DEPARTMENT OF MICROBIOLOGY, TUMOR, AND CELL BIOLOGY
Karolinska Institutet, Stockholm, Sweden

# EXPLORATION OF HOST CELLS DURING OLD WORLD ALPHAVIRUS INFECTION: MODULATION OF RNA GRANULES AND THE PI3K/AKT PATHWAY

Lifeng Liu


Stockholm 2019

The cover: These are two mouse embryonic fibroblast cells: non-infected (top) or Semliki Forest virus (SFV) infected (bottom). The picture shows RNA processing bodies (P-bodies, cyan dots) disappear and stress granules (purple dots) appear upon SFV infection (yellow dots). Picture source: an experiment conducted for paper II of this thesis.


Published by Karolinska Institutet.
Printed by E-Print AB 2018

ISBN 978-91-7831-476-8

# EXPLORATION OF HOST CELLS DURING OLD WORLD ALPHAVIRUS INFECTION: MODULATION OF RNA GRANULES AND THE PI3K/AKT PATHWAY
# THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Lifeng Liu**

*Principal Supervisor:*
Associate Professor Gerald M. McInerney
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

*Co-supervisor(s):*
Dr. Bastian Thaa
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology
Current: Co.faktor GmbH, Berlin, Germany

Professor Adnane Achour
Karolinska Institutet
Department of Medicine, Solna

*Opponent:*
Professor Mark Harris
University of Leeds, United Kingdom
School of Molecular and Cellular Biology

*Examination Board:*
Professor Gunnar Schulte
Karolinska Institutet
Department of Physiology and Pharmacology

Professor Francesca Chiodi
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Docent Tanel Punga
Uppsala University
Department of Medical Biochemistry and Microbiology, Infection biology, antimicrobial resistance and immunology

“If you know the enemy and know yourself, you need not fear the result of a hundred battles”
(Sun Tzu, *The art of war*)

# ABSTRACT

As obligate intracellular parasites, viruses have to explore and modulate cellular pathways for their survival. Mechanistic studies of virus–host interactions provide a better understanding of viral infection and cellular responses and help to identify potential targets for therapeutic intervention. Alphaviruses, a group of small enveloped viruses with positive-sense, single-stranded RNA genomes, are transmitted by mosquitoes and pose threats to human health. Semliki Forest virus (SFV) belongs to this group and has been extensively studied as a model virus for alphaviruses. Infection with alphaviruses, such as the important human pathogens chikungunya virus (CHIKV) or Ross River virus (RRV), is characterized by high fever, rash and debilitating joint pain, which can last for months or even years. The recent outbreaks and expanding spread of CHIKV in many tropical regions of the world as well as the continuous endemic of RRV in Australasia highlighted the significance of alphavirus research.

Stress granules (SGs) are cytoplasmic aggregates of non-translated messenger ribonucleoprotein particles (mRNPs) that can be induced by many types of environmental stress, including viral infection. RNA processing bodies (P-bodies) are cytoplasmic aggregates of translationally silent mRNA and proteins involved in mRNA decay and translation repression. Unlike SGs, P-bodies are constitutively present in the cytoplasm under normal conditions. Both granules can actively respond to environmental stress and associate with each other under certain conditions. The PI3K–Akt–mTOR pathway plays important roles in regulating the transition between cell anabolism and catabolism, responding to changes in the cellular environment. Proper responses of SGs, P-bodies or the PI3K–Akt–mTOR pathway are seen as important adaption for cell survival under stress conditions.

In this thesis, it was investigated how alphaviruses (more specifically, Old World alphaviruses such as SFV, CHIKV and RRV) exploit the SG nucleating protein G3BP, P-bodies and the PI3K–Akt–mTOR pathway upon infection.

Previous studies have demonstrated that the alphavirus non-structural protein nsP3 binds to the SG nucleating protein G3BP via its two FGDF motifs to block SG induction. In **paper I**, we compared the two FGDF motifs in nsP3 with respect to their contribution to G3BP binding. The three-dimensional structure of G3BP1 bound to an SFV nsP3-derived peptide (nsP3-25, containing two FGDF motifs) revealed a poly-assembly of G3BP1 dimers inter-connected by nsP3-25. Both *in vitro* and *in vivo* binding studies demonstrated a hierarchical binding mode of the duplicate FGDF motifs to G3BP. SFV mutants lacking either of the FGDF motifs failed to bind levels of G3BP necessary for efficient replication, clearly demonstrating that both intact FGDF motifs are required for efficient virus replication. The hierarchical binding mode of two FGDF motifs was also observed for CHIKV nsP3. Growth curves showed that the two intact FGDF motifs are critical for viral replication. Mutation of both FGDF motifs was lethal to CHIKV. These results highlight a conserved molecular mechanism of virus-induced G3BP modulation.

In **paper II**, we described that P-bodies are disassembled or reduced in number after infection with SFV or CHIKV in various cell lines. Disassembly of P-bodies occurs at an early stage (3–4h) post infection and is independent of viral structural protein expression. Similar to SGs, P-bodies could not be re-introduced by a second stress (sodium arsenite) in infected cells. However, formation of SGs or communication between P-bodies and SGs is not necessary for P-body disassembly, since P-bodies were still disassembled to a similar extent upon infection of cells incapable of forming SGs. Studies of the translation status by ribopuromycylation showed that P-body disassembly is independent of host translation shutoff, which requires the phosphorylation of the eukaryotic initiation factor eIF2α in cells infected with SFV or CHIKV. By labelling newly synthesized RNA with bromo-UTP, we observed that the timing of host transcription shutoff correlated with P-body disassembly, occurring at the same stage (3–4h) after infection. However, inhibition of transcription with actinomycin D (ActD) failed to disassemble P-bodies as efficiently as the viruses did. Interestingly, the block of nuclear import in non-infected cells with importazole led to an efficient P-body loss. These results reveal that P-bodies are disassembled independently of SG formation at early stages of Old World alphavirus infection and that nuclear import is involved in the dynamics of P-bodies.

Infection with SFV hyperactivates the PI3K–Akt–mTOR pathway. In **paper III**, we show that a tyrosine residue in the sequence context YEPM in nsP3 of SFV is pivotal for this phenotype. When cells were infected with SFV carrying mutations that affect this motif, such as the replacement of the YEPM tyrosine by phenylalanine (SFV-YF), Akt activation was significantly reduced and delayed in comparison to wildtype SFV infection. Ectopically expressed nsP3 of SFV-WT but not SFV-YF activated the pathway, but only when provided with a membrane anchor. Co-immunoprecipitation experiments revealed that nsP3 of SFV-WT, but not SFV-YF, bound to the regulatory subunit p85 of PI3K, dependent on both the tyrosine of nsP3 and the SH2 domains of p85, which specifically interact with phosphorylated tyrosines in YXXM motifs. This indicates tyrosine phosphorylation of SFV nsP3. A similar YETM motif was identified and characterized in RRV nsP3. Similar to SFV, RRV-WT hyperactivated the PI3K–Akt–mTOR pathway while the mutant RRV-YF, in which the YXXM tyrosine in nsP3 was replaced by phenylalanine, failed to do so.. Complementary experiments showed that infection of cells with SFV or RRV reprograms cellular metabolism and that mutation of the critical tyrosine residue attenuates the virus in cell culture and, in case of RRV, also in mice. These results reveal that the hyperactivation of the PI3K–Akt–mTOR pathway by SFV and RRV, mediated by the interaction of nsP3 and p85, contributes to virus pathogenicity.

Taken together, the data from **papers I, II** and **III** highlight and characterize three mechanisms by which Old World alphaviruses subvert cells – interactions with the SG nucleating protein G3BP, P-bodies and the PI3K–Akt–mTOR pathway, respectively. These viral subversion mechanisms are largely independent of each other and represent three different modes by which Old World alphaviruses ensure efficient virus growth.

# LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscripts

I. Schulte, T*., **Liu, L***., Panas, M. D., Thaa, B., Dickson, N., Gotte, B., Achour, A*., & McInerney, G. M*. (2016). Combined structural, biochemical and cellular evidence demonstrates that both FGDF motifs in alphavirus nsP3 are required for efficient replication. *Open Biol, 6*(7). doi:10.1098/rsob.160078. *equal contribution

II. **Liu, L**., Weiss, E., Panas, M., Götte, B., Sellberg, S., Thaa, B., & McInerney, G. M. RNA Processing Bodies are Disassembled during Old World Alphavirus Infection. (Submitted manuscript)

III. Mazzon, M*., Castro, C*., Thaa, B*., **Liu, L**., Mutso, M., Liu, X., Mahalingam, S., Griffin, J. L*., Marsh, M*., & McInerney, G. M*. (2018). Alphavirus-induced hyperactivation of PI3K/AKT directs pro-viral metabolic changes. *PLoS Pathog, 14*(1), e1006835. doi:10.1371/journal.ppat.1006835. *equal contribution

# PUBLICATIONS NOT INCLUDED IN THIS THESIS

IV. Götte, B., Panas, M., Hellström, K., **Liu, L**., Ahola, T., Samreen, B., Larsson, O., & McInerney, G. M. (2019) Separate domains of G3BP promote efficient clustering of alphavirus replication complexes and recruitment of the translation initiation machinery. *PLoS Pathog*. (accepted)

V. Götte, B., **Liu, L**., & McInerney, G. M. (2018). The Enigmatic Alphavirus Non-Structural Protein 3 (nsP3) Revealing Its Secrets at Last. *Viruses, 10*(3). doi:10.3390/v10030105 (Review article)

VI. Sultana, M. A., Du, A., Carow, B., Angbjar, C. M., Weidner, J. M., Kanatani, S., Fuks, J. M., Muliaditan, T., James, J., Mansfield, I. O., Campbell, T. M., **Liu, L**., Kadri, N., Lambert, H., Barragan, A., & Chambers, B. J. (2017). Downmodulation of Effector Functions in NK Cells upon *Toxoplasma gondii* Infection. *Infect Immun, 85*(10). doi:10.1128/iai.00069-17

# CONTENTS

1 Introduction ..... 1
1.1 Studying viruses is important and necessary ..... 1
1.2 Alphaviruses ..... 2
1.2.1 Alphaviruses and challenges for public health ..... 2
1.2.2 The alphavirus virion ..... 4
1.2.3 Infection cycle of alphaviruses ..... 5
1.2.4 Functions of the alphavirus non-structural proteins ..... 8
1.3 Stress granules and virus infection ..... 13
1.4 RNA processing bodies and virus infection ..... 16
1.5 The PI3K–Akt–mTOR pathway and virus infection ..... 20
2 Results ..... 21
2.1 Results Paper I ..... 21
2.2 Results Paper II ..... 27
2.3 Results Paper III ..... 40
3 Discussion ..... 49
4 Future perspectives ..... 60
5 Acknowledgements ..... 65
6 References ..... 69

# LIST OF ABBREVIATIONS

| | |
|---|---|
| aa | Amino acid |
| ActD | Actinomycin D |
| ADP/AMP/ATP | Adenosine diphosphate/monophosphate/triphosphate |
| AIDS | Acquired immune deficiency syndrome |
| ARE | AU-rich element |
| BAP | Biotin acceptor peptide |
| BHK | Baby hamster kidney |
| BFV | Barmah Forest virus |
| CHIKV | Chikungunya virus |
| CHX | cycloheximide |
| CP | Capsid protein |
| CPV-I | Type I cytopathic vacuoles |
| CRM1 | Chromosomal maintenance 1 |
| Dcp1a/2 | mRNA decapping enzyme 1a/2 |
| ds/ssRNA | Double/single-stranded RNA |
| EBSS | Earle's balanced salt solution |
| EEEV | Eastern equine encephalitis virus |
| EGFP | Enhanced green fluorescent protein |
| eIF2α/eIF3b | Eukaryotic translation initiation factor 2α, 3b |
| EYFP | Enhanced yellow fluorescent protein |
| FRAP | Fluorescence recovery after photobleaching |
| G3BP | Ras GTPase-activating protein-binding protein |
| GAP | GTPase-activating protein |
| GCN2 | General control nonderepressible 2 kinase |
| GDP/GMP/GTP | Guanosine diphosphate/monophosphate/triphosphate |
| GETV | Getah virus |
| GFP/RFP | Green/red fluorescent protein |
| g/sgRNA | Genomic/subgenomic RNA |
| HAV/HBV/HCV | Hepatitis virus A/B/C |
| HEK293 | Human embryonic kidney 293 |

| | |
|---|---|
| HIV | Human immunodeficiency virus |
| hpi/t | Hours post infection/transfection |
| HPV | Human papilloma virus |
| HRI | Heme-regulated kinase |
| HSP | Heat shock protein |
| HSV | Herpes simplex virus |
| HVD | Hypervariable domain |
| IAV | Influenza A virus |
| IDPR | Intrinsically disordered protein regions |
| IP | Immunoprecipitation |
| IRES | Internal ribosome entry site |
| MAR | Monomeric ADP ribose |
| MEF | Mouse embryonic fibroblast |
| MOI | Multiplicity of infection |
| mRNA | Messenger ribonucleic acid |
| mRNP | Messenger ribonucleoprotein particles |
| MST | Microscale thermophoresis |
| mTOR | Mammalian target of rapamycin |
| mTORC1/2 | mTOR complex 1/2 (mTOR–raptor complex) |
| Myr-Pal | Myristoylation and palmitoylation |
| NC | Nucleocapsid |
| NLS | Nuclear localization sequence |
| nsP | Non-structural protein |
| NTF2 | Nuclear transport factor 2 |
| NTP/dNTP | Nucleoside triphosphate/deoxynucleoside triphosphate |
| ONNV | O'nyong-nyong virus |
| ORF | Open reading frame |
| PABP | Poly-A binding protein |
| PAR | Polymeric ADP-ribose |
| P-bodies/PBs | RNA processing bodies |
| PDK1 | Phosphoinositide-dependent kinase 1 |

| | |
|---|---|
| PERK | PKR-like endoplasmic reticulum kinase |
| PI3K | Phosphatidylinositol-3-kinase |
| PKR | Protein kinase R |
| RC | Replication complex |
| RdRp | RNA-dependent RNA polymerase |
| RGG | Arginine-glycine-glycine |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RRM | RNA-recognition motif |
| SA | Sodium arsenite |
| SARS | Severe acute respiratory syndrome |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SFV | Semliki Forest virus |
| SG | Stress granule |
| SH | Src homology |
| SINV | Sindbis virus |
| TIA1/R | T-cell-restricted intracellular antigen/related |
| TTP | Tristetraprolin |
| USP10 | Ubiquitin-specific protease 10 |
| UTP | Uridine triphosphate |
| UTR | Untranslated region |
| UV | Ultraviolet |
| VEEV | Venezuelan equine encephalitis virus |
| WEEV | Western equine encephalitis virus |
| WNV | West Nile virus |
| WT | Wildtype |
| U2OS | Human bone osteosarcoma epithelial cells |
| βGal | Beta-galactosidase |

# 1 INTRODUCTION

## 1.1 STUDYING VIRUSES IS IMPORTANT AND NECESSARY

Viruses are important pathogens for all other life forms – humans, animals, plants, bacteria and archaea. The word "virus" is derived from the Latin "vīrus", which refers to poison or noxious liquids. Virus infection in humans can lead to a life-threatening impact on all organs including nervous system, lung, liver and intestines. Many devastating human diseases are caused by viruses, such as rabies, smallpox, influenza and acquired immune deficiency syndrome (AIDS). Disease-causing viruses are usually highly contagious and may lead to an epidemic or even a pandemic. Recent important outbreaks of virus diseases include severe acute respiratory syndrome (SARS) 2002–2003 (Tam 2004), influenza 2009–2010 (Poland 2010), consistently reported Marburg virus disease and Ebola, and Zika virus related microcephaly 2015–2017 (Brady *et al.* 2019). In addition, some viruses are a constant threat for human health, including human immunodeficiency virus (HIV), human papilloma virus (HPV) and hepatitis viruses (HAV, HBV, HCV). Lastly, some viruses, such as chikungunya virus (CHIKV), are re-emerging and expanding their circulation regions (Staples *et al.* 2009, Nasci 2014), which poses threat to human health as well. Virus infections in animals or plants can also have a highly destructive impact on society. Outbreaks of virus diseases in domestic animals, such as avian influenza in poultry and foot-and-mouth disease in cattle, cause huge economic losses. Infected animals and those at risk of infection are usually culled to prevent spread of the disease. Similarly, virus infections in plants, for example in potatoes and tomatoes, can dramatically decrease the production of the crops, which not only reduces the profits of farmers but also threatens food supply. Overall, viruses are adding heavy burdens to human health and society.

Viruses are not only health-threatening, but also extremely abundant. They are the simplest and smallest forms of life on Earth. The virus particles, termed virions, just consist of a nucleic acid genome and a protein coat, and the size of virions varies from 20 to 500 nanometers. According to some estimation, the total mass of only bacterial viruses (bacteriophages) is more than 1000 times that of all elephants on Earth. It is estimated that there are more than $10^{31}$ bacteriophages in the oceans on Earth. If lined up in a row, all the viruses would extend as far as 200 million light-years (Acheson 2011). The enormous abundance of viruses makes it difficult to eliminate or prevent virus diseases. Importantly, some viruses can also spread across different host species, e.g. from animal species to humans, which contributes to the increasing frequency of zoonoses in recent years, such as SARS, Ebola and H5N1 influenza. Viruses are transmitted

by a wide variety of routes, e.g. via aerosols, blood or contaminated surfaces. Some viruses are transmitted by vectors, for instance arthropods (arboviruses).

Replication of viruses is entirely dependent on living cells since they lack basic elements for growth and replication, such as synthesis of molecular building blocks (nucleotides, amino acids, carbohydrates, lipids). Their extreme simplicity hence makes viruses obligatory intracellular parasites. During infection, viruses explore and reprogram intracellular pathways. This is a challenge to develop antiviral drugs, but also makes viruses unique tools for the study of cell biology. Some important discoveries were revealed from the study of viruses, such as the identification of eukaryotic RNA promoters, the internal ribosome entry site (IRES), RNA splicing and the isolation of numerous cellular oncogenes (Flint *et al.* 2015). In addition, some scientific subjects are derived from the study of viruses. For example, bacteriophage studies built the foundation of modern molecular biology and crystallization of tobacco mosaic virus pioneered structural biology. Virus studies have also enabled many applications of virus vectors. In combination with recombinant DNA techniques, virus vectors, such as Semliki Forest virus (SFV), are commonly used for gene delivery and expression in various cells and organisms. Gene therapy based on viral vectors, such as adenovirus or alphavirus, is an emerging area, with over 300 gene therapy projects in clinical trials worldwide (Lundstrom 2018). Knowledge for development of antiviral therapies and application of viruses as study tools or vectors: This all derives from virus–host studies, highlighting the significance of such studies.

## 1.2 ALPHAVIRUSES

### 1.2.1 Alphaviruses and challenges for public health

Alphaviruses are a group of viruses that are transmitted by mosquitoes between vertebrate hosts around the world. Infection in mosquitoes usually leads to life-long persistence, making mosquitoes the main reservoir and vectors for viral transmission. Transmission between species, including transmission to human beings, is often mediated through bites from infected blood-sucking mosquitoes. Alphaviruses belong to the virus family *Togaviridae*, which comprises two genera, *Alphavirus* and *Rubivirus*. “Toga” means “cloak” in Latin, which describes the appearance of the virus envelope. There are 27 members of the genus *Alphavirus* while there is only one member of the genus *Rubivirus*, rubella virus. Alphaviruses are commonly grouped into Old World alphaviruses and New World alphaviruses based on their original isolation sites and geographic distribution. Old World alphaviruses comprise important human pathogens, such as chikungunya virus (CHIKV), Ross River virus (RRV) and O’nyong-

nyong virus (ONNV). New World alphaviruses mainly infect horses and rodents, but can infect humans as well, including Eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV) and Western equine encephalitis virus (WEEV). Symptoms vary after infection with different alphaviruses. Infection with Old World alphaviruses rarely results in fatal disease, but leads to high morbidity in humans, characterized by high fever, rash and debilitating joint pain (Lwande *et al.* 2015), which can last for months or even years (Strauss *et al.* 1994). Infection with New World alphaviruses usually causes serious encephalitis, but with low morbidity in humans.

Alphaviruses pose a significant threat to human health. Increasing outbreaks and spread of CHIKV have been recently reported. Since its first isolation in 1952, CHIKV circulated in African and Asian regions along the Indian Ocean for nearly three decades from the 1960s to the 1980s (Nasci 2014). After a short time of silence, an endemic of CHIKV began in Kenya 2004 and spread to several Indian Ocean islands with over one thousand reported cases (Higgs 2006). During 2005–2006, an outbreak of CHIKV occurred in India with over one million victims (Staples *et al.* 2009). Later, in 2007, the first autochthonous outbreak in Europe was reported in Italy with over two hundred cases (Liumbruno *et al.* 2008). Recently, locally acquired cases of CHIKV infection were also reported in Caribbean islands in 2013 and then the mainland of the USA in 2014 (Morrison 2014, McSweegan *et al.* 2015). Since the first reported case in Florida in 2014, CHIKV infections have been found in most states of the USA and became a nationally notifiable condition in 2015. Since then, infected cases have been reported each year including locally acquired cases (Centers for Disease Control and Prevention 2019, January 8). Due to its recent outbreaks and extended spread in the twenty-first century, CHIKV is considered to be a re-emerging virus (Lwande *et al.* 2015). Millions of cases of CHIKV infection have been reported since its re-emergence (Weaver *et al.* 2015).

The primary mosquito vector of CHIKV is *Aedes aegypti*. However, studies show that the related mosquito species *Aedes albopictus* was responsible for the CHIKV epidemic during 2005–2006 on La Réunion island (Reiter *et al.* 2006). The vector change is attributed to the emergence of a mutation at position 226, alanine to valine, in the E1 envelope glycoprotein (E1-A226V), which enhanced virus infectivity in *Ae. albopictus*, with only marginal effect on its infectivity in *Ae. aegypti* (Tsetsarkin *et al.* 2007). It is surprising how fast the virus can adapt itself to a new vector species, since the mutation E1-A226V was not present in the isolates from the beginning of the outbreak but was dominant in the subsequent isolates (Schuffenecker *et al.* 2006). Compared to *Ae. aegypti*, *Ae. albopictus* is more abundant, leading to the long-lasting and large-scale circulation of the outbreaks. The species *Ae. albopictus* is also widely

distributed in urban areas of Europe and the USA (Gratz 2004), which is considered to contribute to the expansion of CHIKV into the Western hemisphere.

Sporadic outbreaks of other Old World alphaviruses have been consistently reported. ONNV was originally isolated in Northern Uganda in 1959 and re-emerged in 1996 in Southern Uganda. In 2014, high rates of ONNV and CHIKV transmission were reported in Coastal Kenya, indicating constant and re-emerged circulation of both alphaviruses (LaBeaud *et al.* 2015). RRV is endemic in Australia and Papua New Guinea. In Australia, RRV infection is the most common arboviral infection, with approximately 5000 cases reported every year (Harley *et al.* 2001). Similar to RRV, another Old World alphavirus, Barmah Forest virus (BFV), is also endemic in Australia, causing notifiable infection (Suhrbier *et al.* 2012).

The increasing challenge of alphaviruses for human health has brought more attention to research on this virus group. Semliki Forest virus (SFV) and Sindbis virus (SINV), mostly considered as avirulent for humans, are generally studied as laboratory models and are the best-studied viruses in this field (Strauss *et al.* 1994). As model viruses, both SFV and SINV are able to establish infection in a variety of invertebrate and vertebrate cell lines, bringing many options for studies of these viruses. Recombinant infectious clones of several alphaviruses, including SFV and SINV, have been developed, making it easier to create viral mutants and to study the functions of individual viral components (Liljeström and Garoff. 1991a). With the help of infectious clones, many aspects of alphavirus biology have been revealed, regarding processes like viral protein translation and polyprotein processing, RNA replication, virion assembly and virus–host interaction. Generally, cellular processes are modified after alphavirus infection. Therefore, interaction studies of alphaviruses and host cells add new knowledge not only about the viruses but also about cellular processes. Even though most knowledge has been obtained based on SFV or SINV, the processes are largely conserved for other alphaviruses. However, one should be aware that the knowledge from the models is not always transferable to other viruses. This thesis will focus on the study of SFV and the extension of our findings to CHIKV and RRV.

### 1.2.2 The alphavirus virion

Alphaviruses are enveloped viruses with a positive-sense, single-stranded RNA genome. The envelope is an icosahedral lattice, consisting of the viral proteins E1 and E2 embedded in a host-derived lipid bilayer (Strauss *et al.* 2002). Both E1 and E2 are glycoproteins and have a transmembrane helix structure. They form heterodimers with a 1:1 ratio, and a spike complex is assembled with 3 copies of E1-E2 heterodimers, with 80 spikes (240 copies of E1-E2) in

total presented on the envelope surface (Helenius 1995). The envelope of some alphaviruses also contains small amounts of another viral protein, 6K (60 amino acid residues in SFV), which is considered to be an important component for the structure of the viral particle (Gaedigk-Nitschko *et al.* 1990, Lusa *et al.* 1991). Beneath the envelope lies an icosahedral protein shell made of 240 monomers of capsid protein (CP). Through its carboxyl terminus, CP interacts with the cytoplasmic domain of E2 in a perfect 1:1 symmetry match. This interaction is responsible for anchoring the spikes (outer layer of glycoproteins) to the structural capsid (Lee *et al.* 1996, Skoging *et al.* 1996). The amino terminus of CP is linked with viral genomic RNA (gRNA), assembled as nucleocapsid (NC), with viral gRNA folded inside the capsid shell. The gRNA of alphaviruses (42S RNA) contains two open reading frames (ORFs), with an approximate length of 11.7 kb. The first ORF in the 5′ part covers two thirds of the genome and encodes a nonstructural polyprotein (nsP), which gets processed into 4 nonstructural proteins (nsP1–nsP4). The incoming full-length viral genome is directly used for the translation of the nonstructural polyprotein (discussed below). The second ORF, located in the 3′ part of the genome, is under the control of a subgenomic promoter. During infection, a shorter 26S subgenomic RNA (sgRNA), containing the second ORF and is transcribed and is used for the translation of the structural proteins. In addition to the two ORFs, other conserved sequences and structure elements are present in the viral RNA genome (reviewed in (Strauss *et al.* 1994, Jose *et al.* 2009)). For example, efficient transcription of sgRNA requires the presence of a conserved sequence element identified at the junction between the two ORFs (Pushko *et al.* 1997).

### 1.2.3 Infection cycle of alphaviruses

Infection of all viruses starts with the attachment of viral particles to susceptible cells. In case of alphaviruses, E2 is the viral attachment protein that binds to cellular receptors, which are probably different for the various alphaviruses (Smith and Tignor. 1980, Ludwig *et al.* 1996). So far, no specific cellular receptor has been identified for SFV. Virus tropism is attributed to species-specific receptors. Many viruses use more than one factor as receptor. However, it is still unknown why alphaviruses are able to establish infection in such a broad spectrum of vertebrate and invertebrate species. Two possible explanations for the phenomenon have been suggested: either a conserved cell receptor exists in different species; or viruses can alter the components of different cells to make use of them for attachment (Jose *et al.* 2009). Virions successfully attached to cellular receptors are transported into cells through clathrin-mediated endocytosis, which leads to the formation of clathrin-coated vesicles (Marsh *et al.* 1983, DeTulleo and Kirchhausen. 1998). Then, these vesicles lose the clathrin coats quickly and fuse

with early endosomes located near the cell surface. Early endosomes then mature to late endosomes during their transport from the cell surface to the perinuclear area. The main change during maturation is that late endosomes are more acidic than the early ones. The low pH environment destabilizes the E1-E2 heterodimers and causes a conformational re-arrangement of the complex, resulting in the fusion of the viral envelope with the endosome membrane. The fusion is triggered by the exposure of a fusion peptide in E1 (Glomb-Reinmund *et al.* 1998, Gibbons *et al.* 2000). The viral envelope around the NC is peeled off via membrane fusion, and the NC is released into the cell cytoplasm. Once the NC is exposed to the cytoplasm, cellular ribosomes are recruited through interaction with CP, followed by the release of the gRNA into the cytoplasm (Singh and Helenius. 1992). With ribosomes around, viral gRNA is immediately translated into the non-structural proteins.

The non-structural proteins are translated as a polyprotein. For most alphaviruses, including SINV and CHIKV, the polyprotein nsP123 is dominantly translated, together with a smaller proportion of nsP1234 (10–20%). This variation is attributed to a leaky opal termination codon identified at the end of nsP3. For SFV (depicted in Fig. 1), only nsP1234 is translated due to the replacement of this stop codon with an arginine codon (CGA) (Firth *et al.* 2011). Translated nsP1234 is first cleaved in cis by nsP2 to produce nsP123 and nsP4. The complex formed by nsP123 and nsP4 facilitates the synthesis of (−)RNA genome (complimentary strand RNA genome). Subsequently, nsP123 is cleaved into nsP1 plus nsP23. A different complex is then formed by nsP1, nsP23 and nsP4, which is capable of synthesizing both (−)RNA and (+)RNA genomes. This complex, however, only exists shortly, because cleavage between nsP2 and nsP3 happens rapidly after the release of nsP1, resulting in fully cleaved nsPs. Individual nsPs then form a replicase variant that is only used for the synthesis of (+)RNA genome and sgRNA, leading to the cessation of (−)RNA genome synthesis (Strauss *et al.* 1994). All types of the replicases are present in viral replication complexes (RCs), where the viral RNA genome is replicated (Friedman *et al.* 1972). RCs are first assembled at the plasma membrane as clusters of membrane invaginations termed spherules. Later, spherules may also be present in the cytoplasm on the cytoplasmic surface of modified endosomes/lysosomes, termed cytopathic vacuoles of type I (CPV-I) (Grimley *et al.* 1968, Froshauer *et al.* 1988). During SFV infection, the spherules are internalized from the plasma membranes into the cytoplasm, which is dependent on the phosphatidylinositol-3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) signaling pathway, the actin cytoskeleton and the microtubule network. Inhibition of PI3K blocks the internalization of spherules, leading to the loss of CPV-I. These results indicate that CPV-I consist of internalized spherules and modified endosomes/lysosomes (Spuul *et al.*

2010). However, in other alphaviruses, such as CHIKV, RC internalization does not occur efficiently (Thaa *et al.* 2015).

