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DISSECTION OF VIRUS-HOST CELL INTERACTIONS IN THE EARLY RESPONSE TO INFECTION

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About the cover:

This is the human interactome visualized in Cytoscape 2.5. It shows protein-protein interactions, which were constructed from publically available data. Original picture by Andrew Garrow, 2006, https://www.flickr.com/photos/andytrop/. Modified by Marc D. Panas, 2014.

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Like a virus needs a body
As soft tissue feeds on blood
Someday I'll find you, the urge is here…

…Like a virus, patient hunter
I'm waiting for you, I'm starving for you…

Björk – Virus, Biophilia

ABSTRACT

Stress granules (SG) are dynamic RNA/protein assemblies in the cytoplasm of the cell, formed under conditions of oxidative stress, heat shock or viral infections. These stress conditions trigger a sudden translational arrest, leading to a rapid switch of translation from housekeeping genes to stress-related factors. SGs fulfil multiple roles in the cell one of which is acting as triage centres for mRNA, where the mRNA is stored pending either degradation or reinitiation of translation. Many proteins are sequestered to SGs, among them signalling molecules, which make SGs signal centres to communicate a "state of emergency". The importance of SGs is also underlined by the fact that they restrict viral propagation. The assembly of SGs is dependent on many RNA-binding proteins, one of which is G3BP (Ras-GAP SH3 domain binding protein). Semliki Forest virus (SFV) belongs to the alphaviruses, a large group of arthropod-borne animal viruses including several relevant human pathogens such as the re-emerging Chikungunya virus (CHIKV). Alphavirus infections lead to fever, rashes, arthralgia and can be lethal. Recent CHIKV outbreaks in the Caribbean area and the US, brings alphavirus research back on the agenda. Therefore there is a need to understand the molecular mechanisms how alphaviruses interact with their host. The aim of this thesis was to dissect virus-host cell interactions in the early response to alphavirus infection.

Alphavirus infection leads to the formation of SGs at very early time points. Interestingly, they dissolve in the vicinity of viral replication complexes at later time points. In paper I, we showed that the non-structural protein nsP3 of SFV is responsible for sequestration of G3BP to replication complexes, by doing so, actively disassembling SGs and blocking their reformation. We mapped the binding site for G3BP to two C-terminal repeat domains of nsP3. A recombinant virus mutant lacking these repeats showed a longer and more persistent stress response and was attenuated in growth.

In paper II, we extended this finding to the closely related CHIKV. Our results show that nsP3 of both SFV and CHIKV interact with G3BP via two C-terminal repeat domains and that the proline-rich region of nsP3 is dispensable for this interaction.

In paper III we investigated the interaction between nsP3 and G3BP in molecular detail and determined that the residues FGDF in the C-terminal repeats of nsP3 are the G3BP binding motif. We further asked whether other proteins use the same mechanism as nsP3 to bind G3BP and whether this interaction inhibits the formation of SGs. We revealed that the phenylalanines and the glycine in the FGDF are essential for binding G3BP. We further demonstrated that the cellular ubiquitin-specific protease 10 (USP10) and the herpes simplex virus (HSV) protein ICP8 (infected cell protein 8) also bind G3BP via an FGDF motif. In addition we show that the FGDF-mediated binding to G3BP leads to a negative regulation of G3BP's SG-nucleating function. Lastly we present a 3D-model of G3BP bound to an FGDF-containing peptide, which we validated by site-directed mutagenesis.

Our findings present a common FGDF motif to bind G3BP, which has a negative regulatory effect on the SG-nucleating function of G3BP. This molecular mechanism and the presented 3D-model demonstrate the therapeutic potential of targeting this interaction.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscripts.

I. **Panas, M.D.,** Varjak, M., Lulla, A., Eng, K.E., Merits, A., Karlsson Hedestam, G.B., and McInerney, G.M. Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection. *Molecular Biology of the Cell,* 2012, vol. 23, 4701–4712.

II. **Panas, M.D.,** Ahola, T., and McInerney, G.M. The C-terminal repeat domains of nsP3 from the Old World alphaviruses bind directly to G3BP. *Journal of Virology,* 2014, vol. 88, 5888–5893.

III. **Panas, M.D.,** Schulte, T., Thaa, B., Sandalova, T., Kedersha, N., Achour, A., and McInerney, G.M. FGDF motifs mediate the binding of viral and cellular proteins to G3BP. *Submitted manuscript.*

The following additional publications were obtained during the course of the education but are not part of this thesis.

IV. Eng, K.E., **Panas, M.D.,** Karlsson Hedestam, G.B., and McInerney, G.M. A novel quantitative flow cytometry-based assay for autophagy. *Autophagy,* 2010, vol 6. 634–641.

V. Eng, K.E., **Panas, M.D.,** Murphy, D., Karlsson Hedestam, G.B., and McInerney, G. M. Accumulation of autophagosomes in Semliki Forest virus infected cells is dependent on the expression of the viral glycoproteins. *Journal of Virology,* 2012, vol. 86, 5674–5685.

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LIST OF ABBREVIATIONS

aa	Amino acid (s)
BHK	Baby hamster kidney
CHIKV	Chikungunya virus
CPV-I	Type I cytopathic vacuoles
dsRNA	Double-stranded RNA
DUBs	Deubiquitinating enzymes
EGFP	Enhanced green fluorescent protein
eIF2α/eIF3	Eukaryotic translation initiation factor 2α, 3
ER	Endoplasmic reticulum
G3BP	Ras-GAP SH3 domain binding protein
HEK293T	Human embryonic kidney 293 with T antigen of SV40
hpi	Hours post infection
HRI	Heme-regulated inhibitor
HSV-1	Herpes simplex virus type 1
HVD	Hypervariable domain
ICP8	Infected cell protein 8
ICT	Isothermal titration calorimetry
ID	Intrinsically disordered
IP	Immunoprecipitation
IRES	Internal ribosome entry site
ISG	Interferon-stimulated genes
LC	Low complexity
MEF	Mouse embryonic fibroblast
MOI	Multiplicity of infection
mRNPs	Ribonucleoprotein particles
NC	Nucleocapsid
NLS	Nuclear localization sequence
nsP	Non-structural protein
NTF2	Nuclear transport factor 2
PABP	Poly-A binding protein

Pat A	Pateamine A
PB	Processing bodies
PERK	PKR-like endoplasmic reticulum kinase
PKR	Protein kinase R
Q/N	Glutamine asparagine (Q/N)-rich domains
RC	Replication complex
RIG-I	Retinoic acid-inducible gene I
SEC	Size exclusion chromatography
SFV	Semliki Forest virus
SG	Stress granule
SINV	Sindbis virus
ssRNA	Single-stranded RNA
TIA-1/R	T-cell-restricted intracellular antigen/related
TTP	Tristetraprolin
USP10	Ubiquitin-specific protease 10
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
wt	Wild type

1 INTRODUCTION

1.1 VIRUSES

1.1.1 Why we study viruses

The word "information" is derived from the Latin verb *informare*, which originally means "to give form to the mind", "to discipline", "instruct" or "teach". A virus is information in the form of DNA or RNA, surrounded by a protein coat and/or an envelope and delivered to a receiver which understands the syntax and is therefore taught, or instructed, to form new virus particles. We are surrounded by billions of viruses and encounter them every day. Viruses reside in our lungs, gastrointestinal, and urogenital tracts, and other places. The numbers are just astonishing, for example it is estimated that there are more than 10^{30} bacteriophages in the world's water supply. Arranged together head to tail they would extend to outer space more than 200 million light years. The nearest galaxy, Andromeda is only 2.5 million light years away.

With such constant exposure to viruses, it is amazing that they have relatively little impact on our health. This is, at least in part, due to our immune defence systems, which have evolved to fight viruses and other microbial infections. But when these defence systems are compromised even a common cold (caused by rhinoviruses) can be lethal. Nevertheless, there are still viruses which lead to devastating human diseases like AIDS, Ebola hemorrhagic fever, hantavirus pulmonary syndrome, rabies, smallpox, measles, influenza, poliomyelitis and others. Viruses are also thought to be responsible for approximately 15% of human cancers (zur Hausen, 1991). This underlines the biomedical importance of these agents.

Viruses are passive agents which are totally dependent on the mercy of their environment. In an infected cell they act as obligate intracellular parasites. However, the dependency of viruses on their hosts for propagation makes them unique tools to study the biology of cells. Viral infection induces reprogramming of cellular mechanisms and this provides insights into the cellular biology as well as the function of host defence systems. Additionally, viruses can be manipulated with ease to generate useful virus mutants to further study cellular mechanisms. Therefore studies of virus-infected cells have contributed to our understanding of cell biology and for example the protein synthesis machinery. The 5´cap structure was first identified on the viral RNA of the vesicular stomatitis virus (VSV) (Rhodes et al., 1974, Muthukrishnan et al., 1975). New translation initiation mechanisms, such as internal ribosomal entry sites (IRES) were discovered in virus-infected cells (Jang et al., 1988, Pelletier and Sonenberg, 1988), which are now found in cellular mRNA as well. Moreover, the investigation of oncoviruses, which can cause cancer, revealed the genetic basis of the disease. Finally, alphaviruses like Semliki Forest virus (SFV) have been used extensively as model envelope viruses to study the biology of viral infection and the biology of cells. The alphaviruses now represent one of the best-defined animal virus systems. However, there are still interactions between virus and the host cell which are not discovered.

1.2 SEMLIKI FOREST VIRUS

1.2.1 Background

Semliki Forest virus (SFV) is a positive-sense, single-stranded RNA virus and belongs to group IV in the Baltimore classification system. This group represents the largest of all groups. The most important animal and human pathogens and also plant viruses belong to this group, like SARS-CoV, poliovirus, hepatitis C virus, Norwalk virus, potato virus Y and many more. In 1942, SFV was first isolated in the Semliki Forest of Uganda. The virus is spread by mosquitoes and infects small animals and humans. SFV itself is a mild human pathogen, causing fever, rashes and joint pain. But closely related family members like Chikungunya virus (CHIKV) cause severe illness in humans with symptoms like high fever, rashes, headache and severe and persistent joint pain, in rare cases it can be fatal. Generally, CHIKV is spread by the mosquito *Aedes aegypti*, which is distributed in South America, Central, West and East Africa, India and South East Asia (Schwartz and Albert, 2010). In 2005–2006 CHIKV entered South West Indian Ocean Islands and the situation changed suddenly, because CHIKV adapted rapidly to mosquitoes of the species *Aedes albopictus* through a single point mutation in one of the glycoprotein genes of the virus. This mutation (A226V) in the glycoprotein E1 is associated with an increased replication capacity in this worldwide disseminated and invasive vector (Tsetsarkin et al., 2007). *A. albopictus* is also found in Southern Europe, where it seems to be held back by the Alps to reach further north. However due to global warming and heavy traffic in Europe, it could be a matter of time until this mosquito can reach other parts of Europe.

In December 2013 on the island of Saint-Martin, in the French West Indies the first evidence for cases of CHIKV infection in the Western hemisphere was reported. (Leparc-Goffart et al., 2014). Four months later, at the end of March 2014, nine Caribbean islands reported cases of CHIKV infections, and at the end of April 2014, 15 Caribbean islands claimed cases (Morrison, 2014, Nasci, 2014) of CHIKV infections. The Centers for Disease Control and Prevention (CDC) in the US reported in July 2014 the first locally acquired case in Florida, indicating that CHIKV has reached the continental United States.

SFV and CHIKV belong to the family *Togaviridae*, which consists of two virus genera, alphavirus and rubivirus. There is only one member of the rubivirus genus, rubella virus, which infects only humans and for which no insect vector is known. In contrast, more than 30 members of the alphavirus genus are known, some of which are pathogenic for humans and animals (Strauss, 1994). Alphaviruses are distributed worldwide and grouped into New World and Old World alphaviruses. Old World alphaviruses include CHIKV, SFV, Sindbis virus (SINV) and Ross River virus (RRV). They are found in Europe, Asia, Africa and Australia. New World alphaviruses can be found in South and North America, for instance, Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV) and Western equine encephalitis virus (WEEV). Those viruses infect mainly horses and rodents and lead to encephalitis, which can cause death. Human beings can be infected by mosquito bites.

Replication of alphaviruses occurs in invertebrate vectors and vertebrate hosts. The viruses spread between individuals or species by blood-sucking mosquitoes, which makes alphaviruses a member of the group of arboviruses (arthropod borne). An alphaviral infection is asymptomatic and persists life-long in the mosquito. Interestingly, this can be also observed in *in vitro* systems with insect cells, where the acute infection is limited and converted to a persistent infection without killing the host cell (Strauss, 1994). This is not the case for vertebrate cells, where the infection leads to rapid cell death (Strauss, 1994). In vertebrates, an acute infection can occur with symptoms like high fever, rashes and arthritis; in the case of infection with New World alphaviruses, encephalitis can also occur often ending with death. But normally an infection is cleared by the immune system. The recovery from the infection varies by age. Younger people recover in 5–15 days, while elderly people need a longer time, around 1–2.5 months (Simon et al., 2007, Taubitz et al., 2007). There are no specific treatments available nor have any vaccines been developed, tough vaccines against CHIKV are undergoing clinical evaluation (Weaver et al., 2012, Morens and Fauci, 2014). Currently, the best prevention is mosquito control, by using mosquito repellents and wearing appropriate clothing.

The most-studied members of the alphavirus genus are SFV and SINV. Nowadays the re-emerging CHIKV gets more and more attention in the alphavirus research field, and there is a drastic increase in published literature in the CHIKV field. Nevertheless, the knowledge that was gathered by studying SFV and SINV are fundamental for our understanding of alphaviral infections. SFV and SINV are not associated with severe human diseases and therefore considered as safe model system to study alphaviral infections. A wide range of cells from invertebrates and vertebrates can be used to study SFV and SINV replication. The development of SINV (Rice et al., 1987) and SFV (Liljestrom and Garoff, 1991) infectious cDNA, allowed easy reverse genetics. A great deal has been learnt about alphaviral infections regarding RNA replication, transcription and viral polyprotein processing as well as basic cellular processes (Strauss, 1994, Jose et al., 2009). However, SFV, SINV and CHIKV are different viruses, and what has been learnt from one virus is not necessarily true for the others. Therefore care has to be taken if results from studying one virus are translated to explain the effects of another virus. SFV is also well known and used in the biotechnology field. It is used as a viral vector for the expression of heterologous proteins which have a potential use for vaccination or cancer gene therapy (Yamanaka, 2004, Riezebos-Brilman et al., 2006, Atkins et al., 2008, Johansson et al., 2012).

1.2.2 Virion and genome organization

The SFV particle contains a positive-sense single-stranded RNA genome. It has a length of approx. 11.7 kb (Jose et al., 2009). The genome is packed into an icosahedral nucleocapsid (NC), which is composed of 240 capsid monomers. The N-terminal part of the capsid monomer interacts with the genomic RNA. The nucleocapsid, with a diameter of 30 nm, is enveloped, increasing the size of the virion to approximately 70 nm. The envelope is derived

from the host plasma membrane and consists of 240 copies of a heterodimer of the E1-E2 glycoprotein. Three E1-E2 heterodimers form a spike complex, which leads to a total of 80 spike complexes on the surface. A third glycoprotein E3 is associated to each heterodimer. The E2 glycoprotein anchors the spike to the envelope by interacting with the nucleocapsid that lies beneath. Another important role for E2 is binding to the host cell receptor, which has, however, still not been identified.

