

Institute of Biotechnology
Department of Biosciences
Division of General Microbiology
Faculty of Biological and Environmental Sciences
Viikki Graduate School in Molecular Biosciences
University of Helsinki

Functions of Alphavirus Macrodomain- Containing Protein nsP3

Maarit Neuvonen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Biological and Environmental
Sciences,

for public examination at lecture hall 2402, Biocenter 3, Viikinkaari 1,
Helsinki, on March 11th, 2011 at 12 noon.

Helsinki 2011

Supervisor**Docent Tero Ahola**

Institute of Biotechnology, University of Helsinki
Viikinkaari 9, 00790, Helsinki, Finland

Reviewers**Docent Sirkka Keränen**

Faculty of Biological and Environmental Sciences
Department of Biosciences
FI-00014 University of Helsinki
FINLAND

Docent Pirkko Heikinheimo

Biochemistry
Department of Biochemistry and Food Chemistry
Vatselankatu 2, Arcanum, 3rd floor
FI-20014 University of Turku
FINLAND

Opponent**Professor Ari Hinkkanen**

University of Eastern Finland
A.I. Virtanen Institute for Molecular Sciences
Neulaniementie 2
FI-70211 Kuopio
FINLAND

Cover graphics: N2A cells infected with Semliki Forest virus and stained for nsP3 (red) and amphiphysinI (green)

ISBN 978-952-10-6828-7 (paperback)

ISBN 978-952-10-6829-4 (PDF) - <http://ethesis.helsinki.fi>

Printed by Yliopistopaino
Helsinki 2011

*To my family and friends.
It was a good journey,
thank you for travelling with me!*

Abstract

Alphaviruses are positive strand RNA viruses that replicate in association with cellular membranes. The viral RNA replication complex consists of four non-structural proteins nsP1-nsP4 which are essential for viral replication. The functions of nsP1, nsP2 and nsP4 are well established, but the roles of nsP3 are mainly unknown. In this work I have clarified some of the functions of nsP3 in order to better understand the importance of this protein in virus replication. Semliki Forest virus (SFV) has been mostly used as a model alphavirus during this work, but some experiments have also been conducted with Sindbis and Chikungunya viruses.

NsP3 is composed of three different protein domains. The N-terminus of nsP3 contains an evolutionarily conserved macrodomain, the central part of nsP3 contains a domain that is only found in alphaviruses, and the C-terminus of the protein is hypervariable and predicted to be unstructured. In this work I have analyzed the functions of nsP3 macrodomain, and shown that viral macrodomains bind poly(ADP-ribose) and that they do not resemble cellular macrodomains in their properties. Furthermore, I have shown that some macrodomains, including viral macrodomains of SFV and hepatitis E virus, also bind poly(A). Mutations in the ligand binding pocket of SFV macrodomain hamper virus replication but do not confer lethality, indicating that macrodomain function is beneficial but not mandatory for virus replication.

The hypervariable C-terminus of nsP3 is heavily phosphorylated and is enriched in proline residues. In this work I have shown that this region harbors an SH3 domain binding motif (Sh3BM) "PxRxPR" through which cellular amphiphysin is recruited to viral replication sites and to nsP3 containing cytoplasmic aggregate structures. The function of Sh3BM was destroyed by a single point mutation, which led to impaired viral RNA replication in HeLa cells, pointing out the functional importance of amphiphysin recruitment by the Sh3BM. In addition, I have provided evidence that the endosomal localization of alphavirus replication is mediated by nsP3 and that the phosphorylation of hypervariable region might be important for the endosomal targeting.

Together these findings demonstrate that nsP3 contains multiple important host interaction motifs and domains, which facilitate successful viral propagation in host cells.

Acknowledgements

I would like to express my deep gratitude to all the wonderful people I've had an opportunity to work with during my PhD project. For the beginning of this story, thanks belongs to Professor Leevi Kääriäinen and Docent Tero Ahola, who believed in me and took me as a PhD student, a girl from University of Technology, whose background was in microbiology. I also want to thank Tero as my supervisor, for his help and support and for continuous development of my scientific writing skills. For Leevi, I would like to express my gratitude for the discussions, support and encouragement, which have been highly appreciated during these years.

I would like to thank the former and current leaders of the Institute of Biotechnology, Prof. Mart Saarma and Prof. Tomi Mäkelä, for creating such an excellent environment for scientific research, and for being available for us researchers. I've enjoyed a lot of being a member of Viikki Graduate School in Molecular Biosciences. It has offered an inspiring network of people, excellent courses and great meetings, as well as very much appreciated monetary support. A big thanks belongs to the excellent coordinators Eeva Sievi and Sandra Falck. For the follow up of my thesis work, thanks belongs to Dr. Pekka Lappalainen and Dr. Marja Mikkola, who have created a nice and relaxed environment for scientific discussion and given me a lot of useful advice. I want to express my gratitude to the Head of the Microbiology Department, Professor Timo Korhonen, who has been always helpful and available, and to Professor in virology, Dennis Bamford, the custodian of my defence for always having an open door and encouraging attitude.

I would like to thank Professor Kalle Saksela and my co-worker Arunas Kazlauskas for a very fruitful collaboration during the procession of the last manuscript. I think we did a good teamwork, and I'm very thankful for all the interesting discussions, we had during this process. I would like to thank my thesis reviewers, Dr. Sirkka Keränen and Dr. Pirkko Heikinheimo, for critical reading of the manuscript and useful comments and suggestions, which helped to improve the manuscript to become this book we see now.

Last but not least, I want to thank my friends and relatives. The biggest thanks belongs to my colleagues Pirjo Spuul, Giuseppe Balistreri and Andrey Golubtsov, our coffee room team, for your friendship, support and all the inspiring scientific discussions during these years. I would like to express my gratitude to all the SFV group members I've been privileged to work with: Peter, Julia, Javier, Airi, Riikka, Saija, Henna, Leena, Antti, Yaseen, Pasi, Kirsi and Katri. A special thanks belongs to my husband Markus for being always there for me, for your love and support, and your tolerance for my work in science. I want to thank my parents and my sister for all the support now and before, and the very final thanks goes to the most important, my dear son Osma. Thank you for your love!

Maarit Neuvonen,
Helsinki 2011

Contents

| | |
|---|------|
| Abstract..... | iv |
| Acknowledgements | v |
| List of original publications | viii |
| Abbreviations | ix |
| 1 Introduction | 1 |
| 1.1 Positive-strand RNA viruses | 1 |
| 1.2 Alphaviruses | 1 |
| 1.3 Alphavirus life cycle | 2 |
| 1.3.1 Virus entry..... | 2 |
| 1.3.1 RNA replication..... | 3 |
| 1.4 Alphavirus RNA replication machinery..... | 5 |
| 1.4.1 nsP1..... | 5 |
| 1.4.2 nsP2..... | 5 |
| 1.4.3 nsP3..... | 6 |
| 1.4.4 nsP4..... | 6 |
| 1.4.5 Membrane-bound replication complex | 7 |
| 1.5 Host interaction protein nsP3 | 8 |
| 1.5.1 Macrodomain..... | 9 |
| 1.5.2 Alphavirus unique domain (AUD)..... | 10 |
| 1.5.3 Hypervariable region (HVR)..... | 10 |
| 1.6 The conserved macrodomain family..... | 11 |
| 1.6.1 Human macrodomain proteins..... | 12 |
| 1.6.2 Viral macrodomains..... | 14 |
| 2 Aims of the study..... | 16 |
| 3 Materials and methods | 17 |
| 4 Results | 20 |
| 4.1 Characterization of the properties of macrodomains (I, II and unpublished)..... | 20 |
| 4.1.1 Macrodomains in ADPR binding | 20 |
| 4.1.2 Macrodomains as ADPR1-P hydrolyzing enzymes..... | 21 |
| 4.1.3 PAR binding by viral and human macrodomains..... | 21 |
| 4.1.4 Some macrodomains are poly(A) binding modules..... | 21 |

| | | |
|-------|---|----|
| 4.1.5 | Mutations in nsP3 macrodomain cause defects in SFV reproduction (unpublished) | 22 |
| 4.1.6 | Subcellular localization of macrodomain proteins | 23 |
| 4.2 | Virus-host interactions mediated through nsP3 C-terminus (III and unpublished)..... | 24 |
| 4.2.1 | The HVR of nsP3 functions in SFV replication complex internalization (unpublished) | 24 |
| 4.2.2 | Alphavirus nsP3 binds SH3 domain proteins through a proline rich region in HVR..... | 27 |
| 4.2.3 | SH3 domain binding by alphavirus nsP3 is important for virus replication in cell culture | 29 |
| 5 | Discussion | 31 |
| 5.1 | Human and viral macrodomain proteins..... | 32 |
| 5.2 | Multifunctional nsP3 HVR | 34 |
| 5.2.1 | Replication complex internalization mediated by nsP3 HVR | 35 |
| 5.2.2 | Functions of amphiphysin in alphavirus replication..... | 36 |
| | References | 40 |

List of original publications

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

- I. Egloff MP, Malet H, Putics A, **Heinonen M**, Dutartre H, Frangeul A, Gruez A, Campanacci V, Cambillau C, Ziebuhr J, Ahola T, Canard B. (2006) Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. *J. Virol.* 80: 8493-8502.
- II. **Neuvonen M**, Ahola T. (2008) Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites. *J. Mol. Biol.* 385: 212-225.
- III. Kazslauskas A*, **Neuvonen M***, Ahola T, Saksela K. (2011) SH3 domain-mediated recruitment of host cell amphiphysins by alphavirus nsP3 promotes viral RNA replication. *Submitted*.

* These authors contributed equally to this work

Unpublished data are also presented.

Abbreviations

| | |
|--------------------|--|
| 293FT cells | SV40 large T antigen expressing cell line derived from primary embryonal human kidney (293F) cells |
| A1pp | ADPR1-P processing enzyme |
| aa | amino acid |
| ADP | adenosinediphosphate |
| ADP1-P | adenosinediphosphoribose1"phosphate |
| ADPR | ADP-ribose |
| AUD | alphavirus unique domain |
| BAL | B-Aggressive Lymphoma protein |
| BAR | Bin/Amphiphysin/Rvsp |
| BHK cell | baby hamster kidney cell |
| BSA | bovine serum albumin |
| BSR-T7 cell | BHK-21 cell clone, which stably expresses T7 RNA polymerase |
| C6orf130 | Chromosome 6 open reading frame 130 |
| CHIKF | Chikungunya fever |
| CHIKV | Chikungunya virus |
| Cin85 | Cbl interacting protein of 85 kDa |
| CPE | cytopathic effect |
| CPV | cytopathic vacuole type I |
| dsRNA | double stranded RNA |
| EEEV | eastern equine encephalitis virus |
| EGFP | enhanced green fluorescent protein |
| ER | endoplasmic reticulum |
| ESCRT | endosomal sorting complex required for transport |
| G3BP | Ras-GAP SH3 domain binding protein |
| GDAP2 | ganglioside induced differentiation-associated protein 2 |
| HAV | hepatitis A virus |
| HCV | hepatitis C virus |
| HeLa cell | human cervix epitheloid carcinoma cell |
| HEV | hepatitis E virus |
| HIV | human immunodeficiency virus |
| HVR | hypervariable region |
| Jak | janus kinase |
| K _D | dissociation coefficient |
| LRP16 | leukemia related protein 16 |
| MDO | human macrodomain protein |
| mH2A | macrohistone 2A |
| nsP | non-structural protein |
| NTPase | nucleoside triphosphatase |
| P12 ^{CA3} | nsP1-nsP2-nsP3 polyprotein, in which protease activity has been inactivated |
| P13 | Artificial polyprotein nsP1-nsP3 |
| P13ZsG | Artificial polyprotein nsP1-nsP3 with ZsGreen insertion |

| | |
|------------------|--|
| PAR | poly(ADP-ribose) |
| PARP | poly(ADP-ribose) polymerase |
| PBS | phosphate-buffered saline |
| PDK-1 | 3-phosphoinositide-dependent protein kinase-1 |
| pfu | plaque forming unit |
| p.i. | post infection |
| PI3K | phosphoinositide 3-kinase |
| PIP ₂ | phosphatidylinositol 4,5-bisphosphate |
| PIP ₃ | phosphatidylinositol 3,4,5-triphosphate |
| poly(A) | polyadenylate |
| pORF3 | protein encoded by open reading frame 3 in HEV |
| RdRp | RNA dependent RNA polymerase |
| SARS-CoV | severe acute respiratory syndrome coronavirus |
| SFV | Semliki Forest virus |
| SFVΔP | Semliki Forest virus mutant with deletion in proline region in nsP3 (aa 407-440 in nsP3 are deleted) |
| SH3 | SRC Homology 3 |
| Sh3BM | SH3 domain binding motif |
| SINV | Sindbis virus |
| Snf | sucrose non fermentation |
| Stat | signal transducer and activator of transcription |
| SUD | SARS unique domain |
| VEEV | Venezuelan equine encephalitis virus |
| WEEV | western equine encephalitis virus |
| wt | wild type |
| ZsG | ZsGreen |

1 Introduction

1.1 Positive-strand RNA viruses

Viruses are small infectious agents that replicate only inside living cells. They are found everywhere in nature, and they infect organisms from all kingdoms of life. Viruses are classified into six different groups, based on the type of their genetic material. Viral genome can be composed of either DNA or RNA, and it can be either single or double stranded. The largest group of viruses under this classification is positive-strand RNA viruses. The genome of these viruses consists of RNA that is of the same sense as host cell mRNA and can be directly translated to viral proteins by host cell machinery. The RNA genome can consist of a single RNA molecule, or several different RNA molecules that are all needed for viral replication.

Positive-strand RNA viruses include several human pathogens such as poliovirus, severe acute respiratory syndrome coronavirus (SARS-CoV), Rubella virus and Hepatitis A, C and E viruses (HAV, HCV, HEV). Positive-strand RNA viruses do not have a DNA intermediate in their replication cycle, but maintain their genome purely in an RNA form. The replication of the genome is achieved by viral replication proteins, among which is an RNA dependent RNA polymerase (RdRp) that is able to synthesize RNA from an RNA template. The whole replication cycle takes place in the cytoplasm, where positive-strand RNA viruses form membrane-bound replication complexes that are typically surrounded by virus-modified host membranes (160). These membranous structures are thought to concentrate the virus replication factors to the sites of replication and to protect the viral RNA from host cell defence mechanisms (145).

1.2 Alphaviruses

Alphaviruses are small enveloped positive-strand RNA viruses belonging to the family *Togaviridae*. The only other member of *Togaviridae* is Rubella virus, the sole member of genus Rubivirus. Alphaviruses are animal viruses that infect various vertebrate hosts. The genus contains at least 24 species of which many can also infect humans (132). They are transmitted mainly via mosquito vector, in which they cause systemic infection but no symptomatic disease. Thus alphaviruses are often referred to as arthropod borne viruses (arboviruses), although currently it is uncertain whether salmonid alphaviruses are transmitted through lice or can be directly transmitted from fish to fish (128, 140).

Alphaviruses can be divided into two categories based on the geographic area where they spread: Old World and New World viruses. Diseases caused by alphaviruses partially follow the geographical distribution, as many Old World viruses cause a disease characterized by rash and high fever and sometimes painful arthralgia, whereas several New World viruses cause encephalitis. However, there are close genetic relationships between Old World and New World alphaviruses, which probably originate from several transoceanic exchanges mediated by birds (132).

The best characterized alphaviruses are Semliki Forest virus (SFV) and Sindbis virus (SINV). These viruses are the representatives of two of the main alphavirus groups, namely the SFV complex and western equine encephalitis virus (WEEV) complex. They are considered rather safe for laboratory use, since SFV the typical strains of SFV do not cause a disease in humans in nature, and SINV causes a rather mild illness characterized by fever and rash. The third main group of alphaviruses is the Eastern equine encephalitis virus (EEEV) / Venezuelan equine encephalitis virus (VEEV) –complex (132, 161). All the three equine encephalitis viruses reside in the New World and cause a devastating neuronal disease in horses, with occasional outbreaks in humans with high mortality rates (159). Other alphaviruses may also harbour potential for neuropathogenicity. For example, an untypical strain variant of SFV, named Me Tri virus, was associated with encephalitis in children in Vietnam in the early 1970s, and the laboratory strain of SFV has caused a rare case of encephalitis in an immunosuppressed person (66, 103, 164). Arthritogenic alphaviruses may also cause very difficult symptoms, as is the case with Chikungunya virus (CHIKV), a member of SFV complex, that has caused several outbreaks in Africa, Asia and Europe since year 2005 (138, 167).

Chikungunya gets its name from the Kimakonde language of Tanzania and Mozambique and translates in Swahili to “the illness of the bended walker”. It causes a severe fever illness named “Chikungunya fever” (CHIKF). About 95% of the humans that are infected get a symptomatic disease. Acute phase CHIKF symptoms include fever, arthralgia, and in some cases, rash (137). The acute phase inflicts intense joint and muscular pain that causes severe suffering and prostrates its victims. About 40% of patients get a chronic chikungunya arthritis that persists more than 18 months after the onset of the fever. Old people are especially susceptible to CHIKF, and the disease is lethal in about 33 % of victims over 65 years of age (reviewed in (167)).

To date there is no existing vaccine or treatment against alphaviruses, which drives interest in alphavirus research. Another important line of alphavirus work is the development of viral vectors. Currently, these vectors can be used in laboratory as a tool for efficient production of recombinant proteins, but the ultimate goal is to develop vectors for use in gene therapy and anti-cancer treatment (99, 100, 136). My personal interest in alphavirus research lies in the understanding of basic molecular and biological mechanisms behind the interactions between virus and the host cell. This line of research is gaining growing amount of attention, as it becomes evident that many cellular factors play important roles in positive-strand RNA virus replication.

1.3 Alphavirus life cycle

1.3.1 Virus entry

Alphaviruses enter the cell via clathrin-mediated endocytosis. The entry is mediated by two viral spike glycoproteins: the entry protein E2 and the fusion protein E1. The entry begins with interaction between the immunoglobulin-like domains of the spike protein E2 and a specific cell surface receptor (97, 181). It is not known, which

receptors are recognized by alphaviruses in nature, but many laboratory strains have adapted to use heparan sulphate as a receptor, and lectins have been suggested as a possible target upon primary alphavirus infection in animals (85).

Virions that are bound to a receptor are endocytosed in a clathrin-dependent manner, and end up in endocytic vesicles (72). Upon acidification of the endosome, domain B of E2 is released from the fusion loop in E1, which makes the fusion loop exposed (Figure 1A). This triggers a conformational change in the viral spike heterodimer leading to escape of E2 protein and trimerization of E1. This causes the fusion of viral envelope and endosome membrane through a mechanism that involves large conformational changes in viral glycoproteins, and the virus capsid is released into the cytoplasm (Fig. 1A) (97, 181). In the cytoplasm, the unenveloped capsid is disassembled, probably through an interaction with a ribosome, and viral RNA is released (183). The viral genome is then translated into viral replication proteins, and the viral RNA is replicated as will be explained in more detail below. Finally capsid and envelope proteins are produced as a single polypeptide that is subsequently cleaved by host and viral proteases into the structural proteins needed for virus particle formation and assembly (Fig. 1B) (77).

1.3.1 RNA replication

Alphavirus genome consists of a single ~11.5 kb RNA molecule, which contains a cap structure and a polyadenylate (poly(A)) tail. The genome encodes four non-structural proteins (nsP1-4) and five proteins needed for formation and assembly of the virus particle (E1, E2, E3, 6K and capsid). During alphavirus replication three different types of RNA are produced. Initially a minus strand (42S RNA in SFV) is produced from the viral genome. The minus strand serves as a template for synthesis of new plus strand genomes (42S RNA) needed for virus assembly, and for shorter mRNAs (26S RNA) from which the structural proteins are translated (78, 160).