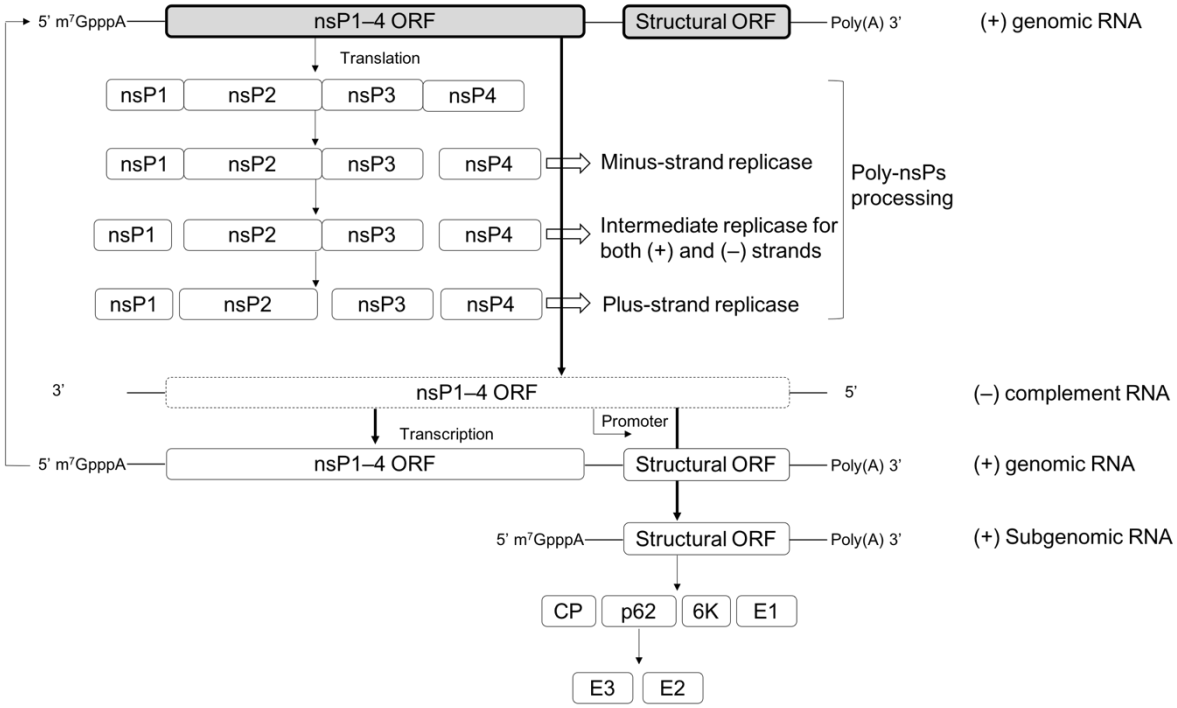


**Fig 1: Schematic sketch of SFV genome and replication.** Once released into a host cell, the incoming positive-sensed, single-stranded RNA genome, ((+) genomic RNA in grey), is directly used as template for translation of nsP1–4. Different viral replicases are produced through polyprotein processing. Replicase nsP123 + nsP4 generates minus complementary viral genome ((−)complement RNA); Replicase nsP1 + nsP23 + nsP4 can replicate both plus and minus genomic RNA; Replicase consists of fully processed nsPs mainly replicates (+) genomic RNA and transcribes the (+) subgenomic RNA, which is used as the template for the translation of structural proteins. Adapted from (sonon 2009).

While replication continues, viral 26S sgRNA is produced under a sub-genomic promoter and then used as template for the translation of the viral structural proteins. Similar to viral nsPs, viral structural proteins are initially translated as a polyprotein and processed into individual proteins, including CP, p62 (named for SFV, as precursor E2 for other alphaviruses, pE2), 6K and E1. During translation of the structural proteins, CP is first released by autoproteolysis (Choi *et al.* 1991). Through the interaction between CP and the viral (+)RNA genome, NC is assembled in a multistep process in the cytoplasm and then transported to the plasma membrane. After the release of CP, the remaining polyprotein is translocated to the endoplasmic reticulum (ER), where further cleavages of the polyprotein into the individual structural proteins are carried out (Strauss *et al.* 1994). For polyprotein translocation, two signal

peptides have been shown to be involved, situated in the p62 and 6K (Liljeström and Garoff. 1991b). Once separated in the ER, p62 and E1 interact to form heterodimers with the help of 6K (Lusa *et al.* 1991). The p62-E1 heterodimers are then transported to the Golgi apparatus. The environment of the Golgi is acidic, thus could trigger the destabilization of p62-E1 heterodimers. This is prevented by the pH-resistance of immature p62-E1 heterodimers. However, before they leave the Golgi, p62-E1 complexes are cleaved by the host cell protease furin to become mature E2-E1 heterodimers with the release of a third glycoprotein E3 from p62. E3 has been suggested to protect E2-E1 from premature activation after furin cleavage (Sjöberg *et al.* 2011), and remains in virion for SFV (Strauss *et al.* 1994). The E2-E1 heterodimeric complexes are ultimately transported to the plasma membrane, where the complexes meet NCs for the assembly of new virions. Budding of newly formed virions at the plasma membrane is the last step of the viral lifecycle, during which the completion of the spike conformation is achieved (Garoff and Simons. 1974).

### 1.2.4 Functions of the alphavirus non-structural proteins

NsP1 (~60 kDa) of alphaviruses has two well-studied functions. During infection, nsP1 catalyzes the capping reaction of newly synthesized viral gRNA and sgRNA and also functions to anchor viral RCs to membranes (Kääriäinen and Ahola. 2002). For newly synthesized viral RNA, the first step of the capping reaction is catalyzed by nsP2, with the removal of the gamma-phosphate from the first nucleotide at the 5′ end (Vasiljeva *et al.* 2000). Like eukaryotic capping enzymes, nsP1 possesses both methyltransferase and guanylyltransferase activity. Through its methyltransferase function, nsP1 first transfers a methyl group to GTP to form 7-methyl-GTP, followed by the removal of di-phosphate from the GTP to form a covalent complex, nsP1-$m^7$GMP, which is attributed to the guanylyltransferase activity of nsP1. The nsP1-$m^7$GMP complex is then used for capping viral RNA. Nevertheless, the capping reaction of viral RNA is different to that of cellular mRNA, in which the GMP is first covalently bound before the methyl group is transferred. In addition, the cap structure of alphavirus mRNA contains only one methyl group on the first guanine base and is termed cap0 structure, which is different from the predominant cap structure of cellular mRNA containing one further methyl group (cap-1) on the ribose of the second nucleotide or two further methyl groups (cap-2), one each on the ribose of the following two nucleotides (Ahola *et al.* 1997, Kääriäinen and Ahola. 2002). The membrane affinity of nsP1 is attributed to the direct binding of nsP1 to anionic membrane phospholipids as well as the palmitoylation of nsP1. The direct interaction is mainly mediated by a conserved polypeptide (residues 245–264) in nsP1; mutations in this polypeptide result in poor palmitoylation of nsP1 and are lethal to SFV (Ahola *et al.* 1999, Spuul *et al.*

2007). The palmitoylation is found on cysteine residues (residues 418–420) in SFV nsP1 (Ahola *et al.* 2000).

Alphavirus nsP2 (~90 kDa) is a multi-functional protein, distributed in both the cytoplasm and nucleus of the infected cells. In the cytoplasm, nsP2 mainly serves as RNA triphosphatase, RNA helicase and protease for the nonstructural polyprotein. As RNA triphosphatase, nsP2 hydrolyses the gamma-phosphate of newly synthesized viral RNA, producing RNA substrates capped by nsP1 (Vasiljeva *et al.* 2000). NsP2 also acts as RNA helicase to unwind double-stranded RNA structures to facilitate viral replication. This helicase activity requires the presence of NTPs and dNTPs (Gomez de Cedron *et al.* 1999). The putative NTP-binding site at the N-terminus of nsP2 is essential for both RNA triphosphatase and RNA helicase activities, since a single mutation (L192S) in this site ablates both activities. For SFV, this mutation abolishes replication completely (Rikkonen *et al.* 1994a, Rikkonen 1996). The protease activity of nsP2 is located at its C-terminus, which contains a papain-like cysteine protease domain (Vasiljeva *et al.* 2001). This domain is functionally active in both individual nsP2 and nsP2 within nsP-polyprotein. Through its protease activity, nsP2 cleaves nonstructural polyproteins at different sites in the designated processing steps, leading to the formation of different replication complexes responsible for the regulation of viral RNA synthesis (Strauss *et al.* 1994). Due to the presence of multiple nuclear localization signals (NLS), nsP2 is partly transported into the nucleus (Peränen *et al.* 1990, Rikkonen *et al.* 1994b). Mutations in NLS delay the nuclear transport of nsP2 and result in attenuated virus mutants. This attenuation has been explained by a failure of type I interferon inhibition and/or the deficiency of protein expression and RNA synthesis (Breakwell *et al.* 2007, Tamm *et al.* 2008). NsP2 is also highly related to virus replication and cytopathic effect. Several mutations in the region of nsP2 have been identified as key residues for efficient virus replication and establishment of persistent virus infection in vertebrate cells in SFV (Perri *et al.* 2000, Casales *et al.* 2008), CHIKV (Fros *et al.* 2013, Utt *et al.* 2015) and SINV (Gorchakov *et al.* 2005, Garmashova *et al.* 2006, Akhrymuk *et al.* 2018). Recent studies have shown that persistence infection of SINV or CHIKV in vertebrate host usually requires extra mutations in nsP1 and/or nsP3 in addition to those in nsP2 (Utt *et al.* 2015, Akhrymuk *et al.* 2018). In addition, studies of SINV suggest that nsP2 manipulates infection-induced shutoff of host transcription and translation (Gorchakov *et al.* 2005). The proposed mechanism is that nsP2 induces rapid degradation of Rpb1 (the catalytic subunit of RNA polymerase II) through a ubiquitin-dependent pathway (Akhrymuk *et al.* 2012).

Alphavirus nsP3 (~60 kDa) is less well studied compared to the other nsPs, but has been the focus of a lot of research recently (Götte *et al.* 2018). The protein has been suggested to play important roles as a scaffold that interacts with viral and host factors. Many nsP3-interacting partners have been identified by immunoprecipitation studies in SINV-infected cells (Cristea *et al.* 2006). The discovered binding partners could also be shared by other alphaviruses. However, further studies are needed to reveal the functions and mechanisms of the interactions. In addition, a recent study has developed a new microscopy approach to live-track CHIKV nsP3 based on the SNAP-tag self-labelling systemin in human cells, which can provide a sensitive and versatile platform for fundamental research in nsP3 functions (Remenyi *et al.* 2017). All these studies will shed more light on the roles of alphavirus nsP3.

NsP3 consists of three domains: the macro domain at the N-terminus, the alpha domain in the middle and the hypervariable domain (HVD) at the C-terminus (depicted in Figure 2, $nsP3_{SFV}$). The first two domains are conserved in alphaviruses. The macro domain, spanning approximately 160–170 amino acids (aa), is evolutionarily conserved and also present in other viruses, e.g. rubella virus, some coronaviruses and hepatitis E virus, and also in some bacteria, archaea and eukaryotes ( Kääriäinen and Ahola. 2002). Two main features have been studied for viral macro domain, binding and hydrolysis of monomeric and polymeric ADP-ribose (MAR and PAR, respectively). The binding of MAR by nsP3 is seen in the macro domain of CHIKV and VEEV, but not SFV, while the binding of PAR is observed for all three alphaviruses (Malet *et al.* 2009, Neuvonen *et al.* 2009). Viral macro domains, such as in nsP3 of CHIKV, SINV, ONNV and VEEV, are also known to be able to remove either ADP or PAR from substrates via hydrolysis. The catalytic efficiency of PAR hydrolysis is lower in CHIKV, SINV and ONNV than in VEEV (Li *et al.* 2016, Eckei *et al.* 2017). Recent studies of the macro domain in CHIKV nsP3 revealed that viral mutants are attenuated in either cell culture or a mouse model when the macro domain's ability to bind or hydrolyze ADP is abolished (McPherson *et al.* 2017, Abraham *et al.* 2018). Interestingly, ADP-ribosylation of cellular proteins is found to be increased with CHIKV infection, which seems to be beneficial for the virus growth (Abraham *et al.* 2018). Also, alterations in macro domain have been shown to contribute the establishment of persistent replication of virus replicons for SINV (deletion of 24-29aa in nsP3) (Akhrymuk *et al.* 2018) or CHIKV (I175L in nsP3) (Utt *et al.* 2015). The persistent replication of CHIKV replicon (CHIKVRepRLuc-FL-5A-PG-IL) is achieved together with alterations in nsP1 (F391L) and nsP2 (insertion of 5aa between 647/648 and P718G) (Utt *et al.* 2015). With help of the SNAP-tag based microscopy method (Remenyi *et al.* 2017), stable CHIKV nsP3 granules are observed to be associated with the persistent

replication of CHIKVRepRLuc-FL-5A-PG-IL, which provides some hints for the pathogenic studies of long-lasting symptoms of CHIKV infection (Remenyi *et al.* 2018).

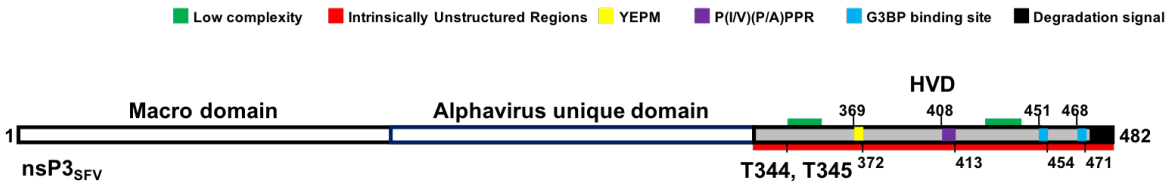


**Fig 2: Schematic sketch of SFV non-structural protein 3 (nsP3$_{SFV}$).** Selected features in nsP3 HVD were displayed with colors as indicated above. T: threonine. Adapted from (Götte *et al.* 2018).

Similarly to the macro domain, the alphavirus unique domain (AUD) also covers ~160 aa ( Kääriäinen and Ahola. 2002). The crystal structure of this domain has been studied as a part of the nsP2-nsP3 polyprotein, revealing a new binding site for zinc ions in the domain. Thus, it is also named zinc-binding domain. The AUD was proven to be essential for viral pathogenesis (Shin *et al.* 2012), and mutational studies correlated the domain to viral RNA synthesis and neurovirulence (De *et al.* 2003, Tuittila and Hinkkanen. 2003). Recently, a mutagenic study revealed that AUD of CHIKV nsP3 plays multiple roles in replication and transcription of virus genome (Gao *et al.* 2019). Some combined mutations on the outer surface of AUD can abolish virus replication in both mammalian and mosquito cells, including R243A/K245A, V260A/P261A or C262A/C264A. The replication of CHIKV mutant harboring mutations P247A/V248A was either abolished or dramatically attenuated, with clear loss of membrane localization of nsP3 complexes and selective and striking reduction in the transcription of CHIKV sgRNA and subsequent synthesis of structural proteins. The reduction of CHIKV sgRNA is partly attributed to the impaired RNA-binding affinity of AUD of CHIKV nsP3 caused by the mutations P247A/V248A (Gao *et al.* 2019).

The HVD of nsP3 is seen as an interaction hub for host factors, considering it is intrinsically unstructured and of low complexity (McInerney 2015). Deletions in the HVD attenuate the virulence of SFV (Vihinen *et al.* 2001, Galbraith *et al.* 2006). The HVD varies in length and sequence between different alphaviruses, but several common features have been studied in this domain as depicted in Fig. 2 (reviewed in (Götte *et al.* 2018)). The N-terminal end of the HVD contains a cluster of phosphorylatable threonine and serine residues and is termed the hyperphosphorylated/acidic region. A mass spectrometry study mapped the major

phosphorylation sites of SFV nsP3 to about 50 residues at the N-terminal end of HVD, with T344 and T345 as the major phosphorylation sites (depicted in Fig. 2) (Vihinen and Saarinen. 2000, Vihinen *et al.* 2001, Galbraith *et al.* 2006). Mutations in the phosphorylation sites showed little effect on viral replication (Foy *et al.* 2013), but the deletion of 50 aa (residues 319–368) from this region in SFV nsP3 resulted in a significant reduction in the activation of the PI3K–Akt–mTOR pathway, an important proviral signaling pathway (discussed below) (Thaa *et al.* 2015). SFV-induced activation of the PI3K–Akt–mTOR pathway is attributed to the YEPM (369–372) motif in the HVD region, which was revealed in this thesis (paper III). Downstream of the hyperphosphorylated/acidic region, a proline-rich region is shared in many alphaviruses. This proline-rich region, including a P[I/V][P/A]PPR motif (Fig. 2), was shown to be the interaction site for the host factors amphiphysin-1 and -2, an interaction that facilitates viral replication (Neuvonen *et al.* 2011). In the rest of the HVD, two conserved sequences, containing two FGDF motifs (depicted in Figure 1), were described (Varjak *et al.* 2010, Panas *et al.* 2015b). These motifs are binding sites for the cellular protein G3BP (Ras-GAP SH3 domain binding protein). Many studies have reported the interaction between nsP3 and G3BP (Cristea *et al.* 2006, Frolova *et al.* 2006, Gorchakov *et al.* 2008) and it is the two FGDF motifs of nsP3 and the NTF2-like domain of G3BP that mediate the interaction (Panas *et al.* 2014, Panas *et al.* 2015b). The main function of G3BP is the nucleation of stress granules (SGs), which have been described as antiviral RNA/protein assemblies in cells. The FGDF-mediated interaction between nsP3 and G3BP sequesters G3BP and counteracts the SG response (Panas *et al.* 2012). In this thesis, the structural and functional relevance of the FGDF-mediated interaction of nsP3 with G3BP was studied in molecular detail (paper I). After the disassembly of SGs, G3BP remains in the viral RCs, indicating its requirement for viral replication beyond the inhibition of the SG response. Knockdown of G3BP results in pronounced inhibition of viral replication for SINV and complete inhibition for CHIKV (Kim *et al.* 2016). The precise roles of G3BP during viral replication are not fully understood, but G3BP has been suggested to facilitate the switch from viral translation to genome amplification by unknown mechanisms (Scholte *et al.* 2015). In addition, other host factors, such as CD2-associated protein (CD2AP) and SH3 domain-containing kinase-binding protein (SH3KBP1), are also interaction partners of SFV and CHIKV nsP3 HVD (Mutso *et al.* 2018).

At the extreme C-terminus, a degradation signal was found in nsP3 of both SFV and SINV and was narrowed down to 6 –10 residues and 36 residues respectively (Varjak *et al.* 2010). This degradation signal is effective when nsP3 is individually expressed, while nsP123 is rather resistant to the degradation. When fused to some reporters, such as EGFP or luciferase, the degradation signal remains functionally effective as well. The biological relevance of the

degradation signal is undefined, but the degradation has been suggested to regulate the ratio of the nsPs (Varjak *et al.* 2010).

NsP3 in Old World alphaviruses has also been suggested as a determinant for mosquito vectors. ONNV is the only alphavirus transmitted by *Anopheles* mosquitos, while most other Old World alphaviruses, such as CHIKV, are transmitted by *Aedes*. A screening study for vector dependence found that a chimeric CHIKV, expressing ONNV nsP3, gained the ability to replicate with a comparable rate as wildtype ONNV in *Anopheles gambiae* mosquitoes (Saxton-Shaw *et al.* 2013).

NsP4 (~70 kDa) mainly serves as the RNA-dependent RNA polymerase (RdRp). The RdRp activity of nsP4, including acting as RNA polymerase and terminal adenylyl-transferase, was demonstrated for SINV by mutational studies and *in vitro* studies with purified nsP4 (Hahn *et al.* 1989, Tomar *et al.* 2006, Rubach *et al.* 2009). The activity of nsP4 is attributed to the C-terminal part (~500 aa) of the protein, which shows sequence homology to other RdRps, containing a conserved GDD motif ( Kääriäinen and Ahola. 2002). NsP4 is less abundant than other nsPs in infected cells for two reasons: the presence of an opal stop codon between the genes for nsP3 and nsP4 in some of the viruses (not SFV) (Strauss *et al.* 1994); and the uncommon tyrosine as the first amino acid of the protein, which leads to rapid degradation of nsP4 through the N-end rule pathway (de Groot *et al.* 1991, Varshavsky 1996). For SINV nsP4, the N-terminal tyrosine is a destabilizing signal, but also necessary for the polymerase activity, since substitutions, except with aromatic residues or histidine, are lethal (Shirako and Strauss. 1998). The N-terminus (~100 aa) of nsP4 is unique in alphaviruses. Mutational studies of SINV nsP4 suggest that the N-terminus of nsP4 mediates the interaction with other nsPs to form different RCs for viral RNA synthesis (Rupp *et al.* 2011).

## 1.3 STRESS GRANULES AND VIRUS INFECTION

Stress granules (SGs) are cytoplasmic, non-membranous aggregates of non-translated messenger ribonucleoprotein particles (mRNPs). SGs are induced by various environmental stresses, including heat shock, oxidative stress, viral infection and UV radiation (Anderson and Kedersha. 2009). Experimentally, sodium arsenite (SA) is frequently used for SG induction. The formation of SGs is seen as an important adaptation of cells exposed to environmental stress (Kedersha and Anderson. 2002). In response to such stress, mRNA is rapidly and selectively sequestered into SGs, leading to the rapid change of translation from housekeeping proteins to stress-related proteins in stressed cells. SGs are treated as sites of mRNA storage and triage, as they are disassembled with the release of mRNA when the stress is removed.

They are highly dynamic structures and keep rapid exchange of mRNPs with the cytoplasm, with some proteins shuttling in and out within seconds (Kedersha *et al.* 2005). The composition of SGs is variable, with over a hundred different identified components. The main components of SGs are translationally stalled mRNA, early translation initiation factors and some RNA binding proteins such as G3BP (discussed below), Caprin-1 and TIA1 (Anderson and Kedersha. 2006). These components and stalled 48S preinitiation complexes are commonly present in all types of SGs, while recruitment of some specific factors to SGs varies with different stress stimuli. For example, Hsp27 was found in SGs induced by heat shock but not SGs induced by arsenite (Kedersha *et al.* 1999). In addition, several signaling molecules were reported to be present in SGs, suggesting SGs as signaling hubs (Kedersha *et al.* 2013).

SG assembly involves multiple steps and usually initiates with the accumulation of non-translated mRNPs. It has been suggested that there is an equilibrium between non-translated mRNA and polysome-bound (translated) mRNA, and that SGs assemble as a result of an excess of non-translated mRNA in the cytoplasm (Kedersha *et al.* 2000). The suggestion is indicated from the modulation of SGs by some drugs. For example, cycloheximide treatment reduces the level of non-translated mRNA by stabilizing polysomes, and consequently, SG induction by sodium arsenite is prevented in the presence of cycloheximide. In contrast, puromycin, which causes premature termination of translation and releases mRNA from polysomes, promotes the assembly of SGs when cells are stressed with sodium arsenite due to the increase of non-translated mRNA ( Kedersha and Anderson. 2002). Translation inhibition can lead to an excess of non-translated mRNPs and hence SG induction. One of the best-studied pathways for SG formation is initiated with the phosphorylation of eIF2α, a subunit of the eukaryotic translation initiation factor eIF2. Phosphorylation of eIF2α affects the formation of the 48S preinitiation complex and inhibits translation initiation, resulting in stalled preinitiation complexes and "run off" of ribosomes. Phosphorylation of eIF2α can be catalyzed by either of four kinases: PKR (double-stranded RNA-activated protein kinase), PERK (PKR-like endoplasmic reticulum kinase), GCN2 (general control nonderepressible 2 kinase) or HRI (heme-regulated kinase) (Dever 2002). Different stimuli activate different kinases that phosphorylate eIF2α. For example, heat shock activates GCN2; oxidative stress activates HRI; and double-stranded RNAs, generated during infection with some RNA viruses, activate PKR. In addition, other pathways have also been described for SG induction, independent of eIF2α phosphorylation (Panas *et al.* 2016). A group of compounds, such as pateamine A, induces SGs via inhibiting the RNA helicase eIF4A (Dang *et al.* 2006). Other compounds, such as hydrogen peroxide, can induce SGs by disrupting the formation of the cap-binding eIF4F complex (Emara *et al.* 2012). Stalled preinitiation complexes have been suggested as the core of SG assembly upon the

inhibition of translation initiation, followed by the recruitment of nucleators for condensation (Panas *et al.* 2016). RNA binding proteins are seen as nucleators of SGs. Some of them can nucleate SGs when overexpressed, even without environmental stress (Kedersha and Anderson. 2007). One common feature of the nucleators is that many of them possess low-complexity and intrinsically disordered protein regions (IDPR) that have high conformational flexibility. These regions can mediate weak interactions, which facilitate the accumulation of IDPR-containing proteins and hence lead to a quick and transient segregation of the proteins into a subcellular compartment (Nott *et al.* 2015). In addition to conformational flexibility, IDPR proteins can also quickly achieve post-translational modifications, which usually leads to a fast change of their structural conformation and subsequent interactions. The fact that they contain IDPR regions enables SG nucleators to convey the highly dynamic processes of SG formation and maintenance (Panas *et al.* 2016).

G3BP is one of the SG-nucleating proteins and an essential component for SG formation. The protein, with the full name "Ras GTPase-activating protein-binding protein", was first described as a binding partner to the SH3 domain of the Ras GTPase-activating protein (GAP) (Parker *et al.* 1996). There are two isoforms, G3BP1 and G3BP2 (collectively referred to as G3BP), sharing 74% amino acid sequences similarity. Both G3BP1 and G3BP2 are ubiquitously expressed in most cells, as homodimers and/or heterodimers (Matsuki *et al.* 2013). Single knockdown of either G3BP1 or G3BP2 results in the increase in expression of the other, indicating a compensaroty relationship of the two isoforms (Matsuki *et al.* 2013). Only simultaneous knockdown of both G3BP1 and G3BP2 renders the cells unable to form stress granules upon eIF2α dependent and eIF2α independent stresses (Kedersha *et al.* 2016). G3BP consists of four domains: a nuclear transport factor 2 (NTF2) like domain, a proline-rich domain, an RNA-recognition motif (RRM) and an arginine-glycine-glycine (RGG) motif (Tourriere *et al.* 2003). The NTF2-like domain displays sequence homology to the cytoplasmic nuclear transport factor 2 (NTF2), which transports cargo into the nucleus (Clarkson *et al.* 1996). However, G3BP does not function as a nuclear transport factor. Instead, the NTF2-like domain is a binding site for proteins containing FGDF motifs, including ubiquitin-specific protease 10 (USP10) and some viral proteins, such as alphavirus nsP3 and herpes simplex virus (HSV) protein ICP8 (Panas *et al.* 2015b). Interaction of G3BP with Caprin-1 or USP10 was shown to be involved in the assembly or disassembly of SGs (Kedersha *et al.* 2016). The viral proteins with FGDF motifs, in particular nsP3 of alphaviruses, mimic this disassembly principle of SG modulation (Panas *et al.* 2015b). The NTF2-like domain has also been suggested to contribute to dimerization of G3BP (Tourriere *et al.* 2001). The proline-rich domain is thought to bind to the SH3 domain of Ras GAP (Parker *et al.* 1996). The RRM motif

mediates the interaction of G3BP with nucleotides of RNA (Nagai *et al.* 1995, Kennedy *et al.* 2001). The RGG domain of G3BP has been recently revealed as essential for SG competence and the interaction with 40S ribosome subunits (Kedersha *et al.* 2016). In a reconstituted cell line expressing only a truncated version of G3BP lacking the RGG domain, induction of SGs by sodium arsenite was blocked and interactions were abolished between G3BP and some factors of 40S ribosome subunits (Kedersha *et al.* 2016).

SG assembly is important for cell adaptation in response to environmental stress, including viral infection. Most viruses have evolved different mechanisms to modulate and subvert SG assembly at some point during infection (reviewed in (Valiente-Echeverria *et al.* 2012, Lloyd 2013, Reineke and LIoyd. 2013)). The viral modulation of the SG response can be categorized into 3 different types: (a) inhibition of SG induction, such as upon infection with some flaviviruses; (b) induction and maintenance of SGs during infection, such as upon vaccinia virus infection; and (c) induction of SGs at early stages of infection followed by disassembly later, e.g. upon poliovirus or alphavirus infection (White and LIoyd. 2012). Our previous work has revealed that alphaviruses antagonize SGs by sequestering the SG nucleating protein G3BP into RCs. This sequestration is mediated by the interaction between FGDF motifs of nsP3 and the NTF2-like domain of G3BP (Panas *et al.* 2012, Panas *et al.* 2014, Panas *et al.* 2015b). Sequestration of G3BP into viral RCs suppresses SG assembly. In addition, this interaction is essential for efficient viral replication, but the reasons for this have remained unclear (Panas *et al.* 2012, Kim *et al.* 2016, Schulte *et al.* 2016). One aim of this thesis is to further characterize this interaction and its functions.

## 1.4 RNA PROCESSING BODIES AND VIRUS INFECTION

RNA processing bodies (P-bodies or PBs) are discrete RNA granules consisting of translationally suppressed mRNPs. The main components enriched in P-bodies are translation-repressed mRNA and factors involved in mRNA decay, such as decapping enzymes, deadenylases, exonucleases, and some RNA-binding proteins involved in nonsense-mediated decay or microRNA-mediated silencing (Decker and Parker. 2012, Lloyd 2013). Thus, P-bodies are usually considered as degradation sites of mRNA (Arribere *et al.* 2011).

Direct mRNA degradation is carried out by three ribonucleases (RNases): endonucleases that initiate cleavage within the body of mRNA, 5′ exonucleases that digest mRNA in 5′ – 3′ direction, and 3′ exonucleases that remove nucleotides from the 3′ end of mRNA (Houseley and Tollervey. 2009). Most mRNA molecules in eukaryotic cells carry a 5′ cap structure and 3′ poly (A) tail, two features which ensure translation and also protect mRNA from digestion

by exonucleases. In order to degrade mRNA, the cap structure and/or the poly (A) tail need to be removed to give access to exonucleases. The majority of mRNA decay is initiated by deadenylation, the removal of the poly (A) tail at the 3′ end. In mammalian cells, the poly (A) tail of mRNA molecules is first shortened by Pan2 in association with Pan3 to about 110 nucleotides, followed by the further shortening mediated by the Ccr4-NOT complex (Chen and Shyu. 2011). Deadenylation of mRNA is seen as the rate-limiting step and also a reversible process. Thus, deadenylation is treated as a critical checkpoint for mRNA before it is committed for decay (Chen and Shyu. 2013). Interestingly, recent studies implicate that uridylation at the 3′ end of mRNA may also serve as a trigger for mRNA degradation (Labno *et al.* 2016).

After removal of the poly (A) tail, mRNA decay can be carried out either by exosomes from 3′ to 5′, or alternatively by the exonuclease XRN1, which requires the removal of the 5′ cap structure (decapping). The whole process of decapping is well regulated with mRNA decapping enzyme 2 (Dcp2), acting together with many decapping enhancers, such as Dcp1a, Hedls, the protein complex Lsm1–7, and the helicase DDX6 (Rck/p54) (Narayanan *et al.* 2013). Notably, decapping is thought to be an irreversible event that targets the mRNA for degradation by XRN1 and hence serves as a backup pathway/checkpoint for mRNA decay via deadenylation (Chen and Shyu. 2013). The endonucleolytic degradation of mRNA can be mediated by the RNA-induced silencing complex (RISC). Other pathways targeting certain types of mRNA for degradation are triggered by deadenylation as well, including degradation of mRNA containing AU-rich elements (ARE), nonsense-containing RNA and microRNA-targeting RNA (Chen and Shyu. 2013).

Several studies provide experimental evidence that P-bodies are mRNA degradation sites: For example, stabilization of mRNA by cycloheximide protects mRNA from degradation and results in dispersion of P-bodies (Cougot *et al.* 2004), and P-bodies are dispersed when deadenylation is inhibited (Zheng *et al.* 2008). In contrast, there are other studies showing that the processes of mRNA degradation also proceed in the absence of P-bodies, including mRNA decay, nonsense-mediated mRNA decay and RNA-mediated gene silencing (Eulalio *et al.* 2007, Stalder and Muhlemann. 2009).

