Realia et naturalia

DISSERTATIONES
BIOLOGICAE
UNIVERSITATIS
TARTUENSIS
259

ANDREI NIKONOV

RNA-Dependent RNA Polymerase Activity as a Basis for the Detection of Positive-Strand RNA Viruses by Vertebrate Host Cells

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
259

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
259

ANDREI NIKONOV

RNA-Dependent RNA Polymerase Activity as a Basis for the Detection of Positive-Strand RNA Viruses by Vertebrate Host Cells

UNIVERSITY OF TARTU
PRESS

Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy (in Virology) on 05.05.2014 by the Council of Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu.

Supervisor:	Dr. Mart Ustav, PhD Department of Biomedical Technology, Institute of Technology, University of Tartu, Estonia
Reviewer:	Dr. Maido Remm, PhD Department of Bioinformatics, Institute of Molecular and Cell Biology, University of Tartu, Estonia
Opponent:	Dr. Volker Lohmann, PhD Molecular Virology, Department of Infectious Diseases University of Heidelberg, Germany
Commencement:	Auditorium 105, Riia 23B, Tartu, Estonia, at 13:00 on June 17th, 2014

Publication of this thesis is granted by the Institute of Molecular and Cell Biology, University of Tartu and by the Graduate School in Biomedicine and Biotechnology created under the auspices of European Social Fund.

ISSN 1024–6479
ISBN 978–9949–32–583–2 (print)
ISBN 978–9949–32–584–9 (pdf)

Copyright: Andrei Nikonov, 2014

University of Tartu Press
www.tyk.ee

To my father and mother,
Viktor Ivanovich and Anna Eduardovna

“It is by logic that we prove, but by intuition that we discover”
– **Henri Poincaré**, Science and Method (1908),
as translated by Francis Maitland (1914).

CONTENTS

LIST OF ORIGINAL PUBLICATIONS 10

LIST OF ABBREVIATIONS 11

1. INTRODUCTION.. 13

2. LITERATURE ANALYSIS 14
2.1. General introduction to RNA viruses 14
2.1.1. RNA viruses and their RdRps 14
2.1.2. Virion coat 15
2.1.3. Domination of +ssRNA viruses 15
2.2. *Flaviviridae* and *Togaviridae* family viruses and the forces they exert on humans 16
2.2.1. Flaviviridae and HCV 16
2.2.2. Togaviridae and SFV.. 17
2.3. SFV and HCV virions: “Beauty and the Beast” 18
2.3.1. SFV virions 18
2.3.2. The “virion” or lipo-viro-particle of HCV 19
2.4. SFV and HCV entry into host cells 21
2.4.1. SFV.. 21
2.4.2. HCV 21
2.5. SFV and HCV genomes 22
2.5.1. SFV genome organization 23
2.5.2. HCV genome organization 24
2.5.3. Stem-loops and their higher-order structures 25
2.6. SFV and HCV replicases.. 25
2.6.1. SFV replicase 26
2.6.2. HCV replicase 27
2.6.3. The structure and function of the HCV RdRp 27
2.6.3.1. The “anatomy” of the HCV RdRp 27
2.6.3.2. Initiation complex formation and catalysis: interplay between motifs (theoretical model) 31
2.6.3.3. The mechanisms of RNA synthesis initiation 32
2.6.3.4. Initiation, elongation, and termination of *de novo* RNA synthesis 33
2.6.3.5. A novel NS5B enzymatic function and bias in error rate 35
2.6.3.6. RNA templates used by HCV RdRp 35
2.6.4. Functions of non-RdRp components of the SFV and HCV replicases 36
2.6.4.1. Other components of the SFV replicase complex 36
2.6.4.2. Non-RdRp components of HCV replicase 37

2.7. Replication of the SFV and HCV genomes 38
2.7.1. SFV 39
2.7.2. HCV 40
2.8. Replication of the genomes of other RNA viruses: appearance of RF 41
2.9. Detection of RNA virus infection by the host cell 42
2.9.1. Viral dsRNA or RF activates the type I interferon (IFN) system 42
2.9.2. Limitations of viral dsRNA/RF detection by RLRs 43
2.9.3. Side effects of replicase components 44
2.9.3.1. Viral protease counteracts the type I IFN system 44
2.9.3.2. Viral RdRp: an activator of the type I IFN system? ... 45

3. AIM OF THE STUDY 46

4. MATERIALS AND METHODS 47
4.1. Paper I 47
4.2. Paper II 47
4.3. Paper III 47

5. RESULTS AND DISCUSSION 49
5.1. Paper I 49
5.1.1. Conserved targets for molecular probes in the HCV RdRp fingers subdomain 49
5.1.2. HCV RdRp fingers subdomain controls the initiation of RNA synthesis and the elongation of RNA chains 49
5.1.3. Motif G is part of the RdRp molecular interaction platform detected in the HCV replicase context 51
5.1.4. Molecular environment-dependent activity of HCV NS5B: from RdRp to replicase 52
5.2. Paper II 52
5.2.1. Side effect evaluation for predicted NS3-4A inhibitors 52
5.3. Papers I and II 53
5.3.1. In pursuit of viral RdRp side effects 53
5.4. Paper III 54
5.4.1. Side effects of viral RdRp activity: HCV NS5B and SFV replicase 54
5.4.2. The SFV dsRNA RF is not accessible to the host cell antiviral machinery 55
5.4.3. The result of SFV replicase RdRp activity side effect in the context of viral infection 56
5.5. Two concurrent processes are driven by viral replicase in infected cells 57
5.5.1. Replicase-based detection of SFV 57
5.5.2. RdRp-based detection of HCV 60

5.5.3. The model: emergence of polymerase-protease antagonism ... 62
5.5.4. Inhibition of HCV replicase from the perspective of the current model ... 62
6. CONCLUSIONS ... 65
6.1. Specific conclusions (experimental observations) ... 65
6.2. General conclusions (model) ... 65
7. REFERENCES ... 66
8. SUMMARY IN ESTONIAN ... 86
ACKNOWLEDGEMENTS ... 90
PUBLICATIONS ... 93
CURRICULUM VITAE ... 193

LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original papers referred to in the text by their Roman numerals:

I. **Nikonov, A.**, Juronen, E., and Ustav, M. (2008). Functional characterization of fingers subdomain-specific monoclonal antibodies inhibiting the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* 283, 24089–24102.

II. Karelson, M., Dobchev, D.A., Karelson, G., Tamm, T., Tämm, K., **Nikonov, A.**, Mutso, M., and Merits, A. (2012). Fragment-based development of HCV protease inhibitors for the treatment of hepatitis C. *Curr Comput Aided Drug Des* 8, 55–61.

III. **Nikonov, A.***, Mölder, T., Sikut, R., Kiiver, K., Männik, A., Toots, U., Lulla, A., Lulla, V., Utt, A., Merits, A., and Ustav, M.* (2013). RIG-I and MDA-5 detection of viral RNA-dependent RNA polymerase activity restricts positive-strand RNA virus replication. *PLoS Pathog* 9, e1003610.
*Corresponding authors

Reprints of the papers listed above were made with the permission of the respective copyright owners.

My contribution to the papers is as follows:

I. I designed most and performed all experiments, except the production of monoclonal antibodies. I analyzed the data and wrote most of the manuscript.

II. I participated in the design of toxicity assays and performed their results analysis. I wrote the corresponding part of the manuscript.

III. I designed and performed most of the experiments. I analyzed the data and wrote most of the manuscript.

LIST OF ABBREVIATIONS

Å	angstrom
aa	amino acid residues
ADP	adenosine diphosphate
Apo	apolipoprotein
CARD	caspase activation and recruitment domain
CARDIF	CARD adaptor inducing IFN-β
DNA	deoxyribonucleic acid
C	capsid protein
CHIKV	Chikungunya virus
{c,m,v}RNA	{complementary, messenger, viral,} RNA
CRC	crude replication complex
CRE	cis-acting RNA element
CSE	conserved sequence element
DI	defective interfering
DMV	double-membrane vesicle
{d,s}sRNA	{double-, single-}stranded RNA
(F)QSAR	(fragment-based) quantitative structure-activity relationship
G{M,D,T}P	guanosine {mono,di,tri}phosphate
GORS	genome-scale ordered RNA structure
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus type 1
hpi	hours post infection
IC_{50}	inhibitory concentration 50%
{I,L,VL}DL	{intermediate, low, very-low}-density lipoprotein
IFN	type I interferon
Ig	immunoglobulin
IRES	internal ribosome entry site
K_i	inhibitory or affinity constant
LCS	low-complexity sequence
LVP	lipo-viro-particles
m7-GMP	7-methylguanosine monophosphate
mAb	monoclonal antibody
MEF	mouse embryonic fibroblast
NA	nucleoside analogue
NANBH	non-A, non-B hepatitis
(N)C	(nucleo)capsid
NDV	Newcastle desease virus
NLR	NOD-like receptor
NLS	nuclear localization signal
nm	nanometer
NNI	non-nucleoside inhibitor
NOD	nucleotide oligomerization domain
NS, nsP	non-structural protein

nt	nucleotides
NTP	nucleoside triphosphate
NTPase	nucleoside triphosphatase
oligo(dT)	homo-oligomeric deoxyribonucleotide, poly(dT)
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
poly(I:C)	dsRNA composed of polyriboinosinic and polyribocytidylic acids
poly(X)	X-rich RNA sequence, where X are nucleotides U, G, A, C or their combination
polyA-	nonpolyadenylated
polyA+	polyadenylated
PPIase	peptidyl-prolyl isomerase
PRR	pathogen recognition receptor
RDR	SFV nsP2 NLS mutation ($RR^{649}R \rightarrow RD^{649}R$)
RdRp	RNA-dependent RNA polymerase
RF	replicative form
ribavirin	1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
Rluc	*Renilla* luciferase
RNA	ribonucleic acid
RNP	ribonucleoprotein
(r)NTP	(ribo)nucleoside triphosphate
RT	reverse transcriptase
RT-CES	real-time cell electronic sensing
S	Svedberg sedimentation unit
SFV	Semliki Forest virus
SINV	Sindbis virus
SL	stem-loop RNA structure
SR-BI	scavenger receptor class B type I
T7	bacteriophage T7
TLR	Toll-like receptor
TMEV	Theiler's murine encephalitis virus
TRIF	Toll-IL1 receptor domain-containing adaptor inducing IFN-β
UTR	un-translated region
VSV	vesicular stomatitis virus
5'-ppp	5'-triphosphate

1. INTRODUCTION

Essentially every living organism studied thus far may serve as a host for the infection and propagation of species-specific RNA viruses. An infectious virus particle, *i.e.* a virion, carries the genome in the form of nucleic acid surrounded by a protein or protein-lipid coat.

RNA virus genome replication is driven by the viral genome-encoded RNA-dependent RNA polymerase (RdRp). More specifically, viral RNA genome replication is performed by a replicase, a multisubunit enzyme complex that possesses a core component with RdRp activity. RdRp first utilizes a viral RNA genome template to catalyze the synthesis of complementary RNA, which subsequently serves as a template for the production of viral genomes. RdRp is a molecular machine that transfers the genetic information embedded in genetic material from one RNA molecule to another. All RNA viruses encode the RdRp component of the replicase because host cells either cannot replicate long RNA genomes of viruses (*e.g.*, invertebrates and plants) or do not possess intrinsic RdRp (*e.g.*, vertebrates). Hepatitis delta virus, a satellite virus, is an exception to this rule. In addition to RdRp, all of the other protein subunits required for replicase assembly are encoded by either the viral RNA genome or the host genome.

This dissertation involved a journey from the test tube to the host cell to analyze the effects of the viral RdRp activities of "yellow" and "mantled" RNA viruses of *Flaviviridae* and *Togaviridae* families. First, we performed structure-function relationship analyses of the hepatitis C virus (HCV) core RdRp component in different molecular environments. Second, by conducting Semliki Forest virus (SFV) replication studies, we found that the SFV replicase not only replicates viral nucleic acids but also has the capacity to transcribe host cell RNA templates. The latter "side effect" of SFV replicase RdRp activity triggers a potent cellular antiviral response. This finding led to the development of a generalized novel model that describes how vertebrate host cells might detect RNA viruses similar to SFV and how viruses counteract this detection.

2. LITERATURE ANALYSIS

2.1. General introduction to RNA viruses

2.1.1. RNA viruses and their RdRps

The virions of all RNA viruses contain RNA genomes, which encode specific viral RdRps required for their replication. RNA extracted from the virions of most RNA viruses is infectious, although exceptions exist. The finding that extracted viral RNA is infectious indicates that translation machinery of the host cell utilizes this RNA directly for the production of viral replicase. The common substrate for translation machinery is single-stranded (ss) messenger RNA (mRNA), which is produced during the transcription of the host cell genome. Thus, the translation machinery utilizes infectious viral RNA extracted from virions as if it were cellular mRNA. This finding led to a proposition to designate infectious viral RNA and cellular mRNA as positive-strand RNAs (or +RNA) (Baltimore, 1971) (Figure 1). Simultaneously, active viral RdRps were found in the virions of Newcastle disease virus (NDV) (Huang et al., 1971), vesicular stomatitis virus (VSV) (Baltimore et al., 1970; Marcus et al., 1971), influenza virus (Bishop et al., 1971), and reoviruses (Borsa and Graham, 1968; Gomatos, 1970; Shatkin and Sipe, 1968). The RNA extracted from the virions of these viruses was noninfectious, thus, the role of virion RdRp in the production of viral mRNA and, subsequently, replicase was evident. Hence, noninfectious RNAs of NDV, VSV, and influenza virus were defined as negative-strand RNAs (or –RNA), whereas the double-stranded (ds) RNA of reoviruses was defined as ±RNA (Baltimore, 1971). Furthermore, the virions of arenaviruses were found to contain RdRp (Carter et al., 1974), however, in contrast to other negative-strand RNA viruses, arenaviruses used both viral genomic RNA (vRNA) and its complementary RNA (cRNA) to encode viral proteins. Hence, the noninfectious RNAs of arenaviruses were defined as single-stranded ±RNA (Bishop, 1986). Thus, virion RdRp is the hallmark of –ssRNA, ±ssRNA, and ±dsRNA viruses, whereas no RdRp is present in the virions of +ssRNA viruses.

Figure 1. Expression (*i.e.*, mRNA production) of RNA virus genomes (modified from (Baltimore, 1971)). See text for details.

2.1.2. Virion coat

The production of a multitude of viral genomic RNA molecules by viral RdRp in a host cell is futile until a protein or a lipid-protein coat envelopes this genetic material. Only then can a fully infectious virion be formed. The presence of a viral coat essentially distinguishes a virus from a replicon, a modified viral genome capable of replication but incapable of virion formation. Accordingly, in addition to encoding replicase, viral genomes must encode proteins that protect their genetic material from the environment.

Animal RNA viruses can be classified into two categories with respect to their virion structure: (i) "enveloped" viruses, which possess an inner protein coat and an outer lipid bilayer membrane, and (ii) "nonenveloped" viruses, which only possess a protein coat, known as a capsid. The capsids of enveloped and nonenveloped viruses, together with the viral RNA they enclose, are referred to as nucleocapsids (NCs). The NCs of the majority of +ssRNA viruses contain naked viral RNA, which electrostatically interacts with the capsid's inner surface. In contrast, the NCs of –ssRNA, ±ssRNA, and ±dsRNA viruses are mainly represented by either single or multiple tightly packed ribonucleoprotein (RNP) complexes composed of viral RNA, RdRp, and nucleoproteins (International Committee on Taxonomy of Viruses. and King, 2012).

2.1.3. Domination of +ssRNA viruses

Currently, 6+1 orders, 87 families, 19 subfamilies, 349 genera, and 2284 species of viruses and viroids are thought to exist (International Committee on Taxonomy of Viruses. and King, 2012). The order, family, subfamily, and genus names of viral taxa are formed by adding the Latin suffixes *-virales*, *-viridae*, *-virinae*, and *–virus*, respectively. The names of species within a genus are typically formed in the following way: [*Disease*] *virus*, [*Characteristic symptom*] *virus* or [*Geographical location*] *virus*. Positive-strand RNA viruses are the largest viral class, represented by 12 families in 3 orders (*Tymovirales*, *Nidovirales*, and *Picornavirales*) and an additional 19 families in the unassigned order designated "+1" above, with a total of 117 genera.

Given the paramount significance of RdRp for the existence of RNA viruses, this subunit is the most conserved of all replicase subunits (Koonin, 1991). Comparative analyses of representative amino acid (aa) sequences of RdRps of eukaryotic +ssRNA viruses have revealed the presence of conserved protein sequences, called motifs (Kamer and Argos, 1984; Koonin, 1991; Poch et al., 1989). Phylogenetic analyses of these motifs enabled the classification of the majority of +ssRNA viruses into three large supergroups: picorna-, flavi-, and alpha-like (Koonin, 1991; Koonin and Dolja, 1993). Subsequent examinations of the viral genes that encode the remaining viral proteins, which together with the core RdRp form the viral replicase, revealed that the relative arrangement of the genes in the viral genome was unique for each supergroup (Koonin and Dolja, 1993).

2.2. *Flaviviridae* and *Togaviridae* family viruses and the forces they exert on humans

The viruses of the *Flaviviridae* and *Togaviridae* families are +ssRNA viruses and belong to an unassigned order (International Committee on Taxonomy of Viruses. and King, 2012).

2.2.1. Flaviviridae and HCV

The *Flaviviridae* family consists of four genera: *Flavivirus* (53 species), *Hepacivirus* (1 species), *Pegivirus* (2 species), and *Pestivirus* (4 species) (International Committee on Taxonomy of Viruses. and King, 2012). Many viruses in the genus *Flavivirus* are arthropod borne (arboviruses); they can be transmitted to humans by arthropod vectors (mosquitoes and ticks) from birds, pigs, or primates. Typically, these viruses persist in arthropods due to vertical transmission and are amplified in vertebrates other than humans, whereas the primary transmission cycles of dengue virus and yellow fever virus (the type species of *Flaviviridae*) include humans (Fields et al., 2013). The *Flaviviridae* family and its genera are far from completely described: molecular evidence of novel members of the *Hepacivirus* genus was recently obtained from wild rodents (four-striped mice and bank voles) (Drexler et al., 2013). However, blood-borne HCV is currently the only species present in the *Hepacivirus* genus. Phylogenetic analyses of HCV RdRp sequences resulted in the identification of six major genotypes, which were further divided into subtypes (Simmonds et al., 1993). Historically, the most important HCV strains are 1a, 1b, 2a, and 2a/2a, a chimera of two different isolates of the same genotype and subtype. The first cloned HCV genome, which was isolated from a patient's plasma and known for over a decade as non-A, non-B hepatitis agent (NANBH), was of genotype 1a (Choo et al., 1989; Choo et al., 1991). Subsequently, it was demonstrated that inoculating the RNA of another HCV clone of genotype 1a into chimpanzees' livers caused disease in chimpanzees (Kolykhalov et al., 1997). The first subgenomic replicon capable of high-level replication in a human hepatoma cell line was of genotype 1b (Lohmann et al., 1999). The availability of this subgenomic replicon system provided insights into HCV replication and antiviral drug discovery. Finally, the first cloned full-length viral genome capable of replication and infectious virus production in human hepatoma cells was of genotype 2a (Wakita et al., 2005). The chimera of two isolates of this genotype significantly increased the titer of obtained virus (up to 1000-fold), which enabled laboratory analyses of the HCV infection cycle (Pietschmann et al., 2006). As the name of the virus implies, strict hepatic tropism is a unique characteristic of HCV. Only humans and chimpanzees can be infected with HCV. Importantly, no small-animal models (including mouse) supporting efficient HCV replication and its complete infection cycle currently exist.

The impact of *Flaviviridae* family viruses on human health is enormous. For example, approximately 50–100 million dengue virus infections occur annually; in some cases (<0.5%), these infections lead to severe hemorrhagic fever, the incidence of which is magnified several-fold upon secondary infection (Gubler, 2002; Halstead, 1990). According to the World Health Organization, 130–170 million individuals worldwide had chronic HCV infections in 2005 (Mohd Hanafiah et al., 2013). Globally, 57% of cirrhosis deaths and 78% of liver cancers in 2002 were attributed to chronic viral infections. Half of these cirrhosis deaths and a third of liver cancers were attributed to chronic HCV infections (Perz et al., 2006). In the majority of cases, HCV and dengue virus produce asymptomatic infections, which become evident only when chronic infection is established or after secondary infection, respectively.

2.2.2. Togaviridae and SFV

Togaviridae family is exemplified by two genera: *Alphavirus* (30 species) and *Rubivirus* (1 species) (International Committee on Taxonomy of Viruses. and King, 2012). Genus *Alphavirus* species are typically divided into two groups according to their geographic distribution: Old World (Eurasia, Africa, and Australia) and New World (Americas) viruses (Hahn et al., 1988). These viruses are exclusively arboviruses, mainly transmitted by mosquitoes to either mammalian or avian hosts. Typically, human diseases caused by alphavirus infections are acute and characterized by specific symptoms. All pathogenic alphaviruses infecting humans cause fevers and, depending on the geographic location, induce arthritis and rashes (Old World) or encephalitis (New World) (Fields et al., 2013). Compared with the *Flaviviridae* family, the impact of the *Togaviridae* family on human health is not as pronounced, and the latter family is generally considered much "safer" for humans. However, a recent virus outbreak in the Indian Ocean region resulted in the infection of 1.4 million individuals with Chikungunya virus (CHIKV) within a year (Pialoux et al., 2007; Ravi, 2006). Thus, even an Old World alphavirus can raise serious public health concerns. CHIKV is serologically closely related to SFV, indicating that these two viruses exhibit antigenic cross-reactivity (Powers et al., 2001). At least six other alphaviruses displaying this same type of relation to SFV are currently known (Fields et al., 2013) and hence belong to the Semliki Forest (SF) antigenic complex group, which primarily consists of Old World viruses (Powers et al., 2001). In sharp contrast to CHIKV, which is a reemerging virus (Padbidri and Gnaneswar, 1979; Ravi, 2006), SFV has caused disease in otherwise healthy humans (22 individuals) during only one outbreak (Mathiot et al., 1990); thus, it does not represent a major concern for human health.

SFV and Sindbis virus (SINV) are the most studied viruses of the *Alphavirus* genus and serve as model viruses. SFV exhibits wide tissue tropism and is easily cultured (produced) in a variety of vertebrate and invertebrate organisms (Strauss and Strauss, 1994). The extremely high replication efficiency and viral titers generated during SFV infection lead to the death of infected cells. The

majority of SFV strains used currently were derived from two original strains of viruses isolated from mosquitoes in Africa. Virulent (L10) (Bradish et al., 1971) and avirulent (A7 and A7[74] (Bradish et al., 1971)) strains of SFV were isolated in western Uganda and Mozambique, respectively (McIntosh et al., 1961; Smithburn and Haddow, 1944). The L10 and L10.H6 strains of SFV were virulent and lethal to mice, hamsters, and rabbits *via* various routes of inoculation (Boulter et al., 1971; Henderson et al., 1967; Smithburn and Haddow, 1944). Many strains have been derived from these original isolates, including the virulent SFV4, which was obtained from the infectious RNA of the L10 virus (Liljestrom et al., 1991). Virulent and avirulent strains differ in their ability to invade and replicate in the central nervous system of adult mice and rats, inducing cell damage (Balluz et al., 1993). For adult mice, infection with a neurovirulent strain (SFV4) leads to death, whereas infection with an avirulent strain (A7 or A7[74]) leads to survival (Balluz et al., 1993; Tuittila et al., 2000).

2.3. SFV and HCV virions: "Beauty and the Beast"

2.3.1. SFV virions

SFV virions have spherical, multilayered structures with a diameter of 70 nm. A single copy of infectious SFV RNA is enclosed in an NC shell composed of 240 copies of capsid protein C, which is then enveloped in a lipid bilayer derived from the host cell's plasma membrane. The SFV lipid bilayer is covered and penetrated by 80 glycoprotein spikes each composed of three E1-E2 glycoprotein heterodimers (Mancini et al., 2000). An additional, small E3 glycoprotein is non-covalently associated with each E1-E2 heterodimer. The symmetry of the NC and virion is icosahedral, with a triangulation number T=4 (Caspar and Klug, 1962; Fuller et al., 1995; Mancini et al., 2000), and can be envisioned in the following way (Figure 2). Every three copies of C protein are arranged in a small "triangle" along the three-fold axis with one C protein in each corner. Subsequently, four such triangles (4x3=12 C copies) are asymmetrically, or "quasi-symmetrically", arranged into a larger triangle (hence T=4). Finally, 20 larger triangles (4x3x20=240 C copies) are symmetrically assembled into an icosahedron, a polyhedron with 12 vertices, 30 edges, and 20 equivalent equilateral triangular faces, possessing five-fold, two-fold, and three-fold rotational symmetries, respectively. The resulting 80 small triangles (80x3=240 C copies) of NC form pentamers at the vertices of the icosahedron and hexamers at intermediate locations. Each C protein in each small triangle interacts directly with an E2 glycoprotein, which in turn interacts with an E1 glycoprotein to form a spike (Mancini et al., 2000). Thus, there is a single spike per single small triangle.

Caspar and Klug proposed the idea of spherical virus shell triangulation as the solution to the problem of how more than 60 C proteins can be arranged on a sphere's surface. By folding different triangular plane (cardboard) nets into

convex surfaces, these authors demonstrated that icosahedral symmetry was the optimal design for spherical NC shells (Caspar and Klug, 1962). Inside a cell infected by +ssRNA virus, the cardboard triangular plane net becomes the cellular membrane plane net, which is formed by the arrays of viral C proteins associated with the membrane. Furthermore, such an analogy can be easily extended to other viral proteins that are not involved in NC construction but that play a role in the formation of spherical surfaces from intracellular membranes.

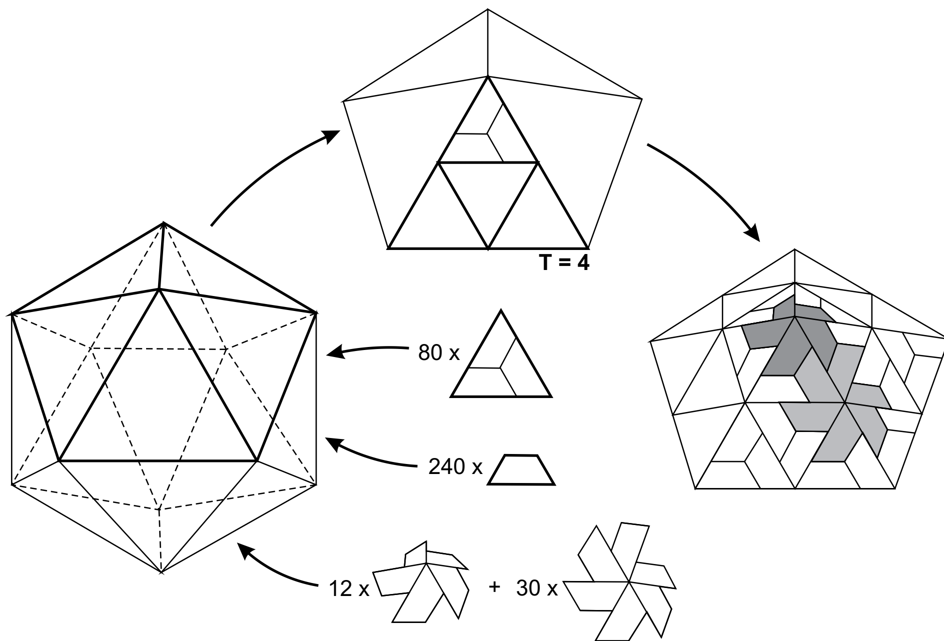

Figure 2. Construction of SFV NC shell structure. See text for details.

2.3.2. The "virion" or lipo-viro-particle of HCV

HCV RNA-containing particles have been found to exist as immunoglobulin conjugates (*e.g.*, IgG and IgM) in low-density fractions of plasma from chronically infected patients. Protein A-coated beads were used to isolate these HCV RNA-containing particles, which were named lipo-viro-particles (LVPs) due to the abundance of lipoproteins in plasma low-density fractions (Andre et al., 2002).

Lipoproteins are synthesized by hepatocytes in the form of very-low-density lipoproteins (VLDLs) and are used to transport lipids and deliver lipid-soluble materials throughout the organism. VLDLs consist of a hydrophobic core of neutral lipids, *i.e.*, triglycerides and cholesteryl esters, surrounded by a monolayer of phospholipids with apolipoproteins B (ApoB) and E (ApoE) on their surfaces, which are buried in free cholesterol (Getz and Reardon, 2009; Segrest et al., 2001). In the human circulatory system, VLDLs use their ApoE to enter

target cells *via* receptor-mediated endocytosis, where lipoprotein lipases release triglycerides, thus reducing the size of the VLDL and giving rise to cholesterol-rich intermediate- and low-density lipoproteins (IDLs and LDLs, respectively), depleted of ApoE (Getz and Reardon, 2009; Mead et al., 2002; Segrest et al., 2001).

It has been shown that HCV LVPs are large, spherical particles larger than 100 nm in diameter and containing internal structures. These triglyceride-rich particles contain HCV RNA and ApoB. Lipid removal produced capsid-like structures that were readily detected by an antibody against HCV capsid protein (Andre et al., 2002).