Viral replication proteins are initially translated as a single polypeptide P1234. The polypeptide is cleaved into viral replication proteins nsP1-4 by a protease domain in nsP2 that recognizes conserved cleavage sites between the nsPs. The protease moiety, when inside the polypeptide, is able to cleave efficiently only the 3/4 site *in cis*. The termini of nsP2 (1/2 and 2/3 sites) can be efficiently cleaved only *in trans*. Consequently, the early replication complex consists of P123 and nsP4 (112, 176). This complex produces the negative strand RNA, and can also produce positive-strand genomic RNA, but not subgenomic RNA. Conformational changes are needed to form the late replicase that produces the genomic and subgenomic RNA, but is no more able to produce negative strand RNA. Polyprotein processing by nsP2 facilitates these conformational changes in the replication complex, and the fully processed replication proteins (nsP1-nsP4) form the late replicase (93, 150, 176). This leads to temporal regulation of viral RNA replication, so that early in the infection negative strands are produced as new replication complexes form. One replication complex probably produces only one negative strand RNA, to be used as a template in positive strand RNA synthesis. The early replication complex can then produce new genomic RNAs for synthesis of nsPs, leading to formation of new replication complexes. When the replication complex matures through *in trans* cleavages, it can recognize the promoter for subgenomic RNA, needed for production of structural proteins, but it maintains

capability of produce also full length genomic RNA. Late in the infection no new replication complexes are formed, probably partially due to efficient cleaving at 2/3 site, which produces P34 that is incapable of forming active replicase. At the late stage, structural proteins are produced very efficiently and genomic RNAs are synthesized for production of new viruses (reviewed in (78, 160)).

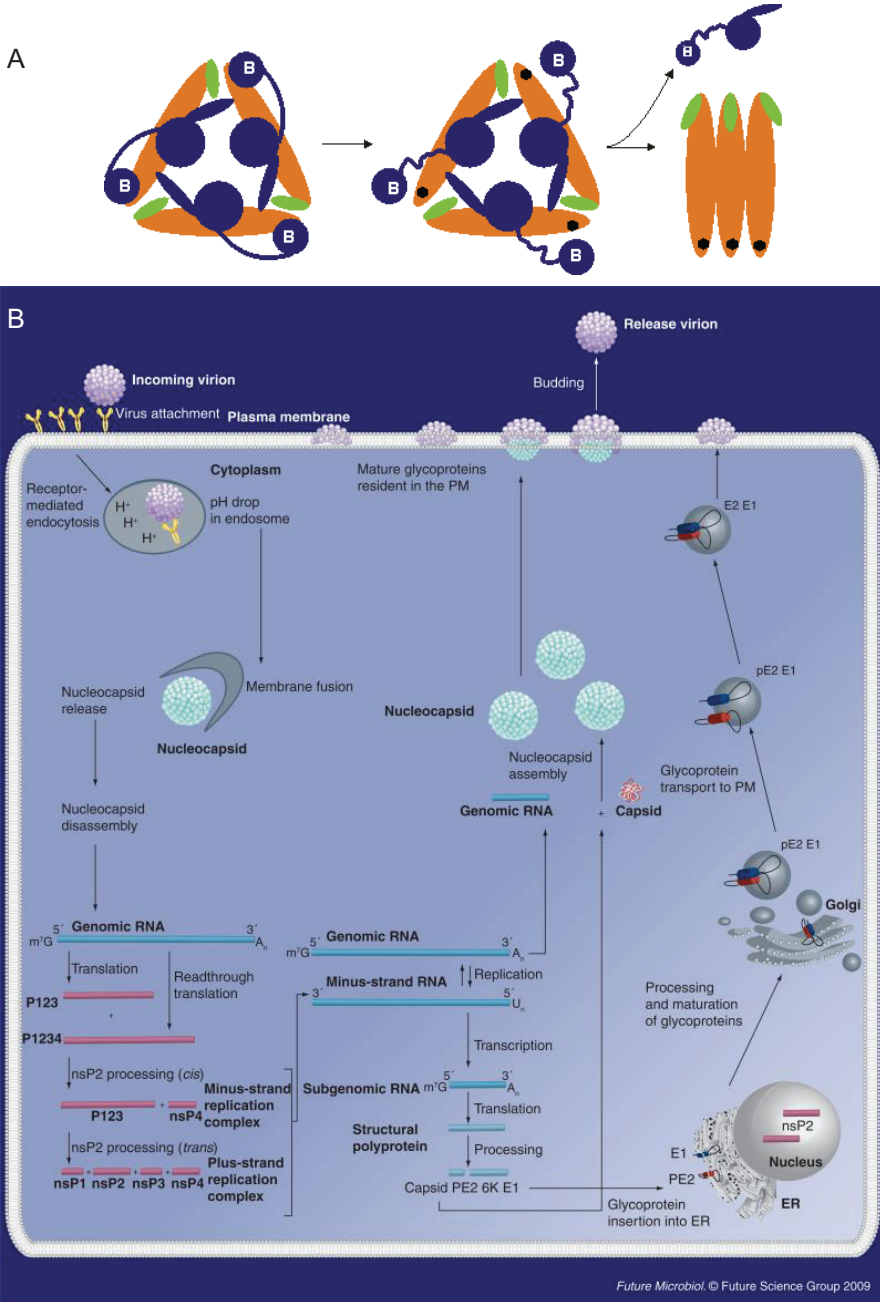


Figure 1. Alphavirus replication cycle. (A) Structural changes in viral glycoproteins during the virus entry. Domain B (B) of E2 (blue) is unattached from E1 (orange-green) and the fusion loop (●) becomes exposed. This leads to disassembly of viral spike and homotrimers of E1 are formed. The model is redrawn based on ref. (97) (B) Alphavirus replication cycle is achieved in the cytoplasm. Incoming RNA is translated into four non-structural proteins which transcribe the viral RNA. RNA transcription and viral protein processing are shown in the left bottom corner. Glycoproteins are further modified in endoplasmic reticulum (ER) and Golgi as shown on the right side. Virus budding appears at the plasma membrane (PM). Reproduced with permission from ref (77).

1.4 Alphavirus RNA replication machinery

Alphavirus replicase consists of four nsPs. The functions and enzymatic activities of these proteins have been well established, except for nsP3, the function of which is still quite poorly understood. All nsPs are needed for formation of functional replication complex. In addition, each replication protein is present in cell as an independent protein that may have important functions other than those directly linked to RNA replication.

1.4.1 nsP1

NsP1 is the protein responsible for the membrane association of the replication complex (125, 146). An amphipathic alpha helix in the central part of the protein is responsible for this membrane association, but palmitoylation of a distant site is needed for tight membrane binding (4, 158) In addition, nsP1 is the viral capping enzyme possessing methyltransferase and guanylyltransferase activities (3, 114). To initiate the capping reaction, the RNA triphosphatase activity of nsP2 is also needed. Formation of the 7-methyl-guanine-cap is achieved in a different way as compared to eukaryote cells, since GTP is first methylated and only after that transferred to the RNA, not vice versa (3).

NsP1 molecules that are not part of replication complex localize to plasma membrane. When overexpressed by transfection, nsP1 causes the formation of filopodia-like protrusions that are devoid of actin filaments (89). In virus infected cells similar structures are seen layered with nsP1 at the inner side of the membrane, but these structures are typically larger and contain actin. Also viral RNA has been detected inside the protrusions (155). It has been proposed that these filopodia-like structures might make connections to neighbouring cells to enable virus RNA to spread in a budding independent manner, similar to what has been recently shown for Rubella virus (107, 155).

1.4.2 nsP2

NsP2 is the protease responsible for processing of the viral replication proteins. The protease domain in the C-terminus is homologous to papain-like cysteine proteinases (13, 60). It has been proposed that the proteolytic cleavage of the polyprotein is the key temporal regulator of viral RNA replication, which controls the promoter

recognition by the viral RNA replicase (93, 176). NsP2 possesses several enzymatic activities in addition to protease activity, namely helicase activity, nucleoside triphosphatase (NTPase) activity and RNA triphosphatase activity. NsP2 helicase is able to unwind partially double-stranded RNA, and the NTPase hydrolysis by the same protein seems to be a driving force for the unwinding (61, 139). RNA triphosphatase activity of nsP2 is needed for removing the gamma-phosphate from the nascent RNA before the cap-structure can be added by nsP1 (175).

Several mutations in nsP2 have been reported to have an effect on negative strand RNA synthesis and sub-genomic RNA synthesis (37, 162). Some, but not all of these mutational effects, can be explained by the fact that they map to nsP2 C-terminus, and affect nsP2 protease domain and polyprotein processing. Some mutational effects may be explained by the fact that early in the infection nsP2 functions in the form of P23 (or P123) to be able to form active negative strand replicase, and many determinants can affect the folding and stability of the precursor protein (36, 37, 149, 162). It has been also suggested that nsP2 – host cell interactions might be important for minus-strand synthesis cessation (149).

When cleaved from the polyprotein, a portion of nsP2 that is not bound to replication complex is transported to nucleus. In SFV the transport is mediated by a nuclear localization signal (NLS) that consists of a PRRRV sequence in the C-terminus of the protein and other determinants in distinct loci (139). NsP2 of Old World viruses SFV and SINV inhibits cellular transcription in nucleus by a yet unknown mechanism (55, 56). However, nsP2 protein of New World viruses VEEV and EEEV does not cause transcription inhibition, but instead the inhibition seems to be mediated by the capsid protein (56). NsP2 also suppresses the interferon response of the host cell, and interestingly this function has been shown to be independent of transcription shut off, but seems to be mediated by specific inhibition of Jak/Stat (Janus kinase/signal transducers and activators of transcription) signaling (50, 152).

1.4.3 *nsP3*

In contrast to other nsPs, no enzymatic function in RNA replication has been shown for nsP3. However, nsP3 is needed for RNA replication, and mutations in nsP3 have been shown to affect negative strand RNA synthesis and subgenomic RNA synthesis (68, 91). Similar defects in RNA synthesis have been detected with mutations in nsP2 or with the mutations either inactivating or hyperactivating 2/3 site cleavage (37, 93, 104), indicating that interactions between nsP3 and nsP2 might be important for the correct formation of the replication complex. The 2/3 site cleavage has been shown to be the most important factor for switching off the negative strand RNA synthesis (83, 93). Thus, nsP3 might function in RNA replication together with nsP2 as a structural component responsible for the conformational changes in the replicase, rather than have a direct enzymatic function on the RNA. However, there is growing evidence for the role of nsP3 in virus-host interaction, which will be discussed in a separate chapter below.

1.4.4 *nsP4*

NsP4 is the catalytic subunit of the RNA polymerase in the alphavirus replication complex. The catalytic core of the enzyme contains a conserved GDD motif that is

found in many viral RdRps (80, 86). The N-terminus of alphavirus nsP4 contains a short non-conserved region that has no similarities with other viral polymerases. This region is probably needed for interaction with other nsPs and viral RNA. The cleavage of nsP4, which exposes the N-terminus, is a prerequisite for functional polymerase, and it has been shown that an aromatic residue in the N-terminus is needed for alphavirus replication *in vivo* (93). NsP4 has also been shown *in vitro* to contain adenylyl transferase activity, which may help to maintain the poly(A) tail of viral positive-sense RNAs (170).

Some alphaviruses like SFV produce nsP4 in equimolar amounts with other nsPs, whereas other viruses like SINV contain an opal termination codon between nsP3 and nsP4. The opal codon in SINV is read through with about 10% efficiency, so that only a minority of non-structural polyproteins contain nsP4 (95). Unlike other nsPs, nsP4 is not known to have independent functions when not bound to replication complex, and nsP4 is efficiently degraded in the N-end rule dependent manner, when released into cytoplasm (35).

1.4.5 Membrane-bound replication complex

Membrane association mediated by nsP1 is essential for the formation of the replication complex, and viral RNA replication correlates with the emergence of spherical membrane invaginations (“spherules”) intimately associated with viral proteins (47, 51, 65, 87) (Fig. 2). Consequently, the viral RNA replication is thought to occur inside of the spherules. How the spherules and the associated viral replication complex are formed, is not exactly known, but membrane binding seems to be an absolute prerequisite for RNA replication, and both the viral nsPs and RNA replication have been shown to be needed for spherule formation (157, 158). It is not known, whether some host protein components are also needed for replication complex assembly and spherule formation. However, several host proteins have been shown to be associated with viral proteins in SINV infected cells (29, 48). In the case of Brome mosaic virus, a positive-strand RNA virus classified into the “Alphavirus-like superfamily”, a membrane shaping host reticulon protein has been shown to be necessary for spherule formation (39).

Alphavirus replication complexes first form at the plasma membrane, where the polyprotein P1234 is targeted by nsP1 (145, 146). NsP1 can adopt its active fold only when associated to a lipid layer (5). Consequently, replication complexes can only form in association with cellular membranes. RNA binding is also thought to be needed for correct formation of replication complexes, and the spherules can not be formed in the absence of viral RNA replication (156). However, replication complexes remain on the plasma membrane only for a short period (157). They are targeted from there to virus induced endo-lysosomal organelles, named cytopathic vacuoles type I (CPVs), where the alphavirus replication is thought to mainly occur (87, 157). It is not known, how the replication complexes are targeted to endosomes, although the transport has been shown to depend on actin and microtubules (157).

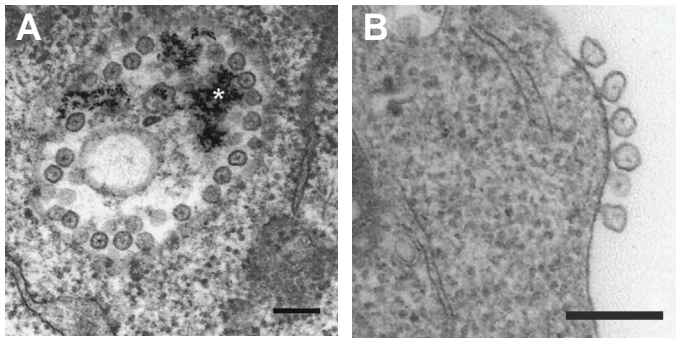


Figure 2. Alphavirus spherules imaged with electron microscopy. (A) Spherules are detected inside of endocytic vesicles labeled with gold-bovine serum albumin (BSA). (B) Side view of spherules detected at the plasma membrane show the connection between spherule and the cytoplasm through narrow neck. Bars 200 nm. Reproduced with permission from reference (87).

1.5 Host interaction protein nsP3

In alphavirus infected cells, nsP3, like all the nsPs (with the possible exception of nsP4), is present in the cytoplasm in two different pools (78). nsP3 can be detected in spherules as a part of the replication complex, and secondly nsP3, when not associated with replication complex, forms aggregates in the cytoplasm (29, 146). Several cellular proteins including heat shock protein 90, Ras-GAP SH3 domain binding protein (G3BP), and tubulin have been found to associate with nsP3 in SINV infected cells (29, 48). Several of these proteins colocalize with nsP3 aggregates when observed by immunofluorescence (29, 48, 63). Many of the proteins found in nsP3 aggregates are probably also present in replication complexes as has been confirmed by colocalization and co-immunoprecipitation of these proteins with the viral RdRp nsP4 (30). Based on these recent findings, it seems likely that nsP3 is interacting with several host proteins. However, none of these studies proved a direct binding between a host protein and nsP3, and thus some of the detected nsP3 – host protein interactions might actually be mediated by other nsPs, associated cellular proteins or viral RNA.

nsP3 can be divided into three different domains based on the conservation of these parts (Fig. 3). The N-terminus of nsP3 consists of a macrodomain that is a globular domain, which has been highly conserved in evolution. The central part of nsP3 consists of a sequence that is conserved between alphaviruses but has no sequence similarity with any of the known protein domains from other organisms (78). Consequently, this domain is here termed alphavirus unique domain (AUD). In contrast to the first two domains, the C-terminus of nsP3 has no sequence similarity even amongst alphaviruses and has been termed the hypervariable region (HVR).

As many alphaviruses can cause a severe brain disease, several studies have aimed to specify the neurovirulence determinants of SFV and SINV. Many of the amino acids contributing to neurovirulence have been found to reside in nsP3, indicating that nsP3 might be an important pathogenicity factor in alphavirus infected

cells (122, 163, 172). Interestingly, mutations attenuating the neurovirulence can be found in each of the three domains in nsP3, and also an opal termination codon in the 3/4 junction causes attenuation (163, 172, 173). One study showed that deletions in SFV HVR cause attenuation of the virus after peripheral inoculation, indicating that pathogenicity factors inside nsP3 are not just neuron specific (53).

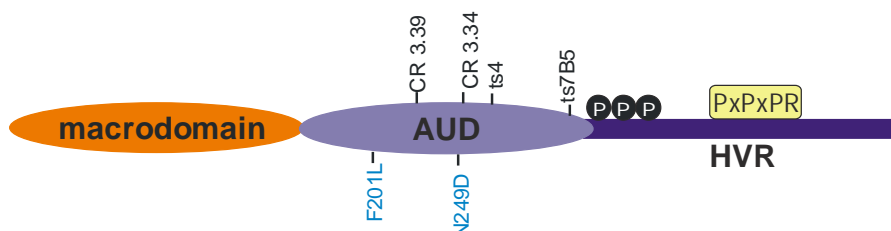


Figure 3. Alphavirus nsP3. N-terminus of nsP3 consists of a globular macrodomain. Central ~160 amino acids form an alphavirus unique domain (AUD) of unknown function. AUD mutants displaying replication defects are depicted in picture in relative positions compared to each other. Mutations in SFV are shown in blue and in SINV in black. C-terminal hypervariable region is characterized by presence of phosphorylation site (black balls with “P”) and proline rich region (PxRxPR). Mutations are depicted with the names given in the original publications (36, 91, 172).

1.5.1 Macrodomain

The N-terminus of alphavirus nsP3 comprises an evolutionarily conserved domain of ~170 amino acids (aa), initially termed “the X domain” for its unknown function in viral replication (62). In addition to alphaviruses, Rubella virus, HEV (the sole member of the genus Hepevirus), and corona and toroviruses in the family of *Coronaviridae* are the only other virus genera known to contain an X-domain. Similar domains sharing the fold of the viral X-domains are found in all the kingdoms of life in various different proteins. Probably the best studied of these is an atypically large histone termed “macrohistone” that is found only in vertebrates (reviewed in (168)). The non-histone domain of the protein was termed macrodomain, and therefore all the conserved domains sharing the same fold, including X-domains, have been termed macrodomains (123). The macrodomains from different organisms are further discussed in chapter 1.6.

The first hint of the macrodomain function came from a yeast protein library screen. A macrodomain from yeast was shown to be able to hydrolyse adenosinediphospho-ribose 1"phosphosphate (ADPR1-P), a by-product from yeast t-RNA splicing, indicating an enzymatic function for a macrodomain (151). This was the first indication that nsP3 might have an enzymatic function through one of its domains. In another study, several macrodomains were shown to bind ADP-ribose (ADPR), and it was suggested that macrodomains are ADPR binding moieties in general (81). These findings could explain the macrodomain conservation in alphaviruses, whether the reason is an enzymatic activity or ADPR binding. In addition to the putative ADPR-related activity of macrodomain, macrodomain may be important for alphavirus

replication also for another reason. When the processing of P1234 polypeptide by viral protease nsP2 was studied with artificial substrates containing short stretches (~40 aa) from each cleavage site, the cleavage of 2/3 site was not detected (177). However, when the whole macro domain (170 aa) was included in the nsP3 side, nsP2 was able to cleave the substrate (59, 177). Thus it seems that the properly folded macrodomain is needed for correct polyprotein processing in alphaviruses.

1.5.2 *Alphavirus unique domain (AUD)*

Very little is known of the functions of AUD, which is a ~160 aa protein region in the central part of nsP3. This domain is conserved in alphaviruses, as can be seen from the sequence homology, but its structure is unknown, and there are no similar proteins in other organisms based on the protein sequence. However, several computational programs predict that at least the C-terminal part of AUD is most likely folded and consequently forms a three dimensional structure (PredictProtein server (142)).

Several mutations that affect viral RNA synthesis and neurovirulence of the virus map to AUD (Fig. 3). These include SINV mutants CR3.34, CR3.39, ts4 and ts7B5 and SFV mutants F201L and N249D (36, 91, 172). Some of the mutants have a defect in subgenomic RNA synthesis (CR3.34), whereas others are incapable of producing negative strand RNA at 40 °C (CR3.39, ts4) or have impaired polyprotein processing (ts7B5) (36, 91, 172). Interestingly CR3.39 and ts4 were also defective in phosphorylation of the HVR at 40 °C, indicating that there is an interplay between AUD and HVR (36). Point mutations F201L and N249D in SFV have been shown to reduce the neurovirulence of the virus when introduced peritoneally into mice, but the mechanism of the reduction is unknown (172).