Both deadenylation and decapping are highly related to P-body dynamics. P-bodies are enriched with factors directly involved in these processes, such as the deadenylation factors Pan2, Pan3 and Ccr4 (Zheng *et al.* 2008) and the decapping factors Dcp1, Hedls and DDX6 (Cougot *et al.* 2004). When deadenylation is impaired by knocking down Caf1, a catalytic enzyme in the Ccr4-NOT complex, or by expressing a dysfunctional Caf1, P-bodies are

dispersed, suggesting that their formation depends on mRNA deadenylation (Zheng *et al.* 2008). Based on these results, a model for P-body formation driven by deadenylation has been suggested: when the poly (A) tail of mRNA is shortened by Pan2-Pan3 as the first step of deadenylation, mRNA is dissociated from the poly (A) binding protein complex, which enables the access of Ccr4-NOT for the second step of deadenylation. The first shortening step is reversible and seen as the start of P-body formation. At this stage, mRNA can either resume translation or proceed to further deadenylation and subsequent decapping, which depends on the recruitment of decapping factors. The complexes consisting of deadenylation and decapping factors are thought to be the core of P-bodies (Chen and Shyu. 2013). However, this model is challenged by a recent study, in which P-bodies were successfully purified via fluorescently labeled key P-body components, followed by transcriptomic profiling, which revealed that mRNA aggregated in P-bodies contains normal poly (A) tails and lacks any sign of degradation (Hubstenberger *et al.* 2017). Two other recent studies tracked the life cycle of tagged mRNAs in live cells and observed that mRNA degradation occurs diffusely in the cytoplasm rather than in P-bodies (Horvathova *et al.* 2017, Tutucci *et al.* 2018). All these results suggest P-bodies as storage sites of translationally repressed mRNPs.

Results of decapping studies however suggest a kinetic model for P-body formation driven by an excess of translationally repressed mRNPs (Franks *et al.* 2008). This model is mainly based on the fact that many decapping factors possess dual functions as both translation suppressors and decapping activators. Observations in favor of this model include: Treatment of cells with cycloheximide reduces free mRNA in the cytoplasm and leads to a rapid dispersion of P-bodies (Cougot *et al.* 2004); in contrast, puromycin causes premature termination of translation and release of polysome-free mRNA, which contributes to P-body formation under stress (Eulalio *et al.* 2007); recent data show that interactions between DDX6 and 4E-transporter (4E-T) mediate translation suppression and are essential for P-body assembly (Ayache *et al.* 2015, Kamenska *et al.* 2016). This model also suggests that P-bodies serve as mRNA decay and storage sites, as translation-suppressed mRNA are not always committed for decay. Nevertheless, regarding the formation and dispersal of P-bodies, the signaling cascades and mechanisms are unknown and less well studied than those for SGs. In addition to deadenylation and decapping pathways, intact cellular transport networks are also important for the transport and dynamic changes of P-bodies (Aizer *et al.* 2008).

P-bodies may have a dual role as mRNA decay and storage sites. Through live cell imaging, tagged mRNA was found to reside in P-bodies under stress conditions and to be gradually released from P-bodies to the cytoplasm when stress was removed (Aizer *et al.* 2014). Similar

to SGs, P-bodies respond to different stimuli and vary in number and size when translation arrest occurs. Furthermore, physical links between P-bodies and SGs have been shown by live cell imaging. Some proteins are found in both SGs and P-bodies (Kedersha *et al.* 2005). When overexpressing tristetraprolin, a shared component of SGs and P-bodies, these two types of cytoplasmic foci are transiently positioned in close proximity with each other, indicating direct communication between these two structures (Kedersha *et al.* 2005). Multiple connections between polysomes, SGs and P-bodies have been observed. This observation provides the experimental basis for the mRNA cycle model, which states that cytoplasmic mRNA can exist and be transported between the three structures (Balagopal and Parker. 2009).

In summary, mRNA deadenylation and decapping are both connected to P-bodies, while the relative contribution of these processes to P-body formation and dynamics is rather unclear. P-bodies may serve as mRNA decay and/or storage sites with potential links to SGs.

Virus infections can manipulate P-bodies in different ways (reviewed in (Pattnaik and Dinh. 2013, Poblete-Duran *et al.* 2016)). Currently, most viruses shown to alter P-bodies during infection are RNA viruses, of which the majority are positive-strand RNA viruses. In cells infected with hepatitis C virus (HCV) or West Nile virus (WNV), P-bodies are dispersed or reduced in number, with some P-body components hijacked or recruited to the virus replication sites (Ariumi *et al.* 2011, Chahar *et al.* 2013). During poliovirus infection, P-bodies are dispersed concomitantly with the accelerated degradation of some P-body scaffold proteins, including the decapping factors Dcp1a and XRN1 as well as the deadenylase complex component Pan3 (Dougherty *et al.* 2011). These results suggest that P-bodies are disassembled during poliovirus infection by inhibition of deadenylation (Dougherty *et al.* 2011), considering that deadenylation has been shown to be required for P-body formation (Zheng *et al.* 2008). Follow-up studies from the same group added that disassembly of P-bodies is mainly attributed to virus proteases, as individual expression of viral proteases, including 3CD, 2A(pro) and 3C(pro), repressed or dispersed P-bodies (Dougherty *et al.* 2015). The negative-strand RNA virus influenza A virus also interferes with the formation of both SGs and P-bodies. Viral non-structural protein 1 (NS1) interacts with RNA-associated protein 55 (RAP55, also known as LSM14A), a shared protein in both SGs and P-bodies. Overexpression of NS1 inhibits the assembly of both RNA granules (Mok *et al.* 2012). So far, little is known about the fate of P-bodies during alphavirus infection, which is investigated in this thesis.

## 1.5 THE PI3K–AKT–MTOR PATHWAY AND VIRUS INFECTION

The PI3K–Akt–mTOR pathway is one of the most important pathways responsible for cell survival in different environments, by regulating the transition between cell anabolism and catabolism. Many cellular processes, such as cell proliferation, cell division, apoptosis and cell metabolism, are controlled or affected by this pathway (reviewed in (Manning and Cantley. 2007, Wong *et al.* 2010, Laplante and Sabatini. 2012)).

The activation of the pathway typically starts with the binding of a growth factor such as insulin to its receptor at the plasma membrane, which induces dimerization of the receptor and phosphorylation of a tyrosine residue in the cytoplasmic tail of the receptor or an associated protein. Phosphorylated tyrosine in the sequence context YXXM leads to activation of PI3K, class IA. Class IA PI3Ks consist of a regulatory subunit p85 and a catalytic subunit p110, with p85 serving as a stabilizer and inhibitor of the p110 subunit. In mammalian cells, there are three isoforms of the p110 subunit (α, β, and δ) and two isoforms of the p85 regulatory subunit (α, β). Subunit p85 contains two Src homology 2 (SH2) domains, which interact with phospho-tyrosine in YXXM motifs (Songyang *et al.* 1993). Through this interaction, active p110 is released and recruited to the plasma membrane, where it converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) to phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$). $PI(3,4,5)P_3$ subsequently facilitates the phosphorylation and activation of the protein kinase Akt, by recruiting both Akt and its kinase phosphoinositide-dependent kinase 1 (PDK1) into close proximity at the plasma membrane. Both proteins contain a pleckstrin-homology domain for binding to $PI(3,4,5)P_3$. Full activation of Akt requires phosphorylation of two amino acids: T308, phosphorylated by PDK1; and S473, the phosphorylation of which is primarily attributed to the mTOR–rictor complex (mTORC2) (Backer *et al.* 1992, Rordorf-Nikolic *et al.* 1995, Yu *et al.* 1998). Full activation of Akt results in phosphorylation of many substrates related to cell metabolism and survival. The mechanistic target of rapamycin (mTOR) in the mTOR–raptor complex (mTORC1) is one of the main downstream substrates of Akt. Activation of mTORC1 promotes synthesis of proteins and lipids and keeps cells in a pro-survival state.

Virus infection often interferes with the PI3K–Akt–mTOR pathway (reviewed in (Diehl and Schaal. 2013, Le Sage *et al.* 2016)). In regards to Old World alphaviruses, CHIKV activates the pathway to a moderate level but SFV hyperactivates the pathway, in which the hyperactivation of the pathway is demonstrated as strong, persistent activation, even sustained in starved cells, where the pathway is normally inactive (Thaa *et al.* 2015). The mechanistic basis of PI3K–Akt–mTOR hyperactivation in SFV is investigated in this thesis.

# 2 RESULTS

## 2.1 RESULTS PAPER I

Previous studies have revealed that the FGDF motif directly binds to the NTF2-like domain of G3BP and is necessary and essential for the interaction (Panas *et al.* 2014, Panas *et al.* 2015b). The motif is found in many cellular proteins and also in some viral proteins, such as alphavirus nsP3 (Panas *et al.* 2015b). There are duplicates of the FGDF motif, $FGDF_N$ and $FGDF_C$, in alphavirus nsP3, including $nsP3_{SFV}$ and $nsP3_{CHIKV}$, in contrast to a single copy of the motif in the cellular proteins. The crystal structure was previously studied for the NTF2-like domain of G3BP2 in complex with a short FGDF containing peptide LT**FGDF**DE, demonstrating that the peptide binds to the hydrophobic pocket of the NTF2-like domain (Kristensen 2015). The peptide used in that study however only corresponds to the $FGDF_N$ in SFV-nsP3 and may not reflect the full binding mode of the two FGDF motifs in nsP3 to the NTF2-like domain of G3BP. Thus, we investigated the structural and biological significance of the duplicate FGDF motifs in alphavirus nsP3. To answer these questions, we started with $nsP3_{SFV}$ studies. Through collaboration, the crystal structure was solved for the NTF2-like domain of G3BP1 (Fig. 3A, left-hand side) in complex with a peptide derived from $nsP3_{SFV}$ harboring both $FGDF_N$ and $FGDF_C$ motifs (residue 449 473, $nsP3_{SFV}$-25, LT**FGDF**DEHEVDALASGIT**FGDF**DD, see Fig. 3A, right-hand side). Considering that the NTF2-like domain of G3BP1 forms homodimers $(G3BP1)_2$, our structure data show that each $nsP3_{SFV}$-25 peptide can engage with two $(G3BP1)_2$, leading to the formation of a poly-complex of $(G3BP1)_2$ interconnected by $nsP3_{SFV}$-25 (Fig. 3B). This structure suggests an oligomer-inducing function of alphavirus nsP3.

To further characterize the two FGDF motifs, recombinant constructs were generated to express the 36 C-terminal residues (447–482) of $nsP3_{SFV}$ fused to the C-terminus of EGFP. These constructs either carry the natural sequence (EGFP-36-WT) or a mutation, Phe to Ala, in the $FGDF_N$ (EGFP-36-$F3A_N$), $FGDF_C$ (EGFP-36-$F3A_C$) or both motifs (EGFP-36-$F3A_{NC}$). Using these constructs, we studied the interaction between the endogenous G3BP1 and the individual FGDF motifs in HEK293 cells after transfection with either of the constructs or the empty EGFP vector. Cells were lysed at 24 hours post transfection (hpt) and the lysates were subjected to immunoprecipitation with anti-GFP antibodies, followed by immunoblotting for GFP, G3BP1 and actin. EGFP-36-WT bound efficiently to G3BP1, while EGFP-36-$F3A_{NC}$ did not bind at a detectable level (Paper I, Fig. 4 (a)), which is consistent with our previous studies

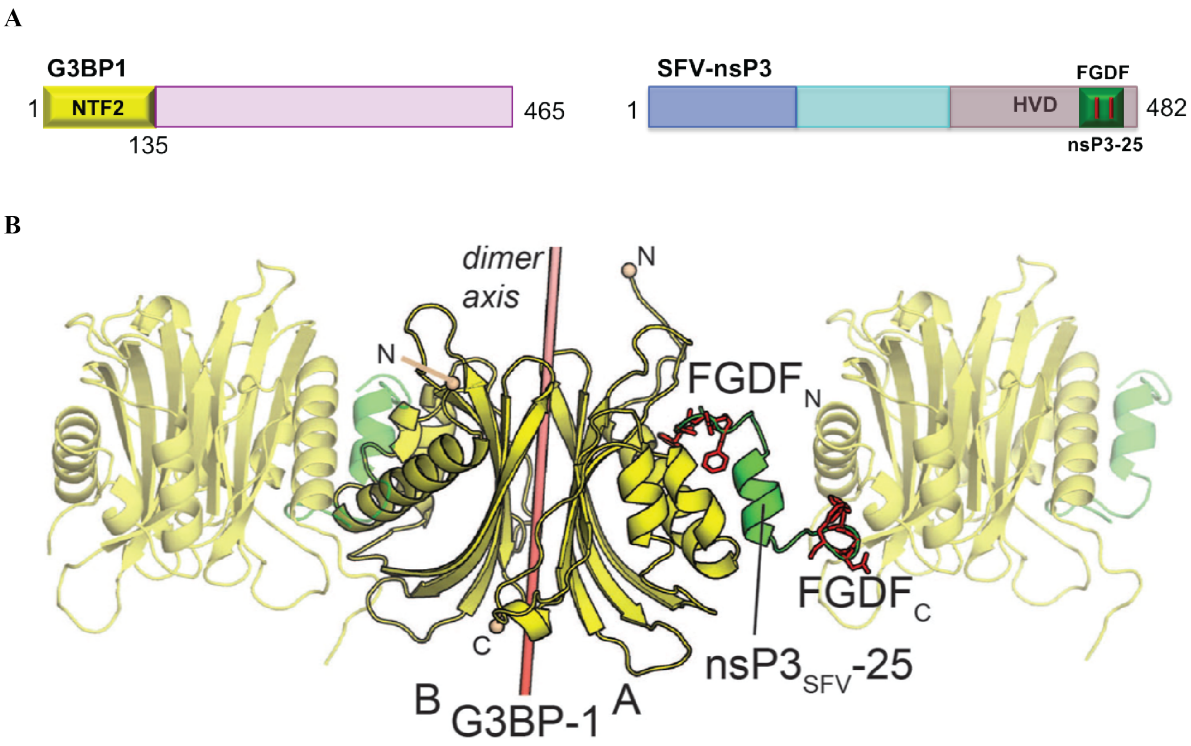


**Fig. 3: The two FGDF motifs in nsP3$_{SFV}$-25 inter-connect (G3BP1)$_2$ into a poly-[(G3BP1)$_2$: nsP3$_{SFV}$-25] complex. (A)** A sketch of G3BP1 and nsP3$_{SFV}$.The NTF2-like domain of G3BP1 and the nsP3-25 region in nsP3 used for crystal structure studies are highlighted. **(B)** The dimer of the NTF2-like domain of G3BP1and a single nsP3$_{SFV}$-25 peptide molecule are displayed in yellow and green, respectively. The pink bar represents the twofold pseudo-symmetry axis between each G3BP1 subunit. While chain A of G3BP1 binds to the N-terminal nsP3$_{SFV}$-25 FGDF motif (FGDF$_N$, highlighted in red) comprising Phe-451 to Phe-454, the chain B of G3BP1 binds to the C-terminal FGDF motif (FGDF$_C$) comprising Phe-468 to Phe-471, thus creating a multimeric assembly of [(G3BP1)$_2$: nsP3$_{SFV}$-25] complexes. The [(G3BP1)$_2$: nsP3$_{SFV}$-25] complexes of two adjacent asymmetric units are displayed semi-transparent. The N- and C-termini of the NTF2-like domain of G3BP1 are highlighted as spheres.

(Panas *et al.* 2015b). The data for G3BP1 and the nsP3 mutants with single FGDF mutations revealed a weak interaction of G3BP1 with EGFP-36-F3A$_N$ but a very strong interaction with EGFP-36-F3A$_C$ (Paper I, Fig. 4 (a)). These different interaction intensities were confirmed with densitometric analysis of the G3BP1 bands. We also studied the effect of other regions in nsP3$_{SFV}$ on the interaction with G3BP1, by expressing either the HVD region (residues 319–482) or the full length of nsP3 carrying the correspondent mutations in the same backbone vector as fusion proteins with EGFP. Highly similar results were observed regardless of the length of nsP3$_{SFV}$ truncation: WT version and single mutant F3A$_C$ always efficiently bound G3BP1, whereas the double mutant F3A$_{NC}$ did not bind to detectable levels and the single mutant F3A$_N$ bound much less G3BP1 than the corresponding nsP3-WT (Paper I, Fig. 4 (b) and (c)). The results of these studies in cells revealed a hierarchical binding mode for the

duplicate FGDF motifs and suggest a higher binding affinity to G3BP1 for $FGDF_N$ than $FGDF_C$.

In addition, we also expressed and purified EGFP-$nsP3_{SFV}$-36, analogous to EGFP-36 used for the studies in cells, in all variants (WT, $F3A_N$, $F3A_C$, $F3A_{NC}$) to investigate the binding to G3BP1 *in vitro*. Through collaboration, the binding affinities were analyzed by microscale thermophoresis (MST) between the purified NTF2-like domain of G3BP1 and the EGFP-$nsP3_{SFV}$-36 proteins. The MST results show that $FGDF_N$ has higher binding affinity to the NTF2-like domain of G3BP1 than $FGDF_C$ (Paper I, Fig. 3 (b)), supporting the observations from our studies in cells.

The next question was whether the FGDF motifs of SFV nsP3 behave similarly in the context of infection. To address this question, the respective mutations were introduced into the SFV genome (as depicted in Fig. 4A) and correspondent recombinant viruses, i.e. SFV-$F3A_N$, SFV-$F3A_C$ and SFV-$F3A_{NC}$, were rescued from infectious clones. The interaction between nsP3 and G3BP1 was studied after infection of baby hamster kidney (BHK) cells with SFV-WT or the FGDF-mutated recombinant viruses. Cells were lysed at 8 hours post infection (hpi) and subjected to immunoprecipitation with nsP3 antiserum. Subsequent immunoblotting showed that WT nsP3 efficiently interacted with G3BP1 and that $F3A_C$ $nsP3_{SFV}$, with the intact $FGDF_N$ motif, bound nearly half (49.5 ± 2.0%) of G3BP1 in comparison to WT nsP3, according to densitometric analysis of the G3BP1 bands (Fig. 4B). The other two nsP3 mutants, $F3A_N$ $nsP3_{SFV}$ and $F3A_{NC}$ $nsP3_{SFV}$ did not bind G3BP1 at any detectable level, as shown in the densitometry results (Fig. 4B). The results from virus infection studies support the model that two FGDF motifs bind two G3BP1 dimers and a single FGDF motif binds one G3BP dimer, with the binding of $FGDF_C$ happening only when $FGDF_N$ binds first.

G3BP1 is recruited to viral replication complexes (RCs) during SFV infection, which is dependent on its interaction with FGDF motifs in nsP3 (Panas *et al.* 2012). Recruitment of G3BP1 to RCs was tested by immunofluorescence in BHK cells after infection with the same panel of viruses as used for the immunoprecipitation studies displayed in Fig 4B. Infected cells were fixed at 8hpi and probed for nsP3 and G3BP. The results show that robust G3BP1 signals accumulated with WT $nsP3_{SFV}$, indicating efficient recruitment of G3BP1, while the colocalization of G3BP1 was intermediate with $F3A_C$ $nsP3_{SFV}$ and only seen with a small proportion of $F3A_N$ $nsP3_{SFV}$ and very weak with $F3A_{NC}$ $nsP3_{SFV}$ (Fig. 4C).

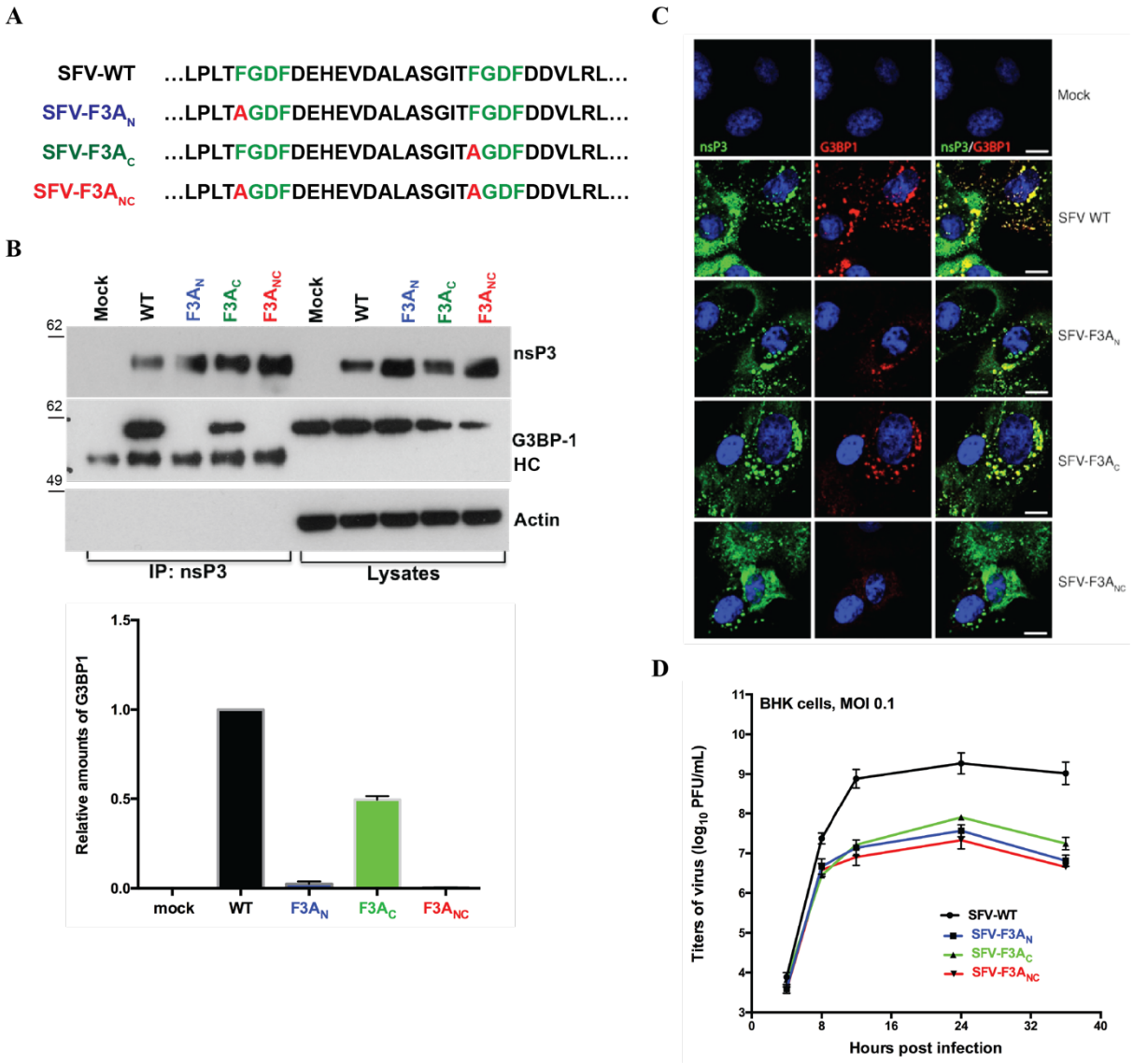


**Fig. 4**: **Both FGDF motifs are necessary for efficient SFV replication. (A)** FGDF motifs in nsP3 of SFV and mutations thereof. **(B)** BHK cells were mock-infected or infected at MOI 10 with SFV WT, -F3A$_N$, -F3A$_C$ or -F3A$_{NC}$. At 8 hours post infection (hpi), cell lysates were prepared and subjected to immunoprecipitation with nsP3 antiserum and separated by SDS–PAGE. Lysates and IPs were probed for nsP3, G3BP1 or actin. Representative result. HC, immunoglobulin heavy chain. Lower panel, densitometry of the intensities of G3BP1 bands in IPs as displayed in the upper panel and displayed relative to SFV WT. Data are averages from two experiments, and error bars are standard deviations. **(C).** Cells infected as in (B) were fixed at 8 hpi and stained for nsP3 (green) and G3BP1 (red). Nuclei in blue. Scale bar 10 µm. **(D).** BHK cells were infected with the indicated viruses at an MOI of 0.1. At 4, 8, 12, 24 and 36 hpi, supernatants were collected and SFV titers were quantified by plaque assay in BHK cells. Data are means of two to four independent experiments. Error bars indicate standard error of the mean (SEM).

Previous studies demonstrated that SFV-F3A$_{NC}$, in which both FGDF motifs are mutated, is attenuated (Panas *et al.* 2015b). We further studied how virus growth was affected with single mutations in the motifs. Multiple and single step growth curves were determined in BHK cells

after infection with SFV-WT, SFV-F3A$_N$, SFV-F3A$_C$ or SFV-F3A$_{NC}$ at low (0.1) and high (10) multiplicity of infection (MOI), respectively. Released viruses were collected at the indicated time points and titrated in BHK cells by plaque assay. Growth curves at both low (Fig. 4D) and high MOI (Paper I, Fig. 5 (c)) show that all three virus mutants were able to grow, but significantly slower than WT virus after 8hpi, and also produced much less progeny than WT virus. Overall, the results from infection studies demonstrate that only duplicate FGDF motifs promote efficient virus growth, which is likely attributed to the efficient interaction with G3BP and subsequent sequestration of the protein to viral replication complexes.

The duplicate FGDF motifs are also conserved in nsP3 of CHIKV (nsP3$_{CHIKV}$) (Varjak *et al.* 2010), mediating the interaction with G3BP (Panas *et al.* 2014). We asked whether the knowledge gained from SFV was transferable to the more important human pathogen CHIKV. Based on the sequence homology, a structural model was built for the NTF2-like domain of G3BP1 in complex with a similar peptide nsP3$_{CHIKV}$-23 derived from nsP3$_{CHIKV}$ via secondary structure predictions (Paper I, Fig. 6). The model predicts that nsP3$_{CHIKV}$-23 binds two G3BP1 molecules in a similar way as demonstrated with nsP3$_{SFV}$-25 (Paper I, Fig. 6). However, the peptide sequence for formation of an alpha helix between the two FGDF motifs are three amino acids longer in nsP3$_{CHIKV}$ than in nsP3$_{SFV}$, which results in an elongated helix and a shift of the FGDF$_C$ motif in nsP3$_{CHIKV}$ (Fig. 5A). This shift of FGDF$_C$ is predicted to cause a less stable interaction with G3BP1, likely due to the exposure of the hydrophobic face of the helix that is covered by G3BP1 in the case of nsP3$_{SFV}$ (Paper I, Fig. 6). The significance of the individual FGDF motifs in nsP3$_{CHIKV}$ regarding binding to G3BP1 was assessed in experiments analogous to those conducted for SFV. Full length nsP3$_{CHIKV}$ was expressed as EGFP fusion protein in HEK293 cells with WT motifs (nsP3$_{CHIKV}$-WT), mutations in either of the duplicate FGDF motifs (F479A, nsP3$_{CHIKV}$-F3A$_N$ or F497A, nsP3$_{CHIKV}$-F3A$_C$) or in both motifs (nsP3$_{CHIKV}$-F3A$_{NC}$). At 24hpt, cells were lysed and subjected to anti-GFP immunoprecipitation. Immunoblotting results for GFP, nsP3$_{CHIKV}$ and actin revealed a similar hierarchical binding mode of nsP3$_{CHIKV}$ to G3BP1, with strong and efficient G3BP1 binding to nsP3$_{CHIKV}$-WT or nsP3$_{CHIKV}$-F3A$_C$, while the binding to nsP3$_{CHIKV}$-F3A$_N$ or nsP3$_{CHIKV}$-F3A$_{NC}$ was much weaker or non-noticeable, which mirrors the results for nsP3$_{SFV}$ (Fig. 5B). The single mutations were then introduced to the CHIKV genome to evaluate the contribution of the individual FGDF motifs to G3BP1 binding in the context of virus infection. During our attempts to rescue the recombinant CHIKV mutants, CHIKV-F3A$_N$, CHIKV-F3A$_C$ or CHIKV-F3A$_{NC}$ after transfection of BHK cells, we noticed huge delays for the development of cytopathic effect, which appeared at 60hpt for CHIKV-F3A$_N$ and CHIKV-F3A$_C$ and 84hpt for CHIKV-F3A$_{NC}$

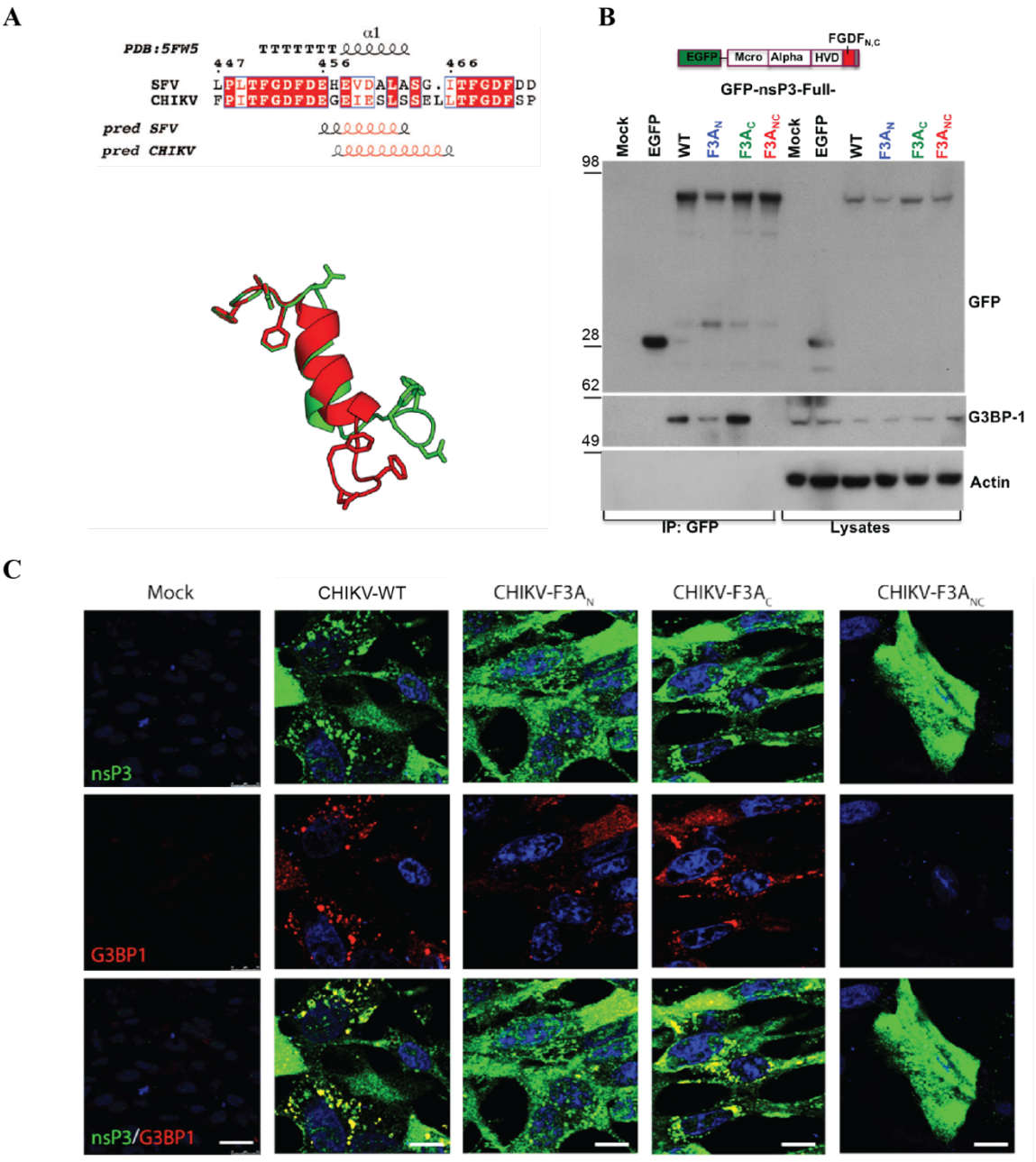


**Fig. 5**: **Both FGDF motifs are necessary for efficient CHIKV replication. (A) Upper panel**: The sequence alignment of the FGDF motif containing residues from nsP3$_{SFV}$ (Uniprot ID P08411) and nsP3$_{CHIKV}$ (Q5XXP4) highlights the two conserved N- and C-terminal (L/I)TFGDFD motifs. Residue numbers indicate nsP3$_{SFV}$. A helical spacer region (highlighted in red) was predicted for both FGDF motif containing residues of nsP3$_{SFV}$ and nsP3$_{CHIKV}$, as revealed in the nsP3-25$_{SFV}$ crystal structure. **Lower panel:** Structural modelling of the FGDF-motif-containing peptide sequence of nsP3$_{CHIKV}$ (red) suggests a longer helical region than that of nsP3$_{SFV}$ (green), with a rotated and shifted FGDFc motif. **(B)** HEK293 cells were mock-transfected or transfected with pEGFP, pEGFP-nsP3$_{CHIKV}$-WT, -F3A$_N$, -F3A$_C$ or -F3A$_{NC}$. Cells were lysed at 24hpt and subjected to immunoprecipitation with anti-GFP antibody. Then, samples were separated by SDS–PAGE and analyzed by immunoblot for GFP, G3BP-1 or actin as indicated. **(C)** BHK cells were mock-transfected or transfected with infectious clone plasmids expressing replication-competent RNA of CHIKV-wt, -F3A$_N$, -F3A$_C$ or -F3A$_{NC}$, followed by fixation and immunofluorescence staining at 24hpt for nsP3$_{CHIKV}$ (green) and G3BP1 (red). Nuclei in blue. Scale bar, 10 μm.

compared to 48hpt for CHIKV-WT. This indicates that mutations in both FGDF motifs are lethal to CHIKV, likely due to the failure to bind and recruit G3BP. After plaque purification of virus from BHK cells transfected with the CHIKV-F3$A_{NC}$ infectious clone, reversion of the N-terminal FGDF motif was noticed by sequence analysis. Later, our collaborator's results revealed another rescue strategy for CHIKV-F3$A_{NC}$, supporting the conclusion that mutations in both FGDF motifs are lethal to CHIKV. The results from our collaborators also suggest the revision of N-terminal FGDF motif could be a consequence of CHIKV-F3$A_{C}$ contamination. This failure to rescue CHIKV-F3$A_{NC}$ precluded any experiment with the full panel of rescued CHIKV mutants. Therefore, the interaction of nsP3$_{CHIKV}$ and G3BP1 in the viral context was investigated by immunofluorescence after transfection with the CHIKV infectious clones in BHK cells. At 24hpt, cells were fixed and probed for nsP3$_{CHIKV}$ and G3BP1. Our results show that the G3BP1 signal colocalized with nsP3$_{CHIKV}$-WT was strong, indicating efficient recruitment of G3BP1 to viral RCs, while the colocalization of the G3BP1 signal was weaker with nsP3$_{CHIKV}$-F3$A_{C}$ and barely detectable with nsP3$_{CHIKV}$-F3$A_{N}$ or nsP3$_{CHIKV}$-F3$A_{NC}$ (Fig. 5C). Thus, the properties of the FGDF-mediated interaction between nsP3 and G3BP are mostly conserved between nsP3$_{SFV}$ and nsP3$_{CHIKV}$. Overall, these results demonstrate that the duplicate FGDF motifs in nsP3 are essential for efficient growth of SFV and CHIKV.

