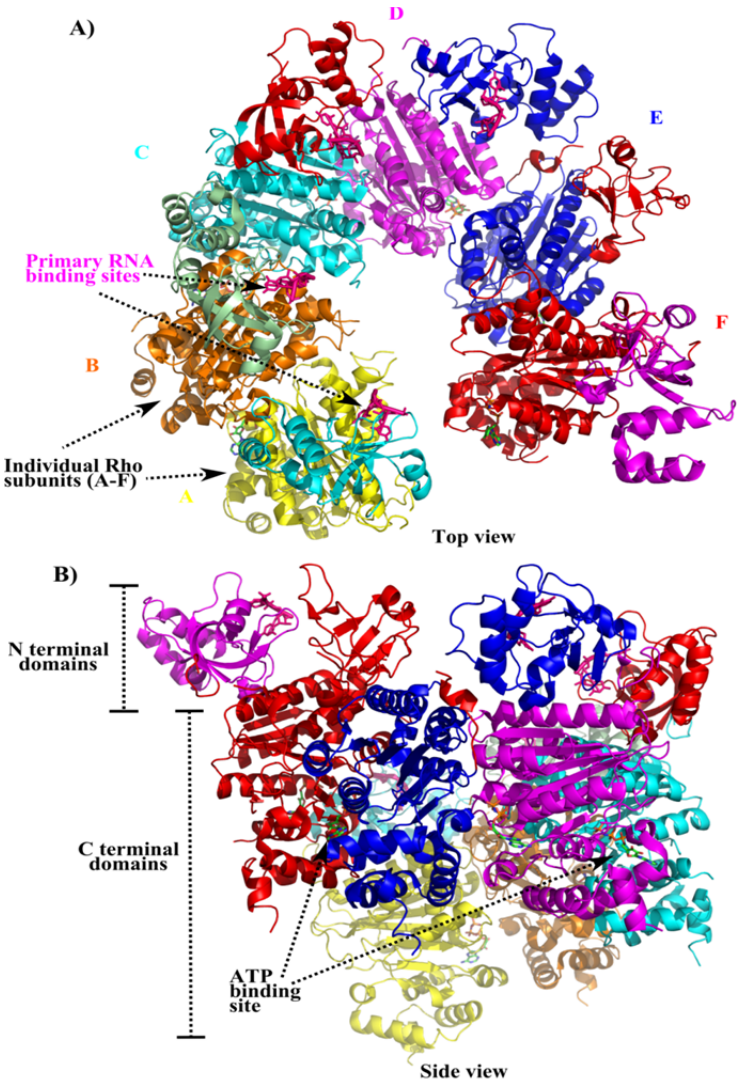


Figure 21. 3D structure of Rho helicase, the transcription terminator factor of bacteria. A) The hexameric arrangement of Rho subunits (A-F). Each subunit is color-coded. The primary RNA binding site is highlighted on the N-terminal domain, while the secondary RNA binding site is present towards the interior (center of the ring), to which the C-terminal domain of each Rho subunit contributes. Note that the Rho is an open hexamer rather a closed hexamer. B) Side view of Rho hexamer showing the N- and C-terminal domains and the ATP binding site (401) and PDB ID 1PVO.

Cryo-electron microscopy suggests that in the absence of RNA, the Rho protein (at a low micromolar concentration range) forms heterogenous complexes. In the presence of RNA, however, a predominantly hexameric arrangement of Rho subunits has been reported. The hexameric state could possess a “closed or

notched" conformation. In the same report, a small subpopulation of Rho was reported to have a dodecamer arrangement (402). However, a subsequent crystallographic study reinforced the hexameric arrangement of Rho subunits and identified two RNA binding sites on each Rho subunit (Fig. 21). The first site is responsible for mRNA recognition, while other contributes towards mRNA translocation. Interestingly, the global conformation of Rho is not closed but is split open (Fig. 21); it has been proposed that this facilitates mRNA loading (401).

3.2.3.4. Superfamily 6

Members of this SF mainly form hexameric arrangements; however, they can be heterohexamers as observed for eukaryotic MCM 2-7 or homohexamers as observed in the case of archaeal MCM. In the case of the eukaryotic complexes, only MCM 4,6,7 heterohexamers have been reported to possess helicase activity; this activity is involved in initiation and elongation during DNA replication (225, 376, 377). The available structural information for this SF is primarily derived from archaeal MCM. *Methanobacterium thermoautotrophicum* has only one MCM-like gene (ORF Mt1770), and it resembles the MCM 4 of eukaryotic cells. The purified archaeal MCM appears to form a double hexamer with an approximate molecular mass of 850 kDa and has ATP-independent DNA binding and DNA-stimulated ATPase activity. It also possesses processive 3'-5' polarity DNA unwinding activity (375). Electron microscopic reconstruction of these molecular machines shows that monomers are arranged to form hexameric complexes with a large central cavity to accommodate dsDNA and lateral holes (403). The X-ray studies, however, revealed that the subunits of MCM are arranged in a dodecamer complex with an unusually long central channel. This channel has a large number of positively charged residues that contribute towards DNA binding (404).

3.2.4. RNA Helicases, chaperones and match makers

RNA molecules have an intrinsic tendency to get misfolded and become kinetically trapped. Due to this basic problem, the specific RNA binding proteins termed "RNA chaperones," which can rescue RNA molecules in an ATP-dependent or independent manner, have been proposed (405–407). The RNA chaperones aid with the correct folding of RNA by preventing misfolding or by interfering with an incorrect rather than a stable RNA structure (405). For a comprehensive classification of the proteins with RNA chaperone activity, please see the relevant review (407) and the hyperlink therein.

In a similar direction, the set of proteins that facilitate the formation of double strands as a result of binding to each complimentary strand has been termed "match makers." These molecules often act in an ATP-independent manner.

However, in some cases ATP can be used to disrupt the secondary structure of RNA; thus, tight regulation and balance between the formation and contortion of a duplex exist (405, 406).

Among all of the SFs, the helicases of SF1 and SF2 are widely considered to be helicases that possess RNA chaperone activity and play an important role in RNA metabolism (328, 406). It has been proposed that RNA helicase activity is a property by which it is possible to estimate the extent to which a protein can perform RNA remodeling. These enzymes can unwind RNA via a basic cycle that comprises multiple consecutive steps involving ATP hydrolysis or by carrying out local strand separation, which requires the binding of ATP but not hydrolysis (328, 351). RNA helicases can also displace protein molecules from RNA in an ATP-dependent manner (408). However, it is not clear which exact functions RNA helicases carry out in a cellular context, where they could be involved in regulated interactions with partner proteins to diversify the complexity of their functions by many fold (328).

3.2.5. Mechanisms employed by RNA Helicases

3.2.5.1. Cooperativity, active and passive mode of unwinding

As mentioned above, the helicases can be divided into two broad categories: those that form a ring structure around the NA and those that do not. Ring-shape helicases require hexameric structures; for example, in a monomeric state the T7 gp4 helicase is not functional. In contrast, for monomeric helicases such as NS3, T4 Dda, UvrD, and RecBCD, the formation of oligomers is not necessary, although the helicase-related activity of some enzymes could be enhanced with the formation of dimers or higher-order oligomers (409, 410). Functional cooperation is often attributed to the monomeric helicase; multiple copies of these motor molecules are loaded onto the tracking strand, which prevents back slippage and enhances the availability of new molecules in case the forward molecule falls off (410, 411).

In a very broad sense, it can be said that helicase can operate in an active or a passive manner. In the active mode, the helicase molecule directly unwinds or destabilizes the ds NA. Under optimal conditions, an active helicase can unwind ds NA as fast as it can translocate onto the NA (412). In case of passive mode, the enzyme waits at the ss and ds junction until a thermal fluctuation occurs, which opens up the duplex. When this happens, helicase molecules go forward to bind to a new junction region, which prevents reannealing.

3.2.5.2. Processivity

It has been proposed that enzymes working on polymeric substrates, e.g., helicases, can carry out multiple rounds of catalysis before dissociating. However, the frequency of the dissociation depends on the specific mode employed by the

enzyme. In case of binding to the NA, the likeliness of dissociation decides whether a particular enzyme is processive or not.

One group of enzymes, which tends to be not so processive, only partially encloses the NA. In contrast, processive enzymes employ their elongated asymmetric structures or toroidal symmetry to fully enclose the NA polymer. The enzymes with toroidal symmetry tend to be more processive as they do not just bind; instead, they gird the NA substrate. However, it should be noted that complete enclosure of a substrate is not the only requirement for processivity. The specific aa residues that interacts with the NA, as well as the overall conformational geometry, must complement the favorable structure of the enzyme (380). Alternatively, processivity can also be increased by increasing the surface area of the enzyme that interacts with the NA. Thus, monomeric viral helicases, such as VV NPH II and HCV NS3, are also processive helicases, although they do not gird their NA substrates. Instead, they have large surfaces to cling to the NA (366, 413).

3.2.5.3. Stepping mechanism; Inchworm and Rolling models

Helicases exhibit directional translocation on their NA substrate. The mechanisms employed by these molecules are poorly understood. The current hypothesis is generally based on changes in enzyme affinity towards NA, which result from conformational alterations due to NTP hydrolysis (410). In a proposed stepping mechanism, the helicase molecule makes contact at least with two sites on the NA, which act accordingly to invoke from NTP hydrolysis. The inchworm model in the stepping mechanism assumes that a monomeric helicase establishes weak contact and firm contact with the NA. In a cycle of NA adherence, discharge and translocation, the weak bound site decouples due to the so-called power stroke, moves away from the firmly bound site and establishes contact at a new position (410). The rolling model proposed for dimeric helicase suggests that the subunit contacts the ssDNA and dsDNA based on the NTP hydrolysis state; however, the subunit switches mutually for the leading or lagging position in the translocation process (414).

3.2.5.4. Brownian motor model

The Brownian motor mechanism was originally proposed for HCV NS3. This model suggests a two stroke cycle in which the helicase adheres to NA alternatively between strong and weak binding modes according to the presence or absence of ATP. The absence of ATP results in a directional translocation and local melting of basepairs, which is fuelled by energy from ssDNA binding, while ATP binding charges the helicase molecule and prepares it for subsequent cycles (364, 410, 415).

3.2.5.5. Model for non-processive Helicase

Apart from the above proposed mechanism for processive helicases, there are also several mechanisms proposed for non-processive helicase, such as the DEAD class of helicase (328, 364). In one of the speculative models, it is proposed that during ATP hydrolysis and release, the helicase adopts a destabilizing conformation that exposes the electrostatic surface towards the duplex and triggers local strand melting. Another model was proposed for the Vasa helicase. It speculates that the helicase can execute force to accommodate only an ssRNA while excluding or tearing apart the other strand of the dsRNA substrate (328, 364).

3.2.6. Role of Helicases in viral lifecycle

It has been evident that positive strand RNA viruses with genomes larger than 7 kb and a number of DNA viruses encode for helicases/putative helicases or outsource cellular helicases to support their replication cycle. However, the role of helicases in the viral replication cycle is still intensely disputed. An immense amount of data, originating from biochemical experiments, is poorly aligned with the biological data. Thus, interpretations of biochemical evidence to extrapolate biological understanding have been extensively speculative in this regard (311, 334, 416, 417).

Viral infection is responsible for approximately 20% of human cancer cases and can cause severe neurological disorders and chronic diseases (418) while eighty percent of all viruses are RNA viruses, presumably coding for helicases or helicase-related enzymes (334, 417). It should be interpreted in a generic way that helicases are important for viruses, and thus it is obvious to predict that mutations in potential motifs in the helicase region or the application of compounds targeting these activities could impair viral replication (324, 419). For representative purpose I will briefly describe the biological roles of few selected viral helicases.

3.2.6.1. NS3 Helicase

HCV NS3 helicase has been extensively studied (358). It belongs to SF2 and unwinds both DNA, RNA and hybrid substrates in a 3'-5' directionally biased manner (413, 420). The helicase-related activity of NS3 is influenced by the presence of a protein cofactor, NS4A (421). Furthermore, alanine scanning of the acidic region of NS4A indicates that the NS4A and NS3 interaction is important for viral replication and particle assembly (422). It has been reported that along with a hydrophobic NS4B protein, NS3 physically and functionally interacts with HCV RdRp (NS5B). NS3 regulates template recognition by NS5B; for this, NTPase and presumably also helicase activities are required. The interaction between NS3 and NS5B is negatively regulated by NS4B (423).

It has been reported that the proline-rich linker region between the protease and helicase portions of NS3 interact with the Src homology 3 (SH3) domain. Indeed, mutation in the linker region leads to substantial defects in viral replication and infectivity, which are independent of polyprotein processing and NS3 stability (424–426).

3.2.6.2. Nidovirus Helicase

Nidovirus helicase, which also possesses RTPase activity, can unwind both DNA and RNA substrates in a 5'-3' directional biased manner (427–431). The helicase is located at the C-terminal region of nsp13, while the N-terminal region of the same protein contains ZBD (428). ZBD has been described as affecting mRNA synthesis, genome replication, and virion biogenesis (432). In the case of SARS-CoV, it has been shown that the RdRp of CoV influences the unwinding activity of nsp13 (427), which localizes to the cytoplasm despite its affinity towards DNA substrates (429). As it is out of the scope of this thesis, the extensive role of Nidovirus helicase in the viral lifecycle may be studied elsewhere (430).

3.2.6.3. Vaccinia virus Helicase

DNA viruses such as VV encode for a RNA helicase (NPH II) (417). NPH II does, however, unwind both DNA and RNA substrates (433). It has been shown that temperature-sensitive mutants of VV, lacking NPH II-related activity such as NTPase (and very likely helicase activity as well), fail to produce infectious progeny and exhibit defects in transcription initiation (434). This corroborates an earlier study that proposed that the DNA helicase activity of the NPH II protein is required for the initiation of early transcription (433). The effects of alanine mutations that were introduced into the classical helicase motifs of NPH II suggest that NTPase and importantly helicase activity of this protein are essential for VV replication (435).

3.2.6.4. HIV-associated Helicase

Retroviruses, including HIV-1, do not encode for a helicase. Instead, these viruses efficiently outsource a number of host cell helicases for their replication process (436). The gene expression of HIV-1 is regulated by interaction between the virus-encoded Tat and Rev Proteins and host factors, which include proteins with helicase-related activities. Rev interacts with pre-spliced mRNA, which is transported out of the nucleus to form genomic RNA for packaging. It has been reported that cellular helicases, such as DDX1 and DDX3, interact with Rev to regulate HIV-1 gene expression during the late stage of viral replication. In particular, DDX1 has a functional role in mRNA splicing,

transcription and translation (417, 437–439). It has also been reported that many other proteins with helicase activity (DHX36, DDX24, DDX17, DHX9, DDX47 and DDX5) interact with the Rev protein. Of these, DDX5 and DDX17 have been shown to affect the production of HIV virions (417, 440). DHX9 is also associated with HIV-1 Gag and becomes encapsulated into the virion. HIV-1 virions, lacking this cellular protein, exhibit reduced reverse transcriptase activity (417, 441, 442). It is also reported that the interaction of DDX24 with Rev and Gag influences the trafficking of the viral genome for packaging (443).

3.2.6.5. Generic role of viral Helicase

Apart from the above mentioned specific roles of helicases in the life cycle of different viruses, few generic functions of helicases have been additionally proposed (334, 430). First, it has been observed that helicases are encoded by viruses with relatively longer genomes. Therefore, it is suggested that unwinding activity is dispensable for viruses with smaller genomes. Second, as viruses with relatively longer genomes encode for helicase, it is possible that helicase might be involved in proofreading activity by interacting with polymerases (444). This hypothesis is supported by a study examining the fidelity of CHIKV replicase with a C483Y mutation in nsP4. It was found that this mutant virus acquires a second supportive mutation in nsP2 (G641D), the helicase protein of CHIKV. However, the role of nsP2 in the fidelity of CHIKV RNA replication still remains unknown (445). In the case of SARS-CoV, another positive strand RNA virus, it has been biochemically shown that nsp12, which is the RdRp, enhances the unwinding activity of nsp13, the viral helicase (427). Third, as suggested for VV, helicases may be involved in RNA-DNA duplex unwinding. This places helicase just behind the DNA-dependent RNA polymerase in this virus; the aim of this arrangement may be to prevent R loop formation (446). Finally, it has been suggested that helicase could play an important role during the initiation of translation as deduced from the helicase activity of eukaryotic initiation factor 4A-4B (327).

3.3. Alphavirus Proteases

The field of proteases is very extensive and composite. As the current thesis reports protease-related activity in the context of CHIKV, it is appropriate to briefly review the literature relevant to alphaviral proteases. As alphavirus proteases have a crucial role in the maturation of structural and ns proteins, they also represent attractive targets for drugs to suppress viral replication (447). Indeed, anti-protease compounds are in use in combinational therapy against HIV and in recent years also in anti-HCV therapy. This demonstrates how the protease enzymes, which are essential for the replication of different viruses, can be efficiently targeted (448–450). In the case of alphavirus, the protease activity has been reported in two different contexts: first for the capsid auto-

protease (chymotrypsin-like serine protease) and then for the nsP2 protease (papain-like protease) (450–452).

3.3.1. Capsid: the autoprotease of alphavirus

The structural polyprotein of alphaviruses, with a capsid protein region at its N-terminal portion, is translated from sg RNA (25, 450). The capsid protein cleaves itself from this polyprotein and folds into a conformation where its active site is blocked (450). In the 264 aa long SINV capsid protein, residues H141, N163 and S263 have been identified as a catalytic triad. A recent study of CHIKV capsid autoprotease activity revealed that W261, situated at the C-terminus of this protein, and I227 also contribute to protease activity (453, 454). The structures for the capsid proteins of SINV, SFV and Aura virus (AV) are informative. In the case of SINV, the residues from 114 to 264 fold into a pattern that resembles the chymotrypsin-like serine protease; furthermore, it is evident that residues 261–264 are situated in the active site of the enzyme, thus resembling the unprocessed peptide substrate. In particular, a bulky aa such as W264 situated at the extreme C-terminus is accommodated back into this catalytic pocket, explaining the auto inhibition mechanism of this protease (153, 450, 455). Recently, a FRET-based *trans* protease assay using AV capsid protease was proposed and used for the screening of inhibitors for alphavirus capsid protease (456).

3.3.2. NsP2 Protease as a target for potential drug candidates

As functions of nsP2 have already been discussed extensively in the previous section, only its potential use as a drug target is reviewed here. Several research studies have attempted to target nsP2 protease activity, which is central for virus replication, with small molecules (457–461). After the 3D structure of the protease portion of nsP2 became available, different techniques were used to predict small molecules, which could dock into the proteinase active site (222, 228). This resulted in the discovery of different molecules with varying degrees of efficiency. It was reported in a non-biologically validated study that Q1039, K1045, E1157, G1176, H1222, K1239, S1293, E1296 and M1297 residues of CHIKV establish interactions with the peptidase substrate and can be targeted with chemical molecules (461). Using a purely bioinformatical platform, a ligand that exhibited potential affinity towards the protease domain has also been reported (458). In another study, it was found that thiazolidine derivatives could act as CHIKV inhibitors in a low micromolar range; it was proposed that these compounds target the protease domain (459). In a comparatively comprehensive study, which initially took into account 5 million compounds (for the assessment of structure-function relationships), a set of 26 compounds was selected for biological validation. In this study, by employing a cytopathic re-

duction assay, a compound with relatively higher anti-CHIKV activity was described, although its anti-nsP2 activity was not directly demonstrated (457, 462). As the nsP2 protease is not the only potential drug target for the treatment of CHIKV infection, researchers have used diversified approaches to address the therapeutic aspects of this highly pathogenic virus. A description of those strategies is beyond the scope of the current thesis and can be obtained elsewhere (463, 464).

4. AIMS OF THE STUDY

NsP2, a replicase protein of alphavirus, has been the focus of a number of research projects, both in our lab and also around the world. These studies have aimed not only to exploit the replicative aspects of this important protein but also to assign important enzymatic activities to the various regions of nsP2. Furthermore, functions of nsP2 those pertaining to viral replication and/or to its catalytic activities have been studied.

Among its enzymatic activities, the helicase activity of nsP2 has been deficiently studied and understood. It was demonstrated only once, more than 15 years ago, for nsP2 of SFV (217). Since then, it has not been confirmed for any other nsP2s from alphaviruses; in contrast, papers reporting failure to demonstrate this activity have also been published (209). There have been almost no detailed studies to follow up on previous conclusions or more detailed helicase-related characterizations such as polarity, substrate specificity and other aspects has been conducted.

Helicase is, by definition, quite a straightforward enzyme. In the simplest terms, it is an enzyme that catalyzes the unwinding of NA in an NTP hydrolysis-dependent manner. Critical observations on this topic led us to imagine that the helicase activities of nsP2 ought to be carried out not only by the so-called classical domains. So we hypothesized that along with these indispensable classical helicase-related domains, which coordinate with each other in a productive manner, accessory domains may exist as described previously for other bona fide helicases. In other words, we expected nsP2 as a helicase to be considerably more complex than the simple so-called "helicase domain protein." It also did not escape our notice that there were increasing amounts of data indicating that the "helicase domain" of nsP2 is likely involved in other activities of nsP2, such as some of its protease activity as well as the cytotoxic functions of nsP2.

Thus, the current thesis set out to understand the functional significance of various domains of nsP2 by carrying out biochemical experiments. This was subsequently extended to experiments aiming to understand how these functions are related to the biological properties of virus. The study integrates several important aspects of alphavirus biology, making it rather difficult to highlight the objectives. Nevertheless the core aims of the thesis can be depicted in the following specific or disassembled manner.

I) Elucidate the structural aspects of the full-length nsP2 protein of Chikungunya virus (CHIKV) using a bioinformatical platform and homology modeling with special attention to identify the key aa residues that could be interacting with NTP molecule.
II) Standardize a protocol for the overexpression and purification of active recombinant nsP2 protein with an authentic N-terminus and various mutant versions of the protein with the aim of obtaining appropriate materials for biochemical analysis.

III) Assess the activity of recombinant protein(s) by employing activity assays to examine helicase, NTPase, and protease activities and attempt to optimize the reaction conditions accordingly.

IV) By employing the knowledge gathered from objectives I, II, and III, try to understand the effects of mutations, presumably unrelated to helicase activity, such as those in alphaviral noncytotoxic (NCT) mutants in the biochemical protease, helicase and NTPase assays.

V) Using experience gathered from alphavirus protein overexpression and purification experiments, try to purify other replicase proteins of CHIKV (and related alphaviruses). This includes the replicase proteins nsP1, nsP3 and nsP4. Use the recombinant proteins for the production of high-grade antibodies that are needed but are unavailable from any commercial source. Use these antibodies for studies carried out by our research team as well as provide them to other groups working with alphaviruses to contribute to the general arena of alphaviral research.

5. RESULTS

The kernels of this thesis have been mostly acquired from the scientific work performed for article II, which extensively addresses the purification and biochemical characterization of CHIKV nsP2. This article also set out to demonstrate the minimal region requirements for helicase activity and requirements for optimal NTPase activity, providing further insight into the functioning of this protein in an enzymatic context. Finally, this article attempted to standardize the optimal conditions for NTPase and helicase activity with a long term aim of proposing or formulating a high-throughput method for screening small molecule inhibitors to suppress or modulate alphavirus infection as proposed for other viruses (465). The core of the results and some data from unpublished studies that could have been part of article II (but were not included for several reasons) are presented in this section.

5.1. CHIKV nsP2 structurally resembles the ToMV Helicase (Article II)

Various research groups around the globe have tried to obtain X-ray crystallographic structures of full-length nsP2 of alphavirus. Despite significant effort, no success has been attained for nsP2 or its N-terminal region. Success has been limited to resolving the structure of the truncated version of nsP2, consisting of C-terminal domains (protease portion) or these domains together with two thirds N- terminal region of nsP3 in a pre-cleavage conformation (222, 228, 234). Therefore, it was imperative to gather more information regarding the structure of full-length and specifically the N-terminal region of nsP2. For structural modeling or predicting the key motifs in CHIKV N470 (fragment consisting of the first 470 aa of the N-terminal region of nsP2), the sequence of this region was processed using various online programs such as PHYRE, PHYRE 2, I-TASSER and MODELLER (466–468). All online programs predicted the structure of N470 to resemble the helicase or NTPase domain of Tomato Mosaic Virus, ToMV, a member of the alphavirus-like SF of helicases. However, the PHYRE server predicted that the structure was comparable to that of the Upf1 helicase, which is also a SF1 helicase. In the latter case, the PHYRE server took into account only residues from 46 to 444 out of 470 aa residues; in contrast, all sequences of N470 (residues 1–470) were accounted for building a structure resembling the ToMV helicase (332, 467, 469). It should also be noted that the core of ToMV helicase is structurally comparable to the Upf1-like, UvrD/Rep and Pif-1-like helicases (469); therefore, the central portions of the N470 models based on different structures (ToMV versus Upf1) were relatively similar.

The structure of the modeled CHIKV N470 had three domains: two RecA-like domains (1A and 2A) and an extreme N-terminal domain (NTD), which was predicted to be disordered (Article II, Fig. 1B). Domains 1A and 2A were

predicted to be pivotal for NTP binding and hydrolysis (223, 469). The function of the alphavirus nsP2 NTD domain has yet to be determined; however, from the structural predictions it can be suggested that this region comprising the loops may be intrinsically disordered; this region lacks clear structural homology with any of the folds in the database, which could indicate that it carries unique viral protein folds (470). At the same time, the confidently predicted RecA-like fold is omnipresent in the helicase-like enzymes (365). These two domains carry the signature motifs for interaction with the NTP molecule and its hydrolysis. Thus, it was relatively obvious to conclude that these motifs share both functional and sequence similarities with corresponding motifs from SF1 helicases such as PcrA, Upf1,ToMV (332, 337, 469). The comparative residue profiles of these motifs are presented as table 2 in article II (215).

When full-length nsP2 was processed through the above mentioned servers, they predicted the structures separately: first, they predicted the structure of N470 in reference to the structure of ToMV (469) and then separately processed the C-terminal region, affiliating it with the unpublished CHIKV protease structure or the VEEV protease structure (222, 228). The limitation with this mode of prediction is that it provides no insight into how the N-terminal region of nsP2 and its C-terminal region are structurally arranged relative to each other.

5.2. Expression and Purification of CHIKV nsP4 (Article I and Unpublished)

During the studies included in this thesis, a number of recombinant ns proteins from different alphaviruses as well as number of other relevant proteins (such as TEV protease, HCV NS3 helicase and Ubl-specific protease 1) were successfully expressed and purified. To avoid redundancy, only some of the proteins that were not reported in the articles are described here. One of these proteins, the purification and use of which was essential for publication (Article I), was CHIKV nsP4, the alphaviral RdRp. Interestingly, this protein turned out to be easy to express but extremely difficult to purify.

The coding region of nsP4 was codon optimized for a bacterial expression system, and the recombinant protein was tagged with a hexahistidine tag at the N-terminus. However, repeated attempts to obtain the protein in the soluble fraction were unsuccessful. The protein yield was high (which could possibly be one factor for insolubility), allowing us to purify the protein from the insoluble pellet. This was carried out via collection of the pellet followed by washes with PBS and “Triton X-100” containing buffer. The pellet was dissolved in a buffer containing 6 M guanidium hydrochloride (to note, the pellet was completely insoluble in 8 M urea, a reagent normally used for the purification of insoluble proteins). After high speed centrifugation, the obtained supernatant was passed through Ni-NTA resin, washed with buffer containing 8 M urea and eluted with the same buffer containing imidazole (under these conditions the protein

remained in solution). The purified protein was processed further to raise anti-nsP4 rabbit polyclonal serum that was used to probe the nsP4 in western blot experiments (Article I). For individual protein purification purposes, readers are advised to peruse the relevant articles associated with the thesis. Of the proteins not described in the article, some mutant versions of nsP2 of CHIKV as well as nsP1 and the N-terminal portions of the nsP2 and nsP3 proteins of ONNV and BFV were obtained, and soluble recombinant proteins were used to raise corresponding antibodies. The technical details of their purification are available from the author.

5.3. NsP2 is largely an alpha-helical protein (Article II)

NsP2 and its N-terminal portion have been refractory to attempts for resolving their 3D structures. In the absence of a 3D structure, CD spectroscopy is an excellent method to estimate the content of the secondary structural elements of a protein. It is a quick and straightforward method and is especially useful to compare the folding patterns of wt and mutant version(s) of the same protein (471). However, it must be considered that the accuracy of secondary structural element estimation is highly dependent on the accuracy of the protein concentration (471, 472). For the current study, CD spectroscopic analysis of nsP2 and its mutant versions indicated that nsP2 has high alpha helical content. A considerable portion of the protein was indicated to be random coil. These data were highly reproducible, indicating that nsP2 is predominately an alpha helical protein. Furthermore, the protein was also often tolerant to point mutations, for example addition and deletion of two aa residues at the N terminus (Article II, Fig. 2B) unless they were situated in a sensitive location of this protein such as the 5A-PG mutation (Article III, Fig. 6B). In contrast, deletions of the NTD or MTL domains resulted in the aggregation of the protein, which was evident from size exclusion chromatography and greatly increased CD signal (473).

5.4. NsP2 hydrolyzes all canonical NTPs and dNTPs (Article II)

NTPase activity assays were carried out to exploit the degree of variability between the wt and the mutant forms of nsP2. In a typical assay, the enzymes were treated with high concentrations (relative to the Km) of NTP molecules such as GTP (150-200 μM), and their continuous reaction progress was monitored on a spectrophotometer. NTPase activity was strongly affected by the presence of a protease domain and the presence of a non-authenticated N-terminus (Article II, Fig. 3D); the defects caused by various NCT mutations were smaller and did not correlate with the cytotoxic/non-cytotoxic properties of replicons containing corresponding mutations (Article III, Fig. 8A). It could also be understood from these experiments that full-length nsP2 was able to hydrolyze all canonical NTPs and dNTPs with comparative catalytic rates.

GTPase activity was selected to compare the data obtained in the present study with that originating from previous works describing the NTPase activity of alphaviral nsP2 (209, 210, 220).

5.5. NsP2 GTPase activity is moderately stimulated by nucleic acid oligomers (Article II)

The GTPase activity of nsP2 was stimulated by 1.75 μM of NA-oligomers depicted in Article II. The degree of stimulation of nsP2 GTPase activity was moderate; a maximum effect (stimulation up to 2.5 times above basal activity) was observed for polyd(C) (Article II, Fig. 3E). Such an effect is highly comparable to SF1 of helicases such as HEV helicase, TGBp1 helicase, and SFV helicase (220, 474, 475). In contrast, the degree of stimulation is almost residual if compared to HCV NS3, which is an SF2 helicase (476). Thus, it could be advocated that the stimulation of NTPase activity by NA-oligomers may be an expected characteristic of nsP2 but may not be a compulsory (or prominent) attribute.