Recently, chimeric HCV virions produced in an immortalized hepatocellular carcinoma cell line (Huh7.5 and its derivatives) (Gastaminza et al., 2010; Merz et al., 2011) and/or in primary human hepatocytes (Catanese et al., 2013) were analyzed. Low-resolution cryo-electron microscopy was used to confirm the presence of ApoE and HCV E2 glycoprotein on the surfaces of these virions (Catanese et al., 2013; Gastaminza et al., 2010; Merz et al., 2011). Although, ApoB and ApoA-I were observed on putative HCV virion surfaces, their colocalization with E2 was not directly demonstrated (Catanese et al., 2013). The number of ApoE molecules on HCV virions was shown to exceed the number of E2 molecules (Catanese et al., 2013; Merz et al., 2011). HCV virions were heterogeneous: often spherical, but sometimes amorphous, with diameter ranging from ~30 to 150 nm (Catanese et al., 2013; Gastaminza et al., 2010; Merz et al., 2011). In some preparations, putative virion spike-like structures were also observed (Catanese et al., 2013). The virions did not exhibit continuous lipid bilayers (Catanese et al., 2013). Mass spectrometry analyses of the HCV virion lipid composition revealed that almost half of the lipids were cholesteryl esters resembling those in VLDL and LDL (Merz et al., 2011). Thus, in accordance with recently proposed model (Bartenschlager et al., 2011), a single phospholipid monolayer-delimited HCV NC appeared to be located inside the VLDL or VLDL-like lipid-rich particle, which was surrounded by another phospholipid monolayer. In this case, functional spikes, consisting of E1-E2 glycoproteins could form only in the partial bilayer regions (Bartenschlager et al., 2011; Catanese et al., 2013).

Primary human and mouse hepatocytes produce VLDLs with remarkably different cholesteryl ester contents. Human VLDLs are rich in cholesteryl esters (Ling et al., 2013), whereas mouse VLDLs have a very low cholesteryl ester content (Li et al., 2012). Given the abundance of cholesteryl esters in HCV virions produced by human hepatocytes (Merz et al., 2011), it is highly unlikely that mouse hepatocytes are capable of efficiently producing similar virions.

The possibility of a detailed HCV virion image reconstruction is precluded by the above unique properties of HCV virions and the current absence of crystal structures for full-length E1 and E2 glycoproteins. However, cryo-electron microscopic reconstructions of native LDL particles at 16Å resolution have recently been reported (Kumar et al., 2011). Thus, obtaining more precise structural information regarding HCV virions should be possible.

2.4. SFV and HCV entry into host cells

SFV and HCV utilize receptors and various attachment factors that are present on their host cells to engage clathrin-mediated endocytosis and gain access to the cellular translation machinery (Doxsey et al., 1987; Helenius et al., 1980; Helle and Dubuisson, 2008; Meertens et al., 2006). However, discriminating between a receptor and an attachment factor is very difficult, because the efficiency of infection depends critically on both (Mercer et al., 2010).

2.4.1. SFV

For SFV, the spike E2 glycoprotein is responsible for virion attachment to the host cell. Due to the broad tissue and cell tropism of SFV, two models of virion-host cell attachment have been proposed: (i) the E2 glycoprotein has binding sites for multiple receptors/attachment factors or (ii) a conserved receptor is present on different cells (Strauss and Strauss, 1994). The attachment of SFV might be mediated non-specifically by negatively charged glycosaminoglycans covalently bound to cell surface proteins, termed proteoglycans. SFV and SINV virions have been shown to interact with liposomes containing lipid-conjugated heparin, a glycosaminoglycan (Smit et al., 2002). Recently, N-glycans linked to SFV E1 were found to differ between rodent and mosquito cells, whereas oligomannose glycosylation of E2 was conserved between species (Crispin et al., 2014). C-type lectins, a type of calcium-dependent, mannose-enriched glycan-binding protein, have been implicated in SINV binding (Klimstra et al., 2003). Recently, an abundantly expressed, transmembrane iron transporter, natural resistance-associated macrophage protein was identified as an SINV-specific receptor in both insect and mammalian cells (Rose et al., 2011); however no SFV-specific receptor was identified.

Upon SFV internalization, clathrin-coated vesicles deliver SFV to early endosomes, which have slightly acidic environments. The subsequent acidification of late endosomes triggers the destabilization of viral spikes (Wahlberg and Garoff, 1992), the trimerization of E1 glycoproteins (Gibbons et al., 2000), and the subsequent association of five such trimers into a ring structure, leading to fusion-pore formation in the endosome membranes (Gibbons et al., 2004). After endosomal membrane barrier penetration, SFV NC becomes unstable and instantly releases infectious viral RNA in the cytosol of the host cell.

2.4.2. HCV

The LVP structure of HCV suggests receptors/attachment factors that might be important for virus entry. In particular, it was demonstrated that ApoB- and ApoE-specific antibodies blocked the entry of HCV LVPs and that the up-regulation of LDL receptors, which recognize ApoB and ApoE (Boren et al., 1998; Weisgraber, 1994), enhanced LVP uptake (Andre et al., 2002). Recently,

a human Niemann-Pick C1-like 1 cellular cholesterol uptake receptor was also demonstrated to act as an HCV attachment factor (Sainz et al., 2012). HCV E2 glycoprotein interacts with the tetraspanin CD81 cell surface protein, and this interaction is important for infection (Pileri et al., 1998), because CD81-specific antibodies neutralize infection (Wakita et al., 2005). The interaction of E2 with heparan sulfate proteoglycans was also demonstrated and was found to be important for HCV attachment (Barth et al., 2003). Moreover, scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002) and tight junction proteins claudin-1 (Evans et al., 2007) and occludin (Ploss et al., 2009) were found to contribute to HCV entry into host cells. In particular, the expression of human CD81 and occludin was minimally required for HCV entry into mouse hepatocytes *in vivo*, whereas the additional expression of SR-BI and claudin-1 resulted in maximal HCV uptake (Dorner et al., 2011).

How HCV penetrates the endosomal membrane barrier remains unknown. Currently, there is no consensus on whether either E1 or E2 glycoprotein is responsible for the fusion of viral and endosomal membranes (Helle and Dubuisson, 2008). Based on *in silico* modeling, HCV E2 was proposed to be a class II fusion protein, in the same class as the SFV E1 glycoprotein (Garry and Dash, 2003; Krey et al., 2010). However, recently published crystal structures revealed that the HCV E2 core region exhibited a more compact structure than that of class II fusion proteins, characterized by extended, three-domain fold (Kong et al., 2013). Moreover, HCV virions are stable at an acidic pH and do not lose their infectivity, suggesting that glycoprotein activation is not required for fusion to occur (Tscherne et al., 2006). This finding may be due to acidification not being sufficient for HCV spike activation (thus necessitating additional factors) or due to E1 and E2 glycoproteins being shielded by LVP (Bartenschlager et al., 2011; Tscherne et al., 2006). If pH-triggered HCV virion spike activation is not required, then this virus must possess a novel fusion mechanism. Such a fusion might occur in early endosomes or in close proximity to the plasma membrane. After fusion occurs, the NC of HCV releases infectious RNA into the cytosol; however, this process is not well understood.

2.5. SFV and HCV genomes

The nucleotide sequences, organization, and structures of the genomes of +ssRNA viruses, including HCV and SFV, contain all of the information required for the utilization and manipulation of the host cell's metabolic machinery. First, conserved RNA structures in the 5' terminus of the viral genome or within the 5'-terminal region enable the"feeding" of the viral genome into the translation machinery of the host cell, leading to massive viral protein production. Second, conserved RNA structures in the 3'-terminal region of the viral genome and full-length complementary anti-genome serve as "semaphores", which attract the "attention" of viral replicases and promote their recruitment to the 3' terminus enabling viral replication. These semaphores, which are present

in HCV and SFV genomes, are often represented by secondary RNA structures, known as RNA stem-loops (SLs), or by unstructured uridine-rich (U-rich) tracts. Three SLs have been predicted in the SFV genome, and at least ten SLs have been experimentally verified in the HCV genome, whereas only a single conserved U-rich tract is present in the genomes of both viruses (Figure 3).

2.5.1. SFV genome organization

SFV positive-strand genomic RNA is approximately 11,500 nucleotides (nt) long and sediments at 42 S in sucrose gradients (Kaariainen and Ahola, 2002; Kaariainen and Soderlund, 1978). Similar to eukaryotic cellular mRNAs, the 5' terminus of the SFV RNA genome is capped, *i.e.*, it contains 7-methylguanosine in a 5' pyrophosphate linkage (with free 2'-, and 3'-hydroxyl groups), and its 3' terminus contains a poly(A) sequence (Sawicki and Gomatos, 1976; Wengler and Gross, 1979). It was demonstrated that the cap structure is required for eukaryotic mRNA translation (Muthukrishnan et al., 1975). Additional capped and polyadenylated subgenomic RNA, which corresponds to the 3' third of 42S RNA and sediments at 26 S in sucrose gradients is abundantly present in SFV-infected cells (Kaariainen and Soderlund, 1978; Sawicki and Gomatos, 1976; Wengler and Gross, 1979). Two open reading frames (ORFs) are encoded by genomic 42S and subgenomic 26S viral RNAs, which upon translation yield nonstructural (ORF1: nsP1, nsP2, nsP3, and nsP4) and structural (ORF2: C, core; E3, E2, 6K, and E1; E3, E2, and E1 are envelope glycoproteins) proteins, respectively. Within the 42S SFV RNA sequence these two ORFs are separated by a ~50-nt-long, internal un-translated region (UTR) containing multiple stop codons (Ou et al., 1982).

Three predicted SLs and two conserved regions containing four regulatory conserved sequence elements (CSEs) are dispersed throughout the SFV genomic 42S RNA. In particular, a ~40 nt element located within the 5'UTR at the 5' terminus of the SFV genome contains a single SL, referred to as CSE1 (Ou et al., 1983). There are two additional SLs in the CSE2 element (~50 nt), which is embedded in the nsP1 coding region and is located ~110 nt downstream of CSE1 (Ou et al., 1983). A 19-nt U-rich tract located within the 3'UTR is juxtaposed to the poly(A) sequence and is termed CSE4 (Ou et al., 1981). The final regulatory 21 nt element, termed CSE3, overlaps with the final codons of nsP4 and serves as a subgenomic promoter (SGP) in the complementary full-length 42S RNA for the synthesis of 26S RNA (ORF2) (Ou et al., 1982).

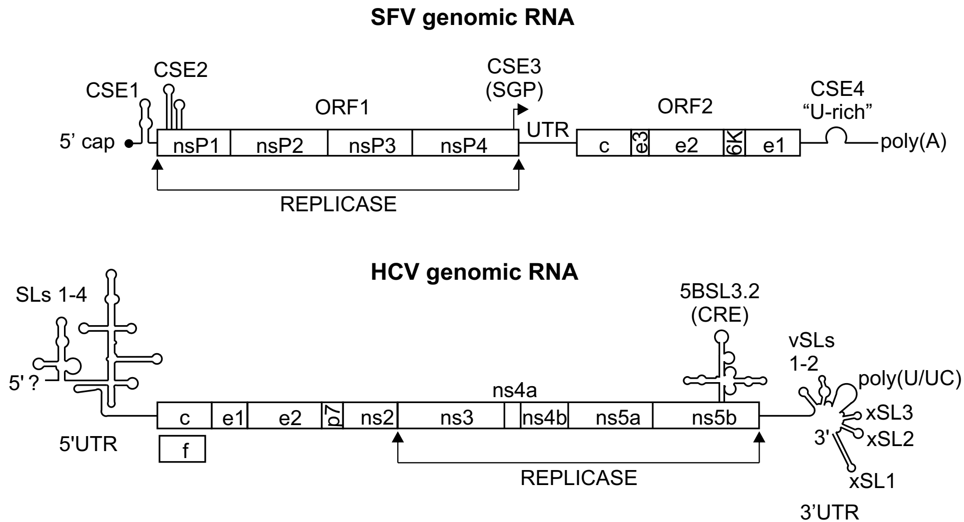

Figure 3. Schematic representation of SFV and HCV genomic RNAs. See text for details.

2.5.2. HCV genome organization

The +ssRNA genome of HCV is approximately 9,600 nt long and contains a single, large ORF flanked by two UTRs, which are indispensable for the translation and replication of the viral genome. The 5'UTR is 341 nt long and contains an internal ribosome entry site (IRES), which is capable of directing the translation of viral RNA in the presence or absence of 5'-terminal cap structure (Rijnbrand et al., 1995; Tsukiyama-Kohara et al., 1992). HCV ORF translation yields a single, large ~ 3,000 aa precursor polyprotein (Grakoui et al., 1993d). This polyprotein is co- and post-translationally cleaved by cellular and viral proteases yielding mature structural and non-structural (NS) proteins (Bartenschlager et al., 1993; Grakoui et al., 1993b; Grakoui et al., 1993d; Selby et al., 1994). The polypeptide begins with structural proteins (C, core; envelope glycoproteins E1 and E2; p7), which are followed by NS proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Bartenschlager et al., 1994; Grakoui et al., 1993c; Han et al., 1991; Kim et al., 1996; Tomei et al., 1993). An alternative small ORF overlaps with C protein, but when it is triggered by a subsequent –2/+1 ribosomal frame shift, an additional viral F protein is generated (Boulant et al., 2003; Xu et al., 2001). The 3'UTR is composed of three consecutive elements: a variable ~40 nt region, a ~30–90 nt poly(U/UC) region, and a highly conserved 98 nt element, termed the X-tail, at which the HCV genome is terminated (Friebe and Bartenschlager, 2002; Kolykhalov et al., 1996; Tanaka et al., 1996). No poly(A) sequence is present at the 3' terminus of the HCV genome.

At least nine conserved SLs have been reported for the HCV genomic RNA strand. In particular, the HCV 5'UTR, the variable region of the 3'UTR, and the X-tail contain four (SL1–4) (Honda et al., 1996), two (vSL1 and vSL2) (Kolykhalov et al., 1996), and three (xSL1, xSL2, and xSL3) (Blight and Rice,

1997) SLs, respectively. Moreover, an additional SL structure, designated 5BSL3.2 and termed cis-acting RNA element (CRE), is located within the coding region of the NS5B protein (You et al., 2004). The 3'-terminal region of the HCV negative RNA strand has seven SLs and differs remarkably from the secondary structure of the corresponding complementary 5'UTR region of the genome (Smith et al., 2002). In addition to the SLs, the poly(U/UC) represents a conserved U-rich tract.

The exact structure of the 5' terminus of the HCV genome, however, is not known. The viral genome is assumed to not be capped, based on the following facts: (i) HCV does not produce the enzymes required for 5' terminus capping; (ii) the cellular apparatus for capping is located in the nucleus and hence is unavailable to cytoplasm-restricted HCV; and (iii) the IRES of HCV can function in a cap-independent manner. Moreover, HCV RNA is postulated to possess a 5'-triphosphate (5'-ppp) at its 5' terminus due to the specific initiation mechanism of RNA synthesis by viral replicase. These assumptions, however, has yet to be verified experimentally.

2.5.3. Stem-loops and their higher-order structures

The number of secondary structures, *i.e.*, SLs, in the HCV genome is large compared with the predicted potential secondary structures within the SFV genome. Bioinformatics analyses of predicted secondary structures in the genomes of +ssRNA viruses have revealed that several viruses (*Pegivirus*, *Aphtovirus*, and *Hepacivirus* genera), including HCV, contain genome-scale ordered RNA structures (GORS), which have not been found in the majority of viruses (including *Alphavirus* genus). Remarkably, compared with the 5'UTR and 3'UTR regions, a significantly greater level of RNA structure was predicted in the coding regions of viruses possessing GORS (Simmonds et al., 2004). These data suggest that the 10 experimentally validated SLs (9 of which are located in the UTRs) that are present in HCV genomic RNA may represent the tip of the iceberg. RNA SLs tend to interact to form higher-order RNA structures, which might prompt the HCV genome to fold into a spatially compact structure, exposing only the 5' and 3' UTR regions.

2.6. SFV and HCV replicases

The minimal genetic units required for the replication of SFV and HCV were defined using self-replicating subgenomic replicons, *i.e.*, genetically engineered viral RNA molecules that are shorter than full-length viral genomes and are capable of replication but incapable of producing virions. The conserved RNA elements and SLs described above have been incorporated into these subgenomic replicons. SFV and HCV devote roughly two-thirds of their protein-coding power to produce the replicases, required for their genome self-replication. The minimal replicase required for SFV replication must contain the

entire ORF1 encoded by 42S viral RNA (Liljestrom and Garoff, 1991; Xiong et al., 1989), whereas for HCV, the NS3-NS5B polyprotein region must be minimally included (Lohmann et al., 1999). For SFV and HCV, the remodeling of the polyprotein (or the region of polyprotein) that is required for the production of fully functional replicase is controlled solely by the viral protease (nsP2) or proteases (NS2-3 and NS3-4A), respectively.

2.6.1. SFV replicase

The ORF1 encoded by 42S SFV RNA is translated into the ~2,400 aa P1234 replicase precursor polyprotein immediately upon viral RNA entry into the host cell cytosol. This precursor polyprotein consists of the covalently bound and uncleaved nsP1, nsP2, nsP3, and nsP4 replicase components. Subsequently, the replicase polyprotein is sequentially cleaved by nsP2 protease to produce at least two replicases with different specificities (Merits et al., 2001; Takkinen et al., 1991; Vasiljeva et al., 2003). In infected cells, a replicase consisting of P123 polyprotein and nsP4 protein is generated autocatalytically (Vasiljeva et al., 2003) and cotranslationally (Takkinen et al., 1991). P123 and nsP4 constitute the "early replicase", which is capable of using 42S genomic RNA as a template to synthesize its complementary anti-genomic strand. The "late replicase" is represented by the complex of fully cleaved nsP1, nsP2, nsP3, and nsP4 proteins, which are capable of utilizing anti-genomic strand to synthesize 42S genomic and 26S subgenomic viral RNAs (Kaariainen and Ahola, 2002). NsP4 is a putative core catalytic RdRp for both the early and late SFV replicases. Despite the presence of a catalytic Gly-Asp-Asp (GDD) triad (Kamer and Argos, 1984), which is characteristic of RdRps, SFV nsP4 RdRp activity has not been demonstrated *in vitro*. Alphavirus nsP4 proteins possess an N-terminal tyrosine (Wellink and van Kammen, 1988), and as demonstrated for SINV, are degraded through the N-end rule pathway in host cells (Degroot et al., 1991). However, ubiquitin dependence has not been established, and no corresponding lysine residues in nsP4s have been identified (Strauss and Strauss, 1994). A recombinant full-length nsP4 with a histidine tag at its C-terminus was demonstrated to be unstable in bacteria, possibly due to a predicted disordered structure in the N-terminal 97 aa region, which was subsequently removed to enable purification (Tomar et al., 2006). Hence, the fusion of an N-terminal small ubiquitin-related modifier tag was used to obtain a full-length, tagless SINV nsP4 with an authentic tyrosine at its N-terminus (Rubach et al., 2009). Both the truncated and full-length forms of nsP4 possessed terminal adenylyl transferase activity, *i.e.*, they were capable of transferring adenosine residues to the 3' end of an RNA substrate in a non-template-dependent manner, whereas neither construct exhibited RdRp activity (Rubach et al., 2009; Tomar et al., 2006). Low-efficiency RdRp activity of SINV nsP4 was demonstrated only when a non-purified cellular P123 polyprotein fraction was supplied (Rubach et al., 2009). Thus, according to available data, SINV nsP4 is not an RdRp, whereas the early and (most likely) late replicase complexes represent active RdRps *in vitro*, hence, the boundary

between replicase and RdRp is erased. A similar situation outcome is highly likely for SFV replicase. Currently, neither crystal structures nor detailed biochemical mechanistic studies for SINV or SFV nsP4s are available.

2.6.2. HCV replicase

The HCV replicase consists of mature NS3, NS4A, NS4B, NS5A, and NS5B proteins, which constitute an approximately 2,000 aa C-terminal region of the HCV polyprotein precursor. The N-terminus of NS3 is liberated autocatalytically by NS2-3 protease (Grakoui et al., 1993a). Subsequently, NS5B is cleaved from the remaining polyprotein to produce a stable NS4AB5A polyprotein, which contains covalently bound and uncleaved NS4A, NS4B, and NS5A replicase components (Pietschmann et al., 2001). These components are subsequently fully processed to NS4A, NS4B, and NS5A (Bartenschlager et al., 1994; Pietschmann et al., 2001). Recent data have suggested that the rate of proteolytic cleavage of the replicase polyprotein, particularly the NS4B5A intermediate, might play an important role in the assembly of functional replicases in the host cell (Herod et al., 2012). RdRp, represented by NS5B protein, is the catalytic component of the HCV replicase responsible for the synthesis of viral RNA. NS5B was originally predicted to be an RdRp due to the presence of a GDD triad in its amino acid sequence (Miller and Purcell, 1990). Direct biochemical evidence of RdRp activity was obtained for a purified, full-length, non-tagged NS5B protein produced in insect cells (Behrens et al., 1996).

HCV NS5B is a membrane-bound protein with a C-terminal transmembrane helix that is essential for HCV replication (Ivashkina et al., 2002; Moradpour et al., 2004). In the majority of biochemical and structural studies, this helix is removed to facilitate purification, because it was demonstrated *in vitro* that the RdRp activity of truncated NS5B was comparable to that of the full-length protein (Lohmann et al., 1997).

2.6.3. The structure and function of the HCV RdRp

2.6.3.1. The “anatomy” of the HCV RdRp

Currently, ~150 various HCV RdRp (NS5B) crystal structures of different HCV genotypes have deposited into the Protein Data Bank (Berman et al., 2000). Crystal structures of truncated HCV NS5B polymerase, produced in bacteria were first reported in 1999 (Bressanelli et al., 1999; Lesburg et al., 1999). NS5B is a heart-shaped molecule with dimensions of approximately 70 x 60 x 40 $Å^3$; it possesses a catalytic domain (~530 aa) followed by a C-terminal domain (~40 aa), and a 21-aa transmembrane helix at its C-terminus (Bressanelli et al., 1999; Lesburg et al., 1999). The catalytic domain of NS5B consists of “fingers”, “palm”, and “thumb” subdomains (NH2-fingers-palm-thumb-COOH, Figure 4A), which were originally described for the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) due to its “anatomical” structural resemblance

to a right hand (Kohlstaedt et al., 1992). The catalytic palm subdomain of the HCV RdRp contains four structural motifs that are present in all polymerase classes (Figure 4B, C). For RdRps, motifs A ($DX_{4-5}D$, where X is a nonconserved aa), B ($GX_{2-3}TX_3N$), and C (XDD) are involved in nucleoside or nucleoside triphosphate recognition and template binding, whereas motifs A and C are also crucial for novel phosphodiester bond formation (Bressanelli et al., 1999; Kamer and Argos, 1984; Lesburg et al., 1999; Poch et al., 1989). The overall structural integrity of the catalytic palm domain is maintained by motif D (Bressanelli et al., 1999; Lesburg et al., 1999). Recent data, however, have suggested that motif D may be involved in discriminating between correct *versus* incorrect nucleotides that are to be incorporated into nascent RNA chain (Yang et al., 2012). RdRps and HIV-1 RT contain an additional motif, E, with no particular consensus sequence, which was identified at boundary of the palm and thumb subdomains and is thought to be important for interactions with the growing nascent strand (primer) synthesized by the polymerase. For HIV-1 RT, this motif is termed "primer-grip" (Huang et al., 1998; Lesburg et al., 1999). The large fragment of *Escherichia coli* DNA polymerase I (Ollis et al., 1985) and HIV-1 RT are "U"-shaped polymerases with large clefts that contain the polymerase active site, whereas the active site of the HCV RdRp is completely encircled (Lesburg et al., 1999). The fingers and thumb subdomains of the HCV RdRp interact extensively with each other. In particular, the fingers subdomain contains a palm-proximal region and a distal region, known as "fingertips" (Bressanelli et al., 1999). Two loops of the fingertips, termed Λ1 (aa 11–45) and Λ2 (aa 139–160), span the fingers and thumb subdomains encircling the active site cavity (Lesburg et al., 1999). Based on NS5B and HIV-1 RT structural alignment, RdRps has been proposed to contain novel conserved motif F ($KX_{1-2}RXI$), which is harbored by the Λ2 loop and is assumed to be important for the proper orientation of both the template and incoming nucleoside triphosphates (Lesburg et al., 1999). Loop Λ2 is conserved between NS5B and RT, whereas the Λ1 loop initially appeared to be a unique feature of the HCV RdRp (Bressanelli et al., 1999). However, the RdRps of other *Flaviviridae* family members were later discovered to exhibit similar loops (Chinnaswamy et al., 2008). A seventh predicted motif, G ($T/SX_{1-2}G$), is assumed to be important for proper template/primer orientation and was found to be conserved in many viral RdRps that are capable of primer-dependent RNA synthesis (Gorbalenya et al., 2002). Mutations in the conserved residues of the A, B, C, and F motifs were found to lead to NS5B RdRp activity inactivation, whereas a mutation ($R \rightarrow K$) in motif D increased RdRp activity both *in vitro* and in the context of self-replicating HCV replicons (Cheney et al., 2002; Lohmann et al., 1997). HCV RdRp motifs E and G and their effects on HCV RdRp activity have not yet been analyzed.

The HIV-1 RT ternary complex (RT/dNTP/DNA) structural alignment with the NS5B apoenzyme indicated that crystallized NS5B exhibits a "closed fingers" conformation, which suggests that the latter polymerase adopts a ribonucleoside triphosphate (rNTP)-bound mode (Bressanelli et al., 1999; Lesburg et al., 1999). For HIV-1 RT, the fingers have been proposed to flex after NTP binding, which is

a critical step for translocating the template to the next base because the structure adopted by RT after novel phosphodiester bond formation is different, with the fingers assuming a more open conformation (Doublie et al., 1999). Thus, the first crystal structures of HCV RdRps contradicted the unliganded (apoenzyme) theory (Bressanelli et al., 1999). However, it is now clear that the "closed fingers" conformation is the rule rather than the exception, it has been additionally demonstrated for many RdRps, including those of bacteriophage φ6 and reoviruses (Butcher et al., 2001; Tao et al., 2002). The fingers subdomains of the RdRps of reoviruses, bacteriophage φ6, and HCV are remarkably similar (Butcher et al., 2001; Tao et al., 2002). The striking ability of reovirus RdRp to catalyze phosphodiester bond formation in a crystalline state (Tao et al., 2002) indicates that no large-scale movements are required for these enzymes, in contrast to U-shaped polymerases (Doublie et al., 1999). These results also underscore the importance of the "closed fingers" conformation for these RdRps. Indeed, the opening of the HCV RdRp closed form by indirect Λ1 loop displacement triggers polymerase inactivation (Biswal et al., 2005; Labonte et al., 2002). Consequently, the HCV RdRp fingers subdomain is a critical component for maintaining overall HCV polymerase fold.

Mapping the double-stranded (template/primer) DNA structure observed in the HIV-1 RT catalytic complex onto the HCV RdRp requires two changes in polymerase conformation (Figure 4D). First, the thumb and C-terminal domain of the enzyme must be moved to accommodate a template/primer molecule. Second, the unique β-loop structural component (aa residues 443-454), which is located in the thumb subdomain, must be displaced from the active site cavity (Bressanelli et al., 1999; Lesburg et al., 1999). It was demonstrated that the C-terminal domain of φ6 RdRp was more mobile than the remaining RdRp molecule (Butcher et al., 2001). It was hypothesized that the C-terminal domain in the closed conformation arrests the incoming 3' end of template RNA in the active site cavity, providing a platform for initiation complex assembly, which is required to begin the synthesis of nascent RNA. Upon nascent RNA synthesis initiation, the C-terminal domain must be displaced to allow the resulting RNA duplex to exit (Butcher et al., 2001). The deletion of the β-loop, however, did not prevent *in vitro* NS5B RdRp activity, whereas in the context of the HCV replicon, such a deletion was deleterious for replication (Cheney et al., 2002). A recent crystal structure of the HCV RdRp with a deleted β-loop and complexed with an RNA primer/template showed that the two changes in the conformation of the polymerase that are required to allow the accommodation of dsRNA might be coupled to each other (Mosley et al., 2012). The exact mechanism of the assembly of the HCV initiation complex RdRp/RNA/rNTP remains unknown, however.

The amino acid residues in the fingers (R32 and S29) and thumb (P495, P496, V499, and R503) subdomains form an rGTP low-affinity binding site, which is located 30 Å away from the catalytic center of HCV RdRp (Bressanelli et al., 2002). This remote rGTP site might serve as either an allosteric site (Dutartre et al., 2005) or a regulator of NS5B homodimerization (Bressanelli et al., 2002; Qin et al., 2002).

