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ALPHAVIRUS-HOST INTERACTIONS: HOW TO UNRAVEL THE PROVIRAL ACTIVITY OF G3BP

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Cover: Electron micrograph of Semliki Forest virus-induced cytopathic vacuoles, edited in Adobe Photoshop. The original image is published in (Götte et al., 2019). Cytopathic vacuoles, which accumulate in the perinuclear region of an infected cell, are indicated in green. Viral replication complexes form as membrane invaginations, termed spherules, and are highlighted in blue. The cytopathic vacuoles are surrounded by electron-dense material, shown in red, indicative for the enrichment of viral and cellular factors at the sites of viral RNA replication.

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Alphavirus-host interactions:
How to unravel the proviral activity of G3BP

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The genus *Alphavirus* belongs to the virus family *Togaviridiae*, a group of arthropod-borne enveloped RNA viruses with single-stranded, positive-sense genome. Members of this genus can be found worldwide and are geographically distinguished into Old World and New World alphaviruses. Old World alphaviruses include the re-emerging human pathogen chikungunya virus (CHIKV) and the model virus Semliki Forest virus (SFV). Upon cellular infection, the released viral genome is directly translated to produce the non-structural polyprotein, which is subsequently processed into four non-structural proteins. The non-structural protein 3 (nsP3) of Old World alphaviruses contains two FGDF motifs, which facilitate binding to the NTF2-like domain of the host protein G3BP and its recruitment to viral replication complexes. G3BP1 and G3BP2 (hereafter jointly referred to as G3BP) are homologous proteins, best characterized for their ability to assemble stress granules in response to a variety of cellular stressors, such as viral infection.

In **paper I** we investigated the structural and functional significance of the two FGDF motifs for the Old World alphaviruses SFV and CHIKV. A 3-dimensional structure of the NTF2-like domain of G3BP1 in complex with a SFV nsP3-derived peptide showed that the two FGDF motifs crosslink dimers of G3BP1 into a nsP3:G3BP1 oligomer. Mutational analysis of the FGDF motifs furthermore revealed that both motifs are required for efficient growth of SFV and suggest a critical role for the formation of nsP3:G3BP oligomeric structures for SFV. CHIKV is non-viable if the nsP3:G3BP1 interaction is abrogated through mutation of both motifs. The presence of a single functional FGDF motif is sufficient to rescue CHIKV replication, albeit to a lesser extent than in the presence of both motifs. Together, the results of this paper highlight similarities, but also discrepancies between SFV and CHIKV for the two G3BP-binding motifs.

In **paper II** we studied potential proviral roles of G3BP for SFV and CHIKV. To this end, we used a panel of human osteosarcoma (U2OS) cell lines lacking endogenous G3BP proteins and stably expressing G3BP1 mutants and truncation variants. SFV replication is attenuated in the absence of G3BP and efficiently rescued by the presence of only the NTF2-like domain of G3BP1, which is accompanied by clustering of replication complexes. On the contrary, CHIKV strictly depends on the presence of the NTF2-like and the RGG domains of G3BP1. By immunoprecipitation we show that the RGG domain of G3BP1 facilitates binding of nsP3:G3BP1 complexes to 40S ribosomal, which correlates with enhanced localized

translational activity in close proximity to viral replication complexes. The results suggest that G3BP exerts several proviral activities by mediating clustering of viral replication complexes and the recruitment of the translation initiation machinery.

The results of **paper III** demonstrate that Old Wold alphaviruses differ remarkably in their dependence on G3BP. We describe a role for the P4 residue of the cleavage site between nsP1 and nsP2 (1/2 site) of the alphavirus non-structural polyprotein in conferring the extent of G3BP sensitivity. An Arg residue at the P4 position of the 1/2 site, as for CHIKV, is associated with fast a cleavage rate at this site and a high sensitivity towards G3BP deletion. An His residue at this position, as for SFV4, confers a slower cleavage rate at this site, accompanied with partial resistance towards G3BP deletion. Arg-to-His substitution of the P4 residue of CHIKV allows partially rescues replication even in the absence of G3BP. However, our data suggest that G3BP proteins do not influence the processing of the non-structural polyprotein. Instead we propose a critical role for G3BP proteins during the initiation of viral RNA replication.

In summary, the work presented in this thesis improves our understanding of alphavirus-host interactions and highlights previously unanticipated proviral roles of G3BP for Old World alphavirus infections. The results mark G3BP as a potential target for the development of antivirals and provide a platform for future investigations.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscript:

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

II. **Götte, B.**, Panas, M.D., Hellström, K., Liu, L., Samreen, B., Larsson, O., Ahola, T., and McInerney, G.M. (2019). Separate domains of G3BP promote efficient clustering of alphavirus replication complexes and recruitment of the translation initiation machinery. *PLoS Pathog* 15, e1007842.

III. **Götte, B.***, Utt, A.*, Fragkoudis, R., Kedersha, N., Merits, A.†, McInerney, G.M.† Sensitivity of alphaviruses to G3BP deletion correlates with efficieny of replicase polyprotein processing. Manuscript in preparation. *,† equal contribution

The following publications were also obtained during the PhD education but are not included in this thesis:

IV. Liu, L. Weiss, E., Panas, M.D., **Götte, B.**, Sellberg, S., Thaa, B., McInerney, G.M. RNA Processing Bodies are Disassembled during Old World Alphavirus Infection. (2019) Accepted for publication in *J Gen Virol*

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

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

CONTENTS

LIST OF ABBREVIATIONS

AUD	Alphavirus unique domain
CHIKV	Chikungunya virus
CPV	Cytopathic vacuole
CSE	Conserved sequence element
EEEV	Eastern equine encephalitis virus
eIF2α-P	Phosphorylated eIF2α
ER	Endoplasmic reticulum
gRNA	Genomic RNA
HCV	Hepatitis C virus
HVD	Hypervariable domain
IAV	Influenza A virus
IDR	Intrinsically disordered region
LLPS	Liquid-liquid phase separation
MAYV	Mayaro virus
MNV	Murine norovirus
MOI	Multiplicity of infection
NLS	Nuclear localization signal
nsP	Non-structural protein
ONNV	O'nyong nyong virus
ORF	Open reading frame
pi	Post infection
RRV	Ross river virus
SAV	Salmonid alphavirus
SFV	Semliki forest virus
SG	Stress granule
sgRNA	Subgenomic RNA

SINV	Sindbis virus
ss(+)RNA	Positive-sense single-stranded RNA
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis
WEEV	Western equine encephalitis virus
WT	Wild-type
ZBD	Zinc-binding domain

1 INTRODUCTION

1.1 A REVISED VIEW ON VIRUSES AND WHY WE STUDY THEM

What is the first thing that comes into our mind when we think about viruses? Is it AIDS, Ebola, smallpox, the Spanish flu, or rinderpest? We've been confronted with the devastating outcome of these diseases and naturally consider viruses primarily a serious health threat, for humans and animals. Consequently, much of our research in virology has been devoted to the development of antiviral treatments and vaccines. In light of this, we might not be aware that most viruses are in fact not harmful to us. The human body is constantly inhabited by trillions of viruses, even in the absence of clinical symptoms (Haynes and Rohwer, 2011). In many cases, viruses just pass the human body without even causing infection. As a result of the evolutionary coexistence with viruses, viral sequences have even become a normal component of the human genome. Approximately 8% of the human genome contains sequences, known as endogenous retroviruses (ERVs), that are related to retroviral DNA sequences (Lander et al., 2001). Some ERV products have been co-opted and serve cellular roles, for example during the morphogenesis of the mammalian placenta (Chuong, 2018).

Some viruses may just be tolerated and are kept under immunological surveillance. However, viruses can also provide clear benefits. The adherence of bacteriophages to mucosal surfaces can reduce bacterial colonization and pathology, which provides enhanced antimicrobial defense to the metazoan host (Barr et al., 2013). In plants, some viruses have been shown to confer improved drought tolerance to the plant they infect (Xu et al., 2008). The mutualistic association of the fungus *Curvularia protuberate* and the plant *Dichanthelium lanuginosum* allows both organisms to grow at elevated temperatures, but only when the fungus is infected with a virus (Marquez et al., 2007). If the virus is cleared from the fungus, both the fungus and the plant are more susceptible to heat stress. A very close mutualistic virus-host relationship can be observed between polydnaviruses and endoparasitoid wasps. Polydnavirus genes involved in viral replication and particle formation have been incorporated into the wasp genome (Bezier et al., 2009). The virions produced by the wasp are used to package wasp genes. When the parasitoid wasp lays its eggs inside a lepitopteran caterpillar, it also deposits polydnavirus particles along with the egg. In the caterpillar, the wasp genes, carried by the virions, are expressed and suppress the caterpillar's immune response. This suppression is required for the survival of the wasp eggs (Edson et al., 1981).

Virus replication must occur inside host cells. Studying the viral life cycle is thus ultimately linked to studying the host itself. Much of our knowledge about cellular functions originates from work on viral infections. For example, our current understanding of eukaryotic DNA replication by the cellular machinery is based on studies of simian virus 40 (SV40) DNA synthesis. Work on the processing of viral RNAs provided fundamental information about mRNA splicing, capping, polyadenylation, and nuclear export. Our understanding of protein synthesis and translation initiation mechanisms has benefited considerably from studies of virus-infected cells. Furthermore, viral membrane glycoproteins proved useful to study the cellular secretory pathway. In some cases, virology may even provide applications of unanticipated scale. For instance, phage therapy, the therapeutic use of bacteriophages to treat bacterial infection, bears great potential in our fight against multi-drug-resistant bacteria. Viral vectors are used in gene therapy for the treatment of various cancers, as well as neurodegenerative and metabolic diseases. In addition, studies on antiviral immune responses in prokaryotes led to the discovery of the CRISPR/Cas system, which has been developed into a technology that revolutionized gene-editing.

Overall, studying the concepts of viral infections has a lot more to it than the mere understanding of a pathogen.

1.2 ALPHAVIRUSES

1.2.1 Background

Alphaviruses are positive-sense single-stranded RNA (ss(+)RNA) viruses and belong to the *Togaviridae* virus family, which are classified into group IV of the Baltimore system (Chen et al., 2018). This genus comprises over 30 species, including several relevant human pathogens (e.g. chikungunya virus (CHIKV) and Ross River virus (RRV)) and veterinary pathogens (e.g. Venezuelan, Western, and Eastern equine encephalitis virus (VEEV, WEEV, EEEV)). Alphaviruses are distributed worldwide and geographically distinguished into ‘Old World’ and ‘New World’ alphaviruses. Most alphavirus species are transmitted to a variety of vertebrate hosts, including humans, via mosquitoes. Exceptions include Eilat virus (Nasar et al., 2012), which is restricted to the insect host, and salmon pancreas disease virus (salmonid alphavirus, SAV), which appears to be transmitted horizontally (Kongtorp et al., 2010). In the mosquito host, alphaviruses establish asymptomatic and persistent infections, whereas infections in the vertebrate host are acute and can be associated with severe disease. Clinically, alphaviruses can

be distinguished into those that cause encephalitis (predominantly New World alphaviruses) and those that typically cause arthritic disease (predominantly Old World alphaviruses).