5.6. NsP2 is a 5'-3' RNA Helicase (Article II)

Historically (or to provide the enzyme with the most conducive substrate), helicases have been mostly studied using artificial substrates to determine directionality, substrate specificity and possible occlusion size. In some cases, biologically relevant substrates have also been studied (326, 420, 477). As the biologically relevant substrate for nsP2 is not known (as the role of helicase activity of nsP2 in the virus life cycle is not clear), the substrates used in the current study were adapted from studies dedicated to the analysis of HEV helicase activity (474). The treatment of appropriate dsNA substrates with nsP2 and its mutant forms in the presence of ATP demonstrated that nsP2 works as a helicase on the RNA substrate in a 5'-3' directionally biased manner. The occlusion size of this enzyme could be predicted to be in between 7 to 12 nts. This enzyme remained unresponsive to DNA substrates with equivalent properties (Article II, Fig. 4).

5.7. NsP2 also unwinds hybrid substrates (Unpublished)

When a hybrid substrate comprising a RNA tracking (longer) strand of 28 nts and a DNA displacing (shorter) strand of 16 nts was presented to CHIKV nsP2, it exhibited quite evident unwinding activity (Fig. 22). When a substrate containing longer DNA and shorter RNA strand was presented, the outcome was inconclusive: a certain amount of displaced ss strand was also observed in the absence of ATP (Fig. 22).

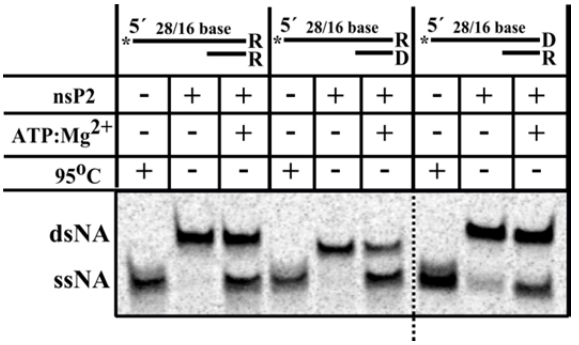


Figure 22. CHIKV nsP2 unwinds both dsRNA and hybrid RNA:DNA substrates where the tracking strand is RNA. In an alternative situation where DNA is used as the tracking strand, a reproducible ATP-independent release of ssRNA was observed. Most likely this could be attributed to the instability of this particular type of hybrid substrate.

It should be noted here that thermodynamically the hybrid substrates such used in this study are less stable than their homogenous counterparts (478). Thus, passive mode of helix destabilization may also take place. Such activity is also observed for the nsp2C of Picorna-like virus, in which the RNA chaperone-like activity can be functionally separated from its ATP hydrolyzing activity (479). At this point we cannot rule out that the same could be true for the last substrate with longer DNA and shorter RNA strand. Indeed, it remains possible that under the tested conditions this particular substrate becomes intrinsically unstable or a passive way of hybrid substrate unwinding could occur. To provide a definitive answer, the ability of nsP2 to unwind such a substrate must be carefully investigated using a panel of control substrates before conclusions are drawn.

5.8. NsP2 possesses RNA annealing or matchmaker activity (Article II)

Rewinding activity is opposite to the unwinding activity, which means that the enzyme can supposedly anneal complementary ssRNA strands to form dsRNA. It was suspected that the RNA annealing properties of nsP2 may be the reason for its low helicase reaction processivity as well as productivity. To verify this hypothesis, the enzyme was mixed with ssRNAs that were complementary to each other, and the reaction was carried out under conditions that were non-optimal for helicase activity: the salt concentration was kept minimal (1 mM) and ATP was not added. It was indeed observed that under these conditions nsP2 works as an RNA strand annealer for the substrates, purportedly forming a fork-like structure (Article II, Fig. 6 C). The enzyme was less effective with substrates supposedly forming 5' overhang products (Article II, Fig. 6 A, B), possibly because binding of nsP2 to a shorter ssRNA strand hampered duplex formation. Interestingly, nsP2 with an N-terminal His-tag was a more efficient

annealer than nsP2 with an authentic N-terminus (Article II, Fig. 6). The obtained results are quite consistent with previous observations with the Ded1 helicase (350). The RNA annealing process is presumably facilitated by the binding of nsP2 to an individual RNA or both RNA strands, which increases their probability of being in close proximity to each other, thereby enhancing macromolecular crowding and the rate of annealing (407).

5.9. Reaction parameters residually influence nsP2 unwinding activity (Article II)

Enzyme reactions are very often influenced by the parameters by which they are driven. In case of the nsP2 helicase assay, the reaction criteria such as time kinetics, enzyme concentration, monovalent salt concentration, pH, nucleotides and temperature were varied, and the effects of these variables on the reaction were observed. It was noticed that the products of nsP2 helicase activity can be detected at very early time points and that reactions (in approximately 30 min) reach equilibrium irrespective of the type of substrate. The considerable increase in the enzyme concentration with respect to the substrate could not make the helicase reaction more productive. These data are consistent with idea that nsP2 as a helicase is an enzyme with moderate processivity. In turn, it implies that the enzyme is unlikely to be involved in unwinding long dsRNA duplexes such as replication intermediates of CHIKV (which have length of approximately 12 kbp). It was also observed that the presence of an N-terminal His-tag decreased the reaction amplitude; most likely this was due to the enhanced ability of this enzyme to catalyze the backward (rewinding) reaction (Article II, Fig. 5 A, B), although a defect in RNA unwinding (for example due to compromised NTPase activity) cannot also be ruled out.

Other reaction parameters, such as those referred to in the published paper (Article II, Fig. 7), did not have notable effects. NsP2 helicase activity was observed to be very sensitive to elevated salt concentrations (Article II, Fig. 7A). The least preferred nucleotides hydrolyzed to fuel unwinding activity were UTP and dGTP (Article II, Fig. 7C), and the enzyme preferred Mg divalent cations over the others tested (Article II, Fig. 7D).

5.10. N-terminus of CHIKV nsP2 is important for 2/3 site processing (Unpublished)

The N-terminus of nsP2 was clearly important for NTPase activity and its intactness also affected helicase activity (Article II). In the case of nsP2 of SFV, it affects the protease activity, more specifically the ability to cleave the 2/3 site. To verify that this was also the case for nsP2 of CHIKV, wt nsP2 was used to process substrates corresponding to the 1/2 site, 2/3 site and 3/4 site (the design of these substrates are described in Article III). In addition, variants of nsP2

containing minimal alterations in the N-terminal region were assayed for their ability to process the 2/3 site.

As expected, nsP2 efficiently processed substrates corresponding to the 1/2 and 3/4 sites. The 2/3 site in its short form was processed very inefficiently (if at all); however, a longer substrate corresponding to this site was efficiently cleaved (Fig. 23). Thus, the basic requirements for protease substrates were similar or comparable to those revealed for nsP2 of SFV (132). NsP2 lacking just two N-terminal aa residues was not able to cleave the 2/3 substrate (Fig. 23). In contrast, the addition of two extra aa residues to the N-terminus did not affect the ability of the enzyme to cleave this substrate (Fig. 23), indicating that nsP2 of CHIKV tolerates the addition of extra aa residues in this region. Based on this property, nsP2 of CHIKV is different from nsP2 of SFV as in the latter case both deletion and insertion of just a single aa residue has a detrimental effect on the ability of nsP2 to process the 2/3 site (132). It should be noted that the 2/3 site of CHIKV has a much more favorable aa sequence for cleavage than the corresponding site in SFV nsP1234. Thus, it is possible that better context of the cleavage site made the enzyme more tolerant to some modifications at its N-terminus. Most likely it indicates that nsP2 of CHIKV can, at least to some extent, cleave the 2/3 site in the nsP123 polyprotein before its N-terminus is liberated by the cleavage of the 1/2 site. Indeed, evidence of this cleavage has been observed in *in vitro* translation experiments (our unpublished data).

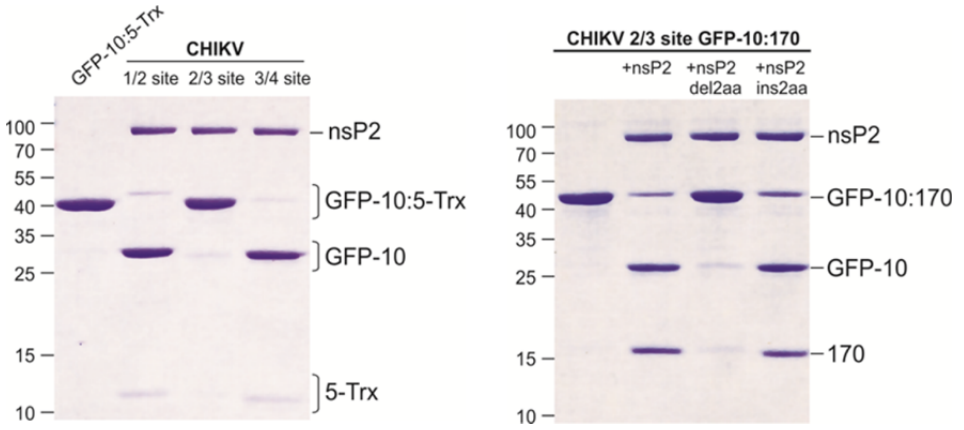


Figure 23. Processing of the 2/3 site from the CHIKV nsP1234 site is sensitive to manipulations of the N-terminal region of nsP2. Left panel: nsP2 is able to process substrates containing short variants of the 1/2 and 3/4 cleavage sites but not similar substrates corresponding to the 2/3 site. Right: substrate containing a longer version of the 2/3 site is cleaved by wt nsP2 but not with nsP2 lacking 2 aa residues in its N-terminus. Please see text for more details.

5.11. N- and C-terminal domains of nsP2 may interact with each other (Unpublished)

It was ascertained during the purification process of various versions of nsP2 that a hexahistidine tag followed by a TEV protease site could not be removed from full-length protein using TEV protease.

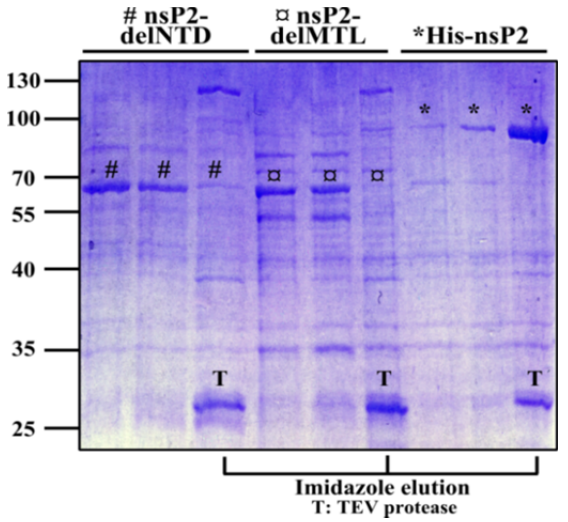


Figure 24. Accessibility of the TEV protease cleavage site for the TEV protease. Recombinant proteins were treated with TEV protease and attempted to bind to Ni-NTA resin. TEV protease-treated nsP2-delNTD (#) and nsP2-delMTL (¤) failed to bind the resin, indicating the removal of the tag. In contrast, His-nsP2 (*) bound the resin efficiently and was eluted from it only in the presence of imidazole.

In contrast, an identically placed tag was easily removed from N470, which lacks the last two C-terminal domains (protease and MTL). This finding provoked the idea that these two domains (or one of them) impose steric hindrance on the TEV protease to process its site at the extreme N-terminus of nsP2 (Article II). To test this possibility, recombinant proteins lacking either NTD or the MTL domain were constructed and expressed. It was found that an N-terminal hexahistidine tag was efficiently removed from both of these truncated proteins using TEV protease (Fig. 24). In the case of a deleted version of NTD, it could be proposed that the N-terminus of the protein fell short of reaching the cavity between the protease and the MTL. Interestingly, in the protein lacking MTL domain, the deletion apparently exposed that cavity, making the tag accessible for TEV cleavage. This observation is critically referred to in article II; however, data affiliated with this thesis was not presented there.

The article III associated with this thesis addresses the characterization of NCT mutants of CHIKV obtained in cell culture selection experiments.

Relevant to this thesis is the part involving the characterization of nsP2 proteins carrying mutations identified in these replicons. The screening resulted in several mutations and biological assays confirmed that mutations critical to the NCT phenotype are located in nsP2. Interestingly, however, these mutations did not affect the localization of nsP2 in infected cells, indicating that these mutations affect certain other functions of nsP2 rather than its subcellular localization. It was found that NCT-related mutations invariably affect almost all enzymatic activity of nsP2 including protease, NTPase and helicase activities. It was proposed that defects in one (or several) of these activities are crucial for the NCT phenotype of corresponding replicons.

5.12. Protease activity is selectively affected by NCT mutants (Article III)

The protease substrates, corresponding to various cleavage sites, were presented to CHIKV wt nsP2 and to enzymes carrying various NCT-related mutants. It was observed that the mutation 5A (insertion of five aa between 647–648 residue) (Article III) had a mild effect on the protease activity of nsP2. In contrast, the PG (P718G) mutation alone or in combination with other tested mutations compromised proteolytic activity towards the 1/2 and 3/4 cleavage sites (Article III, Fig. 7B). None of these mutations compromised cleavage of the 2/3 site in its longer version (Article III, Fig. 7C). These data suggest that NCT-related mutations, especially the PG mutation, slow down the processing of 1/2 and 3/4 cleavages. This effect was, however, not observed in the context of infectious viruses, indicating that cleavage of the same site in infected cells was affected to a much lesser extent; a hypothesis explaining this discrepancy is provided in Article III.

5.13. NCT mutants affect GTPase activity as well as often abolish the stimulatory effects of RNA oligomers (Article III)

The PG (P718G) mutation reduced GTPase activity relative to nsP2 to a considerable degree; the effect was greater for the combination of EK-PG (E117K and P718G) mutations but smaller for 5A-PG (insertion of five aa residue between 647–648 and P718G). As both of these combinations result in an NCT phenotype for the corresponding replicon, there was no clear correlation observed between the degree of GTPase activity reduction and the phenotype of the replicon. When the GTPase activity was stimulated by the addition of poly(U), it was observed that wt nsP2 and nsP2-5A were stimulated. NsP2-PG and all proteins with double mutations, except nsP2-EK-PG, remained insensitive to the addition of poly(U). Again, as nsP2 EK-PG (non-cytotoxic combination) was efficiently stimulated by poly(U), no clear correlation between the effects of the

oligonucleotide on GTPase activity and the phenotype of the replicon was observed (Article III, Fig. 8 A, B).

5.14. Amplitude of RNA unwinding is reduced by NCT-related mutants (Article III)

NsP2 has been demonstrated to unwind RNA molecules with 5' overhangs. It was shown that the helicase activity of nsP2 is also reduced by mutations originating from NCT replicons. Due to the nature of the experimental quantitation, the effects of mutations on nsP2 helicase activity were relatively inaccurate; no statistically significant data were obtained. Nevertheless, helicase activity was the only activity of nsP2 for which a correlation between the NCT phenotype and a functional defect in nsP2 was observed: helicase activity was lowest in the case of nsP2 with 5A-PG or EK-PG mutations (both of these combinations were found to cause the NCT phenotype) (Article III, Fig. 8 C, D).

6. DISCUSSION

6.1. Structurally and functionally, nsP2 could be much more composite

As it is understood now, nsP2 consists of five domains (Article II). It is a complex protein, and our understanding of its functions is therefore complicated as well. For example, nsP2 currently has been reported to possess four enzymatic activities, of which NTPase and RTPase are assigned to the N-terminal region and protease is ascribed to the C-terminal region. However, to what region does the helicase activity belong? Only full length nsP2 has the capacity for RNA unwinding activity, indicating that apart from canonical RecA-like domains, the extreme C-terminal portion of the protein (MTL domain), and most likely the NTD, are involved, but the question is how? An obvious guess is NA binding. This hypothesis is supported by various practical observations. Specifically, during the purification process for the SINV $P23^{Pro\text{-}Zbd}$, it was noticed that a considerable amount of bacterial RNA was intrinsically bound to the allegedly purified protein (234). Thus, MTL along with the ZBD likely provides a RNA binding platform that regulates the functionalities of nsP2, such as the protease activity of the enzyme (480). However, it remains unexplained whether the same could also affect helicase functionalities. It was shown in Article II that full-length nsP2 is sufficient for RNA unwinding. However, if a stretch of nsP3 reaching up to the ZBD was included; would the enzyme become more processive? This outcome seems feasible, as an additional RNA binding platform may indeed increase the processivity of the helicase enzyme. However, just as easily, a similar addition may also render the nsP2 selectively nonfunctional, for example by affecting NTPase activity. Furthermore, NA binding activity is not the only function that could be attributed to the NTD and MTL domains. For example, these domains could function by structurally stabilizing the rest of the nsP2 (131, 209, 220, 222).

NsP2 possesses an abnormally long linker region (~30 aa); an average linker size is approximately 10 aa, but a large linker can be as long as 28 aa (228, 481) between the protease and MTL domains. Why a multitasking protein such as nsP2 should carry such a long stretch of inter-domain loop is unclear. Is it reaching out to something or is it ensuring the MTL has relative flexibility and larger domain motions than the other domains? It is possible (as discussed in Article II) that a positively charged domain such as MTL could provide an RNA binding platform. If so, it may need such a type of loop to cling on to the RNA, which somehow necessitates greater motion. Previous studies with SF2 viral helicases such as NS3 from HCV, DENV, and WEEV have shown that the full-length enzyme harboring the protease domain influences the unwinding activity of the helicase domain, most likely by increasing RNA binding activity (360, 361, 482). The data obtained by manipulating the linker region of DENV NS3 suggest that both the helicase domain and protease domain not only must be present on the same polypeptide but also must rotate in a controlled manner

through the linker region, for which unnecessary flexibility would be counter-productive (363). As alphavirus nsP2 is a multidomain protein, it is likely that a long linker could also be involved in performing equivalent functions. Clearly, further investigation beyond its previously described role in minus strand synthesis (230) of the linker region in nsP2 is necessary.

6.2. The N-terminal domain of nsP2 implements diversified functions

To date, the 3D structure of the NTD of nsP2 remains unresolved. Homology modeling software programs predict it to be "partially disordered," but it is highly unlikely that such a long portion of the protein could indeed lack a definitive structure. Instead, it is conceivable that some part of this stretch is structurally disordered. The secondary structure prediction server forecasts with a reasonable degree of confidence that the NTD contains both β-strand and α-helices with a considerable amount of loops as well (483). Thus, it is very likely that this region is at least partly disordered. Viral proteins are indeed abundant sources for this type of intrinsically disordered region, which assists them with adapting to rapidly changing environments by replenishing interactions with proteins and NA molecules (484, 485). In line with this view, it has been proposed that the N-terminal region of nsP2 acts as a cofactor for protease activity and is involved in interactions with the 5' end of the viral genome (131, 486). Later studies have clearly shown that the exactness of the N-terminal region (length and accurate aa sequence of the N-terminal portion of nsP2) is pivotal for cleavage of the 2/3 site. This process is driven by fine-tuned interactions between this particular domain and the MD of nsP3 (132). Taken together, it could be concluded that the existing evidence supports the notion that NTD (and its N-terminus) is designed for a number of complex functionalities. Thus, it is quite possible that this region could not only be disordered but may also be unique, as it has evolved to support functional diversification.

6.3. The authenticity of the N-terminus is important for nsP2-related activities

Consistent with previous studies from our research group (132), the studies presented in this thesis confirm that the N-terminus is important for several enzymatic functions of nsP2 (Article II and unpublished studies). These observations reinforce previous findings and extend them to functions other than alphaviral nsP2/3 site cleavage. Thus, His-tagged nsP2 demonstrated reduced NTPase and helicase activity and enhanced RNA annealing activity. It may sound obvious to expect that a positively charged tag (such as a hexahistidine tag) should enhance RNA annealing by increasing macromolecular crowding (407). However, it is less obvious and remains undefined how such a small N-terminal tag affects NTPase and RNA helicase activities as well.

It was observed that nsP2 itself could *cis* cleave a His-tag from its N-terminus quite efficiently (assuming there is a suitable cleavage site). The same region was apparently not accessible for *trans* cleavage by the TEV protease (Article II and see the results section). Most probably, it indicates that a foreign cleavage site strained the protease active center and trapped the nsP2 in a "pre-cleavage" conformation. In turn, this prevented optimal domain coordination needed for maximal enzymatic activities, hence a reduction in NTPase activity.

The strict requirement for a native N-terminus complicates the expression of functional proteins, as N-terminal tags are clearly unfavorable. The problem can be circumvented by a His-tag at the C-terminus of nsP2; however, it is still not the solution as an initiation codon-derived extra Met residue will be placed at the N-terminus of the protein (the native N-terminal residue of nsP2 is Gly). It was already demonstrated that for SFV, the addition of just one simple unnatural aa is unfavorable for nsP2 to process the nsP2/3 site (132). There may be fewer problems for nsP2 of CHIKV, as the cleavage of the P2/3 site was more tolerant to small extensions at the N-terminus (Fig. 23) than to deletions. However, NTPase activity was still uniformly and significantly reduced by N-terminal manipulations (Article II). Thus, researchers should be cautious while using N-terminally tagged nsP2 in any study, as claiming that "such a small tag is unlikely to affect the function of the protein" is not true. The same applies for other alphaviral ns proteins as well. The genuineness of the N-terminus is important for nsP4, as substitution of an authentic N-terminal Tyr residue in nsP4, the RdRp of alphavirus, with any non-aromatic aa residue is fatal; even the addition of a single extra Met residue to its native N-terminus strongly affected the ability of SINV to replicate (270). Furthermore, similar examples are known for other viral proteins. For example, helicase-related functions of SARS-CoV nsp13 (or its homolog from HCoV 229E) are also sensitive to tags such as hexahistidine, glutathione S-transferase and maltose binding protein. Furthermore, even the way in which the recombinant protein is overexpressed affects its functions (430). In the case of nsp8 (~22 kDa), the second ns protein carrying RdRp activity in addition to nsp12 (~106 kDa), the main polymerase of coronavirus, it was observed that adding a His or ubiquitin tag at the N-terminus of this relatively small protein affects its ability to perform synthesis of oligonucleotides. More specifically, a His-tagged nsp8 was able to synthesize only very small (< 6 nt) oligomers; in contrast, non-tagged (or C-terminally tagged) nsp8 synthesized much longer products. This finding suggests that the N-terminal fusion partner affects the polymerization capability of nsp8 (487–489). One reason for these effects may be that the presence of an extra tag at the N-terminus may mimic the situation prior to the liberation of the N-terminus of the protein via protease cleavage. If so, the properties of the tagged proteins, may resemble those of proteins still incorporated with its polyprotein precursor. Unfortunately, extrapolating the attachment of a fusion partner for viral enzymes to the presence of viral protein at the same position before polyprotein cleavage has little support. Thus, the 3D polymerase of poliovirus is inactive as long as it is attached to the 3C protease; in contrast, the unprocessed polyprotein

(nsp7-nsp8-His) of SARS-CoV extends the primer with an efficiency comparable to that of nsP7+nsP8-His (both proteins present as individual subunits) (489, 490). Taken together, to determine the dependable functionalities of any pathogen-related enzyme, the recombinant proteins must be as comparable to their natural counterparts as possible.

6.4. The Overlaps: NTPase, RTPase and Helicase

Viral helicases such as alphavirus nsP2 are among the insufficiently studied enzymes. In a way, this reflects underestimation of the extent or magnitude to which the virus replication process could be dependent on these enzymes. The viral RNA helicases or the motifs involved in the process have diversified functions to carry out, such as NTPase, RTPase and helicase (209, 210, 474, 491, 492), but all of these reactions are executed or fuelled from a common reaction center comprising the signature motifs (225, 316, 493). Thus, it is exceedingly difficult to dissect these functions from each other and very complex to understand which activities are performed at which stages of the replication cycle (or if they could happen simultaneously) as well as which of them (or all of them) is critically important. Until now, attempts to analyze this state of affairs have been carried out by putting point mutations in the Walker A or Walker B motifs (213, 226). This is not truly informative; as such mutations silence all three activities of the protein. Thus, caution should be maintained when elucidating the interpretation of these *ad hoc* data, and one of the immediate goals could be to identify key aa residues on nsP2 that can affect RNA unwinding activity but no other activities of this protein. This will allow experiments to be carried out on a completely different level and potentially reveal the true significance of RNA helicase activity for alphaviruses.

6.5. N- and C-terminal domains of nsP2 contribute to Helicase functionality

Alphaviral nsP2 terminal domains are investigated to be of considerable significance in many ways however they exhibit a number of contrasting characteristics. The N-terminal domain is predicted to be disordered (Article II), while the C-terminal MTL domain is folded, and its structure (resembles Ftsj MTL domain) has been resolved (222). TEV protease cleavage accessibility data provide indirect evidence that these two domains interact with each other (Fig. 24). This hypothesis is further supported by the difference in the electrostatic potential of these domains: the calculated pI of the NTD is ~5.4, while the pI of MTL is ~10.13, resulting in an electrostatic interaction between these terminal domains (Fig. 25). It was also found that a manipulated version of nsP2, such as Hel470, or other truncated versions of nsP2 (Article II) or fragments of nsP2

(aa residues 167-630) tagged with maltose binding protein all lack helicase activity and possess compromised NTPase activity (209).

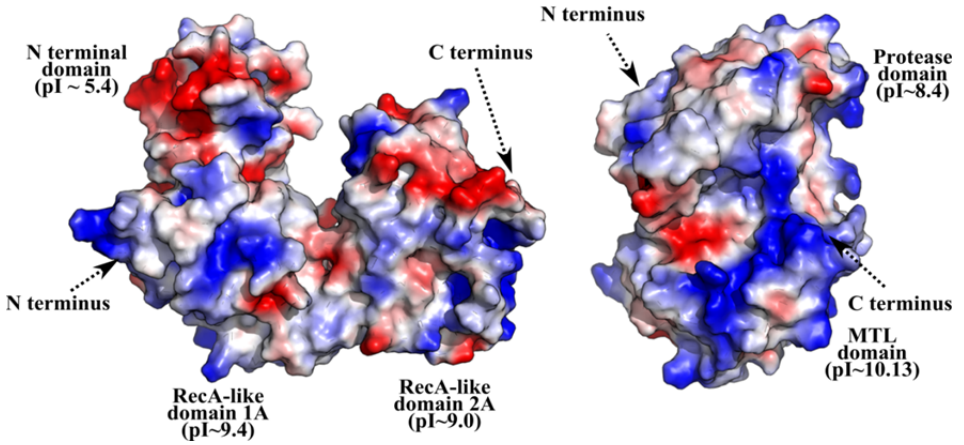


Figure 25. Electrostatic surface of CHIKV nsP2 showing the overall charge distribution across various domains. Note that the NTD (N-terminal domain) is highly acidic (pI 5.4), while the C-terminal MTL domain is highly basic (pI 10.13). Please refer to the text for more detail (Article II and (228)).

It is straightforward to anticipate that basic MTL serves as a binding platform for NA. It is much harder to imagine how the acidic NTD domain contributes to RNA unwinding. For this, some existing data can provide help. It has been previously suggested that the NTD domain of VEEV can interact with RNA. Thus, despite its generally acidic nature, some exposed positively charged (15% of 170 N-terminal aa) residues could be sufficient to catch onto the RNA (486). It has been proposed that for an acidic enzyme such as the helicase domain of NS3 (pI~6), a relatively low pH such as 6.5 considerably activates RNA unwinding, presumably by deprotonating the acidic side chain; this generates a selective and regulated repulsive force for processive translocation along the NA (494). However, it is unclear to what extent this hypothesis holds true for CHIKV nsP2. As for now, we only know that a hexahistidine tag (a highly positively charged patch of aa) attached to this region reduced RNA unwinding activity. This may occur by imposing unfavorable positive charges in a negatively charged domain (Article II). Certainly, the role of the acidic N-terminal domain demands further insightful studies.

6.6. Stimulation of NTPase activity by nucleic acid oligomers may not be a compulsion

Helicase activity is fuelled by the NTPase activity of the same enzyme. Therefore, it is obvious that these two biochemical reactions are coupled. In this line of thought, there is a broad consensus around helicase research community regarding the stimulation of NTPase activity by NA-oligomers; in some cases the effects of stimulation are more than 15-fold. The effects of NA on NTPase activity could thus be correlated to helicase activity. However, this may not be universal for all helicases. To support this assumption, I reviewed the above mentioned properties for a number of helicases studied over the years. It has been observed that the helicases, which exclusively unwind RNA substrates, may not exhibit more than a 2- to 3-fold NTPase stimulation by NA-oligomers as exemplified by helicases from SFV, TGBp1 plant virus, CHIKV, Hepatitis E virus, turnip yellow mosaic virus, rubella virus, and plum pox potyvirus, which all belong to SF1 (209, 220, 474, 475, 495–497). On the other hand, those helicases that unwind both RNA and DNA substrates may exhibit more than 10-fold NTPase stimulation by NA-oligomers as exemplified by helicases from HCV NS3, DENV NS3, WNV NS3, Hepatitis G Virus, YFV, SARS-CoV(SF1), and UPF1(SF1) (476, 498–503). However, there are also exceptions. The NTPase activity of the VV RNA helicase NPH II is 40-fold stimulated by NA-oligomers despite the fact that the enzyme also has high basal activity (9600/min) (504). This may be because this helicase was also shown to unwind DNA:RNA hybrid substrates with an unusual bias towards purine-rich sequences (505). Combined, these data raise an important issue of how the first category of molecular motors could be unwinding the RNA. Low NA stimulation makes the use of an HCV NS3-type model (the Brownian model that suggests that ATP hydrolysis acts as a molecular switch in between high and low affinity DNA binding states (415)) unlikely. Thus, it is likely that exclusive RNA helicases all together should possess a different mechanism for how they interact and translocate on RNA; it is likely that their apparently unprocessive nature is related to this mechanism. This speculation is supported by the fact that while HCV NS3 mostly interact with the phosphodiester backbone (372) and thus does not discriminate between RNA and DNA substrates, exclusive RNA helicases should interact in a unique way that allows them to authenticate their NA substrate. These scientific contemplations clearly require further experimental support; it would be essential to reveal with what part(s) of their substrates these enzymes interact.