## 2.2 RESULTS PAPER II

P-bodies are dynamic, non-membranous RNA granules in the cytoplasm. In order to investigate the fate of P-bodies during alphavirus infection, we first validated two commonly used P-body markers, Dcp1a and DDX6, in the cell lines used in this project, including MEFs (mouse embryo fibroblasts), BHK (baby hamster kidney cells) and U2OS (human bone osteosarcoma epithelial cells). Under normal conditions or after sodium arsenite (SA) treatment, P-bodies could be defined as discrete foci by immunofluorescence with antibodies for Dcp1a and DDX6, with both proteins colocalizing to a large extent in MEFs (Paper II, Fig. 1 (a)), BHK and U2OS cells (data not shown). It is worth mentioning that a small portion of P-bodies were stained only

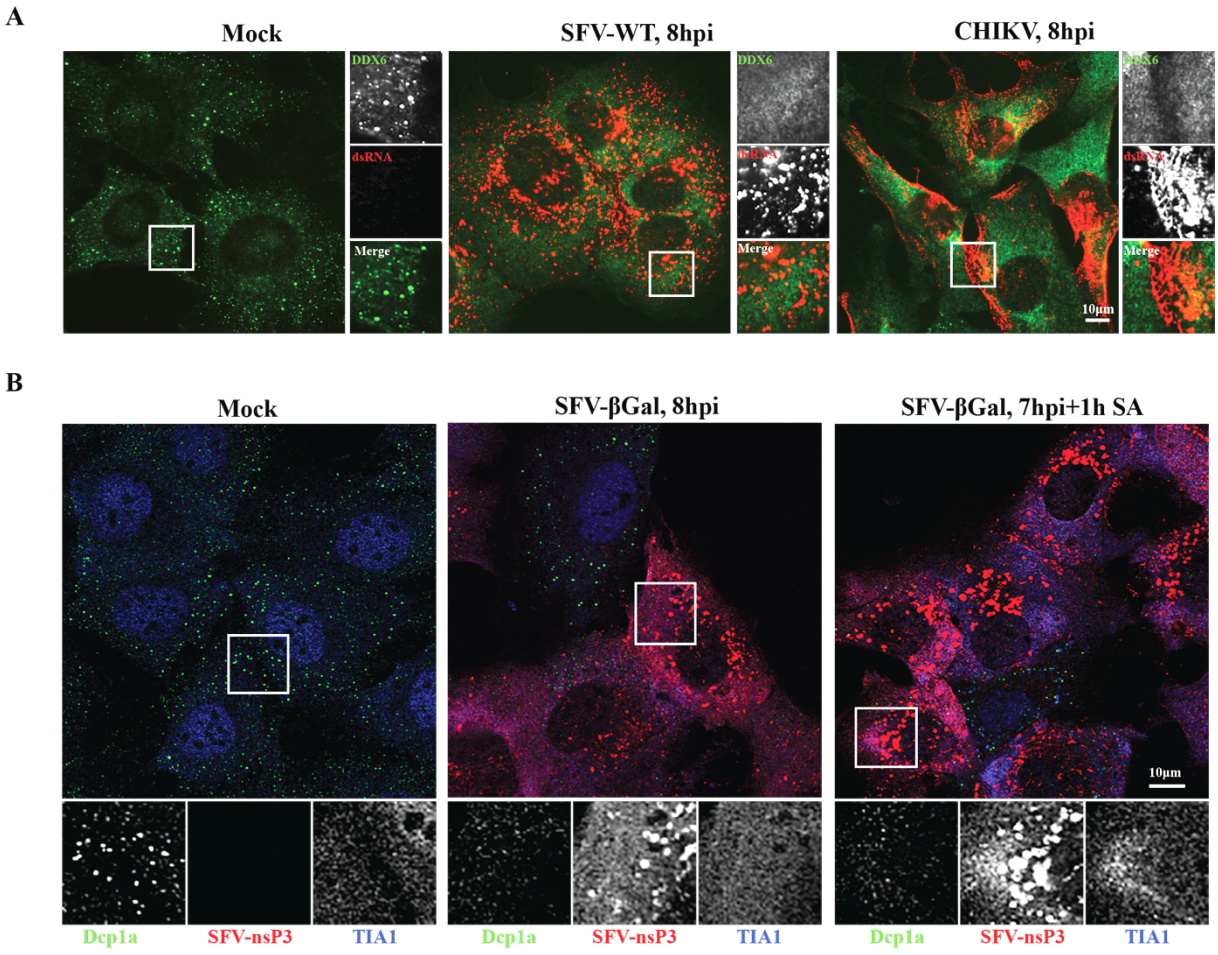


**Fig. 6. Disassembly of P-bodies in cells upon infection with Old World alphaviruses, independent of viral structural proteins. (A)** MEFs were mock-infected or infected with SFV-WT or CHIKV (MOI 1) for 8hpi prior to fixation and immunofluorescence staining of DDX6 (green, P-body marker) and dsRNA(red). **(B)** MEFs were mock-infected or infected with SFV-βGal (SFV expressing β-galactosidase instead of the viral structural proteins) at MOI 1. Cells were mock-treated or treated with SA (500 µM for 1h) before fixation at 8hpi. Fixed cells were stained for Dcp1a (green), SFV-nsP3 (red) and TIA1 (blue). Scale bar, 10 µm. Representative pictures are shown.

by either Dcp1a or DDX6, probably due to the active exchange rate of the markers. In addition, a part of DDX6 was found in TIA1-stained SGs after SA stress, as previously reported (Kedersha *et al.* 2005). The P-body response was then tested upon infection with SFV or CHIKV. At 8hpi, infected MEFs were fixed for immunofluorescence analysis using anti-DDX6 antibodies to probe for P-bodies and anti-dsRNA for viral infection. In contrast to mock-infected cells, where P-bodies were consistently present, P-bodies were almost completely disassembled after infection with either SFV or CHIKV (Fig. 6A). Disassembly of P-bodies was also observed when the granules were defined by Dcp1a in SFV-infected MEFs (paper II, Fig. S1 (a)) or BHK cells (Paper II, Fig. S1 (b)). Previous studies have shown that alphavirus-infected cells are incapable of forming SGs in response to SA (Panas *et al.* 2012). Therefore, we asked if P-bodies could be re-assembled in infected cells when stressed with SA. In response to SA, P-bodies increased in number and SGs were induced in mock-infected MEFs, while neither of the two granules re-appeared in response to SA in MEFs infected with SFV-WT (Paper II, Fig. 1 (c)) or CHIKV (Paper II, Fig. S1 (c)), suggesting that alphavirus-infected cells have lost the ability to react to a second stress by increasing the numbers of P-bodies or SGs. We then sought to determine which part of the virus was responsible for P-body disassembly. To this end, MEFs were mock-infected or infected with the virus recombinant SFV-βGal (Sjöberg *et al.* 1994), which expresses β-galactosidase instead of the viral structural proteins from the viral subgenomic region. Similarly to the situation with SFV-WT, nearly all P-bodies stained by Dcp1a were disassembled after infection with SFV-βGal (Fig. 6B). As seen after SFV-WT infection, re-induction of P-bodies by SA was blocked in SFV-βGal-infected cells (Fig. 6B). This indicates that the structural proteins of the virus are not necessary for the disassembly of P-bodies.

As structural proteins are dispensable for P-body disassembly, we next tested how P-bodies respond to the expression of viral nsPs. An SFV trans-replication system was applied in these experiments. Briefly, the system consists of two plasmids for expression of nsPs (Rep, replicase) and delivery of a viral genome template, respectively (Utt *et al.* 2016). With the help of this system, expression of nsPs and replication of virus genomes can be disconnected by transfection of either only the plasmid for nsPs or both plasmids for nsPs and viral genome template. After transfection, SFV nsPs are expressed either alone or together with the viral genome template, in which the two ORFs were replaced with Firefly and Gaussia luciferase as reporters. MEFs were transfected with either the nsP expression plasmid alone (Rep-wt nsP1–4) or the nsP plasmid plus viral genome template, fixed at 24hpt and subjected to immunofluorescence for dsRNA + nsP3 or Dcp1a + nsP3. The dsRNA signal was used to

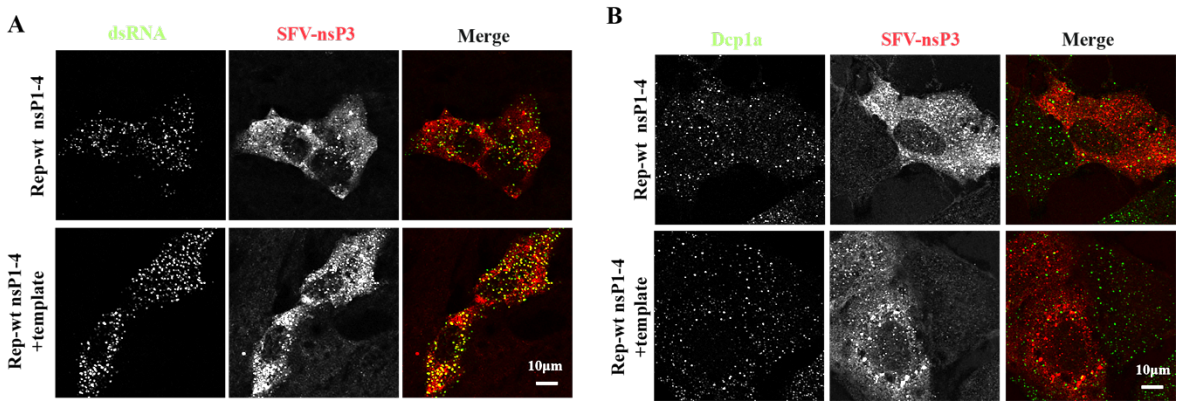


**Fig. 7. P-bodies remain upon expression of alphavirus nsPs.** MEFs were transfected with either Rep-wt or Rep-wt + template. At 24hpi, cells were fixed for immunostaining for dsRNA and SFV-nsP3 **(A)** or Dcp1a and SFV-nsP3 **(B)**. Scale bar, 10 µm. Representative pictures are shown.

assess proper formation of functional viral replicases, which has been shown to replicate both host and viral mRNA and thus generates dsRNA intermediates even in the absence of a viral RNA template (Nikonov *et al.* 2013). Indeed, dsRNA signals were observed and colocalized with nsP3 in cells transfected with either Rep-wt nsP1–4 alone or in combination with template, indicating the presence of functional viral replicases (Fig. 7A). As the viral genome template, but not host mRNA, carries the cis-elements for efficient replication, the dsRNA signal was stronger when viral genome template was provided (Fig. 7A). Nevertheless, there was no noticeable change of P-bodies (Dcp1a channel) in the transfected cells, neither with Rep-wt nsP1–4 alone nor in combination with template, compared to adjacent untransfected cells (Fig. 7B). Our results indicate that expression of viral nsPs alone or together with the replication of viral template analogue is not sufficient to lead to P-body disassembly. Considering that the viral structural proteins are not required, we propose that replication of viral templates that carry proper nsP-encoding regions are required for P-body disassembly.

In order to study at what time after infection P-bodies are disassembled, we performed time course experiments in which P-bodies were stained at different time points after infection with SFV-βGal at MOI 1, using antibodies for Dcp1a, nsP3 and TIA1 (as an SG marker). Disassembly of P-bodies was observed only in a few infected cells at 2.5hpi, and became dominant at 3.5hpi or later (Fig. 8A). At 3.5hpi and 4.5hpi, TIA1-positive SGs were induced in most infected cells concomitantly with P-body disassembly (Fig. 8A). P-body numbers per cell were quantified via CellProfiler for each group. The average number of P-bodies/ cell

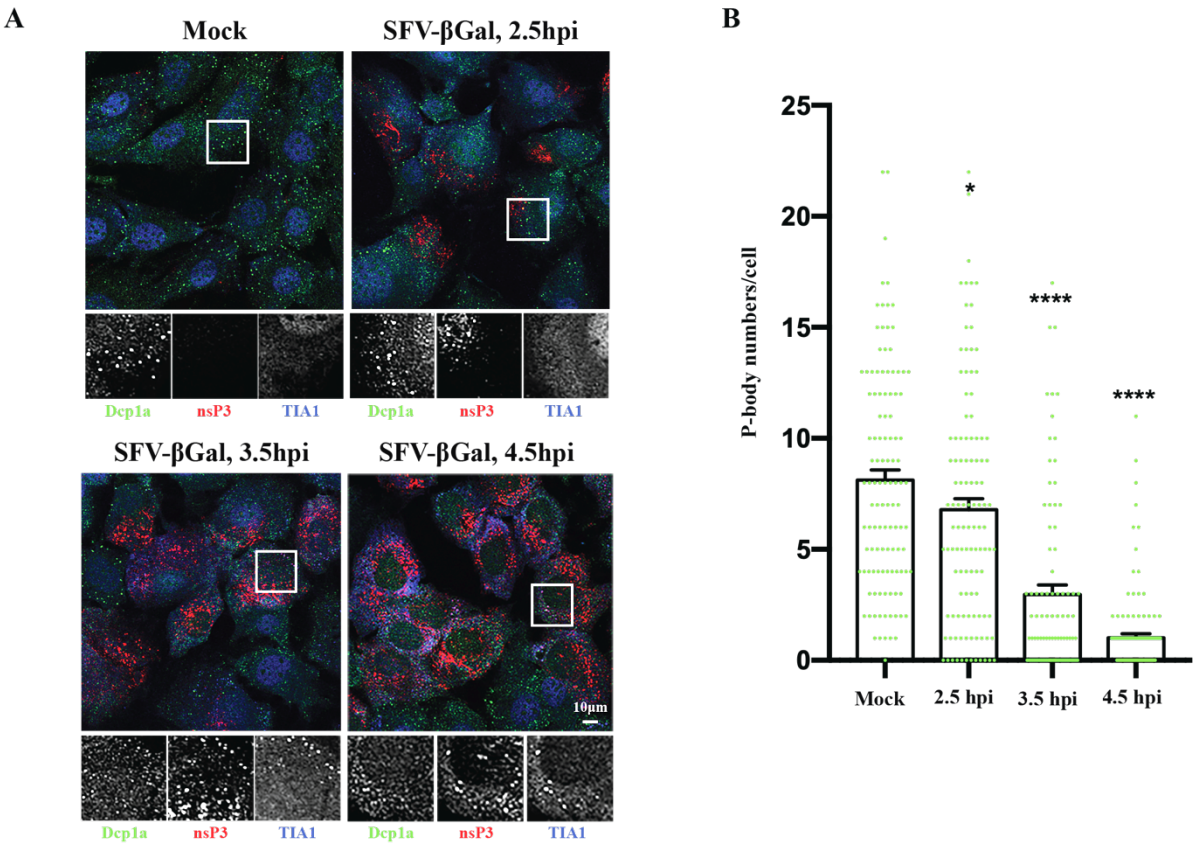


**Fig 8. Disassembly of P-bodies occurs at early stage of viral infection. (A)** MEFs were mock-infected or infected with SFV-βGal (MOI 1) and fixed at the indicated time points, followed by staining for Dcp1a (green), SFV-nsP3 (red) and TIA1 (blue). Representative pictures are shown. **(B)** Mean P-body numbers/cell ± SEM as quantified by CellProfiler based on the experiments shown in (A). The number of P-bodies for each analyzed cell are shown as green dots. Statistical analysis for infected cells in comparison to mock infection by unpaired Student's t test, *$p < 0.05$, ****$p < 0.0001$.

was 8.70 ± 0.56 (mean ± SEM, N=118) in mock-transfected cells, decreased at 2.5hpi (7.0 ± 0.53 (mean ± SEM, N=115)) and dropped sharply and significantly at 3.5hpi and 4.5hpi to 3.42 ± 0.52 (N=93) and 1.15 ± 0.22 (N=120) (Fig. 8B). Thus, the average number of P-bodies/cell compared to mock-infected cells was reduced by 20% within 2.5h, by more than 60% within 3.5h and by more than 80% within 4.5h. Importantly, at 3.5hpi and 4.5hpi the percentages of the infected cells containing ≤ 1 P-body/cell were 53% and 80% (see the distribution of the green dots in Fig. 8B), indicating that almost all P-bodies were disassembled in the majority of infected cells. The quantification results reveal that P-body disassembly in infected cells starts at 2.5hpi and that P-bodies are efficiently disassembled as early as at 3.5hpi.

Similar experiments were conducted with DDX6 as the marker of P-bodies. P-bodies defined by DDX6 were similarly present in mock-infected cells and infected cells at 2.5hpi (Paper II, Fig. 2 (b)). At 3.5hpi and 4.5hpi, bigger DDX6 foci appeared and colocalized with TIA1, indicating the localization of DDX6 in SGs (Paper II, Fig. 2 (b)). These DDX6/TIA1 puncta were confirmed to represent SGs by staining with another SG marker, eukaryotic initiation factor 3b (eIF3b; Paper II, Fig. S2), suggesting redistribution of DDX6 from P-bodies to SGs. These time course studies of P-body response, with either Dcp1a or DDX6 as a P-body marker, revealed that disassembly of P-bodies occurred at early stage (3–4h) post infection when or even before SGs were induced. Thus, the time point 3.5phi was used for the further investigations of the P-body response.

DDX6 has been shown to be required for P-body formation (Ayache *et al.* 2015, Kamenska *et al.* 2016). Thus, we tested if disassembly of P-bodies during infection was a result of redistribution of DDX6 from P-bodies to SGs. To test this, we investigated the P-body response after infection with and without SG induction. Induction of SGs during alphavirus infection is mediated by phosphorylation of translation initiation factor 2 subunit α (eIF2α) on serine 51 (McInerney *et al.* 2005). The P-body response to alphavirus infection was analyzed in SV129 MEFs, where SGs cannot be induced due to the expression of a non-phosphorylatable eIF2α (MEFs-AA, Ser51Ala) (Scheuner *et al.* 2001). MEFs expressing wild-type eIF2α (MEFs-SS, Ser51) were included as controls. Without stress, P-bodies were similarly present in both cell lines (Paper II, Fig. 3 (a)). In response to SA, P-body numbers increased in both cell lines, but SGs stained by eIF3b were induced only in MEFs-SS but not MEFs-AA, as expected (Paper II, Fig. 3 (a)). At 3.5hpi with SFV-βGal, DDX6 foci in MEFs-SS colocalized with eIF3b, indicating induction of SGs and relocation of DDX6 from P-bodies to these SGs (Fig. 9A). However, in MEFs-AA cells, both DDX6 and eIF3b were largely diffused at 3.5hpi with SFV-βGal infection, which indicates efficient disassembly of P-bodies and lack of SG induction (Fig. 9A). Similar results were obtained in MEFs-S/S and MEFs-AA after CHIKV infection (Paper II, Fig 3 (a)). Taken together, these results reveal that P-bodies are always disassembled after Old World alphavirus infection, regardless of SG induction.

A recent study suggests that translation suppression mediated by the interaction between DDX6 and 4E-transporter is required for P-body assembly (Ayache *et al.* 2015, Kamenska *et al.* 2016).

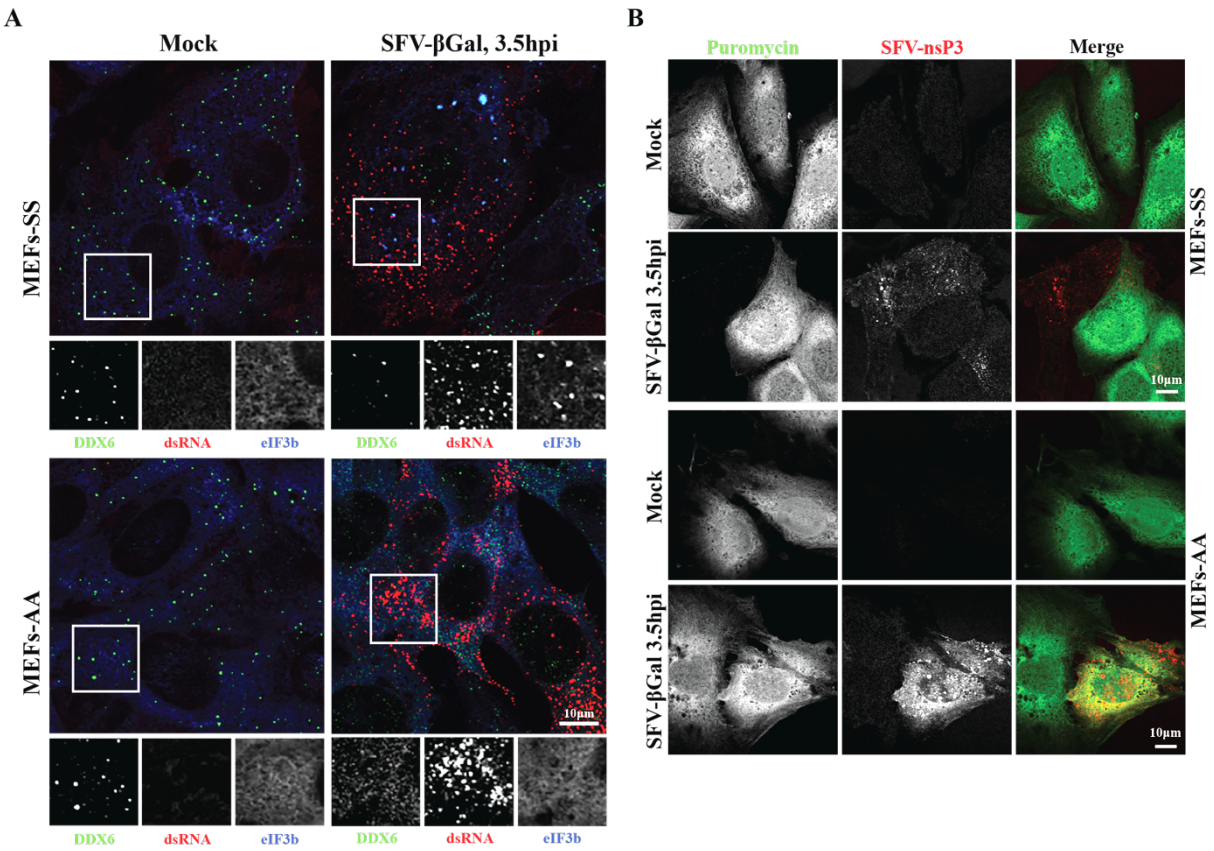


**Fig 9. Disassembly of P-bodies is independent of SG formation or virus induced shutoff of host translation. (A)** MEFs-SS or MEFs-AA were mock-infected or infected with SFV-βGal (MOI 10) and fixed at the indicated times for immunofluorescence. Cells were probed for DDX6 (green), dsRNA (red) and eIF3b (blue, SG marker). **(B)** MEFs-SS or MEFs-AA were mock-infected or infected with SFV-βGal (MOI 10). At the indicated time points, cells were ribopuromycylated (10μg/mL) for 5min before fixation. Fixed cells were probed for puromycin (green) and SFV-nsP3 (red). Scale bar, 10 μm. Representative pictures are shown.

Host translation is dramatically inhibited during alphavirus infection, which was shown to rely on the phosphorylation of eIF2α (McInerney *et al.* 2005). We asked if host translation shutoff was related with P-body disassembly during infection. The translation status was assessed in SFV-βGal-, CHIKV- or mock-infected MEFs-SS and MEFs-AA by ribopuromycylation to assess active translation (David *et al.* 2012, Panas *et al.* 2015a). In the mock group, active translation was observed in each cell of both cell lines, visualized by the strong puromycin signal (Fig. 9B, and Paper II, Fig. 3 (b) and (c)). In MEFs-SS, the puromycin signal was reduced to an undetectable level after infection with either SFV-βGal at 3.5hpi or CHIKV at 5hpi, indicating significant repression of host translation (Fig. 9B, and Paper II, Fig. 3 (b) and (c)). In contrast, SFV-βGal- or CHIKV-infected MEFs-AA displayed similar ribopuromycylation as uninfected cells, due to the failure of efficient host translation shutoff (Paper II, Fig. 3 (b) and (c)). These results support the previous observation that host translation repression at early

times post Old World alphavirus infection is largely dependent on the phosphorylation of eIF2α (McInerney *et al.* 2005). Taken together, the results in Fig. 9 demonstrate that the alphavirus-induced P-body disassembly occurs immediately prior to or coincident with the shutoff of host protein translation and is independent of the phosphorylation of eIF2α.

We next studied if disassembly of P-bodies was a result of alphavirus-infection-induced shutoff of host transcription. Bromo-UTP was used to label newly synthesized RNA (Panas *et al.* 2015a), in order to study the transcription process during the P-body disassembly. We used BHK cells, which have higher transfection efficiency than MEFs, for this experiment. Time course studies of the P-body response in BHK cells revealed that P-bodies are mostly disassembled at 3–4hpi with SFV-βGal infection (Paper II, Fig. S4 (a)) and 4–5hpi with CHIKV infection (Paper II, Fig. S4 (b)). Thus, BHK cells infected with SFV-βGal or CHIKV were labelled by transfection with bromo-UTP at 3.5hpi and 4.5hpi, respectively, for 1h to study the RNA synthesis status. Mock-infected cells were also labelled with bromo-UTP in parallel and the results showed that the bromo-UTP signal was mainly concentrated in nuclei (Fig. 10A and Paper II, Fig. 4 (a) and (b)). In contrast, a sharp shift of the bromo-UTP signal from nucleus to cytoplasm was observed in cells infected with SFV-βGal (Fig. 10A) or CHIKV (Paper II, Fig. 4 (b)). The shift indicates that the active RNA synthesis in infected cells was mainly cytoplasmic, most likely as viral RNA replication. The massive reduction or almost complete lack of nuclear bromo-UTP signal in the infected cells indicates the effective shutoff of host transcription already at this early time in viral infection. The cytoplasmic bromo-UTP staining appeared in most double-positive cells (successfully infected and bromo-UTP labelled) with SFV-βGal infection, assuming that most cells were infected at a similar rate. In CHIKV-infected cells at 4.5hpi, the majority of double-positive cells (successfully infected and bromo-UTP-labelled) had bromo-UTP signals in the cytoplasm, though some double-positive cells still showed nuclear bromo-UTP signals (data not shown), probably due to slower progression of CHIKV infection. These results suggest that shutoff of host transcription caused by virus infection correlates with the disassembly of P-bodies at early stages of Old World alphavirus infection.

Actinomycin D (ActD) at high concentration (5μg/mL) can inhibit transcription in cells by targeting all polymerases (Bensaude 2011). Earlier studies have observed a decrease in P-body numbers with ActD treatment in HEK293 cells (Cougot *et al.* 2004). We therefore tested how

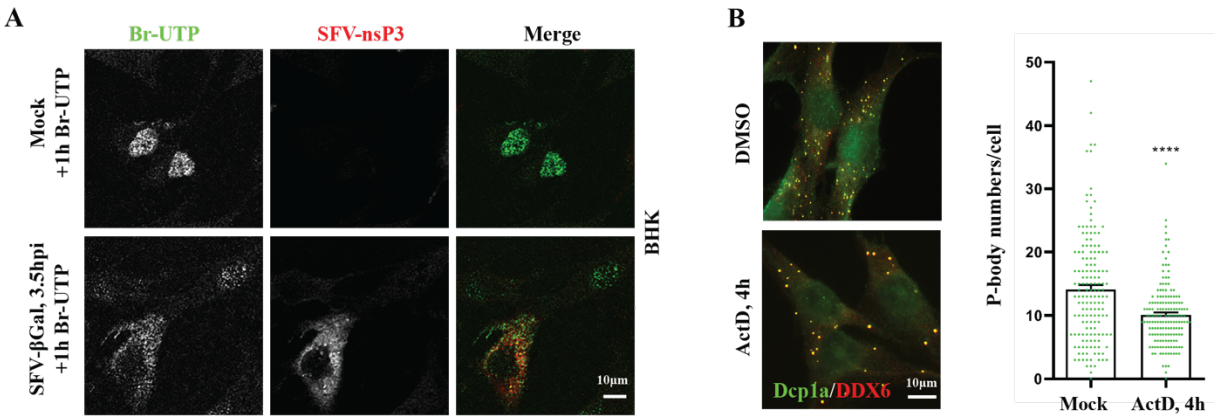


**Figure 10. Virus-induced host transcription shutoff correlates with the disassembly of P-bodies. (A)** BHK cells were mock-infected or infected with SFV-βGal (MOI 10). At 3.5hpi, cells were transfected with bromo-UTP (Br-UTP) to label newly synthesized RNA for 1h before fixation. Cells were then stained for Br-UTP (green) and SFV-nsP3 (red). **(B) Left-hand side:** BHK cells were mock-treated (DMSO) or treated with actinomycin D (5μg/mL) and fixed at different time points (1h, 2h, 4h, 8h, 16h). Fixed cells were stained with dual P-body markers Dcp1a (green) and DDX6 (red). Representative figures after 4h ActD or DMSO treatment were shown. **Right-hand side:** Mean P-body numbers/cell ± SEM as quantified by CellProfiler based on immunofluorescence. Numbers of P-bodies for each analyzed cell are shown as green dots. Statistical analysis by unpaired Student's t test, ****$p < 0.0001$.