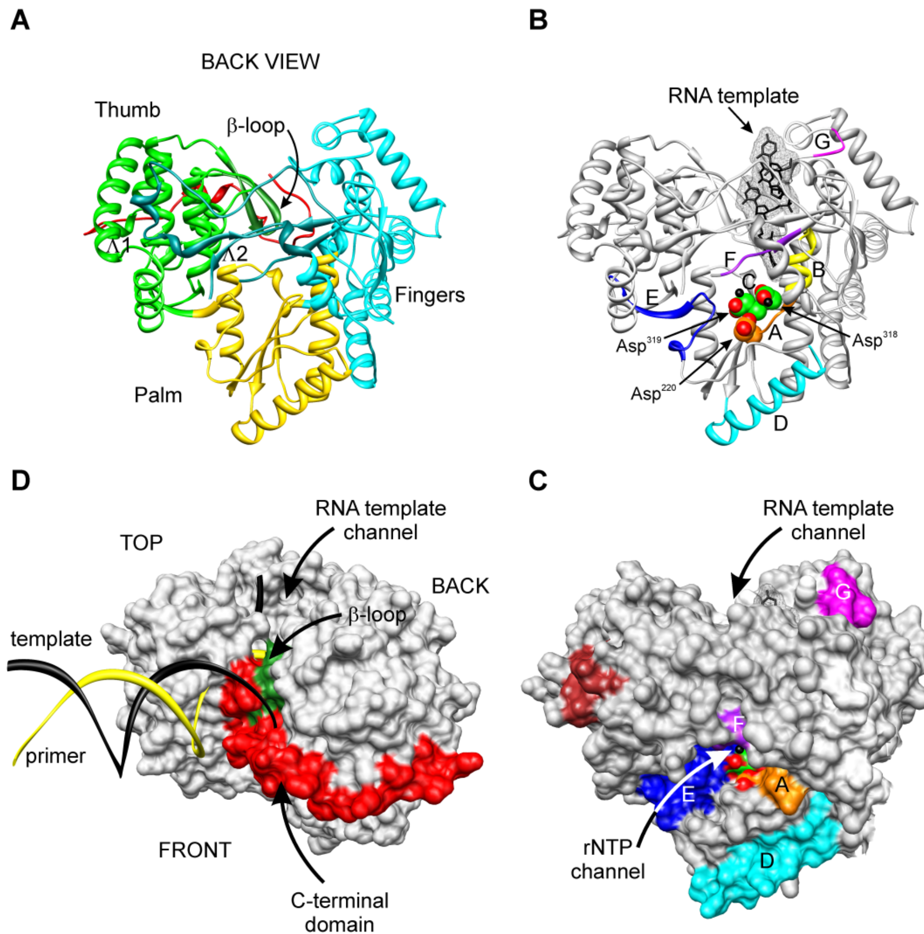

Figure 4. The structure of the HCV RdRp. Subdomains, channels, loops, and motifs are shown on the three-dimensional crystal structure of the NS5B (Protein Data Bank accession code 1c2p, genotype 1b, isolate BK) (Lesburg et al., 1999).

(A–C) Ribbon (A and B) and solid molecular surface (C) representations of the HCV RdRp "back view" are shown.

(A) Fingers, palm, thumb, and C-terminal domain are depicted in *cyan*, *gold*, *green*, and *red*, respectively. Λ1 and Λ2 loops are depicted in *dark cyan*; β-loop is depicted in *forest green*.

(B and C) RNA template and two metal ions were mapped on the 1c2p structure by superimposing the NS5B structures of 1c2p and 1nb7 (O'Farrell et al., 2003), a NS5B-RNA complex structure (genotype 1b, J4 isolate), using MatchMaker tool of the UCSF Chimera package. The RNA template and metal ions are colored *black* and are represented as sticks embedded in transparent mesh molecular surface and as spheres, respectively. Motif C catalytic aspartates and motif A aspartate are represented as *spheres* and depicted in *green* and *orange*, respectively, whereas their oxygen atoms are displayed as *red*. Identical colors are used to depict HCV RdRp motifs in both panels. In addition, rGTP allosteric binding site is colored *brown* (C).

(D) Mapping the template/primer DNA seen in the HIV-1 RT catalytic complex (Protein Data Bank accession code 1rtd) onto the HCV RdRp structure (1c2p) was performed as described in (B and C). The HCV RdRp is represented as solid molecular surface, whereas ribbon representations of HIV-1 RT primer (*yellow*) and template (*black*) are used. The C-terminal domain and β-loop are depicted in *red* and *forest green*, respectively.

See text for details. Figure was prepared with UCSF Chimera package (Pettersen et al., 2004; Sanner et al., 1996).

2.6.3.2. Initiation complex formation and catalysis: interplay between motifs (theoretical model)

The mechanism for all template-dependent polynucleotide polymerases must be both simple and universal (Steitz, 1998). The primary function of the HCV RdRp is the synthesis of a nascent RNA strand, which is complementary to the template RNA strand. The HCV RdRp is essentially a nucleotidyl transferase; it adds successive nucleotidyl moieties of rNTPs that are complementary to a template RNA strand to the 3'-hydroxyl group of a growing nascent RNA strand. Initiation complex formation is driven by the HCV RdRp motifs, which are held in place by the overall polymerase scaffold and can be imagined in the following way. The 3' terminus of the RNA template strand is guided along the surface of motifs F and G into the active site cavity, where it is bound by motif B. The C-terminal domain then blocks the exit of the RNA 3' terminus from the catalytic cavity of the polymerase. The first nucleotide (*i.e.*, the priming nucleotide, termed i) to be incorporated into the nascent RNA strand is then bound by motif E, or "primer-grip". In contrast, the incoming rNTP to be incorporated next, termed i + 1, then bound by motif F. Thus, both the i and i + 1 nucleotides are bound and stabilized by motifs A, B, E, and F, whereas phosphodiester bond formation is mediated by motif C. Motif D provides the structural support for motifs A, B, C, and E, permitting the correct spatial orientation of the catalytic site. In particular, two catalytic aspartate residues of motif C coordinate two metal ions (Mg^{2+} or Mn^{2+}), which in turn bring together the 3' hydroxyl group and the α-phosphate of the i and i + 1 rNTPs, respectively (Figure 5). The destabilization of the 3' hydroxyl group by one of the metal ions would trigger a nucleophilic attack by the hydroxyl group's electron-rich oxygen on the electron-deficient phosphorus atom of the α-phosphate, resulting in phosphodiester bond formation and pyrophosphate release (Steitz, 1998). All known polymerases use this mechanism to synthesize nucleic acids in the 5'→3' direction. Theoretically, this iterative synthetic process is then repeated until the synthesis of a full-length nascent RNA strand is complete.

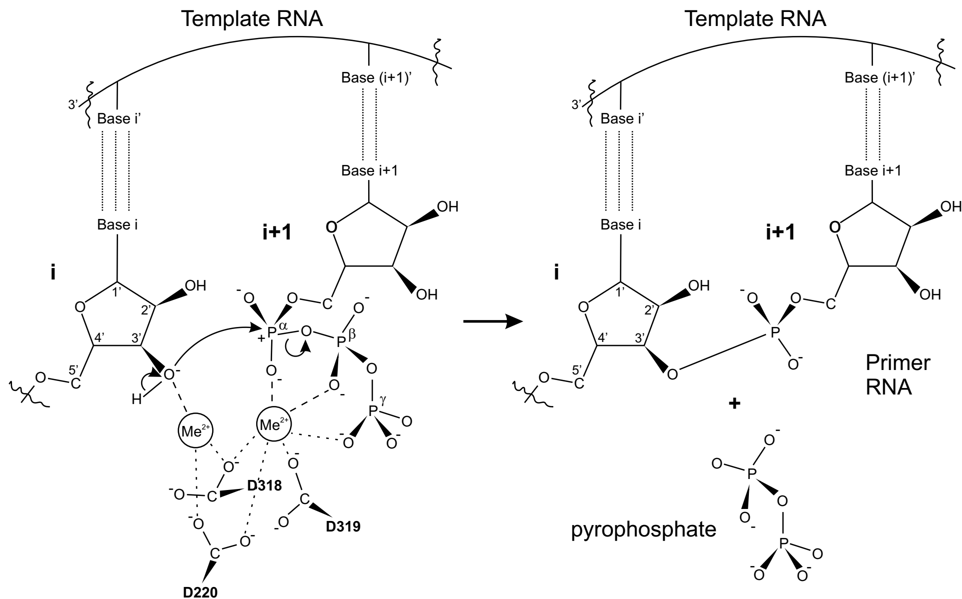

Figure 5. Simplified scheme of putative two-metal-ion mechanism of RdRp, which is based on the HCV NS5B-RNA template complex (O'Farrell et al., 2003) and general mechanism for all polymerases (Steitz, 1998). Two metal ions are ligated to the RdRp by two catalytic aspartate residues of motif C (D318 and D319), assisted by an additional aspartate residue of motif A (D220). See text for details.

2.6.3.3. The mechanisms of RNA synthesis initiation

After binding a template, an RdRp can initiate RNA synthesis *via* two possible mechanisms: primer-dependent and primer-independent (*de novo*). During *de novo* initiation, nucleotide i is represented by a single rNTP, complementary to template. Alternatively, during primer-dependent initiation, nucleotide i is the 3'-terminal nucleotidyl moiety of an oligonucleotide, *i.e.*, primer, that is complementary to the template RNA. A variation on primer-dependent RNA synthesis initiation can also occur, known as a "copy-back" mechanism, during which the 3' end of the template folds back upon itself to allow its use as both the template and the primer. In this situation, nucleotide i is the 3'-terminal nucleotidyl moiety of the template. The HCV RdRp can utilize all three mechanisms; however, the *de novo* route is assumed to be the primary mechanism of RNA synthesis initiation deployed during HCV RNA replication. The first evidence for the copy-back and primer-dependent mechanisms was obtained using both a full-length NS5B purified from insect cells and a histidine-tagged, truncated NS5B (Behrens et al., 1996; Lohmann et al., 1997). It was subsequently demonstrated that bacterially purified NS5B could utilize the *de novo* mechanism to generate full-length copies of HCV genomic RNA (Oh et al., 1999). Subsequently, large amounts of biochemical data have supported the *de novo* mechanism as the primary mechanism utilized by NS5B, revealing new

details regarding the functions of the polymerase and its template requirements (Kao et al., 1999b; Kim et al., 2000; Luo et al., 2000; Ranjith-Kumar et al., 2002; Shim et al., 2002; Sun et al., 2000; Zhong et al., 2000).

Several studies have employed the 3'UTR region of HCV RNA as a template, either alone or in the context of a full-length genome. As previously discussed, extensive SL structures are present in the HCV 3'UTR. In particular, the 3'-terminal nucleotide of the HCV genome is base-paired in the xSL1 SL within the X-tail, theoretically indicating that *de novo* initiation on this template cannot be the primary synthesis mechanism by NS5B. However, in practice it was demonstrated that N-terminally histidine-tagged NS5B full-length enzyme was capable of efficient RNA synthesis on a 3'UTR template (Oh et al., 1999). In contrast, a C-terminally histidine-tagged and truncated NS5B could not efficiently catalyze *de novo* synthesis on a 3'UTR template;aberrant or copy-back products were predominantly generated (Lohmann et al., 1997; Reigadas et al., 2001; Zhong et al., 2000). All C-terminally tagged NS5B proteins contain a deletion of at least 21 aa, which exposes the C-terminal domain, which is thought to be important for *de novo* initiation complex formation, as discussed previously. Thus, C-terminal fusions are likely to destabilize the C-terminal domain and to introduce bias into the selection of an RNA synthesis mechanism by the polymerase. It is known that the location of histidine tag may influence NS5B function. Analyses of different N- and C-terminal versions of histidine-tagged NS5B enzymes have demonstrated that N-terminal tags result in enzymes with a markedly reduced affinity for the template/primer substrate (McKercher et al., 2004). It was demonstrated for HCV NS5B that the affinity for the template RNA substrate was inversely proportional to the efficiency of the nascent RNA synthesis (Lohmann et al., 1997). Thus, N-terminally histidine-tagged NS5B might simply be more active on a 3'UTR template. Moreover, a large majority of 3'UTR RNA templates were prepared using *in vitro* transcription with T7 RNA polymerase, which is known to add nucleotides to the 3' terminus of synthesized RNA in a template-independent fashion (Kao et al., 1999a), thus liberating the 3' nucleotidyl moiety required for optimal *de novo* RNA synthesis initiation *in vitro*.

2.6.3.4. Initiation, elongation, and termination of *de novo* RNA synthesis

Synthetic artificial RNA templates have been used extensively to examine the HCV RdRp preference for initiation nucleotide i. The HCV RdRp was found to show a strong preference for a purine (G or A, particularly G) as the initiating nucleotide (Ranjith-Kumar et al., 2002; Shim et al., 2002). Remarkably, G, GMP, GDP, and GTP have all been used by HCV NS5B as initiating nucleotides (Ranjith-Kumar et al., 2002; Shim et al., 2002), with a particular preference for GDP and GMP (Ranjith-Kumar et al., 2002). These findings indicate that the 5' terminal structures of HCV genomic and antigenomic RNA strands might not, in fact, be 5' triphosphates, contrary to the current widespread belief.

After the rapid synthesis of the first phosphodiester bond between nucleotides i and i + 1, a 5'-ppp(i)p(i + 1)-OH 3' dinucleotide is formed and subsequently used as a primer (Dutartre et al., 2005; Shim et al., 2002). Large amounts of this dinucleotide accumulate during *de novo* initiation by NS5B, suggesting that the initiation complex is fragile and that synthesis is frequently aborted. Hence, it was concluded that the transition from a dinucleotide to a trinucleotide represents the rate-limiting step in the transition from initiation to elongation complex (Shim et al., 2002). Subsequently, it was found that the transition from a pentanucleotide to a highly polymerized product was the second rate-limiting step in elongation complex formation (Dutartre et al., 2005). The introduction of a P495L mutation, which prevents rGTP from binding to its putative allosteric site (Bressanelli et al., 2002), led to a reduction in the formation of pentanucleotides and larger RNA products, suggesting that this site should be occupied to enable the transition from initiation to elongation (Dutartre et al., 2005).

Such mechanistic insights into the functions of NS5B were facilitated by the characterization of the mode of action of "batteries" of small-molecule inhibitors targeting this medically important enzyme. Inhibitors of the HCV RdRp can be classified into two categories: nucleoside analogues (NAs) (Carroll et al., 2003; Dutartre et al., 2005) and nonnucleoside inhibitors (NNIs) (Biswal et al., 2005; Dhanak et al., 2002; Di Marco et al., 2005; Gu et al., 2003a; Love et al., 2003; Wang et al., 2003). NAs are first converted to nucleoside triphosphates by the cell; they then compete with natural counterparts in the active site cavity to terminate RNA synthesis upon incorporation (elongation inhibitors). In contrast, NNIs are allosteric inhibitors that impede the initiation of RNA synthesis. Remarkably, all known binding sites for NNIs are located exclusively in the palm and thumb subdomains of the HCV RdRp (Koch and Narjes, 2006; Pauwels et al., 2007; Sofia et al., 2012). Thus, deeper mechanistic insights into the fingers subdomain have not been enabled by rational drug design and combinatorial chemistry approaches.

A recent technological advance, the *in vitro* directed formation of extremely stable elongation NS5B•template•primer complexes (half-life ~1 week) (Jin et al., 2012; Powdrill et al., 2011), has provided new insights into the function of the HCV RdRp. Both studies employed N-terminally histidine-tagged, truncated NS5B expressed in bacteria. The average rate of nucleotide incorporation was determined to be in the range 4-18.5 nt/second (Jin et al., 2012; Powdrill et al., 2011), which correlated well with the rate determined for long RNA templates, 2.5-11.7 nt/second (Oh et al., 1999; Tomei et al., 2000). Assembled elongation complexes move in the 3'→5' direction relative to the template RNA and synthesize complementary full-length copies in the 5'→3' direction. The termination of RNA synthesis is not currently well understood; however, studies of elongation complexes might illuminate this process.

2.6.3.5. A novel NS5B enzymatic function and bias in error rate

It is generally assumed that viral RdRps are extremely error-prone because these enzymes are unable to correct (proofread) "mistakes", *i.e.*, misincorporated nucleotides. As a result, viral RdRps are considered to be the source of genetic diversity and of mutant viruses, or so-called "quasi-species" (Eigen and Schuster, 1977). However, the absence of RdRp proofreading activity might be complemented by a specialized viral protein with 3'→5' exoribonuclease activity, enabling replicase proofreading, as was found for coronavirus, the +ssRNA virus with the largest nonsegmented genome (~30,000 nt) (Minskaia et al., 2006). However, no studies have reported that viral RdRp is capable of excising misincorporated nucleotides.

Recently, however, NS5B elongation complexes were used to demonstrate that NS5B had the capacity to excise the terminal nucleotide of a growing nascent strand (Jin et al., 2013). In particular, it was demonstrated that the γ-phosphate nucleophilic oxygen of an incoming i + 1 rNTP non-complementary to the template could attack the terminal phosphodiester bond of the i nucleotidyl moiety of nascent RNA. As a result, a dinucleotide tetraphosphate was generated and released from the elongation complex (Jin et al., 2013). HIV-1 RT was demonstrated to catalyze a similar terminal excision reaction (Meyer et al., 1999), although, this reaction was 100-fold less efficient than the excision catalyzed by the HCV NS5B (Jin et al., 2013). The discovery of this novel reaction prompted a re-evaluation of the definition of fidelity for HCV NS5B, suggesting that other viral RdRps may exhibit similar activities.

Another report utilizing NS5B elongation complexes revealed that a mutational bias favoring G:U/U:G mismatches was introduced by viral RdRp (Powdrill et al., 2011). An error rate of $3.2\text{-}8.7 \times 10^{-3}$ per site was found for such mismatches, which was 10- to 100-fold higher compared with other possible mutations. Importantly, such transitions (purine↔purine and pyrimidine↔pyrimidine) are 75-fold more abundant than transversions (purine↔pyrimidine) in HCV-infected patients (Powdrill et al., 2011). Hence, non-Watson-Crick base pairs are formed, preserving the overall RNA secondary structure.

2.6.3.6. RNA templates used by HCV RdRp

In addition to its native HCV RNA templates, NS5B is capable of both primer-dependent and *de novo* RNA synthesis *in vitro* using various templates: homopolymeric (Behrens et al., 1996; Lohmann et al., 1997), heteropolymeric (Ferrari et al., 2008), circular (Ranjith-Kumar and Kao, 2006), and even cellular mRNAs (Behrens et al., 1996). NS5B exhibits no particular sequence specificity when binding to these templates, with the exception of homopolymeric templates; *i.e.*, the HCV RdRp prefers poly(U) over poly(G), poly(A), or poly(C) (Lohmann et al., 1997). Thus, as suggested by Behrens and colleagues, "the NS5B is necessary but not sufficient to direct the replication of the HCV

genome" (Behrens et al., 1996). Other HCV replicase components must play a role in restricting NS5B RdRp activity exclusively to viral RNA.

2.6.4. Functions of non-RdRp components of the SFV and HCV replicases

All of the components of the SFV and HCV replicases are absolutely required for viral RNA replication inside host cells. The components of viral replicase assemble at the intracellular membranes of host cells, recruit viral template RNA, and induce multiple invaginations harboring replication complexes, which are connected to the cytosol (Paul and Bartenschlager, 2013). These numerous virus-induced vesicles are termed "spherules" and "double-membrane vesicles" (DMVs), for SFV and HCV, respectively (Kujala et al., 2001; Romero-Brey et al., 2012). Inside the vesicles, viral RdRp and other non-RdRp replicase components function to generate multiple full-length copies of the viral genome. Although the expression of single HCV replicase components can trigger vesicles formation, the structure of these vesicles differs from that of vesicles generated by the expression of the full replicase (Romero-Brey et al., 2012). For HCV, however, the abundant induction of vesicles by its viral replicase does not depend on RNA replication (no viral RNA template must be present) (Romero-Brey et al., 2012), whereas abundant SFV spherules are produced only when viral RNA is actively replicated (Spuul et al., 2011). More precisely, when SFV replicase was expressed in the absence of replicon RNA template, ~10 spherule-like structures were observed, whereas more than 2,000 spherules were present when replicon RNA was supplied (Spuul et al., 2011).

Importantly, in cells containing subgenomic HCV replicons or in cells infected by SFV or HCV, viral replicases are produced in considerably larger amounts than required for viral replication (Kujala et al., 2001; Quinkert et al., 2005; Romero-Brey et al., 2012; Spuul et al., 2010). For example, more than 95% of HCV replicases are not involved in subgenomic RNA synthesis (Quinkert et al., 2005). Similar phenomena have been observed in the context of SFV and SINV infection (Frolova et al., 2010; Spuul et al., 2010).

The non-RdRp components of the SFV and HCV replicases are briefly described below. There are striking similarities between the penultimate components of the SFV and HCV replicase polyproteins, nsP3 and NS5A, respectively.

2.6.4.1. Other components of the SFV replicase complex

The only membrane-bound protein of the SFV replicase complex is the nsP1 protein, the middle region of which contains a specific lipid-binding region, an amphipathic α-helix, which tethers the replicase to the membrane (Lampio et al., 2000). The nsP1-host cell membrane interactions are enhanced by the S-acylation (palmitoylation) of three C-terminal cysteine residues (Laakkonen et

al., 1996). However, the expression of the entire replicase ORF is required for the formation of spherules (Kujala et al., 2001).

The viral nsP1 and nsP2 proteins are critical for the proper 5' terminus processing of SFV genomic and subgenomic RNAs, known as capping reactions. First, nsP2, a viral RNA triphosphatase, removes the γ-phosphate from the 5' terminal nucleotides of viral genomic and subgenomic RNAs (Vasiljeva et al., 2000). Second, nsP1 transfers a methyl group from S-adenosylmethionine to rGTP (guanine-7-methyltransferase, a unique activity not present in cells), hydrolyzes this methylated rGTP to form rGMP (Laakkonen et al., 1994), and finally transfers the resulting m7-GMP to the nsP2-processed 5' terminus of the template RNA (guanylyltransferase), forming a pyrophosphate linkage (Ahola and Kaariainen, 1995).

The enzymatic activities of nsP2 are not limited to protease, which is required for the cleavage of the replicase polyprotein, and RNA 5'-triphosphatase, which is required for processing viral RNA templates. In addition, nsP2 functions as a nucleoside triphosphatase (NTPase) (Rikkonen et al., 1994) and an RNA helicase (Gomez de Cedron et al., 1999). nsP2 also possesses a nuclear localization signal, which is critical for the pathogenicity of SFV4 in animals (Fazakerley et al., 2002; Peranen et al., 1990; Rikkonen, 1996; Rikkonen et al., 1992).

The variable, natively unfolded C-terminal region of the viral protein nsP3 (Neuvonen and Ahola, 2009) is phosphorylated at threonine and, especially, serine residues (17 potential phosphorylation sites in a ~100-aa region) (Vihinen and Saarinen, 2000). The nsP3 phosphorylation state is higher when this protein is a part of the viral replicase (Peranen et al., 1988). The elimination of phosphorylation greatly reduces the pathogenicity of SFV (Vihinen et al., 2001). The N-terminal region of nsP3 contains an RNA-binding macro domain, which has a high affinity for poly(ADP-ribose) or poly(A) and a low affinity for monomeric ADP-ribose (Neuvonen and Ahola, 2009). A zinc ion-binding domain with four conserved cysteines, which is invariant in all alphaviruses, is located in the middle of SINV nsP3, between the N-terminal macro-domain and C-terminal phosphorylation sites (Shin et al., 2012).

2.6.4.2. Non-RdRp components of HCV replicase

The components of the HCV replicase are either tethered to cellular membranes by in-plane amphipathic and transmembrane helices (NS4A, NS5A, and NS5B) or combine both types of helices in multiple copies to span the membrane (NS4B) (Brass et al., 2008; Brass et al., 2002; Ivashkina et al., 2002; Lundin et al., 2003). NS3 is the only viral protein that is not directly bound to the membrane: the NS4A cofactor tethers NS3 by forming a non-covalent NS3-4A complex (Brass et al., 2008).

In addition to its protease function, which is essential for the cleavage of the replicase polyprotein, NS3 has RNA helicase activity and RNA-stimulated NTPase activity associated with its C-terminal domain. In addition, the binding

of ssRNA to the N-terminal protease domain of NS3 inhibits its protease activity (Gallinari et al., 1998; Vaughan et al., 2012).

NS4A, the NS3 protease cofactor, also regulates the phosphorylation state of NS5A (Asabe et al., 1997). It was demonstrated that certain amino acid substitutions in the C-terminal NS4A acidic domain, which prevented the hyperphosphorylation of NS5A (Koch and Bartenschlager, 1999), also blocked HCV RNA replication (Lindenbach et al., 2007). Because the acidic domain would be unable to form a helical structure at neutral pH, it is assumed that local interactions with positively charged surfaces might induce helix formation; therefore, it is believed that this NS4A domain can act as an electrostatic switch (Lindenbach et al., 2007; Montserret et al., 2000).

The NS4B integral membrane protein was identified as the main viral protein responsible for the formation of tight membrane structures, which consist of vesicles in a membranous matrix, termed a "membranous web" (Egger et al., 2002). NS4B is capable of oligomerization in the membrane environment (Gouttenoire et al., 2009). Further studies revealed that the expression of NS4B (and of NS3-4A and NS5B) led to the formation of single membrane vesicles, whereas the expression of either NS5A or the entire replicase was required for DMV formation, which was associated with HCV replication (Romero-Brey et al., 2012) and nascent RNA synthesis (Paul et al., 2013). In addition, NTPase, GDP hydrolysis, and adenylate kinase activities (Thompson et al., 2009) have been associated with NS4B, which is also capable of binding RNA (Einav et al., 2010).

The NS5A replicase protein contains one structured N-terminal domain and two natively unfolded C-terminal domains (Hanoulle et al., 2009; Verdegem et al., 2011), which are separated by low-complexity sequences (LCS1 and LCS2) (Tellinghuisen et al., 2004). The structured N-terminal domain contains one zinc ion-binding site with four conserved cysteines, which are key for HCV RNA replication (Tellinghuisen et al., 2004). Two forms of NS5A have been described: hypophosphorylated and hyperphosphorylated. The phosphorylation of three serines, which are located in LCS1, is crucial for the transition to the hyperphosphorylated state (Tanji et al., 1995). The crystallization of a structured NS5A domain in the form of a dimer, linked by a disulfide bond revealed a novel structural fold, which was predicted to be capable of interacting with RNA (Tellinghuisen et al., 2005). This structured domain and LCS1 were later demonstrated to be crucial for RNA binding, whereas the remaining portion of NS5A was not involved in RNA binding (Hwang et al., 2010).

2.7. Replication of the SFV and HCV genomes

The replication of +ssRNA viruses is highly asymmetric: the positive genomic RNA strands are always produced in excess, compared with the negative strands. Typically, the number of negative strands present in infected cells is correlated with the number of replication complexes, which are shielded from

the cytosol by structures such as spherules or DMV. Hence, the negative RNA strands of RNA viruses are thought to be protected and to not exist as free molecules, although this situation has not been demonstrated.

A comparison of the data on SFV and HCV replication (see below) indicates that a single SFV-infected cell may contain as many as 7,000 replication complexes, whereas only ~40 replication complexes might be present in a cell infected with HCV (Keum et al., 2012; Wang et al., 1991). The difference in the replication of these viruses translates into different phenotypes observed in cultured cells. In particular, SFV kills a wide spectrum of cells within 24 to 48 hours after infection, whereas HCV is capable of moderate self-reproduction in a single cell line and its derivatives only, and infection by HCV does not typically induce cell death.

2.7.1. SFV

It is assumed that a single molecule of the SFV RNA genome is capable of initiating infection. Upon the entry of the SFV genome into the cytosol of the host cell, the early replicase is produced, which subsequently recognizes CSEs at the 3' end of the genomic RNA and recruits viral RNA into spherules to catalyze the synthesis of complementary anti-genomic 42S negative-strand RNA (42S –ssRNA). After the completion of 42S –ssRNA synthesis, the early replicase is transformed into the late replicase. After the recognition of the corresponding 3' CSEs, the late replicase produces extremely high levels of genomic (42S +ssRNA) and subgenomic (26S +ssRNA) viral RNA in an asymmetric manner. In particular, 42S –ssRNA synthesis is accompanied by 42S +ssRNA synthesis (at a ~1:5 molar ratio), which is then halted after ~4 hours (Sawicki and Sawicki, 1980) due to a change in viral replicase specificity. A single cell has been estimated to contain ~150,000 copies of 42S and 26S viral +ssRNAs 8 hours after infection (Tuomi et al., 1975), whereas the number of negative strands is approximately 7,000 (Wang et al., 1991).

The replicative form (RF) of SFV RNA has been isolated; it is a ±dsRNA consisting of capped 42S +ssRNA and uncapped 42S –ssRNA with an extra unpaired guanosine at its 3' terminus (Wengler and Gross, 1979). The 5' terminus of the negative strand of the alphaviral RF either possesses shorter poly(U) sequences compared with the poly(A) tail of the positive strand (Sawicki and Gomatos, 1976) or has no poly(U) sequences at all (Hardy, 2006; Hardy and Rice, 2005). The number of phosphates at the 5' terminus of 42S –ssRNA remains unknown (Kaariainen and Ahola, 2002; Wengler and Gross, 1982). It is assumed that in SFV-infected cells, all of the 42S –ssRNAs are in the RF configuration, protected by spherules; no evidence for free 42S –ssRNA molecules has been reported.

An interesting SFV replication model has been proposed. According to this model, because the replicase is membrane-bound, the viral RNA template (42S –ssRNA) is hypothesized to move from one replicase to another in a circular manner, accompanied by the synthesis of nascent capped 42S and 26S positive

strands, which are secreted into the cytosol (Kaariainen and Ahola, 2002). This model is particularly attractive in the context of HCV due to several reports describing the short-range (Friebe et al., 2005; Kim et al., 2003) and long-range interactions (Romero-Lopez and Berzal-Herranz, 2009) between conserved RNA regions and SLs of the HCV viral genome, including those present in 5' and 3' UTRs.