6.7. Could there be any biological significance to nsP2 Helicase directionality?

NsP2 of CHIKV demonstrates 5'-3' RNA unwinding activity. However, is there any biological relevance to this directionality or this is just an intrinsic and

biologically disconnected ability of most helicases? Currently, the viral helicase community has only been able to put forward very speculative models that propose that directionality may originate from interactions between viral RNA polymerases and helicases (67, 427, 430). However, the theory regarding polymerase and helicase interactions in the context of alphavirus RNA replication is still very premature and is supported only by limited indirect evidence. Alphavirus RNA polymerase polymerizes nascent RNA in a 5'-3' direction; to do so, it must translocate on the template strand in a 3'-5' direction (268). Thus, if the alphavirus nsP2 helicase had to use the same strand, then there would be a collision between these two oppositely translocating enzymes. Furthermore, it seems highly improbable that the synthesis of a positive strand is carried out from a naked negative strand; instead, the templates for new positive strands are dsRNA replication intermediates (66, 67, 146, 506). It is, however, unclear whether alphavirus RNA replication occurs in a conservative (displacement of nascent strand) or semiconservative (displacement of the old positive strand) manner (67). In any case, however, it appears likely that the RNA polymerases and helicases of positive strand RNA viruses work together. Indeed, the helicase and the polymerase not only interact but also influence each other's complimentary functions (507–511). Thus, it is reasonable to expect that nsP4 RNA polymerase and nsP2 helicase could work together on viral dsRNA intermediates. If so, they translocate on opposite strands but work towards a common goal of synthesizing and displacing a positive strand. Detailed studies are needed to reveal exactly where and how RNA unwinding and/or RNA rewinding activities of nsP2 are utilized.

6.8. NsP2-related enzymatic activities are affected by NCT-associated mutations

In article III, it has been shown that NCT-associated mutations reduce CHIKV nsP2-related functionalities. This is the first study in which such a link has been clearly demonstrated. Nevertheless, it is still vague as to whether CHIKV-induced cytotoxicity is reduced directly due to a decrease in nsP2 enzymatic activities and/or, because the corresponding mutations also negatively affect RNA replication subsequently reducing the concentration of nsP2 in infected cells. As these effects are linked to each other, the entire puzzle is difficult to unravel.

It should also be mentioned that the P718G mutation (originally obtained in studies using SINV replicons) alone is not sufficient for the NCT phenotype in the case of CHIKV replicons. It does, however, serve as an initial starting point for selection to find other related mutations (235, 293). This raises a question: why are secondary mutations needed? It can be observed that the P718G mutation alone results in a decrease in all nsP2 enzymatic activities, and additional mutations such as 5A and EK do not result in significant additional decrease in these nsP2 activities. In contrast, 5A mutations actually increased the NTPase activity of nsP2-PG while EK restored oligonucleotide stimulation of nsP2- PG

(Article II). Taken together these data suggest that the appearance of the NCT phenotype does not exclusively result from a reduction in enzymatic activities. These additional mutations could, at least in the case of CHIKV, underlie among other activities of nsP2. One speculative hypothesis could be that these mutations somehow affect the assembly of optimal replication complexes by altering fine-tuned interactions between various ns proteins. Most probably, these mutations, such as 5A-PG, could render the structure of nsP2, which is cardinal to the regulation of alphavirus replication, overly flexible. Such changes might disrupt certain essential interactions, resulting in suboptimal replication machinery and compromised synthesis of all virus-related components (proteins, RNAs). It remains to be investigated what other viral activities and/or, more importantly, the sequence in which they are affected or must be affected during the process leading to the NCT phenotype.

6.9. Quality and purity of recombinant protein is a key to effective antigenicity

During this study, a number of ns proteins from different alphaviruses (CHIKV, ONNV, and BFV) were expressed and purified. The main objective of this part of the study was to obtain materials needed for the immunization of rabbits (or, in one case, also chickens) to raise polyclonal antisera. It is possible that advanced peptide synthesis would allow avoiding such an "old-fashioned" approach. However, to the best of our knowledge, none of research teams who have attempted to use this approach (of which there were at least several) have been able to obtain antibodies of comparable quality; in many cases, the antisera obtained failed to recognize the viral proteins at all.

The expression of recombinant proteins could also be considered trivial, and as far as the mere yield of recombinant protein is concerned, it is indeed so. The problem is to obtain protein in its native (and most immunogenic) form. It should be noted that there is no universal purification protocol applicable for all proteins; in contrast, individual protocols (or variations of existing protocols) were developed for each and every one. Even then, the quality of proteins remained variable. In retrospect, it could be clearly observed that the purity as well as the nativeness and solubility of a protein are crucial for antibody production. Thus, CHIKV nsP4 was largely insoluble and despite the high amounts and purity of the antigen, the obtained antisera were of the lowest quality (compared to others): they were suitable for western blot (Article I) and immunoprecipitation but not effective enough for making high-quality immunofluorescence images. In contrast, Hel470 is soluble and native protein and the polyclonal serum that resulted from using this protein work perfectly both with western blotting and immunofluorescence staining (Article III). These observations, although they may appear somewhat trivial, reinforce the notion that for good quality antibody production, pure and native high quality protein antigen is necessary.

7. CONCLUSIONS

Alphavirus enzymology and biochemistry has long history. Several important findings in this area date back to the time when the technology of protein expression and purification was still in a very early stage of development. So it was both of utmost curiosity and importance to re-evaluate the conclusions drawn in the previous studies of different alphaviruses using the most medically important member of this group – CHIKV – as research entity. Similarly, it was necessary to attempt to amend our current understandings with new experimental evidence. So this study was carried out to provide in-depth biochemical analysis of nsP2 – convincingly the most complex of alphavirus proteins – using controlled and high quality wt full length protein as well as its variants harboring various point mutations obtained from biological studies. Similarly, various truncated forms of this protein, designed according to previous extensive works corroborating nsP2 and its functions, were prepared, purified and analyzed. In this study I not only examined almost all the enzymatic activity related to nsP2 but also produced recombinant proteins as valuable tools to study different aspect of alphavirology. This allowed, to some extent, connecting biochemical properties of nsP2 to biological properties of the corresponding virus and its mutants. Thus, in its final form this study could be clearly differentiated into two broader parts. The first part extensively addresses the basic biochemistry aspect of nsP2; in this part the enzymatic activities such as NTPase, RNA helicase and novel RNA annealing activity has been demonstrated and conditions for their analysis have been optimized. This first part opens up avenues for second phase of the study which deals with the application of the gathered knowledge (and obtained assays) for understanding role of nsP2 mutations in development of NCT phenotype of CHIKV. Below I would describe key findings which are central to the thesis in an objective manner.

I) Structural homology modeling coupled with existing partial 3D-structural data suggest that CHIKV nsP2 (90 kDa) consists of five distinct domains. These are (enumerating from N-terminus of the protein) NTD, two RecA-like domains, protease and MTL domain. Out of all the domains the NTD was predicted to be partially disordered. Its specific functionalities are unknown however it is likely that this domain with highly acidic pI apparently interacts with the highly basic MTL domain. Experimental data obtained during this study indirectly supports this prediction.

II) The structural predictions were validated by biochemical analysis of the CHIKV nsP2. For this the wt full length protein, its various mutants and truncated versions were expressed and purified to near homogeneity. Functional assays such as NTPase, helicase, RNA rewinding and protease assays were carried out.

III) CD spectroscopic analysis of the purified recombinant wt and various mutant and truncated versions suggest that nsP2 is largely an alpha helical protein however considerable portion of this protein was also suggested to be loops. NsP2 was found to be tolerant to several mutations; however NCT mutation such as the 5A-PG could also contort the structure of nsP2 and deletion of either terminal domains resulted in aggregation of recombinant protein.

IV) The results of NTPase and protease assays suggest that a wt native full length protein (without carrying any additional aa or missing few aa residues) is more active than its mutant forms; in several cases many-fold differences were observed. Furthermore, 5′-3′ RNA helicase activity and RNA strand annealing activity were exclusively properties of full length nsP2. This protein was also able to act on hybrid (RNA:DNA) substrates with a RNA tracking strand. The novel RNA strand annealing activity was most effective on substrates forming fork product.

V) The results of NTPase assay indicate that a full length native nsP2 is much more active than the nsP2 containing a His-tag at its N-terminus. The protease assay carried out using substrates representing 2/3 site revealed that in contrast to the closely related SFV nsP2 the nsP2 of CHIKV is tolerant to addition of two extra aa to its N-terminus.

VI) The understanding accumulated from the basic biochemistry and enzymology of nsP2 was applied towards studying the NCT mutations identified in nsP2 region of CHIKV replicons adapted to non-cytotoxic growth in cell culture. These proteins were expressed, purified and analyzed. It was found that almost all the enzymatic activity such as NTPase, helicase and protease are affected by NCT related mutations. Though no clear correlation between decrement of these enzymatic activities and development of NCT phenotype could be established nevertheless it could be proposed that NCT mutations act by compromising one or more enzymatic (and possibly non-enzymatic) activities of nsP2.

VII) Finally, a number of ns-proteins from different alphaviruses were expressed, purified and used to raise polyclonal antisera. These antisera represent unique and efficient tools for detection of viral proteins using different immunological, such as western blot and immunofluorescence, methods. These samples were not only confined to our lab in Tartu but have been also shared globally with other laboratories studying alphaviruses.

The studies highlighted in this thesis represent important step towards better understanding molecular biology of alphaviruses. In addition, they also provide ground for application towards development of therapeutic compounds against this highly pathogenic virus. As always, not all important questions were answered and, as it is the case for many studies, new questions emerged. For example, currently we do not have any understanding about the residues in nsP2 that contribute towards RNA binding. The answer to this type of question may also be able to clarify unanswered biological questions related to viral helicases in general: identification of RNA binding motif would able researchers to study role of helicase activity without compromising NTPase and RNA triphosphatase activities of the protein (which are inevitably inhibited if mutations are introduced directly into NTPase active site). Determination of phenotype of such viruses would indeed be very important for dissection of RNA unwinding functionalities towards viral replication. It is also clear that in its current form the biochemical assays are robust and suitable for screening and collecting detailed information about effect of particular mutation on the nsP2 enzymatic activities or for screening small molecule inhibitors for their potential anti-enzymatic activity. However, for utilizing their full potential these assays need to be optimized and adapted accordingly. For example, the current helicase and protease assays are gel based which limit the screening of large number of potential antiviral compounds under similar conditions and do not allow continuous measurement of accumulation of reaction products. It can be envisioned that in the coming time these types of assays would be replaced by high throughput systems, for example utilizing FRET based principles and/or other molecular beacons. Although work in this direction has been already started in our lab, describing results of these later studies is clearly beyond the scope of this thesis.

SUMMARY IN ESTONIAN

Alfaviiruse nsP2 valk biokeemilisest vaatekohast: lugu mitmedomäänse valgu ensümaatilisest analüüsist

Chikungunya viirus (CHIKV) on positiivse polaarsusega RNA genoomiga viirus mis kuulub alfaviiruste perekonda (sugukond *Togaviridae*). Sarnaselt teiste alfaviirustega levib CHIKV vektorputukate (enamasti *Aedes* perekonna moskiitod) vahendusel. Võrrelduna teiste alfaviirustega omab CHIKV suurt meditsiinilist tähtsust ning on viimase kümne aasta jooksul põhjustanud kaks suurt epideemiat: alates 2005 aastast India ookeani regioonis ja alates 2013 hilissügisest Kariibi mere saartel ja Ameerikates. Mõlema epideemia käigus on haigestunud miljoneid inimesi. CHIKV infektsiooni iseloomustavad kõrge palavik, lööve ja eriti iseloomulikud on tugevad liigesevalud. Haigus muutub sageli krooniliseks ja liigesevalud võivad kesta kuid või isegi aastaid. CHIKV vastast vaktsiini ega spetsiifilist ravi ei ole.

CHIKV genoom on umbes 12000 nukleotiidi jäägi pikkune. Nakatatud rakus sünteesitakse viiruse genoomselt RNAlt liitvalk, millest moodustuvad neli mittestruktuurset valku: nsP1, nsP2, nsP3 ja nsP4. Replikatsiooni protsessi käigus sünteesitakse ka genoomist lühem (subgenoomne) RNA millelt transleeritakse viiruse struktuursete valkude eelvalk. RNA replikatsioonis struktuursed valgud ei osale, seda protsessi viivad läbi mittestruktuursed valgud. Eriline roll selles protsessi kuulub nsP2 valgule. Ehkki see valk ei ole RNA polümeraas (see funktsioon on nsP4 valgul) on tal mitmed replikatsiooni protsessiks olulised aktiivsused. Selles väitekirjas käsitletud tööde alguses oli teada, et CHIKV nsP2 omab oma N-terminaalses regioonis NTPaaset ja RNA trifosfataaset aktiivsust samal ajal kui valgu C-terminaalne ala omab proteaaset aktiivsust. Samuti eeldati, et nsP2 N-terminaalne region võib funktsioneerida kui RNA helikaas. Tänu sellistele funktsioonidele toimib nsP2 kui CHIKV replikatsiooniprotsessi peamine koordinaator.

Nagu öeldud, CHIKV nsP2 valgu RNA helikaasse aktiivsuse kohta puudusid eksperimentaalsed andmed. Vastupidi, eelnevad tööd, mille eesmärkiks oli selle aktiivsuse demonstreerimine, ei suutnud seda aktiivsust tuvastada. Samuti ei olnud selge ei selle funktsiooni tähtsus viirusele ega ka see miks on ühes ja samas valgus olemas mitmed oma olemuselt väga erinevad aktiivsused: NTPaas, RNA trifiosfataas ja eeldatavasti RNA helikaas valgu N-terminaalses regioonis ja proteaas C-terminaalses alas. Leidmaks vastust nendele küsimustele uuriti nsP2 valgu järjestusi ja tema eeldatavat ruumilist struktuuri bioinformaatilisi meetode kasutades, sellele järgnes valgu puhastamine ja tema funktsioonide uurimine biokeemiliste meetodite abil. Kõige lõpuks uuriti nsP2 valgu funktsioone otse CHIKV nakatatud rakkudes kasutades selleks viiruse mutante, mis sisaldasid mutatsioone nsP2 valku kodeerivas alas.

Läbiviidud molekulaarne modelleerimine näitas, et CHIKV nsP2 valgu esimesed 470 aminohappe jääki pakitakse tõenäoliselt struktuuriks, milles on olemas supersugukonda 1 (SF1) kuuluvatele helikaasidele iseloomulikud

struktuursed motiivid. Loodud CHIKV nsP2 N-terminaalse regiooni ruumiline mudel meenutab oma struktuurilt kõige enam tomati mosaiigiviirus RNA-helikaasi struktuuri. Sellest struktuuris saab eristada kolme domääni. Kõige N-terminaalsem nendest ei oma tõenäoliselt fikseeritud ruumilist struktuuri. Sellele struktureerimata domäänile järgnevad kaks RecA-sarnast domääni, mis on iseloomulikud NTPaasidele ja RNA helikaasidele. Nendes domäänides paiknevad ka konserveerunud aminohappelised järjestused, mis leiduvad ka muudes SF1 RNA helikaasides.

Biokeemiliste analüüside läbiviimiseks ekspresseriti ja puhastati nii nsP2, tema N-terminaalne (470-st aminohappe jäägist koosnev) fragment kui ka mitmed nende valkude mutantsed variandid. Nende rekombinatsete valkude biokeemiline analüüs võimaldas välja selgitada minimaalsed nõuded, mis on vajalikud nsP2 helikaasse aktiivsuse avaldumiseks. Nii selgus, et ainult täispikk nsP2 on RNA mis harutab RNA duplekseid lahti 5'- 3' suunas. Näidati, et lisaks RNA helikaassele aktiivsusele on nsP2 valgul olemas ka RNA dupleksi moodustamise funktsioon; ka see aktiivsus vajab valgu C-terminaalse regiooni olemasolu. Need andmed näitavad, et nn. helikaasi ja proteaasi regiooni olemasolu ühes valgus ei ole kaugeltki juhuslik ja et nende regioonide vahel toimuvad spetsiifilised interaktsioonid tagavad valgu võime läbi viia keerulisi protsesse. Siit saab teha oletuse, et need erinevate ensümaatiliste aktiivsustega domäänid on evolutsioneerunud koos ja moodustavad ühtse funktsionaalse terviku.

NsP2 valgu N- ja C-terminaalsete domäänide ja tema N-terminaalse regiooni esimeste aminohappejääkide olulisust ensümaatilistele aktiivsustele uuriti nsP2 valgu mutantseid variante kasutades. Sarnast lähenemist kasutati ka uurimaks mitmete nsP2 valgus paiknevate muudatuste mõju. Viimane on väga oluline, sest mutatsioonid, mis muudavad viiruse infektsiooni selgroogsete rakkude jaoks mittitsütotoksiliseks (metsikut tüüpi CHIKV infektsioon on nendele rakkudele surmav), paiknevad kõik nsP2 valku kodeerivas alas. Selline seose alfaviiruste mutantide mittetsütotoksilise fenotüübi ja nsP2 regioonis paiknevate mutatsioonide vahel on teada paljudest varasematest uuringutest, küll aga polnud siiani teada, kas ja kuidas sellised mutatsioonid mõjutavad nsP2 valgu aktiivsuseid. Selles töö osas ekspresseriti ja puhastati mitmed nsP2 valgu mutantsed vormid. Biofüüsikalisi meetode (CD spektroskoopia) kasutades näidati, et sellised muudatused ei põhjusta olulisi erinevusi valgu üldises struktuuris, st. mutantsed nsP2 vormid ei kujuta endast valesti pakitud valke. Analüüsid, mis viidi läbi kasutades nsP2 vorme, mis sisaldasid mittetsütotosilist fenotüüpi põhjustavaid mutatsioone, näitasid, et need mutatsioonid (ja nende kombinatsioonid) vähendavad kõiki nsP2 ensümaatilisi aktiivsuseid. Samas osutus konkreetse aktiivsuse vähenemise (või kadumise) korreleerimine mutantse viiruse fenotüübiga keeruliseks; kui üldse, siis näitas sellist korrelatsiooni vaid nsP2 RNA helikaasne aktiivsus.

Üheks täiendavaks tööde suunaks kujunes ka alfaviiruste ns-valkude ekspresserimise ja puhastamise meetodite rakendamine erinevatest alfaviirustest pärinevate ns-valkude tootmiseks. Kõrge kvaliteediga (homogeensed ja enamasti lahustuvad) rekombinantsed valgud osutusid oluliseks vahendiks

efektiivsete polüklonaalsete antiseerumite saamiseks. Saadud töövahendid on palju efektiivsemad kui näiteks sünteetilise peptiide kasutades saadud antiseerumid. Seetõttu on need kasutusel paljudes laborites üle maailma kus neid kasutatakse erinevate analüüside (nt. immunobloti, immuuno-fluorestsents analüüside ja immunosadestamiste) läbiviimiseks. Sellised töövahendid on juba võimaldanud välja selgitada palju uusi fakte CHIKV (ja alfaviiruste üldse) molekulaarbioloogia kohta. Ka strandardiseeritud meetodid aktiivse nsP2 valgu puhastamiseks ja selle ensümaatiliste aktiivsuste mõõtmiseks omavad praktilist väärtust. Muu hulgas saab selliseid töövahendeid kasutada loomaks skriiningsüsteeme, mis võimaldavad leida ja analüüsida nsP2 aktiivsuseid (ja koos sellega viiruse infektsiooni) mahasuruvaid ühendeid.

REFERENCES

1. Horzinek, M. C. (1997) The birth of virology. *Antonie Van Leeuwenhoek*. **71**, 15–20
2. Koonin, E. V., Senkevich, T. G., and Dolja, V. V. (2006) The ancient Virus World and evolution of cells. *Biol. Direct*. **1**, 29
3. Lechevalier, H. (1972) Dmitri Iosifovich Ivanovski (1864-1920). *Bacteriol. Rev.* **36**, 135–145
4. Gelderblom, H. R. (1996) Structure and Classification of Viruses. in *Medical Microbiology*, 4th Ed. (Baron, S. ed), University of Texas Medical Branch at Galveston, Galveston (TX).
5. Baron, S. (ed.) (1996) *Medical Microbiology*, 4th Ed., University of Texas Medical Branch at Galveston, Galveston (TX).
6. Lawrence, C. M., Menon, S., Eilers, B. J., Bothner, B., Khayat, R., Douglas, T., and Young, M. J. (2009) Structural and Functional Studies of Archaeal Viruses. *J. Biol. Chem.* **284**, 12599–12603
7. Suttle, C. A. (2007) Marine viruses--major players in the global ecosystem. *Nat. Rev. Microbiol.* **5**, 801–812
8. Baltimore, D. (1971) Expression of animal virus genomes. *Bacteriol. Rev.* **35**, 235–241
9. King, A. M. Q., Adams, M. J., Lefkowitz, E. J., and Carstens, E. B. (2012) *Virus Taxonomy: Classification and Nomenclature of Viruses : Ninth Report of the International Committee on Taxonomy of Viruses*, Elsevier
10. Yang, G., Cecconi, C., Baase, W. A., Vetter, I. R., Breyer, W. A., Haack, J. A., Matthews, B. W., Dahlquist, F. W., and Bustamante, C. (2000) Solid-state synthesis and mechanical unfolding of polymers of T4 lysozyme. *Proc. Natl. Acad. Sci.* **97**, 139–144
11. Philippe, N., Legendre, M., Doutre, G., Couté, Y., Poirot, O., Lescot, M., Arslan, D., Seltzer, V., Bertaux, L., Bruley, C., Garin, J., Claverie, J.-M., and Abergel, C. (2013) Pandoraviruses: Amoeba Viruses with Genomes Up to 2.5 Mb Reaching That of Parasitic Eukaryotes. *Science*. **341**, 281–286
12. Raoult, D., Scola, B. L., and Birtles, R. (2007) The Discovery and Characterization of Mimivirus, the Largest Known Virus and Putative Pneumonia Agent. *Clin. Infect. Dis.* **45**, 95–102
13. Dennehy, P. H. (2000) Transmission of rotavirus and other enteric pathogens in the home. *Pediatr. Infect. Dis. J.* **19**, S103–105
14. Buck, C. B., Cheng, N., Thompson, C. D., Lowy, D. R., Steven, A. C., Schiller, J. T., and Trus, B. L. (2008) Arrangement of L2 within the papillomavirus capsid. *J. Virol.* **82**, 5190–5197
15. Weir, E. (2005) Parvovirus B19 infection: fifth disease and more. *CMAJ Can. Med. Assoc. J. J. Assoc. Medicale Can.* **172**, 743
16. Robinson, M. C. (1955) An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. I. Clinical features. *Trans. R. Soc. Trop. Med. Hyg.* **49**, 28–32
17. Cavrini, F., Gaibani, P., Pierro, A. M., Rossini, G., Landini, M. P., and Sambri, V. (2009) Chikungunya: an emerging and spreading arthropod-borne viral disease. *J. Infect. Dev. Ctries.* **3**, 744–752
18. Enserink, M. (2006) Infectious diseases. Massive outbreak draws fresh attention to little-known virus. *Science*. **311**, 1085

19. Gérardin, P., Barau, G., Michault, A., Bintner, M., Randrianaivo, H., Choker, G., Lenglet, Y., Touret, Y., Bouveret, A., Grivard, P., Roux, K. L., Blanc, S., Schuffenecker, I., Couderc, T., Arenzana-Seisdedos, F., Lecuit, M., and Robillard, P.-Y. (2008) Multidisciplinary Prospective Study of Mother-to-Child Chikungunya Virus Infections on the Island of La Réunion. *PLoS Med*. **5**, e60
20. Leparc-Goffart, I., Nougairede, A., Cassadou, S., Prat, C., and de Lamballerie, X. (2014) Chikungunya in the Americas. *Lancet*. **383**, 514
21. Manimunda, S. P., Mavalankar, D., Bandyopadhyay, T., and Sugunan, A. P. (2011) Chikungunya epidemic-related mortality. *Epidemiol. Infect.* **139**, 1410–1412
22. Ng, L. F. P., Chow, A., Sun, Y.-J., Kwek, D. J. C., Lim, P.-L., Dimatatac, F., Ng, L.-C., Ooi, E.-E., Choo, K.-H., Her, Z., Kourilsky, P., and Leo, Y.-S. (2009) IL-1beta, IL-6, and RANTES as biomarkers of Chikungunya severity. *PloS One*. **4**, e4261
23. Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M.-C., Lavenir, R., Pardigon, N., Reynes, J.-M., Pettinelli, F., Biscornet, L., Diancourt, L., Michel, S., Duquerroy, S., Guigon, G., Frenkiel, M.-P., Bréhin, A.-C., Cubito, N., Desprès, P., Kunst, F., Rey, F. A., Zeller, H., and Brisse, S. (2006) Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med.* **3**, e263
24. Simon, F., Tolou, H., and Jeandel, P. (2006) The unexpected Chikungunya outbreak. *Rev. Médecine Interne*. **27**, 437–441
25. Strauss, J. H., and Strauss, E. G. (1994) The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.* **58**, 491–562
26. Wangchuk, S., Chinnawirotpisan, P., Dorji, T., Tobgay, T., Dorji, T., Yoon, I.-K., and Fernandez, S. (2013) Chikungunya fever outbreak, Bhutan, 2012. *Emerg. Infect. Dis.* **19**, 1681–1684
27. Watson, R. (2007) Chikungunya fever is transmitted locally in Europe for first time. *BMJ*. **335**, 532–533
28. Carey, D. E. (1971) Chikungunya and dengue: a case of mistaken identity? *J. Hist. Med. Allied Sci.* **26**, 243–262
29. Solignat, M., Gay, B., Higgs, S., Briant, L., and Devaux, C. (2009) Replication cycle of chikungunya: a re-emerging arbovirus. *Virology*. **393**, 183–197
30. Rathore, A. P. S., Haystead, T., Das, P. K., Merits, A., Ng, M.-L., and Vasudevan, S. G. (2013) Chikungunya virus nsP3 & nsP4 interacts with HSP-90 to promote virus replication: HSP-90 inhibitors reduce CHIKV infection and inflammation in vivo. *Antiviral Res.* **103C**, 7–16
31. Waggoner, J. J., and Pinsky, B. A. (2014) How great is the threat of chikungunya virus? *Expert Rev. Anti Infect. Ther.* 10.1586/14787210.2015.995634
32. Higgs, S., and Ziegler, S. A. (2010) A nonhuman primate model of chikungunya disease. *J. Clin. Invest.* **120**, 657–660
33. Powers, A. M., Brault, A. C., Tesh, R. B., and Weaver, S. C. (2000) Re-emergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. *J. Gen. Virol.* **81**, 471–479
34. Higashi, N., Matsumoto, A., Tabata, K., and Nagatomo, Y. (1967) Electron microscope study of development of Chikungunya virus in green monkey kidney stable (VERO) cells. *Virology*. **33**, 55–69

35. Khan, A. H., Morita, K., Parquet Md, M. del C., Hasebe, F., Mathenge, E. G. M., and Igarashi, A. (2002) Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. *J. Gen. Virol.* **83**, 3075–3084
36. Powers, A. M., Brault, A. C., Shirako, Y., Strauss, E. G., Kang, W., Strauss, J. H., and Weaver, S. C. (2001) Evolutionary relationships and systematics of the alphaviruses. *J. Virol.* **75**, 10118–10131
37. Simizu, B., Yamamoto, K., Hashimoto, K., and Ogata, T. (1984) Structural proteins of Chikungunya virus. *J. Virol.* **51**, 254–258
38. Voss, J. E., Vaney, M.-C., Duquerroy, S., Vonrhein, C., Girard-Blanc, C., Crublet, E., Thompson, A., Bricogne, G., and Rey, F. A. (2010) Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. *Nature*. **468**, 709–712
39. Zhang, W., Mukhopadhyay, S., Pletnev, S. V., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2002) Placement of the Structural Proteins in Sindbis Virus. *J. Virol.* **76**, 11645–11658
40. Akahata, W., Yang, Z.-Y., Andersen, H., Sun, S., Holdaway, H. A., Kong, W.-P., Lewis, M. G., Higgs, S., Rossmann, M. G., Rao, S., and Nabel, G. J. (2010) A virus-like particle vaccine for epidemic Chikungunya virus protects non-human primates against infection. *Nat. Med.* **16**, 334–338
41. Sun, S., Xiang, Y., Akahata, W., Holdaway, H., Pal, P., Zhang, X., Diamond, M. S., Nabel, G. J., and Rossmann, M. G. (2013) Structural analyses at pseudo atomic resolution of Chikungunya virus and antibodies show mechanisms of neutralization. *eLife*. **2**, e00435
42. Fuller, S. D., Berriman, J. A., Butcher, S. J., and Gowen, B. E. (1995) Low pH induces swiveling of the glycoprotein heterodimers in the Semliki forest virus spike complex. *Cell*. **81**, 715–725
43. Metz, S. W., Gardner, J., Geertsema, C., Le, T. T., Goh, L., Vlak, J. M., Suhrbier, A., and Pijlman, G. P. (2013) Effective Chikungunya Virus-like Particle Vaccine Produced in Insect Cells. *PLoS Negl Trop Dis*. **7**, e2124
44. Jose, J., Snyder, J. E., and Kuhn, R. J. (2009) A structural and functional perspective of alphavirus replication and assembly. *Future Microbiol.* **4**, 837–856
45. Cheng, R. H., Kuhn, R. J., Olson, N. H., Rossmann, M. G., Choi, H. K., Smith, T. J., and Baker, T. S. (1995) Nucleocapsid and glycoprotein organization in an enveloped virus. *Cell*. **80**, 621–630
46. Gaedigk-Nitschko, K., and Schlesinger, M. J. (1990) The Sindbis virus 6K protein can be detected in virions and is acylated with fatty acids. *Virology*. **175**, 274–281
47. Lusa, S., Garoff, H., and Liljeström, P. (1991) Fate of the 6K membrane protein of Semliki Forest virus during virus assembly. *Virology*. **185**, 843–846
48. Tang, J., Jose, J., Chipman, P., Zhang, W., Kuhn, R. J., and Baker, T. S. (2011) Molecular links between the E2 envelope glycoprotein and nucleocapsid core in Sindbis virus. *J. Mol. Biol.* **414**, 442–459
49. Zhang, R., Hryc, C. F., Cong, Y., Liu, X., Jakana, J., Gorchakov, R., Baker, M. L., Weaver, S. C., and Chiu, W. (2011) 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. *EMBO J.* **30**, 3854–3863
50. Mancini, E. J., Clarke, M., Gowen, B. E., Rutten, T., and Fuller, S. D. (2000) Cryo-electron microscopy reveals the functional organization of an enveloped virus, Semliki Forest virus. *Mol. Cell*. **5**, 255–266