P-bodies respond to ActD in BHK and compared the response with that after alphavirus infection. The inhibition effect of ActD was first validated in bromo-UTP labelling experiments. Without ActD, bromo-UTP was successfully incorporated into newly synthesized RNA and accumulated in the nucleus, while after 1h ActD treatment, there was no cell showing any bromo-UTP signal, indicating the effective suppression of transcription (Paper II, Fig. 4 (c)). ActD treatment for 4h led to a clear decrease of P-body numbers in BHK cells (Fig. 10B), but failed to cause visible changes of P-bodies within a shorter time. P-body numbers/cell was quantified, yielding an average P-body number of 10.09 ± 0.39/cell (mean ± SEM, N=163) in cells treated with ActD for 4h, which is significantly lower than the P-body number in the control group (14.15 ± 0.69/cell, N=158) (Fig. 10B). These results show that P-bodies were decreased in number with ActD, consistent with previous observations in another cell line (Cougot *et al.* 2004). Sixteen hours of treatment with ActD almost depleted P-bodies in BHK cells (data not shown), which likely resulted from the toxicity of ActD, since the majority of cells had died at this time. In summary, the transcription shutoff induced by ActD reduced the average P-body numbers/cell (by 29% within 4h), but failed to disassemble P-bodies as efficiently as virus infection. Thus, these results suggest that alphavirus infection manipulates more pathways than only the transcription shutoff to induce efficient P-body disassembly.

Next, we attempted to mimic the viral effect on P-body disassembly using a panel of pharmacological treatments. Thus, cycloheximide (CHX) and puromycin were used to assess the P-body response to the change of free cytoplasmic mRNA. Also, the P-body response to the modulation of nuclear transport was addressed by using leptomycin B, targeting CRM1-mediated nuclear export (Nishi *et al.* 1994, Kudo *et al.* 1999), and importazole, targeting importin-mediated nuclear import (Soderholm *et al.* 2011). The active cytoplasmic RNA synthesis described above may also indicate potential damages to transport between cytoplasm and nucleus in virus-infected cells, which might affect P-bodies in addition to transcription shutoff. MEFs were mock-treated or treated with either of these drugs and subsequently analyzed by immunofluorescence and quantification of P-body numbers/cell (Fig. 11A/B). P-bodies were consistently present in the control group with 26.77 ± 1.81 P-bodies/cell on average (mean ± SEM, N=62). With CHX treatment, P-bodies were almost completely disassembled, confirming reports in previous studies (Sheth *et al.* 2003, Cougot *et al.* 2004). P-body numbers/cell dropped to 1.83 ± 0.50 after CHX treatment (mean ± SEM, N=58). A robust decrease of P-bodies was only observed after 4h treatment with puromycin (Paper II, Fig. S5 (a), puromycin 4h), but not within a shorter time (1h and 2h, data not shown), a decrease at a similar rate as with ActD (Paper II, Fig. S5 (a), ActD 4h). P-body response to different concentrations of leptomycin B (5ng/mL, 10ng/mL and 20ng/mL) were analyzed for various treatment times (1h, 2h, 4h and 6h). There was no noticeable change of P-bodies between the control cells and leptomycin-B-treated cells (paper II, Fig. S5 (b)). The results suggest that P-bodies are practically insensitive to the interference with nuclear export by leptomycin B, as previously reported (Ferraiuolo *et al.* 2005). In addition, P-body response to treatment with importazole was assessed using different incubation times (15min, 30min, 1h, 2h and 4h) and serial concentrations (25μM, 50μM, 75μM and 100μM). Interestingly, treatment with importazole for 15min resulted in almost complete P-body disassembly at concentrations of 50μM or more, but not 25μM (Fig. 11A). P-body numbers/cell decreased to 1.5 ± 0.30 (mean ± SEM, N=58) after treatment with 75μM importazole for 15min. TIA1 was included as an SG marker in all experiments, and the results showed that SGs were not induced under any treatment, i.e. CHX, Puro, ActD, leptomycin B or importazole (Fig. 11 and paper II, Fig 5a, S5a and S5b, TIA1 channel).

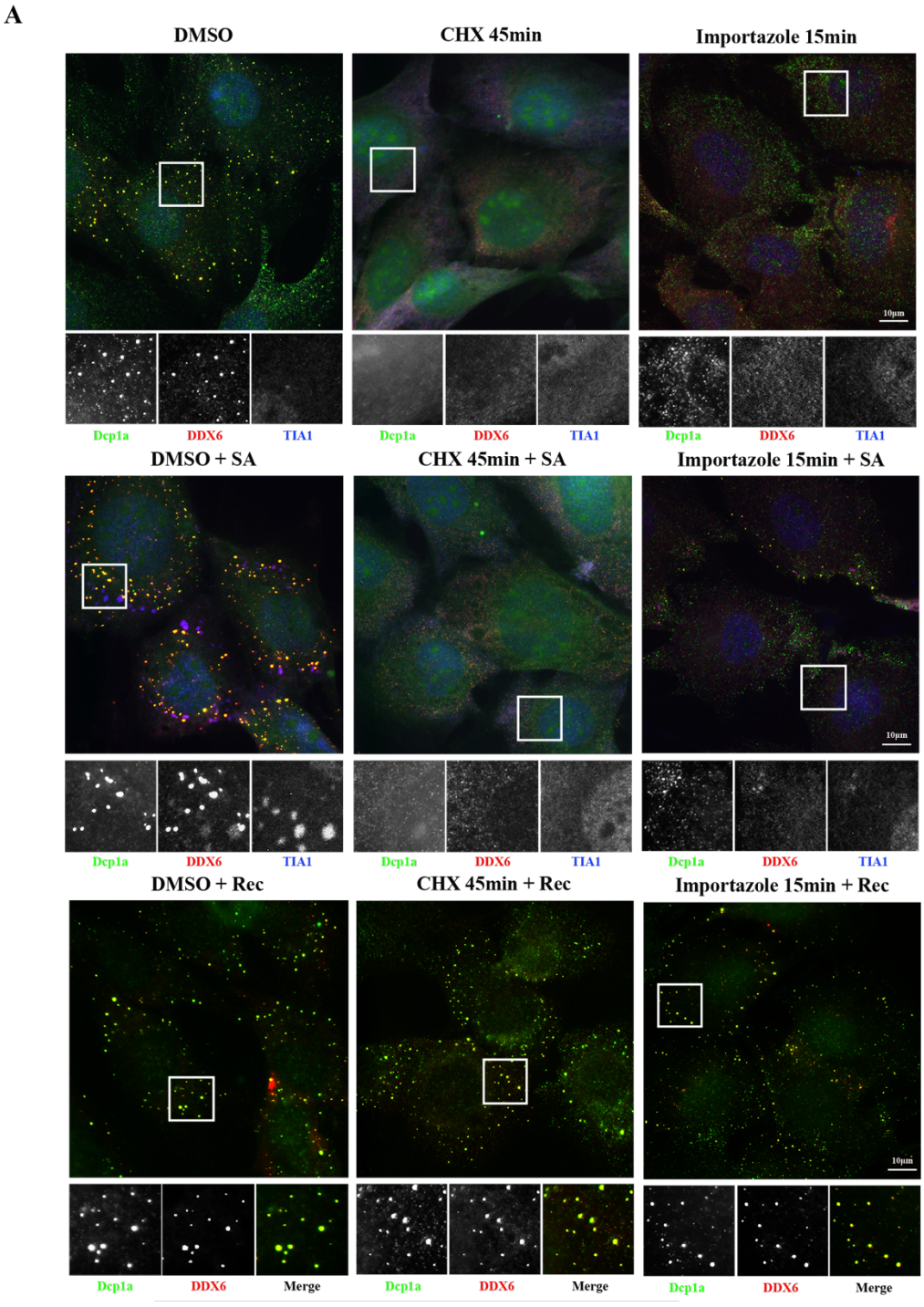
A
DMSO
CHX 45min
Importazole 15min
10μm
Dcp1a
DDX6
TIA1
DMSO + SA
CHX 45min + SA
Importazole 15min + SA
DMSO + Rec
CHX 45min + Rec
Importazole 15min + Rec
Merge
B
P-body numbers/cell
60
40
20
0
ns
**
****
DMSO
CHX
Importazole
+ control
+ SA
+ 15min Recovery

**Fig 11. P-body response to multiple stress conditions. (A) Top panel:** MEFs were treated with CHX (10 µg/mL in DMSO), importazole (75 µM in DMSO) or DMSO (0.15%, contol treatment) for the indicated period of time before fixation, followed by staining for Dcp1a (green), DDX6 (red) and TIA1 (blue). **Middle panel:** After the treatment described in the top panel, MEFs were stressed with SA (500 µM) in the presence of the indicated drugs for 30min, fixed and probed for Dcp1a (green) and DDX6 (red). **Bottom panel:** After the treatment described in the top panel, CHX, importazole or DMSO was removed and replaced with warm normal culture medium. Cells were further cultured and fixed after a recovery time of 15min. Fixed cells were probed for Dcp1a (green) and DDX6 (red). Scale bar, 10 µm. Representative images are shown. **(B)** Mean P-body numbers/cell ± SEM as quantified by CellProfiler based on the experiments shown in **(A)**. Drug-treated groups were compared to the DMSO control group under the same conditions. Numbers of P-bodies for each analyzed cell are shown as green dots. Statistics: unpaired Student's t test, ns $p>0.05$, *$p < 0.05$, ****$p < 0.0001$.

As shown above, re-induction of P-bodies and SGs with additional SA treatment is not observed in alphavirus-infected cells. We next tested whether P-bodies and SGs can be re-induced with SA upon the drug treatments that lead to reductions or efficient disassembly of P-bodies (CHX, puromycin, ActD and importazole). When control cells were stressed with SA, average P-body numbers were almost doubled to 37.18 ± 1.936 (mean ± SEM, N=60) and SGs were induced (Fig. 11A, DMSO + SA, second row; Fig. 11B). In the presence of CHX, re-induction of either P-bodies or SGs by sodium arsenite was efficiently blocked (Fig. 11A, CXH + SA), similarly as previously observed (Kedersha *et al.* 2000). Average numbers of P-body/cell were quantified as 1.5 ± 0.38 (mean ± SEM, N=60) (Fig. 11B). Puromycin treatment, however, did not prevent SG and P-body induction by SA, with similarly increased P-body numbers and formation of SGs as in the control cells (Paper II, Fig S5 (a), puromycin 4h + SA). Cells stressed with sodium arsenite in the presence of ActD showed an increase in P-body numbers, but did not form SGs (Fig S5 (a), ActD 4h + SA). Re-induction of P-bodies and SGs was, however, largely inhibited in the presence of importazole: The response to SA was either completely abolished or dramatically inhibited with no or much lower P-body and SG re-induction compared to the control cells (Fig. 11A, importazle + SA). The average number of P-bodies/cell was 13.63 ± 1.10 in importazole-treated, SA-stressed cells (mean ± SEM, N=71) (Fig. 11B). Even though there was an increase in P-body numbers by SA stress in importazole-treated cells, the average P-body counts only reached approximately one third of the values in SA-stressed control cells. Thus, the capacity of cells to re-form P-bodies was strongly compromised when nuclear import was inhibited with importazole.

Rapid disassembly of P-bodies was observed with either CHX or importazole treatment. We then asked whether the effect of CHX or importazole was reversible and whether P-bodies could re-assemble after drug removal (Fig. 11A, third row). When CHX was removed, P-bodies were restored within 15min to average P-body numbers of 16.90 ± 1.10 (mean ± SEM,

N=73) per cell (Fig 11B). After removal of importazole P-bodies recovered rapidly (within 15min) with 24.42 ± 1.3 (mean ± SEM, N=69) P-bodies/cell (Fig 11B). These values are comparable to the numbers under control conditions (23.32 ± 1.62 P-bodies/cell, mean ± SEM, N=62, Fig. 11B).

The results obtained with importazole reveal nuclear import as a previously unrecognized pathway involved in P-body dynamics. To exclude cell line specificity, we tested the P-body response to importazole also in BHK cells and observed similar P-body disassembly after 15min treatment of importazole (Paper II, Fig. S5 (c)), supporting that nuclear import is a novel pathway involved in the P-body dynamics.

In summary, these results reveal that treatment with either CHX or importazole leads to an efficient and mostly reversible disassembly of P-bodies, which resembles the P-body disassembly efficiency that is observed upon alphavirus infection. This suggests that alphavirus infection may induce P-body disassembly by inhibiting host cell translation (like CHX), transcription (like ActD) as well as nuclear import (like importazole).

## 2.3 RESULTS PAPER III

In our previous work, we showed that infection of cells with SFV but not CHIKV leads to strong and persistent activation ("hyperactivation") of the PI3K–Akt–mTOR pathway and concomitantly internalization of RCs. The phenotype was attributed to the HVD in SFV nsP3 since PI3K–Akt–mTOR hyperactivation and RC internalization are largely abolished in the SFV-Δ50 mutant, in which 50 residues at the beginning of the HVD are deleted (see Fig. 12A) (Thaa *et al*. 2015). We set out to identify a sequence feature in this part of nsP3 that is present in SFV but absent in CHIKV and might be relevant for the phenotypic differences. Upon alignment of the sequences of the C-terminal region of SFV and CHIKV nsP3 (Fig. 12A), we noted that the sequence of SFV but not CHIKV nsP3 contains the sequence Y369-E370-P371-M372 (yellow in Fig. 12A). This corresponds to a YXXM motif, a well-characterized activation motif for PI3K–Akt when the tyrosine is phosphorylated (Fruman *et al.* 1998). Although this motif is present in SFV-Δ50, it is likely to be in an unfavorable structural context because the deletion starts within the structured zinc-binding region of the protein and because Y369 is the first residue after the 50 residues deleted in SFV-Δ50 (grey in Fig. 12A). We hypothesized that hyperactivation of the PI3K–Akt–mTOR pathway during SFV infection could be attributed to the YEPM motif in SFV nsP3. To test the hypothesis, we generated viral mutants containing mutations at Y369, with an exchange of this tyrosine residue either to alanine (SFV-YA) or to phenylalanine (SFV-YF), which is structurally more similar to tyrosine. We mock-infected or infected BHK cells with wild type SFV (SFV-WT), SFV-YA or SFV-YF at an MOI of 10. Cells were lysed at 8hpi for analysis of Akt phosphorylation by Western blotting (Fig. 12B). At 8hpi, Akt was strongly phosphorylated in cells infected with SFV-WT, confirming Akt hyperactivation. Upon infection with SFV-YA or SFV-YF however, the intensity of the phospho-Akt signal was much lower, indicating weaker activation of PI3K–Akt (Fig. 12B, left-handed panel). Infection was firmly established in each case, as judged from the nsP3 signals. Densitometry analysis showed that levels of Akt phosphorylation induced by SFV-YA and SFV-YF were less than half of those for SFV-WT (Fig. 12B, right-handed panel) and only slightly above background. Consistent with low PI3K–Akt activation, viral RCs of SFV-YA and SFV-YF were mainly localized at the cell periphery, while SFV-WT showed efficient RC internalization, based on the results of dsRNA staining (Fig. 12C). Taken together, these results strongly suggest that Y369 in SFV nsP3 is the key residue for the Akt hyperactivation phenotype. We further confirmed that expression of nsP3 alone is sufficient for PI3K–Akt–mTOR hyperactivation when a membrane anchor is provided that mimics

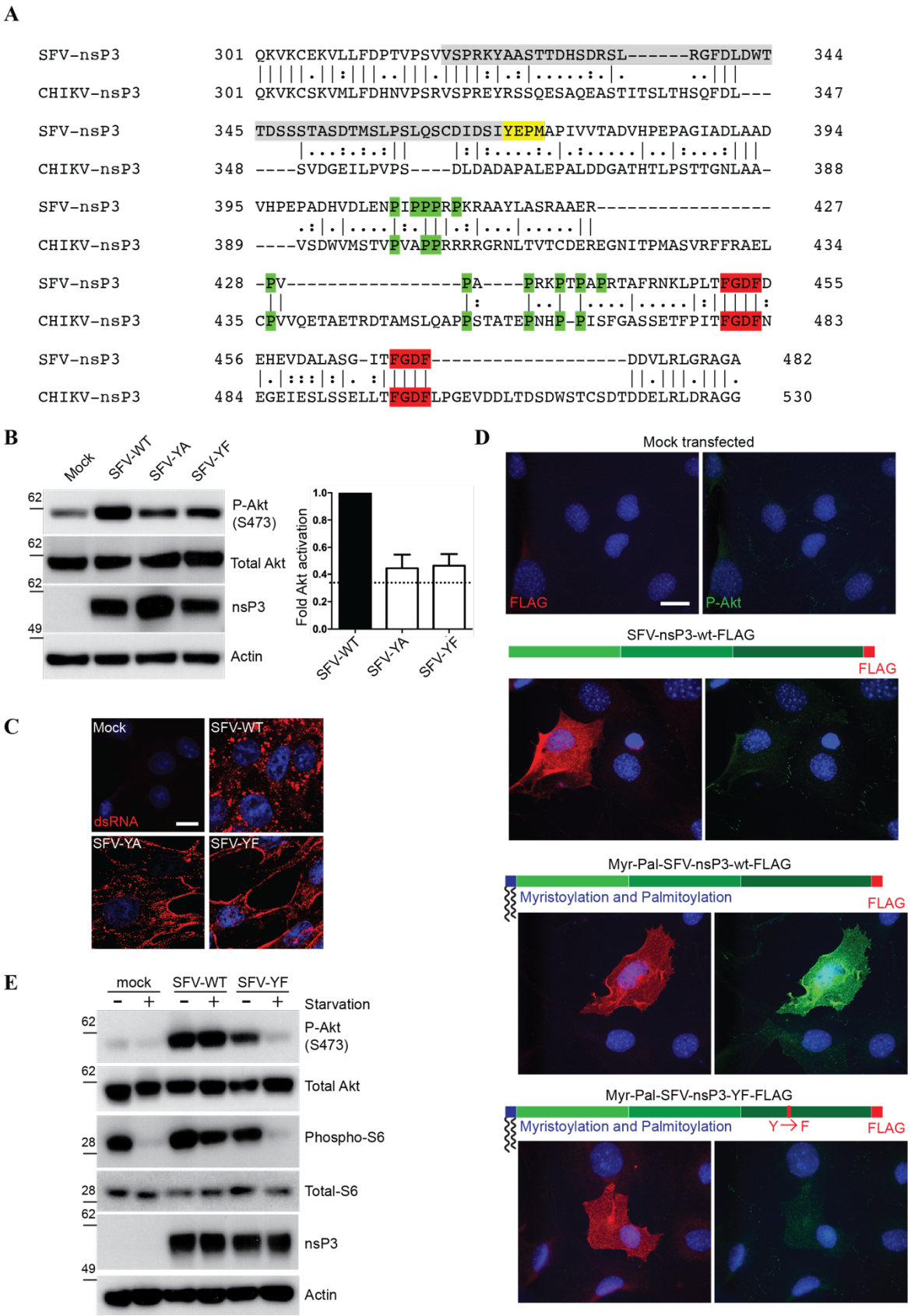


**Fig. 12. (A)** Alignment of the C-terminal regions of SFV-nsP3 (GenBank accession number AKC01667) and CHIKV-nsP3 (GenBank accession number ABX40005). Highlighted sequence features: Deletion of 50 amino acids in SFV-Δ50 (gray background); tyrosine (Y369) in the context of a YXXM motif (yellow, only in SFV); prolines in the proline-rich region (green); FGDF motifs (red). **(B)** BHK cells were infected with SFV-WT, SFV-YA (nsP3 Y369A) or SFV-YF (nsP3 Y369F) at MOI 10 or mock-infected, lysed at 8hpi, followed by Western

blot analysis for the indicated proteins. Representative blot in the top panel; densitometry of the intensities of P-Akt bands (normalized to the respective total Akt signal), shown relative to SFV-WT. Data are averages from two independent experiments ± SEM. Dashed line: Average P-Akt signal intensity in mock-infected controls. **(C)** BHK cells were mock-infected or infected with the indicated viruses at MOI of 10 and fixed at 8hpi followed by immunofluorescence for dsRNA to stain viral replication complexes (red). Nuclei in blue. Scale bar, 10 μm. **(D)** MEFs were mock-transfected or transfected with indicated plasmids encoding FLAG-tagged nsP3-wt, Myr-Pal-nsP3-wt or Myr-Pal-nsP3-YF. Cells were fixed at 24hpt and stained for phospho-Akt (S473, green) and FLAG-nsP3 (red). Nuclei in blue. Scale bar, 20μm. **(E)** BHK cells were mock-infected or infected with SFV-WT or SFV-YF at MOI 10 for 1h; incubation was done in normal growth medium until 4hpi and then continued in normal growth medium (no starvation) or EBSS (starvation) for another 4h prior to lysis at 8hpi and Western blotting for the indicated proteins.

membrane attachment of the RC. Strong Akt phosphorylation was detected by immunofluorescence in cells transfected with Myr-Pal-SFV-nsP3-wt, which carries a membrane anchor (myristoylation and palmitoylation), but neither in cells expressing SFV-nsP3 without this membrane anchor nor in cells expressing Myr-Pal-SFV-nsP3 with mutated Y369 (Fig. 12D). The Y369A and Y369F mutations were similarly effective at destroying the PI3K–Akt–mTOR hyperactivation phenotype. To minimize the potential of unrelated structural changes by the mutation, we selected SFV-YF for further experiments.

In order to test how the mutation Y369F in nsP3 affects viral replication, we next performed growth curve experiments. BHK cells were infected with SFV-WT, SFV-YF or SFV-Δ50 at MOI 0.1 or MOI 10; supernatants were harvested at different time points post infection and used for quantification of viral titers by plaque assay. SFV-YF was clearly attenuated with respect to SFV-WT at later times. SFV-Δ50 was more attenuated than SFV-YF, possibly due to additional malfunction other than the weaker PI3K–Akt activation (Paper III, Fig. 3G and 3H). Previously, we reported that infection with SFV-WT but not SFV-Δ50 keeps the metabolic regulator mTOR active even under conditions of nutrient starvation, where mTOR is normally inactive (Thaa *et al.* 2015). We hence compared the ability of SFV-WT and SFV-YF to activate the PI3K–Akt–mTOR pathway in both normal growth conditions and starvation. BHK cells were mock-infected or infected with SFV-WT or SFV-YF at MOI 10. At 4hpi medium was replaced by either standard growth medium or salt solution without any nutrients or growth factors (EBSS), incubation was continued for an additional 4h until lysis at 8hpi and Western blot analysis. In addition to Akt phosphorylation, phosphorylation of the ribosomal protein S6 was also probed as a downstream readout of mTORC1 activation (Fig. 12E). The results show that in cells infected with SFV-WT, Akt was strongly phosphorylated both under standard nutrient conditions and starvation conditions, and mTOR was active under both of these conditions as evidenced by strong phospho-S6 signals, confirming our earlier

observations (Thaa *et al.* 2015). In cells infected with SFV-YF in standard medium, Akt was again phosphorylated to a lesser degree than with SFV-WT; mTOR was active under these conditions as judged from the strong S6 phosphorylation. Upon starvation however, SFV-YF-infected cells neither showed any Akt phosphorylation above background level nor any S6 phosphorylation, similar to mock-infected starved cells and indicating that mTOR was inactive under these conditions. These results show that Y369 is necessary for efficient viral replication and hyperactivation of PI3K–Akt–mTOR during SFV infection.

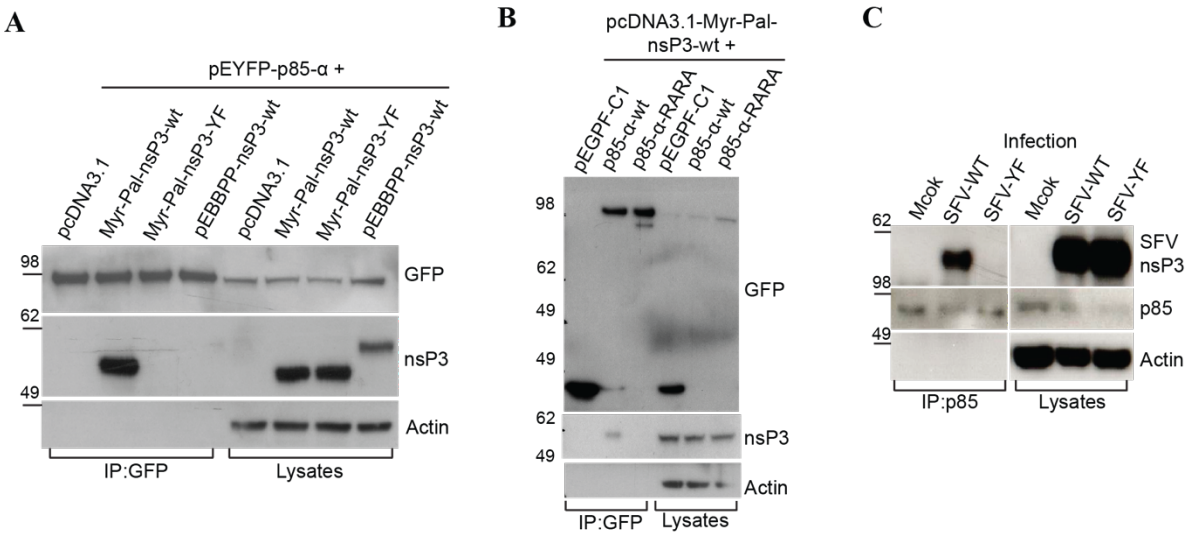


**Fig. 13: Binding of nsP3 and p85, dependent on Y369 in nsP3 and SH2 domain in p85. (A and B)** HEK293 cells were co-transfected with the indicated combinations of plasmids. All p85 constructs were N-terminally tagged with EYFP; pEGFP-C1 served as control. pcDNA3.1-Myr-Pal-nsP3 encodes nsP3 with N-terminal myristoylation and palmitoylation signal; pEBB/PP-nsP3 encodes nsP3 with an N-terminal biotin acceptor peptide (BAP); pcDNA3.1(–) served as empty vector control. At 24hpt, cell lysates were prepared and subjected to immunoprecipitation (IP) with anti-GFP antibody for pulldown of EGFP and EYFP-tagged p85, followed by probing of IPs and lysates for GFP, nsP3 and actin by Western blot. **(C)** BHK cells were mock-infected or infected with SFV-WT or SFV-YF at MOI 10. Cells were lysed at 8 hpi, followed by immunoprecipitation with anti-p85 antibodies, and by Western blot for probing for SFV-nsP3, p85 and actin.

It has been well studied that activation of PI3Ks (class IA) can be mediated through the interaction between the phosphorylated tyrosine in a YXXM motif and the SH2 domain of the p85 subunit of PI3K (Fruman *et al.* 1998). Tyrosine phosphorylation of SFV nsP3 has never been reported (Peränen *et al.* 1988; Vihinen and Saarinen. 2000), but the phenotype of SFV-YF compared to SFV-WT suggests that SFV-nsP3 binds p85 through such an interaction, and that either mutation at Y369 in nsP3 or mutations in the SH2 domains of p85 would abolish the interaction. To test this, we transfected HEK293 cells with a plasmid for expression of the

p85 isoform p85-α with an N-terminal EYFP tag plus plasmids for expression of either Myr-Pal-nsP3-wt, Myr-Pal-nsP3-YF or nsP3-wt without membrane anchor or empty plasmid (pcDNA3.1). We lysed the transfected HEK293 cells at 24hpi, followed by immunoprecipitation with anti-GFP antibodies, which cross-react with the EYFP tag in p85-α. Cell lysates and immunoprecipitation samples were analyzed by Western blot for GFP (to detect EYFP-p85-α), nsP3 and actin (Fig. 13A). The results revealed that Myr-Pal-nsP3-wt was efficiently co-immunoprecipitated with EYFP-p85-α, while both Myr-Pal-nsP3-YF and nsP3-wt without Myr-Pal signal failed to do so, indicating that interaction between nsP3 and p85 requires both Y369 and the membrane localization. Next, we tested whether the interaction between nsP3 and p85-α was dependent on the SH2 domains in p85-α. In the two SH2 domains of p85-α, the arginine residues in the FLVRD/E motifs are required for the interaction between p85 and phospho-tyrosine, and the interaction is abolished when arginine is mutated to alanine in both SH2 domains ("RARA mutant", (Yu *et al.* 1998, Luo *et al.* 2005)). Thus, we transfected HEK293 cells with Myr-Pal-nsP3-wt expression plasmids plus expression plasmids for EGFP, pEYFP-tagged p85-α-wt or the RARA mutant, followed by cell lysis and immunoprecipitation with anti-GFP (Fig. 13B). Myr-Pal-nsP3-wt was bound to EYFP-p85-α-wt, but not EYFP-p85-α-RARA or GFP alone, indicating the requirement of the SH2 domains in p85 for the interaction (Fig. 13B). Since there are two isoforms of p85, we also performed immunoprecipitation experiments for SFV-nsP3 and the other p85 isoform, p85-β, analogous to the experiments shown in Fig. 13B. Similar results were obtained as those of p85-α: Myr-Pal-nsP3-wt was pulled down with the p85-β probe, but not the YF mutant or nsP3 without membrane anchor (Paper III, Fig. 3D). Last, we determined if the interaction between nsP3 and p85 also occurs during viral infection. BHK cells were mock-infected or infected with SFV-WT or SFV-YF at MOI 10 and lysed at 8hpi for immunoprecipitation with anti-p85 antibodies and subsequent Western blot (Fig. 13C). Even though the endogenous levels of p85 in the cell lysates and immunoprecipitation samples were barely detectable by Western blot, probing the p85 immunoprecipitates with anti-nsP3 antibodies showed clear interaction between p85 and nsP3 in SFV-WT infection, while no interaction between p85 and nsP3 was observed in lysates of SFV-YF-infected cells (Fig. 13C). These data collectively demonstrate that SFV hyperactivates the PI3K–Akt–mTOR pathway through the interaction between SFV-nsP3 and the p85 regulatory subunit of PI3K, in a manner that is dependent on membrane localization of nsP3 and on both Y369 in nsP3 and the SH2 domains in p85. The results are also indirect proof that Y369 in SFV nsP3 is phosphorylated, at least transiently, because SH2 domains bind phospho-tyrosines in YXXM motifs (Fruman *et al.* 1998).

Further, the biological relevance of this interaction between SFV nsP3 and p85 was studied in paper III. One such feature is the downstream activation of mTOR, which is involved in translational control, mediated for instance by phosphorylation of the ribosomal protein S6. As shown above in Fig. 12E, mTOR remained active even under conditions of nutrient and growth factor depletion in cells infected with SFV-WT but not SFV-YF, evidenced by sustained S6 phosphorylation. Through collaboration, PI3K–Akt–mTOR hyperactivation upon SFV infection was in addition linked to metabolic reprogramming of cells. Mass-spectrometry-based metabolomics revealed that glycolysis was strongly up-regulated in cells infected with SFV-WT, also leading to increased fatty acid, amino acid and nucleotide levels (Paper III, Fig. 1). This activation of glycolysis was much lower upon inhibition of PI3K–Akt signaling with wortmannin (Paper III, Fig. 2) and upon infection of cells with SFV-YF (Paper III, Fig. 4).