1.5.3 *Hypervariable region (HVR)*

The boundary between AUD and HVR is not very exactly defined. Conserved region in SINV has been estimated to extend up to aa 324 in SINV (325 in SFV), and nsP3 is predicted to be highly unstructured in C-terminus beginning from the amino acid 312 in SFV (91, 142). The HVR of nsP3 is variable in length and amino acid sequence amongst different alphaviruses. The size of HVR varies between ~150-250 aa, and it has been shown to tolerate large deletions when virus is replicated in cell culture (90, 178). However, some similarities exist among the HVR of different alphaviruses; the region is rich in acidic residues, as well as in serine, threonine, and proline, and it doesn't have predicted secondary structure ((178) and references therein). HVRs of SINV and SFV are known to be heavily phosphorylated, and nsP3 is the only alphavirus nsP modified by phosphorylation (96, 178).

NsP3 proteins from SFV and SINV are phosphorylated on serine and threonine residues, but not on tyrosines (96, 127, 180). The phosphorylation sites of SFV are located in the beginning of HVR, and all detectable phosphorylation has been found to be inside of a 50 aa region (aa 319-368), where altogether 11-16 threonines and serines are phosphorylated (180). It has been shown that serine residues are more heavily phosphorylated than threonine residues (SFV) and that serines become first phosphorylated (SINV) (96, 127, 178). In SFV two adjacent threonines (T344 and 345 in nsP3) and one serine (S320) were detected as the main sites responsible for phosphorylation. Point mutation of either of the threonines (T344/345A) or S320

reduced the phosphorylation of nsP3 by 40-50 % when expressed in HeLa cells (178). However, combination of these mutations did not further decrease the phosphorylation, which could indicate that these amino acids have a regulatory role in nsP3 phosphorylation, rather than being the main recipients of phosphorus groups themselves. (178). Interestingly, the serine 320 was not phosphorylated in a construct in which the C-terminus was deleted after aa 328, highlighting the importance of the interplay between different phosphorylation sites (178).

Phosphorylation can modulate biological properties of proteins, such as protein stability, enzymatic activity, subcellular localization, or protein-protein interactions (reviewed in (26)). The functions of nsP3 phosphorylation are difficult to address because of the complicated nature of extensive phosphorylation. However, it has been shown that when phosphorylation is removed by a deletion of the whole phosphorylated region in SFV nsP3 (Δ 319-368; virus mutant “ Δ 50”), viral RNA synthesis is reduced by about 50 % compared to wild type (wt) virus. Interestingly, this mutant virus replicates close to wt titres in cell culture, but is apathogenic in mice (178). Recently it was found that phosphorylation deficient SFV Δ 50 does not form CPVs, but instead the spherules are detected on the plasma membrane throughout infection. This could indicate that the heavily phosphorylated 50 aa region might be linked to signaling that leads to the internalization of the replication complexes from the plasma membrane (10).

The abundance of serines and threonines in nsP3 HVR seems to be explained by their use for phosphorylation, but the proline rich regions present in the HVR of alphaviruses have not been studied until now. Alphaviruses contain 1-3 proline rich regions, conjoined with an arginine residue, in the C-terminus of nsP3. These regions highly resemble an SRC homology 3 domain binding motif (Sh3BM) that is typically defined by presence of prolines and arginines, and can be defined in its most simplistic form as a PxxP motif (108). In this thesis, data is presented to show that the proline clusters in alphaviruses are functional SRC homology 3 (SH3) domain interaction motifs, which bind amphiphysin, and that this function is important for viral replication.

NsP3 may contain yet other functional elements in addition to phosphorylation region and putative Sh3BMs. One such element could be FxxF sequence that is found in the very C-terminus of practically all the alphaviruses. FxxF motif containing region (nt 5637-5667; VITREEFEAF) was found to be the only region in VEEV HVR, which was intolerant to insertions (15). Based on the conservation of the motif and its intolerance to mutations, it would be tempting to hypothesize that this phenylalanine containing region is a functional motif important for alphavirus replication and therefore intolerant to mutations.

1.6 The conserved macrodomain family

Macrodomains constitute an evolutionarily conserved family of globular protein domains, which share a three layer $\alpha/\beta/\alpha$ -fold with a twisted β -sheet surrounded by 4-6 α -helices, and show a limited homology to P-loop nucleotide hydrolases (Fig. 4) (6). Currently, there are 791 proteins in the SMART database with a recognized

macrodomain fold (termed A1pp domain in SMART) (94). Macrodomains are found in all kingdoms of life as well as in some viruses, and they are highly conserved (94). The sequence identity of for example *Escherichia coli* macrodomain (Ymdb) and a human macrodomain (MDO1) is 47 % at the protein level. However, not all the macrodomains share that high sequence identity, but certain highly conserved amino acid regions can be typically detected (see fig. 6). These highly conserved amino acids constitute a positively charged ligand binding pocket on the surface of the domain (I), (42, 81, 88).

It has been shown in this work and by others that macrodomains bind through the ligand binding pocket various NAD^+ metabolites like ADPR, *o*-acetyl-ADPR and poly(ADPribose) (PAR) (I, II), (42, 81, 88, 118). There is strong evidence that certain conserved amino acids in the binding pocket are essential for the specific ligand binding (see: Chapter 4: Results).

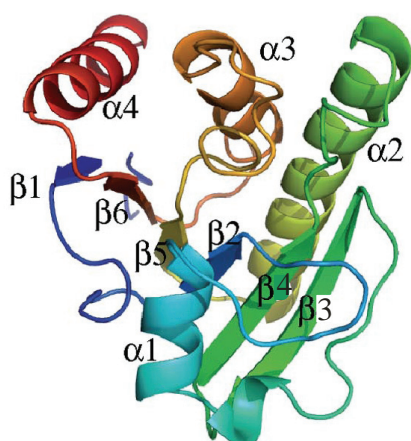


Figure 4. CHIKV macrodomain. X-ray crystallography structure of CHIKV macrodomain shows the conserved $\alpha/\beta/\alpha$ -fold of macrodomains. Structure is colored by secondary structure succession (blue: N terminus), and the secondary structure element numbering is shown. Reproduced from ref. (105).

1.6.1 Human macrodomain proteins

The human genome contains at least 10 genes encoding macrodomains (75). Evolutionary relationships of human macrodomains are shown in figure 5. The most well known of the macrodomain proteins are the macrohistones. There are three different macrohistones in humans: macrohistone 2A1.1 (mH2A1.1) and mH2A1.2 are splice variants from the same gene, and mH2A2 is encoded by another gene located in a different chromosome (28). Macrohistones are atypically large histones, consisting of an N-terminal histone domain and an additional C-terminal macrodomain (123). The mH2A was first detected in inactive X-chromosomes where it is enriched, but it is also found in other subnuclear loci, especially associated with centrosomal heterochromatin (reviewed in (168)). There is growing evidence that the distribution of mH2A variants is linked to their ability to bind PAR. Recent data shows that the splicing variant mH2A1.1, capable of binding PAR, is transiently activated by poly-ADPR polymerase-1 (PARP-1) mediated PARylation upon DNA damage, leading to localization of mH2A1.1 into damage sites, which causes chromatin compaction and Ku70-Ku80 heterodimer recruitment (169). Activation was mediated by PAR binding

through macrodomain, as confirmed by a macrodomain mutant (G224E), in which disruption of ADPR binding pocket led to incapability of sensing the PARP-1 activation (169). In contrast to mH2A1.1, the splicing variant mH2A1.2 does not bind PAR and does not sense PARP-1 activation. MH2A1.1 is only detected in postmitotic and senescent cells, whereas mH2A1.2 is ubiquitously expressed (169). Another nuclear macrodomain protein, Alc1, was shown in two independent studies to be recruited by PARP-1 mediated PARylation to specific areas of chromatin, where it participates in chromatin relaxation through its Snf2-like domain (Snf = Sucrose Non Fermentation) (2, 64).

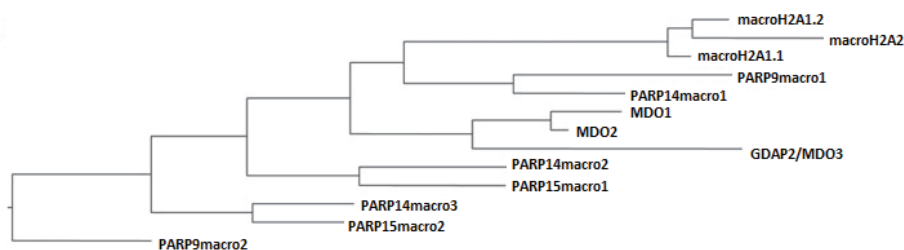


Figure 5. Evolutionary relationships of human macrodomains. Evolutionary tree of human macrodomains including MDO1-3 is shown. MDO1, MDO2 and MDO3 form a cluster based on their evolutionary conservation. All currently recognized human macrodomains except Alc1 and C6orf130 are included in the evolutionary tree. Several macrodomains from the same PARP protein are numbered beginning from the N-terminus. Modified from reference (1).

Many macrodomains have been shown to bind PAR, and interestingly, three human macrodomain proteins (PARP9, PARP14 and PARP15) also contain a PARP domain (I, II), (1, 81). These proteins, formerly known as BAL1-3 (B-Aggressive Lymphoma proteins 1-3), contain two to three N-terminal macrodomains and one C-terminal PARP domain. It has been shown that macrodomain containing PARPs are able to PARylate themselves through activation of their PARP domains in a DNA dependent manner (1). In addition, PAR binding by PARP-9 macrodomains has been verified *in vitro* (81), and PARP-14 has been shown to function as a transcriptional regulator of interleukin-4 mediated Stat6 signalling *in vivo*, in a PARylation dependent manner (23, 109). Consequently, macrodomain containing PARPs are unique proteins, in that can both synthesize and bind PAR.

Most of the human macrodomain proteins seem to localize in the nucleus and have been shown to function in the regulation of chromatin structure and transcriptional activation/repression (1, 2, 64, 70, 169). I was interested to study human macrodomain proteins that might reside in cytoplasm similar to viral macrodomain proteins. Potential candidates for cytoplasmic macrodomain proteins could be macro domain protein 1 (MDO1/ leukemia related protein 16; LRP16), macrodomain protein 2 (MDO2), ganglioside induced differentiation-associated protein 2 (GDAP2/ macro domain protein 3; MDO3) and C6orf130 (Chromosome 6 open reading frame 130) protein. MDO2 and MDO3 have not been reported in any

nuclear processes, except for a very recent study, where MDO2 macrodomain was shown to be recruited to damaged DNA, when overexpressed from a plasmid (169). Altogether, very little is known about the functions of MDO1-3 and C6orf130. MDO1 was initially detected in search for leukemia-relapse related proteins and was later reported to function in regulation of estrogen receptor alpha –mediated signalling (69, 70, 111). One study indicated connection between disruption of MDO2 gene and onset of Kabuki's syndrome (102). However, contradictory results were obtained in a later screen, in which similar gene disruption was detected in three healthy individuals (19). MDO3/GDAP2 was initially found in a screen for genes induced by ganglioside synthase expression in a mouse neuroblastoma cell line (98). It was shown to be expressed mainly in brain and testis, but the functions of MDO3 have not been studied (98). In this work I have shown that MDO1, MDO2 and MDO3 can potentially reside in the cytoplasm, and that they have distinct properties in NAD⁺ metabolite binding, which can to a certain extent be explained by differences in the substrate binding pocket (II). C6orf130 (Uniprot entry Q9Y530, (75)) was found very recently and was therefore not included in my study.

1.6.2 Viral macrodomains

In viruses macrodomains are found only in a small subset of positive-strand RNA viruses, namely coronaviruses, alphaviruses, rubella virus and HEV (42, 62). Macrodomains are found in non-structural proteins of these viruses and they reside inside multi-domain proteins surrounded by different domains depending on the virus. Several structures of viral macrodomains have been recently obtained (42, 106). Viral macrodomains share the conserved features of the ADPR binding pocket, including an aspartate residue (D10 in CHIKV) in close proximity to the adenine moiety of the ligand, flexible glycines (G30, G32 and G112 in CHIKV) in both sides of the phosphates of ADP, and an aromatic residue near the distal ribose (Y114 in CHIKV) (Fig. 6B) (42, 105). We have shown that viral macrodomains bind NAD⁺ metabolites including ADPR and have a modest enzymatic activity on ADPR1-P (I), (105). ADPR1-P hydrolysis activity has been shown to be dispensable for a coronavirus in cell culture (135).

Recently two macrodomains, having no sequence similarity with the earlier recognized members of the family, were discovered (22, 76, 166). These domains from SARS-CoV are called SARS unique domain N and M (SUD-N and SUD-M) (for N-terminus and middle). These proteins were not recognized as macrodomains by computational searches, and they are totally devoid of conserved amino acids that form the ADPR binding pocket (166). Interestingly, these domains reside in the same protein with SARS-CoV X-domain, the more typical macrodomain in SARS-CoV. Although SUD macrodomains share no sequence similarity with X-domain or other known members of the macrodomain family, they have a similar fold consisting of a central twisted six-stranded β sheet surrounded by alpha helices on both sides, and clearly classify as macrodomains (22, 166).

Human macrohistone was detected as the closest homologue of SUD-N and SUD-M with the rmsd 2,5 Å and 2,8 Å when superimposed to SUD-N and SUD-M respectively (112/115 out of 184 of C α atoms) (166). SUD-N and SUD-M macrodomains do not bind ADPR; instead they have been shown to bind G-

quadruplexes and RNA (76, 166). Based on mutational analysis, binding of short RNA seems to be mediated by a site located in a divergent loop between α -helix 2 and β -sheet 3 in SUD-M, which is distinct site from that of ADPR binding site in other macrodomains (166). The loop region corresponds to region between $\alpha 1$ and $\beta 3$ in CHIKV macrodomain that is shown in figure 4.

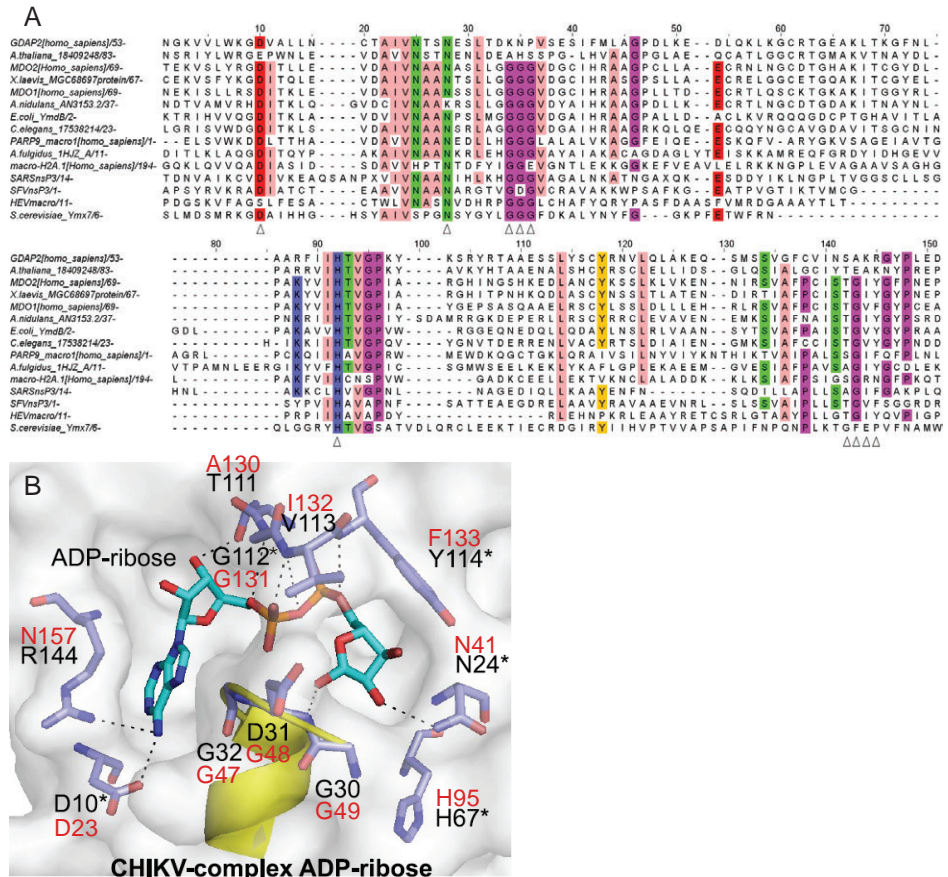


Figure 6. Amino acids in macrodomain ligand binding pocket are highly conserved. (A) Multiple sequence alignment of macrodomain proteins from various origins was made with clustalW and modified with Jalview (182). Amino acids with over 60% conservation amongst aligned proteins are highlighted in colour. Amino acids around the ligand binding pocket, which are shown in (B) are indicated by triangles below the sequences. The sequence names for non-human proteins begin with the organism name and are followed by the database annotation for the protein; SARSnsP3 refers to the “X-domain” of SARS-CoV. (B) CHIKV macrodomain in complex with ADPR ligand is shown with hydrogen bonds are depicted in dotted lines. Amino acids that have been mutated in alphavirus macrodomains (as reported in II and (105)) are marked with asterisk. Corresponding residues in SARS coronavirus are numbered in red. Modified from ref. (105).

2 Aims of the study

The aim of this study was to elucidate the functions of alphavirus nsP3 in alphavirus replication, especially in the context of virus–host interactions. The study was divided into two parts. The first part considers the properties of the nsP3 macrodomain, and the latter part concentrates on the functions of the hypervariable region in nsP3. The specific aims of the projects were:

1. to characterize the biological properties of viral macrodomains
2. to find out whether the properties of macrodomains are conserved and which (if any) host cell macrodomain has the same properties as viral macrodomains
3. to characterize the functions of the proline motifs in nsP3 HVR *in vitro* and *in vivo*
4. to clarify the role of nsP3 HVR in replication complex internalization

3 Materials and methods

Materials and methods are explained in original publications as indicated in table 1 and table 2. Additional methods that have been not used in publications are detailed below. All virus work has been conducted with viruses originating from strains SFV4 and SINV Toto1101.

Table 1. Materials used in this work.

| Materials | Publication |
|---------------------------|--------------------|
| Plasmids | |
| pHAT | I, II |
| pDEST14 | I |
| pEGFP-N1 | II |
| pcDNA4/TO | II |
| pmtYFP | II |
| pEBPP | III |
| pSFV4 (pSP6-SFV4) | III |
| pToto1101 | III |
| Antibodies | |
| anti-MDO1 | II |
| anti-nsP3 (rabbit) | II, III |
| anti-nsP3 (guinea pig) | III |
| anti-EGFP | II |
| anti-dsRNA (J2) | III |
| anti-Amphiphysin-1 (N19) | III |
| anti-Amphiphysin-2 (H100) | III |

Table 2. Research methods used in this work.

| Method | Publication |
|--|--------------------|
| Protein assays | |
| Protein expression and purification | I, II |
| ADP-ribose binding assay | I, II |
| Appr-1´´pase activity assay | I, II |
| Poly(ADP-ribose) synthesis and binding assay | I, II |
| PARG activity assay | II |
| polyA binding assay | II |
| Protein structures | |
| Structure determination and modeling of SARS CoV macro | I |
| Structure prediction for MDO1 | II |
| Cell culture and cell manipulation | |
| Cell lines | II, III |
| Cell culture methods | II, III |
| Transfections | II, III |
| Immunological methods | |
| Western blotting | II, III |
| Immunoprecipitation | III |
| Indirect immunofluorescence | II, III |
| DNA and RNA techniques | |
| Cloning and mutagenesis | II, III |
| Quantitative PCR | III |
| RNA production and virus stocks | III, unpubl. |
| Microscopy and data analysis | |
| Wide field microscopy | II |
| Confocal microscopy | III |
| Image processing (ImageJ) | III |
| Deconvolution (Autoquant) | III |
| Colocalization analysis (Imaris Bitplane) | III |
| 3D whole cell modeling (Imaris Bitplane) | III |

Virus stocks and infectious center assay

BHK (baby hamster kidney) cells were grown on 10-cm plates until nearly confluent. The cells were detached with trypsin and washed once with phosphate-buffered saline (PBS). 1 µg of *in vitro* transcribed viral genomic RNA was used to transfect cells from one plate (~10⁷ cells) collected in 800 µl of PBS. Cells were electroporated with two consecutive pulses of 850 V and 25 µF in a Bio-Rad Gene Pulser apparatus in a 0.4 cm cuvette. A 720 µl aliquot of the electroporation mixture was used to generate the primary virus stock. These cells were immediately diluted with 10 ml of complete BHK medium and seeded onto a 10-cm dish. The medium was harvested when cytopathic effect (CPE) appeared (at 24 h for the wild-type virus and 48 h or 72 h for mutant viruses). The remaining 10% of the electroporation mixture (corresponding to 0.1 µg RNA) was used in an infectious center assay. Tenfold dilutions of the mixture were immediately prepared in minimal essential medium (MEM) containing 0.2% bovine serum albumin (BSA). The dilutions were seeded onto 6 well plates of confluent BHK cells. After two hours, the inoculum was replaced with nutrient agarose in complete BHK medium. After incubation at 37°C for 48 to 72 h, plaques were visualized by staining with neutral red.