51. Paredes, A. M., Brown, D. T., Rothnagel, R., Chiu, W., Schoepp, R. J., Johnston, R. E., and Prasad, B. V. (1993) Three-dimensional structure of a membrane-containing virus. *Proc. Natl. Acad. Sci.* **90**, 9095–9099
52. Jose, J., Przybyla, L., Edwards, T. J., Perera, R., Burgner, J. W., and Kuhn, R. J. (2012) Interactions of the Cytoplasmic Domain of Sindbis Virus E2 with Nucleocapsid Cores Promote Alphavirus Budding. *J. Virol.* **86**, 2585–2599
53. Kielian, M., Chatterjee, P. K., Gibbons, D. L., and Lu, Y. E. (2002) Specific Roles for Lipids in Virus Fusion and Exit Examples from the Alphaviruses. in *Fusion of Biological Membranes and Related Problems* (Hilderson, H., and Fuller, S. eds), pp. 409–455, Subcellular Biochemistry, Springer US.
54. Sousa, I. P., Carvalho, C. A. M., Ferreira, D. F., Weissmüller, G., Rocha, G. M., Silva, J. L., and Gomes, A. M. O. (2011) Envelope Lipid-packing as a Critical Factor for the Biological Activity and Stability of Alphavirus Particles Isolated from Mammalian and Mosquito Cells. *J. Biol. Chem.* **286**, 1730–1736
55. Umashankar, M., Sanchez-San Martin, C., Liao, M., Reilly, B., Guo, A., Taylor, G., and Kielian, M. (2008) Differential Cholesterol Binding by Class II Fusion Proteins Determines Membrane Fusion Properties. *J. Virol.* **82**, 9245–9253
56. Wilkinson, T. A., Tellinghuisen, T. L., Kuhn, R. J., and Post, C. B. (2005) Association of sindbis virus capsid protein with phospholipid membranes and the E2 glycoprotein: implications for alphavirus assembly. *Biochemistry (Mosc.)*. **44**, 2800–2810
57. Kääriäinen, L., Takkinen, K., Keränen, S., and Söderlund, H. (1987) Replication of the genome of alphaviruses. *J. Cell Sci. Suppl.* **7**, 231–250
58. Strauss, E. G., and Strauss, J. H. (1986) Structure and Replication of the Alphavirus Genome. in *The Togaviridae and Flaviviridae* (Schlesinger, S., and Schlesinger, M. J. eds), pp. 35–90, The Viruses, Springer New York. 59. Firth, A. E., Wills, N. M., Gesteland, R. F., and Atkins, J. F. (2011) Stimulation of stop codon readthrough: frequent presence of an extended 3? RNA structural element. *Nucleic Acids Res.* **39**, 6679–6691
60. Frolov, I., Hardy, R., and Rice, C. M. (2001) Cis-acting RNA elements at the 5' end of Sindbis virus genome RNA regulate minus- and plus-strand RNA synthesis. *RNA N. Y. N.* **7**, 1638–1651
61. Wengler, G., Wengler, G., and Gross, H. J. (1982) Terminal sequences of Sindbis virus-specific nucleic acids: Identity in molecules synthesized in vertebrate and insect cells and characteristic properties of the replicative form RNA. *Virology*. **123**, 273–283
62. Wengler, G., Wengler, G., and Gross, H. J. (1979) Replicative form of Semliki Forest virus RNA contains an unpaired guanosine. *Nature*. **282**, 754–756
63. Hyde, J. L., Gardner, C. L., Kimura, T., White, J. P., Liu, G., Trobaugh, D. W., Huang, C., Tonelli, M., Paessler, S., Takeda, K., Klimstra, W. B., Amarasinghe, G. K., and Diamond, M. S. (2014) A viral RNA structural element alters host recognition of nonself RNA. *Science*. **343**, 783–787
64. Ou, J. H., Strauss, E. G., and Strauss, J. H. (1983) The 5'-terminal sequences of the genomic RNAs of several alphaviruses. *J. Mol. Biol.* **168**, 1–15
65. Gorchakov, R., Hardy, R., Rice, C. M., and Frolov, I. (2004) Selection of Functional 5? cis-Acting Elements Promoting Efficient Sindbis Virus Genome Replication. *J. Virol.* **78**, 61–75
66. Kääriäinen, L., and Ahola, T. (2002) Functions of alphavirus nonstructural proteins in RNA replication. *Prog. Nucleic Acid Res. Mol. Biol.* **71**, 187–222

67. Kovalev, N., Pogany, J., and Nagy, P. D. (2014) Template Role of Double-Stranded RNA in Tombusvirus Replication. *J. Virol.* **88**, 5638–5651
68. Niesters, H. G., and Strauss, J. H. (1990) Mutagenesis of the conserved 51-nucleotide region of Sindbis virus. *J. Virol.* **64**, 1639–1647
69. Fayzulin, R., and Frolov, I. (2004) Changes of the Secondary Structure of the 5? End of the Sindbis Virus Genome Inhibit Virus Growth in Mosquito Cells and Lead to Accumulation of Adaptive Mutations. *J. Virol.* **78**, 4953–4964
70. Levis, R., Schlesinger, S., and Huang, H. V. (1990) Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. *J. Virol.* **64**, 1726–1733
71. Ou, J. H., Rice, C. M., Dalgarno, L., Strauss, E. G., and Strauss, J. H. (1982) Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. *Proc. Natl. Acad. Sci.* **79**, 5235–5239
72. Wielgosz, M. M., Raju, R., and Huang, H. V. (2001) Sequence Requirements for Sindbis Virus Subgenomic mRNA Promoter Function in Cultured Cells. *J. Virol.* **75**, 3509–3519
73. Kuhn, R. J., Hong, Z., and Strauss, J. H. (1990) Mutagenesis of the 3' non-translated region of Sindbis virus RNA. *J. Virol.* **64**, 1465–1476
74. Ou, J.-H., Strauss, E. G., and Strauss, J. H. (1981) Comparative studies of the 3′-terminal sequences of several alphavirus RNAs. *Virology*. **109**, 281–289
75. Hardy, R. W. (2006) The role of the 3' terminus of the Sindbis virus genome in minus-strand initiation site selection. *Virology*. **345**, 520–531
76. Frolov, I., and Schlesinger, S. (1994) Translation of Sindbis virus mRNA: effects of sequences downstream of the initiating codon. *J. Virol.* **68**, 8111–8117
77. Sjöberg, E. M., Suomalainen, M., and Garoff, H. (1994) A significantly improved Semliki Forest virus expression system based on translation enhancer segments from the viral capsid gene. *Biotechnol. Nat. Publ. Co.* **12**, 1127–1131
78. Sanz, M. A., Castelló, A., Ventoso, I., Berlanga, J. J., and Carrasco, L. (2009) Dual mechanism for the translation of subgenomic mRNA from Sindbis virus in infected and uninfected cells. *PloS One*. **4**, e4772
79. Ventoso, I., Sanz, M. A., Molina, S., Berlanga, J. J., Carrasco, L., and Esteban, M. (2006) Translational resistance of late alphavirus mRNA to eIF2alpha phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. *Genes Dev.* **20**, 87–100
80. Firth, A. E., Chung, B. Y., Fleeton, M. N., and Atkins, J. F. (2008) Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. *Virol. J.* **5**, 108
81. Kim, D. Y., Atasheva, S., Frolova, E. I., and Frolov, I. (2013) Venezuelan equine encephalitis virus nsP2 protein regulates packaging of viral genome into infectious virions. *J. Virol.* 10.1128/JVI.03142-12
82. White, C. L., Thomson, M., and Dimmock, N. J. (1998) Deletion Analysis of a Defective Interfering Semliki Forest Virus RNA Genome Defines a Region in the nsP2 Sequence That Is Required for Efficient Packaging of the Genome into Virus Particles. *J. Virol.* **72**, 4320–4326
83. Schwartz, O., and Albert, M. L. (2010) Biology and pathogenesis of chikungunya virus. *Nat. Rev. Microbiol.* **8**, 491–500
84. Soonsawad, P., Xing, L., Milla, E., Espinoza, J. M., Kawano, M., Marko, M., Hsieh, C., Furukawa, H., Kawasaki, M., Weerachatyanukul, W., Srivastava, R., Barnett, S. W., Srivastava, I. K., and Cheng, R. H. (2010) Structural Evidence of Glycoprotein Assembly in Cellular Membrane Compartments prior to Alphavirus Budding. *J. Virol.* **84**, 11145–11151

85. Salminen, A., Wahlberg, J. M., Lobigs, M., Liljeström, P., and Garoff, H. (1992) Membrane fusion process of Semliki Forest virus. II: Cleavage-dependent reorganization of the spike protein complex controls virus entry. *J. Cell Biol.* **116**, 349–357
86. Tucker, P. C., and Griffin, D. E. (1991) Mechanism of altered Sindbis virus neurovirulence associated with a single-amino-acid change in the E2 Glycoprotein. *J. Virol.* **65**, 1551–1557
87. Rose, P. P., Hanna, S. L., Spiridigliozzi, A., Wannissorn, N., Beiting, D. P., Ross, S. R., Hardy, R. W., Bambina, S. A., Heise, M. T., and Cherry, S. (2011) Natural resistance-associated macrophage protein is a cellular receptor for sindbis virus in both insect and mammalian hosts. *Cell Host Microbe*. **10**, 97–104
88. Wang, K. S., Kuhn, R. J., Strauss, E. G., Ou, S., and Strauss, J. H. (1992) High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J. Virol.* **66**, 4992–5001
89. Panda, D., Rose, P. P., Hanna, S. L., Gold, B., Hopkins, K. C., Lyde, R. B., Marks, M. S., and Cherry, S. (2013) Genome-wide RNAi screen identifies SEC61A and VCP as conserved regulators of Sindbis virus entry. *Cell Rep.* **5**, 1737–1748
90. Ludwig, G. V., Kondig, J. P., and Smith, J. F. (1996) A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J. Virol.* **70**, 5592–5599
91. Klimstra, W. B., Nangle, E. M., Smith, M. S., Yurochko, A. D., and Ryman, K. D. (2003) DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. *J. Virol.* **77**, 12022–12032
92. Byrnes, A. P., and Griffin, D. E. (1998) Binding of Sindbis virus to cell surface heparan sulfate. *J. Virol.* **72**, 7349–7356
93. Ryman, K. D., Gardner, C. L., Burke, C. W., Meier, K. C., Thompson, J. M., and Klimstra, W. B. (2007) Heparan Sulfate Binding Can Contribute to the Neurovirulence of Neuroadapted and Nonneuroadapted Sindbis Viruses. *J. Virol.* **81**, 3563–3573
94. K S Wang, A. L. S. (1991) Antiidiotypic antibodies as probes for the Sindbis virus receptor. *Virology*. **181**, 694–702
95. Helenius, A., Morein, B., Fries, E., Simons, K., Robinson, P., Schirrmacher, V., Terhorst, C., and Strominger, J. L. (1978) Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3846–3850
96. Leung, J. Y.-S., Ng, M. M.-L., and Chu, J. J. H. (2011) Replication of alphaviruses: a review on the entry process of alphaviruses into cells. *Adv. Virol.* **2011**, 249640
97. Marsh, M., and Helenius, A. (2006) Virus entry: open sesame. *Cell*. **124**, 729–740
98. Mercer, J., Schelhaas, M., and Helenius, A. (2010) Virus entry by endocytosis. *Annu. Rev. Biochem.* **79**, 803–833
99. Pelkmans, L., and Helenius, A. (2003) Insider information: what viruses tell us about endocytosis. *Curr. Opin. Cell Biol.* **15**, 414–422
100. Ooi, Y. S., Stiles, K. M., Liu, C. Y., Taylor, G. M., and Kielian, M. (2013) Genome-wide RNAi screen identifies novel host proteins required for alphavirus entry. *PLoS Pathog.* **9**, e1003835

101. Kononchik, J. P., Hernandez, R., and Brown, D. T. (2011) An alternative pathway for alphavirus entry. *Virol. J.* **8**, 304
102. Kielian, M., Chanel-Vos, C., and Liao, M. (2010) Alphavirus Entry and Membrane Fusion. *Viruses*. **2**, 796–825
103. Sánchez-San Martín, C., Liu, C. Y., and Kielian, M. (2009) Dealing with low pH: entry and exit of alphaviruses and flaviviruses. *Trends Microbiol.* **17**, 514–521
104. Wahlberg, J. M., and Garoff, H. (1992) Membrane fusion process of Semliki Forest virus. I: Low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. *J. Cell Biol.* **116**, 339–348
105. Mrkic, B., Tetaz, T., and Kempf, C. (1997) Cleavage of incoming Semliki Forest virus capsid protein within the endocytotic pathway: a feature common to both invertebrate and mammalian cells. *Arch. Virol.* **142**, 1895–1902
106. Singh, I., and Helenius, A. (1992) Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. *J. Virol.* **66**, 7049–7058
107. Ulmanen, I., Soderlund, H., and Kaariainen, L. (1976) Semliki Forest virus capsid protein associates with the 60S ribosomal subunit in infected cells. *J. Virol.* **20**, 203–210
108. Wengler, G., Würkner, D., and Wengler, G. (1992) Identification of a sequence element in the alphavirus core protein which mediates interaction of cores with ribosomes and the disassembly of cores. *Virology*. **191**, 880–888
109. Garoff, H., Wilschut, J., Liljeström, P., Wahlberg, J. M., Bron, R., Suomalainen, M., Smyth, J., Salminen, A., Barth, B. U., and Zhao, H. (1994) Assembly and entry mechanisms of Semliki Forest virus. *Arch. Virol. Suppl.* **9**, 329–338
110. Wengler, G., Gros, C., and Wengler, G. (1996) Analyses of the Role of Structural Changes in the Regulation of Uncoating and Assembly of Alphavirus Cores. *Virology*. **222**, 123–132
111. Sawicki, D. L., and Sawicki, S. G. (1994) Alphavirus positive and negative strand RNA synthesis and the role of polyproteins in formation of viral replication complexes. *Arch. Virol. Suppl.* **9**, 393–405
112. Li, G. P., and Rice, C. M. (1989) Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. *J. Virol.* **63**, 1326–1337
113. Li, G., and Rice, C. M. (1993) The signal for translational readthrough of a UGA codon in Sindbis virus RNA involves a single cytidine residue immediately downstream of the termination codon. *J. Virol.* **67**, 5062–5067
114. Lulla, A., Tints, K., and Tobi, S. (2007) Optimized recognition site of the alphavirus non-structural protease for tag removal and specific processing of recombinant proteins
115. Myles, K. M., Kelly, C. L. H., Ledermann, J. P., and Powers, A. M. (2006) Effects of an opal termination codon preceding the nsP4 gene sequence in the O'Nyong-Nyong virus genome on Anopheles gambiae infectivity. *J. Virol.* **80**, 4992–4997
116. Tuittila, M., and Hinkkanen, A. E. (2003) Amino acid mutations in the replicase protein nsP3 of Semliki Forest virus cumulatively affect neurovirulence. *J. Gen. Virol.* **84**, 1525–1533
117. Kim, K. H., Rümenapf, T., Strauss, E. G., and Strauss, J. H. (2004) Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. *Virology*. **323**, 153–163

118. Sawicki, D. L., and Sawicki, S. G. (1980) Short-lived minus-strand polymerase for Semliki Forest virus. *J. Virol.* **34**, 108–118
119. Sawicki, D. L., Sawicki, S. G., Keranen, S., and Kaariainen, L. (1981) Specific Sindbis virus-coded function for minus-strand RNA synthesis. *J. Virol.* **39**, 348–358
120. Mai, J., Sawicki, S. G., and Sawicki, D. L. (2009) Fate of minus-strand templates and replication complexes produced by a p23-cleavage-defective mutant of Sindbis virus. *J. Virol.* **83**, 8553–8564
121. Sawicki, D. L., and Sawicki, S. G. (1993) A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. *J. Virol.* **67**, 3605–3610
122. Sawicki, D. L., Perri, S., Polo, J. M., and Sawicki, S. G. (2006) Role for nsP2 Proteins in the Cessation of Alphavirus Minus-Strand Synthesis by Host Cells. *J. Virol.* **80**, 360–371
123. Tuomi, K., Kadaridainen, L., and Soderlund, H. (1975) Quantitation of Semlike Forest virus RNAs in infected cells using 32-P equilibrium labelling. *Nucleic Acids Res.* **2**, 555–565
124. Wang, Y. F., Sawicki, S. G., and Sawicki, D. L. (1994) Alphavirus nsP3 functions to form replication complexes transcribing negative-strand RNA. *J. Virol.* **68**, 6466–6475
125. Glanville, N., Ranki, M., Morser, J., Kaariainen, L., and Smith, A. E. (1976) Initiation of translation directed by 42S and 26S RNAs from Semliki Forest virus in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 3059–3063
126. Keranen, S., and Ruohonen, L. (1983) Nonstructural proteins of Semliki Forest virus: synthesis, processing, and stability in infected cells. *J. Virol.* **47**, 505–515
127. Lachmi, B. E., and Kaariainen, L. (1976) Sequential translation of nonstructural proteins in cells infected with a Semliki Forest virus mutant. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 1936–1940
128. Merits, A., Vasiljeva, L., Ahola, T., Kääriäinen, L., and Auvinen, P. (2001) Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *J. Gen. Virol.* **82**, 765–773
129. Lemm, J. A., Rumenapf, T., Strauss, E. G., Strauss, J. H., and Rice, C. M. (1994) Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. *EMBO J.* **13**, 2925–2934
130. Shirako, Y., and Strauss, J. H. (1994) Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *J. Virol.* **68**, 1874–1885
131. Vasiljeva, L., Merits, A., Golubtsov, A., Sizemskaja, V., Kääriäinen, L., and Ahola, T. (2003) Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J. Biol. Chem.* **278**, 41636–41645
132. Lulla, A., Lulla, V., and Merits, A. (2012) Macromolecular assembly-driven processing of the 2/3 cleavage site in the alphavirus replicase polyprotein. *J. Virol.* **86**, 553–565
133. Sawicki, D. L., Kaariainen, L., Lambek, C., and Gomatos, P. J. (1978) Mechanism for control of synthesis of Semliki Forest virus 26S and 42s RNA. *J. Virol.* **25**, 19–27

134. Summons, D. T., and Steaitss, J. H. (1972) Replication of Sindbis virus: II. Multiple forms of double-stranded RNA isolated from infected cells. *J. Mol. Biol.* **71**, 615–631
135. De Groot, R. J., Hardy, W. R., Shirako, Y., and Strauss, J. H. (1990) Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing in vivo. *EMBO J.* **9**, 2631–2638
136. Salonen, A., Ahola, T., and Kääriäinen, L. (2005) Viral RNA Replication in Association with Cellular Membranes. in *Membrane Trafficking in Viral Replication* (Marsh, D. M. ed), pp. 139–173, Current Topics in Microbiology and Immunology, Springer Berlin Heidelberg.
137. Frolova, E. I., Gorchakov, R., Pereboeva, L., Atasheva, S., and Frolov, I. (2010) Functional Sindbis Virus Replicative Complexes Are Formed at the Plasma Membrane. *J. Virol.* **84**, 11679–11695
138. Spuul, P., Balistreri, G., Hellstrom, K., Golubtsov, A. V., Jokitalo, E., and Ahola, T. (2011) Assembly of Alphavirus Replication Complexes from RNA and Protein Components in a Novel trans-Replication System in Mammalian Cells ▿. *J. Virol.* **85**, 4739–4751
139. Froshauer, S., Kartenbeck, J., and Helenius, A. (1988) Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J. Cell Biol.* **107**, 2075–2086
140. Grimley, P. M., Berezesky, I. K., and Friedman, R. M. (1968) Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. *J. Virol.* **2**, 1326–1338
141. Kallio, K., Hellström, K., Balistreri, G., Spuul, P., Jokitalo, E., and Ahola, T. (2013) Template RNA length determines the size of replication complex spherules for Semliki Forest virus. *J. Virol.* **87**, 9125–9134
142. Kujala, P., Ikaheimonen, A., Ehsani, N., Vihinen, H., Auvinen, P., and Kaariainen, L. (2001) Biogenesis of the Semliki Forest Virus RNA Replication Complex. *J. Virol.* **75**, 3873–3884
143. Peränen, J., and Kääriäinen, L. (1991) Biogenesis of type I cytopathic vacuoles in Semliki Forest virus-infected BHK cells. *J. Virol.* **65**, 1623–1627
144. Peränen, J., Laakkonen, P., Hyvönen, M., and Kääriäinen, L. (1995) The alphavirus replicase protein nsP1 is membrane-associated and has affinity to endocytic organelles. *Virology*. **208**, 610–620
145. Salonen, A., Vasiljeva, L., Merits, A., Magden, J., Jokitalo, E., and Kääriäinen, L. (2003) Properly folded nonstructural polyprotein directs the semliki forest virus replication complex to the endosomal compartment. *J. Virol.* **77**, 1691–1702
146. Spuul, P., Balistreri, G., Kaariainen, L., and Ahola, T. (2010) Phosphatidylinositol 3-Kinase-, Actin-, and Microtubule-Dependent Transport of Semliki Forest Virus Replication Complexes from the Plasma Membrane to Modified Lysosomes. *J. Virol.* **84**, 7543–7557
147. Ahola, T., Lampio, A., Auvinen, P., and Kääriäinen, L. (1999) Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. *EMBO J.* **18**, 3164–3172
148. Laakkonen, P., Ahola, T., and Kääriäinen, L. (1996) The effects of palmitoylation on membrane association of Semliki forest virus RNA capping enzyme. *J. Biol. Chem.* **271**, 28567–28571

149. Spuul, P., Salonen, A., Merits, A., Jokitalo, E., Kääriäinen, L., and Ahola, T. (2007) Role of the Amphipathic Peptide of Semliki Forest Virus Replicase Protein nsP1 in Membrane Association and Virus Replication. *J. Virol.* **81**, 872–883
150. Liljestrom, P., Lusa, S., Huylebroeck, D., and Garoff, H. (1991) In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.* **65**, 4107–4113
151. Rice, C. M., and Strauss, J. H. (1981) Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 2062–2066
152. Sjöberg, E. M., and Garoff, H. (1996) The translation-enhancing region of the Semliki Forest virus subgenome is only functional in the virus-infected cell. *J. Gen. Virol.* **77 (Pt 6)**, 1323–1327
153. Choi, H. K., Tong, L., Minor, W., Dumas, P., Boege, U., Rossmann, M. G., and Wengler, G. (1991) Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. *Nature*. **354**, 37–43
154. Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U., and Liljeström, P. (1990) The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. *J. Cell Biol.* **111**, 867–876
155. Crispin, M., Harvey, D. J., Bitto, D., Bonomelli, C., Edgeworth, M., Scrivens, J. H., Huiskonen, J. T., and Bowden, T. A. (2014) Structural plasticity of the Semliki Forest virus glycome upon interspecies transmission. *J. Proteome Res.* 10.1021/pr401162k
156. Li, L., Jose, J., Xiang, Y., Kuhn, R. J., and Rossmann, M. G. (2010) Structural changes of envelope proteins during alphavirus fusion. *Nature*. **468**, 705–708
157. Liljeström, P., and Garoff, H. (1991) Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J. Virol.* **65**, 147–154
158. Snyder, J. E., Kulcsar, K. A., Schultz, K. L. W., Riley, C. P., Neary, J. T., Marr, S., Jose, J., Griffin, D. E., and Kuhn, R. J. (2013) Functional characterization of the alphavirus TF protein. *J. Virol.* **87**, 8511–8523
159. Niyas, K. P., Abraham, R., Unnikrishnan, R. N., Mathew, T., Nair, S., Manakkadan, A., Issac, A., and Sreekumar, E. (2010) Molecular characterization of Chikungunya virus isolates from clinical samples and adult Aedes albopictus mosquitoes emerged from larvae from Kerala, South India. *Virol. J.* **7**, 189
160. Scholte, F. E. M., Tas, A., Martina, B. E. E., Cordioli, P., Narayanan, K., Makino, S., Snijder, E. J., and van Hemert, M. J. (2013) Characterization of synthetic Chikungunya viruses based on the consensus sequence of recent E1-226V isolates. *PloS One*. **8**, e71047
161. Boege, U., Wengler, G., Wengler, G., and Wittmann-liebold, B. (1981) Primary structures of the core proteins of the alphaviruses semliki forest virus and sindbis virus. *Virology*. **113**, 293–303
162. Hahn, C. S., Strauss, E. G., and Strauss, J. H. (1985) Sequence analysis of three Sindbis virus mutants temperature-sensitive in the capsid protein autoprotease. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4648–4652
163. Melancon, P., and Garoff, H. (1987) Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. *J. Virol.* **61**, 1301–1309

164. Lulla, V., Kim, D. Y., Frolova, E. I., and Frolov, I. (2013) The amino-terminal domain of alphavirus capsid protein is dispensable for viral particle assembly but regulates RNA encapsidation through cooperative functions of its subdomains. *J. Virol.* **87**, 12003–12019
165. Aguilar, P. V., Weaver, S. C., and Basler, C. F. (2007) Capsid Protein of Eastern Equine Encephalitis Virus Inhibits Host Cell Gene Expression. *J. Virol.* **81**, 3866–3876
166. Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E., and Frolov, I. (2007) The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J. Virol.* **81**, 2472–2484
167. Garmashova, N., Atasheva, S., Kang, W., Weaver, S. C., Frolova, E., and Frolov, I. (2007) Analysis of Venezuelan equine encephalitis virus capsid protein function in the inhibition of cellular transcription. *J. Virol.* **81**, 13552–13565
168. Thomas, S., Rai, J., John, L., Schaefer, S., Pützer, B. M., and Herchenröder, O. (2013) Chikungunya virus capsid protein contains nuclear import and export signals. *Virol. J.* **10**, 269
169. Uchime, O., Fields, W., and Kielian, M. (2013) The role of E3 in pH protection during alphavirus assembly and exit. *J. Virol.* **87**, 10255–10262
170. Lobigs, M., Zhao, H. X., and Garoff, H. (1990) Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. *J. Virol.* **64**, 4346–4355
171. Parrott, M. M., Sitarski, S. A., Arnold, R. J., Picton, L. K., Hill, R. B., and Mukhopadhyay, S. (2009) Role of Conserved Cysteines in the Alphavirus E3 Protein. *J. Virol.* **83**, 2584–2591
172. Garoff, H., Frischauf, A.-M., Simons, K., Lehrach, H., and Delius, H. (1980) Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature*. **288**, 236–241
173. Ivanova, L., and Schlesinger, M. J. (1993) Site-directed mutations in the Sindbis virus E2 glycoprotein identify palmitoylation sites and affect virus budding. *J. Virol.* **67**, 2546–2551
174. Mukhopadhyay, S., Zhang, W., Gabler, S., Chipman, P. R., Strauss, E. G., Strauss, J. H., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2006) Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. *Struct. Lond. Engl. 1993*. **14**, 63–73
175. Chua, C. L., Chan, Y. F., and Sam, I.-C. (2014) Characterisation of mouse monoclonal antibodies targeting linear epitopes on Chikungunya virus E2 glycoprotein. *J. Virol. Methods*. **195**, 126–133
176. Goh, L. Y. H., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Pyke, A. T., Suhrbier, A., and Hall, R. A. (2013) Neutralizing monoclonal antibodies to the E2 protein of chikungunya virus protects against disease in a mouse model. *Clin. Immunol. Orlando Fla*. **149**, 487–497
177. Selvarajah, S., Sexton, N. R., Kahle, K. M., Fong, R. H., Mattia, K.-A., Gardner, J., Lu, K., Liss, N. M., Salvador, B., Tucker, D. F., Barnes, T., Mabila, M., Zhou, X., Rossini, G., Rucker, J. B., Sanders, D. A., Suhrbier, A., Sambri, V., Michault, A., Muench, M. O., Doranz, B. J., and Simmons, G. (2013) A

neutralizing monoclonal antibody targeting the acid-sensitive region in chikungunya virus E2 protects from disease. *PLoS Negl. Trop. Dis.* **7**, e2423

178. Arias-Goeta, C., Moutailler, S., Mousson, L., Zouache, K., Thiberge, J.-M., Caro, V., Rougeon, F., and Failloux, A.-B. (2014) Chikungunya virus adaptation to a mosquito vector correlates with only few point mutations in the viral envelope glycoprotein. *Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.* 10.1016/j.meegid.2014.03.015
179. Tsetsarkin, K. A., Vanlandingham, D. L., McGee, C. E., and Higgs, S. (2007) A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential. *PLoS Pathog*. **3**, e201
180. Gaedigk-Nitschko, K., Ding, M. X., Levy, M. A., and Schlesinger, M. J. (1990) Site-directed mutations in the Sindbis virus 6K protein reveal sites for fatty acylation and the underacylated protein affects virus release and virion structure. *Virology*. **175**, 282–291
181. Ivanova, L., Lustig, S., and Schlesinger, M. J. (1995) A pseudo-revertant of a Sindbis virus 6K protein mutant, which corrects for aberrant particle formation, contains two new mutations that map to the ectodomain of the E2 glycoprotein. *Virology*. **206**, 1027–1034
182. Madan, V., Castelló, A., and Carrasco, L. (2008) Viroporins from RNA viruses induce caspase-dependent apoptosis. *Cell. Microbiol.* **10**, 437–451
183. Madan, V., Sanz, M. A., and Carrasco, L. (2005) Requirement of the vesicular system for membrane permeabilization by Sindbis virus. *Virology*. **332**, 307–315
184. Melton, J. V., Ewart, G. D., Weir, R. C., Board, P. G., Lee, E., and Gage, P. W. (2002) Alphavirus 6K proteins form ion channels. *J. Biol. Chem.* **277**, 46923–46931
185. Sanz, M. A., Madan, V., Carrasco, L., and Nieva, J. L. (2003) Interfacial domains in Sindbis virus 6K protein. Detection and functional characterization. *J. Biol. Chem.* **278**, 2051–2057
186. Sanz, M. A., Pérez, L., and Carrasco, L. (1994) Semliki Forest virus 6K protein modifies membrane permeability after inducible expression in Escherichia coli cells. *J. Biol. Chem.* **269**, 12106–12110
187. Schmidt, M., Schmidt, M. F., and Rott, R. (1988) Chemical identification of cysteine as palmitoylation site in a transmembrane protein (Semliki Forest virus E1). *J. Biol. Chem.* **263**, 18635–18639
188. Ryan, C., Ivanova, L., and Schlesinger, M. J. (1998) Effects of site-directed mutations of transmembrane cysteines in sindbis virus E1 and E2 glycoproteins on palmitylation and virus replication. *Virology*. **249**, 62–67
189. Wengler, G., Koschinski, A., Wengler, G., and Dreyer, F. (2003) Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents. *J. Gen. Virol.* **84**, 173–181
190. Qin, Z., Zheng, Y., and Kielian, M. (2009) Role of Conserved Histidine Residues in the Low-pH Dependence of the Semliki Forest Virus Fusion Protein. *J. Virol.* **83**, 4670–4677
191. Vazeille, M., Moutailler, S., Coudrier, D., Rousseaux, C., Khun, H., Huerre, M., Thiria, J., Dehecq, J.-S., Fontenille, D., Schuffenecker, I., Despres, P., and Failloux, A.-B. (2007) Two Chikungunya Isolates from the Outbreak of La Reunion (Indian Ocean) Exhibit Different Patterns of Infection in the Mosquito, Aedes albopictus. *PLoS ONE*. **2**, e1168