The work presented in this thesis focuses on the Old World alphaviruses Semliki Forest virus (SFV) and CHIKV. SFV was first isolated in 1942 from mosquitoes captured in the Semliki Forest in Uganda (Smithburn and Haddow, 1944). All SFV strains are highly neurovirulent in neonatal mice (Allsopp and Fazakerley, 2000; Fazakerley, 2002), but the outcome of infection in adult mice depends on the virus strain (Fazakerley et al., 1993; Fragkoudis et al., 2009). While SFV L10 and SFV4 are virulent in mice of all ages, SFV A7(74) is avirulent in adult mice. Symptoms in humans are, however, less severe (Mathiot et al., 1990) and to date, only one fatal human case has been reported (Willems et al., 1979). CHIKV on the other hand is a re-emerging human pathogen and has gained major public attention within the recent years due to global outbreaks with millions of cases, reviewed in (Morrison, 2014; Weaver and Forrester, 2015; Zeller et al., 2016). CHIKV was first isolated in 1953 in Tanzania; the word chikungunya describes a 'disease that bends up the joints' (Ross, 1956), which refers to the severe joint pain associated with the disease in addition to other symptoms like fever and rash, reviewed in (Schwartz and Albert, 2010). Since its discovery, cases of chikungunya fever have sporadically been reported in Africa and Asia. In 2004, a large outbreak occurred in Kenya, from where it spread to several islands in the Indian Ocean. Isolates from an outbreak on La Réunion Island in 2005 contained an alanine-to-valine mutation in the E1 glycoprotein (A226V) (Schuffenecker et al., 2006). Later it was shown, that this mutation confers increased dissemination rates in *Aedes albopictus* mosquitoes and consequently enhanced transmission by this vector, whereas the original genotype is primarily transmitted by *Aedes aegypti* mosquitoes (Vazeille et al., 2007). After 32 years of absence in India, CHIKV reappeared in 2005, from where it continued spreading to the rest of Asia, Europe, including France, Spain, Italy, and Croatia, and the Americas. Other alphaviruses that are of medical relevance are also emerging. To date, no vaccines or antiviral therapies are available for any alphavirus infection. However, several vaccine candidates are in clinical trials (Erasmus et al., 2016) and development of antivirals and neutralizing antibodies is ongoing (da Silva-Junior et al., 2017; Jin et al., 2015).

1.2.2 Genome and virion structure

The alphavirus genome has a length of about 10 to 12 kb (Figure 1) (Chen et al., 2018). 5′- and 3′-terminal non-coding regions flank two open reading frames (ORF), which are separated by

an internal non-coding region. The 5′ ORF encodes the non-structural proteins, translated directly from the genomic RNA. The 3′ ORF encodes the structural proteins, which are expressed from the subgenomic RNA. The subgenomic RNA is transcribed via a subgenomic promoter from a negative-strand RNA intermediate (see Figure 3 and chapter 1.2.4. for details). Both positive-strand viral RNAs, genomic and subgenomic, contain a 5′ 7-methylguanosine cap and a 3′ poly(A) tail (Ou et al., 1981; Pettersson et al., 1980).

Figure 1: Alphavirus genome. Alphaviruses contain a single-stranded positive-sense RNA genome, with 5′ cap and 3′ poly(A) tail. Two open reading frames code for the non-structural proteins (nsP) 1–4 and the structural proteins (Capsid (C), envelope glycoproteins (E1–3), a 6 kDa protein (6K) and the transframe protein (TF)). TF is an 8-kDa protein, generated through C-terminal extension of 6K after a frameshift event. m^7G—7-methylguanosine; UTR—untranslated region; ORF—open reading frame; SGP—subgenomic promoter; Poly(A)—polyadenylation; adapted from (Götte et al., 2018)

The alphavirus virion is about 70 nm in diameter and consists of the viral RNA genome, surrounded by a capsid shell, and a lipid bilayer with embedded glycoproteins (Figure 2), reviewed in (Jose et al., 2009). The viral genome is encapsidated by 240 copies of the capsid protein. The virion surface is covered by 240 heterodimers of the transmembrane glycoproteins E1 and E2, which assemble into 80 trimeric spikes. The C-terminal endodomain of E2 directly interacts with the nucleocapsid core (Skoging et al., 1996; Tang et al., 2011). The nucleocapsid core and the spike proteins on the surface give the alphavirus particle an icosahedral symmetry.

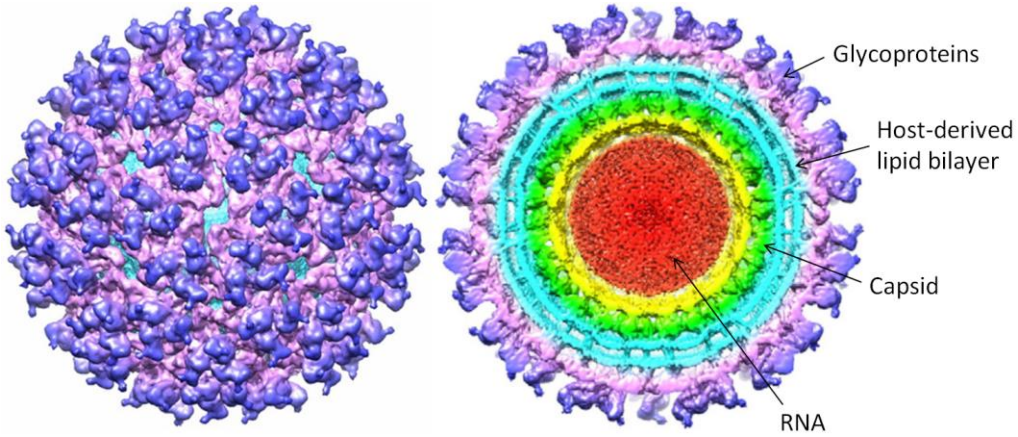

Figure 2: Three-dimensional image reconstruction of Sindbis virus obtained by electron cryo-microscopy at 7 Å resolution. PDB ID: 3J0F; modified from (Tang et al., 2011).

1.2.3 Life cycle

Alphavirus entry into the host cell is initiated by receptor-mediated endocytosis (Figure 3). The entry receptor varies for different alphaviruses. For Sindbis virus (SINV), natural resistance-associated macrophage protein (NRAMP/NRAMP2) was identified as the insect and mammalian host cell receptor (Rose et al., 2011). In contrast, the entry of several other alphaviruses, including CHIKV, RRV, Mayaro virus (MAYV) and O'nyong nyong virus (ONNV) was recently found to be mediated by matrix remodeling-associated protein 8 (Mxra8) (Zhang et al., 2018). Nevertheless, experimental data suggest that additional, yet unidentified host factors contribute to cell binding and entry. After receptor binding, viral particles are internalized through clathrin-mediated endocytosis (DeTulleo and Kirchhausen, 1998). The clathrin coat is subsequently removed and the virus-containing vesicles enter the early endosomal compartment. An ATP-dependent proton pump in the endosomal membrane, termed the vacuolar ATPase, increasingly lowers the pH within the endosomal lumen (Mellman et al., 1986). Acidification of the endosome causes dissociation of the E2/E1 spike and exposure of E1's hydrophobic fusion loop, which triggers membrane fusion and E1 homotrimer formation (Hammar et al., 2003; Wahlberg et al., 1992; Wahlberg and Garoff, 1992). Once the nucleocapsid is released into the cytoplasm, it is disassembled, facilitated through interactions with ribosomes, which liberates the viral genomic RNA (Singh and Helenius, 1992; Wengler et al., 1992). The 5′ ORF of the viral genome is then directly translated into the non-structural polyprotein P1234, which is sequentially processed into the individual non-structural proteins (nsP) 1, 2, 3 and 4. The nsPs form the viral replicase. A more comprehensive description of the functions of the nsPs is given in chapter 1.2.5.

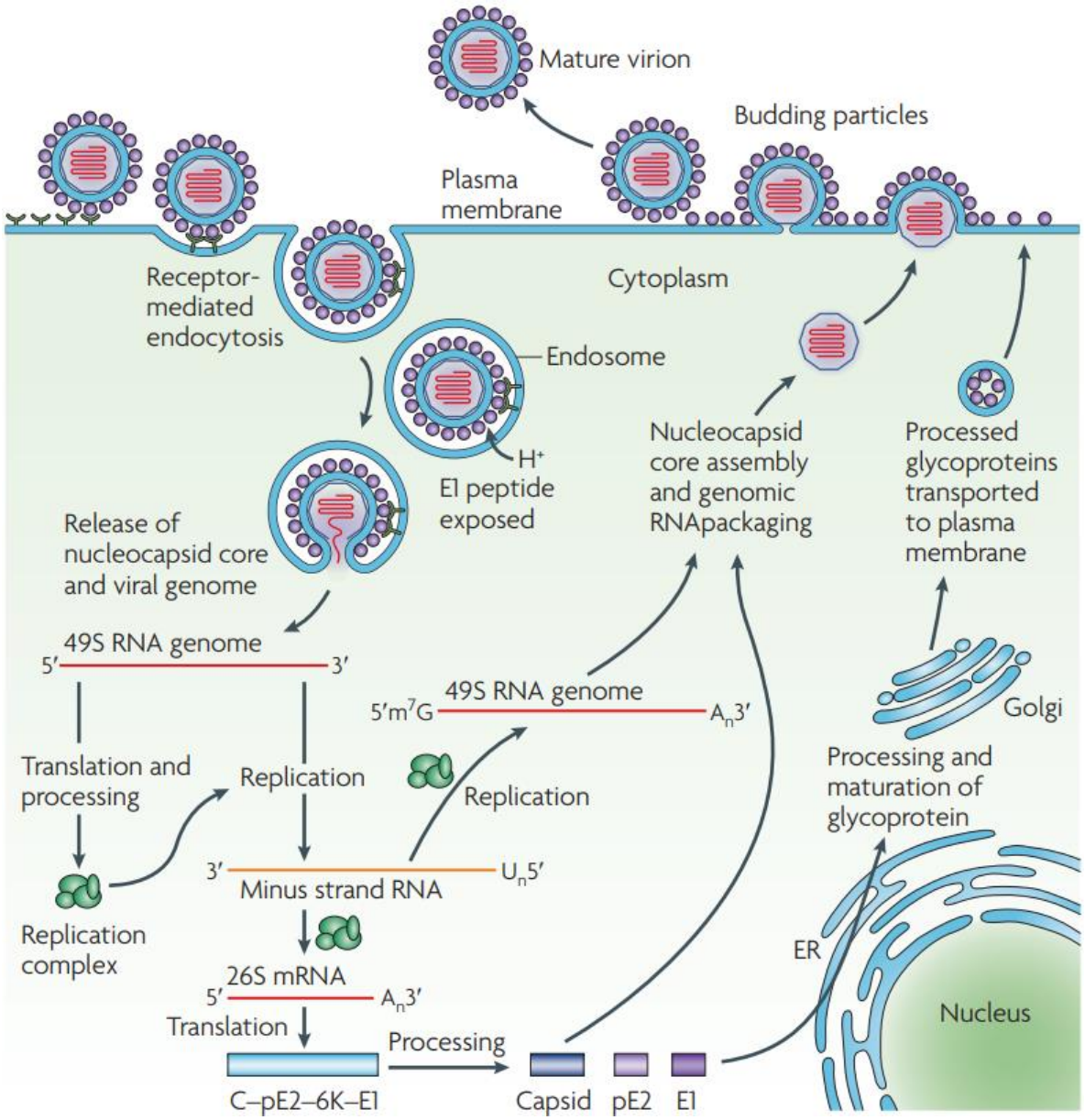

Figure 3: Alphavirus life cycle. Alphaviruses enter the host cell through receptor binding and endocytosis. Acidification of the endosome leads to virus-host membrane fusion and release of the nucleocapsid and viral genome into the cytoplasm. The viral genome is directly translated to produce the non-structural polyprotein, which is sequentially processed into the individual non-structural proteins that form the viral replicase. The replicase assembles into a viral replication complex, which synthesizes a full-length negative-strand RNA intermediate, complementary to the viral genome. The negative-strand RNA serves as template for the synthesis of genomic (42–49S) and subgenomic (26S) RNAs. The subgenomic RNA is translated to produce the structural polyprotein, which is processed into the individual structural proteins. The capsid is autocatalytically cleaved and binds viral genomes to form nucleocapsids. The remaining structural proteins are transported to the plasma membrane via the secretory pathway. Virus particles then assemble at and bud from the plasma membrane. Figure reprinted with permission from Springer Nature: Nature Reviews Microbiology (Schwartz and Albert, 2010).