Virus growth curves

Dishes were prepared as explained above for virus stocks and 200 µl of growth medium was removed at indicated time points and diluted 1:1 in Dulbecco's essential medium + 0,2% BSA. 10 % glycerol was added and the samples were stored in -70 °C. Duplicates of each sample were titrated using plaque assay as described earlier (82).

Imaging of P13 and P13ZsG

Human cervix epitheloid carcinoma (HeLa) cells or BSR-T7 cells (20) cells were transfected by using Exgen 500 (Fermentas), according to manufacturer's instructions, with plasmids P13-pcDNA4/TO or P13ZsG-pTM1 (155). HeLa cells were fixed at 4 h post transfection and visualized by immunofluorescence imaging using rabbit α-nsP1 (87) and guineapig α-nsP3 as described in (III). For live cell imaging, LysoTracker Red DND-99 (Molecular Probes) was added to transfected BSR-T7 cells at 16 h post transfection. BSR-T7 cells were imaged 15 min after LysoTracker addition by using the Olympus IX71 TILL microscope supplemented with a 37°C chamber and CO². Images were taken with UPlanSApo 60x 1.20 numerical aperture water objective.

4 Results

4.1 Characterization of the properties of macrodomains (I, II and unpublished)

4.1.1 Macrodomains in ADPR binding

In order to study the structural basis for ligand binding by a viral macro domain, SARS-CoV macrodomain structure was obtained by X-ray crystallography in the presence and absence of ADPR. Analysis of the data revealed a globular monomer, with approximate dimensions of 40 Å x 40 Å x 35 Å, consisting of a central seven stranded β -sheet flanked by two α -helices on one face and three on the other face (Fig. 1A & B in **I**). Crystals obtained in the presence of ADPR revealed an ADPR molecule in a slightly bent conformation, located at the same side of the central β -sheet as the C-terminal α -helix and partly buried inside of a mainly hydrophobic cleft (Fig. 2A in **I**). The adenine moiety is bound through a hydrogen bond between D23 and the NH₂ group on the adenine ring. This aspartate residue is strictly conserved in macrodomains and seems to serve as a basis for specificity for adenine moiety, as confirmed by structures of different ADPR bound macrodomains (81, 88, 105). Also other amino acids interacting with ADPR belong to motifs that are evolutionarily well conserved (Fig. 5 in **I**). For comparison the structure of CHIKV ADPR binding site is shown in figure 6. ADPR binding of SARS-CoV was confirmed by isothermal titration calorimetry and a dissociation coefficient (K_D) value of ~ 24 μ M was obtained (Fig. 2B in **I**). ADPR binding of other viral macrodomains was also tested. For HEV macrodomain a weaker binding (K_D ~50 μ M) was observed (**I**), but for SFV macrodomain no ADPR binding was detected (**II**).

I wanted to compare the functions of the viral macrodomains with the macrodomains from the host cell. I was especially interested in host macrodomains that might reside in the cytoplasm and thus interact or compete with viral macrodomains. Therefore, I decided to study MDO1, MDO2 and GDAP2 that were not strongly associated with any nuclear processes. Yeast macrodomain protein Poa1p (151) was used as a control. For Poa1p, an enzymatic activity in ADPR1-P hydrolysis has been detected (151), and as expected, Poa1p bound also the end product of the hydrolysis, ADPR (K_D = 2,9 μ M) (Fig. 3a in **II**; Table 1 in **II**). Human macrodomains MDO1 and MDO2 are even more potent ADPR binders than any of the viral macrodomains or the yeast macrodomain Poa1p. MDO1 and MDO2 bound ADPR with a K_D value of 0.9 and 0.15 μ M, respectively. In contrast, GDAP2 had no affinity to ADPR (Fig. 3a in **II**; Table 1 in **II**). ADPR binding of MDO1 depends on the conserved glycines in two sides of the binding pocket (G182; G270) (Fig. 4 in **II**). When these amino acids were mutated to glutamic acid, no binding was detected. Interestingly, in GDAP2 there are no glycines in these positions, but instead K-N-P residues correspond to glycines 180-182 in MDO1 and alanine replaces G 270 (Fig. 1a in **II**).

4.1.2 *Macrodomains as ADPR1-P hydrolyzing enzymes*

All the three viral macrodomains (from HEV, SARS-CoV, SFV) performed ADPR1-P hydrolysis, but the enzymatic activity of SFV macrodomain was rather poor (Fig. 4B & C in **I**; Fig. 2 in **II**). Human macrodomains from MDO1 and MDO2 were also potent hydrolysis enzymes, but GDAP2, which was incapable of ADPR binding, could not hydrolyze ADPR1-P. When mutations were introduced to the binding pocket of SARS-CoV macrodomain in the close vicinity of the distal ribose moiety of the bound ligand, hydrolysis of the phosphate was impaired. Several mutations (N38A, H46A and G47A/G48A) decreased the hydrolysis activity of the protein and two mutants (N41A and F133A) were totally inactive in hydrolysis (Fig. 5A and B in **I**). Some of these mutations probably affect ADPR binding, whereas some could have a direct role in the dissociation of the distal phosphate. For example mutating G47/G48 in SARS-CoV macrodomain probably decreases the hydrolysis activity because of reduced substrate binding. These amino acids correspond to G182 and G183 in MDO1 that were shown to impair ADPR binding by MDO1. In another study we found that macrodomains from three alphaviruses, CHIKV, VEEV and EEEV also performed ADPR1-P hydrolysis, and that Y114A mutation in CHIKV (corresponding to F133A mutation in SARS-CoV) was detrimental to this activity (105). Based on those findings and work presented here, it seems likely that an aromatic amino acid in this position (Y114, Fig. 6) is needed for ADPR1-P hydrolysis.

4.1.3 *PAR binding by viral and human macrodomains*

Most of ADPR in eukaryotic cells is thought to exist in the form of PAR. Consequently, I decided to study whether some of the macrodomains studied (listed in Fig 1b in **II**) could also bind PAR. It turned out that viral macrodomains from SFV, HEV and SARS-CoV bound PAR by an interaction that could not be competed by mono-ADPR as was tested for SFV and HEV (**I**, **II**). Interactions between PAR and SARS-CoV macrodomain were modelled by constructing a di-ADPR-macrodomain combination *in silico* (Fig. 7 in **I**). From the obtained structure, it seems likely that macrodomain would bind to the end of PAR in a capping manner, so that last ADPR would sit inside of the ligand binding pocket, but amino acid residues outside of the pocket might also participate in the binding.

Of the human macrodomains studied here, only MDO1 bound PAR very efficiently, whereas MDO2 and GDAP2 gave only a very weak signal from labelled PAR (Fig. 3b in **II**). For GDAP2 this could be anticipated, since it did have no affinity to monomeric ADPR. The differences between PAR binding by MDO1 and MDO2 are rather intriguing, since they share an almost identical ligand binding pocket, but have very different affinities towards PAR. This indicates that there are other factors, in addition to ligand binding pocket, that contribute to the binding of PAR.

4.1.4 *Some macrodomains are poly(A) binding modules*

I wanted to test whether polymer binding by macrodomains was specific to PAR, or whether other charged molecules could also be bound. It turned out that some of the macrodomains were able to bind also poly(A), but surprisingly the poly(A) binding did not correlate with the PAR binding ability of the proteins. SFV macrodomain and

HEV macrodomain were potent PAR binders and bound also poly(A) with good affinity (II). MDO1 bound PAR efficiently, and was also able to bind poly(A), although not as efficiently as viral macrodomains from SFV, HEV and SARS-CoV. MDO2 was not efficient in PAR binding and had no affinity to poly(A). Interestingly GDAP2, which was found inactive in all ADPR-related assays, including the binding of PAR, was found to be a potent poly(A) binder (Fig. 3c in II).

4.1.5 Mutations in nsP3 macrodomain cause defects in SFV reproduction (unpublished)

SFV macrodomain mutant viruses had been produced in our laboratory before the structure of macrodomain was known. Mutations were designed based on the sequence conservation in the macrodomains, and five of the most highly conserved amino acids were selected for mutation. Four different mutants were produced: a double mutation of N21A and N24A (N21+24A), H67A, P107G and G112S. Based on the comparison with structures of SARS-CoV macrodomain (Fig. 4a in I; 5a in I) and CHIKV macrodomain (105), these mutations map into the ligand binding pocket. N24 and G112 in CHIKV macrodomain form hydrogen bonds with ADPR. N21, H67 and P107 reside in the close vicinity of the ligand bind pocket, but seem not to interact directly with ADPR.

Both mutations (N21+24A, G112S) changing the amino acids participating in the hydrogen bonding of ADPR caused a delay in virus production in BHK cells. P107G mutant virus had only a slight replication delay, whereas H67A mutant was most severely affected (Fig. 7). In an infectious center assay, the infectivity of the corresponding RNAs was assessed in a single round experiment (see materials and methods). Almost no plaque forming viruses were detected for H67A (~12 plaque forming units (pfu)/ μ g RNA). Thus this mutation must be quite destructive for SFV, and the growth of the virus probably arises from compensatory mutations. Other mutants had also reduced infectivity, resulting in two to three logs lower pfu production (Table 3).

Based on the structure comparison with CHIKV macrodomain, these mutations reside in a close vicinity of the ADPR binding pocket, and two of them affect amino acids that have been indicated in the direct binding of ADPR (N24 and G112). Consequently, this data indicates that ligand binding through macrodomain is important for virus multiplication *in vivo*.

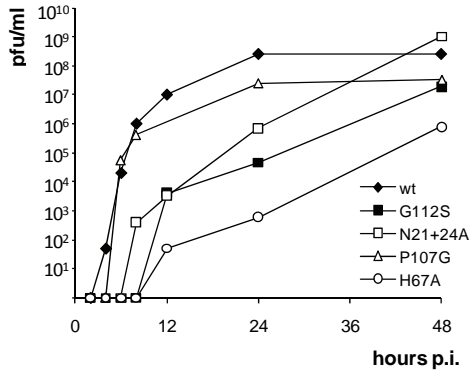


Table 3. Infectivity of SFV macrodomain mutant viruses as measured by infectious center assay.

| virus RNA | Infectivity (pfu) | CPE (days after infection) | Virus stock titer (pfu/ml) |
|-----------|-------------------|----------------------------|----------------------------|
| wt | $3,0 \times 10^6$ | 1 | $1,5 \times 10^9$ |
| G112S | $4,3 \times 10^4$ | 2 | $4,6 \times 10^8$ |
| H67A | ~12,5 | 3 | $1,5 \times 10^9$ |
| N21+24A | $3,7 \times 10^3$ | 2 | $3,7 \times 10^8$ |
| P107G | $3,8 \times 10^4$ | 2 | ND |

Figure 7. Growth curves of the SFV macrodomain mutants. Growth curves were obtained from a single round experiment after RNA transfection. 1 μ g of infectious RNA was electroporated into BHK cells and aliquots of growth media were collected at the indicated time points. Titres of the released viruses were measured by plaque assay.

4.1.6 Subcellular localization of macrodomain proteins

The subcellular localization of human macrodomain proteins was studied in HeLa cells by using either C-terminally EGFP-tagged (enhanced green fluorescent protein tagged) proteins or detection by specific antibodies. Proteins expressed from a plasmid were analysed by Western blot to check that full length proteins were expressed (Fig. 5a in **II**). Expression of MDO1 resulted in two products, the larger of which corresponds to full length protein (62 kDa), and shorter product corresponding to ~56 kDa protein. The shorter protein could be either translation product from the second in-frame methionine (M83) or could be a cleavage product produced from the full length protein *in vivo*. Translation of the shorter protein would give a 243 aa long product, whereas cleavage of a putative mitochondrial localization signal (P = 0.998, Mitoprot (24)) would yield 248 aa product, and thus neither of these possibilities can be ruled out based on the Western blot results (**II**).

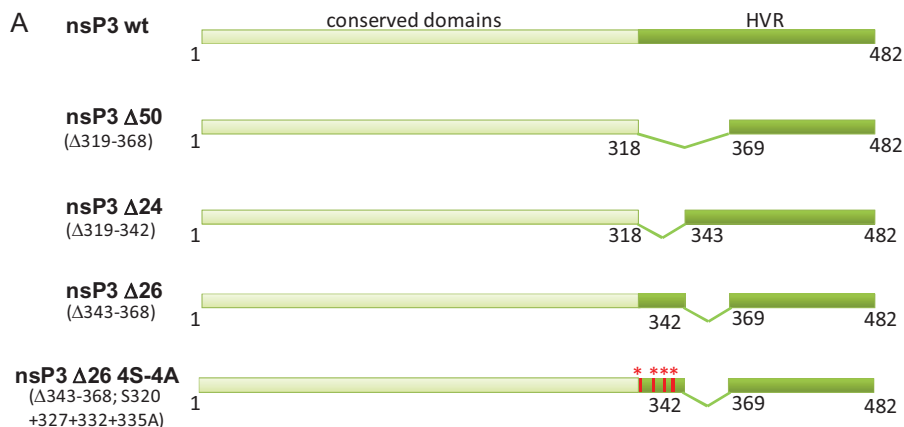
The full length MDO1 (325 aa) was found exclusively localized into mitochondria. When a shorter form (243aa), corresponding the translation from second in-frame methionine, was expressed, a diffuse nuclear-cytoplasmic localization was detected. C-terminally tagged EGFP-MDO2 and EGFP-GDAP2 were also found diffused throughout the whole cell. This indicates that all of these proteins are potentially cytoplasmic residents (**II**).

4.2 Virus-host interactions mediated through nsP3 C-terminus (III and unpublished)

4.2.1 The HVR of nsP3 functions in SFV replication complex internalization (unpublished)

It has been shown that SFV replication complexes first form on plasma membrane, from where they are internalized in a mechanism dependent on phosphoinositide 3-kinase (PI3K), actin cytoskeleton and microtubules, into virus induced endo-lysosomal organelles (157). Recently, it was found that the internalization of replication complexes can be blocked by P13K inhibitors or by deletion of 50 amino acids in the phosphorylation region of nsP3 HVR (aa 319-368) (10, 157). To examine more closely the effect of phosphorylation on the transport of replication complex into CPVs, three different deletion mutants were studied (Fig. 8A). The original 50 amino acid deletion was divided in two halves, $\Delta 24$ and $\Delta 26$, to dissect the role of different phosphorylation determinants in internalization. Vihinen and co-workers (180) reported that when nsP3 $\Delta 26$ 4S-4A mutant was studied, no phosphorylation was detected. Consequently, this mutant was also included in these studies to see whether it could retain the replication complexes on the plasma membrane similar to $\Delta 50$ mutant.

In cells infected with alphaviruses, double stranded RNA (dsRNA) is only detected in the viral replication complexes, where it occurs as a replicative intermediate in RNA replication. The localization of replication complexes was studied by dsRNA staining of BHK cells infected with wt or mutant SFV (Fig. 8 B-F). Replication complexes of $\Delta 50$ virus were detected at the plasma membrane at 8 h p.i., whereas wt replication complexes localized in CPVs. Neither $\Delta 24$ nor $\Delta 26$ mutant viruses affected the localization of replication complexes and the CPVs formed similar to wt. Interestingly, a phosphorylation deficient mutant $\Delta 26$ 4S-4A also formed CPVs at 8 h p.i. However, the transport of the replication complexes of $\Delta 26$ 4S-4A virus was delayed compared to wt (Fig. 9).



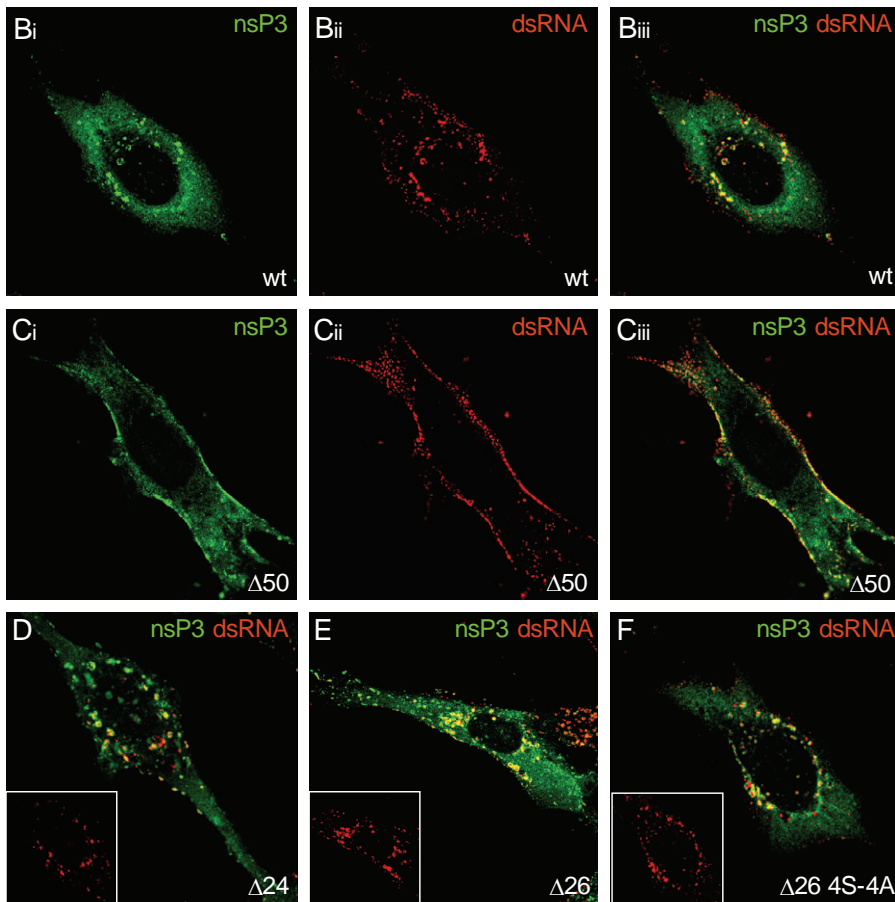


Figure 8. Effects of nsP3 phosphorylation on SFV replication complex intracellular localization. (A) Design of nsP3 mutants with deletions in phosphorylation region. Nomenclature of the mutants and corresponding mutations are indicated in the left. Point mutations are presented with red lines, highlighted with asterisks. (B-F) BHK cells were infected with SFV wt or mutant viruses with 50 plaque forming units (pfu) per cell. Cells were fixed at 8 h post infection (p.i.) and stained with anti-nsP3 and anti-double stranded RNA (dsRNA) antibodies. Wt replication complexes stained with dsRNA and nsP3 antibodies were detected in the CPVs (B), but $\Delta 50$ replication complexes remained at the plasma membrane, (C) whereas shorter deletions ($\Delta 24$, $\Delta 26$) or a combination of deletion and point mutations ($\Delta 26$ 4S-4A) did not affect the localization of the CPVs, and the localization was similar to wt (D-F).

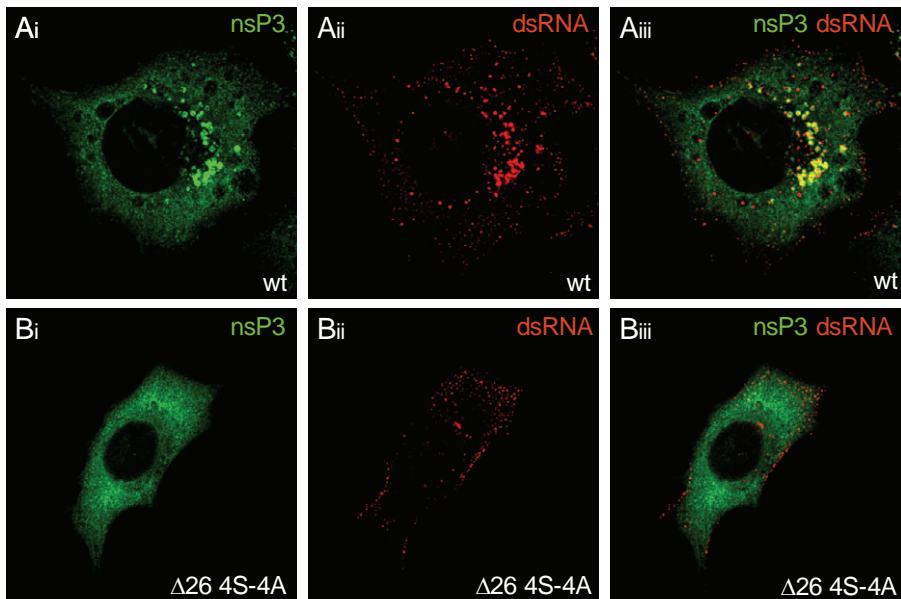


Figure 9. Severe reduction in SFV nsP3 phosphorylation causes delay in CPV formation. BHK cells were infected with 50 pfu of SFV wt and $\Delta 26$ 4S-4A and fixed at 4 h p.i. and stained with anti-nsP3 and anti-dsRNA antibodies. Wt replication complexes were detected in CPVs already at 4 h p.i. (A), whereas $\Delta 26$ 4S-4A remained in small endocytic vesicles and on the plasma membrane (B).