2.7.2. HCV

It is assumed that after the HCV genome enters the cytosol, the viral replicase is produced. Subsequently, the viral replicase recognizes RNA SLs and the conserved regions of the 3'UTR, and it assembles in the proximity of the 3' terminus of the viral genome. Next, the *de novo* initiation mechanism might be utilized by the RdRp component to produce full-length complementary negative-strand RNA, which is subsequently used by the same replicase for the overproduction of positive strands. The existence of viral dsRNA RF has been hypothesized; however, it has not been demonstrated. The exact sequence of events beginning with the viral genome and replicase is speculative and not experimentally well supported, largely because the HCV RF has not been isolated or characterized. The presence of GORS in the HCV genome strongly suggests that the HCV RF might not double-stranded.

HCV replication studies have been made possible largely due to the generation of efficient cell culture systems based on HCV subgenomic replicons (Lohmann et al., 1999); these cell culture systems have since been improved (Frese et al., 2003; Krieger et al., 2001). A recent study of the dynamics of the levels of synthesis of HCV positive and negative RNA strands during virus infection in cell culture (Keum et al., 2012) revealed striking similarities to data obtained using HCV replicons (Binder et al., 2007; Krieger et al., 2001; Quinkert et al., 2005). The viral RNAs entering the cytosol of infected cells were rapidly degraded. In particular, an initial 10-fold decrease in viral RNA genomes was observed (Keum et al., 2012), whereas replicons were decreased by up to ~100-fold (Krieger et al., 2001). The negative strand of viral RNA could be detected only 9 hours after infection (Keum et al., 2012), indicating that a lag-phase was most likely required for both the accumulation of the replicase and the biogenesis of replication complexes. Twelve hours after infection, ~3 copies of each positive and negative viral RNA strand were observed (Keum et al., 2012). The asymmetric replication characteristic for +ssRNA virus was observed only 24 hours after infection, when a ~10:1 ratio of positive (~400 copies) to negative strands (~40 copies) was established (Keum et al., 2012). The identical number of negative strands and approximately the same ratio were obtained using a subgenomic replicon (Binder et al., 2007; Quinkert et al., 2005). A maximum of ~800 and ~2,500 copies of positive strands per cell were produced in the replicon system and during HCV infection, respectively (Keum et al., 2012; Quinkert et al., 2005).

2.8. Replication of the genomes of other RNA viruses: appearance of RF

The replicase function of the viral RdRp is typically associated with the appearance of the viral RF, *i.e.*, double-stranded RNA, in infected cells. In contrast, transcription is associated with the production of single-stranded mRNA molecules. During the infection process, the RdRps of –ssRNA, ±ssRNA, and ±dsRNA viruses function initially as transcriptases and subsequently as replicases, which delays the possible appearance of viral RF dsRNA molecules.

As previously discussed for SFV and HCV, the NCs of +ssRNA viruses are immediately disassembled, and a single “naked” viral RNA molecule is released into the cytosol. This viral RNA is subsequently recruited by the cell-produced replicase to a spherule or DMV, collectively termed “viral factories” (den Boon and Ahlquist, 2010), in which viral RdRps function as replicases to generate multiple full-length copies of the viral genome. In some +ssRNA viruses (*e.g.*, SFV), RdRp simultaneously functions as a replicase and a transcriptase, with the latter generating shorter subgenomic RNAs (*e.g.*, 26S SFV RNA).

In contrast to +ssRNA viruses, the viral RNAs and RdRps of other RNA viruses are held together tightly in partially uncoated virions (subviral particles) or in RNP complexes upon entry into the cytosol or nucleus. For these viruses, virion RdRps, which are associated with incoming RNPs or subviral particles, function initially as transcriptases to produce multiple uncoated, single-stranded mRNA molecules, which are used for the production or viral RdRps, nucleoproteins, and coat proteins. It is assumed that in –ssRNA and ±ssRNA viruses, the replicase function of RdRp critically depends on nucleoprotein synthesis because both newly formed cRNA and vRNA must be coated with nucleoproteins (Medcalf et al., 1999; Newcomb et al., 2009). Recently, RNP containing cRNA (cRNP) was purified from influenza A-infected cells. The synthesis of vRNA by this cRNP was found to critically depend on the addition of exogenous RdRp (York et al., 2013). Thus, it appears that both the newly synthesized RdRp and nucleoprotein molecules are required for the incoming RNP-resident RdRp transcriptase-to-replicase function transition. No “viral factories” are formed during –ssRNA and ±ssRNA virus replication.

The mRNAs produced by ±dsRNA virus RdRps in transcriptase mode are used for the synthesis of protein and complementary negative strands of RNA for the formation of “progeny” ±dsRNA genomes (Patton and Spencer, 2000). However, the RdRp of the incoming subviral particles serves only as a transcriptase; subviral particles containing RdRp that functions as a replicase are assembled later in infection, in sites called “viroplasms” constructed purely of viral proteins (Broering et al., 2004; Parker et al., 2002).

2.9. Detection of RNA virus infection by the host cell

2.9.1. Viral dsRNA or RF activates the type I interferon (IFN) system

By the early 1950s, a large body of *in vivo* evidence had accumulated regarding the successive infection of animals with various viruses. In particular, it was determined that the infection of an animal with one virus frequently prevents or alleviates the subsequent infection of the same animal with another virus, which was termed "interference phenomena" (Henle, 1950). A soluble factor was then found to be released by the membranes of chick embryos *in vitro* in response to coincubation with heat-inactivated influenza virus. This factor interfered with the growth of live influenza virus in pre-treated membranes; thus, it was termed "interferon" (IFN) (Isaacs and Lindenmann, 1957). The identification of IFN inducers represented a clear medical opportunity for treating viral diseases. Antiviral activity was demonstrated in extracts of the fungus *Penicillum funiculosum* (Rytel et al., 1966; Shope, 1953, 1966), and subsequent biochemical analyses of these extracts showed that the molecule responsible for IFN induction was dsRNA. It was suggested that the presence of dsRNA in mold mycelia was due to infection by a hypothetical RNA virus and represented dsRNA RF (Lampson et al., 1967). The dsRNA genomes isolated from reovirus virions (Tytell et al., 1967) and the dsRNA complexes of synthetic polyriboinosinic and polyribocytidylic acids (poly(I:C)) (Field et al., 1968) were also highly active in inducing IFN, which was subsequently identified as a glycoprotein (Dorner et al., 1973). Thus, it was discovered that vertebrate cells had an antiviral defense system, later termed innate immunity, that was capable of recognizing dsRNA molecules and inducing an antiviral state characterized by the production of IFNs.

Nearly 20 years later a "modern" theory of innate immunity was developed (Lemaitre et al., 1996; Medzhitov and Janeway, 1997; Medzhitov et al., 1997; Poltorak et al., 1998). According to this theory, virtually every cell in an organism can utilize germline-encoded receptors to recognize pathogens. These receptors, designated pathogen recognition receptors (PRRs), recognize and bind to fragments of the pathogen's own structural or pathogen-induced molecules, also called pathogen-associated molecular patterns (PAMPs), to trigger an anti-pathogenic response aimed at destroying the pathogen (Janeway, 1989). The universality of the innate immune system and the limited number of PRRs suggests that PAMPs must represent unique molecular hallmarks that are shared by several pathogen groups (Medzhitov and Janeway, 1997). In vertebrates, three PRR families of innate immune sensors that detect virus-specific components have been identified: nucleotide oligomerization domain (NOD)-like receptors (NLRs), Toll-like receptors (TLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Only TLRs and RLRs, however, are essential for type I IFN induction (Takeuchi and Akira, 2010).

Thus, in the 1970s, a subsystem of innate immunity was discovered. Thus, the large class of RNA viruses is recognized through dsRNA RF, which represents the characteristic PAMP of these viruses. RLRs are major germline-

encoded PRRs that recognize the cytosolic dsRNA generated by RNA viruses (Takeuchi and Akira, 2010). This recognition initiates signaling events that lead to the activation of a host cell antiviral defense system mediated by type I IFNs, *e.g.*, IFN-α, IFN-β, and IFN-ω. (Pestka et al., 2004; Samuel, 2001). Currently, it is believed that type I IFNs are triggered exclusively by either viral dsRNA (Kato et al., 2008) or viral dsRNA with a triphosphate at its 5' terminus (5'ppp-dsRNA) (Schlee et al., 2009; Schmidt et al., 2009).

2.9.2. Limitations of viral dsRNA/RF detection by RLRs

Most of the experiments aimed at elucidating of the RNA virus PAMPs that are responsible for activating the innate immune system have been performed either with synthetic model RNAs such as poly(I:C) or with RNAs generated by phage polymerases, *e.g.*, T7 RNA polymerase. The optimal RNA ligands for RLRs, in particular for the RIG-I receptor, were determined through such experiments. These results were subsequently applied to the RNA genomes of viruses and their RFs, typically without direct experimental evidence. Such assumptions require not only the availability of detailed information regarding the RF and but also (preferably) the demonstration of an interaction between the RF and the RLR in the context of viral infection. Obtaining such evidence is extremely challenging experimentally. Such experiments have been attempted for –ssRNA viruses; however, no conclusive data have been obtained, and the natural RIG-I ligands remain unknown.

As previously noted, it is believed that viral dsRNAs or RFs do not exist as free molecules in the cytosol. The following limitations associated with dsRNA detection by RLRs are obvious when considered in the context of RNA virus infections. First, for –ssRNA or ±ssRNA viruses, the formation of dsRNA during replication and transcription is prevented by nucleoproteins that are associated with vRNA and cRNA. Second, for ±dsRNA viruses, no dsRNA is formed during transcription due to the unique structure of viral RdRp and its localization in subviral particles (Tao et al., 2002). dsRNA is formed during the replication of these viruses, but this event occurs in viroplasms or subviral particles. Third, for +ssRNA viruses, dsRNA is formed during replication; because this process occurs in viral factories, these dsRNA molecules are not easily accessible to cytosolic RLRs.

In particular, it was demonstrated that HCV viral factories were connected to the cytosol and enabled access of rNTPs, whereas access by 17–29 kDa proteins was prevented (Quinkert et al., 2005). Moreover, it was demonstrated that dsRNA was detected specifically in DMVs formed during HCV replication (Paul et al., 2013). It remains unknown whether the dsRNA detected in cells infected by HCV (Romero-Brey et al., 2012) or in the DMVs (Paul et al., 2013) of cells harboring HCV replicons corresponds to RF or to an extensive secondary genomic structure (*i.e.*, GORS). For SFV, there is no direct evidence of dsRNA in spherules; however, as previously noted, SFV RFs have been

experimentally isolated from infected cells (Wengler and Gross, 1979). Consequently, the RFs of HCV and SFV are not easily accessible to the innate immune system of the host cell.

2.9.3. Side effects of replicase components

The components of viral replicases are naturally assumed to play a direct role in the replication of HCV and SFV viral RNAs. However, viral replicases and their components are involved in many cellular processes, in addition to viral RNA replication. These processes are typically considered side effects. The side effects of viral proteases and RdRps are discussed below. Although the side effects of HCV and SFV proteases are well established, RdRp side effects are only beginning to emerge.

2.9.3.1. Viral protease counteracts the type I IFN system

It is generally assumed that viral proteins, particularly viral replicase proteins not participating in replicase assembly, either lose their activity or function to counteract the type I IFN system or interfere with the metabolic processes of the cell to promote efficient viral replication. For HCV and SFV, the ability to inhibit the type I IFN response is largely attributed to viral proteases, whereas the contribution of other replicase proteins is not as pronounced.

The HCV NS3-4A protease "cuts off" both "arms" of the type I IFN system. First, it was demonstrated that NS3-4A cleaved the Toll-IL1 receptor domain-containing adaptor inducing IFN-β (TRIF), an adaptor protein that is essential for activating antiviral defenses in response to PAMPs, which are recognized by TLRs (Li et al., 2005b). Second, it was found that NS3-4A protease cleaved the CARD (caspase activation and recruitment domains) adaptor inducing IFN-β (CARDIF), another adaptor protein required for proper RIG-I signaling (Meylan et al., 2005). Finally, NS3-4A was found to cleave yet another host cell target that is responsible for the activation of RIG-I receptor (Oshiumi et al., 2013).

The mechanism of type I IFN system antagonism by the SFV nsP2 protein remains unclear. However, it was demonstrated that a single mutation in nsP2 preventing its nuclear localization affects the neurovirulence of SFV *in vivo* (Fazakerley et al., 2002; Rikkonen, 1996) and affected type I IFN production in cell culture, suggesting a central role for nsP2 in suppressing the cellular antiviral response (Breakwell et al., 2007). In addition, SFV nsP2 has been proposed to induce the degradation (but not in a protease-dependent fashion) of the catalytic unit of a cellular RNA polymerase II (Akhrymuk et al., 2012). Additional functions, such as the global shutoff of host cell macromolecular synthesis and the inhibition of IFN-induced signaling, have been attributed to the nsP2 proteins of other alphaviruses (Frolova et al., 2002; Fros et al., 2010; Garmashova et al., 2006). No cellular targets of nsP2 protease activity have been demonstrated thus far.

2.9.3.2. Viral RdRp: an activator of the type I IFN system?

Accumulating evidence suggests a novel function for the RdRps of +ssRNA viruses. In particular, several studies have demonstrated that the RdRps of vertebrate-infecting viruses are capable of activating the type I IFN system in the host cell. First, the transient expression of HCV NS5B activated the IFN-β promoter in several human cell lines (Dansako et al., 2005; Moriyama et al., 2007; Naka et al., 2006) and induced the secretion of type I IFNs by mouse hepatocytes both in cell culture and *in vivo* (Yu et al., 2012). Second, the activation of the IFN-β promoter was also triggered by the RdRps of human and murine noroviruses (Subba-Reddy et al., 2011). Third, increased basal IFN transcript levels were detected in transgenic mice expressing the RdRp of Theiler's murine encephalitis virus (TMEV), which protected the mice from subsequent TMEV infection (Kerkvliet et al., 2010).

Collectively, these results indicate that the type I IFN system can be activated directly by viral RdRps without a requirement for replication factory-hidden dsRNA RF exposure to cytosolic RLR sensors. However, the mechanism and role of this innate immune system activation in the context of viral infection have not been determined for any of these viruses.

Given that more than 10^6 copies of NS5B are synthesized by a single cell in a replicon system and that 95% of these molecules are not involved in viral replication (Quinkert et al., 2005), the elucidation of this novel HCV RdRp function is of particular interest. However, as previously noted, RLRs and TLRs are critical for the activation of type I IFN system in response to RNA virus infection. The HCV and its subgenomic replicons are capable of self-replication in a single cell line (Huh7) derived from differentiated hepatocellular carcinoma (Nakabayashi et al., 1982), which is either defective for or produces extremely low amounts of the TLR3 receptor, which is critical for dsRNA detection in the endosomes (Li et al., 2005a). Huh7 cell line derivatives (Huh7.5 (Blight et al., 2002) and Huh7.5.1 (Zhong et al., 2005)), which were produced by first establishing cell lines harboring HCV subgenomic replicons and then eliminating these replicons through prolonged treatment with IFN-α, are highly permissive for HCV replication but contain an additional inactivating mutation in the gene encoding RIG-I (Sumpter et al., 2005). Thus, the type I IFN system is severely blunted in Huh7 cells, which prevents analyses of possible role for NS5B in eliciting the host cell antiviral response in the context of HCV replication.

3. AIM OF THE STUDY

HCV NS5B and NS3-4A are components of the multienzyme HCV replicase complex, which is directly involved in viral replication. Thus, NS5B and NS3-4A represent targets for antiviral intervention. In the host cell, however, these enzymes induce side effects that are not directly linked to HCV replication. In particular, the NS3-4A protease counteracts the type I IFN defense system, whereas emerging evidence suggests that NS5B activates this system. Due to the lack of type I IFN system-competent cells that support HCV replication, studies of the potential antagonism between the side effects of NS5B and NS3-4A are not possible. In contrast, SFV is capable of infection and replication in numerous type I IFN system-competent cells. The nsP2 protease component of the SFV replicase can suppress the type I IFN system. Moreover, we found that the SFV replicase activates the type I IFN system. Thus, the HCV and SFV proteases and RdRps produce similar side effects in type I IFN system-competent cells.

The overall aim of this study was to evaluate the potential antagonism between the side effects of viral proteases and RdRps during the infection of type I IFN system-competent cells by +ssRNA viruses. To achieve this aim, the work presented here was divided into the following specific objectives:

1. To generate and employ molecular probes to reveal the crucial functional determinants of RdRp activity within the fingers subdomain of NS5B;
2. To create a computational method (a computer program) to automatically generate molecular probes capable of binding to the NS3-4A protease active site and inhibiting the enzyme;
3. To study the emerging antagonism between the side effect functions of viral protease and replicase in the context of SFV infection of type I IFN system-competent cells;
4. To evaluate antiviral strategies aimed at inhibiting the NS5B and NS3-4A components of the HCV replicase in the context of these enzymes' side effects.

4. MATERIALS AND METHODS

A detailed description of the materials and methods used during this study is provided in the original publications that accompany this dissertation. Below, I will discuss the limitations of the experimental approaches used in the corresponding papers.

4.1. Paper I

In the biochemical part of our study, a full-length NS5B (genotype 1b, Con1) with an N-terminal histidine tag was used. It was previously demonstrated that the histidine tag present at the N-terminus of NS5B decreased its affinity for the template/primer by approximately 8-fold, compared with unmodified or truncated C-terminal histidine-tagged versions of NS5B (McKercher et al., 2004). Hence, *in vitro* RNA synthesis by N-terminally histidine-tagged NS5B represents sensitive system for the isolation of inhibitors that interfere with template/primer binding (McKercher et al., 2004). Additionally, using the N-terminally histidine-tagged NS5B is essential for analyzing the effects of high-molecular-weight probes, *e.g.*, monoclonal antibodies (mAbs), on RdRp activity, because sufficiently high mAb:NS5B molar ratios can be achieved without eliciting nonspecific side effects only in this case.

4.2. Paper II

The compounds used in this study were similar, but not identical, to the compounds predicted using a fragment-based quantitative structure-activity relationship (FQSAR) computation algorithm due to the following reasons. First, commercial libraries containing thousands of synthesized compounds can be searched for structurally similar compounds in a matter of hours. These commercially available compounds are typically inexpensive. Second, custom chemical compounds synthesis is expensive and sometimes require months to complete. After careful consideration of the above reasons, we decided to pursue the approach involving commercial compound libraries.

4.3. Paper III

We applied a mathematical reasoning approach to demonstrate that primary MEF cells infected with wild-type and mutant SFV4 contain large amounts of non-viral RNA PAMPs generated by viral replicase. Specifically, in the context of wild-type SFV4 infection, the following results were demonstrated. First, total RNA was extracted from MEF cells infected with SFV4 and was subsequently fractionated using oligo(dT)-affinity chromatography, resulting in two RNA fractions: polyadenylated (polyA+) and nonpolyadenylated (polyA-). Equal amounts of these RNA fractions were used (polyA+ : polyA- = 1:1, mass

ratio) and the contents of the different viral RNA species in these fractions were subsequently compared using the following methods: (i) an image-based densiometric relative quantitation of 42S ±dsRNA RF; (ii) an infectious center assay relative quantitation of 42S +ssRNA; (iii) a northern blot relative quantitation of 42S –ssRNA; and (iv) reverse transcription polymerization chain reaction (RT-PCR) analyses of dsRNA and ssRNA forms of minor defective interfering RNA (DI-RNA). Method iv showed that the dsRNA form of DI-RNA was present exclusively in the polyA+ RNA fraction, whereas negative RNA strands of DI-RNA were present at nearly equal amounts in both fractions. Utilizing methods i–iii, the polyA+ RNA fraction was found to always contain ~15-fold more of the analyzed viral RNA species compared with the polyA- RNA fraction. Thus, if viral RNAs are the only PAMPs produced during viral infection, then polyA+ RNA fractions must induce 15-fold more IFN-β than the corresponding polyA- RNA fractions, provided that equal amounts of RNA were used.

5. RESULTS AND DISCUSSION

Below, only original contributions, *i.e.*, findings that are not redundant with previously published studies, made specifically during this dissertation (especially Papers I and III) will be discussed in the context of data presented in the "Literature Analysis" section. The overall contributions made by the current work and the analyses performed during the preparation of the current dissertation lead me to propose a novel model for the recognition of positive-strand RNA virus invasion by the host cell.

5.1. Paper I

This paper describes the isolation and characterization of monoclonal antibodies (mAbs) that specifically bind to linear epitopes, *i.e.*, small peptide stretches scattered throughout the surface of the HCV NS5B fingers subdomain. The absence of low-molecular-weight NNIs that bind to the fingers subdomain of NS5B prevented functional analyses of this subdomain in the context of intact or minimally modified RdRps (Sofia et al., 2012). Hence, we used NS5B-specific mAbs as molecular probes to reveal possible functional determinants important for RdRp activity that might have been exposed on the surface of the NS5B fingers subdomain.

5.1.1. Conserved targets for molecular probes in the HCV RdRp fingers subdomain

A recent analysis of 236 HCV NS5B protein sequences belonging to six major genotypes revealed that, in addition to seven RdRp motifs (A–G), there are two more highly conserved regions (Waheed et al., 2012). One of these newly identified conserved regions is bound by mAb 8B2 (aa 1–14 of NS5B). The binding site for mAb 8B2 is located immediately upstream of the NS5B Λ1 loop (aa 11–45) (I, Figure 2). NS5B aa 101–105 comprised the minimal binding site for mAb 7G8, and residues upstream of this sequence were required for optimal binding (I, Figure 1H). In particular, the binding site for mAb 7G8 overlapped with motif G ($[T/S^{99}]X_{1\text{-}2}G^{102}$) of HCV NS5B. Notably, Ser^{99} and Gly^{102} are conserved among all of the HCV genotypes and subtypes (Waheed et al., 2012). Thus, mAbs 8B2 and 7G8 represent unique high-molecular-weight probes that bind to conserved regions in the NS5B fingers subdomain.

5.1.2. HCV RdRp fingers subdomain controls the initiation of RNA synthesis and the elongation of RNA chains

Limited information is available regarding the functions of the NS5B fingers subdomain during RNA synthesis. In addition to the importance of the Λ1 loop

for preserving the NS5B "closed fingers" conformation (Biswal et al., 2005; Labonte et al., 2002), it was demonstrated that the deletion of N-terminal aa 1–19 reduced the polymerase activity by more than 200-fold (Lohmann et al., 1997). In contrast, NS5B and RNA co-crystal structures and cross-linking studies identified several residues in the vicinity of motif G that were potentially involved in the interaction with bound RNA (Deval et al., 2007; Kim et al., 2005; O'Farrell et al., 2003). Although motif G was implicated in the recognition of the template/primer RNA complex, which is crucial for the initiation of RNA synthesis (Barrette-Ng et al., 2002; Gorbalenya et al., 2002; Thompson and Peersen, 2004), the significance of such recognition has never been demonstrated in functional assays for any of these RdRps.

Given the above arguments, we had a unique opportunity to analyze the effects of fingers-specific mAbs on the *in vitro* RdRp activity of HCV NS5B. We found that mAbs 8B2 and 7G8 inhibited the primer-dependent RNA synthesis catalyzed by NS5B in a concentration-dependent manner. These inhibitory effects were specific: mAbs recognizing either the histidine tag or other epitopes in the fingers subdomain had no effect on NS5B-mediated RNA synthesis (I, Figure 3A).

We used the polyanion heparin to gain insights into the mechanism of the inhibition of the HCV RdRp by mAbs 8B2 and 7G8. Specifically, heparin, a nucleic acid mimic that binds free RdRp molecules, was added to the primer-dependent RdRp assays to prevent RNA synthesis re-initiation events (Gu et al., 2003b). We found that only mAb 8B2 retained its inhibitory effect, whereas mAb 7G8 lost its inhibitory potential (I, Figure 4). The presence of heparin did not interfere with the elongation of RNA chains. The dose-dependent inhibition of RNA synthesis by mAb 8B2 in the presence of heparin implied that the elongation of RNA chains was inhibited by this mAb. In contrast, the loss of the mAb 7G8 inhibitory effect indicated that HCV RdRp-mediated RNA synthesis initiation events were restricted in the presence of 7G8. Consequently, we demonstrated that the NS5B fingers subdomain exerts functions that are important for both the initiation and elongation steps of RNA synthesis.

Although heparin provided a convenient way to discriminate between the initiation and elongation steps of RdRp-mediated RNA synthesis, the results we obtained did not explain why mAb 7G8 did not inhibit the first round of initiation. The ability of mAb 7G8 to interfere with RNA synthesis was also found to be template-dependent; the inhibitory potential of mAb 7G8 was virtually eliminated when an HCV replicon RNA template containing strong secondary structures was used (I, Figure 5). Thus, we concluded that the binding sites for mAb 7G8 and the template or template/primer RNA overlapped on the surface of NS5B; however, these binding sites were not mutually exclusive. Hence, the binding of heparin to the polymerase surface, which was driven by the extremely high negative charge density of heparin, might have displaced or dissociated bound mAb 7G8, explaining the absence of an inhibitory effect during the first round of initiation.

5.1.3. Motif G is part of the RdRp molecular interaction platform detected in the HCV replicase context

To characterize the epitope that mAb 7G8 recognized in greater detail, we performed extensive site-directed mutagenesis and analyzed the efficiency of purified mutant RdRps in primer-dependent RNA synthesis assays. The majority of the purified mutant NS5B proteins were active; however, only a single conservative S99T mutation completely abolished primer-dependent RdRp activity, thus corroborating the hypothesis that motif G might be important for template/ primer recognition (I, Table 2). However, the expression level of the S99T mutant NS5B was significantly lower compared with the other mutants, indicating possible defects in overall protein folding (I, Figure 7A). Given that the S99T mutant exhibited compromised protein folding, no conclusions concerning primer/template recognition could be drawn.

According to HCV NS5B X-ray crystal structures, most of the peptide (epitope) bound by mAb 7G8 is solvent-accessible (I, Figure 8E). A major portion (aa 92–103) of this solvent-accessible peptide contains a loop structure. The N-terminal region of the loop possesses two consecutive proline residues (Pro^{93} and Pro^{94}), which might be involved in controlling the loop's structural flexibility. The *cis*/*trans* isomerization of proline, which is catalyzed by peptidyl-prolyl isomerases (PPIases), might serve as a molecular switch to trigger the transition from one enzyme conformation to another (Andreotti, 2003). Cyclophilin B is one such PPIase (a possible replicase host cell factor); it is reported to interact with the 521–591 aa region of NS5B and to be essential for efficient HCV genome replication in cell culture (Watashi et al., 2005).

To analyze the potential functional role of the mAb 7G8 epitope amino acid residues in the context of HCV replication, we generated a panel of subgenomic replicons that encoded mutant NS5B proteins, for which the *in vitro* RdRp activities were previously measured in the primer-dependent NS5B-mediated RNA synthesis assays. Based on subsequent analyses of the replication efficiencies of these mutant replicons in permissive cells, we identified a region consisting of eight consecutive amino acid residues (P^{94}HSAR**S**$^{\mathbf{99}}$KF^{101}) that was extremely sensitive to any kind of substitutions (I, Table 2). In particular, any mutation introduced into this region of NS5B abrogated HCV replication entirely in both Huh7 and Huh7-Lunet cells. Consequently, we identified a platform that was involved in extensive interactions and was important for HCV RNA replication (I, Figure 8). The C-terminal region of this platform contains motif G. This platform might be involved in template recognition and binding, however, this remains to be demonstrated. Importantly, the platform begins with Pro^{94} and ends with Phe^{101}, indicating that the platform resides entirely within a loop (aa 92–103). A plausible hypothesis might be that in the context of HCV replicase, Pro^{94} of NS5B controls the flexibility of the loop that harbors the putative RNA-interaction platform.

5.1.4. Molecular environment-dependent activity of HCV NS5B: from RdRp to replicase

A comparison of the effects of single mutations on primer-dependent NS5B RdRp activity and on the capacity of the corresponding replicons to replicate resulted in one major conclusion. The catalytic activity of the RdRp component (NS5B) does not always guarantee the catalytic activity of the corresponding HCV replicase (I, Table 2). A similar conclusion was reached in a study demonstrating that an NS5B with a deleted β-loop possessed increased replication capacity in *in vitro* assays, whereas in the context of the HCV replicase, the ability of the modified NS5B to replicate viral RNA was completely lost (Cheney et al., 2002). These results not only highlight the essential role of NS5B in driving HCV RNA replication in the host cell but also indicate that the identified molecular interaction platform might be involved in interactions between both the RNA template and other replicase components, including nonviral ones.