The non-structural proteins then associate with the plasma membrane (PM) where they form replication complexes (spherules) (Frolova et al., 2010; Spuul et al., 2010) (Figure 4). Spherules are bulb-shaped membrane evaginations, which provide a segregated environment for viral RNA replication. Inside these spherules, viral RNA is protected from RNase treatment (Kallio et al., 2016; Pietilä et al., 2018). The spherule lumen is connected to the cytoplasm via a pore, through which newly-synthesized RNA is believed to be released (Froshauer et al., 1988). For SFV, but not CHIKV, the replication complexes are internalized during infection and transported in a phosphatidylinositol 3-kinase- (PI3K-), actin-, and microtubule-dependent manner to the perinuclear region, where they mature into type I cytopathic vacuoles (CPV), a modified endolysosomal compartment (Froshauer et al., 1988; Spuul et al., 2010; Thaa et al., 2015).

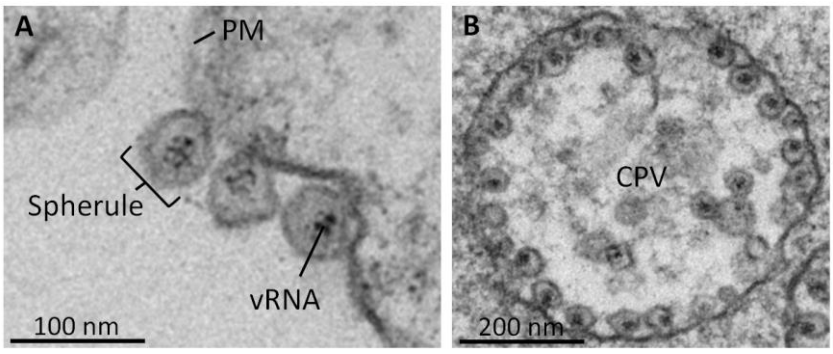

Figure 4: Electron micrographs of SFV-induced spherules and cytopathic vacuoles (CPV). (A) Alphavirus replication induces the formation of replication complexes, termed spherules, at the PM. **(B)** For SFV, spherules are internalized during infection to form cytopathic vacuoles (CPV) in the perinuclear area. PM—plasma membrane; vRNA—viral RNA

During viral RNA replication, a subgenomic RNA is synthesized (see Figure 3 and chapter 1.2.4. for details). Translation of the subgenomic RNA produces the structural polyprotein. The capsid protein is the first to be translated, and autocatalytically and co-translationally cleaves itself from the polyprotein (Nicola et al., 1999). Capsid proteins then package genomic viral RNA into nucleocapsids (Frolova et al., 1997; White et al., 1998). The remaining structural polyprotein is translocated into the endoplasmic reticulum (ER), where it is processed by a signal peptidase. The individual structural proteins are post-translationally modified as they are transported through the secretory pathway to the plasma membrane (Ivanova and Schlesinger, 1993; Mulvey and Brown, 1994; Pletnev et al., 2001). An E3/E2 precursor, termed p62 in SFV (and pE2 in SINV), is not immediately cleaved and remains associated with E1. This prevents

that E1 is activated for membrane fusion at the low pH of the trans-Golgi network (Sjöberg et al., 2011; Wahlberg et al., 1989). p62 is eventually cleaved by the protease furin at a late stage of the secretory pathway, producing mature E2 and E3 proteins (Zhang et al., 2003). At the neutral pH of the extracellular milieu, E3 dissociates, which primes E2/E1 dimers for low-pH-triggered structural rearrangements and hence, activation for fusion and infection (Sjöberg et al., 2011; Wahlberg et al., 1989). Budding of viral particles takes place at the plasma membrane and depends on the interaction of the capsid protein with the endodomain of E2 (Zhao et al., 1994). A (−1) frameshift event during translation of the 6K protein results in a C-terminal extension of 6K, termed transframe protein (TF) (Firth et al., 2008). Both, 6K and TF have been implicated to facilitate budding of viral particles, but their precise functions are not known, as reviewed in (Ramsey and Mukhopadhyay, 2017).

Infection with alphaviruses is accompanied by drastic remodeling of the host cell's cytoskeleton. This process involves formation of intercellular extensions, which contact neighboring cells, but do not fuse with them (Martinez and Kielian, 2016). Expression of the structural proteins alone is sufficient to induce the formation of these extensions, which have been shown to promote cell-to-cell transmission of alphaviruses and may also help the virus to hide from neutralizing antibodies (Lee et al., 2011).

1.2.4 RNA replication

During viral RNA replication, a negative-strand RNA is synthesized, complementary to the genomic RNA, which serves as the template for the production of full-length genomic RNA (gRNA, 42–49S RNA) and for the transcription of a subgenomic RNA (sgRNA, 26S RNA) from an internal promoter. The gRNA serves for translation of the non-structural polyprotein P1234 or is packaged by capsid proteins into nucleocapsids. The sgRNA is not incorporated into virus particles and is translated to produce the structural polyprotein.

Replication and transcription of the viral RNA requires the combined action of all four non-structural proteins (nsPs). Translated as a polyprotein, P1234 is processed into the four mature nsPs (nsP1–4) by the nsP2 protease domain (Kim et al., 2004; Vasiljeva et al., 2001). The three cleavage sites within the polyprotein are processed in a precise sequential order, which regulates the synthesis of the different viral RNA species (Kim et al., 2004; Lemm et al., 1994; Shirako and Strauss, 1994). The first cleavage occurs between nsP3 and nsP4 (3/4 cleavage site), which activates the RNA-dependent RNA polymerase (RdRp) catalytic core of nsP4 (Tomar et al., 2006). The P123-nsP4 replicase predominantly synthesizes full length negative-

strand RNA, complementary to gRNA, whereas synthesis of gRNA and sgRNA remains inefficient. Next, cleavage at the 1/2 site (between nsP1 and nsP2) generates the nsP1-P23-nsP4 replicase, which produces both negative- and positive-strand RNA, but with synthesis of gRNA exceeding synthesis of sgRNA. Finally, cleavage at the 2/3 site occurs to generate completely processed replicase (nsP1-nsP2-nsP3-nsP4), which predominantly synthesizes sgRNA and is incapable of producing negative-strand RNA. As a result, negative-strand RNA synthesis depends on the continued translation of replicase polyproteins (Sawicki and Sawicki, 1980). However, negative-strand RNA synthesis ceases 3–4 hours post infection (pi), which correlates with the increasing levels of nsP2, leading to more rapid polyprotein processing (Vasiljeva et al., 2003). The regulation of RNA replication ensures, that early in infection, vast amounts of gRNA is produced. At a later stage of infection, gRNA is packaged into viral particles, driven by the massive production of structural proteins.

The correct temporal regulation of polyprotein processing is crucial for successful viral replication (Lulla et al., 2018; Strauss et al., 1992). For example, an amino acid substitution in SINV nsP2 (N614D), which is associated with enhanced protease activity, strongly attenuates viral growth. However, this can be compensated for by changing residues at the 1/2 site to be less optimal for protease recognition, which partially rescues the growth defect. For SFV, temporal regulation of polyprotein processing is a major determinant for neurovirulence in mice (Saul et al., 2015). SFV6 (a consensus clone of the L10 strain) and SFV A774 (a consensus clone of the A7(74) strain) differ in their polyprotein processing efficiencies, particularly at the 1/2 site, which can be attributed to different amino acids at positions 534 of nsP1 and 515 of nsP2. The 1/2 site of SFV6 is processed relatively slow and it is highly virulent in adult mice. For SFV A774, on the contrary, the 1/2 site processing occurs relatively fast and it is avirulent in adult mice. When the two processing-associated residues of SFV A774 are substituted with the respective residues naturally present in SFV6, the mutant SFV A774 shows decreased polyprotein processing, associated with strongly enhanced neurovirulence. SFV6 and SFV A774 are described more thoroughly and experimentally examined in paper III.

The alphavirus genome contains four conserved sequence elements (CSEs) which are important for RNA replication. The 5′ UTR contains a CSE which is essential for both negative- and positive-strand RNA synthesis (Frolov et al., 2001; Gorchakov et al., 2004). Based on this observation, it has been proposed that initiation of RNA replication requires the 5′ and 3′ ends to interact. RNA synthesis is furthermore enhanced by a 51-nucleotide CSE within the nsP1-coding region. A CSE covering the 3′ end of the nP4 coding region and part of the junction region between the two ORFs acts as the subgenomic promoter, which is only active in the

negative-strand RNA (Wielgosz et al., 2001). Another short CSE in the 3′ UTR and the poly(A) tail are considered to form the core promoter for negative-strand RNA synthesis (Hardy and Rice, 2005).

1.2.4.1 Trans-replication system

The viral replicase can efficiently replicate a positive-strand template provided *in trans*, which led to the development of trans-replication systems for alphaviruses (Spuul et al., 2011; Utt et al., 2016; Utt et al., 2019). During virus replication, replicase expression is coupled to RNA replication. Mutations negatively affecting RNA replication consequently also reduce replicase expression and often result in reversions or second-site mutations. In a trans-replication system, the replicase and its template are expressed from separate plasmids. The replication-independent replicase expression guarantees genetic stability, which allows the robust analysis of replicase manipulations, including substitutions, deletions, and insertions. Another advantage of this system is that the co-expressed template can be modified to code for reporter proteins, such as luciferases, which allows very sensitive measurement of RNA replication and transcription. A trans-replication system for SFV and CHIKV was applied in paper III.