The RNA genome, which sediments at 42S in a sucrose gradient, hast two open reading frames (ORF). The 5´two thirds of the genome encode a polyprotein that is processed into four non-structural proteins (nsP) to form the viral replicase. A notable difference between different alphaviruses is that some (for example SINV and CHIKV, but not SFV) contain a leaky opal stop codon (UGA) at the end of the sequence of nsP3. Expression of the non-structural polyprotein thus leads primarily to nsP123, but in 10–20% of the cases the read through leads to the expression of nsP1234 (Firth et al., 2011). In SFV, the opal stop codon has been replaced by an arginine codon (CGA), which means that only polyprotein nsP1234 is expressed. The polyprotein undergoes highly regulated processing steps, whereby it is subsequently cleaved into different products with different RNA synthesis capabilities (Fig 1), providing a mechanism for viral RNA synthesis (Fig.1) (Kääriäinen et al., 1987, Merits et al., 2001, Vasiljeva et al., 2001). The 3´ third of the genome, which is under the control of a subgenomic promoter that leads to a 26S subgenomic (sg)-RNA, encodes the structural proteins capsid, E3, E2, 6K, TF and E1. The 5´ end of the capsid protein-coding sequence contains a translational enhancer, which is needed for efficient translation of the structural proteins in an infected cell (Frolov and Schlesinger, 1994, Sjöberg et al., 1994). Another interesting feature of the 26S sg-RNA is that it contains a −1 frame shift signal in the 6K sequence, which leads to the expression of the transframe (TF) protein (Firth et al., 2008).

1.2.3 Replication cycle

SFV enters the cell by receptor-mediated clathrin-dependent endocytosis (Doxsey et al., 1987). The glycoprotein E2 is the receptor-binding ligand. The virus is able to infect a large number of different cell types and also cells from various species. However the receptor is still unknown for most of the alphaviruses. For SINV, the laminin receptor has been proposed to be the binding receptor (Wang et al., 1992), and the binding is dependent on heparin sulphate (Klimstra et al., 1998). To date no specific receptor for SFV has been described.

Once the virion is bound to the receptor on the cell surface, it is internalized by clathrin-mediated endocytosis (Helenius et al., 1980, DeTulleo and Kirchhausen, 1998). Acidification of the endosomal lumen leads to a rearrangement of the $(E1\text{-}E2)_3$ spike complex, which destabilizes the complex and exposes the fusion peptide of E1. This highly hydrophobic peptide gets inserted into the endosomal membrane for membrane fusion to occur (Kielian, 2010). This event releases the nucleocapsid into the cytoplasm (Helenius et al., 1985, Wahlberg et al., 1992, Bron et al., 1993, Justman et al., 1993). Once the nucleocapsid is in the

cytoplasm, it is destabilised, and ribosomes bind to the capsid proteins (Singh and Helenius, 1992). This event releases the viral RNA from the capsid, and the closely located ribosomes immediately start to express the non-structural polyprotein.

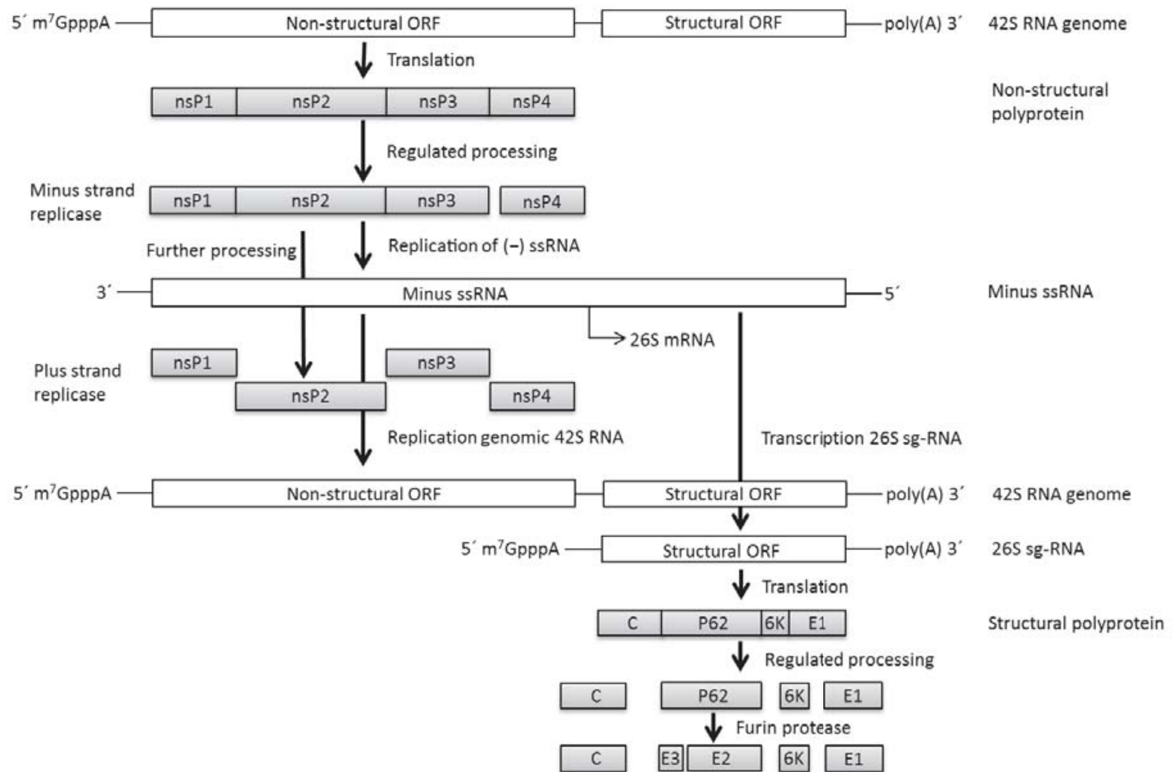

Figure 1: SFV genome organization. The positive single-stranded 42S genome is capped (5´ m^{7}GpppA) and polyadenylated. The incoming 42S genome is translated into the non-structural polyprotein nsP1234. The minus strand replicase nsP123 + nsP4 replicates the 42S genome into a minus strand replicative intermediate. The fully processed plus strand replicase replicates more positive strand 42S RNA and transcribes a 26S subgenomic (sg)-RNA via the 26S RNA promoter. The 26S sg-RNA is then translated into the structural proteins. The RNAs are shown as open boxes whereas the translated ORFs and individual proteins are shown in grey. Adapted from (Strauss, 1994).

The non-structural polyprotein is processed stepwise into four separate proteins. These proteins induce the formation of spherules at the plasma membrane, which are small protrusions sticking out from the plasma membrane. The spherules are then internalized through endocytosis and transported along microtubules to the perinuclear region (Spuul et al., 2010). During transport, they fuse with endosomes and lysosomes, which leads to the formation of CPV-I (type I cytopathic vacuoles). One spherule contains one dsRNA molecule and an unknown number of nsP molecules as well as host proteins (Spuul et al., 2010, Frolova et al., 2010). The processing of the non-structural polyprotein is very well controlled and executed by nsP2, which possesses a protease activity (Vasiljeva, 2003, Lulla et al., 2006). Due to the temporal processing of the polyprotein, the different resulting replication complexes have different RNA synthesis specificities. At an early stage (4–6 h after SFV infection) nsP2 cleaves between nsP3 and 4, leading to the nsP123-nsP4 RC, which is mostly responsible for producing negative-sense ssRNA, the template for genomic viral RNA. Then, nsP1 is cleaved from the nsP123 polyprotein to result in nsP1 + nsP23, upon which both

negative- and positive-strand RNA molecules are synthesised. After further processing of the nsP23 molecule, the replication of full-length 42S genomic RNA and of the 26S (sg)-RNA can occur from the minus strand RNA template. At this point the fully processed non-structural protein complex (nsP1 + 2 + 3 +4) is not able to make minus strand RNA, but the synthesis of positive strand continues until the cell dies (Fig 1). The produced 42S genomic RNA interacts with newly synthesised capsid proteins, and nucleocapsids are formed in the cell (Lemm et al., 1994, Shirako and Strauss, 1994, Vasiljeva, 2003).

1.2.3.1 **Non-structural proteins**

nsP1 is a membrane-binding protein composed of 537 amino acids (aa) and is involved in the synthesis of the m^7GpppA cap0 structure for the 42S RNA and the 26S sg-RNA. nsP1 is tightly bound to membranes in the context of the polyprotein as well as in the mature replicase complex (Salonen et al., 2003). It is essential that the replicase complex is bound to membranes. Studies have revealed that point mutations in the membrane-inserted alpha helix (245–264) are lethal to SFV (Ahola et al., 1999, Spuul et al., 2007). There are also three serial cysteines in the SFV nsP1 protein, spanning amino acids 418–420, which are post-translationally palmitoylated. This modification strengthens the membrane binding but is not required for enzymatic activity. Palmitoylation of nsP1 induces the formation of filopodia-like structures. However the mechanism and function of these structures is still unknown, but they could play a role in transmission of SFV from cell to cell (Laakkonen et al., 1996, Laakkonen et al., 1998). nsP1 catalyses the capping of the 42S genomic RNA and 26S sg-RNA. The first reaction is, however, performed by nsP2, which has an RNA triphosphatase activity to remove the phosphate at the 5´ end of the RNA. The next two steps are performed by nsP1 (Mi and Stollar, 1991, Laakkonen et al., 1994). The guanyltransferase domain of nsP1 forms a complex with guanosine monophosphate (GMP). In the next step, nsP1 transfers a methyl group from S-adenosylmethionine to the nsP1-GMP complex, this is catalysed by the methyltransferase activity of nsP1 and nsP1-m^7GMP is generated. (Ahola and Kääriäinen, 1995). Interestingly, this reaction differs from the reactions in cells to produce the cap0 structure, whereby the GMP is first covalently bound to RNA and subsequently methylated. It is still not known which enzyme performs the last step to covalently bind the alphaviral cap to the RNA.

The non-structural protein 2 is the largest protein of the replication complex, with a size of 799 aa. It has multiple known enzymatic activities and roles. The N-terminus contains helicase (Gomez de Cedrón et al., 1999), RNA triphosphatase and nucleoside triphosphatase activities (Rikkonen et al., 1994a, Vasiljeva et al., 2000). A papain-like cysteine protease domain can be found at the C-terminus of nsP2, as well as an enzymatically non-functional methyltransferase-like domain (Strauss et al., 1992, Vasiljeva et al., 2001). The development of cytopathic effects is caused by the methyltransferase-like domain, which differentially modulates host defence mechanisms (Mayuri et al., 2008). The protease activity is responsible for the processing of the non-structural polyprotein (Strauss et al., 1992). The 3-dimensional structure of the C-terminus spanning residues 468–787 of VEEV-nsP2 has been

solved by X-ray crystallography, revealing that the folding of the protease domain of nsP2 is novel compared to other proteases (Russo et al., 2006). nsP2 whether free or as part of the non-structural polyprotein is catalytically active. The cleavage of the polyprotein is very well orchestrated and depends on the stoichiometry of available products. Another interesting feature of nsP2 is that approximately 50% of the free nsP2 protein is found in the nucleus (Peränen et al., 1990) and that it contains multiple nuclear localization sequences (NLS) at the N-terminal and C-terminal regions (Rikkonen et al., 1994b). Mutational studies on the NLS and the abolishment of nsP2 translocation to the nucleus yielded an attenuated phenotype (Kääriäinen and Ahola, 2002, Fazakerley et al., 2002, Tamm et al., 2008), later shown to be due to a defect in the virus capacity to inhibit the type I interferon response (Breakwell et al., 2007).

nsP3, with a size of 482 aa, has been enigmatic for a long time, but recent discoveries have shed a light on the functions of nsP3. The protein can be divided into three major regions (schematically depicted in Fig. 2). The N-terminal region with 160 aa contains a so-called macro domain, which is conserved among alphaviruses as well as rubiviruses, herpesviruses and coronaviruses (Koonin and Dolja, 1993). The macro domain can bind ADP-ribose, poly(ADP-ribose) and RNA. The latter might be the main function of the nsP3 macro domain (Malet et al., 2009, Neuvonen and Ahola, 2009). It has also been shown that the N-terminal domain of nsP3 has a role in non-structural polyprotein processing (Lulla et al., 2012). The central domain or alpha domain, which also spans around 160 aa, is conserved among alphaviruses (Strauss, 1994). This region was crystallized as part of the nsP2-nsP3 polyprotein. It has been demonstrated that this region binds zinc ions, and mutational studies revealed that this interaction is essential for the virus (Shin et al., 2012). The 3D-structure suggests that this region participates in RNA binding as well. The C-terminal part of nsP3 consists of hypervariable sequences, the length of which is different in the various alphaviruses. The domain is basically unstructured, but it contains areas of functional similarities between the different alphaviruses. Two conserved sequence elements L/ITFGDFD (numbering of the motif: $L/I^1T^2F^3G^4D^5F^6D^7$) close to the C-terminus and a degradation signal in the last 10 aa have been described (Varjak et al., 2010). Some of the motifs are shared among the alphaviruses and some are not. Several reports have shown that nsP3 is involved in interacting with cellular proteins such as Ras-GAP SH3 domain binding protein (G3BP), a SG-nucleating protein (see below) (Cristea et al., 2006, Frolova et al., 2006, Gorchakov et al., 2008, Fros et al., 2012). Nevertheless G3BP was also reported to interact with nsP2 (Atasheva et al., 2007) and nsP4 in SINV-infected cells (Cristea et al., 2010). Recently a proline-rich element in the hypervariable domain (HVD) was shown to interact with amphiphysin-1 and -2. This interaction was shown to promote viral replication but the mechanism was not described (Neuvonen et al., 2011). A cluster of phosphorylated threonines and serines can be found between the second (alpha) and third (hypervariable) domain. 16 phosphorylation sites are found in SFV, located within 50 aa, six of which ($S^{320, 327, 332, 335}$ and $T^{344, 345}$) account for the majority of the phosphorylation of nsP3. Mutation of the phosphorylation sites has a relatively slight effect on replication in mammalian cells

infected with SFV or VEEV (Vihinen, 2000, Foy et al., 2013). On the other hand, it was shown that the phosphorylation plays a role in negative-strand synthesis in SINV-infected cells (de Groot et al., 1990, Dé et al., 2003). If nsP3 is expressed alone, it localizes to cytoplasmic non-membranous granules of different sizes. However, when expressed in the context of the nsP123 polyprotein, nsP3 is first localized at the plasma membrane and then triggers the re-localization to endosomal membranes, which appear similar to CPVs (Salonen et al., 2003, Salonen et al., 2005, Spuul et al., 2010). nsP3 does not have any catalytic activity; it is thus likely that it plays a role as a scaffolding protein. The C-terminus of nsP3 is intrinsically unstructured, which could play a role in interacting with several cellular partners (Cristea et al., 2006, Gorchakov et al., 2008, Neuvonen et al., 2011, Varjak et al., 2013).

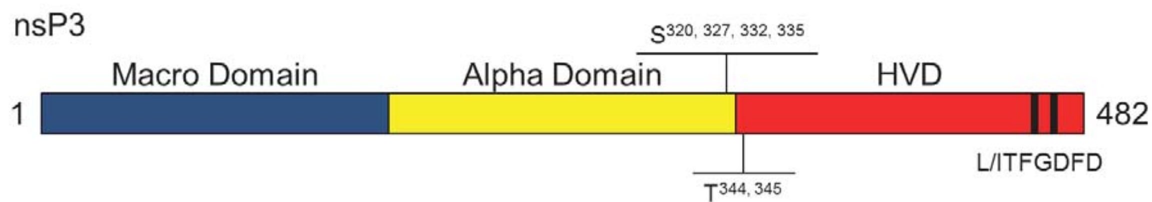

Figure 2: Schematic representation of the non-structural protein 3 (nsP3). HVD: hypervariable domain, S: serine, T: threonine. For details see text.