The YXXM motif is not conserved in all Old World alphaviruses. The motif is absent in nsP3 of CHIKV (Fig. 12A) and most other Old World alphaviruses, but present in the important human pathogen RRV as YETM (Fig. 14A, in yellow). To analyze whether RRV infection also leads to hyperactivation of the PI3K–Akt–mTOR pathway by the YXXM-mediated mechanism, BHK cells were mock-infected or infected with either RRV-WT or RRV-YF, a mutant in which the YXXM tyrosine in nsP3 of RRV was replaced by phenylalanine by means of reverse genetics. Western blot results showed strong and persistent Akt phosphorylation in BHK cells infected with RRV-WT but not RRV-YF (Fig. 14B). Staining for dsRNA at 8 hpi show that RCs of RRV-WT but not RRV-YF were efficiently internalized from the cell periphery (Fig. 14C). Next, we compared the ability of RRV-WT and RRV-YF to activate mTOR upon starvation. BHK cells were mock-infected or infected with RRV-WT or RRV-YF at MOI 5. At 4hpi growth medium was replaced by either standard medium or EBSS. Cells were lysed at 8hpi and processed for Western blot analysis (Fig. 14D). The results showed that infection with RRV-WT led to high phosphorylation levels of both Akt and S6 under normal and starvation conditions, indicating sustained PI3K–Akt–mTOR activation even during starvation. Infection with RRV-YF induced low phosphorylation levels of Akt in non-starved cells and background Akt activation in starved cells. S6 phosphorylation was high under standard conditions and very low under starvation, similar to the situation in mock-infected cells. These data support that Y356 in RRV nsP3 is analogous to Y369 in SFV nsP3 to mediate hyperactivation of the PI3K–Akt–mTOR pathway. Thus, this phenotype is not only seen in SFV, but also in the human pathogen RRV.

Through collaboration, the PI3K–Akt–mTOR hyperactivation by RRV-WT was linked to pathogenesis in an *in vivo* mouse model of infection. RRV-YF was clearly attenuated in

comparison to RRV-WT in mice, evidenced by significantly lower disease scores and thus less pronounced limb weakness and less severe loss of gripping ability, as well as slightly lower viremia (Paper III, Fig. 7).

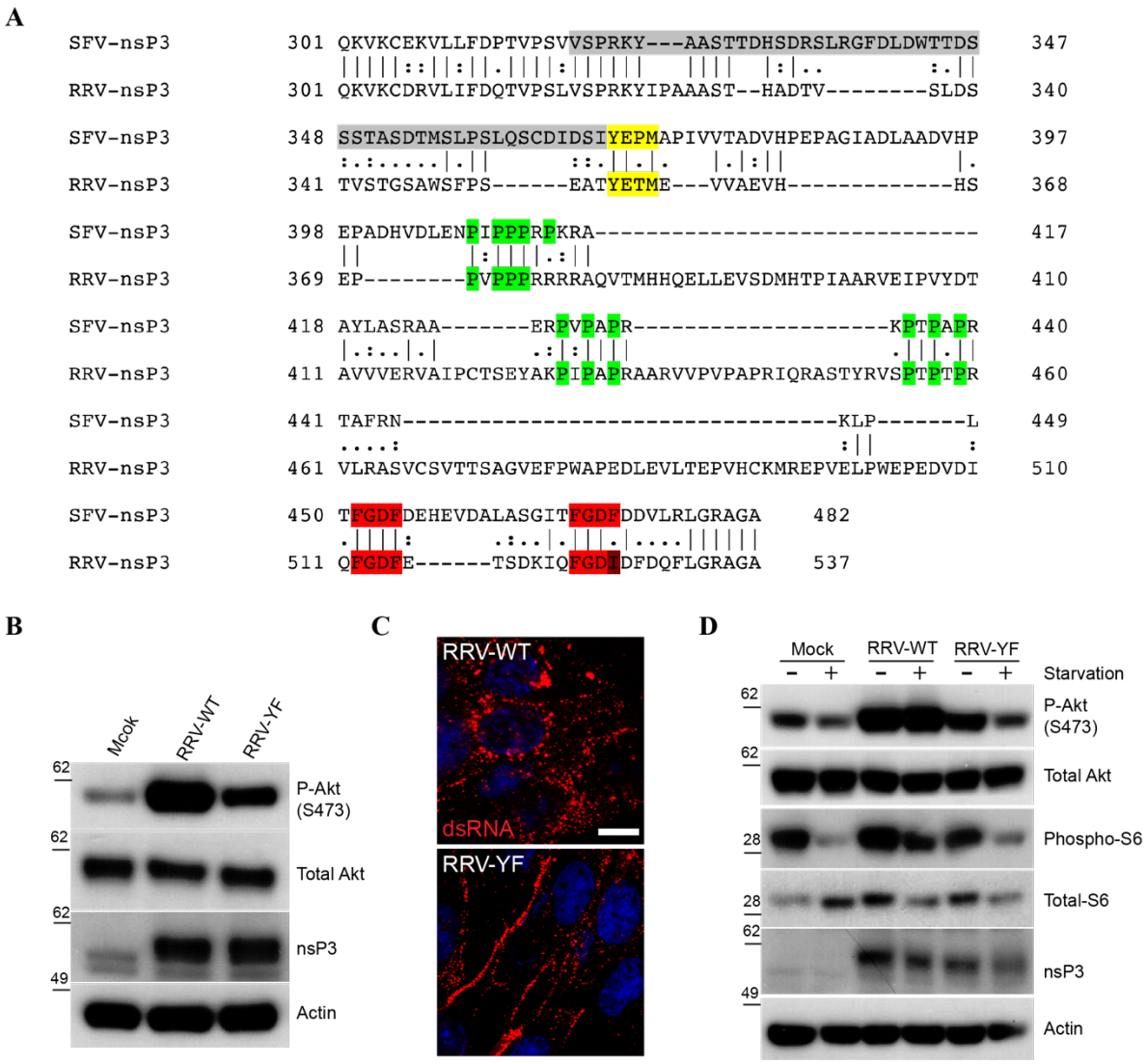


**Fig. 14: Ross River virus has the same phenotype as SFV regarding PI3K–Akt hyperactivation and replication complex internalization. (A)** Alignment of the C-terminal regions of SFV-nsP3 (GenBank accession number AKC01667) and RRV-nsP3 (UniProtKB/Swiss-Prot: P13888.1). Highlighted sequence features as in Fig. 12A. **(B)** BHK cells were mock-infected or infected with RRV-WT or RRV-YF (nsP3 Y356F) at MOI 5 and lysed at 8hpi. Cell were lysed and probed with the indicated antibodies by Western blot. **(C)** BHK cells were infected with RRV-WT or RRV-YF at MOI 5 and fixed at 8hpi., followed by immunofluorescence staining for dsRNA (red). Nuclei in blue. Scale bar, 10 µm. **(D)** BHK cells were mock-infected or infected with RRV-WT or RRV-YF at MOI 5 for 1 h; cells were incubated in standard growth medium until 4hpi and then continued in standard growth medium (no starvation) or EBSS (starvation) for another 4 h, followed by lysis (at 8hpi) and Western blotting for the indicated proteins.

Taken together, the results of paper III show that PI3K–Akt–mTOR hyperactivation, observed with SFV and RRV, is connected to metabolic alterations and pathogenicity. As revealed in paper III, the molecular reason for this PI3K–Akt–mTOR hyperactivation is the interaction of a phospho-tyrosine in the viral protein nsP3 in the context of a YXXM motif with the SH2 domains of p85, the regulatory subunit of PI3K.

# 3 DISCUSSION

The results presented in this thesis demonstrate how Old World alphaviruses explore different cellular pathways in order to create a suitable environment for efficient establishment of virus infection.

In **paper I**, we compared the duplicate motifs $FGDF_N$ and $FGDF_C$ in alphavirus nsP3 with respect to G3BP1 binding, using a panel of four variants (WT and mutations of either or both of the motifs) in different contexts – *in vitro* studies with purified proteins, experiments with transfected cells and with viruses. The results of all the experiments show that the two FGDF motifs consistently differ in their binding capacity to G3BP1. After transient expression in cells, recombinant proteins containing an intact $FGDF_N$ always bound more G3BP1 than those corresponding proteins with just an intact $FGDF_C$ motif or no intact FGDF motif. The results from MST tests with purified recombinant proteins also revealed that $FGDF_N$ has higher binding affinity to G3BP than $FGDF_C$. Interestingly, transiently expressed nsP3-$F3A_C$ variants (containing functional $FGDF_N$) bound similar or even higher levels of G3BP1 than WT nsP3, even though both FGDF motifs are present in WT nsP3. However, in the context of virus infection, only half as much G3BP1 was bound by nsP3-$F3A_C$ compared to WT nsP3. The difference between transient expression and virus infection is likely due to the different expression levels and/or protein processing of nsP3. When transiently expressed, nsP3 is usually overexpressed on its own. Thus, there is no restriction for nsP3 to bind G3BP1, as long as $FGDF_N$ is intact. G3BP1 might be the limiting factor in this case. This may explain why nsP3-$F3A_C$ binds as much G3BP1 as WT nsP3. In contrast, expression levels of nsP3 during infection are well regulated together with the other nsPs, and the expression of the nsPs is overall maintained at a relatively low level. Despite these low expression levels of nsP3 in the context of virus infection, G3BP can still be efficiently recruited by nsP3 during virus infection, which may be attributed to the fact that duplicate FGDF motifs have evolved in alphavirus nsP3.

During virus infection, the efficient recruitment of G3BP1 to nsP3 is dependent on the duplicate FGDF motifs, which leads to the formation of G3BP:nsP3 complexes, probably already at quite early stages of infection. As depicted in our crystal structure, the G3BP:nsP3 complexes may build up higher-order oligomers, but only when both FGDF motifs are present. The biological studies in paper I suggest the formation of oligomers is strongly related to virus growth, which could contribute to establish early virus infection. Virus mutants lacking either or both of the FGDF motifs are all strongly attenuated. Especially for CHIKV, mutations in both FGDF

motifs are lethal for the virus, indicating the extreme dependence of virus infection on the binding of nsP3 to G3BP1. In SFV- or CHIKV-infected cells, some of the nsP3:G3BP puncta also stain for dsRNA (Panas *et al.* 2012), suggesting an important role for G3BP in the replication of viral RNA. Our previous studies have shown that alphavirus infection can efficiently disassemble SGs by capturing G3BP via the FGDF motifs in nsP3 (Panas *et al.* 2012, Panas *et al.* 2015b). The formation of G3BP:nsP3 oligomers probably facilitates a stable sequestration of G3BP, keeping it inaccessible for SG formation. This would help the virus to ensure a rapid and sufficient counteraction of SG formation.

Other host and viral factors could also be involved in or recruited to G3BP:nsP3 complex. Our crystal structure was achieved from the NTF2-like domain of G3BP and a short region of the nsP3 HVD. Both G3BP and nsP3 consist of more domains in addition to those used to obtain the crystal structure. Importantly, both proteins contain various intrinsically disordered regions with quite flexible structural conformation. These regions are seen as interaction hubs, where protein–protein interactions can be adapted. For example, a recent study of G3BP revealed its interaction with 40S ribosome subunits (Kedersha *et al.* 2016), suggesting the recruitment of these translation factors to virus replication sites. This would enable immediate translation of newly produced viral RNA and increase the translation efficiency, which could play important roles for early establishment of infection. Alphavirus nsP3 has been predicted to be largely disordered in its HVD region, and dozens of host factors have been identified as potential interaction partners (reviewed in (Götte *et al.* 2018)). Also, nsP3 is usually seen in virus replication complexes together with other nsPs. Hence, the G3BP:nsP3 complex could also help in the organization of viral replicases, e.g. by optimizing its conformation. Thus, the knowledge derived from paper I, especially the spatial organization of G3BP:nsP3 complexes, provides an important platform for further investigations on the composition of viral replication complexes and the potential roles of G3BP and other cellular factors.

**Paper II**

Many viruses manipulate P-bodies during infection, indicating the P-body response as a conserved and important host reaction against viral infection. In **paper II**, we describe the efficient P-body disassembly in a variety of cells infected with Old World alphaviruses (SFV or CHIKV). Our results add more support to the significance of the P-body response during viral infection. However, the mechanisms of P-body modulation during infection remain largely unknown. Dougherty and colleagues found the accelerated degradation of potential P-body scaffold proteins during poliovirus infection, including Dcp1a, XRN1 and the deadenylase complex component Pan3 (Dougherty *et al.* 2011). Inhibition of deadenylation

upon poliovirus infection was suggested as a potential mechanism of P-body disassembly (Dougherty *et al.* 2011), considering that deadenylation has been shown to be required for P-body formation (Zheng *et al.* 2008). A subsequent study of Dougherty and colleagues proposed that disassembly of P-bodies is mainly attributed to the functions of multiple viral proteases, as individual expression of viral proteases, including 3CD, 2Apro and 3Cpro, repressed or dispersed P-bodies (Dougherty *et al.* 2015). In our studies in the alphavirus context, we tested the protein levels of the P-body components DDX6 and Dcp1a at different time points (2, 4, 6, 8h) after infection with SFV. Both proteins remained rather stable over this period of time and were still present even when P-bodies were disassembled (data not shown). Hence, P-body components are unlikely targeted for accelerated degradation by any viral protease in alphavirus infection.

We investigated what virus parts are required for infection-induced P-body disassembly. Results from SFV-βGal showed that the expression of viral structural proteins is not required for the disassembly of P-bodies during infection with SFV (Fig. 6). Also, the P-body response was tested upon transient expression of all four nsPs, which are capable of forming functional viral replicases (Nikonov *et al.* 2013). This condition resembles the events at the early stage of infection. Upon expression of SFV nsPs, with or without viral genome template, dsRNA could be detected, confirming the formation of functional viral replicases. Yet, functional viral replicases obtained by transient expression of all nsPs failed to induce efficient disassembly of P-bodies (Fig. 7). It has to be noted, though, that these tools (SFV-βGal, trans-replication system) do not efficiently reproduce all aspects of natural infection. For instance, expression levels of viral proteins may differ from those in virus infection, and the replication efficiency in the trans-replication system is probably lower than in virus infection. Further, the regulation of the viral replicase may be different in the trans-replication system because a random reporter mRNA (for Firefly luciferase in this case) is generated instead of the non-structural mRNA that is produced upon infection with SFV-WT or SFV-βGal. Also, the viral non-structural ORF is much longer than the reporter gene, and the length of the virus genome can affect the size of virus-induced spherules and hence possibly the efficiency of downstream effects (Kallio *et al.* 2013). These limitations may have affected the efficiency of replication in the experiments. Proper and/or robust replication of the virus genome and features of the original viral RNA might be required for alphavirus-induced P-body disassembly.

Results from time course studies show that P-bodies are disassembled at a very early stage of infection (Fig. 8). Our previous studies have shown that SGs are induced at a similarly early stage of infection (McInerney *et al.* 2005, Panas *et al.* 2012). Many factors, including DDX6,

are known to be present in both types of granules under stress (reviewed in (Decker and Parker. 2012, Stoecklin *et al.* 2013, Ivanov *et al.* 2018)). DDX6, which has recently been suggested to be critical for P-body formation, was colocalized with SG markers when P-bodies were disassembled during SFV-βGal infection (paper II, Fig. 2 (b)). This all suggests some interdependence of SGs and P-bodies in the context of alphavirus infection. We therefore tested if the virus-induced disassembly of P-bodies was dependent on the virus-induced formation of SGs. To this end, we studied the P-body response in MEFs-AA cells, where SGs cannot be formed after Old World alphavirus infection, in comparison to wildtype MEFs (MEFs-SS). The results of these experiments show that P-bodies were equally disassembled after infection regardless of whether SGs can be induced or not. This, however, does not exclude potential communication between P-bodies and SGs during infection, since both granules are highly dynamic and can change within minutes. In consistence with this feature, an interesting phenomenon in our experiments was that in infected cells, P-bodies were either in their "normal" state (like in non-infected cells) or completely disassembled, but very rarely in an intermediate phase. These results indicate that disassembly of P-bodies happens so fast during infection that time course staining may not be able to catch the process. Thus, live-cell imaging might be a better way to study the dynamics of P-bodies during infection.

Through live-cell imaging studies, P-bodies were observed to be physically correlated with SGs under stress conditions (Kedersha *et al.* 2005). FRAP (fluorescence recovery after photobleaching) results indicate that the P-body factor Dcp1a shuttles between P-bodies and the cytosol as fast as within seconds (Kedersha *et al.* 2005, Aizer *et al.* 2008). The movement of most P-bodies is confined. P-body movement is associated with and also dependent on microtubule networks, as disruption of the microtubule network by nocodazole disrupts P-body movement (Aizer *et al.* 2008). In all these studies, U2OS cells were used, which had been reconstituted with fluorescently tagged P-body components for live-cell imaging. U2OS cells are particularly well-suited for such reconstitutions. However, U2OS cells are not the ideal cell line to study the P-body response because they contain only very few P-bodies (2–5 per cell), with up to 70% of cells not harboring any detectable P-bodies. Therefore, U2OS cells proved unsuitable for the experiments in paper II: The P-body disassembly upon SFV infection was not clearly evident in U2OS cells (data not shown), in contrast to the complete disassembly of P-bodies in virus-infected MEFs or BHK (Paper II, Fig. 1 and S1). The cell lines employed in paper II contain much more P-bodies on average, MEFs around 23 P-bodies/cell and BHK around 15 P-bodies/cell. The reason for the variation of P-body numbers between different cell lines is unknown.

Since reconstituted cell lines such as U2OS carrying RFP-Dcp1a and GFP-G3BP1 (Kedersha *et al.* 2005) are useful to study the communication between P-bodies and SGs, we performed preliminary live-cell imaging experiments with these cells and observed active movement of P-bodies and SGs and physical contact between them during SFV infection (unpublished data). We also attempted to establish reconstituted MEFs or BHK cells with RFP-Dcp1a as P-body marker for live-cell imaging studies. However, MEFs and BHK are both less tolerant than U2OS to such a modification. Furthermore, expression of exogenous Dcp1a turned out to be harmful and led to cell death of MEFs or BHK cells (unpublished data). Overall, a suitable reconstituted cell line for the purpose of live-cell imaging will be beneficial for investigation of P-body dynamics during infection.

P-bodies have been suggested to play important roles in the regulation of cellular translation. Some translation-related factors, such as initiation factor eIF4E and its transporter 4E-T, have been found accumulated in P-bodies (Andrei *et al.* 2005, Ferraiuolo *et al.* 2005). Interaction between eIF4E and 4E-T reduces the binding of eIF4E to eIF4G, leading to translation repression (Kubacka *et al.* 2013, Igreja *et al.* 2014). Recent studies revealed that P-body assembly requires the translation repression complex (Ayache *et al.* 2015) and DDX6–4E-T interaction, which also results in translation repression (Kamenska *et al.* 2016). Alphavirus infection leads to host translation shutoff, but different mechanisms have been suggested (Carrasco *et al.* 2018). Our previous studies have described that host translation shutoff is dominantly mediated through phosphorylation of eIF2α during Old World alphavirus infection (McInerney *et al.* 2005). In paper II, we used ribopuromycylation (David *et al.* 2012, Panas *et al.* 2015a) and found that efficient shutoff of host translation upon SFV or CHIKV infection was only observed in MEFs-SS, but not in MEFs-AA (Fig. 9B), strongly supporting our previous finding.

RNA is one of the essential components for P-body assembly, as P-bodies are disassembled when cellular RNA is depleted with RNAse treatment (Sen and Blau. 2005) or when mRNA is trapped in polysomes with cycloheximide treatment (Cougot *et al.* 2004). Cellular RNA is produced via transcription, a process which is manipulated during alphavirus infection (Garmashova *et al.* 2006, Breakwell *et al.* 2007, Garmashova *et al.* 2007). The transcription status was investigated by labeling newly synthesized RNA with bromo-UTP during infection of cells with SFV or CHIKV. The results show that virus infection induced host transcription shutoff at about the same time as disassembly of P-bodies was observed. When we assessed the P-body response in the context of transcription inhibition by ActD treatment, P-body numbers were reduced within a nontoxic period of time, but ActD treatment did not

disassemble P-bodies as efficiently as virus infection (Fig. 10B). Therefore, it appears likely that the infection-induced P-body disassembly is caused by more than just host transcription shutoff induced by virus infection.

Since a lack of RNA blocks P-body assembly, we hypothesized that changing levels of cytoplasmic mRNA could interfere with P-body dynamics in addition to transcription inhibition. The antibiotics CHX and puromycin are protein synthesis inhibitors and can affect free mRNA levels in the cytoplasm, but through different modes of action: CHX stabilizes mRNA with polysomes, leading to a decrease of free mRNA in cytoplasm, while puromycin causes premature chain termination and mRNA release during translation, resulting in an increase of free mRNA in the cytoplasm (Kedersha *et al.* 2000). Rapid and efficient P-body disassembly was observed after CHX treatment (Paper II, Fig. 5 (a); Fig. 11). However, CHX treatment is unlikely to reflect how P-body disassembly happens during viral infection. Not only does CHX cause disassembly of P-bodies, but it also effectively blocks induction of SGs, as a result of reducing free mRNA. If P-bodies were disassembled by the same mechanism during viral infection, SGs would not be induced after infection, but it is known that they are, which was again confirmed in paper II. It is conceivable but speculative that virus infection somehow makes mRNA selectively unavailable for the formation of P-bodies while not affecting their propensity to move into SGs.

When P-bodies were assessed in response to puromycin treatment, no clear change was observed within a short period of puromycin treatment (2h), while a longer treatment almost eliminated P-bodies (Paper II, Fig. S5 (a)). In contrast, puromycin can elevate mRNA levels in the cytoplasm and contribute to the increase of P-bodies upon stress (Kedersha *et al.* 2000). Here, in the absence of stress, the disassembly of P-bodies after 4h of puromycin treatment most likely indicates that dynamics of P-bodies are related to translation status.

We hypothesized that virus-induced transcription shutoff could indicate potential damages of nuclear transport, which might in turn affect P-body numbers. In order to investigate whether nuclear transport may be involved in P-body disassembly, several selective inhibitors of nuclear transport were used. When importin-mediated nuclear import was blocked with importazole (Soderholm *et al.* 2011), P-bodies were largely disassembled (Fig. 11; Paper II, Fig. 5 (a)), while a block of CRM1-mediated nuclear export with leptomycin B (Nishi *et al.* 1994, Kudo *et al.* 1999) did not affect P-bodies (Paper II, Fig. S5 (b)). Thus, intact nuclear import processes appear to be important to maintain P-bodies, while nuclear export seems to be less relevant. These data reveal nuclear import as a novel P-body regulation pathway, which needs to be explored further.

Nuclear import might be modulated during virus infection and contribute to virus-induced P-body disassembly. It remains to be determined by what mechanism alphavirus infection interferes with nuclear import to affect P-bodies. Alphavirus infection has already been shown to manipulate nuclear import. During infection of Venezuelan equine encephalitis virus (VEEV), various receptor-mediated nuclear import processes are blocked by the viral capsid protein (Atasheva *et al.* 2008). Another hint for nuclear transport modulation originates from the observation that many nuclear proteins are redirected to and stocked in the cytoplasm during SINV infection (Sanz *et al.* 2015).

In summary, paper II shows for the first time that alphavirus infection leads to efficient disassembly of P-bodies early after infection, possibly helping the virus to establish infection. We identified virus-induced inhibition of host cell transcription and translation plus nuclear import inhibition as potential mechanisms by which virus infection could interfere with P-bodies. Nuclear import is a novel mechanism of P-body regulation.

**Paper III**

In this work, we investigated the molecular requirements for alphavirus-induced hyperactivation of the PI3K–Akt–mTOR pathway, an important cellular signaling pathway to promote survival. We identified the key tyrosine residue Y369 in the context of a YXXM motif in nsP3 of SFV to be responsible for this phenotype through interaction with the regulatory subunit p85 of PI3K. We also show that a YXXM motif is functionally present in nsP3 of the important human pathogen RRV to mediate PI3K–Akt–mTOR hyperactivation, with relevance for pathogenesis.

The interaction between p85 and nsP3 of SFV and RRV is a newly discovered feature of nsP3 HVD – a protein region that binds several cellular factors and can be considered to be a hub for molecular interactions: the proline rich region has been shown to bind amphiphysin (Neuvonen *et al.* 2011); FGDF motifs interact with G3BP (Paper I, (Panas *et al.* 2015b)). Such interactions between the nsP3 HVD and cellular proteins play significant roles in viral growth and replication. SFV infection was attenuated by one order of magnitude upon point mutation of the single tyrosine in the YXXM motif, which indicates its high relevance for virus growth, similar to that of other interaction motifs in the HVD such as the FGDF motifs (see Paper I).

According to the well-studied interaction mode between p85 and YXXM motifs, the SH2 domains of p85 recognize phosphorylated tyrosine in the sequence context YXXM (Shoelson *et al.* 1993, Songyang *et al.* 1993, Waksman *et al.* 1993). In line with this binding mode, the results in paper III show that the interaction between p85 and SFV nsP3 is dependent on both

the SH2 domains of p85 and the YXXM tyrosine in nsP3: mutation of either of the two features disrupted the molecular interaction. Replacement of the YXXM tyrosine by phenylalanine – which has the same structure as tyrosine except for the absence of the phosphorylatable –OH group – was enough to abolish the nsP3–p85 interaction and the PI3K–Akt–mTOR hyperactivation phenotype. Even though we have not chemically shown that the YXXM tyrosine in SFV nsP3 is phosphorylated, our results strongly suggest that this tyrosine most likely is phosphorylated, at least to some degree during certain periods of time after infection, probably in a transient manner. This might be the reason why tyrosine phosphorylation of SFV nsP3 has never been reported in biochemical studies (Peränen *et al.* 1988; Vihinen and Saarinen. 2000). It remains to be determined which cellular kinases catalyze phosphorylation of the tyrosine in SFV nsP3 and how their activity is regulated during infection. While membrane targeting of nsP3 is required, the enzymatic activities of the other viral proteins are dispensable for tyrosine phosphorylation and PI3K–Akt–mTOR hyperactivation since expression of Myr-Pal-nsP3-wt (but not -YF) was sufficient to induce the phenotype. A proper position of the YXXM motif however appears to be relevant, based on the results from SFV-Δ50, which does not hyperactivate PI3K–Akt–mTOR even though the YXXM motif is intact. It is conceivable that phosphorylation of some serines and theronines in the region that is deleted in SFV-Δ50 or some other features of this region may be required to allow phosphorylation of the YXXM tyrosine. Most likely, the deletion of the 50 residues immediately preceding Y369 causes steric hindrance of the YXXM motif in SFV-Δ50 and prevents phosphorylation of the tyrosine and/or access of p85.

The YXXM motif is not conserved in all alphaviruses. Sequence analysis of nsP3 of all alphaviruses shows that YXXM motifs are only identified in SFV, RRV and very few other alphaviruse nsP3: Getah virus (GETV) and Sagiyama virus, which are equine pathogens in Asia (Fukunaga *et al.* 2000), as well as Middelburg virus, which mainly infects sheep, goats and horses in Africa (Attoui *et al.* 2007). We hence predict these viruses to induce PI3K–Akt–mTOR hyperactivation as well. YXXM motifs are however absent in CHIKV and many other arthritogenic Old World alphaviruses as well as the New World alphaviruses. CHIKV (and likely the other alphaviruses lacking a functional YXXM motif) only moderately activate PI3K–Akt–mTOR.

It is unlikely that the presence of YXXM is connected to vertebrate host specificity. RRV infects humans and causes clinically relevant disease, while SFV does not, and the other alphaviruses with YXXM motifs in nsP3 mainly infect horses. Other alphaviruses that are pathogenic for humans (e.g. CHIKV) or horses (New World alphaviruses), respectively,

however do not contain the motif. The presence of YXXM might however be related to vector competence. RRV has been isolated from more than 40 different mosquito vectors belonging to the genera *Culex* and *Aedes*, and can be considered a "vector generalist" (Claflin and Webb. 2015). Little is known about SFV vectors in nature, but the virus is known to cause persistent infection in *Aedes* and *Culex* cell lines based on laboratory studies (Davey and Dalgarno. 1974, Davey *et al.* 1979). Alphaviruses without YXXM seem to be more restricted regarding their arthropod vectors. CHIKV, for instance, is carried by *Aedes aegypti* and *Aedes albopictus*, but not *Culex* mosquitoes (Pialoux *et al.* 2007). Perhaps PI3K–Akt–mTOR hyperactivation somehow helps the virus to establish persistent infection in a wider variety of anthropod hosts, which may be beneficial for virus spread – and potentially challenging for public health.

The PI3K–Akt–mTOR pathway is also targeted and exploited by other viruses using different strategies. Some viruses inhibit the pathway during infection (Avota *et al.* 2001, Dunn and Connor. 2011), while others, such as herpes simplex virus (Strunk *et al.* 2016) and influenza A virus (IAV) (Hale *et al.* 2006), activate the pathway. Interestingly, a similar – but not identical – molecular phenotype as the one described in paper III for SFV and RRV was observed for IAV, a segmented negative-strand RNA virus unrelated to alphaviruses: A conserved tyrosine in a YXXM-like motif in the nonstructural protein 1 (NS1) of IAV was shown to be responsible for binding of p85-β (but not p85-α) and activation of the PI3K–Akt–mTOR pathway. In addition, PI3Ks can also be activated by proline-rich domains, which can be recognized by the SH3 domain of p85. Of note, there is a proline-rich region in the HVD of alphavirus nsP3 as well, but deletion of this region in SFV does not markedly affect the PI3K–Akt–mTOR hyperactivation phenotype (Thaa *et al.* 2015). We cannot exclude a potential contributing role of the proline-rich motif in alphavirus nsP3 for PI3K–Akt–mTOR activation. However, the YXXM tyrosine is clearly the dominant feature for p85 binding and PI3K–Akt–mTOR hyperactivation by SFV and RRV.

Paper III provides functional implications of the PI3K–Akt–mTOR hyperactivation phenotype. A very important downstream effect of PI3K–Akt hyperactivation is the sustained activation of mTOR, even under conditions of complete nutrient and growth factor starvation. Activation of mTOR promotes synthesis of proteins and lipids and keeps cells in a state of normal growth, which might ensure viral proliferation even under adverse conditions. Furthermore, metabolomics revealed an up-regulation of glycolysis upon infection with SFV-WT, which is connected to PI3K–Akt–mTOR hyperactivation because it was sensitive to the PI3K inhibitor wortmannin and much less pronounced with SFV-YF. The metabolic changes upon SFV-WT infection led to increased levels of fatty acids, amino acids and nucleotides, which all serve as

building blocks for progeny virions. Similar effects on glycolysis were also observed with RRV. These results suggest that a major effect of PI3K–Akt–mTOR hyperactivation concerns metabolism. The results of the *in vivo* infection experiment with RRV in mice, showing attenuation of RRV-YF compared to RRV-WT, indicates that PI3K–Akt–mTOR hyperactivation is relevant for virulence, likely because of metabolic effects, but potentially due to other additional mechanisms.