1.2.5 Non-structural proteins

Independent of their role as an integral part of the viral replicase, the individual nsPs fulfill additional functions during the viral life cycle, which are described in this section. Many alphaviruses, e.g. SINV and RRV, produce two variants of the non-structural polyproteins. A leaky opal stop codon at the C-terminal end of nsP3 limits the production of nsP4, leading to the predominant production of P123 and to a lesser amount P1234 (Lopez et al., 1985; Strauss et al., 1988). In some strains of CHIKV, ONNV and SFV the opal codon is replaced by an arginine codon and only P1234 is produced (Chen et al., 2013; Strauss et al., 1988; Takkinen, 1986). The presence of either an opal or an arginine codon can provide a host-specific fitness advantage (Myles et al., 2006) and is known to influence virulence *in vivo* (Jones et al., 2017; Tuittila et al., 2000).

1.2.5.1 Non-structural protein 1 (nsP1)

nsP1 (~60 kDa) possesses methyltransferase and guanylyltransferase activity, required for the synthesis of the 5′ cap structures on the genomic and subgenomic viral RNAs (Ahola and Kääriäinen, 1995; Mi and Stollar, 1991; Pettersson et al., 1980). During the addition of the 5′ cap structure, nsP1 catalyzes the transfer of a methyl group from S-adenosylmethionine to a

GTP molecule, which, after hydrolysis, yields m^7GMP. The 5′ γ-phosphate on the nascent viral RNA is removed by the triphosphatase activity of nsP2 (Vasiljeva et al., 2000) and the m^7GMP moiety is transferred to the 5′ end by the guanylyltransferase activity of nsP1, resulting in 5′ cap structure (m^7Gppp) formation.

All positive-strand RNA viruses replicate their genomes in association with cellular membranes (Belov and van Kuppeveld, 2012). For alphaviruses, nsP1 functions as the membrane anchor for the viral replicase. The membrane association of nsP1 is facilitated by an amphipathic helix and through palmitoylation (Ahola et al., 2000; Spuul et al., 2007). The amphipathic helix is crucial for the membrane association of nsP1 and therefore the entire replication complex (Spuul et al., 2007). Mutations that interfere with membrane-binding also block palmitoylation of nsP1 and are lethal for SFV replication. Palmitoylation of nsP1 occurs at specific cysteine residues. Mutations, preventing palmitoylation, still allow for membrane association of nsP1, but with lower affinity (Laakkonen et al., 1996). Conversely, while nsP1 palmitoylation-deficient viruses still replicate to high titres in cell culture, SFV with non-palmitoylated nsP1 is apathogenic in mice (Ahola et al., 2000). Based on these observations, it has been proposed that the amphipathic helix of nsP1 mediates the initial membrane attachment of the replicase complex, which is then tightened through nsP1 palmitoylation.

1.2.5.2 Non-structural protein 2 (nsP2)

nsP2 (~90 kDa) possesses three activities that are critical for viral replication, namely NTPase/helicase, triphosphatase, and protease activity. The helicase activity resides within the N-terminal region and is required for unwinding of RNA duplexes during RNA replication (Gomez de Cedron et al., 1999). NTP hydrolysis (NTPase activity) provides the required energy for the motor activity of the helicase (Karpe et al., 2011). The N-terminal region of nsP2 also contains (RNA-dependent 5′-) triphosphatase (RTPase) activity, which, as mentioned above, is involved in the formation of the 5′ cap structure of the viral positive-strand RNAs (Karpe et al., 2011; Vasiljeva et al., 2000). Since mutations that negatively affect the NTPase activity also reduce the RTPase activity, it is likely that both activities depend on the same active site within nsP2 (Karpe et al., 2011).

The C-terminal domain of nsP2 contains the papain-like cysteine protease which is required for the processing of the non-structural polyprotein and essential for RNA replication (Kim et al., 2004; Vasiljeva et al., 2001). The protease recognizes specific amino acid residues surrounding the cleavage site. Of particular importance are the positions −4 (N-terminal) to +1 (C-terminal) with respect to the cleavage site, referred to as P4 to P1′ (Lulla et al., 2006). The

protease activity is tightly regulated and linked to the cleavage site sequences. Mutations within the cleavage sites that artificially accelerate cleavage rates of the SFV4 non-structural polyprotein were found to strongly impair infectivity and eventually led to the emergence of compensatory mutations (Lulla et al., 2018). Among these, a compensatory substitution within the nsP2 protease domain was found (V515M), which restored the infectivity by decreasing nsP2 activity, thereby normalizing cleavage rates. Nevertheless, it should be noted that the macromolecular architecture of the replicase complex is another factor that likely influences the processing of the non-structural polyprotein by nsP2 (Lulla et al., 2012; Lulla et al., 2013; Shin et al., 2012).

Alphaviruses interfere with the innate antiviral immune response of vertebrate cells by inhibition of cellular transcription (Garmashova et al., 2007). For Old World alphaviruses, this function is mediated by the nsP2-dependent ubiquitination and subsequent degradation of Rpb1, a catalytic subunit of the DNA-directed RNA polymerase II (Akhrymuk et al., 2012). nsP2 contains a nuclear localization signal (NLS) which targets a portion of the fully processed protein for translocation into the nucleus, which is required for the transcriptional shutoff (Gorchakov et al., 2005; Rikkonen et al., 1992).

1.2.5.3 Non-structural protein 3 (nsP3)

nsP3 (~60 kDa) is subdivided into three domains, the N-terminal macro domain, the central zinc-binding or alphavirus-unique domain and the C-terminal hypervariable domain (Figure 5Figure 5).

Figure 5: Domain structure of SFV nsP3. nsP3 can be subdivided into three domains, the N-terminal macro domain, the central alphavirus-unique (AUD) or zinc-binding (ZBD) domain and the C-terminal hypervariable domain (HVD). The hyperphosphorylated region is shown in orange. Green bars indicate the proline-rich regions that mediate binding to amphiphysin-1 and -2 and red bars highlight the positions of the two 'FGDF' motifs, the binding sites for the cellular protein G3BP. aa—amino acids.

Macro domains can be found in viral, bacterial and cellular proteins (Koonin et al., 1992; Rack et al., 2016). This domain is well conserved among alphaviruses and structural data are available for MAYV, VEEV, and CHIKV (Malet et al., 2009; Tsika et al., 2019). The alphavirus macro domain is capable of binding and hydrolyzing monomeric ADP-ribose

(MAR) and poly(ADP-ribose) (PAR) from modified target proteins (Eckei et al., 2017; Li et al., 2016; Neuvonen and Ahola, 2009). For CHIKV it was shown that ADP-ribosylhydrolase activity is absolutely critical for CHIKV replication in vertebrate and mosquito cells (Abraham et al., 2018; McPherson et al., 2017). ADP-ribosylation is a post-translational modification, catalyzed by ADP-ribosyltransferases (also known as the PARP family). The expression of several PARPs is stimulated by interferon and known to inhibit alphavirus replication (Atasheva et al., 2012; Atasheva et al., 2014; Eckei et al., 2017). It is therefore believed that viral macro domains counteract ADP-ribosylation to interfere with an antiviral host response.

The alphavirus-unique domain (AUD) contains four conserved cysteine residues, which together coordinate a zinc ion (Shin et al., 2012). Each of the respective cysteines is essential for virus replication. This domain is therefore also referred to as the zinc-binding domain (ZBD). Mutational studies have shown that this domain plays an important role for subgenomic RNA synthesis and binds viral RNA (Gao et al., 2019).

As the name implies, the hypervariable domain (HVD) shows high variability among alphaviruses with respect to sequence and length. In contrast to the macro and AUD/ZBD domains, the HVD is largely intrinsically disordered, a feature commonly associated with the ability to bind multiple partners (Meshram et al., 2018; Uversky, 2013). Indeed, an accumulating amount of data suggest that nsP3 functions as a hub for multiple interactions with host proteins, mediated in particular via its HVD, reviewed in (Götte et al., 2018). For example, a proline-rich region located within the HVD facilitates binding to the SH3 domain of amphiphysin proteins (Figure 5) (Neuvonen et al., 2011). Other SH3 domain-containing proteins (CD2AP and SH3KBP1) also interact with nsP3, but bind to a region adjacent to the proline-rich motif (Mutso et al., 2018). The functional significance of these interactions, however, has not been elucidated.

Some alphaviruses, such as SFV and RRV, contain a YXXM motif in the HVD of nsP3. This motif facilitates binding to the SH2 domain of the regulatory subunit p85 of phosphatidylinositol 3-kinase (PI3K), followed by hyperactivation of the anti-apoptotic and proliferation-promoting PI3K-Akt-mTOR pathway (Mazzon et al., 2018). For SFV and RRV, hyperactivation of this pathway leads to internalization of viral replication complexes from the plasma membrane (Thaa et al., 2015). On the contrary, for CHIKV, which lacks the YXXM motif, activation of this pathway is much weaker and replication complexes remain predominantly associated with the PM.

The best characterized nsP3-host interaction is its interaction with the cellular stress granule (SG) protein G3BP. SGs and G3BP will be described in detail in chapter 1.3 and 1.4, respectively. Upon binding of nsP3, G3BP is recruited to viral replication complexes and cytoplasmic foci, which correlates with the disassembly of infection-induced G3BP-positive SGs (Panas et al., 2015; Panas et al., 2012). In the absence of the nsP3:G3BP interaction, SFV replication is attenuated in cell culture, and the SG-response is prolonged. In contrast, CHIKV strictly depends on G3BP and is essentially non-viable in its absence (Kim et al., 2016; Schulte et al., 2016). The importance of G3BP is also conserved in the insect host and knockdown of the G3BP-homolog Rasputin in *Aedes albopictus* mosquitoes impairs CHIKV replication (Fros et al., 2015). nsP3 of most Old World alphaviruses binds to G3BP via an FGDF (Phe-Gly-Asp-Phe) motif, located within the C-terminal region of the HVD (Figure 5) (Panas et al., 2015). Some Old World alphaviruses, such as SFV, CHIKV, and ONNV, contain two FGDF motifs, others, like MAYV and RRV, contain only one motif. New World alphaviruses, on the contrary, do not interact with G3BP, but instead, the nsP3 HVD recruits another set of SG-associated proteins, i.e. members of the FXR protein family (Kim et al., 2016). The New World alphavirus EEEV is the only reported example to bind both FXR and G3BP proteins (Frolov et al., 2017). EEEV nsP3 binds G3BP also via its NTF2-like domain, however, the G3BP-binding sequence within the nsP3 HVD is distinct from the FGDF motif of Old World alphaviruses.

nsP3 is the only phosphorylated non-structural protein. A deletion within the HVD of SFV nsP3, which completely abolishes the phosphorylation, does not negatively affect production of infectious virus in cell culture, but is associated with greatly reduced neurovirulence in infected mice (Vihinen et al., 2001).

Most alphaviruses are transmitted to a vertebrate host by mosquitoes. ONNV is the only alphavirus known to be transmitted by anopheline mosquitoes. In contrast, CHIKV is primarily transmitted by *Aedes* mosquitoes, but cannot establish infection in *Anopheles gambiae*. nsP3 plays a particular role in determining this vector specificity, as a chimeric CHIKV, in which CHIKV nsP3 was replaced by that of ONNV, restored infection in the *Anopheles* host (Saxton-Shaw et al., 2013). nsP3 may execute such cell type-dependent functions through specific protein-protein interactions.