192. Stapleford, K. A., Coffey, L. L., Lay, S., Bordería, A. V., Duong, V., Isakov, O., Rozen-Gagnon, K., Arias-Goeta, C., Blanc, H., Beaucourt, S., Haliloğlu, T., Schmitt, C., Bonne, I., Ben-Tal, N., Shomron, N., Failloux, A.-B., Buchy, P., and Vignuzzi, M. (2014) Emergence and transmission of arbovirus evolutionary intermediates with epidemic potential. *Cell Host Microbe*. **15**, 706–716
193. Tsetsarkin, K. A., Chen, R., Yun, R., Rossi, S. L., Plante, K. S., Guerbois, M., Forrester, N., Perng, G. C., Sreekumar, E., Leal, G., Huang, J., Mukhopadhyay, S., and Weaver, S. C. (2014) Multi-peaked adaptive landscape for chikungunya virus evolution predicts continued fitness optimization in Aedes albopictus mosquitoes. *Nat. Commun.* **5**, 4084
194. Tsetsarkin, K. A., Chen, R., Leal, G., Forrester, N., Higgs, S., Huang, J., and Weaver, S. C. (2011) Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 7872–7877
195. Ahola, T., Kujala, P., Tuittila, M., Blom, T., Laakkonen, P., Hinkkanen, A., and Auvinen, P. (2000) Effects of palmitoylation of replicase protein nsP1 on alphavirus infection. *J. Virol.* **74**, 6725–6733
196. Lampio, A., Kilpeläinen, I., Pesonen, S., Karhi, K., Auvinen, P., Somerharju, P., and Kääriäinen, L. (2000) Membrane binding mechanism of an RNA virus-capping enzyme. *J. Biol. Chem.* **275**, 37853–37859
197. Tomar, S., Narwal, M., Harms, E., Smith, J. L., and Kuhn, R. J. (2011) Heterologous production, purification and characterization of enzymatically active Sindbis virus nonstructural protein nsP1. *Protein Expr. Purif.* **79**, 277–284
198. Laakkonen, P., Auvinen, P., Kujala, P., and Kääriäinen, L. (1998) Alphavirus replicase protein NSP1 induces filopodia and rearrangement of actin filaments. *J. Virol.* **72**, 10265–10269
199. Alcover, A., and Thoulouze, M.-I. (2010) Vesicle Traffic to the Immunological Synapse: A Multifunctional Process Targeted by Lymphotropic Viruses. in *Immunological Synapse* (Saito, T., and Batista, F. D. eds), pp. 191–207, Current Topics in Microbiology and Immunology, Springer Berlin Heidelberg.
200. Gurke, S., Barroso, J. F. V., and Gerdes, H.-H. (2008) The art of cellular communication: tunneling nanotubes bridge the divide. *Histochem. Cell Biol.* **129**, 539–550
201. Lehmann, M., Nikolic, D. S., and Piguet, V. (2011) How HIV-1 Takes Advantage of the Cytoskeleton during Replication and Cell-to-Cell Transmission. *Viruses*. **3**, 1757–1776
202. Zusinaite, E., Tints, K., Kiiver, K., Spuul, P., Karo-Astover, L., Merits, A., and Sarand, I. (2007) Mutations at the palmitoylation site of non-structural protein nsP1 of Semliki Forest virus attenuate virus replication and cause accumulation of compensatory mutations. *J. Gen. Virol.* **88**, 1977–1985
203. Fata, C. L., Sawicki, S. G., and Sawicki, D. L. (2002) Modification of Asn374 of nsP1 suppresses a Sindbis virus nsP4 minus-strand polymerase mutant. *J. Virol.* **76**, 8641–8649
204. Shirako, Y., Strauss, E. G., and Strauss, J. H. (2000) Suppressor mutations that allow sindbis virus RNA polymerase to function with nonaromatic amino acids at the N-terminus: evidence for interaction between nsP1 and nsP4 in minus-strand RNA synthesis. *Virology*. **276**, 148–160

205. Decroly, E., Ferron, F., Lescar, J., and Canard, B. (2012) Conventional and unconventional mechanisms for capping viral mRNA. *Nat. Rev. Microbiol.* **10**, 51–65
206. Ahola, T., and Kääriäinen, L. (1995) Reaction in alphavirus mRNA capping: formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 507–511
207. Laakkonen, P., Hyvönen, M., Peränen, J., and Kääriäinen, L. (1994) Expression of Semliki Forest virus nsP1-specific methyltransferase in insect cells and in Escherichia coli. *J. Virol.* **68**, 7418–7425
208. Mi, S., and Stollar, V. (1991) Expression of Sindbis virus nsP1 and methyltransferase activity in Escherichia coli. *Virology*. **184**, 423–427
209. Karpe, Y. A., Aher, P. P., and Lole, K. S. (2011) NTPase and 5′-RNA Triphosphatase Activities of Chikungunya Virus nsP2 Protein. *PLoS ONE*. **6**, e22336
210. Vasiljeva, L., Merits, A., Auvinen, P., and Kääriäinen, L. (2000) Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. *J. Biol. Chem.* **275**, 17281–17287
211. Li, C., Guillén, J., Rabah, N., Blanjoie, A., Debart, F., Vasseur, J.-J., Canard, B., Decroly, E., and Coutard, B. (2015) mRNA capping by Venezuelan Equine Encephalitis Virus nsP1: Functional characterization and implication for antiviral research. *J. Virol.* 10.1128/JVI.00599-15
212. Wang, H. L., O'Rear, J., and Stollar, V. (1996) Mutagenesis of the Sindbis virus nsP1 protein: effects on methyltransferase activity and viral infectivity. *Virology*. **217**, 527–531
213. Akhrymuk, I., Kulemzin, S. V., and Frolova, E. I. (2012) Evasion of the innate immune response: the Old World alphavirus nsP2 protein induces rapid degradation of Rpb1, a catalytic subunit of RNA polymerase II. *J. Virol.* **86**, 7180–7191
214. Breakwell, L., Dosenovic, P., Karlsson Hedestam, G. B., D'Amato, M., Liljeström, P., Fazakerley, J., and McInerney, G. M. (2007) Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. *J. Virol.* **81**, 8677–8684
215. Das, P. K., Merits, A., and Lulla, A. (2014) Functional Crosstalk between Distant Domains of Chikungunya Virus Non-Structural Protein 2 Is Decisive For Its RNA-Modulating Activity. *J. Biol. Chem.* 10.1074/jbc.M113.503433
216. Garmashova, N., Gorchakov, R., Frolova, E., and Frolov, I. (2006) Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J. Virol.* **80**, 5686–5696
217. Gomez de Cedrón, M., Ehsani, N., Mikkola, M. L., García, J. A., and Kääriäinen, L. (1999) RNA helicase activity of Semliki Forest virus replicase protein NSP2. *FEBS Lett.* **448**, 19–22
218. Gorchakov, R., Frolova, E., and Frolov, I. (2005) Inhibition of Transcription and Translation in Sindbis Virus-Infected Cells. *J. Virol.* **79**, 9397–9409
219. Hardy, W. R., and Strauss, J. H. (1989) Processing the nonstructural polyproteins of sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in cis and in trans. *J. Virol.* **63**, 4653–4664
220. Rikkonen, M., Peränen, J., and Kääriäinen, L. (1994) ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *J. Virol.* **68**, 5804–5810

221. Peränen, J., Rikkonen, M., Liljeström, P., and Kääriäinen, L. (1990) Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *J. Virol.* **64**, 1888–1896
222. Russo, A. T., White, M. A., and Watowich, S. J. (2006) The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. *Struct. Lond. Engl. 1993*. **14**, 1449–1458
223. Caruthers, J. M., and McKay, D. B. (2002) Helicase structure and mechanism. *Curr. Opin. Struct. Biol.* **12**, 123–133
224. Story, R. M., Weber, I. T., and Steitz, T. A. (1992) The structure of the E. coli recA protein monomer and polymer. *Nature*. **355**, 318–325
225. Singleton, M. R., Dillingham, M. S., and Wigley, D. B. (2007) Structure and mechanism of helicases and nucleic acid translocases. *Annu. Rev. Biochem.* **76**, 23–50
226. Rikkonen, M. (1996) Functional significance of the nuclear-targeting and NTP-binding motifs of Semliki Forest virus nonstructural protein nsP2. *Virology*. **218**, 352–361
227. Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* **17**, 4713–4730
228. Cheung, J., Franklin, M., Mancia, F., Rudolph, M., Cassidy, M., Gary, E., Burshteyn, F., and Love, J. (2011) Structure of the Chikungunya virus nsP2 protease. *Be Publ.*
229. DeLano, W.L. (2002) The PyMOL Molecular Graphics System
230. Mayuri, Geders, T. W., Smith, J. L., and Kuhn, R. J. (2008) Role for conserved residues of sindbis virus nonstructural protein 2 methyltransferase-like domain in regulation of minus-strand synthesis and development of cytopathic infection. *J. Virol.* **82**, 7284–7297
231. Vasiljeva, L., Valmu, L., Kääriäinen, L., and Merits, A. (2001) Site-specific Protease Activity of the Carboxyl-terminal Domain of Semliki Forest Virus Replicase Protein nsP2. *J. Biol. Chem.* **276**, 30786–30793
232. Lemm, J. A., and Rice, C. M. (1993) Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. *J. Virol.* **67**, 1916–1926
233. Lemm, J. A., and Rice, C. M. (1993) Assembly of functional Sindbis virus RNA replication complexes: requirement for coexpression of P123 and P34. *J. Virol.* **67**, 1905–1915
234. Shin, G., Yost, S. A., Miller, M. T., Elrod, E. J., Grakoui, A., and Marcotrigiano, J. (2012) Structural and functional insights into alphavirus polyprotein processing and pathogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16534–16539
235. Dryga, S. A., Dryga, O. A., and Schlesinger, S. (1997) Identification of mutations in a Sindbis virus variant able to establish persistent infection in BHK cells: the importance of a mutation in the nsP2 gene. *Virology*. **228**, 74–83
236. Frolov, I., Agapov, E., Hoffman, T. A., Pragai, B. M., Lippa, M., Schlesinger, S., and Rice, C. M. (1999) Selection of RNA Replicons Capable of Persistent Noncytopathic Replication in Mammalian Cells. *J. Virol.* **73**, 3854–3865

237. Tamm, K., Merits, A., and Sarand, I. (2008) Mutations in the nuclear localization signal of nsP2 influencing RNA synthesis, protein expression and cytotoxicity of Semliki Forest virus. *J. Gen. Virol.* **89**, 676–686
238. McInerney, G. M., Kedersha, N. L., Kaufman, R. J., Anderson, P., and Liljeström, P. (2005) Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation. *Mol. Biol. Cell.* **16**, 3753–3763
239. Montgomery, S. A., Berglund, P., Beard, C. W., and Johnston, R. E. (2006) Ribosomal Protein S6 Associates with Alphavirus Nonstructural Protein 2 and Mediates Expression from Alphavirus Messages. *J. Virol.* **80**, 7729–7739
240. Toribio, R., and Ventoso, I. (2010) Inhibition of host translation by virus infection in vivo. *Proc. Natl. Acad. Sci.* 10.1073/pnas.1004110107
241. Utt, A., Das, P. K., Varjak, M., Lulla, V., Lulla, A., and Merits, A. (2015) Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2. *J. Virol.* **89**, 3145–3162
242. Frolov, I., Garmashova, N., Atasheva, S., and Frolova, E. I. (2009) Random insertion mutagenesis of sindbis virus nonstructural protein 2 and selection of variants incapable of downregulating cellular transcription. *J. Virol.* **83**, 9031–9044
243. Frolov, I., Akhrymuk, M., Akhrymuk, I., Atasheva, S., and Frolova, E. I. (2012) Early events in alphavirus replication determine the outcome of infection. *J. Virol.* **86**, 5055–5066
244. Frolova, E. I., Fayzulin, R. Z., Cook, S. H., Griffin, D. E., Rice, C. M., and Frolov, I. (2002) Roles of Nonstructural Protein nsP2 and Alpha/Beta Interferons in Determining the Outcome of Sindbis Virus Infection. *J. Virol.* **76**, 11254–11264
245. Fros, J. J., van der Maten, E., Vlak, J. M., and Pijlman, G. P. (2013) The C-terminal domain of chikungunya virus nsP2 independently governs viral RNA replication, cytopathicity, and inhibition of interferon signaling. *J. Virol.* **87**, 10394–10400
246. Fros, J. J., Major, L. D., Scholte, F. E. M., Gardner, J., van Hemert, M. J., Suhrbier, A., and Pijlman, G. P. (2014) Chikungunya virus nsP2-mediated host shut-off disables the unfolded protein response. *J. Gen. Virol.* 10.1099/vir.0.071845-0
247. Gorchakov, R., Frolova, E., Sawicki, S., Atasheva, S., Sawicki, D., and Frolov, I. (2008) A new role for ns polyprotein cleavage in Sindbis virus replication. *J. Virol.* **82**, 6218–6231
248. Montgomery, S. A., and Johnston, R. E. (2007) Nuclear Import and Export of Venezuelan Equine Encephalitis Virus Nonstructural Protein 2. *J. Virol.* **81**, 10268–10279
249. Rikkonen, M., Peränen, J., and Kääriäinen, L. (1992) Nuclear and nucleolar targeting signals of Semliki Forest virus nonstructural protein nsP2. *Virology*. **189**, 462–473
250. Rikkonen, M., Peränen, J., and Kääriäinen, L. (1994) Nuclear targeting of Semliki Forest virus nsP2. *Arch. Virol. Suppl.* **9**, 369–377
251. Schulz, O., Pichlmair, A., Rehwinkel, J., Rogers, N. C., Scheuner, D., Kato, H., Takeuchi, O., Akira, S., Kaufman, R. J., and Reis e Sousa, C. (2010) Protein

kinase R contributes to immunity against specific viruses by regulating interferon mRNA integrity. *Cell Host Microbe*. **7**, 354–361
252. Koonin, E. V., and Dolja, V. V. (1993) Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* **28**, 375–430
253. Fros, J. J., Domeradzka, N. E., Baggen, J., Geertsema, C., Flipse, J., Vlak, J. M., and Pijlman, G. P. (2012) Chikungunya Virus nsP3 Blocks Stress Granule Assembly by Recruitment of G3BP into Cytoplasmic Foci. *J. Virol.* **86**, 10873–10879
254. Neuvonen, M., Kazlauskas, A., Martikainen, M., Hinkkanen, A., Ahola, T., and Saksela, K. (2011) SH3 Domain-Mediated Recruitment of Host Cell Amphiphysins by Alphavirus nsP3 Promotes Viral RNA Replication. *PLoS Pathog*. **7**, e1002383
255. Egloff, M.-P., Malet, H., Putics, A., Heinonen, M., Dutartre, H., Frangeul, A., Gruez, A., Campanacci, V., Cambillau, C., Ziebuhr, J., Ahola, T., and Canard, B. (2006) Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. *J. Virol.* **80**, 8493–8502
256. Neuvonen, M., and Ahola, T. (2009) Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites. *J. Mol. Biol.* **385**, 212–225
257. Malet, H., Coutard, B., Jamal, S., Dutartre, H., Papageorgiou, N., Neuvonen, M., Ahola, T., Forrester, N., Gould, E. A., Lafitte, D., Ferron, F., Lescar, J., Gorbalenya, A. E., de Lamballerie, X., and Canard, B. (2009) The crystal structures of Chikungunya and Venezuelan equine encephalitis virus nsP3 macro domains define a conserved adenosine binding pocket. *J. Virol.* **83**, 6534–6545
258. Gorchakov, R., Garmashova, N., Frolova, E., and Frolov, I. (2008) Different Types of nsP3-Containing Protein Complexes in Sindbis Virus-Infected Cells. *J. Virol.* **82**, 10088–10101
259. Cristea, I. M., Carroll, J.-W. N., Rout, M. P., Rice, C. M., Chait, B. T., and MacDonald, M. R. (2006) Tracking and elucidating alphavirus-host protein interactions. *J. Biol. Chem.* **281**, 30269–30278
260. Foy, N. J., Akhrymuk, M., Akhrymuk, I., Atasheva, S., Bopda-Waffo, A., Frolov, I., and Frolova, E. I. (2013) Hypervariable Domains of nsP3 Proteins of New World and Old World Alphaviruses Mediate Formation of Distinct, Virus-Specific Protein Complexes. *J. Virol.* **87**, 1997–2010
261. Panas, M. D., Ahola, T., and McInerney, G. M. (2014) The C-terminal repeat domains of nsP3 from the Old World alphaviruses bind directly to G3BP. *J. Virol.* 10.1128/JVI.00439-14
262. Varjak, M., Zusinaite, E., and Merits, A. (2010) Novel functions of the alphavirus nonstructural protein nsP3 C-terminal region. *J. Virol.* **84**, 2352–2364
263. Issac, T. H. K., Tan, E. L., and Chu, J. J. H. (2014) Proteomic profiling of chikungunya virus-infected human muscle cells: reveal the role of cytoskeleton network in CHIKV replication. *J. Proteomics*. **108**, 445–464
264. Vihinen, H., and Saarinen, J. (2000) Phosphorylation site analysis of Semliki forest virus nonstructural protein 3. *J. Biol. Chem.* **275**, 27775–27783
265. Vihinen, H., Ahola, T., Tuittila, M., Merits, A., and Kääriäinen, L. (2001) Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J. Biol. Chem.* **276**, 5745–5752

266. Dé, I., Fata-Hartley, C., Sawicki, S. G., and Sawicki, D. L. (2003) Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. *J. Virol.* **77**, 13106–13116
267. Foy, N. J., Akhrymuk, M., Shustov, A. V., Frolova, E. I., and Frolov, I. (2013) Hypervariable domain of nonstructural protein nsP3 of Venezuelan equine encephalitis virus determines cell-specific mode of virus replication. *J. Virol.* **87**, 7569–7584
268. Rubach, J. K., Wasik, B. R., Rupp, J. C., Kuhn, R. J., Hardy, R. W., and Smith, J. L. (2009) Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro. *Virology*. **384**, 201–208
269. Rupp, J. C., Jundt, N., and Hardy, R. W. (2011) Requirement for the Amino-Terminal Domain of Sindbis Virus nsP4 during Virus Infection. *J. Virol.* **85**, 3449–3460
270. Shirako, Y., and Strauss, J. H. (1998) Requirement for an aromatic amino acid or histidine at the N terminus of Sindbis virus RNA polymerase. *J. Virol.* **72**, 2310–2315
271. Koonin, E. V. (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *J. Gen. Virol.* **72 (Pt 9)**, 2197–2206
272. Ferrer-Orta, C., Arias, A., Escarmís, C., and Verdaguer, N. (2006) A comparison of viral RNA-dependent RNA polymerases. *Curr. Opin. Struct. Biol.* **16**, 27–34
273. Ng, K. K.-S., Arnold, J. J., and Cameron, C. E. (2008) Structure-Function Relationships Among RNA-Dependent RNA Polymerases. *Curr. Top. Microbiol. Immunol.* **320**, 137–156
274. Tomar, S., Hardy, R. W., Smith, J. L., and Kuhn, R. J. (2006) Catalytic core of alphavirus nonstructural protein nsP4 possesses terminal adenylyltransferase activity. *J. Virol.* **80**, 9962–9969
275. De Groot, R. J., Rümenapf, T., Kuhn, R. J., Strauss, E. G., and Strauss, J. H. (1991) Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8967–8971
276. Varshavsky, A. (1992) The N-end rule. *Cell*. **69**, 725–735
277. Lemm, J. A., Bergqvist, A., Read, C. M., and Rice, C. M. (1998) Template-Dependent Initiation of Sindbis Virus RNA Replication In Vitro. *J. Virol.* **72**, 6546–6553
278. Barton, D. J., Sawicki, S. G., and Sawicki, D. L. (1991) Solubilization and immunoprecipitation of alphavirus replication complexes. *J. Virol.* **65**, 1496–1506
279. Hardy, W. R., and Strauss, J. H. (1988) Processing the nonstructural polyproteins of Sindbis virus: study of the kinetics in vivo by using monospecific antibodies. *J. Virol.* **62**, 998–1007
280. Shirako, Y., Strauss, E. G., and Strauss, J. H. (2003) Modification of the 5' terminus of Sindbis virus genomic RNA allows nsP4 RNA polymerases with nonaromatic amino acids at the N terminus to function in RNA replication. *J. Virol.* **77**, 2301–2309
281. Raju, R., Hajjou, M., Hill, K. R., Botta, V., and Botta, S. (1999) In Vivo Addition of Poly(A) Tail and AU-Rich Sequences to the 3? Terminus of the Sindbis Virus RNA Genome: a Novel 3?-End Repair Pathway. *J. Virol.* **73**, 2410–2419

282. Thal, M. A., Wasik, B. R., Posto, J., and Hardy, R. W. (2007) Template requirements for recognition and copying by Sindbis virus RNA-dependent RNA polymerase. *Virology*. **358**, 221–232
283. Grdzelishvili, V. Z., Garcia-Ruiz, H., Watanabe, T., and Ahlquist, P. (2005) Mutual interference between genomic RNA replication and subgenomic mRNA transcription in brome mosaic virus. *J. Virol.* **79**, 1438–1451
284. Li, M.-L., and Stollar, V. (2007) Distinct Sites on the Sindbis Virus RNA-Dependent RNA Polymerase for Binding to the Promoters for the Synthesis of Genomic and Subgenomic RNA. *J. Virol.* **81**, 4371–4373
285. Li, M.-L., and Stollar, V. (2004) Identification of the amino acid sequence in Sindbis virus nsP4 that binds to the promoter for the synthesis of the subgenomic RNA. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 9429–9434
286. Hahn, Y. S., Strauss, E. G., and Strauss, J. H. (1989) Mapping of RNA- temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. *J. Virol.* **63**, 3142–3150
287. Lundstrom, K. (1999) Alphaviruses as tools in neurobiology and gene therapy. *J. Recept. Signal Transduct. Res.* **19**, 673–686
288. Baldi, L., Hacker, D. L., Adam, M., and Wurm, F. M. (2007) Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotechnol. Lett.* **29**, 677–684
289. Davis, N. L., Willis, L. V., Smith, J. F., and Johnston, R. E. (1989) In vitro synthesis of infectious venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. *Virology*. **171**, 189–204
290. Liljeström, P., and Garoff, H. (1991) A New Generation of Animal Cell Expression Vectors Based on the Semliki Forest Virus Replicon. *Nat. Biotechnol.* **9**, 1356–1361
291. Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M., and Huang, H. V. (1989) Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science*. **243**, 1188–1191
292. Riezebos-Brilman, A., Mare, A. de, Bungener, L., Huckriede, A., Wilschut, J., and Daemen, T. (2006) Recombinant alphaviruses as vectors for anti-tumour and anti-microbial immunotherapy. *J. Clin. Virol.* **35**, 233–243
293. Pohjala, L., Utt, A., Varjak, M., Lulla, A., Merits, A., Ahola, T., and Tammela, P. (2011) Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays. *PloS One*. **6**, e28923
294. Agapov, E. V., Frolov, I., Lindenbach, B. D., Prágai, B. M., Schlesinger, S., and Rice, C. M. (1998) Noncytopathic Sindbis virus RNA vectors for heterologous gene expression. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12989–12994
295. Galbraith, S. E., Sheahan, B. J., and Atkins, G. J. (2006) Deletions in the hypervariable domain of the nsP3 gene attenuate Semliki Forest virus virulence. *J. Gen. Virol.* **87**, 937–947
296. Perri, S., Driver, D. A., Gardner, J. P., Sherrill, S., Belli, B. A., Dubensky, T. W., and Polo, J. M. (2000) Replicon Vectors Derived from Sindbis Virus and Semliki Forest Virus That Establish Persistent Replication in Host Cells. *J. Virol.* **74**, 9802–9807
297. Tuittila, M. T., Santagati, M. G., Roytta, M., Maatta, J. A., and Hinkkanen, A. E. (2000) Replicase Complex Genes of Semliki Forest Virus Confer Lethal Neurovirulence. *J. Virol.* **74**, 4579–4589

298. Ehrengruber, M. U., Lundstrom, K., Schweitzer, C., Heuss, C., Schlesinger, S., and Gahwiler, B. H. (1999) Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 7041–7046
299. Bredenbeek, P. J., Frolov, I., Rice, C. M., and Schlesinger, S. (1993) Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J. Virol.* **67**, 6439–6446
300. Berglund, P., Sjöberg, M., Garoff, H., Atkins, G. J., Sheahan, B. J., and Liljeström, P. (1993) Semliki Forest virus expression system: production of conditionally infectious recombinant particles. *Biotechnol. Nat. Publ. Co.* **11**, 916–920
301. Frolov, I., Frolova, E., and Schlesinger, S. (1997) Sindbis virus replicons and Sindbis virus: assembly of chimeras and of particles deficient in virus RNA. *J. Virol.* **71**, 2819–2829
302. Smerdou, C., and Liljeström, P. (1999) Two-Helper RNA System for Production of Recombinant Semliki Forest Virus Particles. *J. Virol.* **73**, 1092–1098
303. Weiss, B. G., and Schlesinger, S. (1991) Recombination between Sindbis virus RNAs. *J. Virol.* **65**, 4017–4025
304. Thomas, J. M., Klimstra, W. B., Ryman, K. D., and Heidner, H. W. (2003) Sindbis Virus Vectors Designed To Express a Foreign Protein as a Cleavable Component of the Viral Structural Polyprotein. *J. Virol.* **77**, 5598–5606
305. Atasheva, S., Gorchakov, R., English, R., Frolov, I., and Frolova, E. (2007) Development of Sindbis Viruses Encoding nsP2/GFP Chimeric Proteins and Their Application for Studying nsP2 Functioning. *J. Virol.* **81**, 5046–5057
306. Bick, M. J., Carroll, J.-W. N., Gao, G., Goff, S. P., Rice, C. M., and MacDonald, M. R. (2003) Expression of the zinc-finger antiviral protein inhibits alphavirus replication. *J. Virol.* **77**, 11555–11562
307. Frolova, E., Gorchakov, R., Garmashova, N., Atasheva, S., Vergara, L. A., and Frolov, I. (2006) Formation of nsP3-specific protein complexes during Sindbis virus replication. *J. Virol.* **80**, 4122–4134
308. Fragkoudis, R., Tamberg, N., Siu, R., Kiiver, K., Kohl, A., Merits, A., and Fazakerley, J. K. (2009) Neurons and oligodendrocytes in the mouse brain differ in their ability to replicate Semliki Forest virus. *J. Neurovirol.* **15**, 57–70
309. Kim, D. Y., Atasheva, S., McAuley, A. J., Plante, J. A., Frolova, E. I., Beasley, D. W. C., and Frolov, I. (2014) Enhancement of protein expression by alphavirus replicons by designing self-replicating subgenomic RNAs. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 10708–10713
310. Ylösmäki, E., Martikainen, M., Hinkkanen, A., and Saksela, K. (2013) Attenuation of Semliki Forest virus neurovirulence by microRNA-mediated detargeting. *J. Virol.* **87**, 335–344
311. Fairman-Williams, M. E., Guenther, U.-P., and Jankowsky, E. (2010) SF1 and SF2 helicases: family matters. *Curr. Opin. Struct. Biol.* **20**, 313–324
312. Lohman, T. M., Tomko, E. J., and Wu, C. G. (2008) Non-hexameric DNA helicases and translocases: mechanisms and regulation. *Nat. Rev. Mol. Cell Biol.* **9**, 391–401
313. Byrd, A. K., and Raney, K. D. (2012) Superfamily 2 helicases. *Front. Biosci. Landmark Ed.* **17**, 2070–2088

314. Abdel-Monem, M., Dürwald, H., and Hoffmann-Berling, H. (1976) Enzymic unwinding of DNA. 2. Chain separation by an ATP-dependent DNA unwinding enzyme. *Eur. J. Biochem. FEBS*. **65**, 441–449
315. Hotta, Y., and Stern, H. (1978) DNA unwinding protein from meiotic cells of Lilium. *Biochemistry (Mosc.)*. **17**, 1872–1880
316. Gorbalenya, A. E., and Koonin, E. V. (1993) Helicases: amino acid sequence comparisons and structure-function relationships. *Curr. Opin. Struct. Biol.* **3**, 419–429
317. Cheok, C. F., Bachrati, C. Z., Chan, K. L., Ralf, C., Wu, L., and Hickson, I. D. (2005) Roles of the Bloom's syndrome helicase in the maintenance of genome stability. *Biochem. Soc. Trans.* **33**, 1456–1459
318. Ellis, N. A. (1997) DNA helicases in inherited human disorders. *Curr. Opin. Genet. Dev.* **7**, 354–363
319. Friedberg, E. C., and Wood, R. D. (2007) New insights into the combined Cockayne/xeroderma pigmentosum complex: human XPG protein can function in transcription factor stability. *Mol. Cell*. **26**, 162–164
320. Goto, M. (2000) Werner's syndrome: from clinics to genetics. *Clin. Exp. Rheumatol.* **18**, 760–766
321. Van Brabant, A. J., Stan, R., and Ellis, N. A. (2000) DNA helicases, genomic instability, and human genetic disease. *Annu. Rev. Genomics Hum. Genet.* **1**, 409–459
322. Briguglio, I., Piras, S., Corona, P., and Carta, A. (2010) Inhibition of RNA Helicases of ssRNA +Virus Belonging to Flaviviridae, Coronaviridae and Picornaviridae Families. *Int. J. Med. Chem.* **2011**, e213135
323. Frick, D. N., and Lam, A. M. I. (2006) Understanding helicases as a means of virus control. *Curr. Pharm. Des*. **12**, 1315–1338
324. Kwong, A. D., Rao, B. G., and Jeang, K.-T. (2005) Viral and cellular RNA helicases as antiviral targets. *Nat. Rev. Drug Discov.* **4**, 845–853
325. Lee, C.-G. (2002) RH70, a Bidirectional RNA Helicase, Co-purifies with U1snRNP. *J. Biol. Chem.* **277**, 39679–39683
326. Lohman, T. M., and Bjornson, K. P. (1996) Mechanisms of helicase-catalyzed DNA unwinding. *Annu. Rev. Biochem.* **65**, 169–214
327. Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990) Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol. Cell. Biol.* **10**, 1134–1144
328. Jankowsky, E. (2011) RNA helicases at work: binding and rearranging. *Trends Biochem. Sci.* **36**, 19–29
329. Ye, J., Osborne, A. R., Groll, M., and Rapoport, T. A. (2004) RecA-like motor ATPases--lessons from structures. *Biochim. Biophys. Acta*. **1659**, 1–18
330. Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997) The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science*. **277**, 333–338
331. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945–951