The RNA-dependent RNA-polymerase (RdRp) activity resides in the nsP4. The C-terminus shows sequence homology with other known RdRps, including the highly conserved GDD motif. The N-terminus (approx. 100 aa) does not display any similarities with other known sequences from viruses or cells, but they are conserved between the alphaviruses. Genetic evidence suggests that these sequences are involved in interactions with other non-structural proteins, but the function is still unknown (Rupp et al., 2011). The levels of nsP4 in infected cells are relatively low, for two reasons: First, an opal stop codon is located at the end of the nsP3-encoding sequence of most alphaviruses (but not SFV), which leads to lower levels of the polyprotein nsP1234 (Strauss, 1994). Secondly, the first amino acid of nsP4 is always a tyrosine residue, which is a very unusual feature. The tyrosine at the N-terminus of a protein is a destabilizing amino acid (Varshavsky, 1996), leading to rapid degradation of the protein by the proteasome. Interestingly, nsP4 is protected from degradation when it is incorporated into the replication complex (de Groot et al., 1991). Furthermore the polymerase activity requires a Tyr at the N-terminus, the only acceptable residues being other aromatic residues or histidine. Methionine is tolerated, whereas other residues are lethal for virus replication, resulting in reversions and selection for mutations in nsP4 (Shirako and Strauss, 1998).

1.2.3.2 **Structural proteins**

The formation of the replication complex composed of the individual non-structural proteins initiates the synthesis of the 26S sg-RNA, leading to the expression of the structural polyprotein, comprising the capsid, p62 (E3-E2), 6K, TF and E1 proteins. The capsid protein is autocatalytically cleaved from the structural polyprotein (Choi et al., 1991). This process

exposes a signal peptide in E3, which results in the binding to and translocation of the nascent polypeptide across the endoplasmic reticulum (ER) membrane (Garoff et al., 1990). Several membrane-spanning regions can be found in the p62-6K-E1 polyprotein. In the ER, the p62-6K-E1 is processed (glycosylated, palmitoylated) and cleaved into the proteins p62, 6K and E1 by cellular proteases (Melancon and Garoff, 1987). During the transport from the ER to the Golgi apparatus, p62 is further processed into E3 and E2 (Liljeström and Garoff, 1991, Strauss, 1994). E2 and E1 form heterodimers, which are assembled to a trimeric $(E1\text{-}E2)_3$ spike complex in the rough ER. The spikes are then further transported to the plasma membrane, where the formation of virions occurs (Ziemiecki et al., 1980, Mulvey and Brown, 1996, Lu et al., 1999). The viral RNA, which is bound to multiple copies of the capsid protein, accumulates at the plasma membrane and interacts with the cytoplasmic regions of E2 (Kail et al., 1991, Suomalainen et al., 1992, Skoging et al., 1996). The E3 glycoprotein is also incorporated into the virus particles, but not in all alphaviruses (not SINV) (Sjöberg et al., 2011). The small 6K protein has been found to be incorporated into virions and is important for the correct assembly of the fully infectious SFV particle (Gaedigk-Nitschko and Schlesinger, 1990, Lusa et al., 1991, McInerney et al., 2004). The transframe (TF) protein, which is expressed upon a frameshift during translation, was reported to be incorporated into the virion as well, but the functions are unclear (Snyder et al., 2013).

1.2.4 Host response

Viral infections are detected by the infected cells. Cells have developed certain countermeasures to restrict viral propagation. However, viruses have antagonized these countermeasures, leading to an arms race. SFV infection triggers a strong host response. SFV infection is primarily recognized via melanoma differentiation-associated protein 5 (MDA5) which leads to interferon-α/β production, but for efficient interferon-α/β production PKR is also required. Possibly by maintaining the integrity of the synthesized IFN-α/β mRNA, thereby allowing its translation (Barry et al., 2009, Schulz et al., 2010). PKR is a ubiquitously expressed enzyme, whereas its expression can be greatly enhanced by type I interferons. The enzyme recognizes double-stranded (ds) RNA, which is a replication intermediate formed during the replication of the minus and plus strand synthesis (Clemens and Elia, 1997, Clemens, 1997, Pindel and Sadler, 2011). Activated PKR phosphorylates the eukaryotic translation initiation factor 2 alpha (eIF2α) at serine 51. This is a very important regulation step for the initiation of eukaryotic protein translation. eIF2α is part of the ternary complex composed of eIF2, GTP and initiator Met-tRNA. This activated ternary complex meets the small 40S ribosomal subunit and other eukaryotic translation initiation factors to form the 43S translation preinitiation complex at the cap structure of the mRNA. This complex scans the 5´ untranslated region for the AUG start codon, where the large 60S subunit joins the complex and translation of the protein begins. Once eIF2α is phosphorylated, the guanine nucleotide exchange factor eIF2B gets sequestered to eIF2α and is then inhibited in its

function to exchange GDP for GTP in the ternary complex. Since the cellular concentration of eIF2B is much lower than that of eIF2α, even a small amount of phosphorylated eIF2α can suppress the eIF2B activity completely. The consequences are that the translation comes to a halt (Siekierka et al., 1982, Siekierka et al., 1984, Krishnamoorthy et al., 2001). Other important kinases which phosphorylate eIF2α, besides PKR, are the PKR-like endoplasmic reticulum kinase (PERK), Heme-regulated eukaryotic initiation factor eIF2α kinase (HRI) and general control nonderepressible 2 (GCN2). Misfolded proteins induce ER-stress that is sensed by PERK, a type I membrane protein in the ER, leading to the phosphorylation of eIF2α (Sood et al., 2000). Oxidative stress, for example treatment with sodium arsenite (Lu et al., 2001) leads to the activation of the HRI, which subsequently phosphorylates eIF2α. HRI is also activated by other stress signals like heat shock (Xu et al., 1997), heme depletion (Matts and Hurst, 1992, Chen et al., 1994, Chen and London, 1995) and osmotic shock (Lu et al., 2001). The kinase GCN2 senses amino acid deficiency through binding to uncharged tRNAs in the cytoplasm (Wek et al., 1995).

PKR senses dsRNA via its C-terminal dsRNA-binding domain (dsRBD). Under normal conditions, an autoinhibitory domain blocks the kinase activity. The presence of dsRNA in the cytoplasm leads to dimerization of PKR by binding of two PKR molecules to one stretch of dsRNA. A subsequent autophosphorylation stabilizes the dimer, and conformational changes lead to the binding and subsequent phosphorylation of eIF2α (García et al., 2006). PKR is constitutively expressed in most cells but the expression can be greatly enhanced by the presence of IFN to counteract viral infections. Consequently, viruses have evolved strategies to circumvent the actions of PKR. For instance, the ICP34.5 protein of herpes simplex virus 1 (HSV-1) inhibits downstream effects of PKR by dephosphorylating eIF2α and thereby reverting PKR-induced translational shutoff (Mohr and Gluzman, 1996, He et al., 1997). The NS1 protein of influenza A virus (IAV) (Lu et al., 1995) as well as the omega3 protein from reovirus (Jacobs and Langland, 1998) bind and sequester dsRNA to prevent recognition by PKR. The E3L protein of vaccinia virus (VV) binds tightly to the catalytic cleft of PKR and blocks autophosphorylation (Sharp et al., 1998). Other viruses employ mechanisms independent of eIF2α. Cricket paralysis virus has distinct pseudoknot-like structures on the RNA that form multiple contacts with the ribosome independently of initiation factors (Jan and Sarnow, 2002). The 5′ end of the structural proteins of SFV and SINV contain a genetic element called translational enhancer element (Frolov and Schlesinger, 1994, Sjöberg et al., 1994). RNA sequence analysis predicts a very stable hairpin loop. The translational enhancement only occurs in infected cells (Sjöberg and Garoff, 1996). It was later shown that the translational enhancer element counteracts the inhibition of translation induced by the phosphorylation of eIF2α (McInerney et al., 2005, Ventoso et al., 2006).

The type I interferons, which induce an innate antiviral state in the cell (Stark et al., 1998), were discovered in the supernatant of cells incubated with heat-inactivated virus. The supernatant interfered with the growth of live virus when added to the cells (Isaacs and

Lindemann, 1957). The type I interferons comprise several subtypes (IFN-α/β/ε/κ/ω) (Liu, 2005) . IFN-α/β is primarily induced upon recognition of viral nucleic acids by host pattern recognition receptors (PRRs), such as proteins of the RIG-I-like receptor family, like MDA-5, retinoic acid inducible gene I (RIG-I), or a combination of the two (Takeuchi and Akira, 2009). RIG-I senses viral single-stranded RNA bearing a 5´ triphosphate (Yoo et al., 2014). MDA5 senses long dsRNA intermediates which occur in the replication of positive-sense ssRNA and dsRNA viruses during the minus-strand RNA synthesis (Feng et al., 2012). SFV, for instance, is primarily recognized by MDA5 (Pichlmair et al., 2009) but also RIG-I (Schulz et al., 2010). IAV, Newcastle disease virus and VSV are sensed by RIG-I (Kato et al., 2006) and viruses such as murine norovirus-1, encephalomyocarditis virus (EMCV) Theiler's murine encephalomyelitis virus (TMCV) by MDA5 (Gitlin et al., 2006, McCartney et al., 2008). The receptors signal through IFN-β promoter stimulator 1 (IPS-1), leading to the translocation of interferon regulatory factor 3 (IRF-3) and nuclear factor kappa B (NFκB) to the nucleus leading to the expression of type I interferons. Once induced and secreted, the type I interferons carry out the innate antiviral immune defence of cells by signalling via IFN-α/β receptors in an autocrine and paracrine fashion. This leads to a signalling cascade involving the phosphorylation of the transcription factors STAT1 and 2 and translocation to the nucleus, which induces the transcription of thousands of genes, called interferon-stimulated genes (ISGs). This signalling cascade induces an antiviral state in the cell. However, viruses counteract this response. The phosphatase VH1 of VV interacts and dephosphorylates STAT1 and blocks the interferon gamma signalling transduction (Najarro et al., 2001, Koksal and Cingolani, 2011). Morbillivirus non-structural protein 5 (NS5) blocks the type I interferon signalling pathway by interacting with the host non-receptor tyrosine-protein kinase 2 (TYK2) and thereby inhibiting downstream STAT1 and STAT2 phosphorylation (Chinnakannan et al., 2013).

How does SFV deal with the cellular host response? The synthesis of cellular RNA and proteins is inhibited in vertebrate cells during alphavirus infection, but the synthesis of viral RNA and proteins is maintained. Gorchakov and coworkers showed that the transcriptional and translational shutdowns of the cellular macromolecule synthesis are independent events (Gorchakov et al., 2005). This leads to the limitation of the production of antiviral proteins, including type I interferons, which delays the induction of an antiviral state (Frolova et al., 2002). The shutdowns appear to be driven at least in parts by nsP2 in the Old World alphaviruses and by the capsid protein in the New World alphaviruses (Gorchakov et al., 2005, Garmashova et al., 2006, Aguilar et al., 2007, Breakwell et al., 2007). The presence of nsP2 causes the degradation of the DNA-directed RNA polymerase II subunit (RPB1) within the RNA polymerase complex II. RPB1 is ubiquitinated and then rapidly degraded by the proteasomal degradation pathway, which results in a decrease of host mRNA transcription (Akhrymuk et al., 2012). A different mode of action to downregulate cellular transcription is achieved by the New World alphaviruses. The capsid protein binds to importin α/β and the nuclear export receptor RCM1. This complex then accumulates at the

nuclear pore and eventually inhibits the export of cellular mRNAs into the cytoplasm (Garmashova et al., 2007).

The shutoff of the host translation machinery is influenced by a couple of mechanisms. The association of nsP2 with the ribosomal protein S6 (RpS6) suggests that alphaviruses modify the ribosome, which may contribute to differential translation of mRNA (Montgomery et al., 2006). Host translational shutoff is also mediated by the phosphorylation of eIF2α, via sensing of dsRNA by PKR. Interestingly phosphorylated eIF2α leads to the formation of SGs, which are cytoplasmic sites of aborted translation initiation complexes (Kedersha and Anderson, 2002), as described in detail below (chapter 1.3). SFV infection induces the formation of SGs, but as the infection progresses, the SGs are disassembled in the vicinity of replication complexes. The translation of the 26S sg-RNA is not affected by the phosphorylation of eIF2α. This is due to the translational enhancer residing at the 5′ end of the 26S sg-RNA, rendering the expression of the structural proteins unaffected of highly phosphorylated eIF2α (McInerney et al., 2005).

1.3 STRESS GRANULES

1.3.1 Background

Stress granules (SGs) are cytoplasmic, non-membranous, phase dense structures which assemble in response to environmental stress. They are aggregates of aborted, translationally silenced messenger ribonucleoprotein particles (mRNPs), which are thought to be sites of mRNA storage and triage (Kedersha et al., 2002). They are formed in the cytoplasm upon various stress stimuli, like oxidative stress, heat shock or viral infections. The sequestration of mRNAs into SGs contributes to the rapid change of translation from housekeeping genes to heat shock proteins and other stress-related proteins (Anderson and Kedersha, 2009). Recent reports suggest that SGs could act as signal hubs, similar to transmembrane complexes such as the immunological synapse (Dustin, 2012), by recruiting important signalling proteins (Kedersha et al., 2013). Stress induces translational arrest, which causes the assembly of SGs in the cytoplasm, mediated by the phosphorylation of eIF2α (Kedersha et al., 1999). SGs are very dynamic structures, and their formation is orchestrated by numerous mRNA-binding proteins. The proteins shuttle in and out within seconds, whereas the SGs themselves last for minutes or even hours (Kedersha et al., 2005). The rapidly moving mRNPs create a large stable SG, in which the mRNPs are in a constant flux (like water in a river), whereas the SG as such (“the river”) is stable. SGs are assembled on untranslated mRNA by different mRNA-binding proteins, which specify the fate of the mRNA in the given environmental conditions. The fate can be storage, degradation or reinitiation of translation. Furthermore SGs are in equilibrium with polysomes indicated by treatments with certain drugs: drugs which arrest translation elongation and stabilize polysomes, for example cycloheximide or emetine, lead to disassembly of SGs. Drugs that destabilize polysomes like puromycin promote the assembly of SGs (Kedersha et al., 2000). A very well described pathway of SG formation initiates with

the phosphorylation of eIF2α, which is executed by the kinases PKR, PERK, GCN2 and HRI (Kedersha et al., 1999, Kedersha et al., 2000, McEwen et al., 2005). Sodium arsenite, an oxidative stressor, activates HRI, GCN2 is activated during heat shock and nutrient starvation, PKR senses dsRNA during viral infection and PERK is activated by ER stress. However SG formation can also be induced by alternative pathways, independently of eIF2α phosphorylation, for instance, inhibition of the RNA helicase eIF4A through hippuristanol or pateamine A (Pat A) (Dang et al., 2006) or treatment of cells with 2-deoxyglucose, an inhibitor of glycolysis, leading to ATP depletion and an eIF2α-independent formation of SGs (Kedersha, 2001).