In mice, SFV infection is mostly associated with neurovirulence, another characteristic linked to nsP3 (Tuittila and Hinkkanen, 2003). As mentioned above, SFV A774 is an exception and infection is avirulent in adult mice. However, replacement of the nsP3 region with that of SFV4 or SFV6 introduces lethal neurovirulence (Saul et al., 2015; Tuittila et al., 2000). It should be

noted that nsP3 is not the exclusive determinant for virulence since other components, such as the structural protein E2, also contribute to virulence (Atkins and Sheahan, 2016; Santagati et al., 1995).

1.2.5.4 Non-structural protein 4 (nsP4)

All alphaviruses contain a Tyr at the N-terminus of nsP4 (~70 kDa) and mutation of this residue results in RNA replication defects (Shirako and Strauss, 1998). The Tyr residue has, however, also a destabilizing effect, leading to degradation of nsP4 by the ubiquitin-dependent N-end rule pathway (de Groot et al., 1991). The N-terminal region is predicted to be disordered and has been implied to interact with all three other nsPs to activate replicase activity (Rupp et al., 2011). It has been proposed that these interactions vary during viral RNA replication to mediate synthesis of the different RNA species. nsP4 also possesses the core RdRp activity with the signature GDD (Gly-Asp-Asp) motif, but RNA synthesis activity relies on the presence of the remaining replicase proteins P123 (Rubach et al., 2009; Tomar et al., 2006). In addition, nsP4 possesses terminal adenylyltransferase (TATase), which catalyzes polyadenylation at the 3′ end of an acceptor RNA *in vitro* (Tomar et al., 2006). If this activity is responsible for polyadenylation of viral RNA *in vivo* remains to be verified.

1.3 STRESS GRANULES

SGs are non-membranous dynamic aggregates of translationally silent messenger ribonucleoproteins (mRNPs), reviewed in (Anderson and Kedersha, 2008; Protter and Parker, 2016). SGs are proposed to mediate a variety of stress responses through regulation of host translation, RNA triage, and cellular signaling. Their assembly is triggered by a variety of cellular stresses, such as heat shock, oxidative stress or viral infection. A stress-response cascade then induces phosphorylation of translation initiation factor eIF2α (eIF2α-P), thereby reducing levels of the eIF2-GTP-$tRNA_i^{Met}$ ternary complex, which results in inhibition of global protein translation and polysome disassembly. eIF2α can be phosphorylated by several stress-specific kinases. These include protein kinase R (PKR), which is activated by double-stranded RNA during viral infection (Lemaire et al., 2008), PKR-like endoplasmic reticulum kinase (PERK), activated by ER stress (Marciniak et al., 2006), general control nonderepressible 2 (GCN2), activated by amino acid deprivation (Wek et al., 1995), and heme-regulated eIF2α kinase (HRI), which senses oxidative stress (McEwen et al., 2005).

SGs are in dynamic equilibrium with polysomes. Drugs that cause polysome disassembly, like puromycin, promote SG assembly, whereas drugs that stabilize polysomes, like emetine or

cycloheximide, inhibit SG assembly (Kedersha et al., 2000). SGs are predominantly composed of stalled preinitiation complexes, derived from disassembling ribosomes, and are therefore enriched in mRNA, small (40S) ribosomal subunits and various initiation factors (Anderson and Kedersha, 2009). Additional RNA-binding and non-RNA-binding proteins are recruited to SGs in a cell-type- and stress-dependent manner. RNA-binding proteins, such as G3BP (Tourriere et al., 2003), TIA-1/TIAR (Gilks et al., 2004), and FMRP/FXR1 (Mazroui et al., 2006) promote SG assembly, and overexpression of these proteins induces SG assembly even in the absence of stress (Kedersha and Anderson, 2007). It is believed that SGs form through liquid-liquid phase separation (LLPS), driven by a network of weak interactions between intrinsically disordered regions (IDRs) (Lin et al., 2015; Molliex et al., 2015). Many RNA-binding proteins present in SGs contain IDRs.

Several neuro- or muscular degenerative diseases are linked to mutations that affect SG dynamics, reviewed in (Li et al., 2013; Ramaswami et al., 2013). Such mutations often increase the self-assembly properties of SG proteins or interfere with the clearance of SGs, suggesting that persistence of SGs leads to cellular degeneration.

1.3.1 Stress granules and viral infection

PKR plays a key role in the detection of viral replication and subsequent SG formation via phosphorylation of eIF2α. Influenza A virus (IAV) circumvents PKR-mediated SG assembly via sequestration of dsRNA by non-structural protein 1 (NS1) (Khaperskyy et al., 2012; Lu et al., 1995). Infection with a NS1-mutant IAV, which allows PKR activation and SG formation, correlates with a reduction of viral protein synthesis. Infection with hepatitis C virus (HCV) involves oscillating SG formation. PKR-dependent SG assembly and translation arrest is temporarily antagonized by dephosphorylation of eIF2α (Ruggieri et al., 2012).

Upon viral infection, several antiviral proteins, including PKR, RIG-I, OAS, and RNase L are recruited to SGs, which stimulates their activation and hence, induces an innate immune response (Onomoto et al., 2012; Reineke et al., 2015). Additionally, SG formation involves the sequestration of translation components which are essential for translation of viral transcripts. In order to ensure successful replication under conditions of a cellular stress-response, viruses have developed a diverse set of tools to antagonize SGs, reviewed in (Poblete-Duran et al., 2016; Tsai and Lloyd, 2014).

For example, several flaviviruses block SG formation by sequestration of key SG proteins. In the case of West Nile virus, this is facilitated by a stem loop structure in the 3′ UTR of the

negative-strand RNA, which recruits TIA-1/TIAR to viral replication complexes (Emara and Brinton, 2007; Li et al., 2002).

Other viruses induce SG formation early in infection, but then actively disrupt them later in infection. Examples include poliovirus and other picornaviruses that disassemble SGs through cleavage of the SG-nucleating protein G3BP by the viral 3C proteinase, which is associated with reduced interferon production (Ng et al., 2013; White et al., 2007). Old World alphaviruses also target G3BP and disassemble SGs by nsP3-mediated sequestration of G3BP (Panas et al., 2015; Panas et al., 2012).

1.3.2 Stress-resistant translational of alphavirus subgenomic RNA

The sgRNA of some alphaviruses contains a particular sequence element downstream of the start codon, which enhances translation in infected cells (Frolov and Schlesinger, 1994; Sjöberg et al., 1994). It was later shown that this sequence confers resistance to PKR activation and eIF2α phosphorylation, allowing efficient translation, despite shutoff of host translation (McInerney et al., 2005; Ventoso et al., 2006). The translation enhancing element forms a stable RNA stem-loop structure, which has been proposed to facilitate correct positioning of the translation initiation complex on the start codon without the participation of eIF2 (Toribio et al., 2016). Such stem-loop structures within the coding sequence of the sgRNA have been identified in several alphaviruses, including SFV and SINV, but appears to be absent in CHIKV.

1.4 G3BP

G3BP was originally identified as a protein that binds the Ras-GTPase-activating protein's SH3 domain and was therefore named Ras-GAP (SH3-domain)-binding protein or short G3BP (Parker et al., 1996). G3BP is expressed in three isoforms, G3BP1 and two splice variants G3BP2a and G3BP2b (hereafter jointly referred to as G3BP). G3BP1 shares 66 % amino acid identity with G3BP2a and both have a similar domain structure (Figure 6). All G3BPs comprise, from N- to C-terminus, a nuclear transport factor 2 (NTF2)-like domain, an acidic domain, a proline-rich region with several PxxP motifs, an RNA recognition motif (RRM) and an arginine-/glycine-rich domain (RGG).

Figure 6: G3BP domain structure. G3BP contains an N-terminal NTF2-like domain, an acidic domain, a proline-rich region with several PxxP motifs, an RNA recognition motif (RRM), and an arginine-/glycine-rich domain (RGG) at the C-terminal end. Human G3BP1, 2a, and 2b have a length of 466, 482, and 449 amino acids, respectively. Orange underlining indicates intrinsically disordered regions, as predicted by PONDR®, based on the sequence of human G3BP1 (UniProtKB: Q13283).

The main difference between G3BP1 and G3BP2 can be attributed to the number of PxxP motifs, located in the central region. G3BP1 and G3BP2 form homo- and heterodimers via the N-terminal NTF2-like domain, the most conserved part of the protein (Kristensen, 2015; Matsuki et al., 2013; Vognsen et al., 2013). Early studies showed that *in vitro* G3BP possesses helicase activity (Costa et al., 1999) and endoribonuclease activity (Tourriere et al., 2001). Meanwhile, G3BP is best known for its ability to nucleate SGs, and overexpression of either G3BP1 or G3BP2 nucleates SG formation (Matsuki et al., 2013; Tourriere et al., 2003).

G3BP is essential for eIF2α-P-mediated SG formation and several characteristics of G3BP are linked to SG formation (Kedersha et al., 2016). Except for the NTF2-like and RRM domains, G3BP is predicted to be largely intrinsically disordered (Figure 6), a property commonly shared by SG proteins. For SG competence, G3BP requires its C-terminal RGG domain, which binds 40S ribosomal subunits. Therefore, it has been proposed that SG formation is mediated via the RGG-40S interaction. G3BP1 was found to be methylated on arginine residues within the RGG domain, which influences SG competence (Tsai et al., 2016). G3BP1 is demethylated upon oxidative stress and remethylated during stress recovery. Accordingly, increased methylation represses SG assembly and decreased methylation promotes SG assembly. G3BP-mediated SG formation is furthermore regulated by the mutually exclusive binding of the cellular proteins Caprin-1 and USP10 to G3BP. Binding of Caprin-1 promotes SG formation and binding of USP10 has the opposite effect, inhibiting SG formation (Kedersha et al., 2016). USP10 binds the NTF2-like domain of G3BP via a single FGDF motif, the same binding motif found in alphavirus nsP3 (Panas et al., 2015).

G3BP is evolutionarily conserved and homologs have been identified in yeast, insects and plants. The NTF2-like domain structures of human G3BPs and the *Drosophila* homolog Rasputin are very similar (Vognsen and Kristensen, 2012). Like the human homologs, *Drosophila* Rasputin is also recruited to SGs in monkey-derived COS-7 cells (Tourriere et al., 2003). Furthermore, while G3BP is important for replication of CHIKV in mammalian cells,

Rasputin plays an important role for CHIKV infection and transmissibility in *Aedes albopictus* mosquitoes (Fros et al., 2015). G3BP-like proteins have also been described in plants (Krapp et al., 2017). *Arabidopsis thaliana* G3BP-like proteins (AtG3BP) also localize to plant SGs and are targeted by plant virus proteins, which contain FGDF-like motifs. *Nicotiana benthamiana* plants transiently expressing human G3BP show accumulation of human G3BP in granule-like structures upon heat stress, which stain positive for a plant SG marker. Taken together, these observations suggest that at least some functions of G3BP and the molecular mechanisms of SG formation are conserved across distinct eukaryotes.