The PI3K–Akt–mTOR pathway has been linked to many cellular processes, including SGs (Nitulescu *et al.* 2016, Heberle *et al.* 2019). A recent study has shown that activation of PI3K and mTOR promotes SG formation under stress (Heberle *et al.* 2019), which resembles the events upon SFV-WT infection, where the PI3K–Akt–mTOR pathway is hyperactivated (paper III) and SGs are induced at early stages of infection (McInerney *et al.* 2005). However, the PI3K–Akt–mTOR hyperactivation seen with SFV and RRV, dependent on the YXXM motif in nsP3, is largely unrelated to the induction or modulation of SGs. Alphavirus-induced SG formation proceeds by translation shutoff caused by infection-induced eIF2α phosphorylation early in infection (McInerney *et al.* 2005). With alphavirus infection, eIF2α is phosphorylated by dsRNA-activated protein kinase R (PKR), which is probably not linked to the PI3K–Akt–mTOR pathway. Likely, SFV-YF also induces SGs upon infection by generation of dsRNA. Further, virus-induced SGs are later counteracted by the FGDF-motif-mediated interaction of nsP3 with G3BP (Panas *et al.* 2012, Panas *et al.* 2015b), which is likely conserved between SFV-WT and SFV-YF due to the presence of two intact FGDF motifs. Moreover, SFV-delta789, the nsP3 of which does not interact with G3BP, hyperactivates PI3K–Akt–mTOR to the same extent as SFV-WT (Thaa *et al.* 2015). Upon infection of SFV-WT or SFV-YF, P-bodies were similarly disassembled (data not shown), although the PI3K–Akt–mTOR pathway is activated to different levels. Lastly, SG modulation and G3BP binding (see paper I) and P-body disassembly (see paper II) are conserved between SFV and CHIKV, while the PI3K–Akt–mTOR hyperactivation phenotype is not (Thaa *et al.* 2015). In summary, virus-caused PI3K–Akt–mTOR hyperactivation is unlikely related to the responses of SGs or P-bodies upon alphavirus infection (Table 1).

Potential connections between the PI3K–Akt–mTOR pathway and SGs and/or P-bodies in cells may exist, but these connections are unlikely to be relevant in alphavirus infection and are much weaker than the viral effects on either of these pathways as characterized in this thesis. The effects of alphaviruses on SGs/G3BP, P-bodies and the PI3K–Akt–mTOR pathway can be seen as mostly separate principles (Table 1), each of which is exploited by the virus to ensure efficient establishment of infection and efficient growth.

| | Interaction with G3BP | SG counteraction | P-body disassembly | PI3K–Akt–mTOR hyperactivation |
|---|---|---|---|---|
| SFV-WT | ✓ **a** | ✓ **a** | ✓ **b** | ✓ **c** |
| SFV-F3A$_{NC}$ | ✗ **a** | ✗ **a** | ✓ **d** | ✓ **c** |
| SFV-YF | ✓ **e** | ✓ **e** | ✓ **d** | ✗ **c** |

**Table 1: Feature summary of SFV-WT, -F3A$_{NC}$, -YF regarding interaction with G3BP, SG counteraction, P-body disassembly and PI3K–Akt–mTOR hyperactivation. (✓): positive. (✗)**: negative. **a**: shown in paper I and (Panas *et al.* 2012, Panas *et al.* 2015b); **b**: shown in paper II; **c**: shown in paper III and (Thaa *et al.* 2015); **d**: data not shown; **e**: not determined, but highly likely based on the molecular mechanism of G3BP interaction

# 4 FUTURE PERSPECTIVES

The work described in this thesis characterized three separate mechanisms of how alphaviruses interfere with the host cell to establish infection: binding of the two FGDF motifs in nsP3 to the SG nucleating protein G3BP (paper I), disassembly of P-bodies (paper II) and hyperactivation of the PI3K–Akt–mTOR pathway by interaction of a YXXM motif in nsP3 of SFV and RRV with the p85 subunit of PI3K (paper III). The results in papers I and III demonstrate two specific virus–host interactions in molecular detail and characterize their functional importance for the virus at different levels. In paper II, a novel virus–host interaction was discovered and characterized.

**Paper I**

In paper I, the FGDF-mediated interaction of nsP3 with G3BP was scrutinized for SFV and CHIKV. The FGDF-mediated interaction of nsP3 with G3BP seems to be conserved across the Old World alphaviruses, while the details of this interaction and the subsequent functional sequences may differ among different virus species. The HVD of nsP3, the protein domain where the FGDF motifs are situated, varies greatly between the alphavirus species and even between strains of the same virus. In respect to the binding to G3BP, the surrounding of the two FGDF motifs may matter, as seen in paper I for the comparison between SFV and CHIKV. In addition, the two FGDF sequences are not fully conserved in all Old World alphaviruses. For instance, the second FGDF in nsP3 of RRV is replaced by the sequence FGDIDF. Given the functional importance of the interaction with G3BP, it is very likely that nsP3 of RRV binds to G3BP in a very similar manner as nsP3 of SFV or CHIKV. But the interaction details and subsequent potential impact on virus growth remain unknown, which is worth to be investigated.

Another open issue concerns the difference between the two isoforms of G3BP, G3BP1 and G3BP2, which has been investigated only poorly (Tourriere *et al.* 2003). Most studies focus on either of these isoforms and treat them as backup for each other with redundant functions. It is not easy to study only one of the isoforms individually, as single knockdown of either G3BP1 or G3BP2 results in the increase of the other one (Matsuki *et al.* 2013). In our studies, we observed potentially relevant differences between G3BP1 and G3BP2: Both $FGDF_N$ and $FGDF_C$ bound G3BP2 to equal extents, in contrast to the binding to G3BP1. Moreover, virus infection accelerated the degradation of G3BP2 but not G3BP1 (data not shown). These results suggest that FGDF motifs may have different binding preferences for G3BP1 and G3BP2.

More studies should be conducted to differentiate the roles and functions of the G3BP isoforms, in alphavirus infection and beyond.

The roles of G3BP in alphavirus infection have not been fully elucidated. The results of paper I suggest that G3BP plays an active role in the viral RCs. Such studies have been done in our group to investigate the role of the domains of G3BP to promote efficient translation in viral replication complexes (Götte *et al.* 2019).

The FGDF-mediated interaction may be an attractive target for therapeutic intervention, since it is so important for alphaviruses infection. CHIKV, for instance, is unable to replicate if there is no interaction of nsP3 and G3BP (paper I). Thus, blocking the interaction with some small molecule inhibitor or peptide-based agent may inhibit virus replication.

**Paper II**

In paper II, the timing and extent of alphavirus-induced P-body disassembly was characterized, and some hints to possible mechanisms were suggested (transcription and translation inhibition, nuclear import). This needs to be explored further.

Knowledge on what elements of the virus are responsible for P-body disassembly would facilitate the mechanistic studies of virus-induced P-body responses, as well as P-body dynamics. Our results show that viral structural proteins are dispensable for alphavirus-induced P-body disassembly (Fig. 6B), while expression of nsPs, even though functional virus replicase is formed, is not sufficient (Fig. 7). The trans-replication system is a convenient but somewhat artificial system, which could however still be useful for further studies. In contrast to the virus and SFV-βGal, a reporter gene, instead of the "real" virus genes for nsPs, is replicated by the viral replicase in the trans-replication system. This difference could result in defects in replicase regulation and subsequently aberrant replication in the trans-replication system. To minimize this difference, the reporter gene could be replaced with full length genes for nsPs, to resemble the events during SFV infection more faithfully. The experiment could be further extended by replacing the reporter gene with genes for various combinations of nsPs, e.g nsP12, nsP123, nsP23. The results of such experiments would shed more light on how P-bodies respond to various combinations of nsPs and help to identify what elements of the virus are responsible for P-body disassembly.

The results of paper II reveal and highlight the importance of nuclear import for P-body dynamics. This warrants closer investigation. There may be cellular factors involved in nuclear transport, such as importins and Ran GTPases, that might be targeted by the virus.

Immunoprecipitation and proteomics studies could be helpful to identify interaction partners with potential relevance for P-body disassembly.

Recently, there have been significant technical advances in labelling and tracking of RNA (Horvathova *et al.* 2017, Tutucci *et al.* 2018). It would be interesting to apply these techniques for P-body studies after infection. Such studies would require triple labeling in live cells for random RNA, P-bodies and virus. Such an imaging study in live cells would generate interesting information regarding how infection modulates cellular RNA and how P-bodies respond to virus infection. Specifically, the hypothesis could be tested that at early stages of infection, the virus may cause rapid degradation of cytoplasmic mRNA in the periphery of P-bodies, leading to the disassembly of the granules. Live-cell imaging will probably provide more information, considering that P-bodies are highly dynamic structures.

Lastly, it is currently unclear whether P-bodies could be exploited as an antiviral strategy. Stabilizing P-bodies or preventing their disassembly might restrict virus replication. But first, more research is needed to better understand P-body dynamics and the viral strategies to interfere with P-bodies.

**Paper III**

In paper III, the molecular mechanism of PI3K–Akt–mTOR hyperactivation upon SFV and RRV infection was characterized in molecular detail. The study revealed that these viruses exploit a well-known cellular interaction mechanism, the binding of the SH2 domains of p85 to phospho-tyrosine in a YXXM motif. The PI3K–Akt–mTOR hyperactivation alters cellular metabolism and possibly affects other signaling pathways.

The PI3K–Akt–mTOR hyperactivation phenotype is not conserved across the Old World alphavirus, unlike the FGDF-motif-based interaction of nsP3 with G3BP (paper I) or the P-body disassembly phenotype (paper II). The relevance of PI3K–Akt–mTOR hyperactivation is thus limited to some specific alphaviruses. However, the knowledge about the PI3K–Akt–mTOR hyperactivation phenotype may be helpful to design SFV-based viral vectors for gene therapy or vaccines. Hyperactivation of PI3K–Akt–mTOR could be either beneficial or harmful to the target cells depending on the circumstances, thus either SFV-WT or SFV-YF should be selectively applied.

The PI3K–Akt–mTOR pathway is also activated by alphaviruses that do not carry a functional YXXM motif (SFV-YF, RRV-YF, CHIKV), albeit to a lesser extent. This “basal” activation of the pathway is weaker and slower and can be inhibited by nutrient and growth factor

starvation (paper III, (Thaa *et al.* 2015)). The mechanisms and functions of this "basal" activation have not been determined, but warrant closer investigation. It is unclear whether this "basal" activation is just a cellular response to virus infection at later stages or whether it is actively modulated by the virus. In the latter case, the "basal" activation of this cellular survival pathway may be relevant for virus growth and may thus represent a potential therapeutic target.

**Concluding remarks**

This thesis describes three principles by which alphaviruses modulate mammalian host cells. As arboviruses, alphaviruses have another host – the insect vector. Some of the mechanisms described in this thesis may also be relevant in insects or may even have evolved in the insect host. Future research should therefore investigate these mechanisms in insects as well. It has been shown that nsP3 binds to the insect homolog of G3BP termed "Rasputin" (Fros *et al.* 2015), while alphavirus interactions with P-bodies in insects have not been described. Modulation of the PI3K–Akt–mTOR pathway in insects might be relevant in terms of persistence and vector competence.

The results presented in this thesis hopefully build the basis for follow-up research to learn even more about alphavirus and host cell biology.

# 5 ACKNOWLEDGEMENTS

During my PhD studies, I feel so lucky that I have met so many great people. I am truly grateful to all of you who have helped and supported me in your ways.

**Gerry**, my main supervisor, thank you for recruiting me to your group and trusting me to do a PhD. During my studies, you have taught me so much basic knowledge of virology and cell biology and helped me to develop scientific thinking and talking, to raise up questions and seek solutions and to build up my confidence. I am also grateful to your patience. You give me time to improve my language, give me your support when I made mistakes and give me the freedom to explore my interest and develop the project independently. You made me as a researcher. Most importantly, all these have been happening in a way I feel like I am having fun with friends, instead of studying – you have the best way for teaching and supervising. I am proud of how much I have improved and what I have achieved, which could only happen with your help. I am also influenced by your great passion for science. You have created an environment for "pure research" where we can focus the most on our work. Beyond the work in this thesis, I am most grateful that you have shown me how research is done as the way it should be, which, I believe, will continue guiding me in the future.

**Bastian**, my co-supervisor, thank you for helping me to start my studies and a new life in Stockholm. You have helped me to get into our group and my projects. I am impressed by your knowledge. You always have constructive suggestions and inspiring ideas during our discussion. You are patient and optimistic. Whenever I have questions, you explain in great details and you see the positive sides even when we got negative results. All these experiences helped to formulate my attitude to scientific research, which will of course be beneficial for my future career. Also, I am grateful how much effort you have put to help me with my spoken and written English. During my PhD studies, you have been so supportive all the time. I feel so lucky to be your student.

**Adnane**, my co-supervisor, thank you for your guidance and support for the first project. I am glad I could join the project and collaborate with your group. During our meetings, you often brought up critical questions and gave helpful suggestions, which ensured the progress and the publication of our work. Whenever I need your help and support, you reply my emails within several minutes. I am amazed how decisive and efficient you are. It is so good to have you as a co-supervisor.

All my co-authors, **Tim**, **Marc**, **Ben**, **Nicole**, **Eva**, **Stina**, **Michela**, **Cecilia**, **Margit**, **Xiang**, **Suresh**, **Julian**, **Mark**, it is your enthusiasm and effort to make our work published. I

appreciate all your contribution, although some of you I haven't met yet. For those I meet regularly, **Tim, Ben**, **Marc**, I really enjoyed the discussion with you. I admire how talent and creative you are. I have learned a lot from you. It is so nice to work with you.

**Susanne**, thank you for being my mentor. Although we didn't interact frequently, I feel supported by having you as my mentor.

The former and current members of McInerney group: **Ainhoa**, **Ben**, **Marc**, **Leo**, **Cecilia**, **Juli**, **Timothy**, **Lina**, **Eline**: We are a great team and willing to help each other all the time. Everyone is generous to share their experience and expertise. I have learnt so much from all of you. We voluntarily take lab responsibilities and maintain our lab the most comfortable work place. It is the effort of all your that ensures the work efficiency and productivity in the group. You also made the group as my family in Europe. Every time I walk into our lab, I feel at home. Our weekly group meeting is like a family brunch. All these daily, small things mean a lot to me and can keep me up. It can be our regular gym company, an ironic joke in the lab, a causal chat over a cup of coffee, a share of experience over lunch or even a brief pass-by smile. I cannot image my life without all these enjoyable moments.

I started my studies in the same lab with Nilla's and Jonathon's groups on 4$^{th}$ floor in MTC. Then our group moved to 5$^{th}$ floor and shared the lab with Peter's group. Now we all have moved into the same quarter in Biomedicum, sharing the lab. What a great start, a wonderful experience and a happy big reunion. **Nilla** and **Jonathan**, both of you are great scientists and excellent group leaders. Thank you for creating such a creative work environment for us and sharing the experience of your careers as well as giving advices to mine. Thank you **Peter** for sharing all the equipment with our group. It was a great pleasure to work with the people in the three groups. **Néstor** and **Sharesta**, thank you for helping to improve my English, to get to know the European lifestyle and to cheer me up over all the talks. All these mean a lot to me. **Elina**, thank you for sharing your postdoc experience and help me when it comes to the culture or policies in Sweden. **Martin**, **Gabriel**, **Monika**, **Lotta**, **Ganesh**, **Martina**, **Paola**, thank you for sharing your experience in Sweden and giving nice tips. **Leona**, **Julian**, **Chris**, **Junjie**, thank you for sharing funny stories and keep our small quarter joyful. **Marco**, **Sanjana**, **Pradee**, **Uta**, thank you for being friendly, supportive and joking around in the lab. It's always nice to have you in company in the lab. **Inga** and **Karl**, it is great to share lab with you. Everything goes so smooth all the time. Thank you **Karl** for sharing postdoc information with me and giving suggestions for my defense examination board.

Some friends from MTC, it is great to meet you here. It's always relaxing to talk to you. **Gao**, **Nyosha**, **Minghui**, **Wenwen**, **Shuijie**, thank you for giving me suggestions and helping to have a better overview of my future plan. **Shady**, **Fengyang**, **Benedek**, **Gergana**, **Fan**, **Honglei**, **Wesam**, **Shixing, Xiaogang**, **Chengfei**, **Wenyu**, **Xiaolei**, thank you for providing support to my experiments and sharing interesting experience as well as helpful information for life and career. **Jiawei**, thank you for the causal chat and being a company in the gym. **Vanessa**, thank you for your understanding for the defense schedule and sharing information with me. **Sonia, Gergana, Andrés** and **Marcus**, thank you for your effort to our antiviral project. You are talent and enthusiastic for research. I got much experience from you for small molecule studies. I have to say that all your encouragement have cheered me up from time to time and helped me get over tough moments.

All my friends I have met here in Sweden, thank you all for sharing my life here. There is no doubt I will keep all the happy moments and memories. **Carles**, I'm glad we had lunch together sometime. It's always relaxing and beneficial to talk to you. **Ang**, thank you for sharing your room with me for the first two weeks I came to Sweden. **Ang** and **Wenyi**, it was a wonderful year to share a flat with you. Thank you for being good roommates and teaching me cooking. **Liang**, **Haomin**, **Tenghao**, **Dongmei**, **Xixi**, **Ang**, **wenyi**, and our "group leader" **Liesu**, thank you for great events, good food and wonderful games we have had together. **Yitian**, **Zeyu**, **Muyi**, **Liang**, **Gao**, **Xiaolei**, **JunJie**, it's always fun to play basketball and chat with you. I will miss all the wonderful games. **Weifeng** and **Xu**, it was a good time to live in the same corridor and share the kitchen. Thank you for sharing your experience in Sweden and helping me and Yanzhou with Swedish studying. To all of you, it wouldn't be such a great time without any of you.

**Renhai Cao** and **Li Chen**, you are good seniors. Thank you for your advices for my life and career. It was a great experience to go fishing with you, **Renhai**.

I also want to express my appreciation to all my teachers when I studied in China. Some of you are not only good teachers but also import mentors for my career and life. Many thanks to **白红敏**老师，**孔令军**老师，**赵桂英**老师。

My "brothers" from high school, we have known each other for over 15 years. It's amazing we keep in touch all the time, even after I moved to Sweden. I live far away from all of you now, but every time we chat via our Wechat group, it brings my mind home. You make my life grounded. It's so great to keep the friendship with you: **Qingyun**, **Xiangyu**, **Qingjie**, **Weichao Weitao**, **Zhigang** and **Ruiyan**.

To my family:

**哥**、**嫂子**、**姐**、**姐夫**，谢谢你们一直以来对咱爸、咱妈和家里照顾，也谢谢**刘铭杰**、**刘铭森**和**李怡然**给家里带来的快乐和希望。**叔**和**姑姑们**，谢谢你们对我成长的关心和对我学习的支持。**红军哥**，很幸运经常和你交流，感谢你对我学习的支持和开导。**艳洲妈**，谢谢你对家里的照顾和对我们的支持，使得艳洲和我能在瑞典安心地生活和学习。**知宏**，谢谢你对你姐和我的理解，你的成长是对我们最大的支持，同时也不断激励着我。

**爸**、**妈**，谢谢你们一直以来对我的照顾和关爱，谢谢你们无论什么时候都支持我和鼓励我。不管我做的怎么样，在你们心中我都是最优秀的，每当我遇到困难的时候，你们都会说：”孩子，累了就回家”，这是你们对我最大的安慰和鼓励，我没有辜负你们为我所做的一切，我取得博士学位了。

**艳洲**, du är unik för mig. Tack för att du är här med mig. Jag inspireras av din entusiasm för studier och för livet. Du får mig att känna mig säker och trygg i mig själv. Att vara tillsammans med dig tar fram det bästa hos mig. Den här boken är till dig.

My study was partly supported by Chines Scholarship Council (CSC).

# 6 REFERENCES


Abraham, R., D. Hauer, R. L. McPherson, A. Utt, I. T. Kirby, M. S. Cohen, A. Merits, A. K. L. Leung and D. E. Griffin (2018). "ADP-ribosyl-binding and hydrolase activities of the alphavirus nsP3 macrodomain are critical for initiation of virus replication." Proc Natl Acad Sci U S A **115**(44): E10457-e10466.

Acheson, N. H. (2011). Fundamentals of Molecular Virology WELEY. 2nd edition. Hoboken, NJ, USA: John Wiley & Sons. ISBN 9780470900598.

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**(15): 6725-6733.

Ahola, T., P. Laakkonen, H. Vihinen and L. Kaariainen (1997). "Critical residues of Semliki Forest virus RNA capping enzyme involved in methyltransferase and guanylyltransferase-like activities." J Virol **71**(1): 392-397.

Ahola, T., A. Lampio, P. Auvinen and L. Kaariainen (1999). "Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity." Embo j **18**(11): 3164-3172.

Aizer, A., Y. Brody, L. W. Ler, N. Sonenberg, R. H. Singer and Y. Shav-Tal (2008). "The dynamics of mammalian P body transport, assembly, and disassembly in vivo." Mol Biol Cell **19**(10): 4154-4166.

Aizer, A., A. Kalo, P. Kafri, A. Shraga, R. Ben-Yishay, A. Jacob, N. Kinor and Y. Shav-Tal (2014). "Quantifying mRNA targeting to P-bodies in living human cells reveals their dual role in mRNA decay and storage." J Cell Sci **127**(Pt 20): 4443-4456.

Akhrymuk, I., I. Frolov and E. I. Frolova (2018). "Sindbis Virus Infection Causes Cell Death by nsP2-Induced Transcriptional Shutoff or by nsP3-Dependent Translational Shutoff." J Virol **92**(23): e01388-18.

Akhrymuk, I., S. V. Kulemzin and E. I. Frolova (2012). "Evasion of the innate immune response: the Old World alphavirus nsP2 protein induces rapid degradation of Rpb1, a catalytic subunit of RNA polymerase II." J Virol **86**(13): 7180-7191.

Anderson, P. and N. Kedersha (2006). "RNA granules." J Cell Biol **172**(6): 803-808.

Anderson, P. and N. Kedersha (2009). "Stress granules." Curr Biol **19**(10): R397-398.

Andrei, M. A., D. Ingelfinger, R. Heintzmann, T. Achsel, R. Rivera-Pomar and R. Luhrmann (2005). "A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies." Rna **11**(5): 717-727.

Ariumi, Y., M. Kuroki, Y. Kushima, K. Osugi, M. Hijikata, M. Maki, M. Ikeda and N. Kato (2011). "Hepatitis C virus hijacks P-body and stress granule components around lipid droplets." J Virol **85**(14): 6882-6892.

Arribere, J. A., J. A. Doudna and W. V. Gilbert (2011). "Reconsidering movement of eukaryotic mRNAs between polysomes and P bodies." Mol Cell **44**(5): 745-758.

Atasheva, S., N. Garmashova, I. Frolov and E. Frolova (2008). "Venezuelan equine encephalitis virus capsid protein inhibits nuclear import in Mammalian but not in mosquito cells." J Virol **82**(8): 4028-4041.

Attoui, H., C. Sailleau, F. Mohd Jaafar, M. Belhouchet, P. Biagini, J. F. Cantaloube, P. de Micco, P. Mertens and S. Zientara (2007). "Complete nucleotide sequence of Middelburg virus, isolated from the spleen of a horse with severe clinical disease in Zimbabwe." J Gen Virol **88**(Pt 11): 3078-3088.

Avota, E., A. Avots, S. Niewiesk, L. P. Kane, U. Bommhardt, V. ter Meulen and S. Schneider-Schaulies (2001). "Disruption of Akt kinase activation is important for immunosuppression induced by measles virus." Nat Med **7**(6): 725-731.

Ayache, J., M. Benard, M. Ernoult-Lange, N. Minshall, N. Standart, M. Kress and D. Weil (2015). "P-body assembly requires DDX6 repression complexes rather than decay or Ataxin2/2L complexes." Mol Biol Cell **26**(14): 2579-2595.

Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger and et al. (1992). "Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation." Embo j **11**(9): 3469-3479.

Balagopal, V. and R. Parker (2009). "Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs." Curr Opin Cell Biol **21**(3): 403-408.

Bensaude, O. (2011). "Inhibiting eukaryotic transcription: Which compound to choose? How to evaluate its activity?" Transcription **2**(3): 103-108.

Brady, O. J., A. Osgood-Zimmerman, N. J. Kassebaum, S. E. Ray, V. E. M. de Araujo, A. A. da Nobrega, L. C. V. Frutuoso, R. C. R. Lecca, A. Stevens, B. Zoca de Oliveira, J. M. de Lima, Jr., Bogoch, II, P. Mayaud, T. Jaenisch, A. H. Mokdad, C. J. L. Murray, S. I. Hay, R. C. Reiner, Jr. and F. Marinho (2019). "The association between Zika virus infection and microcephaly in Brazil 2015-2017: An observational analysis of over 4 million births." PLoS Med **16**(3): e1002755.

Breakwell, L., P. Dosenovic, G. B. Karlsson Hedestam, M. D'Amato, P. Liljestrom, J. Fazakerley and G. M. McInerney (2007). "Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response." J Virol **81**(16): 8677-8684.

Carrasco, L., M. A. Sanz and E. Gonzalez-Almela (2018). "The Regulation of Translation in Alphavirus-Infected Cells." Viruses **10**(2): E70.

Casales, E., J. R. Rodriguez-Madoz, M. Ruiz-Guillen, N. Razquin, Y. Cuevas, J. Prieto and C. Smerdou (2008). "Development of a new noncytopathic Semliki Forest virus vector providing high expression levels and stability." Virology **376**(1): 242-251.

Centers for Disease Control and Prevention. (accessed on 19 April 2019). "States reporting chikungunya virus disease cases – United States, 2018." from https://www.cdc.gov/chikungunya/geo/united-states-2018.html.

Chahar, H. S., S. Chen and N. Manjunath (2013). "P-body components LSM1, GW182, DDX3, DDX6 and XRN1 are recruited to WNV replication sites and positively regulate viral replication." Virology **436**(1): 1-7.

Chen, C. Y. and A. B. Shyu (2011). "Mechanisms of deadenylation-dependent decay." Wiley Interdiscip Rev RNA **2**(2): 167-183.

Chen, C. Y. and A. B. Shyu (2013). "Deadenylation and P-bodies." Adv Exp Med Biol **768**: 183-195.

Choi, H. K., L. Tong, W. Minor, P. Dumas, U. Boege, M. G. Rossmann and G. Wengler (1991). "Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion." Nature **354**(6348): 37-43.

Claflin, S. B. and C. E. Webb (2015). "Ross River Virus: Many Vectors and Unusual Hosts Make for an Unpredictable Pathogen." PLoS Pathog **11**(9): e1005070.

Clarkson, W. D., H. M. Kent and M. Stewart (1996). "Separate binding sites on nuclear transport factor 2 (NTF2) for GDP-Ran and the phenylalanine-rich repeat regions of nucleoporins p62 and Nsp1p." J Mol Biol **263**(4): 517-524.

Cougot, N., S. Babajko and B. Seraphin (2004). "Cytoplasmic foci are sites of mRNA decay in human cells." J Cell Biol **165**(1): 31-40.

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**(40): 30269-30278.

Dang, Y., N. Kedersha, W. K. Low, D. Romo, M. Gorospe, R. Kaufman, P. Anderson and J. O. Liu (2006). "Eukaryotic initiation factor 2alpha-independent pathway of stress granule induction by the natural product pateamine A." J Biol Chem **281**(43): 32870-32878.

Davey, M. W. and L. Dalgarno (1974). "Semliki Forest virus replication in cultured Aedes albopictus cells: studies on the establishment of persistence." J Gen Virol **24**(3): 453-463.

Davey, M. W., R. J. Mahon and A. J. Gibbs (1979). "Togavirus interference in Culex annulirostris mosquitoes." J Gen Virol **42**(3): 641-643.

David, A., B. P. Dolan, H. D. Hickman, J. J. Knowlton, G. Clavarino, P. Pierre, J. R. Bennink and J. W. Yewdell (2012). "Nuclear translation visualized by ribosome-bound nascent chain puromycylation." J Cell Biol **197**(1): 45-57.

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**(20): 8967-8971.

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**(24): 13106-13116.

Decker, C. J. and R. Parker (2012). "P-bodies and stress granules: possible roles in the control of translation and mRNA degradation." Cold Spring Harb Perspect Biol **4**(9): a012286.

DeTulleo, L. and T. Kirchhausen (1998). "The clathrin endocytic pathway in viral infection." Embo j **17**(16): 4585-4593.

Dever, T. E. (2002). "Gene-specific regulation by general translation factors." Cell **108**(4): 545-556.

Diehl, N. and H. Schaal (2013). "Make yourself at home: viral hijacking of the PI3K/Akt signaling pathway." Viruses **5**(12): 3192-3212.

Dougherty, J. D., W. C. Tsai and R. E. Lloyd (2015). "Multiple Poliovirus Proteins Repress Cytoplasmic RNA Granules." Viruses **7**(12): 6127-6140.

Dougherty, J. D., J. P. White and R. E. Lloyd (2011). "Poliovirus-mediated disruption of cytoplasmic processing bodies." J Virol **85**(1): 64-75.

Dunn, E. F. and J. H. Connor (2011). "Dominant inhibition of Akt/protein kinase B signaling by the matrix protein of a negative-strand RNA virus." J Virol **85**(1): 422-431.

Eckei, L., S. Krieg, M. Butepage, A. Lehmann, A. Gross, B. Lippok, A. R. Grimm, B. M. Kummerer, G. Rossetti, B. Luscher and P. Verheugd (2017). "The conserved macrodomains of the non-structural proteins of Chikungunya virus and other pathogenic positive strand RNA viruses function as mono-ADP-ribosylhydrolases." Sci Rep **7**: 41746.

Emara, M. M., K. Fujimura, D. Sciaranghella, V. Ivanova, P. Ivanov and P. Anderson (2012). "Hydrogen peroxide induces stress granule formation independent of eIF2alpha phosphorylation." Biochem Biophys Res Commun **423**(4): 763-769.

Eulalio, A., I. Behm-Ansmant, D. Schweizer and E. Izaurralde (2007). "P-body formation is a consequence, not the cause, of RNA-mediated gene silencing." Mol Cell Biol **27**(11): 3970-3981.

Ferraiuolo, M. A., S. Basak, J. Dostie, E. L. Murray, D. R. Schoenberg and N. Sonenberg (2005). "A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay." J Cell Biol **170**(6): 913-924.

Firth, A. E., N. M. Wills, R. F. Gesteland and J. F. Atkins (2011). "Stimulation of stop codon readthrough: frequent presence of an extended 3' RNA structural element." Nucleic Acids Res **39**(15): 6679-6691.

Flint, J., V. R. Racaniello, G. F. Rall, A. M. Skalka and L. W. Enquist (2015). Principles of Virology. 4th edition. London: Taylor & Francis. ISBN 9781555819514.

Foy, N. J., M. Akhrymuk, I. Akhrymuk, S. Atasheva, A. Bopda-Waffo, I. Frolov and E. I. Frolova (2013). "Hypervariable domains of nsP3 proteins of New World and Old World alphaviruses mediate formation of distinct, virus-specific protein complexes." J Virol **87**(4): 1997-2010.

Franks, T. M. and J. Lykke-Andersen (2008). "The control of mRNA decapping and P-body formation." Mol Cell **32**(5): 605-615.

Friedman, R. M., J. G. Levin, P. M. Grimley and I. K. Berezesky (1972). "Membrane-associated replication complex in arbovirus infection." J Virol **10**(3): 504-515.

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**(8): 4122-4134.

Fros, J. J., C. Geertsema, K. Zouache, J. Baggen, N. Domeradzka, D. M. van Leeuwen, J. Flipse, J. M. Vlak, A. B. Failloux and G. P. Pijlman (2015). "Mosquito Rasputin interacts

with chikungunya virus nsP3 and determines the infection rate in Aedes albopictus." Parasit Vectors **8**: 464.

Fros, J. J., E. van der Maten, J. M. Vlak and G. P. Pijlman (2013). "The C-terminal domain of chikungunya virus nsP2 independently governs viral RNA replication, cytopathicity, and inhibition of interferon signaling." J Virol **87**(18): 10394-10400.

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**(6 Pt 1): 2075-2086.

Fruman, D. A., R. E. Meyers and L. C. Cantley (1998). "Phosphoinositide kinases." Annu Rev Biochem **67**: 481-507.