Eukaryotic cells also contain other types of cytoplasmic RNA/protein granules, like processing bodies (PBs), exosome bodies, and neuronal bodies. PBs and exosome bodies are constitutively present and contain proteins of the mRNA decay machinery (Lin et al., 2007). Neuronal bodies are also constitutively present in the neuronal cells; they are foci that concentrate and transport silenced mRNPs along axons to the dendrites of neuronal cells (Knowles et al., 1996, Krichevsky and Kosik, 2001).

1.3.2 SG assembly/disassembly

The signalling cascade which leads to SGs assembly is very well described, but the mechanism how SGs actually assemble in the cytoplasm is less well understood. An essential component for the assembly of SGs is non-translating mRNA, as shown in cells treated with cycloheximide, which stalls polysomes on mRNA (Kedersha et al., 2000). An increasing pool of non-translated mRNA leads to the formation of SGs, a condition which can be induced by puromycin (Kedersha et al., 2000). Recent data on how SGs assemble suggest that low-affinity interactions between intrinsically disordered (ID) regions of SG-nucleating and RNA-binding proteins promote the formation of SGs (Kedersha et al., 2013, Malinovska et al., 2013). One protein that promotes the reversible aggregation of untranslated mRNPs is the T-intracellular antigen 1 (TIA-1). It contains a prion-related domain (PRD), which is a glutamine/asparagine (Q/N)-rich motif of low amino acid complexity that mediates the aggregation of the protein (Gilks et al., 2004). Prion-related sequences and low complexity (LC) regions are subtypes of ID proteins. ID regions can form multiple conformations, mediating transient interactions. These interactions can be influenced by the local environment, post-translational modifications and/or binding to other proteins (Uversky et al., 2013, Malinovska et al., 2013). The SG-nucleating protein G3BP contains a serine residue at position 149 in its intrinsically disordered/low complexity (ID/LC) region. Phosphorylation of this residue alters the association with SGs by inhibiting the dimerization of G3BP (Tourrière et al., 2003). Similarly, tristetraprolin (TTP) can be phosphorylated at serine residues 52 and 178, also located in an ID/LC region. Phosphorylation leads to the exit of TTP from SGs, without altering its targeting to PBs (Stoecklin et al., 2004). Furthermore, the self-interacting domain of G3BP also promotes the formation of SGs. Deletion of this domain impairs SG assembly (Tourrière et al., 2003). An analogous process was shown for the

protein Staufen, which multimerizes by binding dsRNA, but also via protein-protein interactions (Martel et al., 2010). Staufen, G3BP and many others serve as scaffolds, and many of these scaffold proteins also interact with multiple stress granule components. Moreover, the cytoskeleton and associated motor proteins also contribute to the assembly and disassembly of SGs. The microtubule network destabilizing drug nocodazole reduces the appearance of SGs dramatically (Ivanov et al., 2003, Kwon et al., 2007) and dynein and kinesin motorproteins can be localized in SGs and facilitate their assembly (Loschi et al., 2009). Disassembly of SGs occurs by exit of the mRNA from the stress granule and entry into translation. Translation shows an inverse relationship with the formation of SGs (Kedersha et al., 2000). If the translation levels increase, the number of granules drops. The turnover of scaffolding SG proteins may be another way to disassemble SGs. This is suggested by the finding that the inhibition of the ubiquitin-proteasome pathway with MG132 induces SGs, a condition that may, however, have been caused by the activation of GCN2 kinase (Mazroui et al., 2007).

1.3.3 G3BP

The stress granule component G3BP was first described in 1996 as a protein that binds the SH3 domain of Ras-GAP (Parker et al., 1996) and is known to be involved in several signalling pathways, such as Ras signalling (Parker et al., 1996, Pazman et al., 2000), c-myc mRNA turnover (Gallouzi et al., 1998, Tourriere et al., 2001) and NFκB signalling (Prigent et al., 2000). G3BP exists in two isoforms, G3BP-1 and G3BP-2, with 74% similarity in the amino acid sequence. G3BP-1 is encoded on chromosome 5 and G3BP-2 on chromosome 4. There are two splice variants (a and b) of G3BP-2. G3BP-2b lacks 33 amino acids in the proline-rich region (Kennedy et al., 2001). G3BP-1 contains 466 aa and has a predicted size of 52 kDa, but the electrophoretic mobility is lower. The two G3BP proteins have structural similarities, with four distinctive regions (depicted in Fig. 3). The N-terminus was shown by sequence homology to consist of a nuclear transport factor 2 (NTF2)-like domain (Suyama et al., 2000). The cytoplasmic protein nuclear transport factor 2 (NTF2) enables protein transport into the nucleus by binding to FxFG repeat motifs in proteins of the nuclear pore (Clarkson et al., 1996) This binding is required for the NTF2-mediated transport of cargo into the nucleus along the RanGDP/GTP gradient. NTF2 and its bound cargo associate with RanGTP and translocate to the nucleus, whereas the exchange to GDP releases the cargo and leads to back-transport of NTF2 to the cytoplasm (Ribbeck et al., 1998). While there is evidence that G3BP is targeted to the nucleus and to the nuclear envelope (Prigent et al., 2000). G3BP apparently does not function as a nuclear transport factor: an inhibitor of nuclear export, leptomycin B (Ullman et al., 1997), does not affect the subcellular localization of G3BP (Prigent et al., 2000). Furthermore Ran is not associated with G3BP (Tourrière et al., 2003). Rather, the NTF2-like domain of G3BP is probably involved in dimerization (Suyama et al., 2000, Tourriere et al., 2001), but little is known about the functional consequences of such dimerization. In the centre of G3BP, there are conserved

acidic and proline-rich regions (PxxP), which have been suggested to bind SH3 domains and were shown to interact with Ras-GAP (Parker et al., 1996). However, another report questions the concept that G3BP interacts with Ras-GAP (Annibaldi et al., 2011).

The C-terminus of G3BP contains an RNA-recognition motif (RRM), which forms a three-dimensional structure of α-helixes and β-sheets to interact with a stretch of 2–8 nucleotides of the RNA (Nagai et al., 1995, Kennedy et al., 2001). In addition to the RNA-binding function, RRMs of G3BP can interact with proteins which might alter the specificity of the RNA-RRM interaction (Cléry et al., 2008). Further C-terminally in G3BP, there is an arginine-glycine-glycine (RGG) motif, which is a sequence of closely located arginine and glycine residues, which is often unstructured due to the polar arginines next to the glycine residues. This unstructured region influences interactions with proteins and RNA (Rogelj et al., 2012). Additionally post-translational modifications of G3BP, for example the methylation of R435, could regulate the stability of the β-catenin mRNA in a Wnt-dependent manner (Bikkavilli and Malbon, 2011). G3BP was also shown to function as a helicase of both RNA and DNA. Interestingly, G3BP does not contain the canonical DEAD box of helicases (Bork and Koonin, 1993) and is thus a non-canonical ATP-dependent helicase. The RGG motifs may influence the RNA/DNA helicase activity of G3BP (Costa et al., 1999).

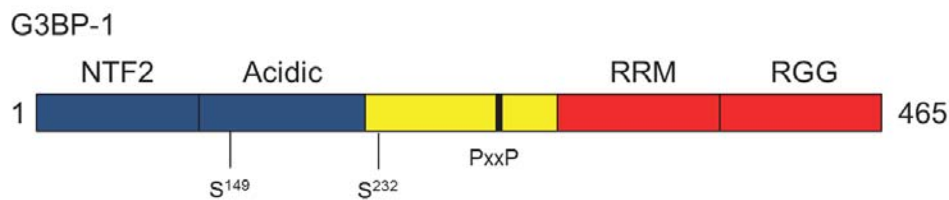

Figure 3: Schematic representation of human G3BP-1. Nuclear transport factor 2 (NTF2)-like domain, RNA-recognition motif (RRM), arginine-glycine-glycine motif (RGG), proline rich motif (PxxP), serine (S). For details see text.

Both G3BP-1 and G3BP-2 are dramatically overexpressed in human cancers such as breast, head, neck, colon and thyroid cancer as well as in human melanoma cell lines (Guitard et al., 2001, Barnes et al., 2002, French et al., 2002, Oi et al., 2014). Growing evidence suggests that deregulated RNA processing is often associated with cell proliferation and cancer (Sonenberg and Gingras, 1998, Sueoka et al., 1999). The observation that G3BPs are specifically overexpressed in several cancers and involved in RNA metabolism makes them a potential target for anti-cancer therapeutics.

Intriguingly, G3BP was also described as a binding partner of ubiquitin-specific protease 10 (USP10), which is a deubiquitinating enzyme (DUB) (Soncini et al., 2001). Recently it has been shown that G3BP interacts with the first 76 N-terminal residues of USP10 (Takahashi et al., 2013). The deubiquitination activity of USP10 leads to the stabilization of several important proteins, including the autophagy regulator Beclin-1 (Liu et al., 2011), the NF-κB essential modulator (NEMO/IKKγ) (Niu et al., 2013) and the tumour suppressor p53 (Yuan et al., 2010, Oi et al., 2014). Interestingly, recent reports show that USP10 regulates p53 activity, localization and stability by directly interacting and deubiquitinating p53 in the

cytoplasm, which is negatively regulated by G3BP (Soncini et al., 2001, Yuan et al., 2010, Oi et al., 2014). G3BP-1 is able to disrupt this interaction *in vitro*, indirectly leading to destabilization of p53 (Oi et al., 2014). However, an extensively described DUB for p53 is HAUSP/USP7, which is mainly found in the nucleus and reported to deubiquitinate p53. It is thought that USP10 deubiquitinates the cytoplasmic fraction of p53, whereas the nuclear fraction is deubiquitinated by HAUSP/USP7 (Yuan et al., 2010). Another report indicates that G3BP binds to the C-terminus of p53 to physically disrupt the normal p53 oligomer assembly or to obscure critical residues which are post-translationally modified (Kim et al., 2007). However, it was not shown whether this interaction is direct, and therefore it may be mediated by USP10, binding both p53 and G3BP.

In addition, G3BP also forms complexes with the cell cycle regulator Caprin-1, a protein which may regulate the transport and translation of mRNAs of proteins involved in cell proliferation and migration. Overexpression of Caprin-1 induces the formation of SGs and eIF2α phosphorylation through a mechanism that depends on its ability to bind mRNA (Solomon et al., 2007).

1.3.4 SG and diseases

SGs are implicated to be involved in many diseases. As described above, viruses target SGs because of their antiviral capabilities (Montero and Trujillo-Alonso, 2011, White and Lloyd, 2012, Valiente-Echeverria et al., 2012). In some cancers, SGs are upregulated (Baguet et al., 2007) and may support cancer survival (Fournier et al., 2010, Thedieck et al., 2013). It is also seen that SG-associated proteins like G3BP or USP10 are dysregulated in cancer (Barnes et al., 2002, Yuan et al., 2010). An emerging area of interest is the connection between mRNP granules, like SG, and degenerative diseases. Cytoplasmic granules are a pathohistological hallmark in neurodegenerative diseases. Recently, mutations were identified in the RNA-binding proteins TDP-43 and FUS (Kwiatkowski et al., 2009, Vance et al., 2009). Interestingly these RNA-binding proteins are found in SGs as well (Parker et al., 2012, Daigle et al., 2013). TDP-43 and FUS interact with SG core components, and mutated forms have the capability to enhance the aggregation of SGs (Bosco et al., 2010, Liu-Yesucevitz et al., 2010). Additionally, SGs containing TDP-43 and FUS tend to be more persistent and larger (Baron et al., 2013, Vance et al., 2013). Therefore, these specific RNA-binding proteins may provide a link between SGs and neurodegeneration.

Further, SGs also play a role in Alzheimer’s disease (Vanderweyde et al., 2012), where the SG components TIA-1 and TTP bind phospho-tau, which is a disease-linked pathological protein that forms stable insoluble protein aggregates. The aggregation of SGs could become pathological when the pro-aggregation state is favoured because of mutations, other disease processes or environmental conditions.

1.3.5 SG and signalling

More and more reports show that signalling molecules are recruited to SGs. The transient assembly of SGs could influence several signalling pathways until the cell is adapted to stress or dies. Therefore SGs could be seen as signal hubs, which sequester signalling components to react to a state of emergency. As described above, SG-associated RNA-binding proteins have an LC/ID region in common, a region that is also found in signalling molecules like protein kinase Cα (PKCα), USP10, PKR and others. PKCα enhances the assembly of SGs in cells subjected to heat shock or sodium arsenite stress, most likely through binding to G3BP-2 (Matsuki et al., 2013). USP10 interacts with G3BP and poly(A)-binding protein (PABP) and deubiquitinates and stabilizes the tumour suppressor p53, influencing apoptosis induction (Yuan et al., 2010, Oi et al., 2014). Knock-down of USP10 dampens the SG response and correlates with increased production of reactive oxygen species and increased apoptosis (Takahashi et al., 2013). Another protein, RACK1, an adaptor molecule that integrates cell adhesion polarity and motility, is also sequestered to SGs. However RACK1 does not contain LC/ID regions, but binds to the multisubunit eIF3, which is a core component of SGs. The recruitment of RACK1 to SGs inhibits stress-induced activation of the p38/JNK signalling pathway, which triggers apoptosis (Arimoto et al., 2008). When RACK1 is sequestered to SGs, it cannot act as a scaffolding protein for MTK1 (mitogen activated kinase 1), which acts upstream of p38 and JNK to initiate apoptosis. Therefore sequestration of RACK1 to SGs inhibits this signalling pathway and promotes cell survival. In addition, two important signalling molecules with a direct link to the detection of viral infections have been found to be sequestered to SGs as well. Upon IAV infection, RIG-I is sequestered to SGs-like structures, which were termed antiviral SGs (avSGs), and this sequestration was found to be critical to activate IFN genes. Furthermore PKR is also recruited to avSGs (Onomoto et al., 2012).

1.3.6 SG and viral infections

SGs have fundamental roles in inhibition of host mRNA translation, which affects viral mRNAs as well. Viruses are totally dependent on the cellular gene expression machinery, and therefore it is evident that viruses interact with SGs to control virus replication and counteract antiviral effects of SGs. A very well described mechanism employed by cells to restrict viral propagation is through the activation of PKR. Activated PKR phosphorylates eIF2α leading to the formation of SGs and translational restriction. Many viruses, such as influenza virus, vaccinia virus, poliovirus, herpes simplex virus, West Nile and dengue virus employ mechanisms to avoid or block the functions of PKR and SGs (Montero and Trujillo-Alonso, 2011, White and Lloyd, 2012, Valiente-Echeverria et al., 2012, Lloyd, 2013). Furthermore the formation of SGs may also lead to the sequestration of viral mRNA and eukaryotic initiation factors, which could limit efficient translation of viral proteins.