It is known that nsP1 is the membrane anchor of the replication complex (125, 146). nsP1 seems to be responsible for directing replication complexes first to plasma membrane, since nsP1 (and P12) localize to plasma membrane when expressed alone (146). Polyprotein P123 is transported to an intracellular vesicular compartment (146) (Fig. 10 A-C). We wanted confirm that nsP3 alone is sufficient to induce the transport to vesicular compartment, and nsP2 is not needed for this purpose. In this study, an artificial protein P13 was constructed and fluorescent reporter ZsGreen (ZsG) was inserted inside of nsP3 in a position that has been shown not to interfere with the formation of replication complex and virus infection, and thus should not affect the folding nsP3(157). Western blot with nsP3 confirmed an expression of protein of correct size producing no degradation products that would be recognised by nsP3 antibody (Fig. 10 D). When expressed in HeLa cells, nsP1 and nsP3 staining colocalized well in immunofluorescence and colocalization was detected in vesicle-like structures (Fig. 10 E). However, over-expression of the construct seemed to lead to aggregation (not shown). When observed in BSR-T7 cells P13ZsG colocalized with acidic vesicles stained with lysotracker, indicating that nsP3 can drive the relocalization of nsP1 from plasma membrane to lysosomes (Fig. 10 F). Together these data indicate that nsP3 is sufficient and necessary for transport of replication complex to the vesicular compartment, and that the transport is dependent on determinants in nsP3 HVR. However, nsP1 is also needed for this process, at least as a membrane anchor of the polyprotein.

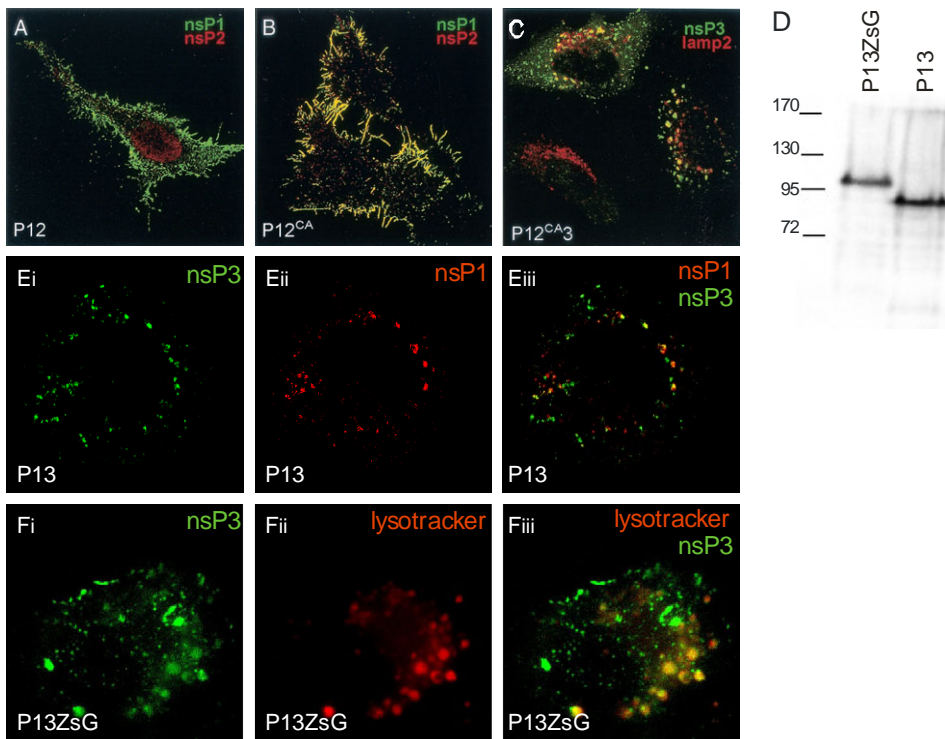


Figure 10. SFV P13 localizes to endosomes similar to P123 polyprotein. Localizations of nsP1 and nsP2 (cleaved from P12), P12CA (uncleavable) and P12CA3 (uncleavable) are shown as detected by Salonen et al. (A-C). Reproduced with permission from ref. (146). HeLa and BSR-T7 cells were transfected with P13 and P13ZsG expression plasmids. Expression of P13 and P13ZsG was detected from transfected BSR-T7 cells by Western blot analysis with anti-nsP3 antibody (D). HeLa cells transfected with P13 were fixed at 24 h p.i. and stained with anti-nsP1 and anti-nsP3 antibodies and analyzed by immunofluorescence microscopy (E). BSR-T7 cells transfected with P13ZsG were analyzed by live cell imaging after an addition of lysotracker for staining the acidic endosomal compartment (F).

4.2.2 Alphavirus nsP3 binds SH3 domain proteins through a proline rich region in HVR

All alphaviruses harbour in the C-terminus of nsP3 one or more proline rich regions of about 6-12 amino acids in length (Fig. 1a in **III** and Fig. 3). In SFV these regions reside 40 amino acids downstream of the phosphorylation area in HVR. Sequences of the proline rich stretches closely resemble an Sh3BM consisting of conserved prolines and arginines. Consequently, we decided to assess whether alphavirus nsP3 could function in SH3 domain binding. For this purpose nsP3 proteins from SFV, SINV and CHIKV were used to screen a phage library containing the SH3 domains from virtually all the human SH3 domain proteins. NsP3 proteins turned out to be excellent

SH3 domain binders, giving over 1000-times enrichment of phages compared to the mock protein used as a control. The SH3 domains fished out by these proteins consisted almost purely of amphiphysin-1 and amphiphysin-2 (Table 4).

Table 4. The identities of individual SH3 domain clones obtained after a single round of panning with SFV, SINV and CHIKV nsP3.

| Full SH3 screen | | SH3 screen w/o Amph2 | |
|--------------------|-------|----------------------|-------|
| SFV nsP3: | | SFV nsP3: | |
| AMPH2 | 10/16 | AMPH1 | 12/16 |
| AMPH1 | 4/16 | CMS 1/3 | 3/16 |
| CMS 1/3 | 1/16 | CIN85 1/3 | 1/16 |
| CIN85 1/3 | 1/16 | | |
| SINV nsP3: | | SINV nsP3: | |
| AMPH2 | 13/16 | AMPH1 | 7/8 |
| AMPH1 | 3/16 | SASH1 1/1 | 1/8 |
| CHIKV nsP3: | | CHIKV nsP3: | |
| AMPH2 | 13/16 | AMPH1 | 8/8 |
| AMPH1 | 3/16 | | |

The relevance of the detected SH3 domain binding was tested by studying full length amphiphysins. Both amphiphysin-1 and amphiphysin-2 bound to alphavirus nsP3 proteins from SFV, CHIKV and SINV (Fig. 2 in **III**). When the proline rich area was removed, no binding of amphiphysins was detected (Fig. 1B in **III**; 2 in **III**). SINV and CHIKV both contain only one proline cluster in their nsP3, whereas SFV contains two separate proline rich regions (Fig. 1A in **III**). When only the latter region in SFV nsP3 was removed, binding of amphiphysin did not decrease, leading to a conclusion that the first region alone is sufficient for efficient amphiphysin binding. However, the latter region alone can also bind amphiphysin, but the affinity is reduced compared to wt, especially in the case of amphiphysin-2. Thus simultaneous removal of both the region was needed for us to be able to study a mutant with no affinity to amphiphysin (Fig. 1B in **III**).

We wanted to characterize in detail the Sh3BM of alphaviruses and for this purpose we decided to use SINV nsP3, which only contains one proline cluster. We mutated separately each amino acid in SINV proline motif: P-I-P-P-P-R-K-K. Mutations on the first proline (P421A) and in the arginine (R426E) totally abolished amphiphysin-1 and amphiphysin-2 binding, whereas P423A and P425A mutations seemed to reduce the binding slightly (Fig. 3 in **III**). These results confirm that the motif recognized by both amphiphysins represents an Sh3BM of the type PxPPxR. We didn't examine the determinants in SFV proline clusters, but the PxPPxR motif is only found in the first of the two clusters, and it is possible that the second motif is specific for recognition of an SH3 domain from another protein, although it also binds amphiphysin *in vitro*.

4.2.3 SH3 domain binding by alphavirus nsP3 is important for virus replication in cell culture

Amphiphysin-1 and amphiphysin-2 colocalize with SFV and SINV replication complexes *in vivo* (Fig. 4-6 in **III**). This binding is mediated by Sh3BM in nsP3 (Fig. 2 in **III**), and the colocalization of nsP3 containing aggregates is lost when the motif is disrupted (Fig. 7 in **III**). However, some amphiphysin is still detected in association with the replication complexes (Fig. 6 in **III**; 7 in **III**). Even though amphiphysin-2 colocalizes faintly with mutant replication complexes, it is not co-immunoprecipitated by nsP3 from these cells, indicating that amphiphysin is not bound to replication complex components of mutant virus (Fig. 2c in **III** and 7 in **III**).

SINV mutant virus with disrupted Sh3BM replicated very poorly in HeLa cells compared to wt (Fig. 11). Replication of SFV Δ P mutant was not as severely impaired, but the mutant virus formed CPVs more slowly than the wt virus, and the recruitment of amphiphysin to SFV Δ P replication complexes was rather poor (Fig. 7A, B in **III**). Data analysis confirmed that there is a strong and significant colocalization between amphiphysin-2 and nsP3/dsRNA at 10 h p.i. in the case of wt SFV (Pearson's correlation coefficient (r) = 0,67/0,75 (for nsP3/dsRNA respectively)). Instead, only a weak colocalization was detected (r = 0,48/0,52) in the case of SFV Δ P (Fig. 7C in **III**). Consequently, the colocalization analysis supports the idea that amphiphysin is directly bound to replication complexes in wt infected cells, whereas in the cells infected with binding deficient mutant interaction between amphiphysin and replication complexes is more indirect, leading to less significant colocalization.

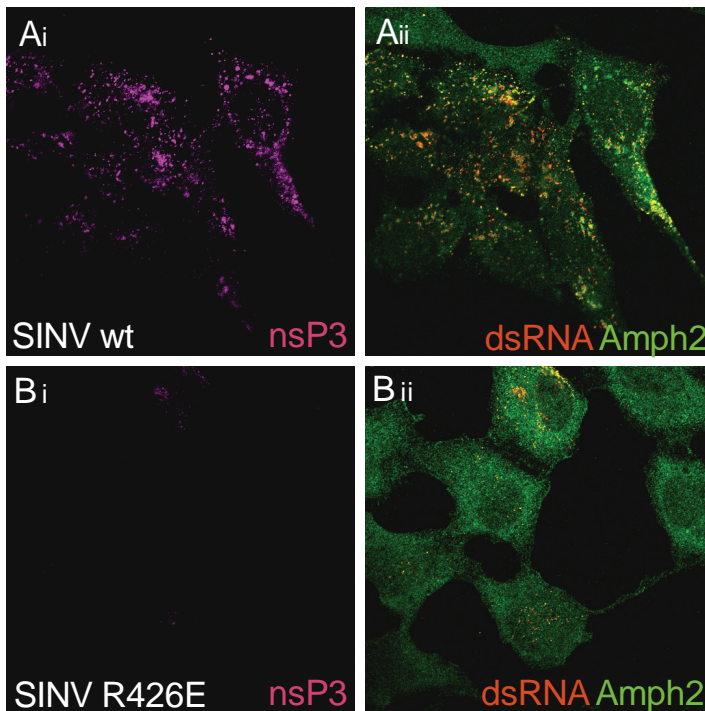


Figure 11. Mutation in SINV proline region site severely reduces virus replication. HeLa cells were infected with SIN wt (A) or R426E (B) at m.o.i. 50, fixed at 6 h p.i. and stained with antibodies against amphiphysin-2 and dsRNA. SIN nsP3 was detected from the fluorescent signal of conjugated cherry (pseudocolored in magenta, A_i and B_i) The overlay of dsRNA (viral replication complexes) and amphiphysin-2 staining is shown in the right (A_{ii} and B_{ii}). Colocalization of viral replication complexes (dsRNA staining) and amphiphysin-2 is seen in yellow.

Since the immunofluorescence data indicated that SH3 domain binding is important for virus replication, we decided to assess the significance of proline rich motif for viral RNA replication. For this purpose total RNA from SINV and SFV infected cells was collected and the virus replication was assessed by using reverse transcription quantitative PCR (RT qPCR). As expected, the RNA replication by proline motif mutants was clearly delayed compared to wt virus. SFV mutant displayed a delay both in HeLa and BHK cells, whereas SINV mutant had a very severe delay in HeLa cells, but replicated close to wt levels in more permissive BHK cells (Fig. 8 in III). Consequently we can conclude that amphiphysin binding mediated by alphavirus nsP3 is important for RNA replication.

5 Discussion

For years alphavirus nsP3 has been the mysterious replication protein, necessary for virus reproduction, but with no obvious clearly defined function in virus replication (78). Based on current evidence, nsP3 is needed for replication as a building block of the replication complex and together with nsP2 it seems to function in regulation of template specificity of the replication machinery (78, 160). However, one can ask whether it would be enough for evolutionary conservation of such a large entity as nsP3 to be just a structural building block.

In the early years, mutational analysis was conducted leading to large amount of information on different complementation groups, temperature sensitive mutants and essential amino acids in all the nsPs including nsP3 ((68) and references therein). When the experimental data was combined with computer aided searches for protein domains, the enzymatic activities of nsP1, nsP2 and nsP4 were uncovered (62, 78). During the early 1990s it was revealed, how alphaviruses were able to achieve the whole replication cycle in cytoplasm, seemingly without the help of host proteins, with as little as four replication proteins on their own (51, 93, 150, 161). Curiously, no specific function for nsP3 was detected. There was not a single recognized domain in nsP3, which made its function difficult to address. Thus nsP3 remained somewhat a puzzle (reviewed in (78)).

Interestingly, some of the nsPs turned out to have other functions not related to RNA replication. Most importantly, nsP2 was shown to shut down the host cell transcription and suppress host immune system (45, 48, 126). However, for nsP3 no function was proposed when cleaved from the replication complex. Rather, its importance remained questionable, since large parts of nsP3 could be removed without compromising the virus replication in cell culture (90). However, nsP3 was revealed as a phospho-protein (96, 127), and when phosphorylation-incapable viruses were introduced into mice, they were apathogenic (178). In fact, there are many other mutations mapping to nsP3 that also turn the virus apathogenic in mice (53, 163, 172). It seems that many pathogenicity factors reside inside nsP3 (172, 178), but since the old studies were mainly done in cell culture, many of these functions could not be revealed, since the host immune system probably plays an important role in these interactions.

In recent years, virus-host interaction has gained growing amount of interest (8, 58, 116). It is becoming evident that the original hypothesis of the host cell independent replication of positive strand RNA viruses is not accurate as such. Although the essential functions of RNA duplication are achieved by the viral machinery, it seems that many host proteins are recruited to viral replication complexes and are needed for efficient virus replication (11, 12, 39). Dissecting the virus-host interactions that determine the virulence and pathogenicity of a certain virus might provide a key for successful antiviral therapy, and analysis of alphavirus nsP3 interactome may be a valuable tool to achieve this goal.

In 1992, an atypical histone variant was found, consisting of a traditional histone core similar to histone 2A and another additional domain, and the new histone was named “macrohistone” for its large size (123). In 1998, it was recognized that the “X-domain” of alphavirus nsP3 belongs to the same evolutionarily conserved protein

family as the non-histone domain of macrohistones (124), and in 2003 the structure of macrodomain was finally revealed (6). However, even though several macrodomain structures have now been solved, the functions of this domain remain somewhat unclear, and we are only getting first insight into the functions of macrodomain. This underlines the fact that information on protein structure does not necessarily reveal the protein function.

In this work I have dissected the functions of different domains of nsP3, thus trying to shed some light into the functions of this protein. During this work it has turned out that the macrodomains might be more diverse group than originally thought. In addition, conserved Sh3BMs were found in the “non-conserved” C-terminus of nsP3 and were shown to be important for virus replication. In the chapters below the macrodomain and the determinants inside nsP3 HVR will be discussed in the context of how they might benefit virus replication, and the functions eukaryotic macrodomains will be also considered.

5.1 Human and viral macrodomain proteins

Recent studies have shown that human macrodomain proteins participate in transcriptional regulation in a PARylation dependent manner, and it has been postulated that PARylation is an epigenetic flag that regulates gene expression in a similar manner compared to methylation or acylation (2, 64, 144, 169). PAR induced chromatin remodelling functions have been confirmed for macrohistone mH2A1, and DNA helicase Alc1 (2, 64, 169). Also MDO1 has been shown to be recruited by chromatin PARylation and indicated in the transcriptional regulation of estrogen receptor (70, 169). Nevertheless, the cellular functions of many macrodomain proteins remain unknown, although most of them bind PAR *in vitro* (II), (1, 81, 88, 169).

It will be interesting to see whether some human macrodomains have other functions than regulation of nuclear chromatin. One interesting case is MDO1, which contains a mitochondrial targeting sequence in its N-terminus (II). As shown in this work, MDO1 is targeted to mitochondria when translated as a full length protein. This would indicate a mitochondrial function for MDO1 (II). Recent evidence suggests that a fraction of PARP-1 is localized to mitochondria and participates in the maintenance of mitochondrial DNA (141). In addition, PAR degrading enzymes have been shown to function in mitochondria, indicating mitochondrial PAR metabolism independent of nuclear PARylation (113, 119, 184). Thus it could be speculated that MDO1 would be recruited to mitochondria upon mitochondrial stress, in a similar way compared to recruitment of macrodomain proteins by PARylated nuclear chromatin.

PARP-1 is the main enzyme producing PAR in mammalian cells (154). For a long time it was thought to be the only enzyme responsible for PARylation in eukaryotic cells (154). Later, altogether 17 PARPs have been recognized in different compartments of the cell (73, 120). These PARylating proteins have been detected in telomere maintenance and in cytoplasmic vault structures as well as in mitochondria (7, 141). Thus it seems likely that many undiscovered functions exist for macrodomain proteins in connection with different PARPs. It should also be noted that despite the current nomenclature, some PARPs like PARP-10 and PARP-14 catalyze mono-ADP-

ribosylation rather than PARylation (84). In addition, several mono-ADPR-transferases exist in eukaryotes, and there is growing evidence for the physiological importance of mono-ADP-ribosylation of eukaryotic proteins (14, 31, 38, 73). This poses a question whether human macrodomains would function also in mono-ADPR binding. Many macrodomains, including proteins from human origin, have been shown to bind mono-ADPR *in vitro* (**I**, **II**), (88, 105, 121). Bacteria contain also macrodomains, and bacterial proteins are known to be modified by mono-ADP-ribosylation, but not by PARylation (27, 43). This is a strong indication that in addition to PAR, monomeric ADPR is a binding partner for some macrodomain proteins. Furthermore, when an archeal macrodomain was artificially expressed in mammalian cells, it was able to pull down mono-ADP-ribosylated proteins (32). Although currently there is no knowledge of the function of eukaryotic macrodomains in binding of mono-ADP-ribosylated proteins, macrodomain interactions with mono-ADP-ribosylated proteins is a promising direction for future research.