5.2. Paper II

This paper describes a computational method applied to predict novel low-molecular-weight inhibitors of the NS3-4A protease component of the HCV replicase. These predictions were based on published structure-activity relationship (SAR) data for various NS3-4A inhibitors. In particular, SAR data were used for extracting the chemical structures of inhibitors and their corresponding inhibitory concentration 50% (IC_{50}) or inhibitory constant (K_i) values. The extracted data were subsequently used to construct a quantitative SAR (QSAR), a system of mathematical equations describing the connection between the properties (*e.g.*, electronic, steric, or hydrophobic) of potential inhibitors (compounds) and their IC_{50} or K_i values. The standard QSAR method can be used to evaluate the overall inhibitor structure, and therefore, to predict the properties of analogous, but more potent, compounds. In contrast, the fragment-based QSAR (FQSAR) method developed in this paper first evaluates the structures of many inhibitors and dissects them into simpler structural "building blocks" or fragments (II, Figure 1). As a result, the properties of these building blocks and their interactions are mathematically described in a more detailed fashion compared with standard QSAR. Subsequently, the building blocks are recombined to generate novel compounds, and their properties are predicted. Finally, the predicted compounds with optimal properties, *e.g.*, the lowest IC_{50} or K_i, are either synthesized or obtained from commercial compound libraries.

5.2.1. Side effect evaluation for predicted NS3-4A inhibitors

One of the side effects associated with low-molecular-weight compounds is cellular toxicity. We developed a cellular assay to measure the toxicity of the

potential NS3-4A protease inhibitors predicted using our FQSAR approach. In particular, we used a novel technology known as real-time cell electronic sensing (RT-CES) (Xing et al., 2006) to monitor the effects of compounds on the growth dynamics of Huh7 cells harboring HCV subgenomic replicons (Frese et al., 2003). The advantage of RT-CES over conventional toxicity end-point assays is that RT-CES is a real-time process that allows direct toxicity detection at any stage during the experimental time course (Xing et al., 2006).

For an RT-CES experiment, cells are first seeded into special plastic chambers containing bottom-integrated microelectrodes, followed by the addition of compounds to be tested. The microelectrodes are used to measure the electrical impedance of the chamber in real time. The relative change in the measured electrical impedance over defined periods of time reflects the rate of cell growth and is used to make conclusions regarding the effect of a compound on the overall cellular growth dynamics.

First, we found that Huh7 cells, harboring HCV subgenomic replicons attached to and grew on the microelectrodes of the chambers. Second, we evaluated the effects of seven FQSAR-predicted potential NS3-4A inhibitors on the cellular growth rate, and we identified two compounds that were not toxic (II, Supplementary Material 3, Figure S1). Thus, RT-CES technology can be used to evaluate the toxicity of compounds in Huh7 cells harboring HCV subgenomic replicons. In addition, we found that the growth of Huh7 replicon cells was decelerated immediately upon the addition of dimethyl sulfoxide, even at very low concentrations, *i.e.*, 0.2%.

5.3. Papers I and II

5.3.1. In pursuit of viral RdRp side effects

The binding sites for mAbs 8B2 and 7G8 on the HCV NS5B surface can be used as target sites for the development of novel inhibitors (Paper I). In contrast, the computational method that was developed to predict novel NS3-4A inhibitors requires experimental evidence to support the inhibitory activity of these compounds (Paper II). The approaches applied in both studies were similar. In Paper I, we used mAbs recognizing various epitopes in the HCV NS5B fingers subdomain to identify the essential epitopes for RdRp function. In Paper II, the "epitopes" were represented by sub-sites in the catalytic center of the protease, which were bound by sub-sets of chemical entities, *i.e.*, the fragments, which were recombined using a computer algorithm to produce final compounds. In this way, the compounds generated by the program could be interpreted as low-molecular-weight "mAbs", which recognized slightly different epitopes in the catalytic center of the protease.

Thus, in Papers I and II, we attempted to inhibit the activities of RdRp (NS5B) or to design inhibitors for the protease (NS3-4A) component of the HCV replicase. Although the approaches we used were not standard, inhibiting

components of the HCV viral replicase is a well-established strategy for interfering with viral replication.

As discussed above, various side effects are associated with the usage of molecular probes. These effects include the following: (i) the nonspecific inhibition of NS5B RdRp activity *in vitro* by high concentrations of mAbs and (ii) the toxicity of potential NS3-4A inhibitor compounds predicted using the FQSAR approach. However, the side effects of viral replicase components are more interesting because these effects can directly influence viral infection.

One well-known side effect of HCV NS3-4A is its interference with the host cell's antiviral response. In contrast, the potential role of NS5B in activating the antiviral response indicates that additional functions (side effects) are associated with viral RdRp. However, the limitations of the available cellular experimental systems for HCV prevented an evaluation of the potential significance of this viral RdRp activity side effect.

In the following study, we modified the protease component of the SFV replicase with a single mutation to uncover a novel mechanism used by host cells to detect RNA virus infection.

5.4. Paper III

This paper demonstrates that similar to the RdRps of HCV (Dansako et al., 2005), norovirus (Subba-Reddy et al., 2011), and TMEV (Kerkvliet et al., 2010), the SFV replicase is capable of type I IFN system activation. By exploiting the unique mutation introduced into the NLS of nsP2 (Peranen et al., 1990), which attenuates the pathogenicity (Rikkonen, 1996) and neuropathogenicity of SFV4 in mice (Fazakerley et al., 2002) and induces higher levels of type I IFNs in MEF cells (Breakwell et al., 2007), we identified a novel mechanism used by host cells to detect SFV4.

5.4.1. Side effects of viral RdRp activity: HCV NS5B and SFV replicase

Given the enormous amount of intracellular viral RdRps that are necessary, but not sufficient, for viral RNA synthesis (Kujala et al., 2001; Quinkert et al., 2005; Romero-Brey et al., 2012; Spuul et al., 2010), the role of these RdRps in the context of viral infection remains puzzling. Due to a type I IFN system defect in Huh7.5 cells, we were unable to pursue this question for HCV NS5B RdRp.

Unexpectedly, we found that the expression of SFV4 replicase (ORF1) from non-replicating RNA caused a robust induction of IFN-β in COP-5 cells, a mouse fibroblast cell line (Tyndall et al., 1981). In addition, we demonstrated that an SFV replicase with a unique NLS mutation in the nsP2 protein induced ~4-fold more IFN-β than the wild-type replicase (III, Figure 1D). Subsequently, we showed that the inactivation of the catalytic GDD motif resulted in a total

abrogation of IFN induction by both SFV replicase and HCV NS5B in COP-5 cells (III, Figure 1F), implying that RdRp activity was both necessary and sufficient for IFN-β induction. These data suggest the following: (i) the SFV replicase expression triggers the accumulation of previously unknown PAMPs, which induce IFN-β; (ii) the induction of IFN-β by the SFV replicase does not depend on the replication or transcription of the viral genome; and (iii) the nsP4 and nsP2 components of the SFV replicase are important for eliciting and limiting the type I IFN response, respectively.

Using RNA interference, we demonstrated that RLRs were important for detecting mutant SFV replicase RdRp activity in COP-5 cells and for detecting a recombinant SFV virus (carrying the same nsP2 mutation) in primary MEF cells (III, Figure 2). We subsequently found that SFV replicase expression in COP-5 cells triggered the accumulation of RNA PAMPs that were capable of inducing IFN-β (III, Figure 3). Analyses of these IFN-inducing RNAs isolated from COP-5 cells, which were transfected with DNA encoding mutant SFV replicase, corroborated the identity of the primary RLR sensor involved in detection. In particular, we found that this RNA contained dsRNA and a 5'-ppp terminal structure, *i.e.*, PAMPs recognized by RIG-I (III, Figures 3C, D and 4B, C). In addition, we found that the majority of IFN-inducing RNAs generated by the SFV replicase were not polyadenylated and had overall lengths exceeding 200 nt (III, Figure 3A, B).

5.4.2. The SFV dsRNA RF is not accessible to the host cell antiviral machinery

Another unexpected finding resulted from the multiparametric analysis of primary MEF cells infected by an SFV4 reporter virus, termed SFV4-Rluc-RDR, which contained the *Renilla* luciferase coding sequence (Tamberg et al., 2007) and the above-mentioned mutation in the nsP2 NLS (III, Figure 5I). In particular, 12 hours post-infection (hpi), a clear replication signal was observed, whereas at 24 and 36 hpi, the complete suppression of viral replication was observed at every multiplicity of infection tested (III, Figure 5E). However, when MEF cells were infected at a lower temperature (28°C), efficient SFV4-Rluc-RDR replication was achieved (III, Figure 5G). Subsequent analyses indicated that a delay in the kinetics of IFN-β secretion allowed efficient SFV4-Rluc-RDR replication.

To directly analyze the viral RNAs present in the infected MEF cells, we used a northern hybridization approach. We found that at 28°C, efficient SFV4-Rluc-RDR replication occurred, and all of the RNA species produced during the infection accumulated in a time-dependent manner. In contrast, at 37°C, we found that the 42S and 26S positive strands of mutant SFV that were detectable at 12 hpi could not be detected at 24 and 36 hpi, whereas the 42S negative strands remained at a steady level (III, Figure 5J).

Collectively, these results suggest that MEF cells mounted a potent antiviral response and destroyed the positive strands of the mutant SFV in an IFN-β-dependent manner, *i.e.*, the MEF cells "cured" themselves of the virus (albeit not entirely). Another important point that we demonstrated is that negative-strand 42S viral RNAs, *i.e.*, dsRNA RFs, remained intact during a full-blown host cell antiviral response. Thus, we provided direct evidence that no free and easily accessible negative-strand RNA molecules of SFV existed in the infected cells. These results also indicated that the spherules formed during SFV4-Rluc-RDR infection were stable and safeguarded the 42S negative strands. Importantly, these results provide a mechanism for IFN-β-mediated SFV replication restriction and indicate that nsP2 indeed plays a central role in disrupting host cell antiviral signaling during SFV infection.

5.4.3. The result of SFV replicase RdRp activity side effect in the context of viral infection

The extraction of total RNA from MEF cells infected by wild-type and mutant SFV4 and its subsequent transfection into COP-5 cells demonstrated that approximately equal amounts of PAMPs were generated during infection with both viruses (III, Figure 6). This result further corroborated the importance of nsP2 in the subversion of the innate immune response to SFV infection.

We subsequently demonstrated that IFN-β induction by SFV could not be explained by the presence of viral RNAs alone. In particular, we performed oligo(dT)-affinity chromatography to separate the total RNA isolated from MEF cells infected with SFV4-Rluc or SFV4-Rluc-RDR into polyadenylated (polyA+) and nonpolyadenylated (polyA-) RNA fractions. This technique enabled us to significantly enrich the polyA+ RNA fraction (~15-fold) for all of the major and minor viral RNA species detected in the SFV-infected cells (III, Figure 7A). The SFV-specific RNAs enriched in the polyA+ RNA fractions included single-stranded 42S and 26S positive-strand RNAs, and double-stranded RF, and defective interfering RNA species. In addition, we demonstrated that no detectable amounts of negative 42S strand degradation products were present in the polyA- fraction (III, Figure 7B and S4). We also found that the IFN-inducing RNAs generated during SFV infection of MEF cells were longer than 200 nt (III, Figure 7D, E) and hence possessed similar characteristics to the IFN-inducing RNAs produced during SFV replicase expression in COP-5 cells (III, Figure 3). Next, we showed that the transfection of polyA+ and polyA- RNA fractions that were isolated from cells infected with wild-type SFV induced approximately the same amounts of IFN-β in COP-5 cells (III, Figure 7C). This finding indicated the presence in the polyA- fraction of large amounts of other IFN-inducing RNAs, which were not derived from viral RNAs.

We devised a method for amplifying the RNAs transcribed by SFV replicase on host cell RNA templates (III, Figure 8A–C). However, due to biases in a

downstream cloning procedure, only one host cell RNA target was identified (III, Figure 8D), suggesting that this approach was inefficient.

5.5. Two concurrent processes are driven by viral replicase in infected cells

The preservation of mutant SFV 42S negative RNA stands, *i.e.*, dsRNA RF, indicated that RIG-I had extremely limited access to viral RF (III, Figure 5J), even during potent type I IFN response induction (III, Figure 5B). The results also suggest that large amounts of PAMPs capable of innate immune system activation were produced during MEF cell infection by both wild-type and mutant SFV (III, Figure 6). These PAMPs are immediately detected by the host cells if the nuclear localization of nsP2, the SFV safeguard protein, is compromised. Analyses of viral RNA species present in the polyA+ and polyA- RNA fractions isolated from MEF cells infected with both wild-type and mutant SFV, suggested that these potent PAMPs were not derived from viral RNAs (III, Figures 7A–C, S4). PAMPs with similar characteristics were produced when the SFV replicase was expressed alone from non-replicating mRNA, supporting the theory that no viral replication is required for SFV detection by host cells (III, Figures 3, 7C–E, 8A). The overall results strongly suggest that the PAMPs that activate the type I IFN system are produced by SFV replicase during the limited transcription of host cell RNA templates. Thus, during SFV infection, two concurrent and spatially separated processes are driven by viral replicase: (i) the replication/transcription of the viral RNA template in spherules and (ii) the limited transcription of host cell RNA templates in the cytosol (III, Figure 9).

The limited transcription of host cell RNA templates transforms these cellular RNAs into PAMPs, which are detected by RLRs and attacked by host cell defense mechanisms as non-self entities. Although the proposed recognition mechanism is PAMP-based, it differs sharply from the widely accepted viral dsRNA RF recognition paradigm. The detection of the viral RdRp activity in vertebrate host cells might be an ancient mechanism for the recognition of non-self enzymatic function. Below, this mechanism is integrated into tentative events that occur during SFV and HCV replication cycles.

5.5.1. Replicase-based detection of SFV

After its entry into the host cell cytosol, the 5’ capped and 3’-polyadenylated largely unstructured SFV RNA genome is translated by the ribosomes, and replicase polyprotein is generated (Figure 6). In the cytosol, this polyprotein precursor is cleaved by nsP2 protease, sequentially generating early and late replicases within ~10 minutes after synthesis completion (Lulla et al., 2012; Merits et al., 2001; Vasiljeva et al., 2003).

In the first process, the early replicase participates in the formation of spherule, where the intensive SFV RNA synthesis is initiated. First, the early replicase generates dsRNA RF (Wengler and Gross, 1979) from positive-strand 42S RNA by synthesizing a single negative 42S RNA strand. Second, the early replicase is cleaved by nsP2, forming the late replicase, which possibly utilizes circularized 42S negative strand that is present in an RF dsRNA configuration in a putative, semi-conservative manner to produce multiple positive viral RNA strands *via* a strand-displacing mechanism (Kaariainen and Ahola, 2002).

In the second concurrent process, the partially or fully processed replicase utilizes polyA- host cell RNAs (III, Figure 7A, C) for transcription, generating partial dsRNA regions with 5'-ppp structures (III, Figures 3B–D, and 8A, B). These PAMP structures are detected by RIG-I sensor (III, Figure 2B–D), which undergoes a conformational change upon binding to both the dsRNA region and 5'-ppp, leading to the exposure of its N-terminal tandem CARD domains (Cui et al., 2008; Myong et al., 2009; Yoneyama and Fujita, 2007). The CARD domains of two RIG-I molecules are subsequently modified, both covalently and noncovalently, by ubiquitination (Gack et al., 2007; Zeng et al., 2010). These modifications lead to the formation of helical "lock-washer" tetramer of ubiquitin-modified RIG-I CARD domains (Peisley et al., 2014), which interacts with the CARD domain of mitochondrial CARDIF protein (Meylan et al., 2005; Seth et al., 2005). This interaction triggers the prion-like aggregation of multiple CARDIF molecules and antiviral signal transmission (Hou et al., 2011), which activates the IFN-β promoter in the nucleus.

The nuclear localization of nsP2 protease is absolutely required for the suppression of the type I IFN response (Breakwell et al., 2007; Fazakerley et al., 2002; Frolova et al., 2002; Peranen et al., 1990). Given that all SFV replicase proteins are present in their mature form simultaneously in the host cell within ~10 minutes after the polyprotein is produced, it is clear that the virus efficiently antagonizes the the appearance of IFN-β induced by the viral replicase. This antagonism directly influences the amount of IFN-β induced in primary MEF cells infected with SFV4-Rluc and SFV4-Rluc-RDR (III, Figure 5B). However, the amount of PAMPs produced during these virus infections is nearly the same (III, Figure 6). By preventing nsP2 access to the nuclei of infected cells, the enormous IFN-β-inducing potential of these PAMPs is unleashed, leading to the shutdown of viral replication (III, Figure 5E, J). More precisely, viral positive RNA strands are destroyed by the cell in an IFN-dependent manner, whereas viral negative RNA strands, *i.e.*, dsRNA RF, are protected (III, Figures 5J and 9).

These data suggest that the nsP2 protease component of SFV replicase counteracts the consequences of SFV replicase RdRp activity, which are not associated with viral replication, *i.e.*, not associated with spherules. Thus, there is an antagonism between the viral replicase RdRp activity and its protease component. Introducing the mutation into nsP2 leads to disequilibrium in this protease-replicase system, resulting in SFV detection by host cells and viral replication restriction.

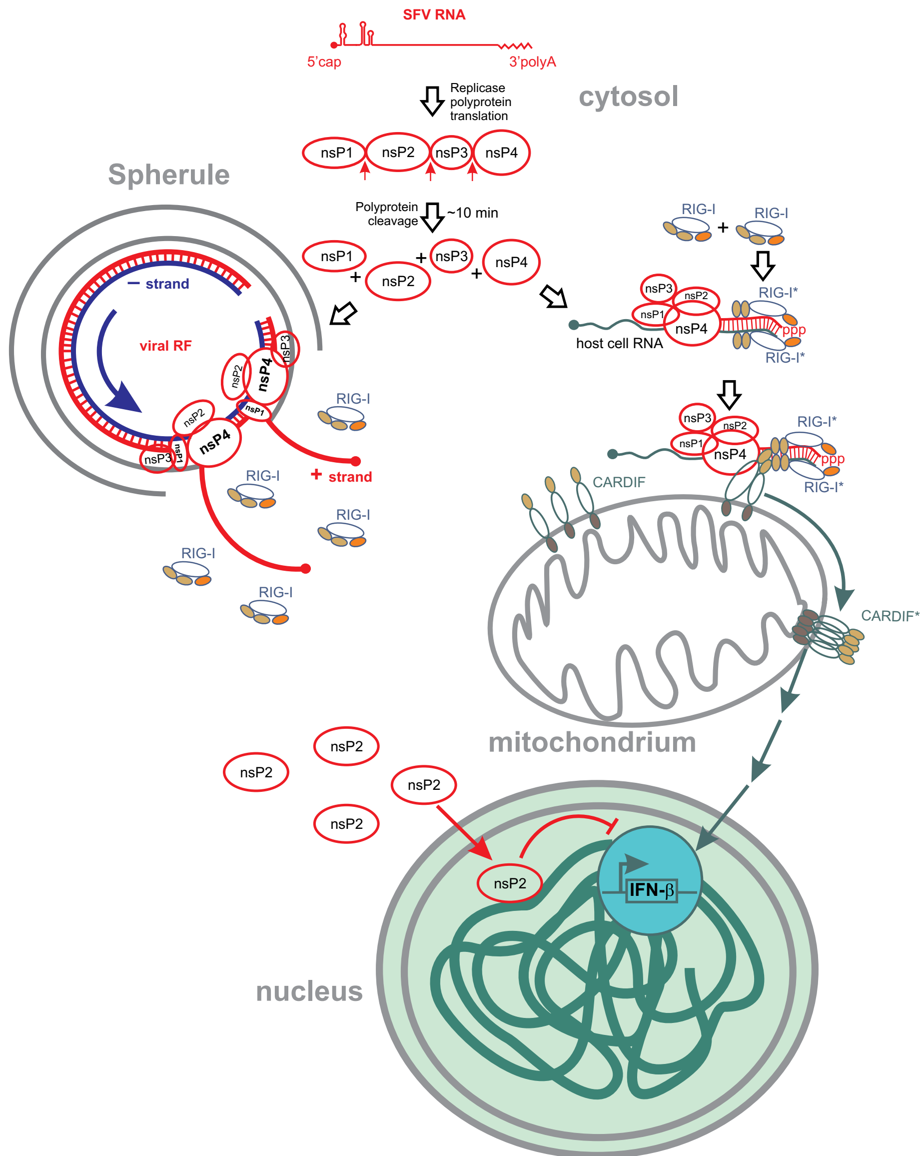

Figure 6. Replicase-based detection of SFV. See text for details. Intracellular membranes are represented by thick *grey* lines. The C-terminal regulatory domain of RIG-I, responsible for 5'-ppp detection (Cui et al., 2008), is depicted in *orange*. The helicase domain of RIG-I, assumed to be important for translocation on dsRNA (Myong et al., 2009), is depicted as a *blue* oval. CARDIF transmembrane domain (Seth et al., 2005) is depicted in *brown*. CARD domains of RIG-I and CARDIF are depicted in *gold*. Asterisks indicate the activated signaling states of the corresponding signal transduction proteins. The timing of SFV polyprotein processing is according to Lulla and colleagues (Lulla et al., 2012). Spherule and SFV dsRNA RF organization is depicted according to the model proposed by Kaariainen and Ahola (Kaariainen and Ahola, 2002). The model for the activation of CARDIF signaling is depicted according to Hou and colleagues (Hou et al., 2011).

5.5.2. RdRp-based detection of HCV

The entry of HCV virus or replicon RNA, presumably containing GORS (Simmonds et al., 2004), which possesses an unknown 5' terminus structure and 3'-hydroxyl buried in the SL, is followed by its translation (Figure 7). No detailed data on polyprotein processing during HCV infection are currently available. However, similarities in the dynamics of RNA synthesis obtained in the HCV replicon system (Binder et al., 2007; Krieger et al., 2001; Quinkert et al., 2005) and during HCV infection (Keum et al., 2012) suggest similarities in polyprotein processing. In particular, in the HCV replicon system, the processing of the HCV replicase polyprotein is much slower compared with SFV ORF1 processing. In the HCV replicon system, the N- and C-terminal replicase components, NS3 and NS5B, are cleaved off the polyprotein precursor within ~10 minutes after its synthesis (Pietschmann et al., 2001).

NS5B RdRp might utilize host cell RNAs as templates for RNA synthesis, *i.e.*, transcription, immediately after it has been cleaved off the polyprotein. The subsequent NS5B-generated PAMP-driven activation of RIG-I receptors and signal transduction relayed by clustered and aggregated CARDIF adaptor molecules would lead to IFN-β promoter activation. In fact, it was demonstrated that the NS5B expressed in IFN-competent mouse cells and *in vivo* utilized host cell RNAs (Yu et al., 2012). We demonstrated that both HCV NS5B and mutant SFV replicase induced comparable levels of IFN-β in mouse cells (III, Figure 1F). Moreover, very recently, it was demonstrated that NS5B bound to specific hepatic mRNAs and had the capacity to use them as templates *in vitro* (Yuhashi et al., 2014).

The NS4A component of viral replicase is cleaved within ~20–30 minutes after polyprotein synthesis completion (Pietschmann et al., 2001), indicating that at this time point, a functional NS3-4A protease complex might be formed. The NS3-4A protease might cleave the CARDIF adaptor signaling protein near its mitochondrial transmembrane insertion sequence (Meylan et al., 2005; Seth et al., 2005), thus antagonizing the consequences of NS5B RdRp transcription activity on host cell RNA.

The replicase polyprotein is fully processed only ~40–50 minutes after the completion of its synthesis (Pietschmann et al., 2001), allowing NS4B to initiate a coordinated DMV formation with other replicase proteins (Egger et al., 2002; Herod et al., 2012; Romero-Brey et al., 2012). Thus, at least in the HCV replicon system, DMV formation can begin only ~40 minutes or after a threshold quantity of polyproteins that are sufficient for DMV formation have been synthesized. Thus, HCV RNA replication is initiated approximately within one hour after infection.

Taken together, the considerations presented above suggest an antagonistic relationship between the NS3-4A protease and the NS5B RdRp components of HCV replicase. This antagonism occurs between the HCV replicase components that are not associated with viral RNA synthesis. In particular, the NS5B RdRp induces IFN-β, whereas NS3-4A cleaves CARDIF, thereby inhibiting IFN-β induction.

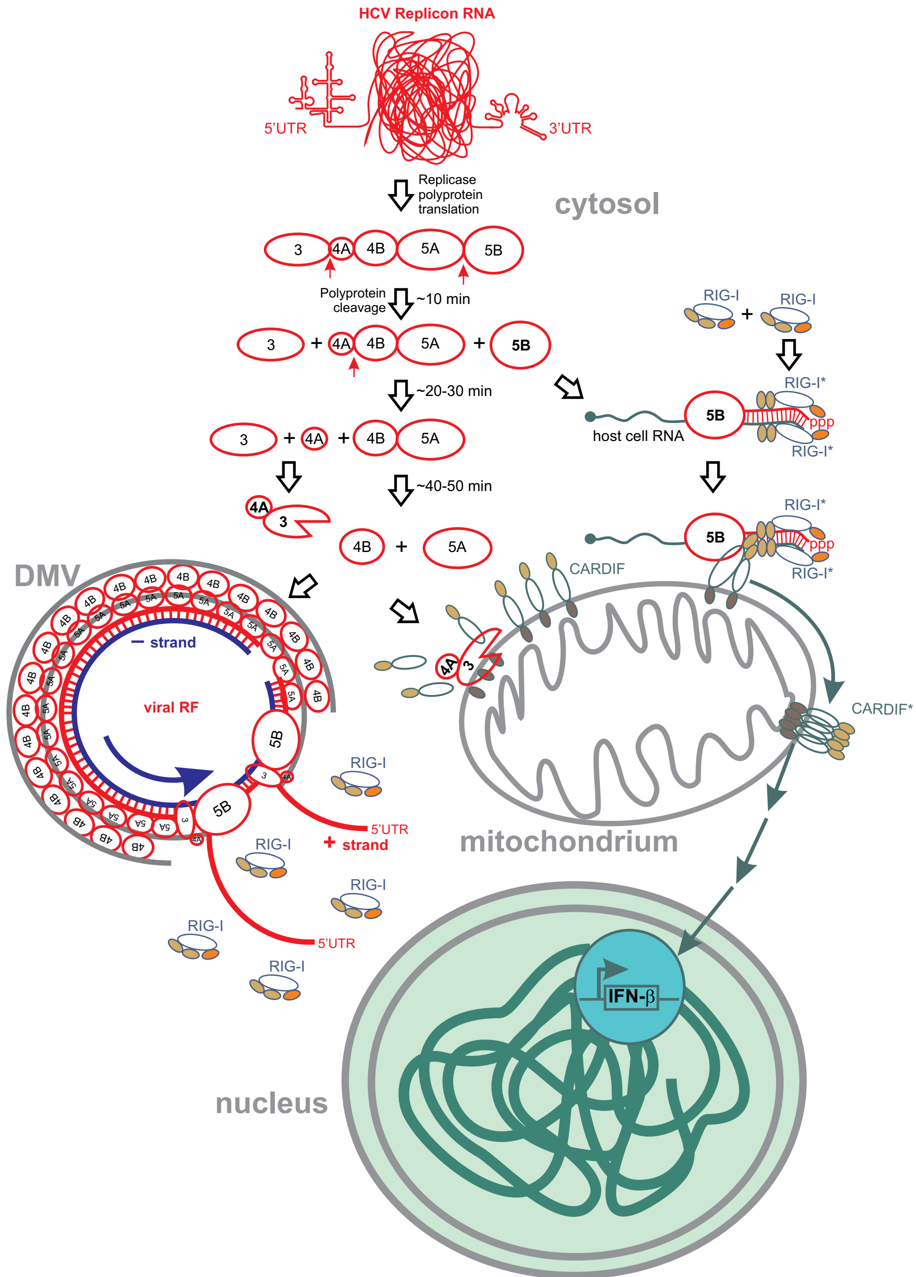

Figure 7. RdRp-based detection of HCV. See text and Figure 1 for details. The timing of HCV replicase polyprotein processing is according to Pietschmann and colleagues (Pietschmann et al., 2001). The model of CARDIF cleavage by NS3-4A is depicted (Meylan et al., 2005; Seth et al., 2005). The model of viral RF within DMV is depicted as proposed for SFV (Kaariainen and Ahola, 2002).

5.5.3. The model: emergence of polymerase-protease antagonism

The fact that NS5B and NS3-4A have differential effects on the type I IFN system is not novel and has been previously established (Dansako et al., 2005). It was proposed that the activation and inhibition of type I IFN by NS5B and NS3-4A, respectively, might be used by HCV to "distract" the host cell and to ultimately establish persistent infection (Dansako et al., 2005). Our studies with SFV strongly suggest that the RdRp activity of viral replicase is used by the host cell for virus detection. In particular, the host cell is not viewed as an inert system that does not respond to viral infection; it represents an active "player" that utilizes viral non-self RdRp enzymatic activity to mount a strong antiviral response. Thus, according to the current model, there is an antagonism between the side effects of viral proteases and RdRps.

To reveal the potential importance of antagonism between the nsP2 protease and the SFV replicase, we had to introduce the mutation into nsP2, *i.e.*, simultaneously into both the protease and replicase. Thus, a highly artificial situation was generated. Apparently, the rapid polyprotein processing of SFV ORF1 limits the significance of the developed model for SFV pathogenesis. However, the infection of heterogeneous bone-marrow-derived dendritic cells with a wild-type SINV leads to potent type I IFN induction, no shutoff of macromolecular synthesis (a function attributed to nsP2), and restriction of infection (Ryman et al., 2005). Moreover, a high level of type I IFN is induced within the first 12 hours of murine SINV infection (Klimstra et al., 1999). Thus, the outcome of Old World alphavirus infection cannot simply be explained with type I IFN system antagonism by nsP2, especially in non-established cell lines and animals (Ryman and Klimstra, 2008).