White and Lloyd reviewed the interactions between viruses and SGs and grouped them into three phenotypic categories, which might be revised in the future: firstly viruses that inhibit SGs, secondly viruses that tolerate or exploit SG responses and lastly viruses that first induce and then inhibit SGs. (White and Lloyd, 2012). West Nile virus and dengue virus, which belong to the family *Flaviviridae*, inhibit SG formation by sequestration of TIA-1 through specific binding to the minus strand 3´ terminal stem loop structure of the viral genome (Emara and Brinton, 2007). Dengue virus 3´ UTR and 5´ UTR physically interact with G3BP, Caprin-1 and USP10, as well as the PB marker DDX6 (Ward et al., 2011). These proteins were found to colocalize with dsRNA, which marks replication sites. IAV also fails to induce SGs, except for mutant viruses that lack non-structural protein 1 (NS1). In the latter case, infection with NS1 mutants, SGs are formed in a PKR-dependent manner. The complete inhibition of SG assembly is dependent on the action of NS1 on PKR (Khaperskyy et al., 2011). The formation of SG represses IAV replication if NS1 is lacking. The induction of SGs is also blocked by HSV via multiple mechanisms. This highlights again the potent anti-viral effect of SGs. Early in HSV infection, activated PKR subsequently phosphorylates eIF2α, which is dephosphorylated by the recruitment of serine/threonine protein phosphatase I by ICP34.5, hence re-activating translation (He et al., 1997). It was shown that HSV-1 mutants lacking the virion host shutoff (vhs) protein, an endoribonuclease that degrades cellular and viral mRNA, induce SGs late in infection (Esclatine et al., 2004, Dauber et al., 2011) A possible role for vhs might be inhibition of SGs or altering expression of other SG-modulating viral gene products. An additional role could be the limitation of available mRNA which is needed to nucleate *bona fide* SGs. Further the closely related family member HSV-2 also blocks the formation of SGs induced by sodium arsenite but not Pat A (Finnen et al., 2012).

The second category of virus/SG interplay is that viruses may tolerate or exploit the SG response. As described above, SGs and active virus replication do not commonly co-exist, because it appears that SGs have a negative effect on viruses and are selected against. However, it is possible that some viruses co-opt or misdirect the SG response for their favour, meaning that SGs have a positive effect on viral replication in this case. For instance, VV sequesters SG proteins into novel aggregates comprised of G3BP, Caprin-1, eIF4G and eIF4E (Katsafanas and Moss, 2007, Simpson-Holley et al., 2011), but surprisingly no silenced cellular mRNA, instead, they contain viral mRNA. The structures are found in close proximity to viral replication factories and may help VV to segregate replication and packaging away from translation (Simpson-Holley et al., 2011).

The third group includes viruses which first induce the formation of SGs, but later disassemble them and inhibit their reformation. Poliovirus for example induces the formation of SGs in an eIF2α-independent manner (White et al., 2007). The formation of SGs peaks 2–3 hours post infection and then declines, by a mechanism that requires viral replication (White et al., 2007). When exogenous stressors (such as sodium arsenite) are applied, the formation of *bona fide* SGs is inhibited in poliovirus infected cells. However in another report, TIA-1-positive SGs were observed late in poliovirus-infection (Piotrowska et al.,

2010), They were later identified as non-canonical SGs because they lacked SG-defining components like initiation factors and mRNA (White and Lloyd, 2011). The mechanism how poliovirus disassembles SGs resides in the activity of the viral protease $3C^{pro}$, which cleaves G3BP. When a cleavage-resistant mutant of G3BP (Q326E) is expressed, the formation of SGs is rescued, along with a sevenfold decrease in viral replication, which also indicates the potential antiviral role of SGs (White et al., 2007, White and Lloyd, 2011). Another virus which first induces SGs and then inhibits their formation is hepatitis C virus. It has been shown that several SG markers colocalize with the HCV core protein, which is likely mediated by the interaction of G3BP and maybe other factors with the viral protein NS5B and the 5´ terminus of the negative-strand RNA (Yi et al., 2011, Khong and Jan, 2011). SFV modulates the SG response as well. At early times of infection, SFV induces the phosphorylation of eIF2α in a PKR-dependent manner and promotes the formation of SGs. Despite the shutoff of host protein synthesis, viral mRNA is still translated due to a translational enhancer, which efficiently works in conditions where eIF2α is phosphorylated. Later in infection, the SGs are disassembled, and the infected cell is not responsive to exogenous stress. This is supported by the observation that areas around the viral RNA are devoid of SGs, which also indicates that viral proteins or RNA may locally disassemble SGs to favour viral replication (McInerney et al., 2005).

The aims of this thesis were to determine how SFV disassembles SGs and what consequences develop from the disassembly. Furthermore we asked if the closely related CHIKV disassembles SGs with a similar mechanism. Lastly we investigated the molecular nature of the SFV-mediated SG disassembly.

1.4 AIMS OF THE THESIS

The overall aim of this thesis was to study virus-host cell interactions in SFV-infected cells.

Paper I

In paper I we addressed the question how SFV achieves the disassembly of SGs very early in infection and how cells are still able to react to other stresses.

Paper II

In paper II we asked whether the closely related alphavirus CHIKV also induces disassembly of SGs in a similar fashion as SFV. Furthermore is this interaction direct or mediated by other factors.

Paper III

In paper III we sought to investigate in molecular detail how the viral protein nsP3 binds G3BP. We further asked whether other cellular and viral proteins use the same mechanism as nsP3 and lastly if this interaction inhibits the formation of SGs.

2 RESULTS

It has been shown in SINV-infected cells that G3BP interacts with nsP3 (Cristea et al., 2006). Nevertheless it was also reported that G3BP interacts with nsP2 (Atasheva et al., 2007) and nsP4 (Cristea et al., 2010), therefore it is not clear if nsP3 is the direct interacting partner of G3BP. Furthermore, G3BP is a defining component of SGs and targeted by other viruses to inhibit the formation of SGs. The poliovirus $3C^{pro}$ protease cleaves G3BP to inhibit the formation of SGs (White et al., 2007). Therefore we hypothesized in SFV-infected cells, G3BP is sequestered to viral replication complexes, which might explain an earlier report by our group showing that regions in the cell in the vicinity of the viral RNA are devoid of SGs (McInerney et al., 2005). Further the sequestration of G3BP may inhibit SG reformation in infected cells. To assess this, we performed immunofluorescence experiments on SFV infected mouse embryonic fibroblasts (MEF). Interestingly we observed a strong colocalization of G3BP with the viral protein nsP3 at 8 hours post infection (hpi) (Fig. 4A, Paper I Fig. 1B). To determine if these foci are viral replication complexes, we stained infected cells against G3BP, nsP1 and dsRNA and confirmed that the foci were positive for G3BP, nsP1 and dsRNA (Paper I Fig. 1C). Other cellular interaction partners of G3BP, like USP10 or Caprin-1, were excluded from these foci (Paper III Fig. S3, and data not shown). We then infected baby hamster kidney (BHK) cells to determine whether nsP3 interacts with G3BP. At 8 hpi, lysates were immunoprecipitated with G3BP or nsP3 antisera and probed for nsP3, G3BP-1 and actin. G3BP-1 was coimmunoprecipitated with nsP3 and *vice versa* (Fig. 4B, Paper I Fig.1A). This suggests that nsP3 forms a complex with G3BP and sequesters it into viral replication complexes.

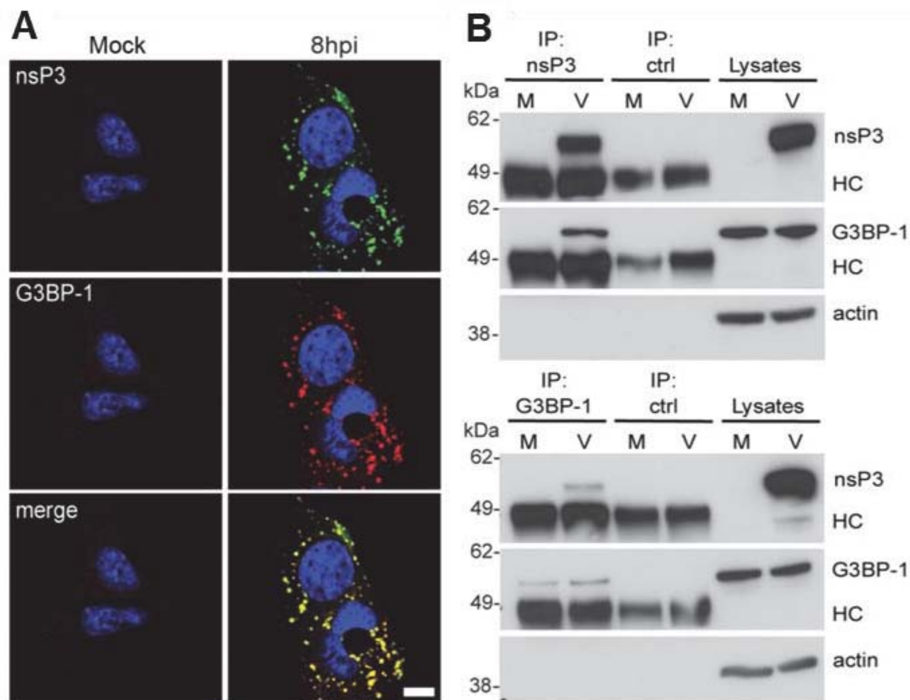

Figure 4: SFV forms a complex with G3BP and sequesters it into viral replication complexes. (A) MEF cells were infected with SFV-wt at an MOI of 1 for 8 h, fixed and stained for nsP3 and G3BP-1. Bar 10 µm. (B) BHK cells were infected at an MOI of 10 with SFV-wt. Cell lysates were prepared 8 hpi and immunoprecipitated (IP) with nsP3- or G3BP-antisera and probed for nsP3, G3BP-1 or actin. The position of the heavy chain (HC) is indicated.

Next, we sought to investigate if nsP3 is the sole interacting partner of G3BP and or if this interaction is mediated by one of the other non-structural proteins and asked what motifs in nsP3 interact with G3BP. At the C-terminal part of nsP3, two seven amino acid repeat sequence elements, L/ITFGDFD (here referred to as C-terminal repeat domain), conserved in the Old World alphaviruses, were identified by Varjak and colleagues (Varjak et al., 2010). The two C-terminal repeat domains are separated by a stretch of 10 amino acids ("spacer"). To biochemically assess if nsP3 is the sole SFV protein to interact with G3BP and to map the interaction domain, we generated two nsP3 mutants for use in transfection experiments in the absence of infection, hence potentially excluding a contribution of the other viral proteins. nsP3-Δ10 lacks 10 amino acids of the non-conserved C-terminal region and nsP3-Δ30 lacking 30 amino acids of the C-terminus, including the C-terminal repeat domains. In paper I (Fig. 2A) we showed in cells expressing individual nsP3-wt and nsP3-Δ10 interacted with G3BP, confirming that indeed, nsP3 is the sole viral interaction partner for G3BP. Furthermore, the nsP3-Δ30 mutant did not, suggesting that the interaction is mediated by sequences within the C-terminal 30 aas. Interestingly a stretch of 31 amino acids containing the two C-terminal repeat domains of nsP3 fused to GFP (EGFP-nsP3-31) efficiently coimmunoprecipitated G3BP (Paper I Fig. 2B). These findings led to the creation of virus mutants with similar truncations in nsP3. The truncation SFV-Δ8 lacks 8 amino acids of the spacer region. SFV-Δ78 lacks the spacer region and the first C-terminal repeat, and SFV-Δ789 lacks both C-terminal repeats (Fig. 5A, Paper I Fig. 3A). We infected BHK cells with wildtype (wt) SFV (SFV-wt) or the virus mutants and lysates were subjected to immunoprecipitation with G3BP antisera, followed by Western blot for nsP3, G3BP and actin. nsP3-wt was efficiently coimmunoprecipitated by G3BP, as expected. nsP3 from the virus mutant SFV-Δ8, also coimmunoprecipitated with G3BP, but less efficiently. SFV-Δ78, lacking the 8 amino acid of the spacer region as well as the first C-terminal repeat showed a drastically reduced interaction with G3BP. Whereas the viral mutant (SFV-Δ789), lacking the both repeats in nsP3 did not coprecipitate G3BP (Fig. 5B, Paper I Fig. 3B). Single-step and multistep growth curve experiments revealed that the virus mutant SFV-Δ789 is attenuated for growth in both MEF cells (Fig. 5C, Paper I Fig 3C) and BHK cells (Paper I Fig. S2). Taken together these results show that nsP3 is the sole SFV protein to interact with G3BP. Furthermore, the two C-terminal repeats of nsP3 are necessary to interact with G3BP and that a virus mutant, lacking these, is limited in viral propagation.

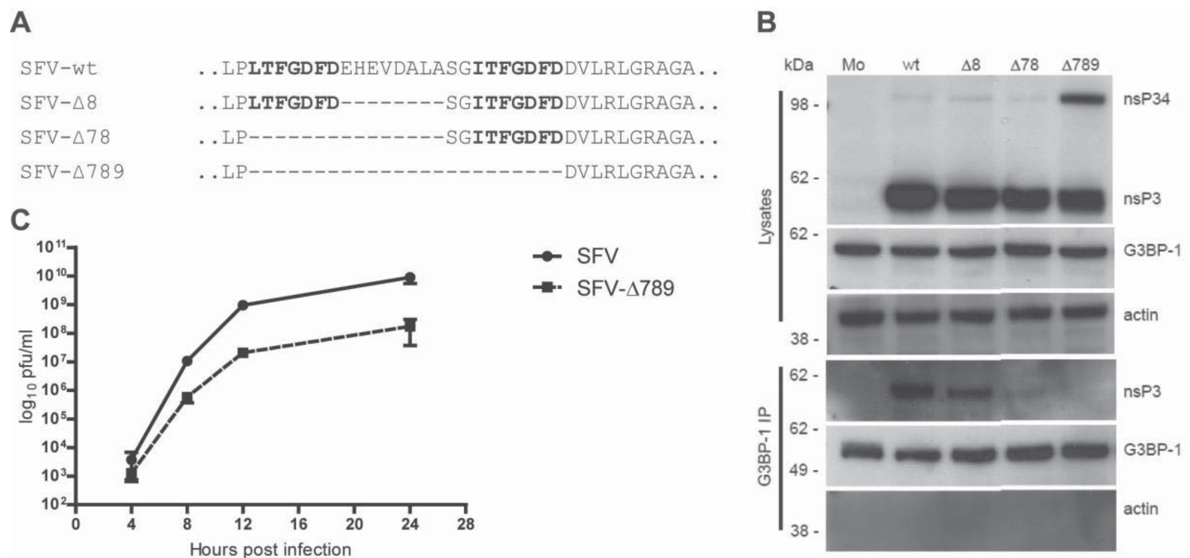

Figure 5: The C-terminal repeats of SFV-nsP3 are required for sequestration of G3BP-1. (A) Representation of the C-terminal sequences of nsP3 from SFV-wt, -Δ8, -Δ78 and -Δ789. The C-terminal repeats are shown in bold. (B) BHK cells were infected with the indicated viruses at an MOI of 10. Cell lysates were prepared at 8 hpi and subjected to immunoprecipitation (IP) with G3BP-1 antisera. Lysates and IPs were probed for the indicated proteins. (C) BHK cells were infected at an MOI of 0.1. At 4, 8, 12, 24 and 38 hpi, supernatants were collected and SFV infectious units were quantified by plaque assay on BHK cells. Error bars indicate SD of two independent experiments.