332. Cheng, Z., Muhlrad, D., Lim, M. K., Parker, R., and Song, H. (2007) Structural and functional insights into the human Upf1 helicase core. *EMBO J.* **26**, 253–264
333. Ivessa, A. S., Zhou, J.-Q., Schulz, V. P., Monson, E. K., and Zakian, V. A. (2002) Saccharomyces Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA. *Genes Dev.* **16**, 1383–1396
334. Kadare, G., and Haenni, A. L. (1997) Virus-encoded RNA helicases. *J. Virol.* **71**, 2583–2590
335. Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) Major domain swiveling revealed by the crystal structures of complexes of E. coli Rep helicase bound to single-stranded DNA and ADP. *Cell*. **90**, 635–647
336. Singleton, M. R., Dillingham, M. S., Gaudier, M., Kowalczykowski, S. C., and Wigley, D. B. (2004) Crystal structure of RecBCD enzyme reveals a machine for processing DNA breaks. *Nature*. **432**, 187–193
337. Subramanya, H. S., Bird, L. E., Brannigan, J. A., and Wigley, D. B. (1996) Crystal structure of a DExx box DNA helicase. *Nature*. **384**, 379–383
338. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) Crystal structures of complexes of PcrA DNA helicase with a DNA substrate indicate an inchworm mechanism. *Cell*. **97**, 75–84
339. Brendza, K. M., Cheng, W., Fischer, C. J., Chesnik, M. A., Niedziela-Majka, A., and Lohman, T. M. (2005) Autoinhibition of Escherichia coli Rep monomer helicase activity by its 2B subdomain. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10076–10081
340. Arslan, S., Khafizov, R., Thomas, C. D., Chemla, Y. R., and Ha, T. (2015) Protein structure. Engineering of a superhelicase through conformational control. *Science*. **348**, 344–347
341. Byrd, A. K., Matlock, D. L., Bagchi, D., Aarattuthodiyil, S., Harrison, D., Croquette, V., and Raney, K. D. (2012) Dda helicase tightly couples translocation on single-stranded DNA to unwinding of duplex DNA: Dda is an optimally active helicase. *J. Mol. Biol.* **420**, 141–154
342. He, X., Byrd, A. K., Yun, M.-K., Pemble, C. W., Harrison, D., Yeruva, L., Dahl, C., Kreuzer, K. N., Raney, K. D., and White, S. W. (2012) The T4 phage SF1B helicase Dda is structurally optimized to perform DNA strand separation. *Struct. Lond. Engl. 1993*. **20**, 1189–1200
343. Nanduri, B., Byrd, A. K., Eoff, R. L., Tackett, A. J., and Raney, K. D. (2002) Pre-steady-state DNA unwinding by bacteriophage T4 Dda helicase reveals a monomeric molecular motor. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 14722–14727
344. Taylor, A. F., and Smith, G. R. (1995) Monomeric RecBCD enzyme binds and unwinds DNA. *J. Biol. Chem.* **270**, 24451–24458
345. Jankowsky, E., and Fairman, M. E. (2007) RNA helicases--one fold for many functions. *Curr. Opin. Struct. Biol.* **17**, 316–324
346. Grohman, J. K., Del Campo, M., Bhaskaran, H., Tijerina, P., Lambowitz, A. M., and Russell, R. (2007) Probing the mechanisms of DEAD-box proteins as general RNA chaperones: the C-terminal domain of CYT-19 mediates general recognition of RNA. *Biochemistry (Mosc.)*. **46**, 3013–3022
347. Yang, Q., and Jankowsky, E. (2006) The DEAD-box protein Ded1 unwinds RNA duplexes by a mode distinct from translocating helicases. *Nat. Struct. Mol. Biol.* **13**, 981–986

348. Hilbert, M., Karow, A. R., and Klostermeier, D. (2009) The mechanism of ATP-dependent RNA unwinding by DEAD box proteins. *Biol. Chem.* **390**, 1237–1250
349. Liu, F., Putnam, A., and Jankowsky, E. (2008) ATP hydrolysis is required for DEAD-box protein recycling but not for duplex unwinding. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 20209–20214
350. Yang, Q., and Jankowsky, E. (2005) ATP- and ADP-dependent modulation of RNA unwinding and strand annealing activities by the DEAD-box protein DED1. *Biochemistry (Mosc.)*. **44**, 13591–13601
351. Yang, Q., Del Campo, M., Lambowitz, A. M., and Jankowsky, E. (2007) DEAD-box proteins unwind duplexes by local strand separation. *Mol. Cell*. **28**, 253–263
352. Tanner, N. K., Cordin, O., Banroques, J., Doère, M., and Linder, P. (2003) The Q motif: a newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. *Mol. Cell*. **11**, 127–138
353. Andersen, C. B. F., Ballut, L., Johansen, J. S., Chamieh, H., Nielsen, K. H., Oliveira, C. L. P., Pedersen, J. S., Séraphin, B., Le Hir, H., and Andersen, G. R. (2006) Structure of the exon junction core complex with a trapped DEAD-box ATPase bound to RNA. *Science*. **313**, 1968–1972
354. Collins, R., Karlberg, T., Lehtiö, L., Schütz, P., van den Berg, S., Dahlgren, L.-G., Hammarström, M., Weigelt, J., and Schüler, H. (2009) The DEXD/H-box RNA helicase DDX19 is regulated by an {alpha}-helical switch. *J. Biol. Chem.* **284**, 10296–10300
355. Chen, Y., Potratz, J. P., Tijerina, P., Del Campo, M., Lambowitz, A. M., and Russell, R. (2008) DEAD-box proteins can completely separate an RNA duplex using a single ATP. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 20203–20208
356. Cordin, O., Banroques, J., Tanner, N. K., and Linder, P. (2006) The DEAD-box protein family of RNA helicases. *Gene*. **367**, 17–37
357. Lorsch, J. R., and Herschlag, D. (1998) The DEAD box protein eIF4A. 1. A minimal kinetic and thermodynamic framework reveals coupled binding of RNA and nucleotide. *Biochemistry (Mosc.)*. **37**, 2180–2193
358. Frick, D. N. (2006) HCV Helicase: Structure, Function, and Inhibition. in *Hepatitis C Viruses: Genomes and Molecular Biology* (Tan, S.-L. ed), Horizon Bioscience, Norfolk (UK).
359. Kim, J. L., Morgenstern, K. A., Griffith, J. P., Dwyer, M. D., Thomson, J. A., Murcko, M. A., Lin, C., and Caron, P. R. (1998) Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Struct. Lond. Engl. 1993*. **6**, 89–100
360. Beran, R. K. F., Serebrov, V., and Pyle, A. M. (2007) The serine protease domain of hepatitis C viral NS3 activates RNA helicase activity by promoting the binding of RNA substrate. *J. Biol. Chem.* **282**, 34913–34920
361. Chernov, A. V., Shiryaev, S. A., Aleshin, A. E., Ratnikov, B. I., Smith, J. W., Liddington, R. C., and Strongin, A. Y. (2008) The two-component NS2B-NS3 proteinase represses DNA unwinding activity of the West Nile virus NS3 helicase. *J. Biol. Chem.* **283**, 17270–17278
362. Fairman-Williams, M. E., and Jankowsky, E. (2012) Unwinding initiation by the viral RNA helicase NPH-II. *J. Mol. Biol.* **415**, 819–832
363. Luo, D., Wei, N., Doan, D. N., Paradkar, P. N., Chong, Y., Davidson, A. D., Kotaka, M., Lescar, J., and Vasudevan, S. G. (2010) Flexibility between the

protease and helicase domains of the dengue virus NS3 protein conferred by the linker region and its functional implications. *J. Biol. Chem.* **285**, 18817–18827
364. Pyle, A. M. (2008) Translocation and unwinding mechanisms of RNA and DNA helicases. *Annu. Rev. Biophys.* **37**, 317–336
365. Frick, D. N. (2007) The hepatitis C virus NS3 protein: a model RNA helicase and potential drug target. *Curr. Issues Mol. Biol.* **9**, 1–20
366. Jankowsky, E., Gross, C. H., Shuman, S., and Pyle, A. M. (2000) The DExH protein NPH-II is a processive and directional motor for unwinding RNA. *Nature*. **403**, 447–451
367. Kawaoka, J., and Pyle, A. M. (2005) Choosing between DNA and RNA: the polymer specificity of RNA helicase NPH-II. *Nucleic Acids Res.* **33**, 644–649
368. Kim, J. W., Seo, M. Y., Shelat, A., Kim, C. S., Kwon, T. W., Lu, H., Moustakas, D. T., Sun, J., and Han, J. H. (2003) Structurally Conserved Amino Acid W501 Is Required for RNA Helicase Activity but Is Not Essential for DNA Helicase Activity of Hepatitis C Virus NS3 Protein. *J. Virol.* **77**, 571–582
369. Raney, K. D., Sharma, S. D., Moustafa, I. M., and Cameron, C. E. (2010) Hepatitis C virus non-structural protein 3 (HCV NS3): a multifunctional antiviral target. *J. Biol. Chem.* **285**, 22725–22731
370. Beran, R. K. F., Bruno, M. M., Bowers, H. A., Jankowsky, E., and Pyle, A. M. (2006) Robust translocation along a molecular monorail: the NS3 helicase from hepatitis C virus traverses unusually large disruptions in its track. *J. Mol. Biol.* **358**, 974–982
371. Kawaoka, J., Jankowsky, E., and Pyle, A. M. (2004) Backbone tracking by the SF2 helicase NPH-II. *Nat. Struct. Mol. Biol.* **11**, 526–530
372. Appleby, T. C., Anderson, R., Fedorova, O., Pyle, A. M., Wang, R., Liu, X., Brendza, K. M., and Somoza, J. R. (2011) Visualizing ATP-dependent RNA translocation by the NS3 helicase from HCV. *J. Mol. Biol.* **405**, 1139–1153
373. Gu, M., and Rice, C. M. (2010) Three conformational snapshots of the hepatitis C virus NS3 helicase reveal a ratchet translocation mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 521–528
374. Slabinski, L., Jaroszewski, L., Rychlewski, L., Wilson, I. A., Lesley, S. A., and Godzik, A. (2007) XtalPred: a web server for prediction of protein crystallizability. *Bioinforma. Oxf. Engl.* **23**, 3403–3405
375. Chong, J. P., Hayashi, M. K., Simon, M. N., Xu, R. M., and Stillman, B. (2000) A double-hexamer archaeal minichromosome maintenance protein is an ATP-dependent DNA helicase. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1530–1535
376. Kaplan, D. L., and O'Donnell, M. (2004) Twin DNA pumps of a hexameric helicase provide power to simultaneously melt two duplexes. *Mol. Cell*. **15**, 453–465
377. Labib, K., Tercero, J. A., and Diffley, J. F. (2000) Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science*. **288**, 1643–1647
378. Hickman, A. B., and Dyda, F. (2005) Binding and unwinding: SF3 viral helicases. *Curr. Opin. Struct. Biol.* **15**, 77–85
379. Patel, S. S., and Picha, K. M. (2000) Structure and function of hexameric helicases. *Annu. Rev. Biochem.* **69**, 651–697
380. Breyer, W. A., and Matthews, B. W. (2001) A structural basis for processivity. *Protein Sci. Publ. Protein Soc.* **10**, 1699–1711
381. Davey, M. J., and O'Donnell, M. (2003) Replicative helicase loaders: ring breakers and ring makers. *Curr. Biol. CB*. **13**, R594–596

382. Singleton, M. R., Sawaya, M. R., Ellenberger, T., and Wigley, D. B. (2000) Crystal structure of T7 gene 4 ring helicase indicates a mechanism for sequential hydrolysis of nucleotides. *Cell*. **101**, 589–600
383. Yu, X., Hingorani, M. M., Patel, S. S., and Egelman, E. H. (1996) DNA is bound within the central hole to one or two of the six subunits of the T7 DNA helicase. *Nat. Struct. Biol.* **3**, 740–743
384. Abbate, E. A., Berger, J. M., and Botchan, M. R. (2004) The X-ray structure of the papillomavirus helicase in complex with its molecular matchmaker E2. *Genes Dev.* **18**, 1981–1996
385. Gorbalenya, A. E., Koonin, E. V., and Wolf, Y. I. (1990) A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. *FEBS Lett.* **262**, 145–148
386. James, J. A., Aggarwal, A. K., Linden, R. M., and Escalante, C. R. (2004) Structure of adeno-associated virus type 2 Rep40-ADP complex: insight into nucleotide recognition and catalysis by superfamily 3 helicases. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 12455–12460
387. James, J. A., Escalante, C. R., Yoon-Robarts, M., Edwards, T. A., Linden, R. M., and Aggarwal, A. K. (2003) Crystal structure of the SF3 helicase from adeno-associated virus type 2. *Struct. Lond. Engl. 1993*. **11**, 1025–1035
388. Li, D., Zhao, R., Lilyestrom, W., Gai, D., Zhang, R., DeCaprio, J. A., Fanning, E., Jochimiak, A., Szakonyi, G., and Chen, X. S. (2003) Structure of the replicative helicase of the oncoprotein SV40 large tumour antigen. *Nature*. **423**, 512–518
389. Koonin, E. V. (1993) A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. *Nucleic Acids Res.* **21**, 2541–2547
390. Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999) AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9**, 27–43
391. Ogura, T., and Wilkinson, A. J. (2001) AAA+ superfamily ATPases: common structure-diverse function. *Genes Cells*. **6**, 575–597
392. Snider, J., Thibault, G., and Houry, W. A. (2008) The AAA+ superfamily of functionally diverse proteins. *Genome Biol.* **9**, 216
393. Enemark, E. J., and Joshua-Tor, L. (2006) Mechanism of DNA translocation in a replicative hexameric helicase. *Nature*. **442**, 270–275
394. Liu, X., Schuck, S., and Stenlund, A. (2010) Structure-based mutational analysis of the bovine papillomavirus E1 helicase domain identifies residues involved in the nonspecific DNA binding activity required for double trimer formation. *J. Virol.* **84**, 4264–4276
395. Lee, S.-J., Syed, S., Enemark, E. J., Schuck, S., Stenlund, A., Ha, T., and Joshua-Tor, L. (2014) Dynamic look at DNA unwinding by a replicative helicase. *Proc. Natl. Acad. Sci. U. S. A.* 10.1073/pnas.1322254111
396. Ilyina, T. V., Gorbalenya, A. E., and Koonin, E. V. (1992) Organization and evolution of bacterial and bacteriophage primase-helicase systems. *J. Mol. Evol.* **34**, 351–357
397. Toth, E. A., Li, Y., Sawaya, M. R., Cheng, Y., and Ellenberger, T. (2003) The crystal structure of the bifunctional primase-helicase of bacteriophage T7. *Mol. Cell*. **12**, 1113–1123

398. Sawaya, M. R., Guo, S., Tabor, S., Richardson, C. C., and Ellenberger, T. (1999) Crystal structure of the helicase domain from the replicative helicase-primase of bacteriophage T7. *Cell*. **99**, 167–177
399. Niedenzu, T., Röleke, D., Bains, G., Scherzinger, E., and Saenger, W. (2001) Crystal structure of the hexameric replicative helicase RepA of plasmid RSF1010. *J. Mol. Biol.* **306**, 479–487
400. Yu, X., Jezewska, M. J., Bujalowski, W., and Egelman, E. H. (1996) The hexameric E. coli DnaB helicase can exist in different Quaternary states. *J. Mol. Biol.* **259**, 7–14
401. Skordalakes, E., and Berger, J. M. (2003) Structure of the Rho transcription terminator: mechanism of mRNA recognition and helicase loading. *Cell*. **114**, 135–146
402. Gogol, E. P., Seifried, S. E., and von Hippel, P. H. (1991) Structure and assembly of the Escherichia coli transcription termination factor rho and its interaction with RNA. I. Cryoelectron microscopic studies. *J. Mol. Biol.* **221**, 1127–1138
403. Pape, T., Meka, H., Chen, S., Vicentini, G., van Heel, M., and Onesti, S. (2003) Hexameric ring structure of the full-length archaeal MCM protein complex. *EMBO Rep.* **4**, 1079–1083
404. Fletcher, R. J., Bishop, B. E., Leon, R. P., Sclafani, R. A., Ogata, C. M., and Chen, X. S. (2003) The structure and function of MCM from archaeal M. Thermoautotrophicum. *Nat. Struct. Biol.* **10**, 160–167
405. Herschlag, D. (1995) RNA Chaperones and the RNA Folding Problem. *J. Biol. Chem.* **270**, 20871–20874
406. Jarmoskaite, I., and Russell, R. (2014) RNA helicase proteins as chaperones and remodelers. *Annu. Rev. Biochem.* **83**, 697–725
407. Rajkowitsch, L., Chen, D., Stampfl, S., Semrad, K., Waldsich, C., Mayer, O., Jantsch, M. F., Konrat, R., Bläsi, U., and Schroeder, R. (2007) RNA chaperones, RNA annealers and RNA helicases. *RNA Biol.* **4**, 118–130
408. Jankowsky, E., and Bowers, H. (2006) Remodeling of ribonucleoprotein complexes with DExH/D RNA helicases. *Nucleic Acids Res.* **34**, 4181–4188
409. Levin, M. K., Wang, Y.-H., and Patel, S. S. (2004) The functional interaction of the hepatitis C virus helicase molecules is responsible for unwinding processivity. *J. Biol. Chem.* **279**, 26005–26012
410. Patel, S. S., and Donmez, I. (2006) Mechanisms of Helicases. *J. Biol. Chem.* **281**, 18265–18268
411. Lee, N.-R., Kwon, H.-M., Park, K., Oh, S., Jeong, Y.-J., and Kim, D.-E. (2010) Cooperative translocation enhances the unwinding of duplex DNA by SARS coronavirus helicase nsP13. *Nucleic Acids Res.* **38**, 7626–7636
412. Betterton, M. D., and Jülicher, F. (2005) Opening of nucleic-acid double strands by helicases: active versus passive opening. *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.* **71**, 011904
413. Pang, P. S., Jankowsky, E., Planet, P. J., and Pyle, A. M. (2002) The hepatitis C viral NS3 protein is a processive DNA helicase with cofactor enhanced RNA unwinding. *EMBO J.* **21**, 1168–1176
414. Wong, I., and Lohman, T. M. (1992) Allosteric Effects of Nucleotide Cofactors on Escherichia coli Rep Helicase&DNA Binding. *Science*. **256**, 350–355

415. Levin, M. K., Gurjar, M., and Patel, S. S. (2005) A Brownian motor mechanism of translocation and strand separation by hepatitis C virus helicase. *Nat. Struct. Mol. Biol.* **12**, 429–435
416. Gorbalenya, A. E., and Koonin, E. V. (1989) Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Res.* **17**, 8413–8440
417. Steimer, L., and Klostermeier, D. (2012) RNA helicases in infection and disease. *RNA Biol.* **9**, 751–771
418. Ahlquist, P. (2006) Parallels among positive-strand RNA viruses, reverse-transcribing viruses and double-stranded RNA viruses. *Nat. Rev. Microbiol.* **4**, 371–382
419. Ahola, T., den Boon, J. A., and Ahlquist, P. (2000) Helicase and capping enzyme active site mutations in brome mosaic virus protein 1a cause defects in template recruitment, negative-strand RNA synthesis, and viral RNA capping. *J. Virol.* **74**, 8803–8811
420. Tai, C. L., Chi, W. K., Chen, D. S., and Hwang, L. H. (1996) The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J. Virol.* **70**, 8477–8484
421. Frick, D. N., Rypma, R. S., Lam, A. M. I., and Gu, B. (2004) The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently. *J. Biol. Chem.* **279**, 1269–1280
422. Phan, T., Kohlway, A., Dimberu, P., Pyle, A. M., and Lindenbach, B. D. (2011) The acidic domain of hepatitis C virus NS4A contributes to RNA replication and virus particle assembly. *J. Virol.* **85**, 1193–1204
423. Piccininni, S., Varaklioti, A., Nardelli, M., Dave, B., Raney, K. D., and McCarthy, J. E. G. (2002) Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. *J. Biol. Chem.* **277**, 45670–45679
424. Kohlway, A., Pirakitikulr, N., Ding, S. C., Yang, F., Luo, D., Lindenbach, B. D., and Pyle, A. M. (2014) The linker region of NS3 plays a critical role in the replication and infectivity of hepatitis C virus. *J. Virol.* **88**, 10970–10974
425. Kwofie, S. K., Schaefer, U., Sundararajan, V. S., Bajic, V. B., and Christoffels, A. (2011) HCVpro: Hepatitis C virus protein interaction database. *Infect. Genet. Evol.* **11**, 1971–1977
426. Mayer, B. J. (2001) SH3 domains: complexity in moderation. *J. Cell Sci.* **114**, 1253–1263
427. Adedeji, A. O., Marchand, B., Te Velthuis, A. J. W., Snijder, E. J., Weiss, S., Eoff, R. L., Singh, K., and Sarafianos, S. G. (2012) Mechanism of nucleic acid unwinding by SARS-CoV helicase. *PloS One*. **7**, e36521
428. Ivanov, K. A., and Ziebuhr, J. (2004) Human Coronavirus 229E Nonstructural Protein 13: Characterization of Duplex-Unwinding, Nucleoside Triphosphatase, and RNA 5′-Triphosphatase Activities. *J. Virol.* **78**, 7833–7838
429. Ivanov, K. A., Thiel, V., Dobbe, J. C., Meer, Y. van der, Snijder, E. J., and Ziebuhr, J. (2004) Multiple Enzymatic Activities Associated with Severe Acute Respiratory Syndrome Coronavirus Helicase. *J. Virol.* **78**, 5619–5632
430. Lehmann, K. C., Snijder, E. J., Posthuma, C. C., and Gorbalenya, A. E. (2014) What we know but do not understand about nidovirus helicases. *Virus Res.* 10.1016/j.virusres.2014.12.001

431. Seybert, A., Hegyi, A., Siddell, S. G., and Ziebuhr, J. (2000) The human coronavirus 229E superfamily 1 helicase has RNA and DNA duplex-unwinding activities with 5'-to-3' polarity. *RNA*. **6**, 1056–1068
432. Van Dinten, L. C., van Tol, H., Gorbalenya, A. E., and Snijder, E. J. (2000) The predicted metal-binding region of the arterivirus helicase protein is involved in subgenomic mRNA synthesis, genome replication, and virion biogenesis. *J. Virol.* **74**, 5213–5223
433. Bayliss, C. D., and Smith, G. L. (1996) Vaccinia virion protein I8R has both DNA and RNA helicase activities: implications for vaccinia virus transcription. *J. Virol.* **70**, 794–800
434. Gross, C. H., and Shuman, S. (1996) Vaccinia virions lacking the RNA helicase nucleoside triphosphate phosphohydrolase II are defective in early transcription. *J. Virol.* **70**, 8549–8557
435. Gross, C. H., and Shuman, S. (1998) The nucleoside triphosphatase and helicase activities of vaccinia virus NPH-II are essential for virus replication. *J. Virol.* **72**, 4729–4736
436. Bolinger, C., and Boris-Lawrie, K. (2009) Mechanisms employed by retroviruses to exploit host factors for translational control of a complicated proteome. *Retrovirology*. **6**, 8
437. Ishaq, M., Ma, L., Wu, X., Mu, Y., Pan, J., Hu, J., Hu, T., Fu, Q., and Guo, D. (2009) The DEAD-box RNA helicase DDX1 interacts with RelA and enhances nuclear factor kappaB-mediated transcription. *J. Cell. Biochem.* **106**, 296–305
438. Malim, M. H., Hauber, J., Le, S. Y., Maizel, J. V., and Cullen, B. R. (1989) The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature*. **338**, 254–257
439. Tanaka, K., Okamoto, S., Ishikawa, Y., Tamura, H., and Hara, T. (2009) DDX1 is required for testicular tumorigenesis, partially through the transcriptional activation of 12p stem cell genes. *Oncogene*. **28**, 2142–2151
440. Naji, S., Ambrus, G., Cimermančič, P., Reyes, J. R., Johnson, J. R., Filbrandt, R., Huber, M. D., Vesely, P., Krogan, N. J., Yates, J. R., Saphire, A. C., and Gerace, L. (2012) Host cell interactome of HIV-1 Rev includes RNA helicases involved in multiple facets of virus production. *Mol. Cell. Proteomics MCP*. **11**, M111.015313
441. Fujii, R., Okamoto, M., Aratani, S., Oishi, T., Ohshima, T., Taira, K., Baba, M., Fukamizu, A., and Nakajima, T. (2001) A Role of RNA Helicase A in cis-Acting Transactivation Response Element-mediated Transcriptional Regulation of Human Immunodeficiency Virus Type 1. *J. Biol. Chem.* **276**, 5445–5451
442. Roy, B. B., Hu, J., Guo, X., Russell, R. S., Guo, F., Kleiman, L., and Liang, C. (2006) Association of RNA Helicase A with Human Immunodeficiency Virus Type 1 Particles. *J. Biol. Chem.* **281**, 12625–12635
443. Ma, J., Rong, L., Zhou, Y., Roy, B. B., Lu, J., Abrahamyan, L., Mouland, A. J., Pan, Q., and Liang, C. (2008) The requirement of the DEAD-box protein DDX24 for the packaging of human immunodeficiency virus type 1 RNA. *Virology*. **375**, 253–264
444. Koonin, E. V. (1991) Similarities in RNA helicases. *Nature*. **352**, 290–290
445. Coffey, L. L., Beeharry, Y., Bordería, A. V., Blanc, H., and Vignuzzi, M. (2011) Arbovirus high fidelity variant loses fitness in mosquitoes and mice. *Proc. Natl. Acad. Sci.* **108**, 16038–16043