G3BP is targeted by viruses from diverse virus families, including *Caliciviridae* (Fritzlar et al., 2019; Humoud et al., 2016), *Flaviviridae* (Bidet et al., 2014; Hou et al., 2017; Ward et al., 2011; Yi et al., 2011), *Geminiviridae* (Krapp et al., 2017), *Nanoviridae* (Krapp et al., 2017), *Picornaviridae* (Ng et al., 2013; White et al., 2007; Visser et al., 2019), *Poxviridae* (Katsafanas and Moss, 2004), and *Togaviridae* (Cristea et al., 2006; Fros et al., 2015; Kim et al., 2016; Panas et al., 2015). A variety of possible functions of G3BP during viral infection have been proposed. In some cases, it is considered as an antiviral factor, mainly because of its propensity to form (antiviral) SGs. Hence, some viruses have evolved mechanisms of sequestration or cleavage of G3BP to interfere with SG formation (Panas et al., 2015; White et al., 2007; Yang et al., 2019). However, for a variety of viruses, proviral activities of G3BP have been suggested and G3BP has been proposed to be involved in roles such as the switch from translation to replication (Scholte et al., 2015), protection of viral genomic RNA and formation of viral replication complexes (Kim et al., 2016), or could function as a component of the viral replication complex (Fritzlar et al., 2019; Götte et al., 2019; Yi et al., 2011) and as transcription factor (Katsafanas and Moss, 2004). The mechanisms of potential proviral roles of G3BP during viral infection are, nonetheless, still insufficiently understood.

2 AIMS OF THIS THESIS

The aim of this thesis was to investigate the functional role of the cellular protein G3BP during Old World alphavirus infection, in particular for SFV and CHIKV.

Paper I: To determine the functional importance of the two G3BP-binding motifs, present in SFV and CHIKV nsP3, for viral replication.

Paper II: To identify and functionally characterize G3BP1 domains, which are critical for viral replication.

Paper III: To investigate the influence of G3BP on viral replicase activity.

3 RESULTS

The Old World alphaviruses SFV and CHIKV contain two FGDF motifs in the C-terminal region of the nsP3 HVD, designated $FGDF_N$ and $FGDF_C$ to distinguish the N-terminal and C-terminal motif, respectively. These motifs facilitate binding to the NTF2-like domain of the host protein G3BP. Mutation of either Phe or the Gly residue in both motifs completely disrupts this interaction (Panas et al., 2014; Panas et al., 2015). A crystal structure of the NTF2-like domain of G3BP2 in complex with an LT**FGDF**DE peptide, corresponding to $FGDF_N$ of SFV nsP3, shows that the peptide occupies a hydrophobic pocket on the surface of the NTF2-like domain (Kristensen, 2015). Substitution of G3BP residue Phe33 with Trp (F33W), located within the peptide-binding site of the NTF2-like domain, disrupts the interaction with nsP3 (Panas et al., 2015).

In **paper I**, we investigated the structural and biological role of the two FGDF motifs for alphavirus infection. Through collaboration, we solved the crystal structure of the NTF2-like domain of human G3BP1 (residues 1-139) in complex with a 25-residue peptide of SFV nsP3, covering both FGDF motifs (termed $nsP3_{SFV}$-25, residues 449-473, LT**FGDF**DEHEVDALASGIT**FGDF**DD), at a resolution of 1.9 Å. The NTF2-like domain of G3BP forms dimers (Kristensen, 2015; Vognsen et al., 2013) and each of the two FGDF motifs binds to a monomer on separate dimers (Figure 7). $NsP3_{SFV}$-25 thereby interconnects G3BP1-dimers, inducing the formation of an nsP3:G3BP oligomer. We aimed to verify the biological relevance of the nsP3:G3BP oligomer formation and reasoned that if it was important to link G3BP dimers together, then mutation of either FGDF motif would give a similar phenotype as the double mutant.

First, we assessed the relative efficiencies of the individual FGDF motifs to bind G3BP through immunoprecipitations (paper I, Figure 4). For instance, we fused the HVD of SFV nsP3 (residues 319 to 482) to the C-terminal end of EGFP and introduced mutations in either one or both FGDF motifs. Phe-to-Ala substitution of the first Phe residues in both FGDF motifs ($F3A_{NC}$, numbering based on $L/I^1T^2F^3G^4D^5F^6D^7$ repeat sequences) is sufficient to completely disrupt the nsP3:G3BP interaction (Panas et al., 2015). Accordingly, we also generated constructs with Phe-to-Ala substitutions in either the $FGDF_N$ or $FGDF_C$ motif, designated $F3A_N$ and $F3A_C$, respectively (illustrated in Figure 8A). HEK293 cells were then transfected with WT, $F3A_N$, $F3A_C$, or $F3A_{NC}$ constructs of EGFP-$HVD_{SFV\text{-}nsP3}$ and 24 h post-transfection, cell lysates were subjected to anti-GFP immunoprecipitation and immunoblotting for GFP, G3BP1,

and actin (Figure 8B). WT and the C-terminal mutant F3A$_C$ strongly co-precipitated G3BP1, whereas the N-terminal mutant F3A$_N$ only weakly interacted with G3BP1, and for F3A$_{NC}$ the interaction was not detectable. This binding pattern was also observed with full-length SFV nsP3 (paper I, Figure 4) and was furthermore validated by *in vitro* binding studies using microscale thermophoresis (paper I, Figure 3). It can be concluded, that the N-terminal FGDF motif has a higher affinity for G3BP than the C-terminal motif. This suggests a hierarchical binding model, in which FGDF$_N$ binding to G3BP precedes binding of FGDF$_C$.

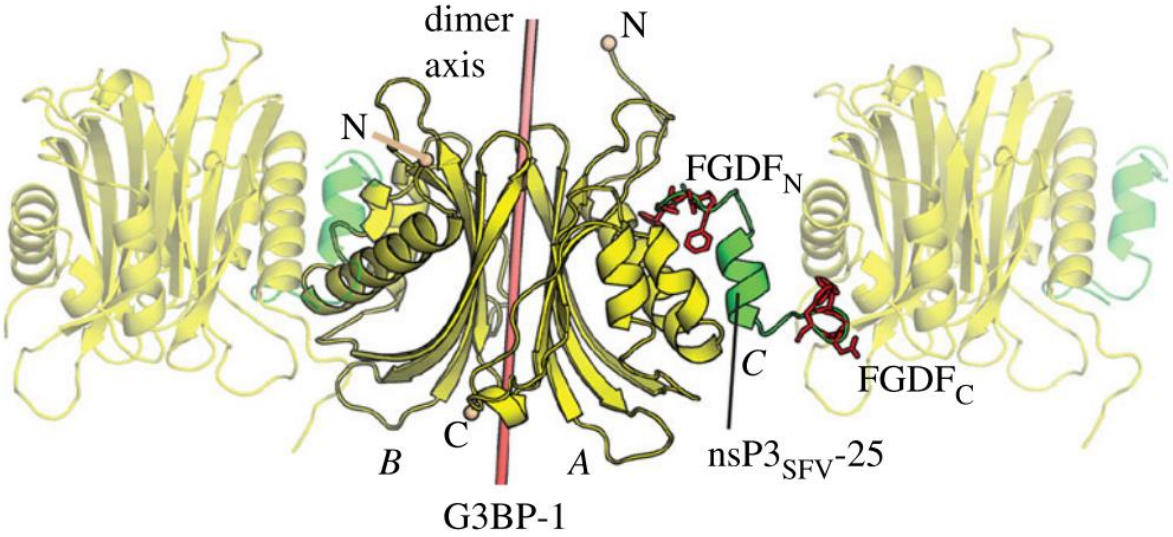

Figure 7: SFV nsP3 and G3BP1 dimers form a nsP3:G3BP poly-complex. The NTF2-like domain of G3BP1 (residues 1-139), shown in yellow, was crystallized with a 25-residue of SFV nsP3 (nsP3$_{SFV}$-25), shown in green. The two-fold symmetry axis of the G3BP dimer is indicated. FGDF$_N$ and FGDF$_C$ (highlighted in red) of nsP3$_{SFV}$-25 bind to G3BP subunit A and subunit B on separate dimers, respectively. NsP3$_{SFV}$-25 thereby interconnects dimers of the NTF2-like domain of G3BP1, inducing the formation of an oligomeric structure.

To characterize the importance of having two G3BP-binding motifs and potentially the formation of poly-complexes for SFV infection, we generated recombinant viruses, containing the F3A$_N$, F3A$_C$, or F3A$_{NC}$ substitutions. Unless otherwise stated, SFV refers to the SFV4 strain. BHK-21 cells were infected with WT or recombinant SFV at a multiplicity of infection (MOI) of 0.1 and virus titres from various times post infection (pi) were quantified by plaque assay (Figure 8C). In agreement with previous reports, SFV replication is attenuated in the absence of the nsP3:G3BP interaction (SFV-F3A$_{NC}$) (Panas et al., 2015; Panas et al., 2012). The two single FGDF mutants, SFV-F3A$_N$ and SFV-F3A$_C$, were also attenuated and both replicated very similar to the double mutant, SFV-F3A$_{NC}$. The growth curve result shows that, despite the different affinities of FGDF$_N$ and FGDF$_C$ for G3BP-binding, SFV requires both

motifs for efficient replication. Naturally, this also suggests a crucial role for nsP3:G3BP oligomers, which can only form, if both FGDF motifs in nsP3 are functional.