Fukunaga, Y., T. Kumanomido and M. Kamada (2000). "Getah virus as an equine pathogen." Vet Clin North Am Equine Pract **16**(3): 605-617.

Gaedigk-Nitschko, K., M. X. Ding, M. A. Levy and M. J. Schlesinger (1990). "Site-directed mutations in the Sindbis virus 6K protein reveal sites for fatty acylation and the underacylated protein affects virus release and virion structure." Virology **175**(1): 282-291.

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**(Pt 4): 937-947.

Gao, Y., N. Goonawardane, J. Ward, A. Tuplin and M. Harris (2019). "Multiple roles of the non-structural protein 3 (nsP3) alphavirus unique domain (AUD) during Chikungunya virus genome replication and transcription." PLoS Pathog **15**(1): e1007239.

Garmashova, N., R. Gorchakov, E. Frolova and I. Frolov (2006). "Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription." J Virol **80**(12): 5686-5696.

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**(5): 2472-2484.

Garoff, H. and K. Simons (1974). "Location of the spike glycoproteins in the Semliki Forest virus membrane." Proc Natl Acad Sci U S A **71**(10): 3988-3992.

Gibbons, D. L., A. Ahn, P. K. Chatterjee and M. Kielian (2000). "Formation and characterization of the trimeric form of the fusion protein of Semliki Forest Virus." J Virol **74**(17): 7772-7780.

Glomb-Reinmund, S. and M. Kielian (1998). "The role of low pH and disulfide shuffling in the entry and fusion of Semliki Forest virus and Sindbis virus." Virology **248**(2): 372-381.

Gomez de Cedron, M., N. Ehsani, M. L. Mikkola, J. A. Garcia and L. Kaariainen (1999). "RNA helicase activity of Semliki Forest virus replicase protein NSP2." FEBS Lett **448**(1): 19-22.

Gorchakov, R., E. Frolova and I. Frolov (2005). "Inhibition of transcription and translation in Sindbis virus-infected cells." J Virol **79**(15): 9397-9409.

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**(20): 10088-10101.

Götte, B., L. Liu and G. M. McInerney (2018). "The Enigmatic Alphavirus Non-Structural Protein 3 (nsP3) Revealing Its Secrets at Last." Viruses **10**(3): 105.

Götte, B., Panas, M., Hellström, K., Liu, L., Ahola, T., Samreen, B., Larsson, O., & McInerney, G. M. (2019) Separate domains of G3BP promote efficient clustering of alphavirus replication complexes and recruitment of the translation initiation machinery. PloS Pathogens, accepted, (BioRxiv; https://doi.org/10.1101/600338).

Gratz, N. G. (2004). "Critical review of the vector status of Aedes albopictus." Med Vet Entomol **18**(3): 215-227.

Grimley, P. M., I. K. Berezesky and R. M. Friedman (1968). "Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis." J Virol **2**(11): 1326-1338.

Hahn, Y. S., A. Grakoui, C. M. Rice, E. G. Strauss and J. H. Strauss (1989). "Mapping of RNA- temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4." J Virol **63**(3): 1194-1202.

Hale, B. G., D. Jackson, Y. H. Chen, R. A. Lamb and R. E. Randall (2006). "Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling." Proc Natl Acad Sci U S A **103**(38): 14194-14199.

Harley, D., A. Sleigh and S. Ritchie (2001). "Ross River virus transmission, infection, and disease: a cross-disciplinary review." Clin Microbiol Rev **14**(4): 909-932, table of contents.

Heberle, A. M., P. Razquin Navas, M. Langelaar-Makkinje, K. Kasack, A. Sadik, E. Faessler, U. Hahn, P. Marx-Stoelting, C. A. Opitz, C. Sers, I. Heiland, S. Schauble and K. Thedieck (2019). "The PI3K and MAPK/p38 pathways control stress granule assembly in a hierarchical manner." Life Sci Alliance **2**(2): e201800257.

Helenius, A. (1995). "Alphavirus and flavivirus glycoproteins: structures and functions." Cell **81**(5): 651-653.

Higgs, S. (2006). "The 2005-2006 Chikungunya epidemic in the Indian Ocean." Vector Borne Zoonotic Dis **6**(2): 115-116.

Horvathova, I., F. Voigt, A. V. Kotrys, Y. Zhan, C. G. Artus-Revel, J. Eglinger, M. B. Stadler, L. Giorgetti and J. A. Chao (2017). "The Dynamics of mRNA Turnover Revealed by Single-Molecule Imaging in Single Cells." Mol Cell **68**(3): 615-625.e619.

Houseley, J. and D. Tollervey (2009). "The many pathways of RNA degradation." Cell **136**(4): 763-776.

Hubstenberger, A., M. Courel, M. Benard, S. Souquere, M. Ernoult-Lange, R. Chouaib, Z. Yi, J. B. Morlot, A. Munier, M. Fradet, M. Daunesse, E. Bertrand, G. Pierron, J. Mozziconacci, M. Kress and D. Weil (2017). "P-Body Purification Reveals the Condensation of Repressed mRNA Regulons." Mol Cell **68**(1): 144-157.e145.

Igreja, C., D. Peter, C. Weiler and E. Izaurralde (2014). "4E-BPs require non-canonical 4E-binding motifs and a lateral surface of eIF4E to repress translation." Nat Commun **5**: 4790.

Ivanov, P., N. Kedersha and P. Anderson (2018). "Stress Granules and Processing Bodies in Translational Control." Cold Spring Harb Perspect Biol. a032813

Jose, J., J. E. Snyder and R. J. Kuhn (2009). "A structural and functional perspective of alphavirus replication and assembly." Future Microbiol **4**(7): 837-856.

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

Kallio, K., K. Hellstrom, G. Balistreri, P. Spuul, E. Jokitalo and T. Ahola (2013). "Template RNA length determines the size of replication complex spherules for Semliki Forest virus." J Virol **87**(16): 9125-9134.

Kamenska, A., C. Simpson, C. Vindry, H. Broomhead, M. Benard, M. Ernoult-Lange, B. P. Lee, L. W. Harries, D. Weil and N. Standart (2016). "The DDX6-4E-T interaction mediates translational repression and P-body assembly." Nucleic Acids Res **44**(13): 6318-6334.

Kedersha, N. and P. Anderson (2002). "Stress granules: sites of mRNA triage that regulate mRNA stability and translatability." Biochem Soc Trans **30**(Pt 6): 963-969.

Kedersha, N. and P. Anderson (2007). "Mammalian stress granules and processing bodies." Methods Enzymol **431**: 61-81.

Kedersha, N., M. R. Cho, W. Li, P. W. Yacono, S. Chen, N. Gilks, D. E. Golan and P. Anderson (2000). "Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules." J Cell Biol **151**(6): 1257-1268.

Kedersha, N., P. Ivanov and P. Anderson (2013). "Stress granules and cell signaling: more than just a passing phase?" Trends Biochem Sci **38**(10): 494-506.

Kedersha, N., M. D. Panas, C. A. Achorn, S. Lyons, S. Tisdale, T. Hickman, M. Thomas, J. Lieberman, G. M. McInerney, P. Ivanov and P. Anderson (2016). "G3BP-Caprin1-USP10 complexes mediate stress granule condensation and associate with 40S subunits." J Cell Biol **212**(7): 845-860.

Kedersha, N., G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M. J. Fritzler, D. Scheuner, R. J. Kaufman, D. E. Golan and P. Anderson (2005). "Stress granules and processing bodies are dynamically linked sites of mRNP remodeling." J Cell Biol **169**(6): 871-884.

Kedersha, N. L., M. Gupta, W. Li, I. Miller and P. Anderson (1999). "RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules." J Cell Biol **147**(7): 1431-1442.

Kennedy, D., J. French, E. Guitard, K. Ru, B. Tocque and J. Mattick (2001). "Characterization of G3BPs: tissue specific expression, chromosomal localisation and rasGAP(120) binding studies." J Cell Biochem **84**(1): 173-187.

Kim, D. Y., J. M. Reynaud, A. Rasalouskaya, I. Akhrymuk, J. A. Mobley, I. Frolov and E. I. Frolova (2016). "New World and Old World Alphaviruses Have Evolved to Exploit Different Components of Stress Granules, FXR and G3BP Proteins, for Assembly of Viral Replication Complexes." PLoS Pathog **12**(8): e1005810.

Kristensen, O. (2015). "Crystal structure of the G3BP2 NTF2-like domain in complex with a canonical FGDF motif peptide." Biochem Biophys Res Commun **467**(1): 53-57.

Kubacka, D., A. Kamenska, H. Broomhead, N. Minshall, E. Darzynkiewicz and N. Standart (2013). "Investigating the consequences of eIF4E2 (4EHP) interaction with 4E-transporter on its cellular distribution in HeLa cells." PLoS One **8**(8): e72761.

Kudo, N., N. Matsumori, H. Taoka, D. Fujiwara, E. P. Schreiner, B. Wolff, M. Yoshida and S. Horinouchi (1999). "Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region." Proc Natl Acad Sci U S A **96**(16): 9112-9117.

LaBeaud, A. D., T. Banda, J. Brichard, E. M. Muchiri, P. L. Mungai, F. M. Mutuku, E. Borland, G. Gildengorin, S. Pfeil, C. Y. Teng, K. Long, M. Heise, A. M. Powers, U. Kitron and C. H. King (2015). "High rates of o'nyong nyong and Chikungunya virus transmission in coastal Kenya." PLoS Negl Trop Dis **9**(2): e0003436.

Labno, A., R. Tomecki and A. Dziembowski (2016). "Cytoplasmic RNA decay pathways - Enzymes and mechanisms." Biochim Biophys Acta **1863**(12): 3125-3147.

Laplante, M. and D. M. Sabatini (2012). "mTOR signaling in growth control and disease." Cell **149**(2): 274-293.

Le Sage, V., A. Cinti, R. Amorim and A. J. Mouland (2016). "Adapting the Stress Response: Viral Subversion of the mTOR Signaling Pathway." Viruses **8**(6): 152.

Lee, S., K. E. Owen, H. K. Choi, H. Lee, G. Lu, G. Wengler, D. T. Brown, M. G. Rossmann and R. J. Kuhn (1996). "Identification of a protein binding site on the surface of the alphavirus nucleocapsid and its implication in virus assembly." Structure **4**(5): 531-541.

Li, C., Y. Debing, G. Jankevicius, J. Neyts, I. Ahel, B. Coutard and B. Canard (2016). "Viral Macro Domains Reverse Protein ADP-Ribosylation." J Virol **90**(19): 8478-8486.

Liljeström, P. and H. Garoff (1991a). "A new generation of animal cell expression vectors based on the Semliki Forest virus replicon." Biotechnology (N Y) **9**(12): 1356-1361.

Liljeström, P. and H. Garoff (1991b). "Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor." J Virol **65**(1): 147-154.

Liumbruno, G. M., D. Calteri, K. Petropulacos, A. Mattivi, C. Po, P. Macini, I. Tomasini, P. Zucchelli, A. R. Silvestri, V. Sambri, S. Pupella, L. Catalano, V. Piccinini, G. Calizzani and G. Grazzini (2008). "The Chikungunya epidemic in Italy and its repercussion on the blood system." Blood Transfus **6**(4): 199-210.

Lloyd, R. E. (2013). "Regulation of stress granules and P-bodies during RNA virus infection." Wiley Interdiscip Rev RNA **4**(3): 317-331.

Ludwig, G. V., J. P. Kondig and J. F. Smith (1996). "A putative receptor for Venezuelan equine encephalitis virus from mosquito cells." J Virol **70**(8): 5592-5599.

Lundstrom, K. (2018). "Viral Vectors in Gene Therapy." Diseases **6**(2).

Luo, J., S. J. Field, J. Y. Lee, J. A. Engelman and L. C. Cantley (2005). "The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex." J Cell Biol **170**(3): 455-464.

Lusa, S., H. Garoff and P. Liljestrom (1991). "Fate of the 6K membrane protein of Semliki Forest virus during virus assembly." Virology **185**(2): 843-846.

Lwande, O. W., V. Obanda, G. Bucht, G. Mosomtai, V. Otieno, C. Ahlm and M. Evander (2015). "Global emergence of Alphaviruses that cause arthritis in humans." Infect Ecol Epidemiol **5**: 29853.

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**(13): 6534-6545.

Manning, B. D. and L. C. Cantley (2007). "AKT/PKB signaling: navigating downstream." Cell **129**(7): 1261-1274.

Marsh, M., E. Bolzau and A. Helenius (1983). "Penetration of Semliki Forest virus from acidic prelysosomal vacuoles." Cell **32**(3): 931-940.

Matsuki, H., M. Takahashi, M. Higuchi, G. N. Makokha, M. Oie and M. Fujii (2013). "Both G3BP1 and G3BP2 contribute to stress granule formation." Genes Cells **18**(2): 135-146.

McInerney, G. M. (2015). "FGDF motif regulation of stress granule formation." DNA Cell Biol **34**(9): 557-560.

McInerney, G. M., N. L. Kedersha, R. J. Kaufman, P. Anderson and P. Liljestrom (2005). "Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation." Mol Biol Cell **16**(8): 3753-3763.

McPherson, R. L., R. Abraham, E. Sreekumar, S. E. Ong, S. J. Cheng, V. K. Baxter, H. A. Kistemaker, D. V. Filippov, D. E. Griffin and A. K. Leung (2017). "ADP-ribosylhydrolase activity of Chikungunya virus macrodomain is critical for virus replication and virulence." Proc Natl Acad Sci U S A **114**(7): 1666-1671.

McSweegan, E., S. C. Weaver, M. Lecuit, M. Frieman, T. E. Morrison and S. Hrynkow (2015). "The Global Virus Network: Challenging chikungunya." Antiviral Res **120**: 147-152.

Mok, B. W., W. Song, P. Wang, H. Tai, Y. Chen, M. Zheng, X. Wen, S. Y. Lau, W. L. Wu, K. Matsumoto, K. Y. Yuen and H. Chen (2012). "The NS1 protein of influenza A virus interacts with cellular processing bodies and stress granules through RNA-associated protein 55 (RAP55) during virus infection." J Virol **86**(23): 12695-12707.

Morrison, T. E. (2014). "Reemergence of chikungunya virus." J Virol **88**(20): 11644-11647.

Mutso, M., A. M. Morro, C. Smedberg, S. Kasvandik, M. Aquilimeba, M. Teppor, L. Tarve, A. Lulla, V. Lulla, S. Saul, B. Thaa, G. M. McInerney, A. Merits and M. Varjak (2018).

"Mutation of CD2AP and SH3KBP1 Binding Motif in Alphavirus nsP3 Hypervariable Domain Results in Attenuated Virus." Viruses **10**(5): 226.

Nagai, K., C. Oubridge, N. Ito, J. Avis and P. Evans (1995). "The RNP domain: a sequence-specific RNA-binding domain involved in processing and transport of RNA." Trends Biochem Sci **20**(6): 235-240.

Narayanan, K. and S. Makino (2013). "Interplay between viruses and host mRNA degradation." Biochim Biophys Acta **1829**(6-7): 732-741.

Nasci, R. S. (2014). "Movement of chikungunya virus into the Western hemisphere." Emerg Infect Dis **20**(8): 1394-1395.

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**(1): 212-225.

Neuvonen, M., A. Kazlauskas, M. Martikainen, A. Hinkkanen, T. Ahola and K. Saksela (2011). "SH3 domain-mediated recruitment of host cell amphiphysins by alphavirus nsP3 promotes viral RNA replication." PLoS Pathog **7**(11): e1002383.

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

Nishi, K., M. Yoshida, D. Fujiwara, M. Nishikawa, S. Horinouchi and T. Beppu (1994). "Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression." J Biol Chem **269**(9): 6320-6324.

Nitulescu, G. M., D. Margina, P. Juzenas, Q. Peng, O. T. Olaru, E. Saloustros, C. Fenga, D. Spandidos, M. Libra and A. M. Tsatsakis (2016). "Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use (Review)." Int J Oncol **48**(3): 869-885.

Nott, T. J., E. Petsalaki, P. Farber, D. Jervis, E. Fussner, A. Plochowietz, T. D. Craggs, D. P. Bazett-Jones, T. Pawson, J. D. Forman-Kay and A. J. Baldwin (2015). "Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles." Mol Cell **57**(5): 936-947.

Panas, M. D., T. Ahola and G. M. McInerney (2014). "The C-terminal repeat domains of nsP3 from the Old World alphaviruses bind directly to G3BP." J Virol **88**(10): 5888-5893.

Panas, M. D., P. Ivanov and P. Anderson (2016). "Mechanistic insights into mammalian stress granule dynamics." J Cell Biol **215**(3): 313-323.

Panas, M. D., N. Kedersha and G. M. McInerney (2015a). "Methods for the characterization of stress granules in virus infected cells." Methods **90**: 57-64.

Panas, M. D., T. Schulte, B. Thaa, T. Sandalova, N. Kedersha, A. Achour and G. M. McInerney (2015b). "Viral and cellular proteins containing FGDF motifs bind G3BP to block stress granule formation." PLoS Pathog **11**(2): e1004659.

Panas, M. D., M. Varjak, A. Lulla, K. E. Eng, A. Merits, G. B. Karlsson Hedestam and G. M. McInerney (2012). "Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection." Mol Biol Cell **23**(24): 4701-4712.

Parker, F., F. Maurier, I. Delumeau, M. Duchesne, D. Faucher, L. Debussche, A. Dugue, F. Schweighoffer and B. Tocque (1996). "A Ras-GTPase-activating protein SH3-domain-binding protein." Mol Cell Biol **16**(6): 2561-2569.

Pattnaik, A. K. and P. X. Dinh (2013). "Manipulation of cellular processing bodies and their constituents by viruses." DNA Cell Biol **32**(6): 286-291.

Perri, S., D. A. Driver, J. P. Gardner, S. Sherrill, B. A. Belli, T. W. Dubensky, Jr. and J. M. Polo (2000). "Replicon vectors derived from Sindbis virus and Semliki forest virus that establish persistent replication in host cells." J Virol **74**(20): 9802-9807.

Peränen, J., M. Rikkonen, P. Liljeström and L. Kääriäinen (1990). "Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2." J Virol **64**(5): 1888-1896.

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.

Pialoux, G., B. A. Gauzere, S. Jaureguiberry and M. Strobel (2007). "Chikungunya, an epidemic arbovirosis." Lancet Infect Dis **7**(5): 319-327.

Plaskon, N. E. (2009). "The Development of New Tools to Investigate Alphavirus Replication Kinetics." Virginia Polytechnic Institute and State University, Blacksburg, VA, USA: Master Thesis. Available at: https://vtechworks.lib.vt.edu/handle/10919/34787, accessed on 19 April 2019

Poblete-Duran, N., Y. Prades-Perez, J. Vera-Otarola, R. Soto-Rifo and F. Valiente-Echeverria (2016). "Who Regulates Whom? An Overview of RNA Granules and Viral Infections." Viruses **8**(7): 180.

Poland, G. A. (2010). "The 2009-2010 influenza pandemic: effects on pandemic and seasonal vaccine uptake and lessons learned for seasonal vaccination campaigns." Vaccine **28 Suppl 4**: D3-13.

Pushko, P., M. Parker, G. V. Ludwig, N. L. Davis, R. E. Johnston and J. F. Smith (1997). "Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo." Virology **239**(2): 389-401.

Reineke, L. C. and R. E. Lloyd (2013). "Diversion of stress granules and P-bodies during viral infection." Virology **436**(2): 255-267.

Reiter, P., D. Fontenille and C. Paupy (2006). "Aedes albopictus as an epidemic vector of chikungunya virus: another emerging problem?" Lancet Infect Dis **6**(8): 463-464.

Remenyi, R., Y. Gao, R. E. Hughes, A. Curd, C. Zothner, M. Peckham, A. Merits and M. Harris (2018). "Persistent Replication of a Chikungunya Virus Replicon in Human Cells Is Associated with Presence of Stable Cytoplasmic Granules Containing Nonstructural Protein 3." J Virol **92**(16) e00477-18.

Remenyi, R., G. C. Roberts, C. Zothner, A. Merits and M. Harris (2017). "SNAP-tagged Chikungunya Virus Replicons Improve Visualisation of Non-Structural Protein 3 by Fluorescence Microscopy." Sci Rep **7**(1): 5682.

Rikkonen, M. (1996). "Functional significance of the nuclear-targeting and NTP-binding motifs of Semliki Forest virus nonstructural protein nsP2." Virology **218**(2): 352-361.

Rikkonen, M., J. Peranen and L. Kaariainen (1994a). "ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2." J Virol **68**(9): 5804-5810.

Rikkonen, M., J. Peranen and L. Kaariainen (1994b). "Nuclear targeting of Semliki Forest virus nsP2." Arch Virol Suppl **9**: 369-377.

Rordorf-Nikolic, T., D. J. Van Horn, D. Chen, M. F. White and J. M. Backer (1995). "Regulation of phosphatidylinositol 3'-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85-kDa regulatory subunit." J Biol Chem **270**(8): 3662-3666.

Rubach, J. K., B. R. Wasik, J. C. Rupp, R. J. Kuhn, R. W. Hardy and J. L. Smith (2009). "Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro." Virology **384**(1): 201-208.

Rupp, J. C., N. Jundt and R. W. Hardy (2011). "Requirement for the amino-terminal domain of sindbis virus nsP4 during virus infection." J Virol **85**(7): 3449-3460.

Sanz, M. A., M. Garcia-Moreno and L. Carrasco (2015). "Inhibition of host protein synthesis by Sindbis virus: correlation with viral RNA replication and release of nuclear proteins to the cytoplasm." Cell Microbiol **17**(4): 520-541.

Saxton-Shaw, K. D., J. P. Ledermann, E. M. Borland, J. L. Stovall, E. C. Mossel, A. J. Singh, J. Wilusz and A. M. Powers (2013). "O'nyong nyong virus molecular determinants of unique vector specificity reside in non-structural protein 3." PLoS Negl Trop Dis **7**(1): e1931.

Scheuner, D., B. Song, E. McEwen, C. Liu, R. Laybutt, P. Gillespie, T. Saunders, S. Bonner-Weir and R. J. Kaufman (2001). "Translational control is required for the unfolded protein response and in vivo glucose homeostasis." Mol Cell **7**(6): 1165-1176.

Scholte, F. E., A. Tas, I. C. Albulescu, E. Zusinaite, A. Merits, E. J. Snijder and M. J. van Hemert (2015). "Stress granule components G3BP1 and G3BP2 play a proviral role early in Chikungunya virus replication." J Virol **89**(8): 4457-4469.

Schuffenecker, I., I. Iteman, A. Michault, S. Murri, L. Frangeul, M. C. Vaney, R. Lavenir, N. Pardigon, J. M. Reynes, F. Pettinelli, L. Biscornet, L. Diancourt, S. Michel, S. Duquerroy, G. Guigon, M. P. Frenkiel, A. C. Brehin, N. Cubito, P. Despres, F. Kunst, F. A. Rey, H. Zeller and S. Brisse (2006). "Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak." PLoS Med **3**(7): e263.

Schulte, T., L. Liu, M. D. Panas, B. Thaa, N. Dickson, B. Gotte, A. Achour and G. M. McInerney (2016). "Combined structural, biochemical and cellular evidence demonstrates that both FGDF motifs in alphavirus nsP3 are required for efficient replication." Open Biol **6**(7): 160078.

Sen, G. L. and H. M. Blau (2005). "Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies." Nat Cell Biol **7**(6): 633-636.

Sheth, U. and R. Parker (2003). "Decapping and decay of messenger RNA occur in cytoplasmic processing bodies." Science **300**(5620): 805-808.

Shin, G., S. A. Yost, M. T. Miller, E. J. Elrod, A. Grakoui and J. Marcotrigiano (2012). "Structural and functional insights into alphavirus polyprotein processing and pathogenesis." Proc Natl Acad Sci U S A **109**(41): 16534-16539.

Shirako, Y. and J. H. Strauss (1998). "Requirement for an aromatic amino acid or histidine at the N terminus of Sindbis virus RNA polymerase." J Virol **72**(3): 2310-2315.

Shoelson, S. E., M. Sivaraja, K. P. Williams, P. Hu, J. Schlessinger and M. A. Weiss (1993). "Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation." Embo j **12**(2): 795-802.

Singh, I. and A. Helenius (1992). "Role of ribosomes in Semliki Forest virus nucleocapsid uncoating." J Virol **66**(12): 7049-7058.

Sjöberg, E. M., M. Suomalainen and H. Garoff (1994). "A significantly improved Semliki Forest virus expression system based on translation enhancer segments from the viral capsid gene." Biotechnology (N Y) **12**(11): 1127-1131.

Sjöberg, M., B. Lindqvist and H. Garoff (2011). "Activation of the alphavirus spike protein is suppressed by bound E3." J Virol **85**(11): 5644-5650.

Skoging, U., M. Vihinen, L. Nilsson and P. Liljestrom (1996). "Aromatic interactions define the binding of the alphavirus spike to its nucleocapsid." Structure **4**(5): 519-529.

Smith, A. L. and G. H. Tignor (1980). "Host cell receptors for two strains of Sindbis virus." Arch Virol **66**(1): 11-26.

Soderholm, J. F., S. L. Bird, P. Kalab, Y. Sampathkumar, K. Hasegawa, M. Uehara-Bingen, K. Weis and R. Heald (2011). "Importazole, a small molecule inhibitor of the transport receptor importin-beta." ACS Chem Biol **6**(7): 700-708.

Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider and et al. (1993). "SH2 domains recognize specific phosphopeptide sequences." Cell **72**(5): 767-778.

Spuul, P., G. Balistreri, L. Kaariainen 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**(15): 7543-7557.

Spuul, P., A. Salonen, A. Merits, E. Jokitalo, L. Kaariainen 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**(2): 872-883.

Stalder, L. and O. Muhlemann (2009). "Processing bodies are not required for mammalian nonsense-mediated mRNA decay." Rna **15**(7): 1265-1273.

Staples, J. E., R. F. Breiman and A. M. Powers (2009). "Chikungunya fever: an epidemiological review of a re-emerging infectious disease." Clin Infect Dis **49**(6): 942-948.

Stoecklin, G. and N. Kedersha (2013). "Relationship of GW/P-bodies with stress granules." Adv Exp Med Biol **768**: 197-211.

Strauss, E. G., E. M. Lenches and J. H. Strauss (2002). "Molecular genetic evidence that the hydrophobic anchors of glycoproteins E2 and E1 interact during assembly of alphaviruses." J Virol **76**(20): 10188-10194.

Strauss, J. H. and E. G. Strauss (1994). "The alphaviruses: gene expression, replication, and evolution." Microbiol Rev **58**(4): 806.

Strunk, U., D. G. Ramos, H. A. Saffran and J. R. Smiley (2016). "Role of Herpes simplex virus 1 VP11/12 tyrosine-based binding motifs for Src family kinases, p85, Grb2 and Shc in activation of the phosphoinositide 3-kinase-Akt pathway." Virology **498**: 31-35.

Suhrbier, A., M. C. Jaffar-Bandjee and P. Gasque (2012). "Arthritogenic alphaviruses--an overview." Nat Rev Rheumatol **8**(7): 420-429.

Tam, A. (2004). "The SARS epidemic in 2002-2003 shocked the world." Paediatr Respir Rev **5**(4): 261.

Tamm, K., A. Merits and I. Sarand (2008). "Mutations in the nuclear localization signal of nsP2 influencing RNA synthesis, protein expression and cytotoxicity of Semliki Forest virus." J Gen Virol **89**(Pt 3): 676-686.

Thaa, B., R. Biasiotto, K. Eng, M. Neuvonen, B. Gotte, L. Rheinemann, M. Mutso, A. Utt, F. Varghese, G. Balistreri, A. Merits, T. Ahola and G. M. McInerney (2015). "Differential Phosphatidylinositol-3-Kinase-Akt-mTOR Activation by Semliki Forest and Chikungunya Viruses Is Dependent on nsP3 and Connected to Replication Complex Internalization." J Virol **89**(22): 11420-11437.

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**(20): 9962-9969.

Tourriere, H., K. Chebli, L. Zekri, B. Courselaud, J. M. Blanchard, E. Bertrand and J. Tazi (2003). "The RasGAP-associated endoribonuclease G3BP assembles stress granules." J Cell Biol **160**(6): 823-831.

Tourriere, H., I. E. Gallouzi, K. Chebli, J. P. Capony, J. Mouaikel, P. van der Geer and J. Tazi (2001). "RasGAP-associated endoribonuclease G3Bp: selective RNA degradation and phosphorylation-dependent localization." Mol Cell Biol **21**(22): 7747-7760.

Tsetsarkin, K. A., D. L. Vanlandingham, C. E. McGee and S. Higgs (2007). "A single mutation in chikungunya virus affects vector specificity and epidemic potential." PLoS Pathog **3**(12): e201.

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**(Pt 6): 1525-1533.

Tutucci, E., M. Vera, J. Biswas, J. Garcia, R. Parker and R. H. Singer (2018). "An improved MS2 system for accurate reporting of the mRNA life cycle." Nat Methods **15**(1): 81-89.

Utt, A., P. K. Das, M. Varjak, V. Lulla, A. Lulla and A. Merits (2015). "Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2." J Virol **89**(6): 3145-3162.

Utt, A., T. Quirin, S. Saul, K. Hellstrom, T. Ahola and A. Merits (2016). "Versatile Trans-Replication Systems for Chikungunya Virus Allow Functional Analysis and Tagging of Every Replicase Protein." PLoS One **11**(3): e0151616.

Valiente-Echeverria, F., L. Melnychuk and A. J. Mouland (2012). "Viral modulation of stress granules." Virus Res **169**(2): 430-437.

Varjak, M., E. Zusinaite and A. Merits (2010). "Novel functions of the alphavirus nonstructural protein nsP3 C-terminal region." J Virol **84**(5): 2352-2364.

Varshavsky, A. (1996). "The N-end rule: functions, mysteries, uses." Proc Natl Acad Sci U S A **93**(22): 12142-12149.

Vasiljeva, L., A. Merits, P. Auvinen and L. Kaariainen (2000). "Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2." J Biol Chem **275**(23): 17281-17287.

Vasiljeva, L., L. Valmu, L. Kaariainen and A. Merits (2001). "Site-specific protease activity of the carboxyl-terminal domain of Semliki Forest virus replicase protein nsP2." J Biol Chem **276**(33): 30786-30793.

Vihinen, H., T. Ahola, M. Tuittila, A. Merits and L. Kaariainen (2001). "Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3." J Biol Chem **276**(8): 5745-5752.

Vihinen, H. and J. Saarinen (2000). "Phosphorylation site analysis of Semliki forest virus nonstructural protein 3." J Biol Chem **275**(36): 27775-27783.

Waksman, G., S. E. Shoelson, N. Pant, D. Cowburn and J. Kuriyan (1993). "Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms." Cell **72**(5): 779-790.

Weaver, S. C. and N. L. Forrester (2015). "Chikungunya: Evolutionary history and recent epidemic spread." Antiviral Res **120**: 32-39.

White, J. P. and R. E. Lloyd (2012). "Regulation of stress granules in virus systems." Trends Microbiol **20**(4): 175-183.

Wong, K. K., J. A. Engelman and L. C. Cantley (2010). "Targeting the PI3K signaling pathway in cancer." Curr Opin Genet Dev **20**(1): 87-90.

Yu, J., Y. Zhang, J. McIlroy, T. Rordorf-Nikolic, G. A. Orr and J. M. Backer (1998). "Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit." Mol Cell Biol **18**(3): 1379-1387.

Zheng, D., N. Ezzeddine, C. Y. Chen, W. Zhu, X. He and A. B. Shyu (2008). "Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells." J Cell Biol **182**(1): 89-101.