In contrast, the timing of HCV polyprotein processing seems to fit the current model better. The rapid initial cytosolic NS5B RdRp accumulation indicates that the activation of the type I IFN system might precede the appearance of functional NS3-4A protease. A recent report demonstrated that multiple CARDIF molecules aggregate in a prion-like mode for signal transduction (Hou et al., 2011). Thus, the ability of NS3-4A to cleave aggregated and activated CARDIF molecules awaits confirmation. Conversely, if both NS5B and NS3-4A are generated simultaneously, then according to the current model, the antagonism between these enzymes might play a decisive role in establishing viral infection.

5.5.4. Inhibition of HCV replicase from the perspective of the current model

Here, I use the current model to analyze the effects of different inhibitors on HCV RNA replication.

It was demonstrated that the membrane-bound HCV replicase, as isolated from replicon cells in the form of crude replication complexes (CRCs), was not inhibited by allosteric NNIs, which interfere with NS5B RNA synthesis

initiation. In contrast, chain-terminating NAs efficiently inhibited CRC-resident HCV replicase in a dose-dependent manner. These findings demonstrated that stable and productive elongation replicase complexes are formed in CRCs (Ma et al., 2005). This is also in line with results demonstrating that NA chain-terminators are incorporated with almost the same efficiency during RNA elongation (Dutartre et al., 2005). In the same way, the inhibitory properties of mAbs 8B2 and 7G8 were lost when HCV NS5B was in elongation mode, highlighting the allosteric properties of these mAbs and indicating that their epitopes are not accessible in a synthesizing RdRp (I, Figure 3D).

Recently, the NS5B•template•primer elongation complexes were demonstrated to be extremely stable (Jin et al., 2012), indicating that the stability of HCV replicase•template•primer elongation complexes might be even higher. According to the current model, HCV-infected cells contain both replicase and NS5B elongation complexes, with the former hidden in DMV and the latter exposed to the cytosol. Consequently, the NNIs present in the cytosol and in DMVs cannot interfere with any of the above-described elongation complex RdRp activities, whereas they might prevent the formation of new initiation complexes. Alternatively, NAs can interfere with both elongation complex RdRp activities, producing stalled elongation complexes.

The broad-spectrum antiviral ribonucleoside ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (Sidwell et al., 1972) is incorporated into the nascent RNA chain synthesized by RdRps and induces mutations. The accumulation of ribavirin-induced mutations leads to the “error catastrophe” (Eigen, 1971), which precludes RNA virus replication (Crotty et al., 2000). The errors introduced into the HCV genome will have the maximum impact on the DMV-resident HCV replicase function, but not on cytosolic NS5B RdRp activity. This statement is corroborated by our findings that mutations in the vicinity of motif G of HCV NS5B, which preserve *in vitro* RdRp activity, can incapacitate the RdRp activity of DMV-resident HCV replicase (I, Figure 8 and Table 2). Thus, cytosolic NS5B RdRps will generate the PAMPs irrespectively of ribavirin incorporation.

The identification of novel rNTP-mediated excision activity for HCV NS5B implies that viral RdRp might be an extremely active molecular machine that will use any available RNA template to perform an RNA synthesis. In particular, this newly identified rNTP-mediated NS5B excision activity (Jin et al., 2013) will have the capacity to remove the chain-terminator (NA), thus re-enabling the RNA synthesis. In contrast, NS3-4A protease inhibitors would stop both HCV replicase polyprotein processing and CARDIF cleavage. However, it has been reported that compared with NS5B-specific NAs, drug-resistant mutations are more easily accumulated for NS3-4A inhibitors (McCown et al., 2008). This result highlights the relevance of novel approaches aimed at designing new chimeric types of NS3-4A inhibitors that might possess a higher barrier to resistance (II, Figure 1). Targeting NS3-4A would not only inhibit HCV polyprotein processing but would also block CARDIF cleavage. Thus, according to the current model, a disequilibrium in RdRp-protease antagonism

would be created, allowing the activation of the type I IFN system by the PAMPs generated by the cytosolic NS5B elongation complexes.

Finally, according to our results, stalling the viral replicase elongation complexes is not sufficient to eliminate viral infection in cells with an intact type I IFN system. The protection of SFV dsRNA RF during a potent type I IFN response mounted by infected primary MEF cells indicates that host cells are unable to destroy the spherules (III, Figure 5J). The antiviral defense capacity of MEF cells appears to be limited to clearing cytosolic viral nucleic acids and proteins (III, Figure 5D). It is highly likely that the protection of the HCV RF, having an unknown structure, within the DMVs, is as efficient as in the case of dsRNA RF protection by SFV spherules. Thus, to clear the viral infection completely it seems that the infected cells have to be destroyed, most likely by other cells of either innate or adaptive immunity, or by both.

6. CONCLUSIONS

6.1. Specific conclusions (experimental observations)

1. The binding sites for mAbs 7G8 and 8B2 in the fingers subdomain of HCV NS5B are involved in the regulation of RNA synthesis initiation and elongation of RNA chains in *in vitro* RdRp assays.
2. Motif G of HCV replicase-resident NS5B is involved in the formation of a molecular interaction platform during the replication of HCV replicon in permissive type I IFN system-deficient host cells.
3. The binding sites for mAbs 7G8 and 8B2 are potential target sites for novel NS5B inhibitors, and the FQSAR algorithm represents a novel method for designing potential NS3-4A-specific inhibitors.
4. The expression of SFV replicase in type I IFN system-competent cells results in the accumulation of IFN-inducing RNA PAMPs, which are detected by the host cell RLR sensors.
5. Host cells are capable of mounting potent antiviral responses and the destruction of SFV positive RNA strands, while dsRNA RFs are not accessible to antiviral machinery. This replication shutdown mechanism is only possible when an NLS mutation is present in the nsP2 protease.
6. During SFV infection, the SFV replicase generates large amounts of RNA PAMPs, which are not derived from viral RNA.

6.2. General conclusions (model)

1. *In vitro*, viral RdRp can utilize virtually any template RNA.
2. In a host cell, at least two populations of viral RdRps exist. First, a minor population is involved in the replication/transcription of viral RNA genomes while hidden in viral factories. Second, a major population is involved in limited host cell RNA transcription. Cytosolic RdRp activity on host cell RNA templates represents a side effect of HCV NS5B and the SFV replicase.
3. The side effects of SFV replicase and HCV NS5B are functionally “invisible” in host cells deficient for the type I IFN system, but they are “visible” in cells with an intact type I IFN system.
4. In the context of viral infection, the side effect of viral RdRp activity is counteracted by the side effect of the viral protease. The specific mechanism of protease antagonism might be different, as is seen for HCV and SFV.
5. The antagonism between the viral RdRp and protease side effects might determine the outcome of positive-strand RNA virus infection

.

7. REFERENCES

Ahola, T., and Kaariainen, 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.

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.

Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J.L., Sodoyer, M., Pol, S., Brechot, C., Paranhos-Baccala, G., and Lotteau, V. (2002). Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol* 76, 6919–6928.

Andreotti, A.H. (2003). Native state proline isomerization: an intrinsic molecular switch. *Biochemistry* 42, 9515–9524.

Asabe, S.I., Tanji, Y., Satoh, S., Kaneko, T., Kimura, K., and Shimotohno, K. (1997). The N-terminal region of hepatitis C virus-encoded NS5A is important for NS4A-dependent phosphorylation. *J Virol* 71, 790–796.

Balluz, I.M., Glasgow, G.M., Killen, H.M., Mabruk, M.J., Sheahan, B.J., and Atkins, G.J. (1993). Virulent and avirulent strains of Semliki Forest virus show similar cell tropism for the murine central nervous system but differ in the severity and rate of induction of cytolytic damage. *Neuropathol Appl Neurobiol* 19, 233–239.

Baltimore, D. (1971). Expression of animal virus genomes. *Bacteriol Rev* 35, 235–241.

Baltimore, D., Huang, A.S., and Stampfer, M. (1970). Ribonucleic acid synthesis of vesicular stomatitis virus, II. An RNA polymerase in the virion. *Proc Natl Acad Sci U S A* 66, 572–576.

Barrette-Ng, I.H., Ng, K.K., Mark, B.L., Van Aken, D., Cherney, M.M., Garen, C., Kolodenko, Y., Gorbalenya, A.E., Snijder, E.J., and James, M.N. (2002). Structure of arterivirus nsp4. The smallest chymotrypsin-like proteinase with an alpha/beta C-terminal extension and alternate conformations of the oxyanion hole. *J Biol Chem* 277, 39960–39966.

Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. (1994). Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J Virol* 68, 5045–5055.

Bartenschlager, R., Ahlbornlaake, L., Mous, J., and Jacobsen, H. (1993). Nonstructural Protein-3 of the Hepatitis-C Virus Encodes a Serine-Type Proteinase Required for Cleavage at the Ns3/4 and Ns4/5 Junctions. *J Virol* 67, 3835–3844.

Bartenschlager, R., Penin, F., Lohmann, V., and Andre, P. (2011). Assembly of infectious hepatitis C virus particles. *Trends Microbiol* 19, 95–103.

Barth, H., Schafer, C., Adah, M.I., Zhang, F., Linhardt, R.J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., Van Kuppevelt, T.H., Depla, E., *et al.* (2003). Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* 278, 41003–41012.

Behrens, S.E., Tomei, L., and De Francesco, R. (1996). Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J* 15, 12–22.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., and Bourne, P.E. (2000). The Protein Data Bank. *Nucleic Acids Res* 28, 235–242.

Binder, M., Quinkert, D., Bochkarova, O., Klein, R., Kezmic, N., Bartenschlager, R., and Lohmann, V. (2007). Identification of determinants involved in initiation of

hepatitis C virus RNA synthesis by using intergenotypic replicase chimeras. *J Virol* 81, 5270–5283.

Bishop, D.H. (1986). Ambisense RNA viruses: positive and negative polarities combined in RNA virus genomes. *Microbiol Sci* 3, 183–187.

Bishop, D.H., Obijeski, J.F., and Simpson, R.W. (1971). Transcription of the influenza ribonucleic acid genome by a virion polymerase. II. Nature of the in vitro polymerase product. *J Virol* 8, 74–80.

Biswal, B.K., Cherney, M.M., Wang, M., Chan, L., Yannopoulos, C.G., Bilimoria, D., Nicolas, O., Bedard, J., and James, M.N. (2005). Crystal structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. *J Biol Chem* 280, 18202–18210.

Blight, K.J., McKeating, J.A., and Rice, C.M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 76, 13001–13014.

Blight, K.J., and Rice, C.M. (1997). Secondary structure determination of the conserved 98-base sequence at the 3' terminus of hepatitis C virus genome RNA. *J Virol* 71, 7345–7352.

Boren, J., Lee, I., Zhu, W., Arnold, K., Taylor, S., and Innerarity, T.L. (1998). Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. *J Clin Invest* 101, 1084–1093.

Borsa, J., and Graham, A.F. (1968). Reovirus: RNA polymerase activity in purified virions. *Biochem Biophys Res Commun* 33, 895–901.

Boulant, S., Becchi, M., Penin, F., and Lavergne, J.P. (2003). Unusual multiple recoding events leading to alternative forms of hepatitis C virus core protein from genotype 1b. *J Biol Chem* 278, 45785–45792.

Boulter, E.A., Zlotnik, I., and Maber, H.B. (1971). A lethal respiratory infection of rabbits with a strain of Semliki Forest virus (SFV). *Br J Exp Pathol* 52, 638–645.

Bradish, C.J., Allner, K., and Maber, H.B. (1971). The virulence of original and derived strains of Semliki forest virus for mice, guinea-pigs and rabbits. *J Gen Virol* 12, 141–160.

Brass, V., Berke, J.M., Montserret, R., Blum, H.E., Penin, F., and Moradpour, D. (2008). Structural determinants for membrane association and dynamic organization of the hepatitis C virus NS3–4A complex. *Proc Natl Acad Sci U S A* 105, 14545–14550.

Brass, V., Bieck, E., Montserret, R., Wolk, B., Hellings, J.A., Blum, H.E., Penin, F., and Moradpour, D. (2002). An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J Biol Chem* 277, 8130–8139.

Breakwell, L., Dosenovic, P., Karlsson Hedestam, G.B., D'Amato, M., Liljestrom, 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.

Bressanelli, S., Tomei, L., Rey, F.A., and De Francesco, R. (2002). Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides. *J Virol* 76, 3482–3492.

Bressanelli, S., Tomei, L., Roussel, A., Incitti, I., Vitale, R.L., Mathieu, M., De Francesco, R., and Rey, F.A. (1999). Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc Natl Acad Sci U S A* 96, 13034–13039.

Broering, T.J., Kim, J., Miller, C.L., Piggott, C.D., Dinoso, J.B., Nibert, M.L., and Parker, J.S. (2004). Reovirus nonstructural protein mu NS recruits viral core surface proteins and entering core particles to factory-like inclusions. *J Virol* 78, 1882–1892.

Butcher, S.J., Grimes, J.M., Makeyev, E.V., Bamford, D.H., and Stuart, D.I. (2001). A mechanism for initiating RNA-dependent RNA polymerization. *Nature* 410, 235–240.

Carroll, S.S., Tomassini, J.E., Bosserman, M., Getty, K., Stahlhut, M.W., Eldrup, A.B., Bhat, B., Hall, D., Simcoe, A.L., LaFemina, R., *et al.* (2003). Inhibition of hepatitis C virus RNA replication by 2'-modified nucleoside analogs. *J Biol Chem* 278, 11979–11984.

Carter, M.F., Biswal, N., and Rawls, W.E. (1974). Polymerase activity of Pichinde virus. *J Virol* 13, 577–583.

Caspar, D.L., and Klug, A. (1962). Physical principles in the construction of regular viruses. *Cold Spring Harb Symp Quant Biol* 27, 1–24.

Catanese, M.T., Uryu, K., Kopp, M., Edwards, T.J., Andrus, L., Rice, W.J., Silvestry, M., Kuhn, R.J., and Rice, C.M. (2013). Ultrastructural analysis of hepatitis C virus particles. *Proc Natl Acad Sci U S A* 110, 9505–9510.

Cheney, I.W., Naim, S., Lai, V.C., Dempsey, S., Bellows, D., Walker, M.P., Shim, J.H., Horscroft, N., Hong, Z., and Zhong, W. (2002). Mutations in NS5B polymerase of hepatitis C virus: impacts on in vitro enzymatic activity and viral RNA replication in the subgenomic replicon cell culture. *Virology* 297, 298–306.

Chinnaswamy, S., Yarbrough, I., Palaninathan, S., Kumar, C.T., Vijayaraghavan, V., Demeler, B., Lemon, S.M., Sacchettini, J.C., and Kao, C.C. (2008). A locking mechanism regulates RNA synthesis and host protein interaction by the hepatitis C virus polymerase. *J Biol Chem* 283, 20535–20546.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244, 359–362.

Choo, Q.L., Richman, K.H., Han, J.H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P.J., *et al.* (1991). Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci U S A* 88, 2451–2455.

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* in press.

Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R., and Cameron, C.E. (2000). The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. *Nature Medicine* 6, 1375–1379.

Cui, S., Eisenacher, K., Kirchhofer, A., Brzozka, K., Lammens, A., Lammens, K., Fujita, T., Conzelmann, K.K., Krug, A., and Hopfner, K.P. (2008). The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. *Molecular Cell* 29, 169–179.

Dansako, H., Naka, K., Ikeda, M., and Kato, N. (2005). Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells. *Biochem Biophys Res Commun* 336, 458–468.

Degroot, R.J., Rumenapf, 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.

den Boon, J.A., and Ahlquist, P. (2010). Organelle-like membrane compartmentalization of positive-strand RNA virus replication factories. *Annu Rev Microbiol* 64, 241–256.

Deval, J., D'Abramo, C.M., Zhao, Z., McCormick, S., Coutsinos, D., Hess, S., Kvaratskhelia, M., and Gotte, M. (2007). High resolution footprinting of the hepatitis C virus polymerase NS5B in complex with RNA. *J Biol Chem* 282, 16907–16916.

Dhanak, D., Duffy, K.J., Johnston, V.K., Lin-Goerke, J., Darcy, M., Shaw, A.N., Gu, B., Silverman, C., Gates, A.T., Nonnemacher, M.R., *et al.* (2002). Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* 277, 38322–38327.

Di Marco, S., Volpari, C., Tomei, L., Altamura, S., Harper, S., Narjes, F., Koch, U., Rowley, M., De Francesco, R., Migliaccio, G., *et al.* (2005). Interdomain communication in hepatitis C virus polymerase abolished by small molecule inhibitors bound to a novel allosteric site. *J Biol Chem* 280, 29765–29770.

Dorner, F., Scriba, M., and Weil, R. (1973). Interferon: evidence for its glycoprotein nature. *Proc Natl Acad Sci U S A* 70, 1981–1985.

Dorner, M., Horwitz, J.A., Robbins, J.B., Barry, W.T., Feng, Q., Mu, K., Jones, C.T., Schoggins, J.W., Catanese, M.T., Burton, D.R., *et al.* (2011). A genetically humanized mouse model for hepatitis C virus infection. *Nature* 474, 208–211.

Doublie, S., Sawaya, M.R., and Ellenberger, T. (1999). An open and closed case for all polymerases. *Structure Fold Des* 7, R31–35.

Doxsey, S.J., Brodsky, F.M., Blank, G.S., and Helenius, A. (1987). Inhibition of endocytosis by anti-clathrin antibodies. *Cell* 50, 453–463.

Drexler, J.F., Corman, V.M., Muller, M.A., Lukashev, A.N., Gmyl, A., Coutard, B., Adam, A., Ritz, D., Leijten, L.M., van Riel, D., *et al.* (2013). Evidence for novel hepaciviruses in rodents. *PLoS Pathog* 9, e1003438.

Dutartre, H., Boretto, J., Guillemot, J.C., and Canard, B. (2005). A relaxed discrimination of 2'-O-methyl-GTP relative to GTP between de novo and Elongative RNA synthesis by the hepatitis C RNA-dependent RNA polymerase NS5B. *J Biol Chem* 280, 6359–6368.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H.E., Moradpour, D., and Bienz, K. (2002). Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 76, 5974–5984.

Eigen, M. (1971). Selforganization of matter and the evolution of biological macromolecules. *Naturwissenschaften* 58, 465–523.

Eigen, M., and Schuster, P. (1977). The hypercycle. A principle of natural self-organization. Part A: Emergence of the hypercycle. *Naturwissenschaften* 64, 541–565.

Einav, S., Sobol, H.D., Gehrig, E., and Glenn, J.S. (2010). The hepatitis C virus (HCV) NS4B RNA binding inhibitor clemizole is highly synergistic with HCV protease inhibitors. *J Infect Dis* 202, 65–74.

Evans, M.J., von Hahn, T., Tscherne, D.M., Syder, A.J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J.A., Bieniasz, P.D., and Rice, C.M. (2007). Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446, 801–805.

Fazakerley, J.K., Boyd, A., Mikkola, M.L., and Kaariainen, L. (2002). A single amino acid change in the nuclear localization sequence of the nsP2 protein affects the neurovirulence of Semliki Forest virus. *J Virol* 76, 392–396.

Ferrari, E., He, Z., Palermo, R.E., and Huang, H.C. (2008). Hepatitis C virus NS5B polymerase exhibits distinct nucleotide requirements for initiation and elongation. *J Biol Chem* 283, 33893–33901.

Field, A.K., Tytell, A.A., Lampson, G.P., and Hilleman, M.R. (1968). Inducers of interferon and host resistance, V. In vitro studies. *Proc Natl Acad Sci U S A* 61, 340–346.

Fields, B.N., Knipe, D.M., and Howley, P.M. (2013). *Fields Virology*, 6th edn (Philadelphia, Wolters Kluwer/Lippincott Williams & Wilkins Health).

Frese, M., Barth, K., Kaul, A., Lohmann, V., Schwarzle, V., and Bartenschlager, R. (2003). Hepatitis C virus RNA replication is resistant to tumour necrosis factor-alpha. *J Gen Virol* 84, 1253–1259.

Friebe, P., and Bartenschlager, R. (2002). Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. *J Virol* 76, 5326–5338.

Friebe, P., Boudet, J., Simorre, J.P., and Bartenschlager, R. (2005). Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *J Virol* 79, 380–392.

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.

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.

Fros, J.J., Liu, W.J., Prow, N.A., Geertsema, C., Ligtenberg, M., Vanlandingham, D.L., Schnettler, E., Vlak, J.M., Suhrbier, A., Khromykh, A.A.*, et al.* (2010). Chikungunya virus nonstructural protein 2 inhibits type I/II interferon-stimulated JAK-STAT signaling. *J Virol* 84, 10877–10887.

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.

Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S.*, et al.* (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446, 916–920.

Gallinari, P., Brennan, D., Nardi, C., Brunetti, M., Tomei, L., Steinkuhler, C., and De Francesco, R. (1998). Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. *J Virol* 72, 6758–6769.

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.

Garry, R.F., and Dash, S. (2003). Proteomics computational analyses suggest that hepatitis C virus E1 and pestivirus E2 envelope glycoproteins are truncated class II fusion proteins. *Virology* 307, 255–265.

Gastaminza, P., Dryden, K.A., Boyd, B., Wood, M.R., Law, M., Yeager, M., and Chisari, F.V. (2010). Ultrastructural and biophysical characterization of hepatitis C virus particles produced in cell culture. *J Virol* 84, 10999–11009.

Getz, G.S., and Reardon, C.A. (2009). Apoprotein E as a lipid transport and signaling protein in the blood, liver, and artery wall. *Journal of Lipid Research* 50, S156-S161.

Gibbons, D.L., Ahn, A., Chatterjee, P.K., and Kielian, M. (2000). Formation and characterization of the trimeric form of the fusion protein of Semliki Forest Virus. *J Virol* 74, 7772–7780.

Gibbons, D.L., Vaney, M.C., Roussel, A., Vigouroux, A., Reilly, B., Lepault, J., Kielian, M., and Rey, F.A. (2004). Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. *Nature* 427, 320–325.

Gomatos, P.J. (1970). Comparison of the virion polymerase of reovirus with the enzyme purified from reovirus-infected cells. *J Virol* 6, 610–620.

Gomez de Cedron, M., Ehsani, N., Mikkola, M.L., Garcia, J.A., and Kaariainen, L. (1999). RNA helicase activity of Semliki Forest virus replicase protein NSP2. *FEBS Lett* 448, 19–22.

Gorbalenya, A.E., Pringle, F.M., Zeddam, J.L., Luke, B.T., Cameron, C.E., Kalmakoff, J., Hanzlik, T.N., Gordon, K.H.J., and Ward, V.K. (2002). The palm subdomain-based active site is internally permuted in viral RNA-dependent RNA polymerases of an ancient lineage. *J Mol Biol* 324, 47–62.

Gouttenoire, J., Montserret, R., Kennel, A., Penin, F., and Moradpour, D. (2009). An amphipathic alpha-helix at the C terminus of hepatitis C virus nonstructural protein 4B mediates membrane association. *J Virol* 83, 11378–11384.

Grakoui, A., Mccourt, D.W., Wychowski, C., Feinstone, S.M., and Rice, C.M. (1993a). A 2nd Hepatitis-C Virus-Encoded Proteinase. *Proc Natl Acad Sci U S A* 90, 10583–10587.

Grakoui, A., Mccourt, D.W., Wychowski, C., Feinstone, S.M., and Rice, C.M. (1993b). Characterization of the Hepatitis-C Virus-Encoded Serine Proteinase - Determination of Proteinase-Dependent Polyprotein Cleavage Sites. *J Virol* 67, 2832–2843.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., and Rice, C.M. (1993c). Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* 67, 1385–1395.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., and Rice, C.M. (1993d). Expression and Identification of Hepatitis C Virus Polyprotein Cleavage Products. *J Virol* 67, 1385–1395.

Gu, B., Johnston, V.K., Gutshall, L.L., Nguyen, T.T., Gontarek, R.R., Darcy, M.G., Tedesco, R., Dhanak, D., Duffy, K.J., Kao, C.C*., et al.* (2003a). Arresting initiation of hepatitis C virus RNA synthesis using heterocyclic derivatives. *J Biol Chem* 278, 16602–16607.

Gu, B.H., Johnston, V.K., Gutshall, L.L., Nguyen, T.T., Gontarek, R.R., Darcy, M.G., Tedesco, R., Dhanak, D., Duffy, K.J., Kao, C.C*., et al.* (2003b). Arresting initiation of hepatitis C virus RNA synthesis using heterocyclic derivatives. *J Biol Chem* 278, 16602–16607.

Gubler, D.J. (2002). Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol* 10, 100–103.

Hahn, C.S., Lustig, S., Strauss, E.G., and Strauss, J.H. (1988). Western Equine Encephalitis-Virus Is a Recombinant Virus. *Proc Natl Acad Sci U S A* 85, 5997–6001.

Halstead, S.B. (1990). Global epidemiology of dengue hemorrhagic fever. *Southeast Asian J Trop Med Public Health* 21, 636–641.

Han, J.H., Shyamala, V., Richman, K.H., Brauer, M.J., Irvine, B., Urdea, M.S., Tekamp-Olson, P., Kuo, G., Choo, Q.L., and Houghton, M. (1991). Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved

sequences in the 5' untranslated region and poly(A) tails at the 3' end. *Proc Natl Acad Sci U S A* 88, 1711–1715.
Hanoulle, X., Badillo, A., Wieruszeski, J.M., Verdegem, D., Landrieu, I., Bartenschlager, R., Penin, F., and Lippens, G. (2009). Hepatitis C virus NS5A protein is a substrate for the peptidyl-prolyl cis/trans isomerase activity of cyclophilins A and B. *J Biol Chem* 284, 13589–13601.
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.
Hardy, R.W., and Rice, C.M. (2005). Requirements at the 3' end of the sindbis virus genome for efficient synthesis of minus-strand RNA. *J Virol* 79, 4630–4639.
Helenius, A., Kartenbeck, J., Simons, K., and Fries, E. (1980). On the entry of Semliki forest virus into BHK-21 cells. *J Cell Biol* 84, 404–420.
Helle, F., and Dubuisson, J. (2008). Hepatitis C virus entry into host cells. *Cellular and Molecular Life Sciences* 65, 100–112.
Henderson, D.W., Peacock, S., and Randles, W.J. (1967). On the pathogenesis of Semliki forest virus (SFV) infection in the hamster. *Br J Exp Pathol* 48, 228–234.
Henle, W. (1950). Interference phenomena between animal viruses; a review. *J Immunol* 64, 203–236.
Herod, M.R., Jones, D.M., McLauchlan, J., and McCormick, C.J. (2012). Increasing Rate of Cleavage at Boundary between Non-structural Proteins 4B and 5A Inhibits Replication of Hepatitis C Virus. *J Biol Chem* 287, 568–580.
Honda, M., Ping, L.H., Rijnbrand, R.C., Amphlett, E., Clarke, B., Rowlands, D., and Lemon, S.M. (1996). Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. *Virology* 222, 31–42.
Hou, F., Sun, L., Zheng, H., Skaug, B., Jiang, Q.X., and Chen, Z.J. (2011). MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell* 146, 448–461.
Huang, A.S., Baltimore, D., and Bratt, M.A. (1971). Ribonucleic acid polymerase in virions of Newcastle disease virus: comparison with the vesicular stomatitis virus polymerase. *J Virol* 7, 389–394.
Huang, H., Chopra, R., Verdine, G.L., and Harrison, S.C. (1998). Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* 282, 1669–1675.
Hwang, J., Huang, L., Cordek, D.G., Vaughan, R., Reynolds, S.L., Kihara, G., Raney, K.D., Kao, C.C., and Cameron, C.E. (2010). Hepatitis C virus nonstructural protein 5A: biochemical characterization of a novel structural class of RNA-binding proteins. *J Virol* 84, 12480–12491.
International Committee on Taxonomy of Viruses., and King, A.M.Q. (2012). *Virus taxonomy: classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses* (London; Waltham, MA, Academic Press).
Isaacs, A., and Lindenmann, J. (1957). Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* 147, 258–267.
Ivashkina, N., Wolk, B., Lohmann, V., Bartenschlager, R., Blum, H.E., Penin, F., and Moradpour, D. (2002). The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. *J Virol* 76, 13088–13093.
Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1, 1–13.

Jin, Z., Leveque, V., Ma, H., Johnson, K.A., and Klumpp, K. (2012). Assembly, purification, and pre-steady-state kinetic analysis of active RNA-dependent RNA polymerase elongation complex. *J Biol Chem* 287, 10674–10683.

Jin, Z., Leveque, V., Ma, H., Johnson, K.A., and Klumpp, K. (2013). NTP-mediated nucleotide excision activity of hepatitis C virus RNA-dependent RNA polymerase. *Proc Natl Acad Sci U S A* 110, E348–357.

Kaariainen, L., and Ahola, T. (2002). Functions of alphavirus nonstructural proteins in RNA replication. *Prog Nucleic Acid Res Mol Biol* 71, 187–222.

Kaariainen, L., and Soderlund, H. (1978). Structure and replication of alpha-viruses. *Curr Top Microbiol Immunol* 82, 15–69.

Kamer, G., and Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res* 12, 7269–7282.