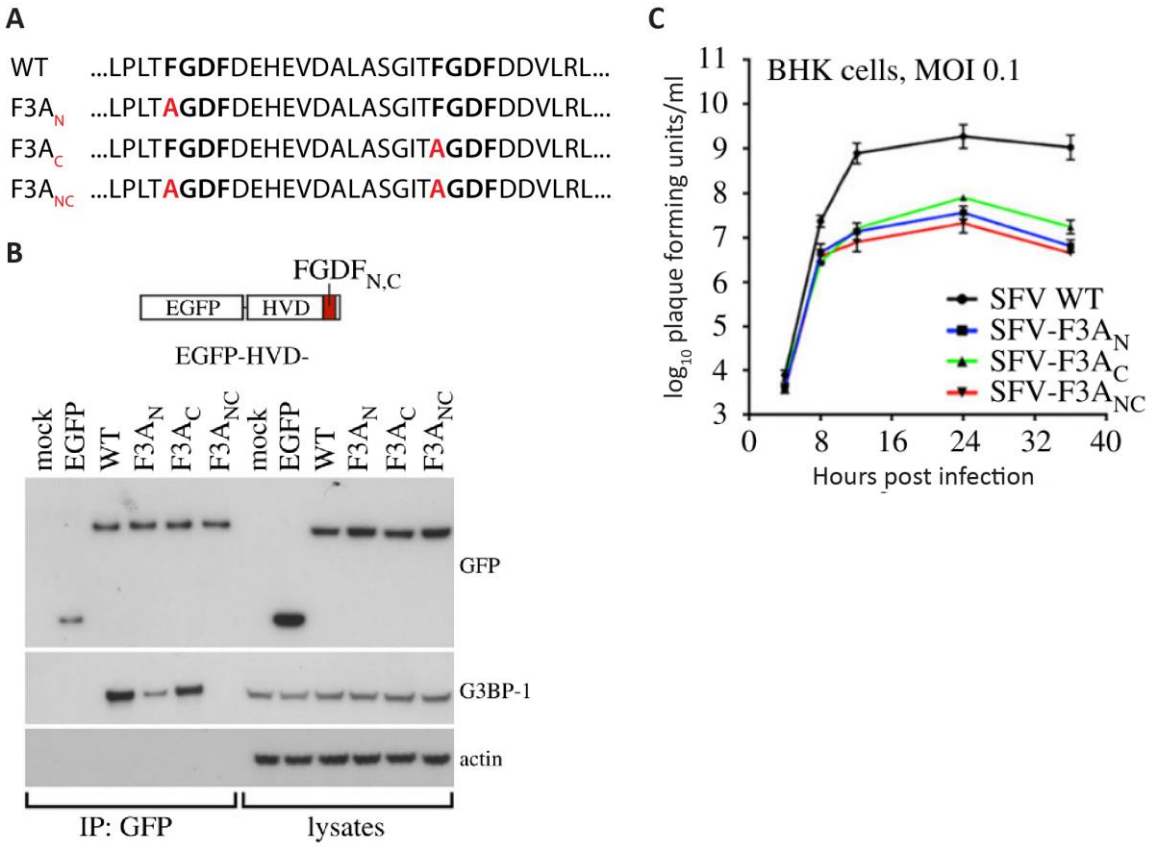

Figure 8: SFV requires both FGDF motifs for efficient replication. (A) Illustration of FGDF motif mutations and corresponding designations. **(B)** The nsP3 HVD was fused to EGFP and WT, F3A$_N$, F3A$_C$, and F3A$_{NC}$ constructs transfected in HEK239 cells. Cell lysates were prepared 24 h post transfection and subjected to anti-GFP immunoprecipitation. Western blots were stained for GFP, G3BP1, and actin. **(C)** BHK-21 cells were infected with WT or recombinant SFV at MOI 0.1 and titres from indicated timepoints quantified by plaque assay. Data are means of four independent experiments. Error bars represent standard error of mean (SEM).

CHIKV also contains two FGDF motifs in the nsP3 HVD, which mediate binding to G3BP (Panas et al., 2014). Rescue of CHIKV F3A$_{NC}$ was not possible, indicating that the G3BP interaction is crucial for CHIKV replication. Similar to SFV, immunoprecipitation of transiently transfected EGFP-nsP3$_{CHIKV}$ constructs showed that the N-terminal FGDF motif has a higher affinity for G3BP1 than the C-terminal motif (paper I, Figure 7(a)). This suggests that the hierarchical binding mode is conserved among SFV and CHIKV.

The results of **paper I** show that the Old World alphaviruses SFV and CHIKV differ in their requirements for G3BP. In absence of G3BP-binding, SFV is attenuated, but replication

proceeds to detectable level. In contrast, CHIKV replication strictly depends on the nsP3:G3BP interaction. Moreover, mutation of a single FGDF mutation ($F3A_N$ or $F3A_C$) has the same effect on SFV replication as when both motifs are mutated ($F3A_{NC}$). For CHIKV, a single functional FGDF motif is sufficient to allow for infectious virus production, although not to levels of WT CHIKV, whereas mutation of both motifs is lethal.

In **paper II**, we aimed to obtain a better understanding of the proviral role(s) of the nsP3:G3BP interaction for SFV and CHIKV replication. We utilized a set of cell lines that was generated by stable transfection of U2OS ΔΔG3BP1/G3BP2 double-knockout cells (hereafter referred to as U2OS ΔΔ) with various GFP-fused G3BP1 mutants (Figure 9A) (Kedersha et al., 2016). U2OS ΔΔ cells expressing GFP only or full-length GFP-G3BP1 WT served as controls. The F33W mutation in the NTF2-like domain disrupts the interaction with nsP3 but maintains SG competence (Kedersha et al., 2016). Expression of only the NTF2-like domain (GFP-G3BP1 1-135) or deletion of the RGG domain blocks SG formation. Deletion of the RRM domain does not interfere with nsP3-binding or SG formation.

The different cell lines were infected with either SFV or CHIKV at MOI 0.1 and production of infectious virus was determined by plaque assay (Figure 9B). In agreement with our previous observations, SFV growth was attenuated but detectable in the absence of the nsP3:G3BP1 interaction (GFP, F33W). Deletion of the RGG domain (ΔRGG) caused a slight delay in growth, whereas the RRM domain was dispensable (ΔRRM). Of note, the NTF2-like domain of G3BP1 (1-135) alone was sufficient to promote efficient replication of SFV to levels very similar to full-length G3BP1 WT. For CHIKV, virus production was not detectable in most cell lines, including cells expressing only the NTF2-like domain (1-135). CHIKV growth was only detected in cells expressing GFP-G3BP1 WT and to a lesser extent GFP-G3BP1 ΔRRM. Taken together, SFV replicates efficiently in presence of the NTF2-like domain of G3BP1, whereas CHIKV requires both the NTF2-like and RGG domains.

In infected cells, nsP3 recruits G3BP to viral replication complexes. To characterize if the G3BP1 mutants affect formation and organization of viral replication complexes, we performed immunofluorescence and electron microscopy experiments. Since CHIKV is non-viable in most of the cell lines, we used primarily SFV for our analysis. For immunofluorescence experiments, we stained SFV-infected cells for nsP3 and dsRNA, a viral RNA replication intermediate and marker for replication complexes (Figure 10A). In GFP-G3BP1 WT and 1-135 expressing cells, nsP3 and GFP-G3BP1 colocalize and accumulate around clusters of dsRNA-positive foci. In the absence of the nsP3:G3BP1 interaction (GFP, F33W), GFP signals do not colocalize with nsP3 but are more diffuse and are associated with

weaker and more dispersed dsRNA signals. Deletion of either the RRM (ΔRRM) or the RGG (ΔRGG) domain of G3BP1 still produced strong dsRNA signals, although less strongly clustered. The recruitment of G3BP1 via the NTF2-like domain thus induces efficient formation of clustered dsRNA-positive replication complexes.

For SFV, replication complexes first assemble at the plasma membrane and are then internalized during infection and accumulate in the perinuclear area as cytopathic vacuoles (Spuul et al., 2010). Electron micrographs of SFV-infected cells at 8 h pi showed that formation of CPVs was strongly impaired in the absence of the nsP3:G3BP1 interaction (Figure 10B). Under those conditions, CPVs were few in number and mostly isolated. In contrast, CPVs were readily detectable if the nsP3:G3BP1 interaction was intact and frequently appeared in clusters, which was particularly apparent in GFP-G3BP1 WT and 1–135 expressing cells.

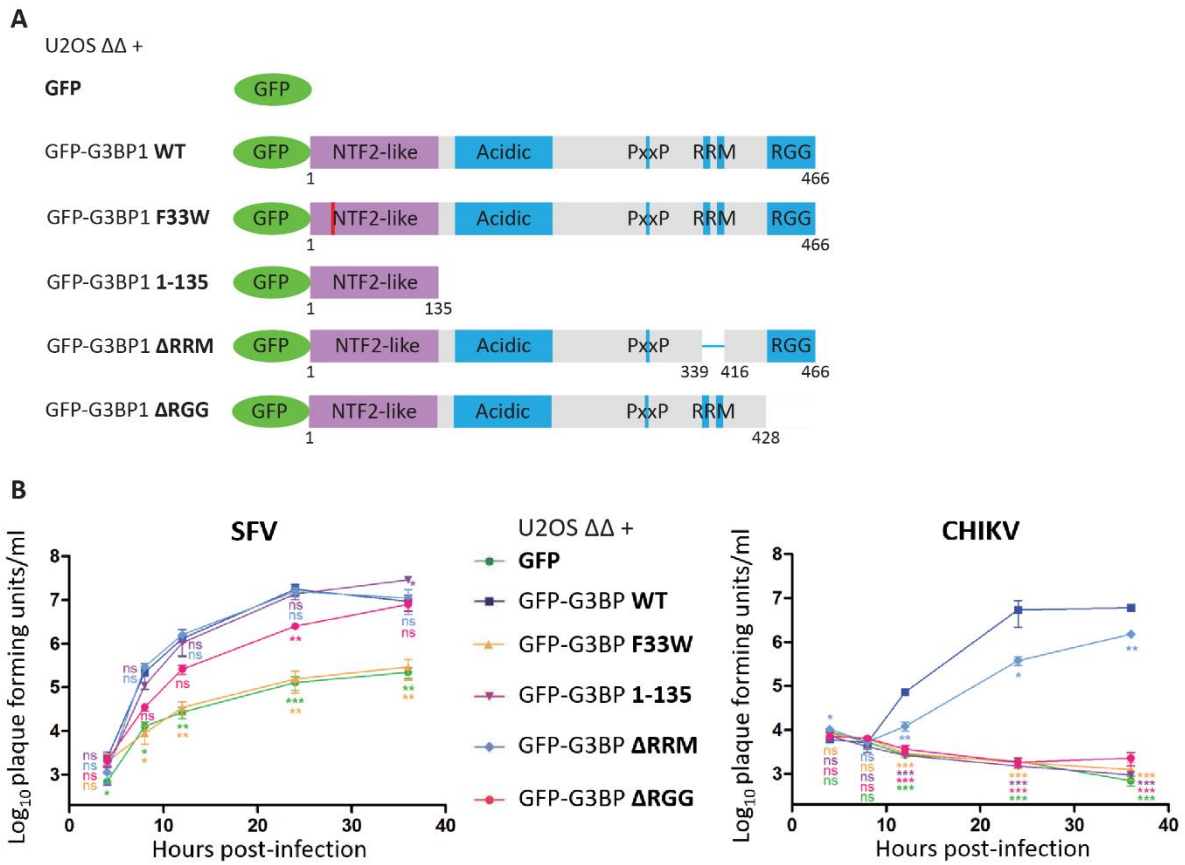

Figure 9: The NTF2-like and RGG domains of G3BP1 are important for Old World alphavirus replication. **(A)** Schematic representation of GFP-fused G3BP1 constructs used for stable transfection of U2OS ΔΔ cells. **(B)** Indicated cell lines were infected with SFV or CHIVK at MOI 0.1 and titres from indicated timepoints quantified by plaque assay on BHK-21 cells. Data are means of three independent experiments. Error bars represent standard error of mean (SEM). For statistical analysis samples from each timepoint were compared to the respective titre obtained in U2OS ΔΔ + GFP-G3BP1 WT cells. ns P>0.05, *P≤0.05, **P≤0.01, ***P≤0.001.