At the same time, Fros and colleagues (Fros et al., 2012) also showed, that nsP3 colocalizes with G3BP, using CHIKV-infected Vero cells. Their results however suggest that the proline-rich region, spanning residues 398–406 of nsP3, is essential for colocalization of CHIKV-nsP3 and G3BP. This is in contrast to our work and it is unexpected that two Old World alphaviruses would differ in the region to bind G3BP. In Paper II, we hence addressed the question if the proline-rich sequence and the C-terminal repeat domains are both necessary to colocalize and interact with G3BP. Immunostaining of MEF cells, infected with the SFV-ΔP1+P2 virus mutant that lacks the proline-rich sequence in nsP3 (Neuvonen et al., 2011), revealed colocalization of nsP3 and G3BP (Paper II Fig. 1A). Furthermore, in transfection experiments with SFV-nsP3-ΔP1+P2 and CHIKV-nsP3-ΔP1, lacking the proline-rich sequences, the nsP3 mutants efficiently coprecipitated G3BP (Fig. 6B, Paper II Fig. 1C). Additionally, Vero cells transfected with CHIKV-nsP3-Δ398–406 efficiently colocalized and coprecipitated G3BP confirming that the proline-rich region is not required for the interaction of CHIKV-nsP3 with G3BP (Paper II Fig. 3B and C). We conclude from these results that the G3BP binding site in nsP3 of the Old World alphaviruses SFV and CHIKV resides in the C-terminal repeats of nsP3 and that the proline-rich region is not required for the interaction.

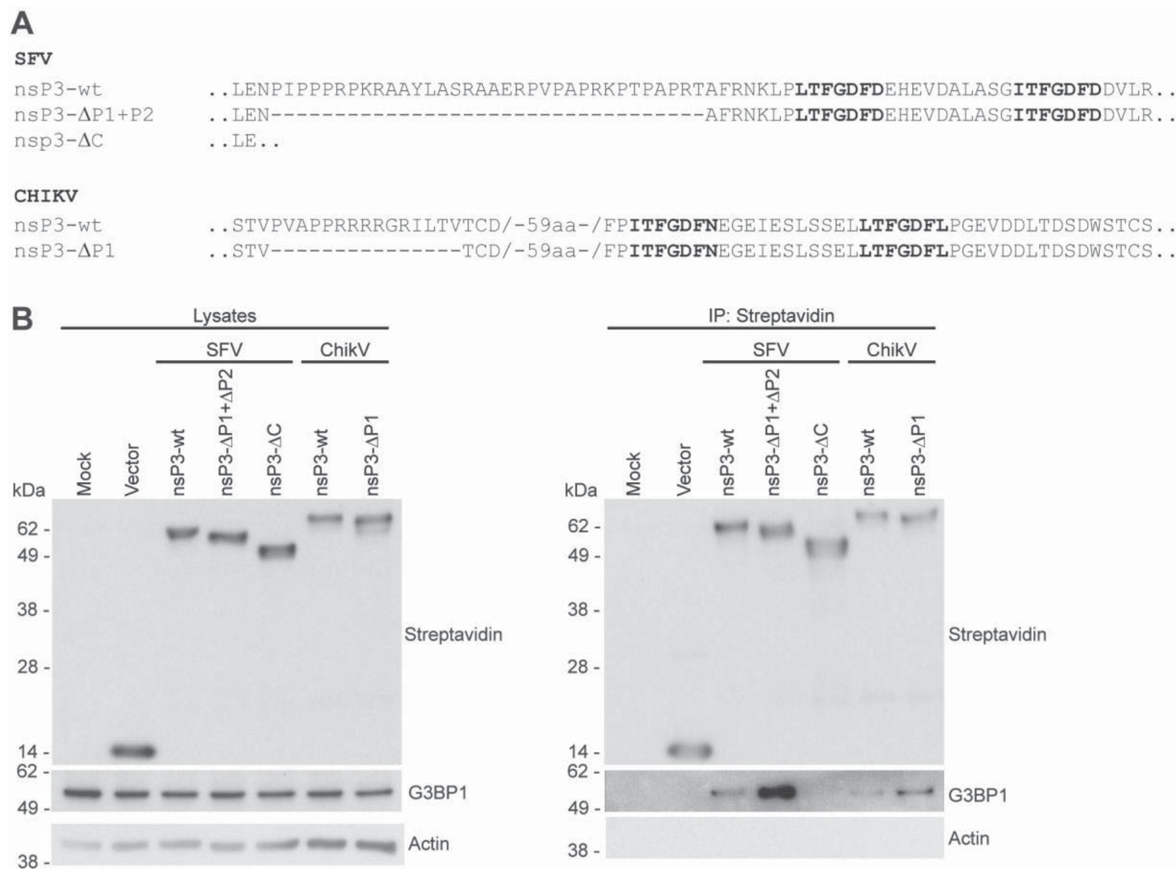

Figure 6: Proline-rich sequences of SFV and CHIKV nsP3 are not required for G3BP-1 binding. (A) Extreme C-terminal sequences of SFV-nsP3-wt, nsP3-ΔP1+P2, nsP3-ΔC, and CHIKV-nsP3-wt and nsP3-ΔP1. (B) BHK cells were mock-transfected or transfected with the indicated constructs. The nsP3 proteins were tagged with a biotin acceptor peptide (BAP). Cell lysates were prepared, precipitated with streptavidin-coated beads and probed with the indicated antibodies**.**

Previous studies have shown that after 8 h of infection with SFV, cells are not capable of forming SGs in response to exogenous stress such as sodium arsenite (McInerney et al., 2005). The work presented here suggests that a possible mechanism to block SGs is the sequestration of G3BP to nsP3-containing replication complexes. Thus we asked whether cells respond to exogenous stress and form SGs if G3BP is sequestered to replication complexes. Therefore we infected cells with SFV-wt or the mutant SFV-Δ789 for 7 h and subsequently treated the cells for 1 h either with sodium arsenite or Pat A. The cells were fixed and stained for G3BP-1 and TIA-1 and the number of SGs positive cells was evaluated. We used Pat A because sodium arsenite signals through eIF2α phosphorylation, which does not have an effect in in SFV-infected cells due to sustained high levels of infection-induced phospho-eIF2α. Pat A on the other hand is an eIF2α-independent stress and stalls translation by interacting with the helicase eIF4A (Bordeleau et al., 2006). As expected, SFV-wt infected cells were not able to mount a stress response to exogenous stressors such as sodium arsenite or Pat A. On the other hand SFV-Δ789 did not mount a stress response upon sodium arsenite treatment but mounted a significant stress response upon Pat A treatment (Fig 7, Paper I Fig. 6B). This shows that SFV has an active mechanism to dissolve SGs and to block their reformation on viral mRNA later in infection.

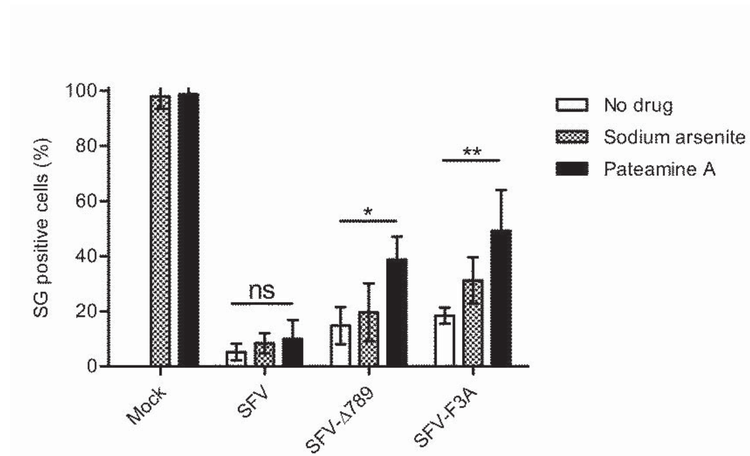

Figure 7: G3BP-1 sequestration into SFV RCs inhibits Pat A- induced SGs. MEFs were infected with SFV-wt, SFV-Δ789 or SFV-F3A at an MOI of 50 for 7 h before 1 h treatment with sodium arsenite or pateamine A. Cells were fixed and stained for G3BP-1, TIA-1 and nsP3. Fifty cells per treatment were scored SG+ based on G3BP-1 and TIA-1 colocalization. Data are presented as mean +/- SD from three independent experiments. Student's t test: * < 0.05, ** < 0.001.

Interestingly, experiments with cells expressing nsP3 alone indicated that nsP3 on its own does not have the capacity to block the formation of SGs. In colocalization studies, we showed that cells expressing nsP3 in the absence of the other viral proteins and stressed with Pat A formed SGs and that nsP3 was found in SGs along with G3BP-1 and TIA-1 (Paper I Fig. 7A). However, experiments with the polyprotein nsP123 revealed that the formation of CPV-like structures containing nsP1, nsP2 and nsP3 was able to block SGs and that nsP3 was not found in *bona fide* SGs under stress conditions (Paper I Fig. 7B). Taken together, these results show that the formation of CPV-like structures and the interaction of these with G3BP is necessary to block the assembly of SGs.

We next sought to determine if the binding of nsP3 to G3BP is direct and if so which amino acids mediate this binding. In paper II we purified the His-tagged protein of the NTF2-like domain of G3BP-1 (His-G3BP1-NTF2) and the 31 aa of the C-terminus of nsP3 fused to GST (GST-31) expressed in *E. coli.* We showed in *in vitro* binding studies that GST-31 efficiently binds His-G3BP1-NTF2, but not GST alone. This verifies that the interaction is direct and not mediated by cellular or viral proteins. Furthermore the His-G3BP1-NTF2 construct lacks the RNA-binding domain of G3BP. It can hence be concluded that RNA is not involved in the nsP3-G3BP binding either (Paper II Fig. 4C). To investigate which amino acids mediate binding of nsP3 to G3BP, we performed an alanine scan of the C-terminal repeats. We used the EGFP-nsP3-31 construct created for paper I and replaced each of the amino acids 2–7 in both C-terminal repeats ($L/I^1T^2F^3G^4D^5F^6D^7$) consecutively with alanine (Fig. 8A, Paper III Fig. 1A). We then transfected BHK cells with these constructs followed by cell lysis, immunoprecipitation with GFP antisera and probed for G3BP. The results (Fig. 8B, Paper III Fig. 1B) clearly show that the mutation of the phenylalanines at positions 3 and 6 of the motif to alanines disrupted the binding to G3BP. Also the mutation of glycine 4 destroyed the interaction. The mutation of the aspartate residue at position 5 and threonine at position 2 led to a drastically reduced binding. In summary, these data reveal a core G3BP-

binding motif of FGDF in nsP3, in which the residues F3, G4 and F6 are essential. Furthermore there is a strong preference for threonine at position 2 and for aspartic acid at position 5.

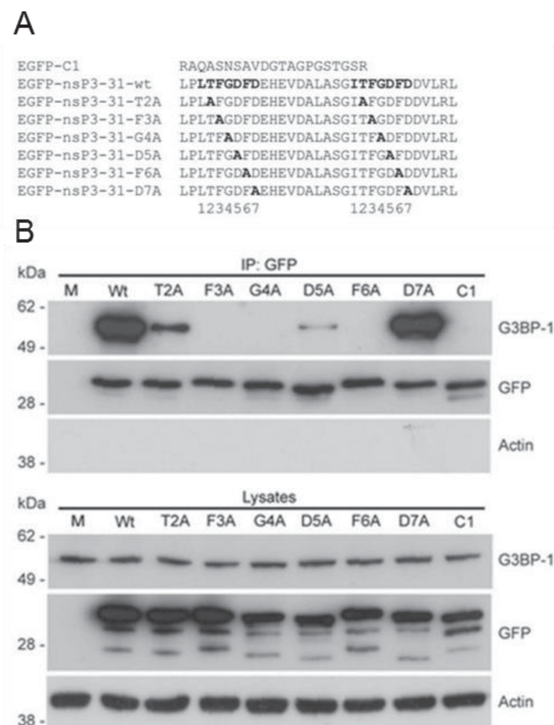

Figure 8: Mutagenesis of the G3BP-binding domain in SFV-nsP3 reveals a core binding motif of FGDF. (A) C-terminal sequences of pEGFP-C1, pEGFP-nsP3-31-wt, -T2A, -F3A, -G4A, -D5A, -F6A or -D7A. Alanine mutations are shown in bold. (B) BHK cells were mock-transfected (M) or transfected with pEGFP-nsP3-31-wt, -T2A, -F3A, -G4A, -D5A, -F6A, -D7A or pEGFP-C1. Cell lysates were prepared 16 h after transfection and immunoprecipitated (IP) with anti-GFP. Lysates and IPs were probed for G3BP-1, GFP or actin.

Based on the alanine scanning experiments of the C-terminal repeats we created an infectious clone of SFV (referred to as SFV-F3A) where the residues F451 and F468 in the two C-terminal repeats were exchanged to alanines (Fig. 9A), to test if the phenylalanines at position 3 are essential for binding of nsP3 to G3BP in the context of virus infection. Infection experiments with SFV-F3A revealed that, in contrast to SFV-wt, G3BP did not colocalize with nsP3 nor coprecipitate with nsP3 (Fig. 9B, Paper III Fig. 2A and B). Moreover, cells infected with SFV-F3A were able to respond to exogenous stress induced by treatment with Pat A (Fig. 7, Paper III Fig. 2C), while SFV-wt-infected cells were not. Finally, the F3A mutant virus was attenuated for growth in MEF and BHK cells (Fig. 9C, Paper III Fig. 2D and S2) to a similar extent as SFV-Δ789 (Fig. 5C). This demonstrates that the sequestration of G3BP to replication complexes by the FGDF motifs of nsP3 inhibits the SG response and is important for the efficient replication of SFV.

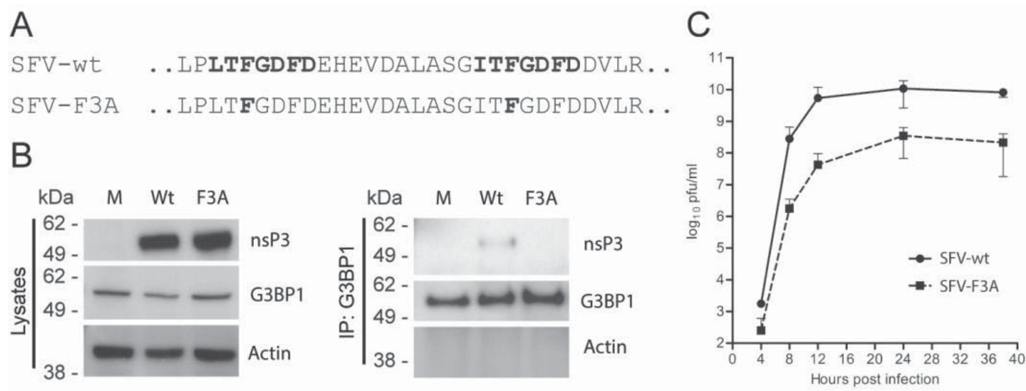

Figure 9: The C-terminal repeat domains of SFV-nsP3 are required for sequestration of G3BP-1. (A) Representation of the C-terminal sequences of nsP3 from SFV-wt and -F3A. The C-terminal repeats are shown in bold, whereas the F3A mutation is shown in bold. (B) BHK cells were infected with the indicated viruses at an MOI of 10. Cell lysates were prepared at 8 hpi infection and subjected to immunoprecipitation with G3BP-1 antisera. Lysates and IPs were probed for the indicated proteins. (C) BHK cells were infected at an MOI of 0.1. At 4, 8, 12, 24 and 38 hpi supernatants were collected and SFV infectious units were quantified by plaque assay on BHK cells. Error bars indicate SD of two independent experiments.