Kao, C., Zheng, M., and Rudisser, S. (1999a). A simple and efficient method to reduce nontemplated nucleotide addition at the 3 ' terminus of RNAs transcribed by T7 RNA polymerase. *Rna-a Publication of the Rna Society* 5, 1268–1272.

Kao, C.C., Del Vecchio, A.M., and Zhong, W. (1999b). De novo initiation of RNA synthesis by a recombinant flaviviridae RNA-dependent RNA polymerase. *Virology* 253, 1–7.

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T.S., Fujita, T., and Akira, S. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* 205, 1601–1610.

Kerkvliet, J., Papke, L., and Rodriguez, M. (2010). Antiviral Effects of a Transgenic RNA-Dependent RNA-Polymerase. *J Virol* 85, 621–625.

Keum, S.J., Park, S.M., Park, J.H., Jung, J.H., Shin, E.J., and Jang, S.K. (2012). The specific infectivity of hepatitis C virus changes through its life cycle. *Virology* 433, 462–470.

Kim, J.L., Morgenstern, K.A., Lin, C., Fox, T., Dwyer, M.D., Landro, J.A., Chambers, S.P., Markland, W., Lepre, C.A., O'Malley, E.T*., et al.* (1996). Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* 87, 343–355.

Kim, M.J., Zhong, W., Hong, Z., and Kao, C.C. (2000). Template nucleotide moieties required for de novo initiation of RNA synthesis by a recombinant viral RNA-dependent RNA polymerase. *J Virol* 74, 10312–10322.

Kim, Y.C., Russell, W.K., Ranjith-Kumar, C.T., Thomson, M., Russell, D.H., and Kao, C.C. (2005). Functional analysis of RNA binding by the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* 280, 38011–38019.

Kim, Y.K., Lee, S.H., Kim, C.S., Seol, S.K., and Jang, S.K. (2003). Long-range RNA-RNA interaction between the 5' nontranslated region and the core-coding sequences of hepatitis C virus modulates the IRES-dependent translation. *RNA* 9, 599–606.

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.

Klimstra, W.B., Ryman, K.D., Bernard, K.A., Nguyen, K.B., Biron, C.A., and Johnston, R.E. (1999). Infection of neonatal mice with sindbis virus results in a systemic inflammatory response syndrome. *J Virol* 73, 10387–10398.

Koch, J.O., and Bartenschlager, R. (1999). Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J Virol* 73, 7138–7146.

Koch, U., and Narjes, F. (2006). Allosteric inhibition of the hepatitis C virus NS5B RNA dependent RNA polymerase. *Infectious disorders drug targets* 6, 31–41.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A., and Steitz, T.A. (1992). Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 256, 1783–1790.

Kolykhalov, A.A., Agapov, E.V., Blight, K.J., Mihalik, K., Feinstone, S.M., and Rice, C.M. (1997). Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 277, 570–574.

Kolykhalov, A.A., Feinstone, S.M., and Rice, C.M. (1996). Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *J Virol* 70, 3363–3371.

Kong, L., Giang, E., Nieusma, T., Kadam, R.U., Cogburn, K.E., Hua, Y., Dai, X., Stanfield, R.L., Burton, D.R., Ward, A.B*., et al.* (2013). Hepatitis C virus E2 envelope glycoprotein core structure. *Science* 342, 1090–1094.

Koonin, E.V. (1991). The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *J Gen Virol* 72 (Pt 9), 2197–2206.

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.

Krey, T., d'Alayer, J., Kikuti, C.M., Saulnier, A., Damier-Piolle, L., Petitpas, I., Johansson, D.X., Tawar, R.G., Baron, B., Robert, B*., et al.* (2010). The Disulfide Bonds in Glycoprotein E2 of Hepatitis C Virus Reveal the Tertiary Organization of the Molecule. *Plos Pathogens* 6, e1000762.

Krieger, N., Lohmann, V., and Bartenschlager, R. (2001). Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J Virol* 75, 4614–4624.

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.

Kumar, V., Butcher, S.J., Oorni, K., Engelhardt, P., Heikkonen, J., Kaski, K., Ala-Korpela, M., and Kovanen, P.T. (2011). Three-Dimensional cryoEM Reconstruction of Native LDL Particles to 16 angstrom Resolution at Physiological Body Temperature. *PLoS One* 6.

Laakkonen, P., Ahola, T., and Kaariainen, L. (1996). The effects of palmitoylation on membrane association of Semliki forest virus RNA capping enzyme. *J Biol Chem* 271, 28567–28571.

Laakkonen, P., Hyvonen, M., Peranen, J., and Kaariainen, L. (1994). Expression of Semliki Forest virus nsP1-specific methyltransferase in insect cells and in Escherichia coli. *J Virol* 68, 7418–7425.

Labonte, P., Axelrod, V., Agarwal, A., Aulabaugh, A., Amin, A., and Mak, P. (2002). Modulation of hepatitis C virus RNA-dependent RNA polymerase activity by structure-based site-directed mutagenesis. *J Biol Chem* 277, 38838–38846.

Lampio, A., Kilpelainen, I., Pesonen, S., Karhi, K., Auvinen, P., Somerharju, P., and Kaariainen, L. (2000). Membrane binding mechanism of an RNA virus-capping enzyme. *J Biol Chem* 275, 37853–37859.

Lampson, G.P., Tytell, A.A., Field, A.K., Nemes, M.M., and Hilleman, M.R. (1967). Inducers of interferon and host resistance. I. Double-stranded RNA from extracts of Penicillium funiculosum. *Proc Natl Acad Sci U S A* 58, 782–789.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86, 973–983.

Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., and Weber, P.C. (1999). Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* 6, 937–943.

Li, G., Thomas, A.M., Williams, J.A., Kong, B., Liu, J., Inaba, Y., Xie, W., and Guo, G.L. (2012). Farnesoid X receptor induces murine scavenger receptor Class B type I via intron binding. *PLoS One* 7, e35895.

Li, K., Chen, Z., Kato, N., Gale, M., Jr., and Lemon, S.M. (2005a). Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J Biol Chem* 280, 16739–16747.

Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C., Ikeda, M., Ray, S.C., Gale, M., Jr., and Lemon, S.M. (2005b). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A* 102, 2992–2997.

Liljestrom, P., and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology (N Y)* 9, 1356–1361.

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.

Lindenbach, B.D., Pragai, B.M., Montserret, R., Beran, R.K.F., Pyle, A.M., Penin, F., and Rice, C.M. (2007). The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphospholylation and viral replication. *J Virol* 81, 8905–8918.

Ling, J., Lewis, J., Douglas, D., Kneteman, N.M., and Vance, D.E. (2013). Characterization of lipid and lipoprotein metabolism in primary human hepatocytes. *Biochim Biophys Acta* 1831, 387–397.

Lohmann, V., Korner, F., Herian, U., and Bartenschlager, R. (1997). Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J Virol* 71, 8416–8428.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285, 110–113.

Love, R.A., Parge, H.E., Yu, X., Hickey, M.J., Diehl, W., Gao, J., Wriggers, H., Ekker, A., Wang, L., Thomson, J.A., *et al.* (2003). Crystallographic identification of a noncompetitive inhibitor binding site on the hepatitis C virus NS5B RNA polymerase enzyme. *J Virol* 77, 7575–7581.

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.

Lundin, M., Monne, M., Widell, A., Von Heijne, G., and Persson, M.A. (2003). Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol* 77, 5428–5438.

Luo, G., Hamatake, R.K., Mathis, D.M., Racela, J., Rigat, K.L., Lemm, J., and Colonno, R.J. (2000). De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. *J Virol* 74, 851–863.

Ma, H., Leveque, V., De Witte, A., Li, W., Hendricks, T., Clausen, S.M., Cammack, N., and Klumpp, K. (2005). Inhibition of native hepatitis C virus replicase by nucleotide and non-nucleoside inhibitors. *Virology* 332, 8–15.

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. *Molecular Cell* 5, 255–266.

Marcus, P.I., Engelhardt, D.L., Hunt, J.M., and Sekellick, M.J. (1971). Interferon action: inhibition of vesicular stomatitis virus RNA synthesis induced by virion-bound polymerase. *Science* 174, 593–598.

Mathiot, C.C., Grimaud, G., Garry, P., Bouquety, J.C., Mada, A., Daguisy, A.M., and Georges, A.J. (1990). An Outbreak of Human Semliki Forest Virus-Infections in Central-African-Republic. *American Journal of Tropical Medicine and Hygiene* 42, 386–393.

McCown, M.F., Rajyaguru, S., Le Pogam, S., Ali, S., Jiang, W.R., Kang, H., Symons, J., Cammack, N., and Najera, I. (2008). The hepatitis C virus replicon presents a higher barrier to resistance to nucleoside analogs than to nonnucleoside polymerase or protease inhibitors. *Antimicrobial agents and chemotherapy* 52, 1604–1612.

McIntosh, B.M., Worth, C.B., and Kokernot, R.H. (1961). Isolation of Semliki Forest virus from Aedes (Aedimorphus) argenteopunctatus (Theobald) collected in Portuguese East Africa. *Trans R Soc Trop Med Hyg* 55, 192–198.

McKercher, G., Beaulieu, P.L., Lamarre, D., LaPlante, S., Lefebvre, S., Pellerin, C., Thauvette, L., and Kukolj, G. (2004). Specific inhibitors of HCV polymerase identified using an NS5B with lower affinity for template/primer substrate. *Nucleic Acids Research* 32, 422–431.

Mead, J.R., Irvine, S.A., and Ramji, D.P. (2002). Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med (Berl)* 80, 753–769.

Medcalf, L., Poole, E., Elton, D., and Digard, P. (1999). Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein. *J Virol* 73, 7349–7356.

Medzhitov, R., and Janeway, C.A., Jr. (1997). Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91, 295–298.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388, 394–397.

Meertens, L., Bertaux, C., and Dragic, T. (2006). Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. *J Virol* 80, 11571–11578.

Mercer, J., Schelhaas, M., and Helenius, A. (2010). Virus entry by endocytosis. *Annu Rev Biochem* 79, 803–833.

Merits, A., Vasiljeva, L., Ahola, T., Kaariainen, L., and Auvinen, P. (2001). Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *J Gen Virol* 82, 765–773.

Merz, A., Long, G., Hiet, M.S., Brugger, B., Chlanda, P., Andre, P., Wieland, F., Krijnse-Locker, J., and Bartenschlager, R. (2011). Biochemical and morphological properties of hepatitis C virus particles and determination of their lipidome. *J Biol Chem* 286, 3018–3032.

Meyer, P.R., Matsuura, S.E., Mian, A.M., So, A.G., and Scott, W.A. (1999). A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Molecular Cell* 4, 35–43.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437, 1167–1172.

Miller, R.H., and Purcell, R.H. (1990). Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc Natl Acad Sci U S A* 87, 2057–2061.

Minskaia, E., Hertzig, T., Gorbalenya, A.E., Campanacci, V., Cambillau, C., Canard, B., and Ziebuhr, J. (2006). Discovery of an RNA virus 3 '-> 5 ' exoribonuclease that is critically involved in coronavirus RNA synthesis. *Proc Natl Acad Sci U S A* 103, 5108–5113.

Mohd Hanafiah, K., Groeger, J., Flaxman, A.D., and Wiersma, S.T. (2013). Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology* 57, 1333–1342.

Montserret, R., McLeish, M.J., Bockmann, A., Geourjon, C., and Penin, F. (2000). Involvement of electrostatic interactions in the mechanism of peptide folding induced by sodium dodecyl sulfate binding. *Biochemistry* 39, 8362–8373.

Moradpour, D., Brass, V., Bieck, E., Friebe, P., Gosert, R., Blum, H.E., Bartenschlager, R., Perlin, F., and Lohmann, V. (2004). Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. *J Virol* 78, 13278–13284.

Moriyama, M., Kato, N., Otsuka, M., Shao, R.X., Taniguchi, H., Kawabe, T., and Omata, M. (2007). Interferon-beta is activated by hepatitis C virus NS5B and inhibited by NS4A, NS4B, and NS5A. *Hepatol Int* 1, 302–310.

Mosley, R.T., Edwards, T.E., Murakami, E., Lam, A.M., Grice, R.L., Du, J., Sofia, M.J., Furman, P.A., and Otto, M.J. (2012). Structure of hepatitis C virus polymerase in complex with primer-template RNA. *J Virol* 86, 6503–6511.

Muthukrishnan, S., Both, G.W., Furuichi, Y., and Shatkin, A.J. (1975). 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. *Nature* 255, 33–37.

Myong, S., Cui, S., Cornish, P.V., Kirchhofer, A., Gack, M.U., Jung, J.U., Hopfner, K.P., and Ha, T. (2009). Cytosolic viral sensor RIG-I is a 5'-triphosphate-dependent translocase on double-stranded RNA. *Science* 323, 1070–1074.

Naka, K., Dansako, H., Kobayashi, N., Ikeda, M., and Kato, N. (2006). Hepatitis C virus NS5B delays cell cycle progression by inducing interferon-beta via Toll-like receptor 3 signaling pathway without replicating viral genomes. *Virology* 346, 348–362.

Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., and Sato, J. (1982). Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res* 42, 3858–3863.

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.

Newcomb, L.L., Kuo, R.L., Ye, Q., Jiang, Y., Tao, Y.J., and Krug, R.M. (2009). Interaction of the influenza a virus nucleocapsid protein with the viral RNA polymerase potentiates unprimed viral RNA replication. *J Virol* 83, 29–36.

O'Farrell, D., Trowbridge, R., Rowlands, D., and Jager, J. (2003). Substrate complexes of hepatitis C virus RNA polymerase (HC-J4): structural evidence for nucleotide import and de-novo initiation. *J Mol Biol* 326, 1025–1035.

Oh, J.W., Ito, T., and Lai, M.M. (1999). A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. *J Virol* 73, 7694–7702.

Ollis, D.L., Kline, C., and Steitz, T.A. (1985). Domain of E. coli DNA polymerase I showing sequence homology to T7 DNA polymerase. *Nature* 313, 818–819.

Oshiumi, H., Miyashita, M., Matsumoto, M., and Seya, T. (2013). A distinct role of Riplet-mediated K63-Linked polyubiquitination of the RIG-I repressor domain in human antiviral innate immune responses. *PLoS Pathog* 9, e1003533.

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 U S A* 79, 5235–5239.

Ou, J.H., Strauss, E.G., and Strauss, J.H. (1981). Comparative studies of the 3'-terminal sequences of several alpha virus RNAs. *Virology* 109, 281–289.

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.

Padbidri, V.S., and Gnaneswar, T.T. (1979). Epidemiological investigations of chikungunya epidemic at Barsi, Maharashtra state, India. *J Hyg Epidemiol Microbiol Immunol* 23, 445–451.

Parker, J.S., Broering, T.J., Kim, J., Higgins, D.E., and Nibert, M.L. (2002). Reovirus core protein mu2 determines the filamentous morphology of viral inclusion bodies by interacting with and stabilizing microtubules. *J Virol* 76, 4483–4496.

Patton, J.T., and Spencer, E. (2000). Genome replication and packaging of segmented double-stranded RNA viruses. *Virology* 277, 217–225.

Paul, D., and Bartenschlager, R. (2013). Architecture and biogenesis of plus-strand RNA virus replication factories. *World J Virol* 2, 32–48.

Paul, D., Hoppe, S., Saher, G., Krijnse-Locker, J., and Bartenschlager, R. (2013). Morphological and biochemical characterization of the membranous hepatitis C virus replication compartment. *J Virol* 87, 10612–10627.

Pauwels, F., Mostmans, W., Quirynen, L.M., van der Helm, L., Boutton, C.W., Rueff, A.S., Cleiren, E., Raboisson, P., Surleraux, D., Nyanguile, O., *et al.* (2007). Binding-site identification and genotypic profiling of hepatitis C virus polymerase inhibitors. *J Virol* 81, 6909–6919.

Peisley, A., Wu, B., Xu, H., Chen, Z.J., and Hur, S. (2014). Structural basis for ubiquitin-mediated antiviral signal activation by RIG-I. *Nature* in press.

Peranen, J., Rikkonen, M., Liljestrom, P., and Kaariainen, L. (1990). Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *J Virol* 64, 1888–1896.

Peranen, J., Takkinen, K., Kalkkinen, N., and Kaariainen, L. (1988). Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *J Gen Virol* 69 (Pt 9), 2165–2178.

Perz, J.F., Armstrong, G.L., Farrington, L.A., Hutin, Y.J., and Bell, B.P. (2006). The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 45, 529–538.

Pestka, S., Krause, C.D., and Walter, M.R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunological Reviews* 202, 8–32.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera – a visualization system for exploratory research and analysis. *J Comput Chem* 25, 1605–1612.

Pialoux, G., Gauzere, B.A., Jaureguiberry, S., and Strobel, M. (2007). Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis* 7, 319–327.

Pietschmann, T., Kaul, A., Koutsoudakis, G., Shavinskaya, A., Kallis, S., Steinmann, E., Abid, K., Negro, F., Dreux, M., Cosset, F.L., *et al.* (2006). Construction and

characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* 103, 7408–7413.

Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., and Bartenschlager, R. (2001). Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *J Virol* 75, 1252–1264.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A.J., Houghton, M., Rosa, D., Grandi, G.*, et al.* (1998). Binding of hepatitis C virus to CD81. *Science* 282, 938–941.

Ploss, A., Evans, M.J., Gaysinskaya, V.A., Panis, M., You, H.N., de Jong, Y.P., and Rice, C.M. (2009). Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457, 882–886.

Poch, O., Sauvaget, I., Delarue, M., and Tordo, N. (1989). Identification of 4 Conserved Motifs among the Rna-Dependent Polymerase Encoding Elements. *Embo J* 8, 3867–3874.

Poltorak, A., Smirnova, I., He, X., Liu, M.Y., Van Huffel, C., McNally, O., Birdwell, D., Alejos, E., Silva, M., Du, X.*, et al.* (1998). Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood Cells Mol Dis* 24, 340–355.

Powdrill, M.H., Tchesnokov, E.P., Kozak, R.A., Russell, R.S., Martin, R., Svarovskaia, E.S., Mo, H., Kouyos, R.D., and Gotte, M. (2011). Contribution of a mutational bias in hepatitis C virus replication to the genetic barrier in the development of drug resistance. *Proc Natl Acad Sci U S A* 108, 20509–20513.

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.

Qin, W., Luo, H., Nomura, T., Hayashi, N., Yamashita, T., and Murakami, S. (2002). Oligomeric interaction of hepatitis C virus NS5B is critical for catalytic activity of RNA-dependent RNA polymerase. *J Biol Chem* 277, 2132–2137.

Quinkert, D., Bartenschlager, R., and Lohmann, V. (2005). Quantitative analysis of the hepatitis C virus replication complex. *J Virol* 79, 13594–13605.

Ranjith-Kumar, C.T., Gutshall, L., Kim, M.J., Sarisky, R.T., and Kao, C.C. (2002). Requirements for de novo initiation of RNA synthesis by recombinant flaviviral RNA-dependent RNA polymerases. *J Virol* 76, 12526–12536.

Ranjith-Kumar, C.T., and Kao, C.C. (2006). Recombinant viral RdRps can initiate RNA synthesis from circular templates. *Rna-a Publication of the Rna Society* 12, 303–312.

Ravi, V. (2006). Re-emergence of chikungunya virus in India. *Indian J Med Microbiol* 24, 83–84.

Reigadas, S., Ventura, M., Sarih-Cottin, L., Castroviejo, M., Litvak, S., and Astier-Gin, T. (2001). HCV RNA-dependent RNA polymerase replicates in vitro the 3 ' terminal region of the minus-strand viral RNA more efficiently than the 3 ' terminal region of the plus RNA. *European Journal of Biochemistry* 268, 5857–5867.

Rijnbrand, R., Bredenbeek, P., van der Straaten, T., Whetter, L., Inchauspe, G., Lemon, S., and Spaan, W. (1995). Almost the entire 5' non-translated region of hepatitis C virus is required for cap-independent translation. *FEBS Lett* 365, 115–119.

Rikkonen, M. (1996). Functional significance of the nuclear-targeting and NTP-binding motifs of Semliki Forest virus nonstructural protein nsP2. *Virology* 218, 352–361.

Rikkonen, M., Peranen, J., and Kaariainen, L. (1992). Nuclear and nucleolar targeting signals of Semliki Forest virus nonstructural protein nsP2. *Virology* 189, 462–473.

Rikkonen, M., Peranen, J., and Kaariainen, L. (1994). ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *J Virol* 68, 5804–5810.

Romero-Brey, I., Merz, A., Chiramel, A., Lee, J.Y., Chlanda, P., Haselman, U., Santarella-Mellwig, R., Habermann, A., Hoppe, S., Kallis, S.*, et al.* (2012). Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication. *PLoS Pathog* 8, e1003056.

Romero-Lopez, C., and Berzal-Herranz, A. (2009). A long-range RNA-RNA interaction between the 5 ' and 3 ' ends of the HCV genome. *Rna-a Publication of the Rna Society* 15, 1740–1752.

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.

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.

Ryman, K.D., and Klimstra, W.B. (2008). Host responses to alphavirus infection. *Immunol Rev* 225, 27–45.

Ryman, K.D., Meier, K.C., Nangle, E.M., Ragsdale, S.L., Korneeva, N.L., Rhoads, R.E., MacDonald, M.R., and Klimstra, W.B. (2005). Sindbis virus translation is inhibited by a PKR/RNase L-independent effector induced by alpha/beta interferon priming of dendritic cells. *J Virol* 79, 1487–1499.

Rytel, M.W., Shope, R.E., and Kilbourne, E.D. (1966). An antiviral substance from Penicillium funiculosum. V. Induction of interferon by helenine. *J Exp Med* 123, 577–584.

Sainz, B., Barretto, N., Martin, D.N., Hiraga, N., Imamura, M., Hussain, S., Marsh, K.A., Yu, X.M., Chayama, K., Alrefai, W.A.*, et al.* (2012). Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nature Medicine* 18, 281–285.

Samuel, C.E. (2001). Antiviral actions of interferons. *Clin Microbiol Rev* 14, 778–809.

Sanner, M.F., Olson, A.J., and Spehner, J.C. (1996). Reduced surface: an efficient way to compute molecular surfaces. *Biopolymers* 38, 305–320.

Sawicki, D.L., and Gomatos, P.J. (1976). Replication of semliki forest virus: polyadenylate in plus-strand RNA and polyuridylate in minus-strand RNA. *J Virol* 20, 446–464.

Sawicki, D.L., and Sawicki, S.G. (1980). Short-lived minus-strand polymerase for Semliki Forest virus. *J Virol* 34, 108–118.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R.M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R., and Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *Embo J* 21, 5017–5025.

Schlee, M., Roth, A., Hornung, V., Hagmann, C.A., Wimmenauer, V., Barchet, W., Coch, C., Janke, M., Mihailovic, A., Wardle, G.*, et al.* (2009). Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 31, 25–34.

Schmidt, A., Schwerd, T., Hamm, W., Hellmuth, J.C., Cui, S., Wenzel, M., Hoffmann, F.S., Michallet, M.C., Besch, R., Hopfner, K.P.*, et al.* (2009). 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proc Natl Acad Sci U S A* 106, 12067–12072.

Segrest, J.P., Jones, M.K., De Loof, H., and Dashti, N. (2001). Structure of apolipoprotein B-100 in low density lipoproteins. *J Lipid Res* 42, 1346–1367.
Selby, M.J., Glazer, E., Masiarz, F., and Houghton, M. (1994). Complex Processing and Protein-Protein Interactions in the E2-Ns2 Region of Hcv. *Virology* 204, 114–122.
Seth, R.B., Sun, L., Ea, C.K., and Chen, Z.J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122, 669–682.
Shatkin, A.J., and Sipe, J.D. (1968). RNA polymerase activity in purified reoviruses. *Proc Natl Acad Sci U S A* 61, 1462–1469.
Shim, J.H., Larson, G., Wu, J.Z., and Hong, Z. (2002). Selection of 3'-template bases and initiating nucleotides by hepatitis C virus NS5B RNA-dependent RNA polymerase. *J Virol* 76, 7030–7039.
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.
Shope, R.E. (1953). An antiviral substance from Penicillium funiculosum. I. Effect upon infection in mice with swine influenza virus and Columbia SK encephalomyelitis virus. *J Exp Med* 97, 601–625.
Shope, R.E. (1966). An antiviral substance from Penicillium funiculosum. VI. Prevention of the establishment of passive immunity to Semliki Forest virus infection in mice by Helenine. *J Exp Med* 124, 15–31.
Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witkowski, J.T., and Robins, R.K. (1972). Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* 177, 705–706.
Simmonds, P., Holmes, E.C., Cha, T.A., Chan, S.W., McOmish, F., Irvine, B., Beall, E., Yap, P.L., Kolberg, J., and Urdea, M.S. (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* 74 (Pt 11), 2391–2399.
Simmonds, P., Tuplin, A., and Evans, D.J. (2004). Detection of genome-scale ordered RNA structure (GORS) in genomes of positive-stranded RNA viruses: Implications for virus evolution and host persistence. *RNA* 10, 1337–1351.
Smit, J.M., Waarts, B.L., Kimata, K., Klimstra, W.B., Bittman, R., and Wilschut, J. (2002). Adaptation of alphaviruses to heparan sulfate: interaction of Sindbis and Semliki forest viruses with liposomes containing lipid-conjugated heparin. *J Virol* 76, 10128–10137.
Smith, R.M., Walton, C.M., Wu, C.H., and Wu, G.Y. (2002). Secondary structure and hybridization accessibility of hepatitis C virus 3'-terminal sequences. *J Virol* 76, 9563–9574.
Smithburn, K.C., and Haddow, A.J. (1944). Semliki Forest Virus: I. Isolation and Pathogenic Properties. *J Immunol* 49, 141–157.
Sofia, M.J., Chang, W., Furman, P.A., Mosley, R.T., and Ross, B.S. (2012). Nucleoside, nucleotide, and non-nucleoside inhibitors of hepatitis C virus NS5B RNA-dependent RNA-polymerase. *J Med Chem* 55, 2481–2531.
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.
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.
Steitz, T.A. (1998). A mechanism for all polymerases. *Nature* 391, 231–232.
Strauss, J.H., and Strauss, E.G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* 58, 491–562.
Subba-Reddy, C.V., Goodfellow, I., and Kao, C.C. (2011). VPg-primed RNA synthesis of norovirus RNA-dependent RNA polymerases by using a novel cell-based assay. *J Virol* 85, 13027–13037.
Sumpter, R., Jr., Loo, Y.M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S.M., and Gale, M., Jr. (2005). Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 79, 2689–2699.
Sun, X.L., Johnson, R.B., Hockman, M.A., and Wang, Q.M. (2000). De novo RNA synthesis catalyzed by HCV RNA-dependent RNA polymerase. *Biochem Biophys Res Commun* 268, 798–803.
Zeng, W., Sun, L., Jiang, X., Chen, X., Hou, F., Adhikari, A., Xu, M., and Chen, Z.J. (2010). Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. *Cell* 141, 315–330.
Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., and Chisari, F.V. (2005). Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* 102, 9294–9299.
Zhong, W., Uss, A.S., Ferrari, E., Lau, J.Y., and Hong, Z. (2000). De novo initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase. *J Virol* 74, 2017–2022.
Takeuchi, O., and Akira, S. (2010). Pattern Recognition Receptors and Inflammation. *Cell* 140, 805–820.
Takkinen, K., Peranen, J., and Kaariainen, L. (1991). Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein. *J Gen Virol* 72 (Pt 7), 1627–1633.
Tamberg, N., Lulla, V., Fragkoudis, R., Lulla, A., Fazakerley, J.K., and Merits, A. (2007). Insertion of EGFP into the replicase gene of Semliki Forest virus results in a novel, genetically stable marker virus. *J Gen Virol* 88, 1225–1230.
Tanaka, T., Kato, N., Cho, M.J., Sugiyama, K., and Shimotohno, K. (1996). Structure of the 3' terminus of the hepatitis C virus genome. *J Virol* 70, 3307–3312.
Tanji, Y., Kaneko, T., Satoh, S., and Shimotohno, K. (1995). Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5A. *J Virol* 69, 3980–3986.
Tao, Y., Farsetta, D.L., Nibert, M.L., and Harrison, S.C. (2002). RNA synthesis in a cage--structural studies of reovirus polymerase lambda3. *Cell* 111, 733–745.
Tellinghuisen, T.L., Marcotrigiano, J., Gorbalenya, A.E., and Rice, C.M. (2004). The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* 279, 48576–48587.
Tellinghuisen, T.L., Marcotrigiano, J., and Rice, C.M. (2005). Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* 435, 374–379.
Thompson, A.A., and Peersen, O.B. (2004). Structural basis for proteolysis-dependent activation of the poliovirus RNA-dependent RNA polymerase. *EMBO J* 23, 3462–3471.
Thompson, A.A., Zou, A., Yan, J., Duggal, R., Hao, W., Molina, D., Cronin, C.N., and Wells, P.A. (2009). Biochemical characterization of recombinant hepatitis C virus nonstructural protein 4B: evidence for ATP/GTP hydrolysis and adenylate kinase activity. *Biochemistry* 48, 906–916.

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.

Tomei, L., Failla, C., Santolini, E., De Francesco, R., and La Monica, N. (1993). NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J Virol* 67, 4017–4026.