446. Gross, C. H., and Shuman, S. (1996) Vaccinia virus RNA helicase: nucleic acid specificity in duplex unwinding. *J. Virol.* **70**, 2615–2619
447. Gould, E. A., Coutard, B., Malet, H., Morin, B., Jamal, S., Weaver, S., Gorbalenya, A., Moureau, G., Baronti, C., Delogu, I., Forrester, N., Khasnatinov, M., Gritsun, T., de Lamballerie, X., and Canard, B. (2010) Understanding the alphaviruses: Recent research on important emerging pathogens and progress towards their control. *Antiviral Res.* **87**, 111–124
448. Lawitz, E., Sulkowski, M. S., Ghalib, R., Rodriguez-Torres, M., Younossi, Z. M., Corregidor, A., DeJesus, E., Pearlman, B., Rabinovitz, M., Gitlin, N., Lim, J. K., Pockros, P. J., Scott, J. D., Fevery, B., Lambrecht, T., Ouwerkerk-Mahadevan, S., Callewaert, K., Symonds, W. T., Picchio, G., Lindsay, K. L., Beumont, M., and Jacobson, I. M. (2014) Simeprevir plus sofosbuvir, with or without ribavirin, to treat chronic infection with hepatitis C virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: the COSMOS randomised study. *Lancet*. **384**, 1756–1765
449. Sanford, M. (2015) Simeprevir: A Review of Its Use in Patients with Chronic Hepatitis C Virus Infection. *Drugs*. 10.1007/s40265-014-0341-2
450. Tong, L. (2002) Viral proteases. *Chem. Rev.* **102**, 4609–4626
451. Atkinson, H. J., Babbitt, P. C., and Sajid, M. (2009) The global cysteine peptidase landscape in parasites. *Trends Parasitol.* **25**, 573–581
452. Rawlings, N. D., Barrett, A. J., and Bateman, A. (2012) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* **40**, D343–350
453. Strauss, J. ., and Strauss, E. . (1990) Alphavirus proteinases. *Semin. Virol.* **1**, 347–356
454. Thomas, S., Rai, J., John, L., Günther, S., Drosten, C., Pützer, B. M., and Schaefer, S. (2010) Functional dissection of the alphavirus capsid protease: sequence requirements for activity. *Virol. J.* **7**, 327
455. Choi, H. K., Lu, G., Lee, S., Wengler, G., and Rossmann, M. G. (1997) Structure of Semliki Forest virus core protein. *Proteins*. **27**, 345–359
456. Aggarwal, M., Dhindwal, S., Kumar, P., Kuhn, R. J., and Tomar, S. (2014) trans-Protease activity and structural insights into the active form of the alphavirus capsid protease. *J. Virol.* **88**, 12242–12253
457. Bassetto, M., De Burghgraeve, T., Delang, L., Massarotti, A., Coluccia, A., Zonta, N., Gatti, V., Colombano, G., Sorba, G., Silvestri, R., Tron, G. C., Neyts, J., Leyssen, P., and Brancale, A. (2013) Computer-aided identification, design and synthesis of a novel series of compounds with selective antiviral activity against chikungunya virus. *Antiviral Res.* **98**, 12–18
458. Bora, L. (2012) Homology Modeling and Docking to Potential Novel Inhibitor for Chikungunya (37997) Protein nsP2 Protease. *J. Proteomics Bioinform.* 10.4172/jpb.1000213
459. Jadav, S. S., Sinha, B. N., Hilgenfeld, R., Pastorino, B., de Lamballerie, X., and Jayaprakash, V. (2015) Thiazolidone derivatives as inhibitors of chikungunya virus. *Eur. J. Med. Chem.* **89**, 172–178
460. Russo, A. T., Malmstrom, R. D., White, M. A., and Watowich, S. J. (2010) Structural basis for substrate specificity of alphavirus nsP2 proteases. *J. Mol. Graph. Model.* **29**, 46–53
461. Singh, K. D., Kirubakaran, P., Nagarajan, S., Sakkiah, S., Muthusamy, K., Velmurgan, D., and Jeyakanthan, J. (2012) Homology modeling, molecular

dynamics, e-pharmacophore mapping and docking study of Chikungunya virus nsP2 protease. *J. Mol. Model.* **18**, 39–51
462. Khan, M., Santhosh, S. R., Tiwari, M., Lakshmana Rao, P. V., and Parida, M. (2010) Assessment of in vitro prophylactic and therapeutic efficacy of chloroquine against Chikungunya virus in vero cells. *J. Med. Virol.* **82**, 817–824
463. Parashar, D., and Cherian, S. (2014) Antiviral Perspectives for Chikungunya Virus. *BioMed Res. Int.* **2014**, e631642
464. Rashad, A. A., Mahalingam, S., and Keller, P. A. (2014) Chikungunya virus: emerging targets and new opportunities for medicinal chemistry. *J. Med. Chem.* **57**, 1147–1166
465. Borowski, P., Schalinski, S., and Schmitz, H. (2002) Nucleotide triphosphatase/ helicase of hepatitis C virus as a target for antiviral therapy. *Antiviral Res.* **55**, 397–412
466. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M.-Y., Pieper, U., and Sali, A. (2007) Comparative protein structure modeling using MODELLER. *Curr. Protoc. Protein Sci. Editor. Board John E Coligan Al.* **Chapter 2**, Unit 2.9
467. Kelley, L. A., and Sternberg, M. J. E. (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* **4**, 363–371
468. Zhang, Y. (2008) I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*. **9**, 40
469. Nishikiori, M., Sugiyama, S., Xiang, H., Niiyama, M., Ishibashi, K., Inoue, T., Ishikawa, M., Matsumura, H., and Katoh, E. (2012) Crystal structure of the superfamily 1 helicase from Tomato mosaic virus. *J. Virol.* **86**, 7565–7576
470. Abroi, A., and Gough, J. (2011) Are viruses a source of new protein folds for organisms? – Virosphere structure space and evolution. *BioEssays*. **33**, 626–635
471. Greenfield, N. J. (2006) Using circular dichroism spectra to estimate protein secondary structure. *Nat. Protoc.* **1**, 2876–2890
472. Kelly, S. M., and Price, N. C. (2000) The use of circular dichroism in the investigation of protein structure and function. *Curr. Protein Pept. Sci.* **1**, 349–384
473. Kelly, S. M., Jess, T. J., and Price, N. C. (2005) How to study proteins by circular dichroism. *Biochim. Biophys. Acta*. **1751**, 119–139
474. Karpe, Y. A., and Lole, K. S. (2010) NTPase and 5' to 3' RNA duplex-unwinding activities of the hepatitis E virus helicase domain. *J. Virol.* **84**, 3595–3602
475. Leshchiner, A. D., Solovyev, A. G., Morozov, S. Y., and Kalinina, N. O. (2006) A minimal region in the NTPase/helicase domain of the TGBp1 plant virus movement protein is responsible for ATPase activity and cooperative RNA binding. *J. Gen. Virol.* **87**, 3087–3095
476. Lam, A. M. I., Keeney, D., Eckert, P. Q., and Frick, D. N. (2003) Hepatitis C Virus NS3 ATPases/Helicases from Different Genotypes Exhibit Variations in Enzymatic Properties. *J. Virol.* **77**, 3950–3961
477. Simpson, D. A., and Condit, R. C. (1995) Vaccinia virus gene A18R encodes an essential DNA helicase. *J. Virol.* **69**, 6131–6139
478. Lesnik, E. A., and Freier, S. M. (1995) Relative thermodynamic stability of DNA, RNA, and DNA:RNA hybrid duplexes: relationship with base composition and structure. *Biochemistry (Mosc.)*. **34**, 10807–10815
479. Cheng, Z., Yang, J., Xia, H., Qiu, Y., Wang, Z., Han, Y., Xia, X., Qin, C.-F., Hu, Y., and Zhou, X. (2013) The nonstructural protein 2C of a Picorna-like

virus displays nucleic acid helix destabilizing activity that can be functionally separated from Its ATPase activity. *J. Virol.* **87**, 5205–5218
480. Berg, J. M. (1990) Zinc fingers and other metal-binding domains. Elements for interactions between macromolecules. *J. Biol. Chem.* **265**, 6513–6516
481. George, R. A., and Heringa, J. (2002) An analysis of protein domain linkers: their classification and role in protein folding. *Protein Eng.* **15**, 871–879
482. Yon, C., Teramoto, T., Mueller, N., Phelan, J., Ganesh, V. K., Murthy, K. H. M., and Padmanabhan, R. (2005) Modulation of the nucleoside triphosphatase/ RNA helicase and 5'-RNA triphosphatase activities of Dengue virus type 2 nonstructural protein 3 (NS3) by interaction with NS5, the RNA-dependent RNA polymerase. *J. Biol. Chem.* **280**, 27412–27419
483. Buchan, D. W. A., Minneci, F., Nugent, T. C. O., Bryson, K., and Jones, D. T. (2013) Scalable web services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Res.* **41**, W349–W357
484. Xue, B., Williams, R. W., Oldfield, C. J., Goh, G. K.-M., Dunker, A. K., and Uversky, V. N. (2010) Viral disorder or disordered viruses: do viral proteins possess unique features? *Protein Pept. Lett.* **17**, 932–951
485. Xue, B., Blocquel, D., Habchi, J., Uversky, A. V., Kurgan, L., Uversky, V. N., and Longhi, S. (2014) Structural Disorder in Viral Proteins. *Chem. Rev.* **114**, 6880–6911
486. Michel, G., Petrakova, O., Atasheva, S., and Frolov, I. (2007) Adaptation of Venezuelan equine encephalitis virus lacking 51-nt conserved sequence element to replication in mammalian and mosquito cells. *Virology*. **362**, 475–487
487. Imbert, I., Guillemot, J.-C., Bourhis, J.-M., Bussetta, C., Coutard, B., Egloff, M.-P., Ferron, F., Gorbalenya, A. E., and Canard, B. (2006) A second, non-canonical RNA-dependent RNA polymerase in SARS Coronavirus. *EMBO J.* **25**, 4933–4942
488. Sevajol, M., Subissi, L., Decroly, E., Canard, B., and Imbert, I. (2014) Insights into RNA synthesis, capping, and proofreading mechanisms of SARS-coronavirus. *Virus Res.* **194**, 90–99
489. Te Velthuis, A. J. W., van den Worm, S. H. E., and Snijder, E. J. (2012) The SARS-coronavirus nsp7+nsp8 complex is a unique multimeric RNA polymerase capable of both de novo initiation and primer extension. *Nucleic Acids Res.* **40**, 1737–1747
490. Marcotte, L. L., Wass, A. B., Gohara, D. W., Pathak, H. B., Arnold, J. J., Filman, D. J., Cameron, C. E., and Hogle, J. M. (2007) Crystal structure of poliovirus 3CD protein: virally encoded protease and precursor to the RNA-dependent RNA polymerase. *J. Virol.* **81**, 3583–3596
491. Karpe, Y. A., and Lole, K. S. (2010) RNA 5′-Triphosphatase Activity of the Hepatitis E Virus Helicase Domain. *J. Virol.* **84**, 9637–9641
492. Wang, C.-C., Huang, Z.-S., Chiang, P.-L., Chen, C.-T., and Wu, H.-N. (2009) Analysis of the nucleoside triphosphatase, RNA triphosphatase, and unwinding activities of the helicase domain of dengue virus NS3 protein. *FEBS Lett.* **583**, 691–696
493. Jankowsky, E., and Fairman-Williams, M. E. (2010) Chapter 1. An Introduction to RNA Helicases: Superfamilies, Families, and Major Themes. in *RNA Helicases* (Jankowsky, E. ed), pp. 1–31, Royal Society of Chemistry, Cambridge.

494. Lam, A. M. I., Rypma, R. S., and Frick, D. N. (2004) Enhanced nucleic acid binding to ATP-bound hepatitis C virus NS3 helicase at low pH activates RNA unwinding. *Nucleic Acids Res.* **32**, 4060–4070
495. Fernández, A., Laín, S., and García, J. A. (1995) RNA helicase activity of the plum pox potyvirus Cl protein expressed in Escherichia coli. Mapping of an RNA binding domain. *Nucleic Acids Res.* **23**, 1327–1332
496. Gros, C., and Wengler, G. (1996) Identification of an RNA-stimulated NTPase in the predicted helicase sequence of the Rubella virus nonstructural poly-protein. *Virology*. **217**, 367–372
497. Kadaré, G., David, C., and Haenni, A. L. (1996) ATPase, GTPase, and RNA binding activities associated with the 206-kilodalton protein of turnip yellow mosaic virus. *J. Virol.* **70**, 8169–8174
498. Benarroch, D., Selisko, B., Locatelli, G. A., Maga, G., Romette, J.-L., and Canard, B. (2004) The RNA helicase, nucleotide 5'-triphosphatase, and RNA 5'-triphosphatase activities of Dengue virus protein NS3 are Mg2+-dependent and require a functional Walker B motif in the helicase catalytic core. *Virology*. **328**, 208–218
499. Bhattacharya, A., Czaplinski, K., Trifillis, P., He, F., Jacobson, A., and Peltz, S. W. (2000) Characterization of the biochemical properties of the human Upf1 gene product that is involved in nonsense-mediated mRNA decay. *RNA*. **6**, 1226–1235
500. Borowski, P., Niebuhr, A., Mueller, O., Bretner, M., Felczak, K., Kulikowski, T., and Schmitz, H. (2001) Purification and characterization of West Nile virus nucleoside triphosphatase (NTPase)/helicase: evidence for dissociation of the NTPase and helicase activities of the enzyme. *J. Virol.* **75**, 3220–3229
501. Gwack, Y., Yoo, H., Song, I., Choe, J., and Han, J. H. (1999) RNA-Stimulated ATPase and RNA helicase activities and RNA binding domain of hepatitis G virus nonstructural protein 3. *J. Virol.* **73**, 2909–2915
502. Tanner, J. A., Watt, R. M., Chai, Y.-B., Lu, L.-Y., Lin, M. C., Peiris, J. S. M., Poon, L. L. M., Kung, H.-F., and Huang, J.-D. (2003) The severe acute respiratory syndrome (SARS) coronavirus NTPase/helicase belongs to a distinct class of 5' to 3' viral helicases. *J. Biol. Chem.* **278**, 39578–39582
503. Warrener, P., Tamura, J. K., and Collett, M. S. (1993) RNA-stimulated NTPase activity associated with yellow fever virus NS3 protein expressed in bacteria. *J. Virol.* **67**, 989–996
504. Shuman, S. (1992) Vaccinia virus RNA helicase: an essential enzyme related to the DE-H family of RNA-dependent NTPases. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10935–10939
505. Taylor, S. D., Solem, A., Kawaoka, J., and Pyle, A. M. (2010) The NPH-II helicase displays efficient DNA x RNA helicase activity and a pronounced purine sequence bias. *J. Biol. Chem.* **285**, 11692–11703
506. Schwartz, M., Chen, J., Janda, M., Sullivan, M., den Boon, J., and Ahlquist, P. (2002) A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol. Cell*. **9**, 505–514
507. Epshtein, V., Kamarthapu, V., McGary, K., Svetlov, V., Ueberheide, B., Proshkin, S., Mironov, A., and Nudler, E. (2014) UvrD facilitates DNA repair by pulling RNA polymerase backwards. *Nature*. **505**, 372–377
508. Kulczyk, A. W., Akabayov, B., Lee, S.-J., Bostina, M., Berkowitz, S. A., and Richardson, C. C. (2012) An interaction between DNA polymerase and helicase

is essential for the high processivity of the bacteriophage T7 replisome. *J. Biol. Chem.* **287**, 39050–39060

509. Manosas, M., Spiering, M. M., Ding, F., Croquette, V., and Benkovic, S. J. (2012) Collaborative coupling between polymerase and helicase for leading-strand synthesis. *Nucleic Acids Res.* **40**, 6187–6198

510. Spacciapoli, P., and Nossal, N. G. (1994) Interaction of DNA polymerase and DNA helicase within the bacteriophage T4 DNA replication complex. Leading strand synthesis by the T4 DNA polymerase mutant A737V (tsL141) requires the T4 gene 59 helicase assembly protein. *J. Biol. Chem.* **269**, 447–455

511. Zhang, H., Lee, S.-J., Zhu, B., Tran, N. Q., Tabor, S., and Richardson, C. C. (2011) Helicase-DNA polymerase interaction is critical to initiate leading-strand DNA synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 9372–9377

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor Prof Andres Merits for accepting me as a PhD student in his laboratory and giving me the very essential freedom which made my day to day scientific endeavor enjoyable and tireless. I still do remember the magnitude of email exchanges and paper works you carried out to bring me here. I would like to convey my deepest appreciation to Aleksei Lulla from whom I learned the prowess of protein biochemistry and especial thank for not being a spoon feeder then. I would like to express gratitude to Prof Juhan Sedman for taking his valuable time to critically review this thesis. I would like to acknowledge all the co-authors of my articles around the world, some of whom I haven't yet met but many thanks for being cooperative and recognizing. All the alumni and present members of Merits group are commended for their salient scientific work which cumulated and influenced this thesis. I would like to specially thank Galina Halus for providing apropos and effective technical support during the progress of this work. I am indebted to all the workers of the fourth floor of TUIT for being always so welcoming and accommodative. I would like to acknowledge the members of doctoral commission for keeping a rigorous track on my work progress on a yearly basis. I would like to extend my deepest esteems for Prof Arvi Freiburg and Dr Margus Rätsep from Biophysics and Plant Physiology unit for letting me to use the CD spectroscopic facility in their laboratory on a recurring basis. I would like to acknowledge Merike and Inge for excellent administrative assistance. I would like to thank Piret Pumm and Kadri Orula of International Student Service of University of Tartu for their help and assistance. The financial support from European Union Social Fund through Activity 4 of the Doctoral Studies and Internationalization Program (DoRa) through the Archimedes foundation and travel grants from the Graduate School in Biomedicine and Biotechnology is deeply acknowledged. I extend special greetings to University of Tartu for providing me with opportunities to use its scientific infrastructure and noetic resources. All the instructors of my course-works are remembered for teaching me the unexplored however important directories of science. I would like to thank all the past and present friends from the Raatuse International dormitory from all over the world for all the nice time and talks. I would like to dhanyabaad my friends Gyanesh, Chandana and Nuppudu for all the nice scientific and non-scientific talks and chokha parties in desi bhati in the bidesi place☺. I would like to thank my Estonian friends for encouraging me to take dips in the ice hole in the winter times which is a moment to cheer forever. I would like to thank Kadi for tolerating my extremely boring experimental talks and understanding why we may not able to get kilograms amount of purified protein from supermarket☺. I would also like to thank Estonia and the lovely city of Tartu or as they say the "city of good thoughts" for providing me with such a friendly and tranquil habitat to carry out my research work. I express deepest gratitude to my friends, teachers, lecturers who inspired and stood by me to pursue a carrier of curiosity and adventure. I would like to convey my heartfelt respect to my mother and

late father for their graceful blessings and unconditional love which supported me all the way through. I consider that the beginning of next scientific journey would be exciting and challenging so I will continue to feel the gravity of the words of great Greek philosopher that *"The only true wisdom is in knowing you know nothing"*.

Pratyush Kumar Das
Tartu, 15.06.2015

CURRICULUM VITAE

Name: Pratyush Kumar Das
Date of Birth: 20.11.1985
Citizenship: Indian
Email: pratyushdas@hotmail.com
Address: Institute of Technology, Nooruse 1-435, Tartu, 50411, Estonia

Education and professional employment

2010–	PhD Student in Gene Technology, Institute of Technology, University of Tartu, Estonia.
2008–2010	Research Assistant (Virology), Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, India.
2006–2008	Master of Science in Biotechnology, (First class first) Fakir Mohan University, Balasore, India.
2003–2006	Bachelor of Science with Botany Honours (First Class) Utkal University, Bhubaneswar, India.
2001–2003	Intermediate of Science, CHSE Board, Bhubaneswar, India.
2001	High School, BSE Board, Cuttack, India.

Research Interests:
Biochemical Virology

Publications

I. Rathore, A.P.S., Haystead, T., **Das, P.K.**, Merits, A., Ng, M.-L., and Vasudevan, S.G. (2013). Chikungunya virus nsP3 & nsP4 interacts with HSP-90 to promote virus replication: HSP-90 inhibitors reduce CHIKV infection and inflammation in vivo. Antiviral Res. *103C*, 7–16.
II. **Das, P.K**., Merits, A., and Lulla, A. (2014). Functional Crosstalk between Distant Domains of Chikungunya Virus Non-Structural Protein 2 Is Decisive For Its RNA-Modulating Activity. J. Biol. Chem. jbc.M113.503433.
III. Utt, A., **Das, P.K.**, Varjak, M., Lulla, V., Lulla, A., and Merits, A. (2015). Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2. J. Virol. 89, 3145–3162.

Awards and Honours

2013	Harden Bursary to participate in EMBO Conference – Helicases and nucleic acid translocases Co-sponsored by the Harden Conferences at University of Cambridge, UK.
2010	Dora full time Doctoral Scholarship, Archimedes Foundation, Estonia
2008	Gold Medal, for standing first class first among Masters of Biotechnology

ELULOOKIRJELDUS

Nimi: Pratyush Kumar Das
Sünniaeg: 20.11.1985
Kodakondsus: India
Aadress: TÜ Tehnoloogiainstituut, Nooruse 1-435, Tartu, 50411, Eesti
E-post: pratyushdas@hotmail.com

Akadeemiline elukäik:

2010–	Doktoriõpe geenitehnoloogia erialal Tartu Ülikooli Tehnoloogiainsittuudis.
2008–2010	Teadusassitent (viroloogia), Pediaatria õppetool, All India Institute of Medical Sciences, New Delhi, India.
2006–2008	Biotehnoloogia magistrikraad (M.Sc.) kiitusega, Fakir Mohan Ülikool, Balasore, India.
2003–2006	Botaanika bakalaureusekraad (B.Sc.) kiitusega, Utkali Ülikool, Bhubaneswar, India.
2001–2003	Gümnaasium, CHSE Board, Bhubaneswar, India.
2001	Keskkool, BSE Board, Cuttack, India

Peamised uurimisvaldkonnad:
Virolooga, biokeemia

Avaldatud teadusartiklid:

I. Rathore, A.P.S., Haystead, T., **Das, P.K.**, Merits, A., Ng, M.-L., and Vasudevan, S.G. (2013). Chikungunya virus nsP3 & nsP4 interacts with HSP-90 to promote virus replication: HSP-90 inhibitors reduce CHIKV infection and inflammation in vivo. Antiviral Res. 103C, 7–16.

II. **Das, P.K**., Merits, A., and Lulla, A. (2014). Functional Crosstalk between Distant Domains of Chikungunya Virus Non-Structural Protein 2 Is Decisive For Its RNA-Modulating Activity. J. Biol. Chem. jbc.M113.503433.

III. Utt, A., **Das, P.K.**, Varjak, M., Lulla, V., Lulla, A., and Merits, A. (2015). Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2. J. Virol. 89, 3145–3162.

Tunnustused ja auhinnad:

2013	Reisistipendium EMBO konverentsile “Helicases and nucleic acid translocases”, *Cambridge'i Ülikool,* UK.
2010	Eesti Teadusfondi DoRa doktoriõppe stipendiaat.
2008	Lõpetanud ülikooli kuldmedaliga: Biotehnoloogia magistrikraad.

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

1. **Toivo Maimets**. Studies of human oncoprotein p53. Tartu, 1991, 96 p.
2. **Enn K. Seppet**. Thyroid state control over energy metabolism, ion transport and contractile functions in rat heart. Tartu, 1991, 135 p.
3. **Kristjan Zobel**. Epifüütsete makrosamblike väärtus õhu saastuse indikaatoritena Hamar-Dobani boreaalsetes mägimetsades. Tartu, 1992, 131 lk.
4. **Andres Mäe**. Conjugal mobilization of catabolic plasmids by transposable elements in helper plasmids. Tartu, 1992, 91 p.
5. **Maia Kivisaar**. Studies on phenol degradation genes of *Pseudomonas* sp. strain EST 1001. Tartu, 1992, 61 p.
6. **Allan Nurk**. Nucleotide sequences of phenol degradative genes from *Pseudomonas sp.* strain EST 1001 and their transcriptional activation in *Pseudomonas putida.* Tartu, 1992, 72 p.
7. **Ülo Tamm**. The genus *Populus* L. in Estonia: variation of the species biology and introduction. Tartu, 1993, 91 p.
8. **Jaanus Remme**. Studies on the peptidyltransferase centre of the *E.coli* ribosome. Tartu, 1993, 68 p.
9. **Ülo Langel**. Galanin and galanin antagonists. Tartu, 1993, 97 p.
10. **Arvo Käärd**. The development of an automatic online dynamic fluorescense-based pH-dependent fiber optic penicillin flowthrought biosensor for the control of the benzylpenicillin hydrolysis. Tartu, 1993, 117 p.
11. **Lilian Järvekülg**. Antigenic analysis and development of sensitive immunoassay for potato viruses. Tartu, 1993, 147 p.
12. **Jaak Palumets**. Analysis of phytomass partition in Norway spruce. Tartu, 1993, 47 p.
13. **Arne Sellin**. Variation in hydraulic architecture of *Picea abies* (L.) Karst. trees grown under different enviromental conditions. Tartu, 1994, 119 p.
13. **Mati Reeben**. Regulation of light neurofilament gene expression. Tartu, 1994, 108 p.
14. **Urmas Tartes**. Respiration rhytms in insects. Tartu, 1995, 109 p.
15. **Ülo Puurand.** The complete nucleotide sequence and infections *in vitro* transcripts from cloned cDNA of a potato A potyvirus. Tartu, 1995, 96 p.
16. **Peeter Hõrak**. Pathways of selection in avian reproduction: a functional framework and its application in the population study of the great tit (*Parus major*). Tartu, 1995, 118 p.
17. **Erkki Truve**. Studies on specific and broad spectrum virus resistance in transgenic plants. Tartu, 1996, 158 p.
18. **Illar Pata**. Cloning and characterization of human and mouse ribosomal protein S6-encoding genes. Tartu, 1996, 60 p.
19. **Ülo Niinemets**. Importance of structural features of leaves and canopy in determining species shade-tolerance in temperature deciduous woody taxa. Tartu, 1996, 150 p.

20. **Ants Kurg**. Bovine leukemia virus: molecular studies on the packaging region and DNA diagnostics in cattle. Tartu, 1996, 104 p.
21. **Ene Ustav**. E2 as the modulator of the BPV1 DNA replication. Tartu, 1996, 100 p.
22. **Aksel Soosaar**. Role of helix-loop-helix and nuclear hormone receptor transcription factors in neurogenesis. Tartu, 1996, 109 p.
23. **Maido Remm**. Human papillomavirus type 18: replication, transformation and gene expression. Tartu, 1997, 117 p.
24. **Tiiu Kull**. Population dynamics in *Cypripedium calceolus* L. Tartu, 1997, 124 p.
25. **Kalle Olli**. Evolutionary life-strategies of autotrophic planktonic microorganisms in the Baltic Sea. Tartu, 1997, 180 p.
26. **Meelis Pärtel**. Species diversity and community dynamics in calcareous grassland communities in Western Estonia. Tartu, 1997, 124 p.
27. **Malle Leht**. The Genus *Potentilla* L. in Estonia, Latvia and Lithuania: distribution, morphology and taxonomy. Tartu, 1997, 186 p.
28. **Tanel Tenson**. Ribosomes, peptides and antibiotic resistance. Tartu, 1997, 80 p.
29. **Arvo Tuvikene**. Assessment of inland water pollution using biomarker responses in fish *in vivo* and *in vitro*. Tartu, 1997, 160 p.
30. **Urmas Saarma**. Tuning ribosomal elongation cycle by mutagenesis of 23S rRNA. Tartu, 1997, 134 p.
31. **Henn Ojaveer**. Composition and dynamics of fish stocks in the gulf of Riga ecosystem. Tartu, 1997, 138 p.
32. **Lembi Lõugas**. Post-glacial development of vertebrate fauna in Estonian water bodies. Tartu, 1997, 138 p.
33. **Margus Pooga**. Cell penetrating peptide, transportan, and its predecessors, galanin-based chimeric peptides. Tartu, 1998, 110 p.
34. **Andres Saag**. Evolutionary relationships in some cetrarioid genera (Lichenized Ascomycota). Tartu, 1998, 196 p.
35. **Aivar Liiv**. Ribosomal large subunit assembly *in vivo*. Tartu, 1998, 158 p.
36. **Tatjana Oja**. Isoenzyme diversity and phylogenetic affinities among the eurasian annual bromes (*Bromus* L., Poaceae). Tartu, 1998, 92 p.
37. **Mari Moora**. The influence of arbuscular mycorrhizal (AM) symbiosis on the competition and coexistence of calcareous grassland plant species. Tartu, 1998, 78 p.
38. **Olavi Kurina**. Fungus gnats in Estonia (*Diptera: Bolitophilidae, Keroplatidae, Macroceridae, Ditomyiidae, Diadocidiidae, Mycetophilidae*). Tartu, 1998, 200 p.
39. **Andrus Tasa**. Biological leaching of shales: black shale and oil shale. Tartu, 1998, 98 p.
40. **Arnold Kristjuhan.** Studies on transcriptional activator properties of tumor suppressor protein p53. Tartu, 1998, 86 p.

41. **Sulev Ingerpuu.** Characterization of some human myeloid cell surface and nuclear differentiation antigens. Tartu, 1998, 163 p.
42. **Veljo Kisand.** Responses of planktonic bacteria to the abiotic and biotic factors in the shallow lake Võrtsjärv. Tartu, 1998, 118 p.
43. **Kadri Põldmaa.** Studies in the systematics of hypomyces and allied genera (Hypocreales, Ascomycota). Tartu, 1998, 178 p.
44. **Markus Vetemaa.** Reproduction parameters of fish as indicators in environmental monitoring. Tartu, 1998, 117 p.
45. **Heli Talvik.** Prepatent periods and species composition of different *Oesophagostomum* spp. populations in Estonia and Denmark. Tartu, 1998, 104 p.
46. **Katrin Heinsoo.** Cuticular and stomatal antechamber conductance to water vapour diffusion in *Picea abies* (L.) karst. Tartu, 1999, 133 p.
47. **Tarmo Annilo.** Studies on mammalian ribosomal protein S7. Tartu, 1998, 77 p.
48. **Indrek Ots.** Health state indicies of reproducing great tits (*Parus major*): sources of variation and connections with life-history traits. Tartu, 1999, 117 p.
49. **Juan Jose Cantero.** Plant community diversity and habitat relationships in central Argentina grasslands. Tartu, 1999, 161 p.
50. **Rein Kalamees.** Seed bank, seed rain and community regeneration in Estonian calcareous grasslands. Tartu, 1999, 107 p.
51. **Sulev Kõks.** Cholecystokinin (CCK) — induced anxiety in rats: influence of environmental stimuli and involvement of endopioid mechanisms and serotonin. Tartu, 1999, 123 p.
52. **Ebe Sild.** Impact of increasing concentrations of O_3 and CO_2 on wheat, clover and pasture. Tartu, 1999, 123 p.
53. **Ljudmilla Timofejeva.** Electron microscopical analysis of the synaptonemal complex formation in cereals. Tartu, 1999, 99 p.
54. **Andres Valkna.** Interactions of galanin receptor with ligands and G-proteins: studies with synthetic peptides. Tartu, 1999, 103 p.
55. **Taavi Virro.** Life cycles of planktonic rotifers in lake Peipsi. Tartu, 1999, 101 p.
56. **Ana Rebane.** Mammalian ribosomal protein S3a genes and intron-encoded small nucleolar RNAs U73 and U82. Tartu, 1999, 85 p.
57. **Tiina Tamm.** Cocksfoot mottle virus: the genome organisation and translational strategies. Tartu, 2000, 101 p.
58. **Reet Kurg.** Structure-function relationship of the bovine papilloma virus E2 protein. Tartu, 2000, 89 p.
59. **Toomas Kivisild.** The origins of Southern and Western Eurasian populations: an mtDNA study. Tartu, 2000, 121 p.
60. **Niilo Kaldalu.** Studies of the TOL plasmid transcription factor XylS. Tartu 2000. 88 p.