Earlier we showed that G3BP was recruited to replication complexes in SFV infected cells; however the G3BP interaction partner USP10 was not (Paper III Fig. S3). This suggests that USP10 is excluded from the complex with G3BP by the nsP3/G3BP interaction, proposing a competition of nsP3 and USP10 for G3BP. Sequence analysis showed that USP10 also contains an FGDF motif, which is situated at the N-terminus (Paper III Fig. 3A). We hypothesized that the FGDF motif of USP10 interacts with G3BP in a similar fashion as described above for nsP3. It has been shown that G3BP interacts with the first 76 N-terminal residues of USP10 (Takahashi et al., 2013). To test this experimentally, we fused the N-terminal 40 amino acids of USP10 to GFP, transfected cells, performed immunoprecipitations with GFP antisera and blotted for G3BP. Indeed, G3BP was found to interact with the N-terminal region of USP10 (Paper III Fig. 3B). Further, alanine mutations which replace F10, G11 or F13 disrupted the interaction with G3BP, strikingly similar to nsP3. This strongly suggests that G3BP binding of USP10 is also mediated by an FGDF motif (Paper III Fig. 3C). Interestingly the overexpression of full-length USP10, fused to GFP, had the capacity to block the formation of SGs induced by exogenous stress, whereas a non-interacting GFP-USP10-F10A failed to do so (Paper III Fig. 3D). Taken together, these data suggest that GFP-USP10 acts as a negative regulator of SG formation by binding G3BP via an FGDF motif.

In addition we hypothesized that G3BP may have other binding partners that also use FGDF motifs. Alignments of USP10 proteins from different species and alignments of the nsP3 sequences of different Old World alphaviruses suggested that the aspartate residue at position 5 in the FGDF motif (numbering of the motif: L/I^{1}T^{2}F^{3}G^{4}D^{5}F^{6}D^7) can be replaced by a serine or a glutamate. We also noted that the FGDF motifs of USP10 and nsP3 are followed by at least two acidic residues within the downstream four residues. In order to identify candidates which might bind G3BP, a bioinformatic query for the following motifs F-G-[DES]-F-[DE], F-G-[DES]-F-X-[DE], F-G-[DES]-F-X-X-[DE], F-G-[DES]-F-X-X-X-[DE], F-G-[DES]-F-X-X-X-X-[DE] (X = any aa) in human and viral proteins in the UniProt database was performed. We identified 34 human proteins and 32 viral proteins (Paper III Tables S1 and

S2) that contain an FGxF (x = D, E, S) and may bind G3BP. One of these is the major DNA-binding protein (also referred to as ICP8) of HSV that contains an FGDF motif in an unstructured region at the C-terminal end. In order to determine if ICP8 is capable of forming a complex with G3BP, we cotransfected cells with ICP8 and EGFP-G3BP, performed immunoprecipitation with GFP antiserum and blotted for ICP8. As predicted, ICP8 also interacted with G3BP (Paper III Fig. 7A). In addition, overexpression experiments with ICP8 revealed, similarly to the USP10 overexpression experiments (Paper III Fig. 3D), that the cytoplasmic fraction of ICP8 harbours the ability to block the formation of SGs induced by exogenous stress (Paper III Fig. 7B). This indicates that ICP8 can act, similarly to USP10, as a negative regulator of the formation of SGs by binding to G3BP via its FGDF motif. A function for the cytoplasmic fraction of ICP8 in SG disassembly has not been described yet.

The NTF2-like domain of G3BP in complex with an FxFG-containing peptide has been recently crystallized (Vognsen et al., 2013). The FxFG peptide shows similarities to the FGDF motif found in nsP3, USP10 and ICP8 and is bound into a hydrophobic cleft on the surface of G3BP. Based on that structure, we modelled an FGDF-containing peptide into the same hydrophobic cleft of G3BP (Fig 10, Paper III Fig 5 A and B).

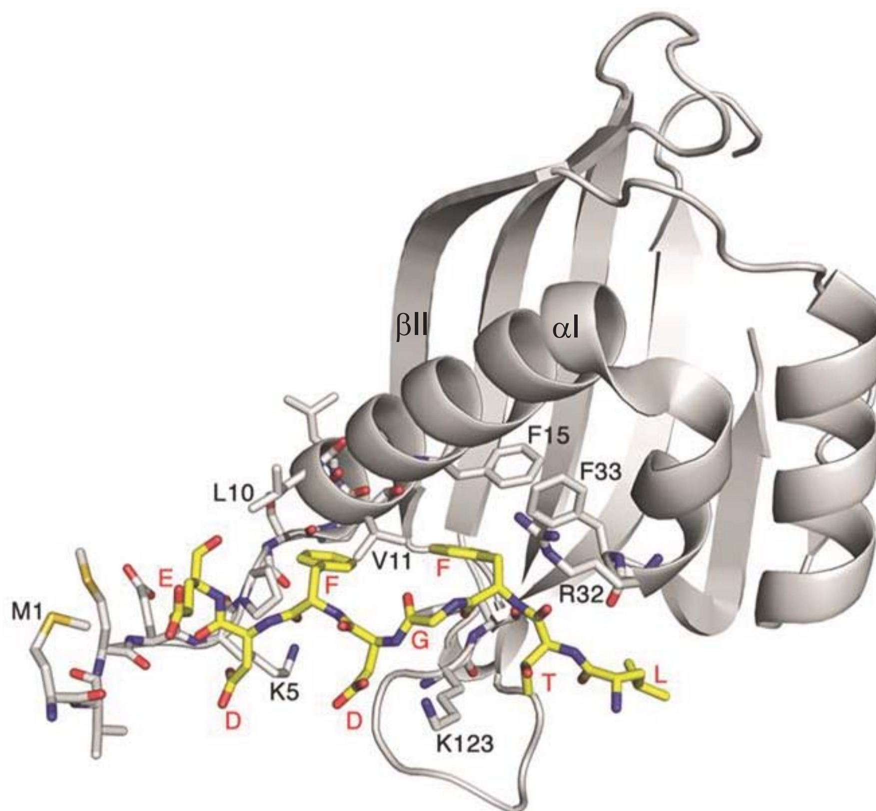

Figure 10: Molecular model of G3BP/FGDF interaction. (A) Ribbon representation of the G3BP-NTF2 with the manually docked LTFGDFDE peptide. The N-terminal 11 residues of the protein as well as several residues lining the peptide-binding groove are displayed as grey sticks. The LTFGDFDE peptide is displayed as sticks with yellow carbon atoms, residues are labelled in red. Residues of G3BP are labelled in black.

According to this model, there is a deep pocket at the base of the hydrophobic cleft formed by F15, F33 and F124, where the side chain of F3 is buried. A shallower pocket is formed by

F124, V11 and L10, where the side chain of F6 is localized. Both phenylalanines of the FGDF motif point into these two pockets. This binding model, presented in paper III Fig. 5, led to the prediction that the phenylalanine F33 is important for FGDF binding. To experimenttally validate the model we performed site-directed mutagenesis of the hydrophobic cleft in G3BP (Fig. 11A, Paper III Fig. 6A). The F33 residue, which is proximal to the F3 in the $L/I^1T^2F^3G^4D^5F^6D^7$ peptide, was mutated to the bulkier tryptophan to make the hydrophobic cleft smaller and to introduce steric hindrance. As a control we mutated F124 also to tryptophan, because this residue is not located in the binding cleft and is also solvent-accessible. To test the proposed binding model, we cotransfected cells with nsP3 (tagged with a biotin acceptor peptide (BAP)) and either with EGFP-C1, EGFP-G3BP-wt, -F33W or -F124W. Cell lysates were subjected to immunoprecipitation with GFP antisera and blotted for nsP3, GFP, or actin (Fig. 11B, Paper III Fig. 6B). EGFP-G3BP-wt interacted with nsP3, as expected. The mutant EGFP-G3BP-F124W also interacted with nsP3, whereas EGFP-G3BP-F33W did not. Importantly, similar experiments with the HSV protein ICP8 (Paper III Fig. 7A) showed analogous results, as well as experiments with endogenous USP10 (Fig. 11C, Paper III Fig. 6C). The combination of these results shows a striking similarity, which strongly supports our model how FGDF motif-containing proteins bind to G3BP.

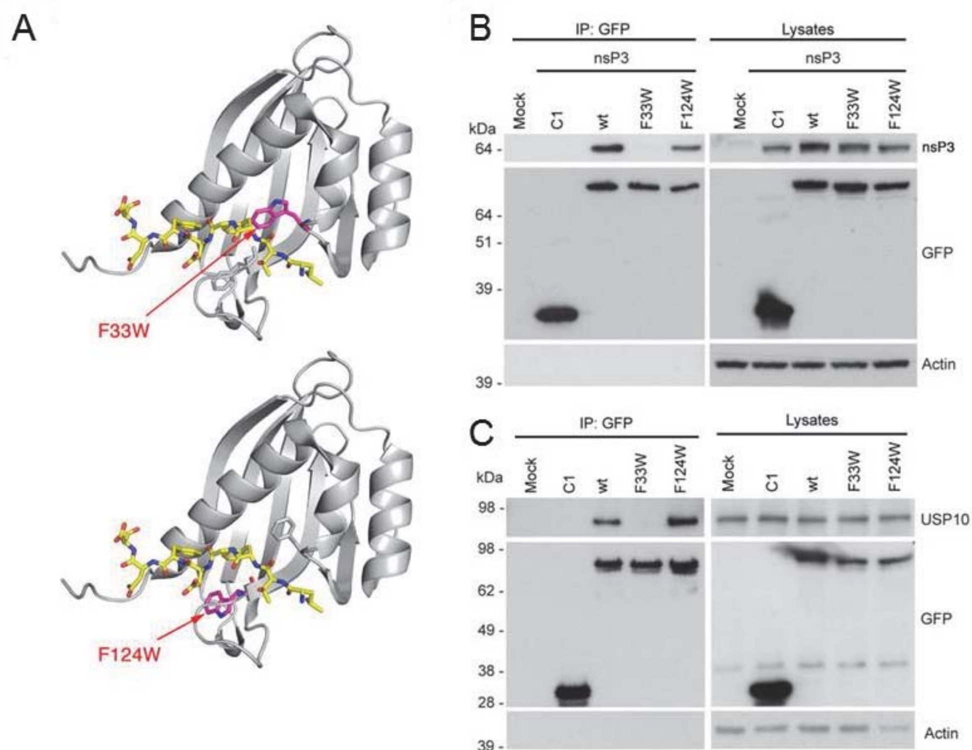

Figure 11: Site-directed mutagenesis in the FGDF peptide binding cleft of G3BP. (A) Schematic representation of the G3BP-F33W mutant (upper panel) and the G3BP-F124W mutant (lower panel) with the modelled LTFGDFDE peptide. The peptide is displayed as sticks with yellow carbon atoms. Mutated tryptophan residues are shown in magenta. (B) HEK293T cells were mock-transfected or cotransfected with pEBB/PP-SFV-nsP3 (nsP3-BAP) and either pEGFP-C1, pEGFP-G3BP-wt, -F33W or -F124W. Cell lysates were prepared 24 h after transfection and subjected to immune-precipitation with anti-GFP. Lysates and IPs were probed with streptavidin-HRP (nsP3) or antibodies against GFP or actin. (C) HEK293T cells were mock-transfected or transfected with pEGFP-C1, pEGFP-G3BP1-wt, -F33W or -F124W. Cell lysates were prepared 24 h after transfection and immunoprecipitated with GFP antisera. Lysates and IPs were probed for USP10, GFP or actin

3 DISCUSSION

In this thesis we showed that the SFV and CHIKV nsP3 proteins directly bind G3BP. Complex formation of G3BP with nsP3 has been observed previously (Cristea et al., 2006, Frolova et al., 2006, Gorchakov et al., 2008), but we are the first to show that this interaction is direct and to describe it in molecular detail. The interaction is mediated by two C-terminal repeats of nsP3 (Paper I and II). These repeats are conserved in nsP3 of the Old World alphaviruses but not in the New World alphaviruses. Accordingly, nsP3 of the New World alphavirus VEEV does not colocalize with G3BP (Foy et al., 2013). In Paper III we investigated the binding sequences in molecular detail and found that the binding of nsP3 to G3BP is mediated by FGDF motifs (numbering of the motif $L/I^1T^2F^3G^4D^5F^6D^7$). An alanine scan revealed that the amino acids F3, G4 and F6 of the FGDF motif are necessary to bind G3BP. This interaction leads to sequestration of G3BP to replication complexes, as confirmed by staining for nsP1 and dsRNA. These results also revealed that the cellular interaction partners of G3BP, USP10 and Caprin-1 are excluded from the replication complexes. We show that like nsP3, USP10 also contains an FGDF motif that mediates binding to G3BP (Paper III). This suggests that USP10 and nsP3 bind to G3BP in the same manner and thus compete for the binding site, thus explaining the exclusion of USP10 from the viral replication complexes. It was reported that Caprin-1 interacts with the NTF2-like domain of G3BP via a conserved peptide motif spanning amino acid 372–380 (Solomon et al., 2007). Interestingly, Vognsen and colleagues modelled this peptide into a binding cleft of G3BP which lies between the α1 helix and the βII sheet (Fig 10), thus besides the FGDF-binding site (Vognsen et al., 2013). This implies that Caprin-1 does not compete directly with the FGDF binding site on G3BP. Yet, immunoprecipitation experiments revealed that Caprin-1 efficiently coprecipitated with G3BP but not USP10 while USP10 efficiently coprecipitated with G3BP but not Caprin-1 (Nancy Kedersha, Harvard Medical School, personal communication) suggesting that there are two complexes present in the cell, G3BP/USP10 and G3BP/Caprin-1, but no ternary complex G3BP/Caprin-1/USP10. It is plausible that FGDF-mediated binding (of USP10 or nsP3) changes the conformation of G3BP to exclude Caprin-1 from the complex, which may be the reason why Caprin-1 is not found in the G3BP/USP10 complex or the viral replication complexes.

As we showed in paper I, SFV-nsP3 binds G3BP via two C-terminal repeats (comprising FGDF motifs as shown in paper III) sequesters it into replication complexes and in doing so inhibits the assembly of SGs. Another report by Fros and colleagues (Fros et al., 2012) presented that the closely related virus CHIKV also recruits G3BP to replication complexes and blocks SGs. In that study however, the G3BP-interacting domain was mapped to the proline-rich domain of nsP3. This domain had previously been shown to interact with amphiphysins (Neuvonen et al., 2011). The proline-rich domain and the C-terminal repeats in the hypervariable domain (HVD) of nsP3 are well conserved in the Old World alphaviruses, and therefore it is surprising that the two viruses should differ in the region used for recruiting G3BP. Fros and colleagues showed in transfection experiments that G3BP does not colocalize with an nsP3 construct missing the proline-rich domain. On the other hand, in our

work, transfection experiments using mutants of SFV and CHIKV nsP3 lacking the proline-rich region presented in paper II showed that G3BP strongly colocalized in nsP3-positive foci. Biochemical analysis revealed that G3BP is efficiently coprecipitated with nsP3 that lacks the proline-rich sequences. Nevertheless an nsP3 mutant which lacks both the proline-rich sequence and two C-terminal repeats did not colocalize or coprecipitate with G3BP. Interestingly amino acid sequences derived from the C-terminal part of SFV- and CHIKV-nsP3, containing the C-terminal repeats but not the proline rich region, efficiently coprecipitated G3BP, suggesting that the C-terminal repeats are necessary and sufficient to bind G3BP. Recently published work with VEEV and chimeric SINV virus showed that the HVD domain of VEEV nsP3, containing a proline-rich sequence but not the two C-terminal repeats, does not form a complex with G3BP thus supporting our findings (Foy et al., 2013). In summary the work from Fros and colleagues showed that the sequestration of G3BP to CHIKV replication complexes inhibits the formation of SG as we have reported in paper I for SFV. However we showed in paper II that that the described CHIKV-nsP3 proline-rich sequence is unnecessary for recruiting G3BP to CHIKV replication complexes. Furthermore our results in paper III indicate that nsP3 proteins of the Old World alphaviruses bind G3BP via their FGDF motifs, whereas the New World alphavirus nsP3 does not interact with G3BP.