Based on the current model, as evidenced by the SARS-CoV macrodomain structure analysed in this work, macrodomains bind PAR in a capping manner, and can only interact with the last ADPR unit in the chain (**I**), (169). Since both ADPR and PAR seem to be bound to macrodomains in a similar manner mediated by the conserved amino acids in the ligand binding pocket, it will be interesting to see how the binding specificity towards PAR or ADPR is regulated in macrodomains. *In vitro* both ADPR and PAR are bound by the same macrodomain proteins, but it is not known whether this happens also *in vivo* (**I**, **II**). It is possible that some amino acids in macrodomain surface participate in the binding of PAR chain and thus could differentiate the PAR binding macrodomains from ADPR binding macrodomains. The binding partners of macrodomain proteins may also be regulated by multi-protein interactions in macrodomain containing complex, and recognition sites in the PARylated protein might be involved in the interaction. Altogether, more data is needed for the determination of the binding specificities of macrodomains.

In this work I have shown that some macrodomains can bind also poly(A) (**III**). This finding was confirmed by our collaborators in another study, where CHIKV macrodomain-triadenine complexes were examined by X-ray crystallography (105). It was shown that adenine rich RNA binds to the same site as the adenine moiety of ADPR, but also interacts with residues outside of the pocket (105). As indicated by the results of my work, different determinants seem to be needed for poly(A) and ADPR binding, evidenced by proteins such as GDAP2 that bind poly(A) but not ADPR (**III**). This implies that poly(A) binding is a specific feature of certain macrodomains. Interestingly, two new macrodomains with no sequence similarity with “traditional” macrodomains were recently discovered in SARS-CoV (22, 166). These macrodomains do not have a conserved ligand binding pocket, but they bind RNA through a loop structure in a distinct site (166). These findings indicate that the macrodomain family might be more diversified than thought before, and different lineages might be found sharing a similar fold but different substrate binding determinants.

Macrodomain function is important but not obligatory for alphavirus replication (Fig 7, Table 3), (122). Although none of the tested mutations conferred total lethality, macrodomain function seems to be beneficial for virus replication, since single point

mutations in the binding pocket restrained viral replication (this work; 4.1.5). It is interesting to speculate whether the functions of viral macrodomains are related to PAR/ADPR binding, poly(A) binding or whether they both could be relevant substrates. It could be hypothesized that poly(A) binding would have a function in viral RNA replication, since the viral RNA from alphaviruses, coronaviruses and HEV is polyadenylated. PAR binding mediated by viral macrodomains could be related to recruiting of PARylated host proteins for replication purposes, or it could aim to sequester PARylated proteins in a similar way that will be discussed for amphiphysin below. Various host proteins have been shown to bind to nsP3 containing protein complexes and some of these proteins, such as vimentin and 14-3-3 are known to be ADP-ribosylated (25, 32, 148). Whether macrodomain plays a role in binding of these host proteins will be a topic for a further study.

Based on the differences between macrodomains studied in this work, it seems that in contrast to our initial hypothesis, viral macrodomains do not have the same properties as a certain host cell macrodomain. None of the eukaryotic macrodomains analysed in this work has the same functional characteristics as the viral macrodomains; high affinity towards PAR and poly(A), and low affinity for ADPR (II), and others have functions associated with nuclear chromatin (2, 64, 169). Furthermore, from a structural point of view, viral macrodomains are not related to each other, and have been most likely acquired by different virus groups independently during their evolution (22, 32, 105). Since viral macrodomain functions have been obtained independent of each other, they might originate from different eukaryotic proteins and are likely to have diverse binding partners. Macrodomains with a conserved binding pocket may bind to different ADP-ribosylated target proteins and the non-conserved macrodomain proteins SUD-M and SUD-N from SARS coronavirus are very likely to have a different substrate.

5.2 Multifunctional nsP3 HVR

Earlier, it was thought that natively disordered protein stretches would act mainly as domain linkers, without any function of their own (41). In this context, it would be difficult to understand, why such a large unstructured sequence as alphavirus nsP3 HVR would be conserved throughout virus generations. However, quite recently people have started to realize that these disordered regions often carry important signalling and interaction motifs. Various cellular proteins recognize their interaction partners based on short linear motifs of 4-8 amino acids in length that are present in unstructured regions (52). Many eukaryotic networking proteins, which interact with multiple proteins, have long segments of intrinsic disorder, and this probably allows them to interact more flexibly with various partners (71). Through these proteins having regulatory short motifs many important cellular functions involving actin dynamics, endocytosis and exocytosis are regulated. Examples of proteins binding to their short linear motifs include Src kinases, amphiphysins, endosomal sorting complex required for transport (ESCRT) components and many others (40).

Large natively disordered protein segments are a typical for many viral proteins. One example is human immune deficiency virus (HIV) Gag protein that has been

shown to possess functional short linear motifs including “the late domains” for ESCRT binding (54, 131). Other viruses have been shown to contain short motifs for binding host proteins such as clathrin, dynein, cyclins and caspases (16, 74, 101, 165). Most likely, the currently recognized linear motifs in viral proteins are just the tip of an iceberg, and many more are to be found now that tools for linear motif search are available (33, 134). Hijacking host pathways through short linear motifs may be a very prominent tool for viruses, since usually only one or two mutations would be necessary for the formation of a novel motif. The mutation frequency of RNA viruses is 10^{-6} – 10^{-4} mutations per position per generation, and thus the appearance of a new motif can be a very rapid process (147). However, although in some cases a short linear motif alone is enough for functional protein-protein interaction, it should be kept in mind that often intrinsically disordered proteins do obtain a secondary structure upon ligand binding and regions other than the short linear motif are needed for folding and tight binding (41).

Alphavirus HVR is an unstructured protein region that tolerates large deletions (15, 53, 90). However, even though the mutants with deletions in HVR replicate in cell culture, they often show reduced pathogenicity in animals (53, 178). Consequently, it seems likely that host-interaction factors are located in alphavirus HVR. Based on sequence data, there are short motifs in HVR that are conserved in all the alphaviruses, although there is some variation in the localization and number of certain motifs. In this thesis one of the motifs in HVR, namely the Sh3BM, has been characterized, and we have shown that phosphorylation region in nsP3 HVR is responsible for endosomal localization of the replication complex.

5.2.1 *Replication complex internalization mediated by nsP3 HVR*

The work presented in this thesis confirms that nsP3 alone without accompanying nsP2 is sufficient to direct the membrane bound polyprotein to endosomal compartment, and that a region inside HVR is responsible for the targeting. I have shown that there is not a unique phosphorylation site in nsP3 that would drive the internalization of replication complexes, but rather phosphorylation of nsP3 in general is needed. Interestingly, SFV mutant (Δ 26 4S-4A) that has earlier been shown to lack any recognizable phosphorylation (180) was not able to stop internalization, in contrast to unphosphorylated SFV Δ 50. However, the internalization of Δ 26 4S-4A replication complexes was delayed compared to the wt. This could be explained by the hypothesis, supported by unpublished data from our group, that in this mutant virus a low level of phosphorylation is remaining, but it falls below the sensitivity of the labelling assay used in the early phosphorylation study (179, 180). This remaining phosphorylation could serve as a basis for internalization although with lower efficiency than the more prominent phosphorylation in wt virus.

It is not known, which cellular kinase is responsible for phosphorylation of HVR, but *in silico* searches for kinase binding motifs might provide a hint for host proteins associated with this process. Practically all alphaviruses contain an FxxF motif, in one or two repeats, close to the nsP3 C-terminus. In VEEV, a 10 amino acid region “VITREEFEAF” that contains the FxxF motif, has been shown to be the only region (other than the 3/4 cleavage site) in HVR intolerant to amino acid insertions (15). This is a strong indication for functional motif at this location. Interestingly, a 30

aa C-terminal deletion in SFV nsP3, that removed the FxxF motifs, was shown to disrupt the endosomal localization of an uncleavable polyprotein P12^{CA3} (174). Thus it could be hypothesized that the FxxF motif is needed for the endosomal targeting of alphavirus replication complexes.

In eukaryotic cells a C-terminal hydrophobic FxxF motif is found exclusively in the targets of 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (18). PDK-1 is a serine/threonine kinase responsible for phosphorylation of several downstream kinases of phosphoinositide 3-kinase (PI3K) signalling pathway, most notably the central effector Akt, and the efficiency of phosphorylation often depend on the FxxF sequence present in the substrate (17). PDK-1 binds through its pleckstrin homology domain to phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-triphosphate (PIP₃), created by PI3K. The activity of PDK-1 is strictly regulated by PI3K that is needed for creating critical threshold of PIP₂/ PIP₃ (26). Interestingly, PI3K has been recently shown to be crucial for SFV replication complex internalization, and there are strong indications that the internalization is caveolin dependent (10, 157). Furthermore, PI3K and PDK-1 have been shown to associate with caveolae in several cell lines including fibroblasts, the primary target cells of alphavirus infection (ref. (153) and references therein). Consequently as a serine/threonine kinase that is intimately linked to PI3K pathway, PDK-1 is a promising candidate for a kinase participating in nsP3 phosphorylation.

An interesting exception in the endosomal localization of alphavirus replication complexes was found in this work and by others (III), (49). The replication complexes of SINV are detected predominantly at the plasma membrane throughout infection in mammalian cell culture (HeLa, Neuro2A, BHK and 3T3 cells) even though SINV is phosphorylated in nsP3 and contains intact FxxF motif (III), (49). However, SINV replication complexes localize to CPVs in C₇10 insect cells (49), which shows that the basic determinants needed for replication complex internalization are intact, but only some factor needed in mammalian cells is missing. Currently, this is the only case indicating that alphaviruses could maintain their whole replication cycle on the plasma membrane, and more studies are needed in order to determine whether some alphaviruses do not form CPVs in their natural mammalian hosts, or whether this is a feature seen only with a specific virus strain or cell lines.

5.2.2 Functions of amphiphysin in alphavirus replication

In this work I have shown that amphiphysin-1 and amphiphysin-2 colocalize with the forming replication complexes early in infection and that association between amphiphysin-2 and replication complexes is maintained throughout the infection. Furthermore, it was proven that amphiphysins are bound to viral replication complexes through direct interaction with a specific motif in nsP3 HVR (III).

Amphiphysins are membrane binding proteins that contain a banana shaped membrane bending BAR (Bin/Amphiphysin/Rvsp) domain. There are two amphiphysin genes in the human genome: Amphiphysin-1 and Bin1/Amphiphysin-2 (133). Amphiphysin-1 is expressed almost solely in neurons, whereas amphiphysin-2 is ubiquitously expressed and spliced into more than 10 isoforms (133). All amphiphysins have an N-terminal N-BAR domain and a C-terminal SH3 domain. The N-BAR domain contains in addition to BAR an N-terminal amphipathic helix that

binds lipid membranes and is thought to make N-BAR capable of inducing membrane curvature (34). Amphiphysin-1 has also a clathrin binding domain that is needed for binding of clathrin containing vesicle buds at the plasma membrane, and its functions are thought to be related with neuronal endocytosis (186). Amphiphysins bind through their SH3 domains proteins such as c-myc and dynamin, and SH3 domain mediated interactions are required for regulation of amphiphysin functions (44, 130, 133, 143, 185).

SH3 domain mediated interactions have been detected for few other viral proteins, including HIV Nef and Kaposi's sarcoma-associated herpesvirus' K15, which bind Src-family kinases (92, 129, 171). Amphiphysin-2 binding has been shown for HCV non-structural protein 5A (117, 187). Many viruses are known to modulate host cell signalling pathways through binding host regulatory proteins in order to create a more beneficial environment for viral replication (79). Interestingly, positive strand RNA virus HEV, distantly related to alphaviruses, has been shown to bind Cin85 (Cbl interacting protein of 85 kDa), another SH3 domain protein that was detected also in our screen, through its protein ORF3 (open reading frame 3) (21). HEV pORF3 localizes to recycling endosomes and delays the trafficking and degradation of epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (c-Met) (21). It is possible that alphavirus nsP3 functions in a similar manner as it segregates amphiphysin and other SH3 domain proteins including Cin85 into the replication complexes and nsP3 induced aggregates. This could help the virus to escape the host immune defences, by down scaling the signalling cascades needed for immune reaction. This could also explain why it would be beneficial for the virus to have stable nsP3 protein complexes aggregated in the cytoplasm, since these structures were detected to bind large amount of amphiphysin.

Although amphiphysin-1 has been indicated in endocytosis, current data doesn't support a similar role for amphiphysins in replication complex intake. First of all, amphiphysin-1 is expressed almost only in neuronal cells, but replication complexes are effectively internalized also in non-neuronal cells (III), (133). In contrast, amphiphysin-2 doesn't function in endocytosis, but it may rather regulate recycling of cargo for exocytosis (115). Secondly, there was only a mild delay in CPV formation by Sh3BM_d viruses, but no accumulation of replication complexes detected on the plasma membrane, as would be expected if amphiphysin binding by viral proteins would be crucial for internalization (III). This kind of accumulation at the plasma membrane has been seen with internalization deficient SFV Δ50 mutant (Fig. 8B) as well as with P13K inhibitors (157).

Since amphiphysin binding has a considerable effect on viral RNA replication, it is tempting to speculate that amphiphysins might have a role in replication complex formation. As amphiphysins are membrane bending proteins, their role could be related to spherule formation. The BAR domain of amphiphysins can sense and induce membrane curvature with an outer radius of 11–15 nm (34). This could correspond to the curvature in the spherule neck. There are two sites in the spherule neck where the curvature could be favourable for amphiphysin binding (Fig.12). The curvature around the spherule neck is physically analogous to the binding site of amphiphysin-1 in endocytic vesicles, but it is difficult to imagine how amphiphysin could be located to this site, since spherules are formed in opposite direction compared to endocytic

vesicles, and are initially protruding outward from the plasma membrane (Fig. 2; fig.12). Another site where membrane curvature might be favourable for BAR domain is in the very beginning of the neck where positive curvature is formed on the inner side of the spherule (yellow dotted line in Fig. 12).

The defect in recruitment of amphiphysin for spherule formation could explain the impaired RNA replication of amphiphysin binding deficient mutants. However, the binding deficient mutant viruses were viable, although replicating slowly and presented a weak colocalization between replication sites and amphiphysin (III). Thus it is possible that other factors in addition to nsP3 can drive the recruitment of amphiphysin. These factors could include other nsPs, viral RNA or host proteins that bind amphiphysin in a weak association that is not maintained in immunoprecipitation. It is possible that these indirect interactions would be able to recruit enough amphiphysin for a lower level of spherule formation. It is under a debate whether the function of amphiphysin in membrane morphogenesis is active induction of membrane bending or mere stabilization of preformed membrane curvature (67). If amphiphysin is not needed for induction of membrane curvature in spherule formation, BAR domains may be recruited to spherules independent of nsP3 binding, based on their affinity for extreme membrane curvature. However, amphiphysin binding through nsP3 would create a local concentration of amphiphysin and would thus promote formation of stable spherule structures. Another possibility is that amphiphysin is not directly needed replication complex formation, but the interaction between amphiphysin and viral replication is more complex and may include factors like reorganization of host cell pathways.

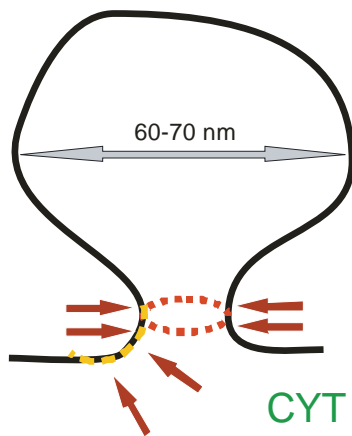


Figure 12. Membrane curvature in alphavirus spherules. BAR domains sense positive curvature and preferably bind to sites of steep extreme membrane curvature with outer radius of 7-15 nm. In alphavirus spherule there are two sites with a curvature close to this radius. The spherule neck has been estimated to have radius of ~19-20 nm (depicted with red dotted line) (51). Steep positive curvature is also formed at site where spherule membrane protrudes outward from the cytoplasm (yellow dotted line). Potential BAR domain binding sites are depicted with red arrows. Cytoplasmic side is marked with "CYT".

In future, it would be interesting to examine the importance of amphiphysin binding through nsP3 in animal studies, to assess whether amphiphysin binding has a role in fighting the host immune defences and facilitating the viral pathogenesis. The role of amphiphysin-2 in viral RNA replication could be studied in more detail also by electron microscopy analysis of spherule formation to detect whether there are structural defects in spherules formed by amphiphysin binding incompetent viruses. Knockout/knockdown studies could be used to analyse whether amphiphysin has a

direct role in RNA replication or whether replication defects are secondary effects resulting from reduced inhibition of host cell immune signalling. If amphiphysin-2 is needed for RNA replication, alphaviruses should not be capable of replicating in knockout cells, whereas if amphiphysin-2 recruitment is targeted to reduce host immune defence, knockout might be even beneficial to viruses and should not impair virus replication.

References

1. **Aguiar, R. C., K. Takeyama, C. He, K. Kreinbrink, and M. A. Shipp.** 2005. B-aggressive lymphoma family proteins have unique domains that modulate transcription and exhibit poly(ADP-ribose) polymerase activity. *J. Biol. Chem.* **280**:33756-33765.
2. **Ahel, D., Z. Horejsi, N. Wiechens, S. E. Polo, E. Garcia-Wilson, I. Ahel, H. Flynn, M. Skehel, S. C. West, S. P. Jackson, T. Owen-Hughes, and S. J. Boulton.** 2009. Poly(ADP-ribose)-dependent regulation of DNA repair by the chromatin remodeling enzyme ALC1. *Science.* **325**:1240-1243.
3. **Ahola, T., and L. Kääriäinen.** 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.
4. **Ahola, T., P. Kujala, M. Tuittila, T. Blom, P. Laakkonen, A. Hinkkanen, and P. Auvinen.** 2000. Effects of palmitoylation of replicase protein nsP1 on alphavirus infection. *J. Virol.* **74**:6725-6733.
5. **Ahola, T., A. Lampio, P. Auvinen, and L. Kääriäinen.** 1999. Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. *EMBO J.* **18**:3164-3172.
6. **Allen, M. D., A. M. Buckle, S. C. Cordell, J. Lowe, and M. Bycroft.** 2003. The crystal structure of AF1521 a protein from *Archaeoglobus fulgidus* with homology to the non-histone domain of macroH2A. *J. Mol. Biol.* **330**:503-511.
7. **Ame, J. C., C. Spenlehauer, and G. de Murcia.** 2004. The PARP superfamily. *Bioessays.* **26**:882-893.
8. **Andeweg, A. C., B. L. Haagmans, and A. D. Osterhaus.** 2008. Virogenomics: the virus-host interaction revisited. *Curr. Opin. Microbiol.* **11**:461-466.
9. **Antoine, A. F., C. Montpellier, K. Cailliau, E. Browaey-Poly, J. P. Vilain, and J. Dubuisson.** 2007. The alphavirus 6K protein activates endogenous ionic conductances when expressed in *Xenopus* oocytes. *J. Membr. Biol.* **215**:37-48. doi: 10.1007/s00232-007-9003-6.
10. **Balistreri, G.** 2010. Structure, function and intracellular dynamics of alphavirus replication complexes. PhD Thesis. University of Helsinki, Helsinki.
11. **Barajas, D., Y. Jiang, and P. D. Nagy.** 2009. A unique role for the host ESCRT proteins in replication of Tomato bushy stunt virus. *PLoS Pathog.* **5**:e1000705.
12. **Barajas, D., and P. D. Nagy.** 2010. Ubiquitination of tombusvirus p33 replication protein plays a role in virus replication and binding to the host Vps23p ESCRT protein. *Virology.* **397**:358-368.