61. **Dina Lepik.** Modulation of viral DNA replication by tumor suppressor protein p53. Tartu 2000. 106 p.
62. **Kai Vellak.** Influence of different factors on the diversity of the bryophyte vegetation in forest and wooded meadow communities. Tartu 2000. 122 p.
63. **Jonne Kotta.** Impact of eutrophication and biological invasionas on the structure and functions of benthic macrofauna. Tartu 2000. 160 p.
64. **Georg Martin.** Phytobenthic communities of the Gulf of Riga and the inner sea the West-Estonian archipelago. Tartu, 2000. 139 p.
65. **Silvia Sepp.** Morphological and genetical variation of *Alchemilla L.* in Estonia. Tartu, 2000. 124 p.
66. **Jaan Liira.** On the determinants of structure and diversity in herbaceous plant communities. Tartu, 2000. 96 p.
67. **Priit Zingel.** The role of planktonic ciliates in lake ecosystems. Tartu 2001. 111 p.
68. **Tiit Teder.** Direct and indirect effects in Host-parasitoid interactions: ecological and evolutionary consequences. Tartu 2001. 122 p.
69. **Hannes Kollist.** Leaf apoplastic ascorbate as ozone scavenger and its transport across the plasma membrane. Tartu 2001. 80 p.
70. **Reet Marits.** Role of two-component regulator system PehR-PehS and extracellular protease PrtW in virulence of *Erwinia Carotovora* subsp. *Carotovora*. Tartu 2001. 112 p.
71. **Vallo Tilgar.** Effect of calcium supplementation on reproductive performance of the pied flycatcher *Ficedula hypoleuca* and the great tit *Parus major,* breeding in Nothern temperate forests. Tartu, 2002. 126 p.
72. **Rita Hõrak.** Regulation of transposition of transposon Tn*4652* in *Pseudomonas putida*. Tartu, 2002. 108 p.
73. **Liina Eek-Piirsoo.** The effect of fertilization, mowing and additional illumination on the structure of a species-rich grassland community. Tartu, 2002. 74 p.
74. **Krõõt Aasamaa.** Shoot hydraulic conductance and stomatal conductance of six temperate deciduous tree species. Tartu, 2002. 110 p.
75. **Nele Ingerpuu.** Bryophyte diversity and vascular plants. Tartu, 2002. 112 p.
76. **Neeme Tõnisson.** Mutation detection by primer extension on oligonucleotide microarrays. Tartu, 2002. 124 p.
77. **Margus Pensa.** Variation in needle retention of Scots pine in relation to leaf morphology, nitrogen conservation and tree age. Tartu, 2003. 110 p.
78. **Asko Lõhmus.** Habitat preferences and quality for birds of prey: from principles to applications. Tartu, 2003. 168 p.
79. **Viljar Jaks.** p53 — a switch in cellular circuit. Tartu, 2003. 160 p.
80. **Jaana Männik.** Characterization and genetic studies of four ATP-binding cassette (ABC) transporters. Tartu, 2003. 140 p.
81. **Marek Sammul.** Competition and coexistence of clonal plants in relation to productivity. Tartu, 2003. 159 p

82. **Ivar Ilves.** Virus-cell interactions in the replication cycle of bovine papillomavirus type 1. Tartu, 2003. 89 p.
83. **Andres Männik.** Design and characterization of a novel vector system based on the stable replicator of bovine papillomavirus type 1. Tartu, 2003. 109 p.
84. **Ivika Ostonen.** Fine root structure, dynamics and proportion in net primary production of Norway spruce forest ecosystem in relation to site conditions. Tartu, 2003. 158 p.
85. **Gudrun Veldre.** Somatic status of 12–15-year-old Tartu schoolchildren. Tartu, 2003. 199 p.
86. **Ülo Väli.** The greater spotted eagle *Aquila clanga* and the lesser spotted eagle *A. pomarina*: taxonomy, phylogeography and ecology. Tartu, 2004. 159 p.
87. **Aare Abroi.** The determinants for the native activities of the bovine papillomavirus type 1 E2 protein are separable. Tartu, 2004. 135 p.
88. **Tiina Kahre.** Cystic fibrosis in Estonia. Tartu, 2004. 116 p.
89. **Helen Orav-Kotta.** Habitat choice and feeding activity of benthic suspension feeders and mesograzers in the northern Baltic Sea. Tartu, 2004. 117 p.
90. **Maarja Öpik.** Diversity of arbuscular mycorrhizal fungi in the roots of perennial plants and their effect on plant performance. Tartu, 2004. 175 p.
91. **Kadri Tali.** Species structure of *Neotinea ustulata*. Tartu, 2004. 109 p.
92. **Kristiina Tambets.** Towards the understanding of post-glacial spread of human mitochondrial DNA haplogroups in Europe and beyond: a phylogeographic approach. Tartu, 2004. 163 p.
93. **Arvi Jõers.** Regulation of p53-dependent transcription. Tartu, 2004. 103 p.
94. **Lilian Kadaja.** Studies on modulation of the activity of tumor suppressor protein p53. Tartu, 2004. 103 p.
95. **Jaak Truu.** Oil shale industry wastewater: impact on river microbial community and possibilities for bioremediation. Tartu, 2004. 128 p.
96. **Maire Peters.** Natural horizontal transfer of the *pheBA* operon. Tartu, 2004. 105 p.
97. **Ülo Maiväli.** Studies on the structure-function relationship of the bacterial ribosome. Tartu, 2004. 130 p.
98. **Merit Otsus.** Plant community regeneration and species diversity in dry calcareous grasslands. Tartu, 2004. 103 p.
99. **Mikk Heidemaa.** Systematic studies on sawflies of the genera *Dolerus, Empria,* and *Caliroa* (Hymenoptera: Tenthredinidae). Tartu, 2004. 167 p.
100. **Ilmar Tõnno.** The impact of nitrogen and phosphorus concentration and N/P ratio on cyanobacterial dominance and N_2 fixation in some Estonian lakes. Tartu, 2004. 111 p.
101. **Lauri Saks.** Immune function, parasites, and carotenoid-based ornaments in greenfinches. Tartu, 2004. 144 p.
102. **Siiri Rootsi.** Human Y-chromosomal variation in European populations. Tartu, 2004. 142 p.

103. **Eve Vedler.** Structure of the 2,4-dichloro-phenoxyacetic acid-degradative plasmid pEST4011. Tartu, 2005. 106 p.
104. **Andres Tover.** Regulation of transcription of the phenol degradation *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 126 p.
105. **Helen Udras.** Hexose kinases and glucose transport in the yeast *Hansenula polymorpha*. Tartu, 2005. 100 p.
106. **Ave Suija.** Lichens and lichenicolous fungi in Estonia: diversity, distribution patterns, taxonomy. Tartu, 2005. 162 p.
107. **Piret Lõhmus.** Forest lichens and their substrata in Estonia. Tartu, 2005. 162 p.
108. **Inga Lips.** Abiotic factors controlling the cyanobacterial bloom occurrence in the Gulf of Finland. Tartu, 2005. 156 p.
109. **Kaasik, Krista.** Circadian clock genes in mammalian clockwork, metabolism and behaviour. Tartu, 2005. 121 p.
110. **Juhan Javoiš.** The effects of experience on host acceptance in ovipositing moths. Tartu, 2005. 112 p.
111. **Tiina Sedman.** Characterization of the yeast *Saccharomyces cerevisiae* mitochondrial DNA helicase Hmi1. Tartu, 2005. 103 p.
112. **Ruth Aguraiuja.** Hawaiian endemic fern lineage *Diellia* (Aspleniaceae): distribution, population structure and ecology. Tartu, 2005. 112 p.
113. **Riho Teras.** Regulation of transcription from the fusion promoters generated by transposition of Tn*4652* into the upstream region of *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 106 p.
114. **Mait Metspalu.** Through the course of prehistory in india: tracing the mtDNA trail. Tartu, 2005. 138 p.
115. **Elin Lõhmussaar.** The comparative patterns of linkage disequilibrium in European populations and its implication for genetic association studies. Tartu, 2006. 124 p.
116. **Priit Kupper.** Hydraulic and environmental limitations to leaf water relations in trees with respect to canopy position. Tartu, 2006. 126 p.
117. **Heili Ilves.** Stress-induced transposition of Tn*4652* in *Pseudomonas Putida.* Tartu, 2006. 120 p.
118. **Silja Kuusk.** Biochemical properties of Hmi1p, a DNA helicase from *Saccharomyces cerevisiae* mitochondria. Tartu, 2006. 126 p.
119. **Kersti Püssa.** Forest edges on medium resolution landsat thematic mapper satellite images. Tartu, 2006. 90 p.
120. **Lea Tummeleht.** Physiological condition and immune function in great tits (*Parus major* l.): Sources of variation and trade-offs in relation to growth. Tartu, 2006. 94 p.
121. **Toomas Esperk.** Larval instar as a key element of insect growth schedules. Tartu, 2006. 186 p.
122. **Harri Valdmann.** Lynx (*Lynx lynx*) and wolf (*Canis lupus*) in the Baltic region: Diets, helminth parasites and genetic variation. Tartu, 2006. 102 p.

123. **Priit Jõers.** Studies of the mitochondrial helicase Hmi1p in *Candida albicans* and *Saccharomyces cerevisia*. Tartu, 2006. 113 p.
124. **Kersti Lilleväli.** Gata3 and Gata2 in inner ear development. Tartu, 2007. 123 p.
125. **Kai Rünk.** Comparative ecology of three fern species: *Dryopteris carthusiana* (Vill.) H.P. Fuchs, *D. expansa* (C. Presl) Fraser-Jenkins & Jermy and *D. dilatata* (Hoffm.) A. Gray (Dryopteridaceae). Tartu, 2007. 143 p.
126. **Aveliina Helm.** Formation and persistence of dry grassland diversity: role of human history and landscape structure. Tartu, 2007. 89 p.
127. **Leho Tedersoo.** Ectomycorrhizal fungi: diversity and community structure in Estonia, Seychelles and Australia. Tartu, 2007. 233 p.
128. **Marko Mägi.** The habitat-related variation of reproductive performance of great tits in a deciduous-coniferous forest mosaic: looking for causes and consequences. Tartu, 2007. 135 p.
129. **Valeria Lulla.** Replication strategies and applications of Semliki Forest virus. Tartu, 2007. 109 p.
130. **Ülle Reier**. Estonian threatened vascular plant species: causes of rarity and conservation. Tartu, 2007. 79 p.
131. **Inga Jüriado**. Diversity of lichen species in Estonia: influence of regional and local factors. Tartu, 2007. 171 p.
132. **Tatjana Krama.** Mobbing behaviour in birds: costs and reciprocity based cooperation. Tartu, 2007. 112 p.
133. **Signe Saumaa.** The role of DNA mismatch repair and oxidative DNA damage defense systems in avoidance of stationary phase mutations in *Pseudomonas putida.* Tartu, 2007. 172 p.
134. **Reedik Mägi**. The linkage disequilibrium and the selection of genetic markers for association studies in european populations. Tartu, 2007. 96 p.
135. **Priit Kilgas.** Blood parameters as indicators of physiological condition and skeletal development in great tits (*Parus major*): natural variation and application in the reproductive ecology of birds. Tartu, 2007. 129 p.
136. **Anu Albert**. The role of water salinity in structuring eastern Baltic coastal fish communities. Tartu, 2007. 95 p.
137. **Kärt Padari.** Protein transduction mechanisms of transportans. Tartu, 2008. 128 p.
138. **Siiri-Lii Sandre.** Selective forces on larval colouration in a moth. Tartu, 2008. 125 p.
139. **Ülle Jõgar.** Conservation and restoration of semi-natural floodplain meadows and their rare plant species. Tartu, 2008. 99 p.
140. **Lauri Laanisto.** Macroecological approach in vegetation science: generality of ecological relationships at the global scale. Tartu, 2008. 133 p.
141. **Reidar Andreson**. Methods and software for predicting PCR failure rate in large genomes. Tartu, 2008. 105 p.
142. **Birgot Paavel.** Bio-optical properties of turbid lakes. Tartu, 2008. 175 p.

143. **Kaire Torn.** Distribution and ecology of charophytes in the Baltic Sea. Tartu, 2008, 98 p.
144. **Vladimir Vimberg.** Peptide mediated macrolide resistance. Tartu, 2008, 190 p.
145. **Daima Örd.** Studies on the stress-inducible pseudokinase TRB3, a novel inhibitor of transcription factor ATF4. Tartu, 2008, 108 p.
146. **Lauri Saag.** Taxonomic and ecologic problems in the genus *Lepraria* (*Stereocaulaceae*, lichenised *Ascomycota*). Tartu, 2008, 175 p.
147. **Ulvi Karu.** Antioxidant protection, carotenoids and coccidians in greenfinches – assessment of the costs of immune activation and mechanisms of parasite resistance in a passerine with carotenoid-based ornaments. Tartu, 2008, 124 p.
148. **Jaanus Remm.** Tree-cavities in forests: density, characteristics and occupancy by animals. Tartu, 2008, 128 p.
149. **Epp Moks.** Tapeworm parasites *Echinococcus multilocularis* and *E. granulosus* in Estonia: phylogenetic relationships and occurrence in wild carnivores and ungulates. Tartu, 2008, 82 p.
150. **Eve Eensalu.** Acclimation of stomatal structure and function in tree canopy: effect of light and CO_2 concentration. Tartu, 2008, 108 p.
151. **Janne Pullat**. Design, functionlization and application of an *in situ* synthesized oligonucleotide microarray. Tartu, 2008, 108 p.
152. **Marta Putrinš.** Responses of *Pseudomonas putida* to phenol-induced metabolic and stress signals. Tartu, 2008, 142 p.
153. **Marina Semtšenko.** Plant root behaviour: responses to neighbours and physical obstructions. Tartu, 2008, 106 p.
154. **Marge Starast.** Influence of cultivation techniques on productivity and fruit quality of some *Vaccinium* and *Rubus* taxa. Tartu, 2008, 154 p.
155. **Age Tats.** Sequence motifs influencing the efficiency of translation. Tartu, 2009, 104 p.
156. **Radi Tegova.** The role of specialized DNA polymerases in mutagenesis in *Pseudomonas putida.* Tartu, 2009, 124 p.
157. **Tsipe Aavik.** Plant species richness, composition and functional trait pattern in agricultural landscapes – the role of land use intensity and landscape structure. Tartu, 2009, 112 p.
158. **Kaja Kiiver.** Semliki forest virus based vectors and cell lines for studying the replication and interactions of alphaviruses and hepaciviruses. Tartu, 2009, 104 p.
159. **Meelis Kadaja.** Papillomavirus Replication Machinery Induces Genomic Instability in its Host Cell. Tartu, 2009, 126 p.
160. **Pille Hallast.** Human and chimpanzee Luteinizing hormone/Chorionic Gonadotropin beta (*LHB/CGB*) gene clusters: diversity and divergence of young duplicated genes. Tartu, 2009, 168 p.
161. **Ain Vellak.** Spatial and temporal aspects of plant species conservation. Tartu, 2009, 86 p.

162. **Triinu Remmel.** Body size evolution in insects with different colouration strategies: the role of predation risk. Tartu, 2009, 168 p.
163. **Jaana Salujõe.** Zooplankton as the indicator of ecological quality and fish predation in lake ecosystems. Tartu, 2009, 129 p.
164. **Ele Vahtmäe.** Mapping benthic habitat with remote sensing in optically complex coastal environments. Tartu, 2009, 109 p.
165. **Liisa Metsamaa.** Model-based assessment to improve the use of remote sensing in recognition and quantitative mapping of cyanobacteria. Tartu, 2009, 114 p.
166. **Pille Säälik.** The role of endocytosis in the protein transduction by cell-penetrating peptides. Tartu, 2009, 155 p.
167. **Lauri Peil.** Ribosome assembly factors in *Escherichia coli.* Tartu, 2009, 147 p.
168. **Lea Hallik.** Generality and specificity in light harvesting, carbon gain capacity and shade tolerance among plant functional groups. Tartu, 2009, 99 p.
169. **Mariliis Tark.** Mutagenic potential of DNA damage repair and tolerance mechanisms under starvation stress. Tartu, 2009, 191 p.
170. **Riinu Rannap.** Impacts of habitat loss and restoration on amphibian populations. Tartu, 2009, 117 p.
171. **Maarja Adojaan.** Molecular variation of HIV-1 and the use of this knowledge in vaccine development. Tartu, 2009, 95 p.
172. **Signe Altmäe.** Genomics and transcriptomics of human induced ovarian folliculogenesis. Tartu, 2010, 179 p.
173. **Triin Suvi.** Mycorrhizal fungi of native and introduced trees in the Seychelles Islands. Tartu, 2010, 107 p.
174. **Velda Lauringson.** Role of suspension feeding in a brackish-water coastal sea. Tartu, 2010, 123 p.
175. **Eero Talts.** Photosynthetic cyclic electron transport – measurement and variably proton-coupled mechanism. Tartu, 2010, 121 p.
176. **Mari Nelis.** Genetic structure of the Estonian population and genetic distance from other populations of European descent. Tartu, 2010, 97 p.
177. **Kaarel Krjutškov.** Arrayed Primer Extension-2 as a multiplex PCR-based method for nucleic acid variation analysis: method and applications. Tartu, 2010, 129 p.
178. **Egle Köster.** Morphological and genetical variation within species complexes: *Anthyllis vulneraria* s. l. and *Alchemilla vulgaris* (coll.). Tartu, 2010, 101 p.
179. **Erki Õunap.** Systematic studies on the subfamily Sterrhinae (Lepidoptera: Geometridae). Tartu, 2010, 111 p.
180. **Merike Jõesaar.** Diversity of key catabolic genes at degradation of phenol and *p*-cresol in pseudomonads. Tartu, 2010, 125 p.
181. **Kristjan Herkül.** Effects of physical disturbance and habitat-modifying species on sediment properties and benthic communities in the northern Baltic Sea. Tartu, 2010, 123 p.

182. **Arto Pulk.** Studies on bacterial ribosomes by chemical modification approaches. Tartu, 2010, 161 p.
183. **Maria Põllupüü.** Ecological relations of cladocerans in a brackish-water ecosystem. Tartu, 2010, 126 p.
184. **Toomas Silla.** Study of the segregation mechanism of the Bovine Papillomavirus Type 1. Tartu, 2010, 188 p.
185. **Gyaneshwer Chaubey.** The demographic history of India: A perspective based on genetic evidence. Tartu, 2010, 184 p.
186. **Katrin Kepp.** Genes involved in cardiovascular traits: detection of genetic variation in Estonian and Czech populations. Tartu, 2010, 164 p.
187. **Virve Sõber.** The role of biotic interactions in plant reproductive performance. Tartu, 2010, 92 p.
188. **Kersti Kangro.** The response of phytoplankton community to the changes in nutrient loading. Tartu, 2010, 144 p.
189. **Joachim M. Gerhold.** Replication and Recombination of mitochondrial DNA in Yeast. Tartu, 2010, 120 p.
190. **Helen Tammert.** Ecological role of physiological and phylogenetic diversity in aquatic bacterial communities. Tartu, 2010, 140 p.
191. **Elle Rajandu.** Factors determining plant and lichen species diversity and composition in Estonian *Calamagrostis* and *Hepatica* site type forests. Tartu, 2010, 123 p.
192. **Paula Ann Kivistik.** ColR-ColS signalling system and transposition of Tn*4652* in the adaptation of *Pseudomonas putida.* Tartu, 2010, 118 p.
193. **Siim Sõber.** Blood pressure genetics: from candidate genes to genome-wide association studies. Tartu, 2011, 120 p.
194. **Kalle Kipper.** Studies on the role of helix 69 of 23S rRNA in the factor-dependent stages of translation initiation, elongation, and termination. Tartu, 2011, 178 p.
195. **Triinu Siibak.** Effect of antibiotics on ribosome assembly is indirect. Tartu, 2011, 134 p.
196. **Tambet Tõnissoo.** Identification and molecular analysis of the role of guanine nucleotide exchange factor RIC-8 in mouse development and neural function. Tartu, 2011, 110 p.
197. **Helin Räägel.** Multiple faces of cell-penetrating peptides – their intracellular trafficking, stability and endosomal escape during protein transduction. Tartu, 2011, 161 p.
198. **Andres Jaanus.** Phytoplankton in Estonian coastal waters – variability, trends and response to environmental pressures. Tartu, 2011, 157 p.
199. **Tiit Nikopensius.** Genetic predisposition to nonsyndromic orofacial clefts. Tartu, 2011, 152 p.
200. **Signe Värv.** Studies on the mechanisms of RNA polymerase II-dependent transcription elongation. Tartu, 2011, 108 p.
201. **Kristjan Välk.** Gene expression profiling and genome-wide association studies of non-small cell lung cancer. Tartu, 2011, 98 p.

202. **Arno Põllumäe.** Spatio-temporal patterns of native and invasive zooplankton species under changing climate and eutrophication conditions. Tartu, 2011, 153 p.
203. **Egle Tammeleht.** Brown bear (*Ursus arctos*) population structure, demographic processes and variations in diet in northern Eurasia. Tartu, 2011, 143 p.
205. **Teele Jairus.** Species composition and host preference among ectomycorrhizal fungi in Australian and African ecosystems. Tartu, 2011, 106 p.
206. **Kessy Abarenkov.** PlutoF – cloud database and computing services supporting biological research. Tartu, 2011, 125 p.
207. **Marina Grigorova.** Fine-scale genetic variation of follicle-stimulating hormone beta-subunit coding gene (*FSHB*) and its association with reproductive health. Tartu, 2011, 184 p.
208. **Anu Tiitsaar.** The effects of predation risk and habitat history on butterfly communities. Tartu, 2011, 97 p.
209. **Elin Sild.** Oxidative defences in immunoecological context: validation and application of assays for nitric oxide production and oxidative burst in a wild passerine. Tartu, 2011, 105 p.
210. **Irja Saar**. The taxonomy and phylogeny of the genera *Cystoderma* and *Cystodermella* (Agaricales, Fungi). Tartu, 2012, 167 p.
211. **Pauli Saag.** Natural variation in plumage bacterial assemblages in two wild breeding passerines. Tartu, 2012, 113 p.
212. **Aleksei Lulla.** Alphaviral nonstructural protease and its polyprotein substrate: arrangements for the perfect marriage. Tartu, 2012, 143 p.
213. **Mari Järve.** Different genetic perspectives on human history in Europe and the Caucasus: the stories told by uniparental and autosomal markers. Tartu, 2012, 119 p.
214. **Ott Scheler**. The application of tmRNA as a marker molecule in bacterial diagnostics using microarray and biosensor technology. Tartu, 2012, 93 p.
215. **Anna Balikova**. Studies on the functions of tumor-associated mucin-like leukosialin (CD43) in human cancer cells. Tartu, 2012, 129 p.
216. **Triinu Kõressaar.** Improvement of PCR primer design for detection of prokaryotic species. Tartu, 2012, 83 p.
217. **Tuul Sepp.** Hematological health state indices of greenfinches: sources of individual variation and responses to immune system manipulation. Tartu, 2012, 117 p.
218. **Rya Ero.** Modifier view of the bacterial ribosome. Tartu, 2012, 146 p.
219. **Mohammad Bahram.** Biogeography of ectomycorrhizal fungi across different spatial scales. Tartu, 2012, 165 p.
220. **Annely Lorents.** Overcoming the plasma membrane barrier: uptake of amphipathic cell-penetrating peptides induces influx of calcium ions and downstream responses. Tartu, 2012, 113 p.

221. **Katrin Männik.** Exploring the genomics of cognitive impairment: whole-genome SNP genotyping experience in Estonian patients and general population. Tartu, 2012, 171 p.
222. **Marko Prous.** Taxonomy and phylogeny of the sawfly genus *Empria* (Hymenoptera, Tenthredinidae). Tartu, 2012, 192 p.
223. **Triinu Visnapuu.** Levansucrases encoded in the genome of *Pseudomonas syringae* pv. tomato DC3000: heterologous expression, biochemical characterization, mutational analysis and spectrum of polymerization products. Tartu, 2012, 160 p.
224. **Nele Tamberg.** Studies on Semliki Forest virus replication and pathogenesis. Tartu, 2012, 109 p.
225. **Tõnu Esko.** Novel applications of SNP array data in the analysis of the genetic structure of Europeans and in genetic association studies. Tartu, 2012, 149 p.
226. **Timo Arula.** Ecology of early life-history stages of herring *Clupea harengus membras* in the northeastern Baltic Sea. Tartu, 2012, 143 p.
227. **Inga Hiiesalu.** Belowground plant diversity and coexistence patterns in grassland ecosystems. Tartu, 2012, 130 p.
228. **Kadri Koorem.** The influence of abiotic and biotic factors on small-scale plant community patterns and regeneration in boreonemoral forest. Tartu, 2012, 114 p.
229. **Liis Andresen.** Regulation of virulence in plant-pathogenic pectobacteria. Tartu, 2012, 122 p.
230. **Kaupo Kohv.** The direct and indirect effects of management on boreal forest structure and field layer vegetation. Tartu, 2012, 124 p.
231. **Mart Jüssi.** Living on an edge: landlocked seals in changing climate. Tartu, 2012, 114 p.
232. **Riina Klais.** Phytoplankton trends in the Baltic Sea. Tartu, 2012, 136 p.
233. **Rauno Veeroja.** Effects of winter weather, population density and timing of reproduction on life-history traits and population dynamics of moose (*Alces alces*) in Estonia. Tartu, 2012, 92 p.
234. **Marju Keis.** Brown bear (*Ursus arctos*) phylogeography in northern Eurasia. Tartu, 2013, 142 p.
235. **Sergei Põlme.** Biogeography and ecology of *alnus*- associated ectomycorrhizal fungi – from regional to global scale. Tartu, 2013, 90 p.
236. **Liis Uusküla.** Placental gene expression in normal and complicated pregnancy. Tartu, 2013, 173 p.
237. **Marko Lõoke.** Studies on DNA replication initiation in *Saccharomyces cerevisiae.* Tartu, 2013, 112 p.
238. **Anne Aan.** Light- and nitrogen-use and biomass allocation along productivity gradients in multilayer plant communities. Tartu, 2013, 127 p.
239. **Heidi Tamm.** Comprehending phylogenetic diversity – case studies in three groups of ascomycetes. Tartu, 2013, 136 p.

240. **Liina Kangur.** High-Pressure Spectroscopy Study of Chromophore-Binding Hydrogen Bonds in Light-Harvesting Complexes of Photosynthetic Bacteria. Tartu, 2013, 150 p.
241. **Margus Leppik.** Substrate specificity of the multisite specific pseudouridine synthase RluD. Tartu, 2013, 111 p.
242. **Lauris Kaplinski.** The application of oligonucleotide hybridization model for PCR and microarray optimization. Tartu, 2013, 103 p.
243. **Merli Pärnoja.** Patterns of macrophyte distribution and productivity in coastal ecosystems: effect of abiotic and biotic forcing. Tartu, 2013, 155 p.
244. **Tõnu Margus.** Distribution and phylogeny of the bacterial translational GTPases and the Mqsr/YgiT regulatory system. Tartu, 2013, 126 p.
245. **Pille Mänd**. Light use capacity and carbon and nitrogen budget of plants: remote assessment and physiological determinants. Tartu, 2013, 128 p.
246. **Mario Plaas.** Animal model of Wolfram Syndrome in mice: behavioural, biochemical and psychopharmacological characterization. Tartu, 2013, 144 p.
247. **Georgi Hudjašov.** Maps of mitochondrial DNA, Y-chromosome and tyrosinase variation in Eurasian and Oceanian populations. Tartu, 2013, 115 p.
248. **Mari Lepik**. Plasticity to light in herbaceous plants and its importance for community structure and diversity. Tartu, 2013, 102 p.
249. **Ede Leppik**. Diversity of lichens in semi-natural habitats of Estonia. Tartu, 2013, 151 p.
250. **Ülle Saks.** Arbuscular mycorrhizal fungal diversity patterns in boreonemoral forest ecosystems. Tartu, 2013, 151 p.
251. **Eneli Oitmaa**. Development of arrayed primer extension microarray assays for molecular diagnostic applications. Tartu, 2013, 147 p.
252. **Jekaterina Jutkina.** The horizontal gene pool for aromatics degradation: bacterial catabolic plasmids of the Baltic Sea aquatic system. Tartu, 2013, 121 p.
253. **Helen Vellau.** Reaction norms for size and age at maturity in insects: rules and exceptions. Tartu, 2014, 132 p.
254. **Randel Kreitsberg.** Using biomarkers in assessment of environmental contamination in fish – new perspectives. Tartu, 2014, 107 p.
255. **Krista Takkis.** Changes in plant species richness and population performance in response to habitat loss and fragmentation.Tartu, 2014, 141 p.
256. **Liina Nagirnaja.** Global and fine-scale genetic determinants of recurrent pregnancy loss. Tartu, 2014, 211 p.
257. **Triin Triisberg.** Factors influencing the re-vegetation of abandoned extracted peatlands in Estonia. Tartu, 2014, 133 p.
258. **Villu Soon.** A phylogenetic revision of the *Chrysis ignita* species group (Hymenoptera: Chrysididae) with emphasis on the northern European fauna. Tartu, 2014, 211 p.

259. **Andrei Nikonov.** RNA-Dependent RNA Polymerase Activity as a Basis for the Detection of Positive-Strand RNA Viruses by Vertebrate Host Cells. Tartu, 2014, 207 p.
260. **Eele Õunapuu-Pikas**. Spatio-temporal variability of leaf hydraulic conductance in woody plants: ecophysiological consequences. Tartu, 2014, 135 p.
261. **Marju Männiste**. Physiological ecology of greenfinches: information content of feathers in relation to immune function and behavior. Tartu, 2014, 121 p.
262. **Katre Kets.** Effects of elevated concentrations of CO_2 and O_3 on leaf photosynthetic parameters in *Populus tremuloides*: diurnal, seasonal and interannual patterns. Tartu, 2014, 115 p.
263. **Külli Lokko.** Seasonal and spatial variability of zoopsammon communities in relation to environmental parameters. Tartu, 2014, 129 p.
264. **Olga Žilina**. Chromosomal microarray analysis as diagnostic tool: Estonian experience. Tartu, 2014, 152 p.
265. **Kertu Lõhmus**. Colonisation ecology of forest-dwelling vascular plants and the conservation value of rural manor parks. Tartu, 2014, 111 p.
266. **Anu Aun.** Mitochondria as integral modulators of cellular signaling. Tartu, 2014, 167 p.
267. **Chandana Basu Mallick.** Genetics of adaptive traits and gender-specific demographic processes in South Asian populations. Tartu, 2014, 160 p.
268. **Riin Tamme.** The relationship between small-scale environmental heterogeneity and plant species diversity. Tartu, 2014, 130 p.
269. **Liina Remm.** Impacts of forest drainage on biodiversity and habitat quality: implications for sustainable management and conservation. Tartu, 2015, 126 p.
270. **Tiina Talve.** Genetic diversity and taxonomy within the genus *Rhinanthus*. Tartu, 2015, 106 p.
271. **Mehis Rohtla**. Otolith sclerochronological studies on migrations, spawning habitat preferences and age of freshwater fishes inhabiting the Baltic Sea. Tartu, 2015, 137 p.
272. **Alexey Reshchikov.** The world fauna of the genus *Lathrolestes* (Hymenoptera, Ichneumonidae). Tartu, 2015, 247 p.
273. **Martin Pook.** Studies on artificial and extracellular matrix protein-rich surfaces as regulators of cell growth and differentiation. Tartu, 2015, 142 p.
274. **Mai Kukumägi.** Factors affecting soil respiration and its components in silver birch and Norway spruce stands. Tartu, 2015, 155 p.
275. **Helen Karu.** Development of ecosystems under human activity in the North-East Estonian industrial region: forests on post-mining sites and bogs. Tartu, 2015, 152 p.
276. **Hedi Peterson.** Exploiting high-throughput data for establishing relationships between genes. Tartu, 2015, 186 p.

277. **Priit Adler.** Analysis and visualisation of large scale microarray data, Tartu, 2015, 126 p.
278. **Aigar Niglas.** Effects of environmental factors on gas exchange in deciduous trees: focus on photosynthetic water-use efficiency. Tartu, 2015, 152 p.
279. **Silja Laht.** Classification and identification of conopeptides using profile hidden Markov models and position-specific scoring matrices. Tartu, 2015, 100 p.
280. **Martin Kesler.** Biological characteristics and restoration of Atlantic salmon *Salmo salar* populations in the Rivers of Northern Estonia. Tartu, 2015, 97 p.