In this thesis, we present data that show that the FGDF domain of SFV-nsP3, CHIKV-nsP3 and USP10 are essential and sufficient to bind G3BP. We noted from alignments of USP10 from different species and alignments of the Old World alphavirus nsP3 sequences suggested that the aspartate in the FGDF motif could also be a glutamate or a serine, which leads to the description of an FGxF core motif to bind G3BP. We also noticed that the FGDF motifs of SFV-nsP3 and USP10 are followed by at least two acidic residues within the downstream 5 residues. A bioinformatic search revealed 34 human proteins and 32 viral proteins containing such FGxF motifs. Biochemical analysis conducted for one of these, the HSV-1 protein ICP8, showed that it is indeed capable of interacting with G3BP, which hasn´t been reported yet. Even though ICP8 is a predominantly nuclear protein during HSV-1 infection, a sizeable fraction of the protein remains in the cytoplasm (Knipe and Spang, 1982), but functions for the cytoplasmic fraction are not well described. Our results suggest that the cytoplasmic fraction of ICP8 has the potential to inhibit SG assembly or alter other functions of G3BP. HSV infection blocks the induction of SGs via multiple mechanisms, also highlighting the potent anti-viral effect of SGs (see introduction chapter 1.3.6). ICP8-G3BP interaction may represent another mechanism by which HSV infection blocks SG formation. This needs to be determined in the context of HSV infection.

On the basis of our biochemical data on the FGDF-mediated binding of nsP3, USP10 and ICP8 to G3BP, we created a molecular model and manually docked the octapeptide $L^1T^2F^3G^4D^5F^6D^7E^8$ peptide into a hydrophobic cleft of G3BP. The NTF2-like domain of G3BP was crystallized and described by Vognsen and colleagues to bind a DSGFSFGSK peptide derived from nucleoporins (Vognsen et al., 2013). Our model shows that both phenylalanines fit snugly into the G3BP binding cleft and that the glycine at position 4 ensures flexibility in the motif such that the phenylalanines point in the same direction.

Interestingly the aspartate at position 5 may form a salt bridge with K123 of G3BP. The model presented in paper III also allows predictions how to disturb the interaction by mutating G3BP. The amino acid F33 of G3BP is the closest amino acid to the first phenylalanine of the FGDF motif. F33 is buried at the bottom of the hydrophobic cleft and we hypothesized that a mutation to a tryptophan would make the pocket smaller and therefore inhibit binding. As a control, we mutated F124 to a tryptophan since this residue is not part of the hydrophobic cleft. Immunoprecipitation experiments with a mutant carrying the F33W mutation clearly showed the loss of binding of nsP3, USP10 and ICP8, whereas the F124W mutant still bound all three proteins. This confirms our three-dimensional model, presented in paper III; it provides a deep structural understanding of the interaction of the G3BP/FGDF complex and could give insights how to design specific drugs to target this interaction.

Several RNA viruses transiently induce SGs. Early in the replication of such viruses, dsRNA is generated which leads to the activation of PKR and subsequent phosphorylation of eIF2α. This phosphorylation induces the assembly of SGs on abortive translation complexes. Poliovirus for example induces SG assembly early in infection; however the signals for the induction are less clear. Later in infection the SGs are efficiently disassembled by the cleavage of G3BP, mediated by the viral protease 3C^{pro}. This was nicely confirmed using a non-cleavable version of G3BP, the expression of which led to a significant reduction in titre (White et al., 2007). West Nile virus and dengue virus, which belong to the family of *Flaviviridae*, recruit the SG protein TIA-1 to viral replication sites, which leads to the inability to respond to exogenous stress and to a block of SG formation (Emara and Brinton, 2007). SFV induces the formation of SGs early in infection and during the course of infection they are disassembled in the vicinity of replication complexes (McInerney et al., 2005). In paper I we presented that nsP3 interacts with G3BP. Deletions of the C-terminal repeat domains of nsP3 or the introduction of two single point mutations F451A and F468A in nsP3 led to two virus mutants which were not able to form a complex with G3BP. Experiments confirmed that the mutants were delayed in the disassembly of SGs compared to SFV-wt. Furthermore, both viruses SFV-Δ789 and SFV-F3A were attenuated in growth by 1.5 to 2 logs in titre (Papers I and III). Additionally, cells infected with the mutant viruses were able to react to exogenous stress, whereas cells infected with SFV-wt were not (Papers I and III). Also infections with viral vectors that lack the translational enhancer (Sjöberg and Garoff, 1996) showed SGs that persist longer in infected cells compared to viruses that contain the translational enhancer. It is possible that the translational enhancer, which is a hairpin loop (Frolov and Schlesinger, 1994, Sjöberg and Garoff, 1996, Ventoso et al., 2006), allows more efficient formation of polysomes and therefore shifts the equilibrium from SGs to polysomes (Kedersha and Anderson, 2002) which may contribute to the disassembly of SGs in infected cells. This appears to be a passive mechanism to disassemble SGs. On the other hand, the FGDF-mediated interaction of nsP3 with G3BP is a targeted, active mechanism. Notably, nsP3 contains two FGDF motifs and efficiently binds two molecules of G3BP, whereas USP10, which contains one FGDF motif, only binds one G3BP molecule (Paper III Fig. 4). The difference in the stoichiometry of the G3BP/USP10 complex compared to the

G3BP/nsP3 complex might also explain the rapid disassembly of SGs in alphavirus-infected cells.

The fact that SFV infection leads to disassembly of SG strongly suggests an antiviral role of SGs. Interestingly, a direct antiviral role for SGs was shown in VV-infected cells. Cells infected with a VV mutant that lacks the PKR antagonist E3L displayed granules which were G3BP-, TIA-1- and eIF3-positive, surrounding viral factories and having a direct role in restricting viral replication (Simpson-Holley et al., 2011). Our experiments did not show a significant difference in the number and appearance of CPVs in cells infected with SFV-wt and the mutant viruses SFV-Δ789 or -F3A, despite the lack of recruitment of G3BP to CPVs in cells infected with the mutants. Infection with SFV-Δ789 led to the formation of SGs at similar times post-infection with SFV-wt, which suggests that the kinetics of PKR activation and eIF2α phosphorylation are comparable. However it was shown in SINV-infected cells that protein production was increased if G3BP expression was knocked down (Cristea et al., 2010), which suggest an antiviral role for G3BP by limiting gene expression. Other possibilities are also likely, for example the restricted production of the 26S sg-RNA, which could also lead to a limited gene expression. Furthermore other functions of G3BP could be compromised by the knockdown of G3BP, for instance the negative regulation of the DUB activity of USP10, which could lead to a stabilization of cellular and viral proteins. Interestingly this would suggest that the DUB activity of USP10 may be enhanced in SFV-wt-infected cells, whereas this activity may be blocked upon infection with the virus mutants SFV-Δ789 and -F3A, where G3BP is not sequestered by nsP3 and therefore free to inhibit USP10. Another possible role for G3BP in the replication complex could be related to the helicase activity, which resides in G3BP and may dissolve secondary structures in the viral RNA. Further experiments are needed to determine if G3BP has a specific role in the replication complex of SFV.

In paper I, we showed that overexpression of nsP3 in the absence of other viral proteins does not inhibit the formation of SGs. However overexpression of the polyprotein complex nsP123 blocked SGs, which suggests that sequestration of G3BP and the formation of replication complex-like CPVs is required to block SGs induced by exogenous stress. Experiments with overexpressed USP10 revealed similar effects on the formation on SGs. The cells could not mount a stress response to exogenous stress, in contrast to cells that expressed the USP10 mutant F10A, which does not bind G3BP. The HSV-1 ICP8 protein, which we showed to bind G3BP as well, is also able to block the formation of SGs. This suggests that proteins binding G3BP via an FGDF motif have an inhibitory effect on the SG-nucleating function of G3BP. Recent reports indicate that the drug resveratrol is able to inhibit the interaction of G3BP with USP10 by interacting with the NTF2-like domain of G3BP, thereby activating the DUB activity of USP10 (Oi et al., 2014). Interestingly, resveratrol has two benzene rings that sit in similar positions as the phenylalanines of the FGDF motif. It seems that the FGDF-mediated interaction between G3BP and USP10 is of a mutually inhibitory fashion. USP10 inhibits the SG-nucleating function of G3BP and G3BP inhibits the DUB activity of USP10.

4 FUTURE DIRECTIONS

So, where can we go from here? In this section, I would like to suggest experiments and directions how this work could be expanded.

In papers I and III, we designed the virus mutants SFV-Δ789 and SFV-F3A which are unique tools to study the stress response *in vivo* and *in vitro*, since the two viruses are viable. For instance, similar experiments with poliovirus would not be feasible. It was shown that the viral protease $3C^{pro}$ cleaves G3BP to inhibit the formation of SGs (White et al., 2007). Virus mutants with defective $3C^{pro}$ are not viable, because $3C^{pro}$ is needed for the processing of the poliovirus polyprotein. We showed that both SFV-Δ789 and SFV-F3A are defective in their interaction with G3BP, induced more persistent SGs during the course of infection and were attenuated for growth in cell culture, by 1.5 and 2 orders of magnitude, respectively; compared to SFV-wt. Therefore is to ask would the mutants differ from SFV-wt in their capacity to establish viremia in mice? This could be assessed by generating survival curves in mice after infection with either SFV-wt or SFV-F3A, possibly accompanied with determination of immune parameters. This would provide important information about the role of the nsP3-G3BP interaction and the longer persistence of SGs for virus infection *in vivo*. It is possible that the mutant viruses are not causing encephalitis in mice and are not lethal.

Furthermore, it will be interesting to investigate if G3BP has a distinctive role in the viral replication complex, to which it is recruited by binding to nsP3. Does the described helicase function (Costa et al., 1999) of G3BP have a role in viral RNA synthesis? Infection of cells with SFV-wt or SFV-F3A and investigation of the viral RNA synthesis would be appropriate experiments to conduct. Furthermore introduction of stem loops, with variable stabilities, in the 5′ region of the viral genome or near the subgenomic promoter could give clues if the helicase function of G3BP is necessary for efficient replication. Another interesting aspect would be to compare the New World and Old World alphaviruses, because New World alphaviruses do not sequester G3BP to the replication complexes. Are there any differences in the secondary structure of the genomic RNA?

It has been shown that in uninfected, unstressed cells, G3BP is in complex with the cell cycle-associated protein Caprin-1 and the deubiquitination enzyme USP10. In this thesis we observed in SFV-wt infected cells that G3BP is recruited to replication complexes, but not USP10 or Caprin-1. Continuing this work, it will be interesting to find out what impact this exclusion has on the cellular interaction partners USP10 or Caprin-1. It can be hypothesized that in the event of G3BP sequestration to viral replication complexes, the interaction partners USP10 and Caprin-1 become free to perform reactions. In case of USP10, we could investigate whether the ubiquitination levels of its substrates, including the tumour suppressor p53, the autophagy modulator Beclin-1 or the NFκB signalling regulator NEMO are changed in cells infected with either SFV-wt or SFV-F3A. Analysis on the cell-cycle and migration

would be useful to investigate effects on Caprin-1 in infected cells. It is conceivable that the sequestration of G3BP enables the activity of USP10 and Caprin-1 to favour viral replication.

It was shown that Caprin-1 interacts with the NTF2-like domain of G3BP. Interestingly Vognsen and colleagues (Vognsen et al., 2013) modelled a conserved peptide of Caprin-1 into a hydrophobic cleft in G3BP that is situated besides the USP10 and nsP3 binding site. G3BP was reported to form dimers but G3BP interacts either with Caprin-1 or with USP10, but never both (Nancy Kedersha, Harvard Medical School, personal communication). However the G3BP-F33W mutation fails to bind either USP10 or Caprin-1, although the suspected Caprin-1 binding cleft was not mutated. This is surprising and raises a number of questions. For instance: Why are both interactions disturbed by this mutation? Do both Caprin-1 and USP10 bind in the same hydrophobic cleft? Does the F33W mutant affect the folding on a larger scale, affecting both binding sites? Tryptophan is a bulkier amino acid than phenylalanine, which could mean that the N-terminal αI helix (Fig. 10, Paper III, Fig. 5) is pushed aside, making the predicted binding cleft for Caprin-1 smaller and therefore preventing the interaction. Creation of further G3BP mutants, which bind either USP10 or Caprin-1, would be very helpful to study the two complexes (G3BP/USP10 and G3BP/Caprin-1) in terms of their influence on SG formation.

A regulatory role of these two complexes could be played by the protein OGFOD1, which hydroxylates proline residues (Loenarz et al., 2014). OGFOD1 is known to be coprecipitated with USP10, Caprin-1 and G3BP (Wehner et al., 2010). Interestingly G3BP contains a proline residue at position 8 before the αI helix and at position 27 after the α1 helix. These residues could be a target for hydroxylation by OGFOD1, leading for example to displacement of the αI helix, which could change the size of the two hydrophobic clefts. Therefore the hydroxylation could act as a molecular switch to favour either the G3BP/USP10 complex or the G3BP/Caprin-1 complex. This mechanistic switch could regulate disassembly (G3BP/USP10) or assembly of SGs (G3BP/Caprin-1). Firstly, biochemical studies to determine whether G3BP is hydroxylated by OGFOD should be performed as well as interaction studies. Furthermore the G3BP-F33W mutant or other G3BP mutants that do not bind USP10 and/or Caprin-1 could be very helpful to study this complex. Another newly developed system to study such interactions would be the CRISPR/Cas9 system which gives the possibility to introduce single point mutations in the genome of cells (Jinek et al., 2012, Hsu et al., 2014). With such a tool in hand, the stoichiometry of the proteins would be preserved, leading to fewer artefacts than by overexpression or knockdown.

In Paper III we investigated in molecular detail how nsP3 and USP10 bind to G3BP with an FGDF motif, where the F3, F6 and the G4 are necessary. Furthermore we discovered that a substitution of the residue F33 in G3BP by a tryptophan disrupts the binding to nsP3 and USP10. The molecular model that we have presented in this work could lead to the design of antiviral drugs that inhibit this interaction. Recently it was shown that the drug resveratrol, an

anti-cancer drug, inhibits the interaction between USP10 and G3BP (Oi et al., 2014). Our data on the precise molecular nature of the G3BP–FGDF interaction could lead to improved derivatives of resveratrol with a stronger binding to G3BP and an improved solubility. Furthermore small peptides on the basis of an FGDF motif could be designed to compete for G3BP binding and restrict viral replication.

In summary this work gives valuable information of relevance for a number of human diseases, including infection and cancer, both of which involve alterations in stress response pathways by providing a detailed molecular mechanism how the SG-nucleating factor G3BP is bound by FGDF-containing proteins, such as nsP3, USP10 or HSV ICP8. The mutants that have been created during this work are valuable tools to study the G3BP/USP10 and G3BP/Caprin-1 complexes. Moreover the virus mutants created in this thesis, where nsP3 does not sequester G3BP, will also unravel new insights into virus-host-cell interactions and the antiviral role of SGs. A lot of work lies ahead of us.

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