13. **Barrett, A. J., and N. D. Rawlings.** 2001. Evolutionary lines of cysteine peptidases. *Biol. Chem.* **382**:727-733.
14. **Bartz, R., J. Seemann, J. K. Zehmer, G. Serrero, K. D. Chapman, R. G. Anderson, and P. Liu.** 2007. Evidence that mono-ADP-ribosylation of CtBP1/BARS regulates lipid storage. *Mol. Biol. Cell.* **18**:3015-3025.
15. **Beitzel, B. F., R. R. Bakken, J. M. Smith, and C. S. Schmaljohn.** 2010. High-resolution functional mapping of the venezuelan equine encephalitis virus genome by insertional mutagenesis and massively parallel sequencing. *PLoS Pathog.* **6**:e1001146.
16. **Best, S. M., J. F. Shelton, J. M. Pompey, J. B. Wolfenbarger, and M. E. Bloom.** 2003. Caspase cleavage of the nonstructural protein NS1 mediates replication of Aleutian mink disease parvovirus. *J. Virol.* **77**:5305-5312.
17. **Biondi, R. M., P. C. Cheung, A. Casamayor, M. Deak, R. A. Currie, and D. R. Alessi.** 2000. Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *EMBO J.* **19**:979-988.
18. **Biondi, R. M., and A. R. Nebreda.** 2003. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem. J.* **372**:1-13.
19. **Bradley, W. E., J. V. Raelson, D. Y. Dubois, E. Godin, H. Fournier, C. Prive, R. Allard, V. Pinchuk, M. Lapalme, R. J. Paulussen, and A. Belouchi.** 2010. Hotspots of large rare deletions in the human genome. *PLoS One.* **5**:e9401.
20. **Buchholz, U. J., S. Finke, and K. K. Conzelmann.** 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J. Virol.* **73**:251-259.
21. **Chandra, V., M. Kalia, K. Hajela, and S. Jameel.** 2010. The ORF3 protein of hepatitis E virus delays degradation of activated growth factor receptors by interacting with CIN85 and blocking formation of the Cbl-CIN85 complex. *J. Virol.* **84**:3857-3867.
22. **Chatterjee, A., M. A. Johnson, P. Serrano, B. Pedrini, J. S. Joseph, B. W. Neuman, K. Saikatendu, M. J. Buchmeier, P. Kuhn, and K. Wuthrich.** 2009. Nuclear magnetic resonance structure shows that the severe acute respiratory syndrome coronavirus-unique domain contains a macrodomain fold. *J. Virol.* **83**:1823-1836.
23. **Cho, S. H., S. Goenka, T. Henttinen, P. Gudapati, A. Reinikainen, C. M. Eischen, R. Lahesmaa, and M. Boothby.** 2009. PARP-14, a member of the B aggressive lymphoma family, transduces survival signals in primary B cells. *Blood.* **113**:2416-2425.
24. **Claros, M. G., and P. Vincens.** 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem.* **241**:779-786.

25. **Coburn, J., S. T. Dillon, B. H. Iglewski, and D. M. Gill.** 1989. Exoenzyme S of *Pseudomonas aeruginosa* ADP-ribosylates the intermediate filament protein vimentin. *Infect. Immun.* **57**:996-998.
26. **Cohen, P.** 2000. The regulation of protein function by multisite phosphorylation-- a 25 year update. *Trends Biochem. Sci.* **25**:596-601.
27. **Corda, D., and M. Di Girolamo.** 2003. Functional aspects of protein mono-ADP-ribosylation. *EMBO J.* **22**:1953-1958.
28. **Costanzi, C., and J. R. Pehrson.** 2001. MACROH2A2, a new member of the MARCOH2A core histone family. *J. Biol. Chem.* **276**:21776-21784.
29. **Cristea, I. M., J. W. Carroll, M. P. Rout, C. M. Rice, B. T. Chait, and M. R. MacDonald.** 2006. Tracking and elucidating alphavirus-host protein interactions. *J. Biol. Chem.* **281**:30269-30278.
30. **Cristea, I. M., H. Rozjabeck, K. R. Molloy, S. Karki, L. L. White, C. M. Rice, M. P. Rout, B. T. Chait, and M. R. MacDonald.** 2010. Host factors associated with the Sindbis virus RNA-dependent RNA polymerase: role for G3BP1 and G3BP2 in virus replication. *J. Virol.* **84**:6720-6732.
31. **Dani, N., E. Mayo, A. Stilla, A. Marchegiani, S. Di Paola, D. Corda, and M. Di Girolamo.** 2010. Mono-ADP-ribosylation of the G-protein $\beta\gamma$ dimer is modulated by hormones and inhibited by ARF6. *J. Biol. Chem.*
32. **Dani, N., A. Stilla, A. Marchegiani, A. Tamburro, S. Till, A. G. Ladurner, D. Corda, and M. Di Girolamo.** 2009. Combining affinity purification by ADP-ribose-binding macro domains with mass spectrometry to define the mammalian ADP-ribosyl proteome. *Proc. Natl. Acad. Sci. U. S. A.* **106**:4243-4248.
33. **Davey, N. E., N. J. Haslam, D. C. Shields, and R. J. Edwards.** 2010. SLiMfinder: a web server to find novel, significantly over-represented, short protein motifs *Nucleic Acids Res.* **38 Suppl**:W534-9.
34. **Dawson, J. C., J. A. Legg, and L. M. Machesky.** 2006. Bar domain proteins: a role in tubulation, scission and actin assembly in clathrin-mediated endocytosis *Trends Cell Biol.* **16**:493-498.
35. **de Groot, R. J., T. Rumenapf, R. J. Kuhn, E. G. Strauss, and J. H. Strauss.** 1991. Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc. Natl. Acad. Sci. U. S. A.* **88**:8967-8971.
36. **De, I., C. Fata-Hartley, S. G. Sawicki, and D. L. Sawicki.** 2003. Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. *J. Virol.* **77**:13106-13116.
37. **De, I., S. G. Sawicki, and D. L. Sawicki.** 1996. Sindbis virus RNA-negative mutants that fail to convert from minus-strand to plus-strand synthesis: role of the nsP2 protein. *J. Virol.* **70**:2706-2719.

38. **Di Girolamo, M., N. Dani, A. Stilla, and D. Corda.** 2005. Physiological relevance of the endogenous mono(ADP-ribosyl)ation of cellular proteins. *FEBS J.* **272**:4565-4575.
39. **Diaz, A., X. Wang, and P. Ahlquist.** 2010. Membrane-shaping host reticulin proteins play crucial roles in viral RNA replication compartment formation and function. *Proc. Natl. Acad. Sci. U. S. A.* **107**:16291-16296.
40. **Diella, F., N. Haslam, C. Chica, A. Budd, S. Michael, N. P. Brown, G. Trave, and T. J. Gibson.** 2008. Understanding eukaryotic linear motifs and their role in cell signaling and regulation *Front. Biosci.* **13**:6580-6603.
41. **Dyson, H. J., and P. E. Wright.** 2005. Intrinsically unstructured proteins and their functions *Nat. Rev. Mol. Cell Biol.* **6**:197-208.
42. **Egloff, M. P., H. Malet, A. Putics, M. Heinonen, H. Dutartre, A. Frangeul, A. Gruez, V. Campanacci, C. Cambillau, J. Ziebuhr, T. Ahola, and B. Canard.** 2006. Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. *J. Virol.* **80**:8493-8502.
43. **Faraone-Mennella, M. R., A. De Maio, A. Petrella, M. Romano, P. Favaloro, A. Gambacorta, L. Lama, B. Nicolaus, and B. Farina.** 2006. The (ADP-ribosyl)ation reaction in thermophilic bacteria. *Res. Microbiol.* **157**:531-537.
44. **Farsad, K., V. Slepnev, G. Ochoa, L. Daniell, V. Haucke, and P. De Camilli.** 2003. A putative role for intramolecular regulatory mechanisms in the adaptor function of amphiphysin in endocytosis *Neuropharmacology.* **45**:787-796.
45. **Fazakerley, J. K., A. Boyd, M. L. Mikkola, and L. Kääriäinen.** 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.
46. **Firth, A. E., B. Y. Chung, M. N. Fleeton, and J. F. Atkins.** 2008. Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. *Virol. J.* **5**:108.
47. **Friedman, R. M., J. G. Levin, P. M. Grimley, and I. K. Berezsky.** 1972. Membrane-associated replication complex in arbovirus infection. *J. Virol.* **10**:504-515.
48. **Frolova, E., R. Gorchakov, N. Garmashova, S. Atasheva, L. A. Vergara, and I. Frolov.** 2006. Formation of nsP3-specific protein complexes during Sindbis virus replication. *J. Virol.* **80**:4122-4134.
49. **Frolova, E. I., R. Gorchakov, L. Pereboeva, S. Atasheva, and I. Frolov.** 2010. Functional Sindbis virus replicative complexes are formed at the plasma membrane. *J. Virol.* **84**:11679-11695.
50. **Fros, J. J., W. J. Liu, N. A. Prow, C. Geertsema, M. Ligtenberg, D. L. Vanlandingham, E. Schnettler, J. M. Vlak, A. Suhrbier, A. A. Khromykh, and G. P. Pijlman.** 2010. Chikungunya virus nonstructural protein 2 inhibits type I/II interferon-stimulated JAK-STAT signaling. *J. Virol.* **84**:10877-10887.

51. **Froshauer, S., J. Kartenbeck, and A. Helenius.** 1988. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J. Cell Biol.* **107**:2075-2086.
52. **Fuxreiter, M., P. Tompa, and I. Simon.** 2007. Local structural disorder imparts plasticity on linear motifs *Bioinformatics.* **23**:950-956.
53. **Galbraith, S. E., B. J. Sheahan, and G. J. Atkins.** 2006. Deletions in the hypervariable domain of the nsP3 gene attenuate Semliki Forest virus virulence. *J. Gen. Virol.* **87**:937-947.
54. **Gamble, T. R., S. Yoo, F. F. Vajdos, U. K. Von Schwedler, D. K. Worthylake, H. Wang, J. P. McCutcheon, W. I. Sundquist, and C. P. Hill.** 1997. Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. *Science.* **278**:849-853.
55. **Garmashova, N., R. Gorchakov, E. Frolova, and I. Frolov.** 2006. Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J. Virol.* **80**:5686-5696.
56. **Garmashova, N., R. Gorchakov, E. Volkova, S. Paessler, E. Frolova, and I. Frolov.** 2007. The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J. Virol.* **81**:2472-2484.
57. **Garoff, H., M. Sjoberg, and R. H. Cheng.** 2004. Budding of alphaviruses. *Virus Res.* **106**:103-116.
58. **Georgel, P., C. Schuster, M. B. Zeisel, F. Stoll-Keller, T. Berg, S. Bahram, and T. F. Baumert.** 2010. Virus-host interactions in hepatitis C virus infection: implications for molecular pathogenesis and antiviral strategies. *Trends Mol. Med.* **16**:277-286.
59. **Golubtsov, A.** 2008. Mechanisms for Alphavirus Nonstructural polyprotein processing. PhD Thesis. University of Helsinki, Helsinki.
60. **Golubtsov, A., L. Kääriäinen, and J. Caldentey.** 2006. Characterization of the cysteine protease domain of Semliki Forest virus replicase protein nsP2 by in vitro mutagenesis. *FEBS Lett.* **580**:1502-1508.
61. **Gomez de Cedron, M., N. Ehsani, M. L. Mikkola, J. A. Garcia, and L. Kääriäinen.** 1999. RNA helicase activity of Semliki Forest virus replicase protein NSP2. *FEBS Lett.* **448**:19-22.
62. **Gorbalenya, A. E., E. V. Koonin, and M. M. Lai.** 1991. Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses. *FEBS Lett.* **288**:201-205.

63. **Gorchakov, R., N. Garmashova, E. Frolova, and I. Frolov.** 2008. Different types of nsP3-containing protein complexes in Sindbis virus-infected cells. *J. Virol.* **82**:10088-10101.
64. **Gottschalk, A. J., G. Timinszky, S. E. Kong, J. Jin, Y. Cai, S. K. Swanson, M. P. Washburn, L. Florens, A. G. Ladurner, J. W. Conaway, and R. C. Conaway.** 2009. Poly(ADP-ribosylation) directs recruitment and activation of an ATP-dependent chromatin remodeler. *Proc. Natl. Acad. Sci. U. S. A.* **106**:13770-13774.
65. **Grimley, P. M., I. K. Berezsky, and R. M. Friedman.** 1968. Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. *J. Virol.* **2**:1326-1338.
66. **Ha, D. Q., C. H. Calisher, P. H. Tien, N. Karabatsos, and D. J. Gubler.** 1995. Isolation of a newly recognized alphavirus from mosquitoes in Vietnam and evidence for human infection and disease. *Am. J. Trop. Med. Hyg.* **53**:100-104.
67. **Habermann, B.** 2004. The BAR-domain family of proteins: a case of bending and binding? *EMBO Rep.* **5**:250-255.
68. **Hahn, Y. S., E. G. Strauss, and J. H. Strauss.** 1989. Mapping of RNA-temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. *J. Virol.* **63**:3142-3150.
69. **Han, W. D., Y. M. Mu, X. C. Lu, Z. M. Xu, X. J. Li, L. Yu, H. J. Song, M. Li, J. M. Lu, Y. L. Zhao, and C. Y. Pan.** 2003. Up-regulation of LRP16 mRNA by 17beta-estradiol through activation of estrogen receptor alpha (ERalpha), but not ERbeta, and promotion of human breast cancer MCF-7 cell proliferation: a preliminary report. *Endocr. Relat. Cancer.* **10**:217-224.
70. **Han, W. D., Y. L. Zhao, Y. G. Meng, L. Zang, Z. Q. Wu, Q. Li, Y. L. Si, K. Huang, J. M. Ba, H. Morinaga, M. Nomura, and Y. M. Mu.** 2007. Estrogenically regulated LRP16 interacts with estrogen receptor alpha and enhances the receptor's transcriptional activity. *Endocr. Relat. Cancer.* **14**:741-753.
71. **Haynes, C., C. J. Oldfield, F. Ji, N. Klitgord, M. E. Cusick, P. Radivojac, V. N. Uversky, M. Vidal, and L. M. Iakoucheva.** 2006. Intrinsic disorder is a common feature of hub proteins from four eukaryotic interactomes *PLoS Comput. Biol.* **2**:e100.
72. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki forest virus into BHK-21 cells. *J. Cell Biol.* **84**:404-420.
73. **Hottiger, M. O., P. O. Hassa, B. Luscher, H. Schuler, and F. Koch-Nolte.** 2010. Toward a unified nomenclature for mammalian ADP-ribosyltransferases. *Trends Biochem. Sci.* **35**:208-219.
74. **Huang, C., S. C. Chang, I. C. Yu, Y. G. Tsay, and M. F. Chang.** 2007. Large hepatitis delta antigen is a novel clathrin adaptor-like protein *J. Virol.* **81**:5985-5994.

75. **Jain, E., A. Bairoch, S. Duvaud, I. Phan, N. Redaschi, B. E. Suzek, M. J. Martin, P. McGarvey, and E. Gasteiger.** 2009. Infrastructure for the life sciences: design and implementation of the UniProt website. *BMC Bioinformatics*. **10**:136.
76. **Johnson, M. A., A. Chatterjee, B. W. Neuman, and K. Wuthrich.** 2010. SARS coronavirus unique domain: three-domain molecular architecture in solution and RNA binding. *J. Mol. Biol.* **400**:724-742.
77. **Jose, J., J. E. Snyder, and R. J. Kuhn.** 2009. A structural and functional perspective of alphavirus replication and assembly. *Future Microbiol.* **4**:837-856.
78. **Kääriäinen, L., and T. Ahola.** 2002. Functions of alphavirus nonstructural proteins in RNA replication. *Prog. Nucleic Acid Res. Mol. Biol.* **71**:187-222.
79. **Kadaveru, K., J. Vyas, and M. R. Schiller.** 2008. Viral infection and human disease--insights from minimotifs. *Front. Biosci.* **13**:6455-6471.
80. **Kamer, G., and P. Argos.** 1984. Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res.* **12**:7269-7282.
81. **Karras, G. I., G. Kustatscher, H. R. Buhecha, M. D. Allen, C. Pugieux, F. Sait, M. Bycroft, and A. G. Ladurner.** 2005. The macro domain is an ADP-ribose binding module. *EMBO J.* **24**:1911-1920.
82. **Keränen, S., and L. Kääriäinen.** 1974. Isolation and basic characterization of temperature-sensitive mutants from Semliki Forest virus; *Acta Pathol. Microbiol. Scand. B. Microbiol. Immunol.* **82**:810-820.
83. **Kim, K. H., T. Rumenapf, E. G. Strauss, and J. H. Strauss.** 2004. Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. *Virology.* **323**:153-163.
84. **Kleine, H., E. Poreba, K. Lesniewicz, P. O. Hassa, M. O. Hottiger, D. W. Litchfield, B. H. Shilton, and B. Lüscher.** 2008. Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation. *Mol. Cell.* **32**:57-69.
85. **Klimstra, W. B., E. M. Nangle, M. S. Smith, A. D. Yurochko, and K. D. Ryman.** 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.
86. **Koonin, E. V., and V. V. Dolja.** 2006. Evolution of complexity in the viral world: the dawn of a new vision. *Virus Res.* **117**:1-4.
87. **Kujala, P., A. Ikäheimonen, N. Ehsani, H. Vihinen, P. Auvinen, and L. Kääriäinen.** 2001. Biogenesis of the Semliki Forest virus RNA replication complex. *J. Virol.* **75**:3873-3884.

88. **Kustatscher, G., M. Hothorn, C. Pugieux, K. Scheffzek, and A. G. Ladurner.** 2005. Splicing regulates NAD metabolite binding to histone macroH2A. *Nat. Struct. Mol. Biol.* **12**:624-625.
89. **Laakkonen, P., P. Auvinen, P. Kujala, and L. Kääriäinen.** 1998. Alphavirus replicase protein NSP1 induces filopodia and rearrangement of actin filaments. *J. Virol.* **72**:10265-10269.
90. **Lastarza, M. W., A. Grakoui, and C. M. Rice.** 1994. Deletion and duplication mutations in the C-terminal nonconserved region of Sindbis virus nsP3: effects on phosphorylation and on virus replication in vertebrate and invertebrate cells. *Virology.* **202**:224-232.
91. **LaStarza, M. W., J. A. Lemm, and C. M. Rice.** 1994. Genetic analysis of the nsP3 region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. *J. Virol.* **68**:5781-5791.
92. **Lee, C. H., K. Saksela, U. A. Mirza, B. T. Chait, and J. Kuriyan.** 1996. Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain *Cell.* **85**:931-942.
93. **Lemm, J. A., T. Rumenapf, E. G. Strauss, J. H. Strauss, and C. M. Rice.** 1994. Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. *EMBO J.* **13**:2925-2934.
94. **Letunic, I., T. Doerks, and P. Bork.** 2009. SMART 6: recent updates and new developments. *Nucleic Acids Res.* **37**:D229-32.
95. **Li, G., and C. M. Rice.** 1993. The signal for translational readthrough of a UGA codon in Sindbis virus RNA involves a single cytidine residue immediately downstream of the termination codon. *J. Virol.* **67**:5062-5067.
96. **Li, G. P., M. W. La Starza, W. R. Hardy, J. H. Strauss, and C. M. Rice.** 1990. Phosphorylation of Sindbis virus nsP3 in vivo and in vitro. *Virology.* **179**:416-427.
97. **Li, L., J. Jose, Y. Xiang, R. J. Kuhn, and M. G. Rossmann.** 2010. Structural changes of envelope proteins during alphavirus fusion. *Nature.* **468**:705-708.
98. **Liu, H., T. Nakagawa, T. Kanematsu, T. Uchida, and S. Tsuji.** 1999. Isolation of 10 differentially expressed cDNAs in differentiated Neuro2a cells induced through controlled expression of the GD3 synthase gene. *J. Neurochem.* **72**:1781-1790.
99. **Lundström, K.** 2003. Semliki Forest virus vectors for rapid and high-level expression of integral membrane proteins. *Biochim. Biophys. Acta.* **1610**:90-96.
100. **Lundström, K.** 2005. Biology and application of alphaviruses in gene therapy. *Gene Ther.* **12 Suppl 1**:S92-7.