Tomei, L., Vitale, R.L., Incitti, I., Serafini, S., Altamura, S., Vitelli, A., and De Francesco, R. (2000). Biochemical characterization of a hepatitis C virus RNA-dependent RNA polymerase mutant lacking the C-terminal hydrophobic sequence. *J Gen Virol* 81, 759–767.

Tscherne, D.M., Jones, C.T., Evans, M.J., Lindenbach, B.D., McKeating, J.A., and Rice, C.M. (2006). Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J Virol* 80, 1734–1741.

Tsukiyama-Kohara, K., Iizuka, N., Kohara, M., and Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *J Virol* 66, 1476–1483.

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.

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.

Tyndall, C., La Mantia, G., Thacker, C.M., Favaloro, J., and Kamen, R. (1981). A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. *Nucleic Acids Res* 9, 6231–6250.

Tytell, A.A., Lampson, G.P., Field, A.K., and Hilleman, M.R. (1967). Inducers of interferon and host resistance. 3. Double-stranded RNA from reovirus type 3 virions (reo 3-RNA). *Proc Natl Acad Sci U S A* 58, 1719–1722.

Waheed, Y., Saeed, U., Anjum, S., Afzal, M.S., and Ashraf, M. (2012). Development of Global Consensus Sequence and Analysis of Highly Conserved Domains of the HCV NS5B Protein. *Hepatitis Monthly* 12.

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.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M.*, et al.* (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Medicine* 11, 791–796.

Wang, M., Ng, K.K., Cherney, M.M., Chan, L., Yannopoulos, C.G., Bedard, J., Morin, N., Nguyen-Ba, N., Alaoui-Ismaili, M.H., Bethell, R.C.*, et al.* (2003). Non-nucleoside analogue inhibitors bind to an allosteric site on HCV NS5B polymerase. Crystal structures and mechanism of inhibition. *J Biol Chem* 278, 9489–9495.

Wang, Y.F., Sawicki, S.G., and Sawicki, D.L. (1991). Sindbis virus nsP1 functions in negative-strand RNA synthesis. *J Virol* 65, 985–988.

Vasiljeva, L., Merits, A., Auvinen, P., and Kaariainen, L. (2000). Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. *J Biol Chem* 275, 17281–17287.

Vasiljeva, L., Merits, A., Golubtsov, A., Sizemskaja, V., Kaariainen, L., and Ahola, T. (2003). Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J Biol Chem* 278, 41636–41645.

Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyanari, Y., and Shimotohno, K. (2005). Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Molecular Cell* 19, 111–122.

Vaughan, R., Li, Y., Fan, B., Ranjith-Kumar, C.T., and Kao, C.C. (2012). RNA binding by the NS3 protease of the hepatitis C virus. *Virus Res* 169, 80–90.

Weisgraber, K.H. (1994). Apolipoprotein E: structure-function relationships. *Adv Protein Chem* 45, 249–302.

Wellink, J., and van Kammen, A. (1988). Proteases involved in the processing of viral polyproteins. Brief review. *Arch Virol* 98, 1–26.

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.

Wengler, G., and Gross, H.S. (1979). Replicative form of Semliki Forest virus RNA contains an unpaired guanosine. *Nature* 282, 754–756.

Verdegem, D., Badillo, A., Wieruszeski, J.M., Landrieu, I., Leroy, A., Bartenschlager, R., Penin, F., Lippens, G., and Hanoulle, X. (2011). Domain 3 of NS5A protein from the hepatitis C virus has intrinsic alpha-helical propensity and is a substrate of cyclophilin A. *J Biol Chem* 286, 20441–20454.

Vihinen, H., Ahola, T., Tuittila, M., Merits, A., and Kaariainen, L. (2001). Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J Biol Chem* 276, 5745–5752.

Vihinen, H., and Saarinen, J. (2000). Phosphorylation site analysis of Semliki forest virus nonstructural protein 3. *J Biol Chem* 275, 27775–27783.

Xing, J.Z., Zhu, L., Gabos, S., and Xie, L. (2006). Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity. *Toxicol In Vitro* 20, 995–1004.

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.

Xu, Z., Choi, J., Yen, T.S., Lu, W., Strohecker, A., Govindarajan, S., Chien, D., Selby, M.J., and Ou, J. (2001). Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *Embo J* 20, 3840–3848.

Yang, X., Smidansky, E.D., Maksimchuk, K.R., Lum, D., Welch, J.L., Arnold, J.J., Cameron, C.E., and Boehr, D.D. (2012). Motif D of viral RNA-dependent RNA polymerases determines efficiency and fidelity of nucleotide addition. *Structure* 20, 1519–1527.

Yoneyama, M., and Fujita, T. (2007). RIG-I family RNA helicases: cytoplasmic sensor for antiviral innate immunity. *Cytokine Growth Factor Rev* 18, 545–551.

York, A., Hengrung, N., Vreede, F.T., Huiskonen, J.T., and Fodor, E. (2013). Isolation and characterization of the positive-sense replicative intermediate of a negative-strand RNA virus. *Proc Natl Acad Sci U S A* 110, E4238–4245.

You, S., Stump, D.D., Branch, A.D., and Rice, C.M. (2004). A cis-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. *J Virol* 78, 1352–1366.

Yu, G.Y., He, G.B., Li, C.Y., Tang, M., Grivennikov, S., Tsai, W.T., Wu, M.S., Hsu, C.W., Tsai, Y., Wang, L.H.C., *et al.* (2012). Hepatic Expression of HCV RNA-Dependent RNA Polymerase Triggers Innate Immune Signalling and Cytokine Production. *Molecular Cell* 48, 313–321.

Yuhashi, K., Ohnishi, S., Kodama, T., Koike, K., and Kanamori, H. (2014). In vitro selection of the 3'-untranslated regions of the human liver mRNA that bind to the HCV nonstructural protein 5B. *Virology* 450–451, 13–23.

8. SUMMARY IN ESTONIAN

RNA-sõltuva RNA polümeraasi aktiivsuse kaudu on selgroogsete rakud võimelised tuvastama pluss ahelaga RNA viiruste nakkuse

Pluss ahelaga RNA viirused ((+)RNA viirused) on kõige rohkemaarvulisem inimesi nakatavate viiruste klass. Nende viiruste hulka kuuluvad sealhulgas hepatiit C viirus (HCV) ning Semliki Forest viirus (SFV). HCV nakkused tekitavad kroonilist maksapõletikku, maksatsirroosi, ja maksavähki. HCV nakkus on väga levinud – vastavalt Maailma Tervise Organisatsiooni (WHO) andmetele põevad C-hepatiiti 130–170 miljonit inimest. Teisalt, SFV on samuti võimeline nakatama inimesi. See viirus ei ole eriti ohtlik inimesele, samas on SFV väga effektiivne hiirte, hamstrite ning küülikute patogeen. Nende kahe viiruse poolt nakatatavate rakkude spekter on väga erinev – HCV on võimeline nakatama ja replitseeruma vaid inimese ja šimpansi maksarakkudes, kuid SFV nakatab ja paljuneb paljudes erinevate organismide rakkudes. Vaatamata HCV ja SFV patogeensuse erinevustele toimub nende viiruste RNA genoomi replikatsioon üldiselt sarnase mehhanismi alusel ja see on omane ka teistele (+)RNA viirustele. Nende genoomi replikatsiooni viib läbi viiruse genoomi poolt kodeeritud RNA-sõltuv RNA polümeraas, mis toimib mitmesubühikulise replikaasi koostises. Funktsionaalse replikaasi moodustavad lisaks polümeraasse aktiivsusega subühikule terve rida teisi valke, milledel on kindlad struktuursed või ensümaatilised aktiivsused ja mis on väga olulised viirusnakkuse produktiivseks toimumiseks. Üheks nendest replikaasi koostisesse kuuluvatest komponentidest on proteaasse aktiivsusega subühik. Nii HCV kui ka SFV puhul, selle proteaasi aktiivsus vajalik üheltpoolt algse polüproteiini protsessinguks. Teisalt, sellele proteaasile omistatakse olulist rolli raku viirusvastase kaasasündinud immuunvastuse tasalülituses.

Käesoleva töö üldeesmärgiks oli uurida (+)RNA viiruste RNA-sõltuva RNA polümeraasse ja proteaasse aktiivsuse potentsiaalset antagonismi rakulises tüüp I interferoonide aktivatsioonis. Selle töö kaigus me:

1. tekitasime spetsiifilised monokloonsed antikehad, mida kasutasime kui molekulaarseid tööriistu, mõjutamaks HCV RNA-sõltuva RNA polümeraasi mittestruktuurse valgu 5B (NS5B) aktiivsust selle ensüümi "sõrmede domeeni" kaudu;
2. töötasime välja algoritmi ette ennustamaks uusi HCV NS3-4A aktiivtsentrisse seonduvaid proteaasi inhibiitoreid ja kontrollisime eksperimentaalselt nende ühendite toksilisuse;
3. uurisime viiruse proteaasi ja RNA-sõltuva RNA polümeraasi aktiivsuste antagonismi tüüp I interferoonide aktiveerimisel SFV nakkuse kontekstis;
4. hindasime NS5B ja NS3-4A ensümaatiliste eriaktiivsustega kaasnevate mõjutuste kasutamist erinevate viirusvastaste ravimite väljatöötamiseks.

Struktuurselt meenutab HCV RNA-sõltuv RNA polümeraas paremat käelaba, mille pöial ja sõrmed on paindunud üksteiste suhtes sellel moel, et pöial puudutab nimetissõrme. Sellesse käe pihku, pöidla ja nimetissõrme vahele, siseneb üheahelaline viiruse genoomne RNA. RNA 3'-otsa peopessa seondumise järgselt tõmbuvad sõrmed kokku ja pigistavad RNA 3'-otsa polümeraasi aktiivtsentrisse, kus polümeraas algatab matriitsi alusel komplementaarse RNA ahela sünteesi, millest saab alguse matriitsiga antiparalleelne RNA ahel. Sünteesi etapi järgselt toimub haarde lõdvenemine ja viiruse genoomse RNA ahela ja sellel sünteesitud RNA ahela liikumine üks samm edasi. Kokkutõmbe, sünteesi ja elongatsiooni etappe korratakse kuni viiruse RNA genoom on kopeeritud (-) ahelaks. Sünteesitud RNA ahel väljub ensüümi aktiivtsentrist peopesaga perpendikulaarselt. RNA-sõltuva RNA polümeraasset aktiivsust kannab HCV puhul NS5B replikaasi komponent. Mõistetavalt on see ensüüm olnud viirusvastaste ainete (inhibiitorite) väljatöötamisel oluline märklaud. HCV NS5B inhibiitorid on reeglina madalmolekulaarsed ained, mis seonduvad selle ensüümiga ja blokeerivad RNA sünteesi. Huvitaval kombel seonduvad kõik kirjeldatud HCV NS5B inhibiitorid kas pöidla või peopesa regiooni. Siiani pole kirjeldatud ühtegi madalmolekulaarset inhibiitorit, mis seonduks polümeraasi nn. "sõrmede domeeniga".

Käesoleva töö eksperimentaalses osas tekitasime hiirte immuniseerimisega antikehasid, mis seonduvad HCV polümeraasile ja blokeerivad selle ensüümi RNA sünteesi võime. Antikehade epitoopide kaardistamisega näitasime, et need antikehad seonduvad HCV polümeraasi "sõrmede domeeni" piirkonda. Me selgitasime välja mehhanismid, kuidas meie poolt isoleeritud antikehad pärast seondumist blokeerisid HCV polümeraasi aktiivsust. Meie töö tulemused näitasid, et antikeha (7G8) seondudes HCV polümeraasiga takistab viiruse genoomse RNA algset seondumist ensüümiga. Samas, teine antikeha (8B2), seondudes polümeraasi sõrmedele sunnib neid tugevamalt "pigistama" ensüümi sees olevat ribonukleiinhapet ja sel viisil takistub ensüümi funktsioneerimine. Seega meie andmed näitavad, et suunates ravimid polümeraasi „sõrmede domeeni" vastu nagu vastavad antikeha molekulid tegid, on võimalik mõjutada kahte erinevat etappi polümeraasi reaktsioonis. Seega on HCV NS5B "sõrmede domeen" oluline RNA sünteesi initsiatsiooni ning RNA ahelate elongatsiooni määrav ravimimärklaud.

Replikaasi ensümaatilised komponendid on kodeeritud (+)RNA viiruse genoomi poolt, mida peremeesraku ribosoomid kasutavad sünteesides viiruse terviklikku polüproteiini. Proteaas – üks replikaasi aktiveerimiseks vajalikke ensümaatilisi komponente, protsessib viiruse algse polüproteiini küpseteks funktsionaalseteks valkudeks. Viiruse polüproteiini lõikamist proteaasi poolt kontrollitakse nii ajaliselt kui ka ruumiliselt. Selle protsessi kulgemine määrab RNA viiruse replikaasi pakkumise ja efektiivsuse replikatsioonil.

Käesoleva töö eksperimentaalse töö teises osas kasutasime me erinevaid HCV proteaasi (NS3-4A kompleks) vastaseid madalmolekulaarseid inhibiitoreid selleks, et ette ennustada uusi potentsiaalseid inhibiitoreid, mis oleksid suunatud sellele märklauale. Selleks töötasime välja spetsiaalse algoritmi.

Erinevad HCV NS3-4A inhibiitorite struktuurid olid jaotatud lihtsamateks fragmentideks, mida kasutati kombinatoorikas uute ühendite etteennustamiseks kasutades selleks väljatöötatud spetsiaalset arvutusliku algoritmi. Antud arvutuslikul algoritmil on kaks iseärasust. Esiteks, see ennustab ette ühendite eriomadusi – keemilisi deskriptoreid (elektrostaatilised, steerilised ja hüdrofoobsed parameetrid) mitte kogu ühendi jaoks keskmiselt, vaid vastavate kasutatud fragmentide jaoks. Selline lähenemine võimaldab kirjeldada ühendite omadusi täpsemini, eriti kindlate märklauaga interakteeruvate külgradikaalide osas. Seega suudab antud algoritm ennustada uusi ühendite klasse, ületades sellega kasutatud sünteesitud ühendite raamatukogude piiranguid. HCV NS5B ja NS3-4A on viiruse replikaasi osised ning on otseselt seotud viiruse replikatsiooniga. Seega, mõlemad ensüümid on märklauad viirusevastasete inhibiitorite väljatöötamisel.

Kirjanduse andmetel on kõigist peremeesraku poolt sünteesitud NS5B ja NS3-4A valkudest vaid ~5% hõlmatud HCV genoomi replikatsioonis. Seega on õigustatud küsimus, kas ülejäänul 95% ensüümidel on mingit rolli viiruse elutegevuses, mis ehk otseselt ei ole seotud HCV genoomse RNA replikatsiooniga? Kaasasündinud immuunsüsteem on organismi esmane patogeenide vastane kaitseliin, mis toimetades raku tasemel omab üldist patogeenide vastast aktiivsust. Selle kaasasündinud immuunsüsteemi olulisteks komponentideks on retseptorid, mis tunnevad ära erinevaid patogeenidele omaseid molekulaarseid struktuure ja sellisel viisil eristavad "oma" "võõrast". Juhul kui kaasasündinud immuunsüsteemi poolt on avastatud "võõras" molekulaarne struktuur s.t. retseptori ligandi seondumistsentrisse on sisenenud vastav molekul, käivitub signaali rada, mis edastab selle kohta koheselt signaali rakutuuma. Seejärel aktiveeritakse vastavate geenide ekspressioon, mille tulemuseks on tüüp I interferoonide ekspressioon ja sekretsioon rakust. Interferooni kui signaalmolekuli rakuväline ilmumine on otseseks ohusignaaliks kõigile ümbritsevatele rakkudele, käivitades terve rea piiranguid transkriptsioonile ja translatsioonile.See on ka signaaliks omandatud immuunsüsteemile, mis aktiveerub ja seejärel ründab nakatunud rakku, mis selle signaali väljutas.

Iga konkreetse patogeeni, sealhulgas kõigi erinevate (+)RNA viiruste jaoks, ei saa olla eraldi spetsiaalset molekulaarset struktuuri, mille järgi antud patogeeni saaks tuvastada. See järeldub asjaolust, et peremeesraku genoomi poolt kodeeritud sensoreid on piiratud arvul. Järelikult peab olema üldine, paljudele patogeenidele omane struktuur, mille järgi peremeesrakk saaks neid tuvastada. Üheks ühiseks ja väga võimsaks patogeeniga assotsieeritud molekulaarseks struktuuriks on kaksikahelaline RNA molekul, mis tekib (+)RNA viiruste paljunemise käigus. Iseenesest on see fakt teada aastast 1967, mil "Merck"-i Terapeutiliste Uuringute Instituudis avastati, et interferooni sekretsiooni kutsub rakkudes esile kaksikahelaline RNA. See viiruse replikatsioonil tekkiv viirusspetsiifilise replikatsiooni intermediaat on ideaalseks viirusomaseks struktuuriks, mille tunneb ära peremeesrakk. Peremeesrakus toimub (+)RNA viiruste replikatsioon erilistes membraaniga piiratud struktuurides, mida nimetatakse "viiruse vabrikuteks", mis kaitsevad viiruse kaksikahelalist RNA replikatsiooni-

vormi ning ei lase kaasasündinud immuunsüsteemi sensoritel seda molekulit tuvastada.

Hepatiit C viiruse NS3-4A proteaasi funktsioon, lisaks viiruse replikaasi protsessimisele, on takistada interferooni sekretsiooni signaaliraja katkestamise kaudu. Samas mitmed hiljuti avaldatud tööd vihjavad sellele, et HCV NS5B aktiivsus on võimeline interferooni sekretsiooni esile kutsuma. Praeguseks ei ole identifitseeritud rakke, mis oleksid tervikliku kaasasündinud immuunsüsteemiga ning samas toetaksid efektiivset HCV replikatsiooni. See asjaolu ei võimalda uurida NS5B ja NS3-4A kõrvaleffekte ja potentsiaalset antagonismi HCV nakkuse ja replikatsiooni kontekstis. Teisalt on SFV võimeline nakatama mitmeid eri tüüpi rakke, milledel on intaktne kaasasündinud immunsüsteem. On teada, et mittestruktuurne valk 2 (nsP2) proteaas on SFV replikaasi komponent ning pärssib interferooni sekretsiooni. Pealegi, oma töös me tuvastasime, et SFV replikaas üksi ilma viiruse genoomi replitseerimata on võimeline esile kutsuma interferooni sekretsiooni. See interferooni sekretsioon aktiveeriti SFV replikaasi kaksikahelalise RNA sünteesi tõttu. Järelikult, HCV ja SFV proteaasid ning RNA-sõltuvad RNA polümeraasid omavad sarnaseid kõrvalefekte rakkudes, kus on intaktne kaasasündinud immunsüsteem.

Seega uurisime me eksperimentaalse töö kolmandas osas SFV proteaasi ja replikaasi kõrval-efektide vahelist potentsiaalset antagonismi antud viiruse nakkuse ja genoomi replikatsiooni kontekstis. Esiteks, me näitasime, et SFV replikaas on üksi rakku sattudes võimeline tekitama patogeeniga assotsieeritud molekulaarseid struktuuri – kaksikahelalist RNAd, mis tuntakse ära vastavate retseptorite poolt, mis omakorda indutseerivad interferooni sekretsiooni rakust. Tähelepanuväärne on asjaolu, et selleks ei ole vajalik viiruse paljunemine, kuna aktiivse RdRp olemasolu iseenesest on piisav, et kaasasündinud immuunsüsteem detekteeriks patogeeni. Teiseks, me näitasime, et nendes rakkudes on SFV replikaas võimeline kasutama rakulist RNA matriitsiks, et tekitada kaksikahelalist RNA, mis ongi patogeeniga assotsieeritud molekulaarne struktuur. Sellise struktuuri tuvastabki peremeesrakk kasutades spetsiaalseid tsütosooli retseptoreid. Kolmandaks, me demonstreerisime et nsP2 proteaas on oluline selleks, et piirata SFV replikaasi RNA-sõltuva RNA polümeraasse aktiivsuse tuvastamist peremeesraku poolt. Neljandaks, me näitasime, et kui nsP2 interferooni induktsiooni pärssimisvõimet blokeerida, siis viiruse üheahelalised RNA molekulid hävitatakse peremeesraku poolt, kuid kaksikahelalised RNA replikatsiooni intermediaadid on kaitstud viiruseliste vabrikute poolt. See asjaolu toetab otseselt seisukohta, et viiruseline kaksikahelaline RNA ei saa olla lihtsasti tuvastatav peremeesrakku sensorite poolt.

Toetudes ülaltoodud tulemustele, käesolevas väitekirjas pakutakse välja viiruselise RNA-sõltuva RNA polümeraasi ning proteaasi kõrval-efektide vahelise antagonismi üldise mudeli. Lähtudes sellest mudelist on analüüsitud erinevaid viirusvastaste ühendite väljatöötamise strateegiaid, millede märklauaks on ennekõike HCV NS5B ja NS3-4A ensümaatilised aktiivsused.

ACKNOWLEDGEMENTS

Most of all, I would like to thank my mentor Mart Ustav. It was an extremely difficult task to publish all the RNA virus research we made in the DNA virus laboratory. You were the most critical reviewer and the best motivator at the same time – incredible! One of the many things You taught me is that the quality is the most important factor in science. During this work I tried hard to accomplish that by driving two big projects essentially from the scratch. Thank You for this experience – I learned a lot, and importantly – I have survived!

I would like to thank Andres Merits, for sharing with me his published and unpublished data and the expertise on the genus *Alphavirus*. This was extremely important to finalize the last big project – thank You.

Age Utt, my young colleague, Your contribution to the last big project was remarkable! Thank You!

I thank all the coauthors of the papers: Erkki Juronen, Tarmo Mölder, Rein Sikut, Kaja Kiiver, Andres Männik, Urve Toots, Aleksei Lulla, Valeria Lulla, Age Utt, Mati Karelson, Dimitar A. Dobchev, Gunnar Karelson, Tarmo Tamm, Kaido Tämm, Margit Mutso, Andres Merits, and Mart Ustav.

I would like to thank the technicians of our lab, especially Jelena Kiprovskaja and Galina Halus, for saving my time with super-quality solutions and competent cells I could rely on every time.

I am grateful to Volker Lohmann and Ralf Bartenschlager for providing the HCV replicon system.

I would like to thank Siim Sõber, Meelis Kadaja, Dmitri Lubenets, Age Utt, Andrei Safjanov, Kalle Kipper, Aksel Soosaar, Mardo Kõivomägi, Toomas Silla, Valeria Lulla, Aleksei Lulla, Liisi Võsa, Marit Orav, Anne Kalling, Vallo Varik, Mart Loog, Aivar Liiv, and Reet Kurg for all scientific discussions and especially for the non-scientific ones. My special thanks goes to my first "unofficial" mentor in the lab – Mart Speek – You taught me the principles and the etiquette of work in the molecular biology laboratory. I consider this a privilege.

I highly appreciate the help and guidance from Tiiu Rootslane and Merike Petuhhov during my education process at the University of Tartu. I am grateful to Katrin Kepp and Lilian Kadaja-Saarepuu for letting me know about the opportunity of being enrolled in the Graduate School of Biomedicine and Biotechnology.

I would like to thank Kalle Kiiranen for saving my damaged laptop's hard drive, which was extremely important during writing of this thesis.

I am indebted to the following people for the contribution to the foundations of my academic education: Peeter Toomik and Jaan Simisker (biochemistry), Ain Heinaru (genetics), Ants Tuulmets and Viktor Palm (organic chemistry), Jaanus Remme (RNA chemistry), Toivo Maimets (molecular cell biology), Toomas Kivisild (the clockwork of evolution trees), Agu Laisk (fundamentals of biophysics), Ain Isotamm (the clockwork of computers and algorithms),

Mart Ustav (generalizations and systems virology), and Richard Villems (the chess of evolution).

This wouldn't have been possible without my teachers from the gymnasium – "Tallinna Tõnismäe Reaalkool". I want to thank especially Tamara Potapova, Lidia Rõbnikova, Tatjana Beloussova, and Ljubov Kirilahha. You taught me how to extract the most important information from the vast data (either mathematical, chemical, or physical) and how to express myself.

I am grateful to the Library of University of Tartu for almost unrestricted access to the Web of Knowledge (20 years depth) and Scopus (unrestricted) databases.

I am grateful to the organizations that provided multiple scholarships for my studies and for attending the symposia and conferences: Archimedes Foundation together with Estonian Ministry of Education and Research (national scholarship program "Kristjan Jaak"), Estonian-Revelia Academic Fund, Rotalia Foundation, Estonian Students Fund, Graduate School of Biomedicine and Biotechnology (European Social Grant No 1.2.0401.09-0073), and Tartu University Foundation.

The work published in Papers I, II, and III was financially supported by Estonian Science Foundation (Grants 5998 and 5999), Estonian Ministry of Education and Research (Target Financial Projects SF0182566, SF0180175As08 and SF0140031As09), and by the European Regional Development Fund through the Center of Excellence in Chemical Biology.

Finally, I would like to thank my wife Regina and children Vladislav & Viktoria for their support and for tolerating my workaholic life-style.

PUBLICATIONS

CURRICULUM VITAE

Name: Andrei Nikonov
Date of birth: August 5th, 1981, Tallinn, Estonia
Telephone: +372 5656 5415
E-mail: andrei.nikonov@outlook.com
Citizenship: Estonian

Languages: Estonian, English, Russian

Education:

1996–1999	Tallinna Tõnismäe Reaalkool (high school), Tallinn, Estonia.
1999–2002	University of Tartu; *baccalaureus scientiarum* (B.Sc.). *Summa cum laude*. Gene technology (Molecular Diagnostics)
2002–2004	University of Tartu, *magister scientiarum* (M.Sc.). Gene technology (Molecular Diagnostics).
2004–2009, 2011–	University of Tartu, doctorate studies. Molecular and Cell biology (Virology).

Work experience:

2005–2008	University of Tartu, Faculty of Science and Technology, Institute of Technology; specialist.
2008–31.08.2013	University of Tartu, Faculty of Science and Technology, Institute of Technology; scientist.
02.09.–31.12.2013	Icosagen Cell Factory OÜ, Research and Development Department; scientist.
01.01.–25.03.2014	Doctoral dissertation writing and preparation (unemployed).

List of publications:

I. **Nikonov, A.**, Juronen, E., and Ustav, M. (2008). Functional characterization of fingers subdomain-specific monoclonal antibodies inhibiting the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* 283, 24089–24102.

II. Karelson, M., Dobchev, D.A., Karelson, G., Tamm, T., Tämm, K., **Nikonov, A.**, Mutso, M., and Merits, A. (2012). Fragment-based development of HCV protease inhibitors for the treatment of hepatitis C. *Curr Comput Aided Drug Des* 8, 55–61.

III. **Nikonov, A.**, Mölder, T., Sikut, R., Kiiver, K., Männik, A., Toots, U., Lulla, A., Lulla, V., Utt, A., Merits, A., and Ustav, M. (2013). RIG-I and MDA-5 detection of viral RNA-dependent RNA polymerase activity restricts positive-strand RNA virus replication. *PLoS Pathog* 9, e1003610.

ELULOOKIRJELDUS

Nimi:	Andrei Nikonov
Sünniaeg:	5^{s} August 1981, Tallinn, Eesti
Telefon:	+372 5656 5415
E-mail:	andrei.nikonov@outlook.com
Kodakondsus:	Eesti

Keelteoskus: Eesti, Inglise, Vene

Haridustee:

1996–1999	Tallinna Tõnismäe Reaalkool, Tallinn, Eesti Vabariik
1999–2002	Tartu Ülikool; *baccalaureus scientiarum* (B.Sc.). *Summa cum laude*. Geenitehnoloogia (Molekulaarne Diagnostika).
2002–2004	Tartu Ülikool, *magister scientiarum* (M.Sc.). Geenitehnoloogia (Molekulaarne Diagnostika).
2004–2009, 2011–	Tartu Ülikool, doktorantuur. Molekulaar- ja rakubioloogia (Viroloogia).

Teenistuskäik:

2005–2008	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Tartu Ülikooli Tehnoloogiainstituut; spetsialist.
2008–2013	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Tartu Ülikooli Tehnoloogiainstituut; teadur.
02.09.–31.12.2013	Icosagen Cell Factory OÜ, Teadus- ja Arendusosakond; teadur.
01.01.–25.03.2014	Doktoriväitekirja ettevalmistamine (töötu).

Publikatsioonide loetelu:

I. **Nikonov, A.**, Juronen, E., and Ustav, M. (2008). Functional characterization of fingers subdomain-specific monoclonal antibodies inhibiting the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* 283, 24089–24102.

II. Karelson, M., Dobchev, D.A., Karelson, G., Tamm, T., Tämm, K., **Nikonov, A.**, Mutso, M., and Merits, A. (2012). Fragment-based development of HCV protease inhibitors for the treatment of hepatitis C. *Curr Comput Aided Drug Des* 8, 55–61.

III. **Nikonov, A.**, Mölder, T., Sikut, R., Kiiver, K., Männik, A., Toots, U., Lulla, A., Lulla, V., Utt, A., Merits, A., and Ustav, M. (2013). RIG-I and MDA-5 detection of viral RNA-dependent RNA polymerase activity restricts positive-strand RNA virus replication. *PLoS Pathog* 9, e1003610.

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 erotonin. 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 synaptone-mal 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 transla-tional 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 popula-tions: 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.