101. **Ma, T., N. Zou, B. Y. Lin, L. T. Chow, and J. W. Harper.** 1999. Interaction between cyclin-dependent kinases and human papillomavirus replication-initiation protein E1 is required for efficient viral replication *Proc. Natl. Acad. Sci. U. S. A.* **96**:382-387.
102. **Maas, N. M., T. Van de Putte, C. Melotte, A. Francis, C. T. Schrandt-Stumpel, D. Sanlaville, D. Genevieve, S. Lyonnet, B. Dimitrov, K. Devriendt, J. P. Fryns, and J. R. Vermeesch.** 2007. The C20orf133 gene is disrupted in a patient with Kabuki syndrome. *J. Med. Genet.* **44**:562-569.
103. **Mackenzie, J. S.** 2005. Emerging zoonotic encephalitis viruses: lessons from Southeast Asia and Oceania. *J. Neurovirol.* **11**:434-440.
104. **Mai, J., S. G. Sawicki, and D. L. Sawicki.** 2009. Fate of minus-strand templates and replication complexes produced by a p23-cleavage-defective mutant of Sindbis virus. *J. Virol.* **83**:8553-8564.
105. **Malet, H., B. Coutard, S. Jamal, H. Dutartre, N. Papageorgiou, M. Neuvonen, T. Ahola, N. Forrester, E. A. Gould, D. Lafitte, F. Ferron, J. Lescar, A. E. Gorbalenya, X. de Lamballerie, and B. Canard.** 2009. The crystal structures of Chikungunya and Venezuelan equine encephalitis virus nsP3 macro domains define a conserved adenosine binding pocket. *J. Virol.* **83**:6534-6545.
106. **Malet, H., K. Dalle, N. Bremond, F. Tocque, S. Blangy, V. Campanacci, B. Coutard, S. Grisel, J. Lichiere, V. Lantez, C. Cambillau, B. Canard, and M. P. Egloff.** 2006. Expression, purification and crystallization of the SARS-CoV macro domain. *Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun.* **62**:405-408.
107. **Matthews, J. D., W. P. Tzeng, and T. K. Frey.** 2010. Analysis of the function of cytoplasmic fibers formed by the rubella virus nonstructural replicase proteins. *Virology.* **406**:212-227.
108. **Mayer, B. J.** 2001. SH3 domains: complexity in moderation. *J. Cell. Sci.* **114**:1253-1263.
109. **Mehrotra, P., J. P. Riley, R. Patel, F. Li, L. Voss, and S. Goenka.** 2010. PARP-14 functions as a transcriptional switch for STAT6 dependent gene activation. *J. Biol. Chem.*
110. **Melton, J. V., G. D. Ewart, R. C. Weir, P. G. Board, E. Lee, and P. W. Gage.** 2002. Alphavirus 6K proteins form ion channels. *J. Biol. Chem.* **277**:46923-46931. doi: 10.1074/jbc.M207847200.
111. **Meng, Y., Z. Wu, X. Yin, Y. Zhao, M. Chen, Y. Si, J. Yang, X. Fu, and W. Han.** 2009. Keratin 18 attenuates estrogen receptor alpha-mediated signaling by sequestering LRP16 in cytoplasm. *BMC Cell Biol.* **10**:96.
112. **Merits, A., L. Vasiljeva, T. Ahola, L. Kääriäinen, and P. Auvinen.** 2001. Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *J. Gen. Virol.* **82**:765-773.

113. **Meyer, R. G., M. L. Meyer-Ficca, C. J. Whatcott, E. L. Jacobson, and M. K. Jacobson.** 2007. Two small enzyme isoforms mediate mammalian mitochondrial poly(ADP-ribose) glycohydrolase (PARG) activity. *Exp. Cell Res.* **313**:2920-2936.
114. **Mi, S., and V. Stollar.** 1991. Expression of Sindbis virus nsP1 and methyltransferase activity in *Escherichia coli*. *Virology.* **184**:423-427.
115. **Muller, A. J., J. F. Baker, J. B. DuHadaway, K. Ge, G. Farmer, P. S. Donover, R. Meade, C. Reid, R. Grzanna, A. H. Roach, N. Shah, A. P. Soler, and G. C. Prendergast.** 2003. Targeted disruption of the murine Bin1/Amphiphysin II gene does not disable endocytosis but results in embryonic cardiomyopathy with aberrant myofibril formation. *Mol. Cell. Biol.* **23**:4295-4306.
116. **Nagy, P. D., and J. Pogany.** 2010. Global genomics and proteomics approaches to identify host factors as targets to induce resistance against Tomato bushy stunt virus. *Adv. Virus Res.* **76**:123-177.
117. **Nanda, S. K., D. Herion, and T. J. Liang.** 2006. The SH3 binding motif of HCV [corrected] NS5A protein interacts with Bin1 and is important for apoptosis and infectivity. *Gastroenterology.* **130**:794-809.
118. **Neuvonen, M., and T. Ahola.** 2009. Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites. *J. Mol. Biol.* **385**:212-225.
119. **Niere, M., S. Kernstock, F. Koch-Nolte, and M. Ziegler.** 2008. Functional localization of two poly(ADP-ribose)-degrading enzymes to the mitochondrial matrix. *Mol. Cell. Biol.* **28**:814-824.
120. **Otto, H., P. A. Reche, F. Bazan, K. Dittmar, F. Haag, and F. Koch-Nolte.** 2005. In silico characterization of the family of PARP-like poly(ADP-ribosyl)transferases (pARTs). *BMC Genomics.* **6**:139.
121. **Ouararhni, K., R. Hadj-Slimane, S. Ait-Si-Ali, P. Robin, F. Mietton, A. Harel-Bellan, S. Dimitrov, and A. Hamiche.** 2006. The histone variant mH2A1.1 interferes with transcription by down-regulating PARP-1 enzymatic activity. *Genes Dev.* **20**:3324-3336.
122. **Park, E., and D. E. Griffin.** 2009. The nsP3 macro domain is important for Sindbis virus replication in neurons and neurovirulence in mice. *Virology.* **388**:305-314.
123. **Pehrson, J. R., and V. A. Fried.** 1992. MacroH2A, a core histone containing a large nonhistone region. *Science.* **257**:1398-1400.
124. **Pehrson, J. R., and R. N. Fuji.** 1998. Evolutionary conservation of histone macroH2A subtypes and domains. *Nucleic Acids Res.* **26**:2837-2842.

125. **Peränen, J., P. Laakkonen, M. Hyvonen, and L. Kääriäinen.** 1995. The alphavirus replicase protein nsP1 is membrane-associated and has affinity to endocytic organelles. *Virology*. **208**:610-620.
126. **Peränen, J., M. Rikkonen, P. Liljeström, and L. Kääriäinen.** 1990. Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *J. Virol.* **64**:1888-1896.
127. **Peränen, J., K. Takkinen, N. Kalkkinen, and L. Kääriäinen.** 1988. Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *J. Gen. Virol.* **69 (Pt 9)**:2165-2178.
128. **Petterson, E., M. Sandberg, and N. Santi.** 2009. Salmonid alphavirus associated with *Lepeophtheirus salmonis* (Copepoda: Caligidae) from Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **32**:477-479.
129. **Pietrek, M., M. M. Brinkmann, I. Glowacka, A. Enlund, A. Havemeier, O. Dittrich-Breiholz, M. Kracht, M. Lewitzky, K. Saksela, S. M. Feller, and T. F. Schulz.** 2010. Role of the Kaposi's sarcoma-associated herpesvirus K15 SH3 binding site in inflammatory signaling and B-cell activation. *J. Virol.* **84**:8231-8240.
130. **Pineda-Lucena, A., C. S. Ho, D. Y. Mao, Y. Sheng, R. C. Laister, R. Muhandiram, Y. Lu, B. T. Seet, S. Katz, T. Szyperski, L. Z. Penn, and C. H. Arrowsmith.** 2005. A structure-based model of the c-Myc/Bin1 protein interaction shows alternative splicing of Bin1 and c-Myc phosphorylation are key binding determinants. *J. Mol. Biol.* **351**:182-194.
131. **Pornillos, O., D. S. Higginson, K. M. Stray, R. D. Fisher, J. E. Garrus, M. Payne, G. P. He, H. E. Wang, S. G. Morham, and W. I. Sundquist.** 2003. HIV Gag mimics the Tsg101-recruiting activity of the human Hrs protein. *J. Cell Biol.* **162**:425-434.
132. **Powers, A. M., A. C. Brault, Y. Shirako, E. G. Strauss, W. Kang, J. H. Strauss, and S. C. Weaver.** 2001. Evolutionary relationships and systematics of the alphaviruses. *J. Virol.* **75**:10118-10131.
133. **Prendergast, G. C., A. J. Muller, A. Ramalingam, and M. Y. Chang.** 2009. BAR the door: cancer suppression by amphiphysin-like genes. *Biochim. Biophys. Acta.* **1795**:25-36.
134. **Puntervoll, P., R. Linding, C. Gemund, S. Chabanis-Davidson, M. Mattingsdal, S. Cameron, D. M. Martin, G. Ausiello, B. Brannetti, A. Costantini, F. Ferre, V. Maselli, A. Via, G. Cesareni, F. Diella, G. Superti-Furga, L. Wyrwicz, C. Ramu, C. McGuigan, R. Gudavalli, I. Letunic, P. Bork, L. Rychlewski, B. Kuster, M. Helmer-Citterich, W. N. Hunter, R. Aasland, and T. J. Gibson.** 2003. ELM server: A new resource for investigating short functional sites in modular eukaryotic proteins. *Nucleic Acids Res.* **31**:3625-3630.

135. **Putics, A., W. Filipowicz, J. Hall, A. E. Gorbalenya, and J. Ziebuhr.** 2005. ADP-ribose-1"-monophosphatase: a conserved coronavirus enzyme that is dispensable for viral replication in tissue culture. *J. Virol.* **79**:12721-12731.
136. **Quetglas, J. I., M. Ruiz-Guillen, A. Aranda, E. Casales, J. Bezunartea, and C. Smerdou.** 2010. Alphavirus vectors for cancer therapy. *Virus Res.* **153**:179-196.
137. **Queyriaux, B., F. Simon, M. Grandadam, R. Michel, H. Tolou, and J. P. Boutin.** 2008. Clinical burden of chikungunya virus infection. *Lancet Infect. Dis.* **8**:2-3.
138. **Rezza, G., L. Nicoletti, R. Angelini, R. Romi, A. C. Finarelli, M. Panning, P. Cordioli, C. Fortuna, S. Boros, F. Magurano, G. Silvi, P. Angelini, M. Dottori, M. G. Ciufolini, G. C. Majori, A. Cassone, and CHIKV study group.** 2007. Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet.* **370**:1840-1846.
139. **Rikkonen, M., J. Peränen, and L. Kääriäinen.** 1994. ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *J. Virol.* **68**:5804-5810.
140. **Rodger, H., and S. Mitchell.** 2007. Epidemiological observations of pancreas disease of farmed Atlantic salmon, *Salmo salar* L., in Ireland. *J. Fish Dis.* **30**:157-167.
141. **Rossi, M. N., M. Carbone, C. Mostocotto, C. Mancone, M. Tripodi, R. Maione, and P. Amati.** 2009. Mitochondrial localization of PARP-1 requires interaction with mitofilin and is involved in the maintenance of mitochondrial DNA integrity. *J. Biol. Chem.* **284**:31616-31624.
142. **Rost, B., G. Yachdav, and J. Liu.** 2004. The PredictProtein server. *Nucleic Acids Res.* **32**:W321-6.
143. **Sakamuro, D., K. J. Elliott, R. Wechsler-Reya, and G. C. Prendergast.** 1996. BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. *Nat. Genet.* **14**:69-77.
144. **Sala, A., and D. F. Corona.** 2009. Poly-ADP-ribose (PAR) as an epigenetic flag. *Epigenetics.* **4**:287-290.
145. **Salonen, A., T. Ahola, and L. Kääriäinen.** 2005. Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* **285**:139-173.
146. **Salonen, A., L. Vasiljeva, A. Merits, J. Magden, E. Jokitalo, and L. Kääriäinen.** 2003. Properly folded nonstructural polyprotein directs the semliki forest virus replication complex to the endosomal compartment. *J. Virol.* **77**:1691-1702.
147. **Sanjuan, R., M. R. Nebot, N. Chirico, L. M. Mansky, and R. Belshaw.** 2010. Viral mutation rates. *J. Virol.* **84**:9733-9748.

148. **Satchell, M. A., X. Zhang, P. M. Kochanek, C. E. Dixon, L. W. Jenkins, J. Melick, C. Szabo, and R. S. Clark.** 2003. A dual role for poly-ADP-ribosylation in spatial memory acquisition after traumatic brain injury in mice involving NAD⁺ depletion and ribosylation of 14-3-3gamma. *J. Neurochem.* **85**:697-708.
149. **Sawicki, D. L., S. Perri, J. M. Polo, and S. G. Sawicki.** 2006. Role for nsP2 proteins in the cessation of alphavirus minus-strand synthesis by host cells. *J. Virol.* **80**:360-371.
150. **Shirako, Y., and J. H. Strauss.** 1994. Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *J. Virol.* **68**:1874-1885.
151. **Shull, N. P., S. L. Spinelli, and E. M. Phizicky.** 2005. A highly specific phosphatase that acts on ADP-ribose 1"-phosphate, a metabolite of tRNA splicing in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **33**:650-660.
152. **Simmons, J. D., L. J. White, T. E. Morrison, S. A. Montgomery, A. C. Whitmore, R. E. Johnston, and M. T. Heise.** 2009. Venezuelan equine encephalitis virus disrupts STAT1 signaling by distinct mechanisms independent of host shutoff. *J. Virol.* **83**:10571-10581.
153. **Smythe, G. M., and T. A. Rando.** 2006. Altered caveolin-3 expression disrupts PI(3) kinase signaling leading to death of cultured muscle cells *Exp. Cell Res.* **312**:2816-2825.
154. **Sodhi, R. K., N. Singh, and A. S. Jaggi.** 2010. Poly(ADP-ribose) polymerase-1 (PARP-1) and its therapeutic implications. *Vascul Pharmacol.* **53**:77-87.
155. **Spuul, P.** 2010. Cellular Membranes as a Playground for Semliki Forest Virus Replication Complex. PhD Thesis. University of Helsinki, Helsinki.
156. **Spuul, P., G. Balistreri, K. Hellström, A. Golubtsov, E. Jokitalo, and T. Ahola.** 2011. Assembly of Alphavirus Replication Complexes from RNA and Protein Components in a Novel Trans-replication System in Mammalian Cells. Manuscript.
157. **Spuul, P., G. Balistreri, L. Kääriäinen, and T. Ahola.** 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.
158. **Spuul, P., A. Salonen, A. Merits, E. Jokitalo, L. Kääriäinen, and T. Ahola.** 2007. Role of the amphipathic peptide of Semliki forest virus replicase protein nsP1 in membrane association and virus replication. *J. Virol.* **81**:872-883.
159. **Steele, K. E., and N. A. Twenhafel.** 2010. REVIEW PAPER: pathology of animal models of alphavirus encephalitis. *Vet. Pathol.* **47**:790-805.

160. **Strauss, J. H., and E. G. Strauss.** 2008. *Viruses and human disease*. Academic Press, USA.
161. **Strauss, J. H., and E. G. Strauss.** 1994. The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.* **58**:491-562.
162. **Suopanki, J., D. L. Sawicki, S. G. Sawicki, and L. Kääriäinen.** 1998. Regulation of alphavirus 26S mRNA transcription by replicase component nsP2. *J. Gen. Virol.* **79 (Pt 2)**:309-319.
163. **Suthar, M. S., R. Shabman, K. Madric, C. Lambeth, and M. T. Heise.** 2005. Identification of adult mouse neurovirulence determinants of the Sindbis virus strain AR86. *J. Virol.* **79**:4219-4228.
164. **Tan le, V., Q. Ha do, V. M. Hien, L. van der Hoek, J. Farrar, and M. D. de Jong.** 2008. Me Tri virus: a Semliki Forest virus strain from Vietnam? *J. Gen. Virol.* **89**:2132-2135.
165. **Tan, G. S., M. A. Preuss, J. C. Williams, and M. J. Schnell.** 2007. The dynein light chain 8 binding motif of rabies virus phosphoprotein promotes efficient viral transcription. *Proc. Natl. Acad. Sci. U. S. A.* **104**:7229-7234.
166. **Tan, J., C. Vornrhein, O. S. Smart, G. Bricogne, M. Bollati, Y. Kusov, G. Hansen, J. R. Mesters, C. L. Schmidt, and R. Hilgenfeld.** 2009. The SARS-unique domain (SUD) of SARS coronavirus contains two macrodomains that bind G-quadruplexes. *PLoS Pathog.* **5**:e1000428.
167. **Thiboutot, M. M., S. Kannan, O. U. Kawalekar, D. J. Shedlock, A. S. Khan, G. Sarangan, P. Srikanth, D. B. Weiner, and K. Muthumani.** 2010. Chikungunya: a potentially emerging epidemic? *PLoS Negl Trop. Dis.* **4**:e623.
168. **Till, S., and A. G. Ladurner.** 2009. Sensing NAD metabolites through macro domains. *Front. Biosci.* **14**:3246-3258.
169. **Timinszky, G., S. Till, P. O. Hassa, M. Hothorn, G. Kustatscher, B. Nijmeijer, J. Colombelli, M. Altmeyer, E. H. Stelzer, K. Scheffzek, M. O. Hottiger, and A. G. Ladurner.** 2009. A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation. *Nat. Struct. Mol. Biol.* **16**:923-929.
170. **Tomar, S., R. W. Hardy, J. L. Smith, and R. J. Kuhn.** 2006. Catalytic core of alphavirus nonstructural protein nsP4 possesses terminal adenylyltransferase activity. *J. Virol.* **80**:9962-9969.
171. **Trible, R. P., L. Emert-Sedlak, and T. E. Smithgall.** 2006. HIV-1 Nef selectively activates Src family kinases Hck, Lyn, and c-Src through direct SH3 domain interaction. *J. Biol. Chem.* **281**:27029-27038.
172. **Tuittila, M., and A. E. Hinkkanen.** 2003. Amino acid mutations in the replicase protein nsP3 of Semliki Forest virus cumulatively affect neurovirulence. *J. Gen. Virol.* **84**:1525-1533.

173. **Tuittila, M. T., M. G. Santagati, M. Røyttä, J. A. Määttä, and A. E. Hinkkanen.** 2000. Replicase complex genes of Semliki Forest virus confer lethal neurovirulence. *J. Virol.* **74**:4579-4589.
174. **Varjak, M., E. Zusinaite, and A. Merits.** 2010. Novel functions of the alphavirus nonstructural protein nsP3 C-terminal region. *J. Virol.* **84**:2352-2364.
175. **Vasiljeva, L., A. Merits, P. Auvinen, and L. Kääriäinen.** 2000. Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. *J. Biol. Chem.* **275**:17281-17287.
176. **Vasiljeva, L., A. Merits, A. Golubtsov, V. Sizemskaja, L. Kääriäinen, and T. Ahola.** 2003. Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J. Biol. Chem.* **278**:41636-41645.
177. **Vasiljeva, L., L. Valmu, L. Kääriäinen, and A. Merits.** 2001. Site-specific protease activity of the carboxyl-terminal domain of Semliki Forest virus replicase protein nsP2. *J. Biol. Chem.* **276**:30786-30793.
178. **Vihinen, H., T. Ahola, M. Tuittila, A. Merits, and L. Kääriäinen.** 2001. Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J. Biol. Chem.* **276**:5745-5752.
179. **Vihinen, H., and G. Balistreri.** 2010. Personal communication. Helsinki.
180. **Vihinen, H., and J. Saarinen.** 2000. Phosphorylation site analysis of Semliki forest virus nonstructural protein 3. *J. Biol. Chem.* **275**:27775-27783.
181. **Voss, J. E., M. C. Vaney, S. Duquerroy, C. Vonrhein, C. Girard-Blanc, E. Crublet, A. Thompson, G. Bricogne, and F. A. Rey.** 2010. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. *Nature.* **468**:709-712.
182. **Waterhouse, A. M., J. B. Procter, D. M. Martin, M. Clamp, and G. J. Barton.** 2009. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics.* **25**:1189-1191.
183. **Wengler, G., A. Koschinski, G. Wengler, and F. Dreyer.** 2003. Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents. *J. Gen. Virol.* **84**:173-181.
184. **Whatcott, C. J., M. L. Meyer-Ficca, R. G. Meyer, and M. K. Jacobson.** 2009. A specific isoform of poly(ADP-ribose) glycohydrolase is targeted to the mitochondrial matrix by a N-terminal mitochondrial targeting sequence. *Exp. Cell Res.* **315**:3477-3485.
185. **Wigge, P., and H. T. McMahon.** 1998. The amphiphysin family of proteins and their role in endocytosis at the synapse. *Trends Neurosci.* **21**:339-344.

186. **Wu, Y., H. Matsui, and K. Tomizawa.** 2009. Amphiphysin I and regulation of synaptic vesicle endocytosis. *Acta Med. Okayama.* **63**:305-323.
187. **Zech, B., A. Kurtenbach, N. Krieger, D. Strand, S. Blencke, M. Morbitzer, K. Salassidis, M. Cotten, J. Wissing, S. Obert, R. Bartenschlager, T. Herget, and H. Daub.** 2003. Identification and characterization of amphiphysin II as a novel cellular interaction partner of the hepatitis C virus NS5A protein. *J. Gen. Virol.* **84**:555-560.
188. **Ziemiacki, A., H. Garoff, and K. Simons.** 1980. Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. *J. Gen. Virol.* **50**:111-123.