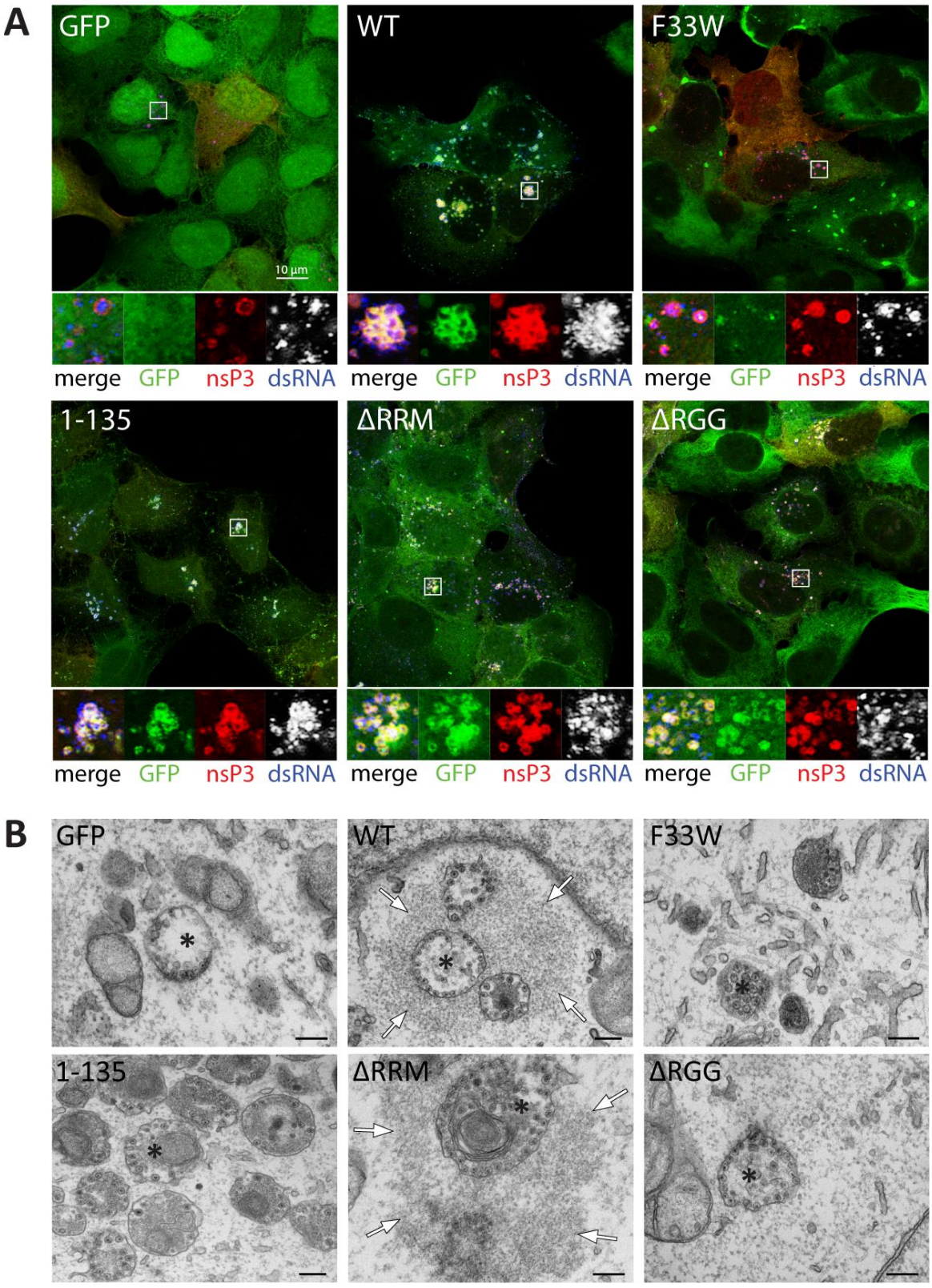

Figure 10: The NTF2-like domain of G3BP1 promotes clustering of replication complexes and the RGG domain is associated with accumulation of electron-dense patches around CPVs. (A) Indicated cell lines were infected with SFV at MOI 10 and at 8 h pi fixed and stained for nsP3 (red) and dsRNA (blue). **(B)** Indicated cell lines were infected with SFV at MOI 100 and at 8 h pi fixed and analysed by transmission electron microscopy. Internalization of PM-associated spherules leads to formation of CPVs (indicated by asterisks) in the perinuclear region. The CPVs in cells expressing GFP-G3BP1 WT or ΔRRM are associated with electron-dense material (indicated by white arrows). Scale bar: 200nm.

We noticed that the CPVs in cells expressing GFP-G3BP1 WT or GFP-G3BP1 ΔRRM, but not any of the other mutants, were associated with patches of electron-dense material (indicated by white arrows in Figure 10B). The presence of those patches was thus dependent on the recruitment of G3BP via the NTF2-like domain and on the RGG domain. This drew our attention, as it were the same domains that were crucial for CHIKV replication. The NTF2-like and RGG domains are also essential for the formation of SGs, which can be induced by overexpression of G3BP (Kedersha et al., 2016; Matsuki et al., 2013; Tourriere et al., 2003). Furthermore, electron microscopy images of SGs show a striking similarity to the CPV-associated patches that we had observed (Souquere et al., 2009). Consequently, we hypothesized that the enrichment of G3BP around SFV-induced CPVs could generate a similar liquid-liquid phase separation and recruit additional host factors. SGs are enriched in translation initiation factors and 40S ribosomal subunits and G3BP specifically interacts with 40S subunits via its RGG domain (Kedersha et al., 2016). By immunofluorescence, we confirmed that several translation initiation factors, including eIF2D, eIF3B, and eIF4A, were enriched around dsRNA-positive replication complexes in SFV-infected cells (paper II, Figure 4). Furthermore, we performed several immunoprecipitations to validate that nsP3:G3BP complexes associate with 40S ribosomal subunits (paper II, Figure 5). For instance, a selected set of cell lines was transfected with biotin-acceptor peptide (BAP)-tagged CHIKV nsP3 and nsP3 complexes were subsequently immunoprecipitated using streptavidin beads (Figure 11A). Each of the cell lines used expressed GFP-G3BP1 variants with intact NTF2-like domain and consequently all co-precipitated with CHIKV nsP3. Co-precipitation of 40S ribosomal subunit proteins rpS3 and rpS6, however, depended on the presence of the RGG domain and was only detected for GFP-G3BP1 WT and ΔRRM expressing cells but not for ΔRGG. Altogether, these results suggest that the CPV-associated electron-dense patches contain nsP3:G3BP:40S complexes and translation initiation factors.

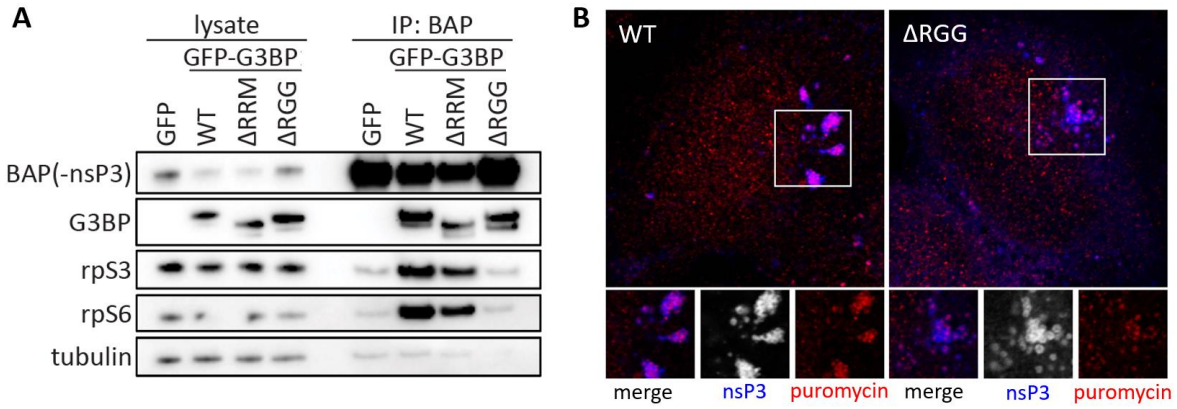

Figure 11: nsP3:G3BP1 complexes bind 40S ribosomal subunits, which correlates with enhanced translational activity. (A) Indicated cell lines were transfected with biotinylated CHIKV nsP3 (BAP-nsP3) for 24 h. and lysates were prepared 24 h after transfection. nsP3 complexes were precipitated using streptavidin-coated beads. Western blots were probed for nsP3, GFP, 40S ribosomal subunit proteins rpS3 and rpS6, or β-tubulin. **(B)** GFP-G3BP1 WT and ΔRGG expressing cells were infected with SFV at MOI 10 for 8 h, pulse-labelled with puromycin for 5 min, and immunostained for nsP3 (blue/white) and puromycin (red).

The recruitment of translation initiation factors to the sites of viral RNA replication suggests a mechanism to guarantee efficient translation of newly synthesized viral RNA. We applied the ribopuromycylation method to determine if translational activity is enhanced in close proximity to viral replication complexes. Puromycin is a compound that in treated cells covalently attaches to nascent polypeptide chains and subsequent detection, using a puromycin-specific antibody, allows for visualization of localized translational activity. We infected GFP-G3BP1 WT or ΔRGG cells with SFV for 8 h, followed by a 5 min puromycin pulse, before fixation and immunostaining (Figure 11B). In GFP-G3BP1 WT expressing cells, dsRNA-positive foci were associated with strong localized puromycin staining, indicative for enhanced translational activity around viral replication complexes. Replication complexes in GFP-G3BP1 ΔRGG expressing cells, however, were associated with noticeably weaker puromycin signals. The absence of nsP3:G3BP:40S complexes was therefore associated with reduced localized translational activity.

In conclusion, our findings of **paper II** show that the NTF2-like domain of G3BP1 is sufficient to promote efficient replication of SFV, which correlates with profound clustering of viral replication complexes. On the contrary, CHIKV depends on the NTF2-like and RGG domains and we propose a role for these domains in the active recruitment of the translation initiation machinery to the sites of viral RNA production.

In contrast to CHIKV, SFV is partially resistant towards G3BP deletion (paper II). In **paper III**, we wanted to determine if this characteristic is shared among different SFV strains. For

this, we infected U2OS WT or U2OS ΔΔ cells with either SFV4 or SFV A774 and compared viral growth over a period of 48 h by plaque assay (Figure 12A). In the presence of G3BP (U2OS WT), replication of both viruses was very similar. In contrast, viral growth differed significantly in G3BP knockout cells (U2OS ΔΔ). SFV4 was attenuated but replication was detectable, consistent with our previous observations, whereas SFV A774 did not show any signs of active replication within 24 h pi and only a slight increase in titre was detected at 48 h pi.

This observation encouraged us to expand the investigation of G3BP sensitivity to other alphaviruses. To this end, we made use of a trans-replication system and compared the replication activity of various alphavirus trans-replicases between U2OS WT or U2OS ΔΔ cells (paper III, Figure 1C). Based on these results we grouped the tested replicases into sensitive and resistant viruses with respect to G3BP dependence. Subsequent analysis showed that all of the replicases that we categorized as G3BP deletion sensitive, contain an Arg residue at the P4 position of the cleavage site between nsP1 and nsP2 (1/2 site) of the non-structural polyprotein (Figure 12B). In resistant replicases, this residue is always altered.

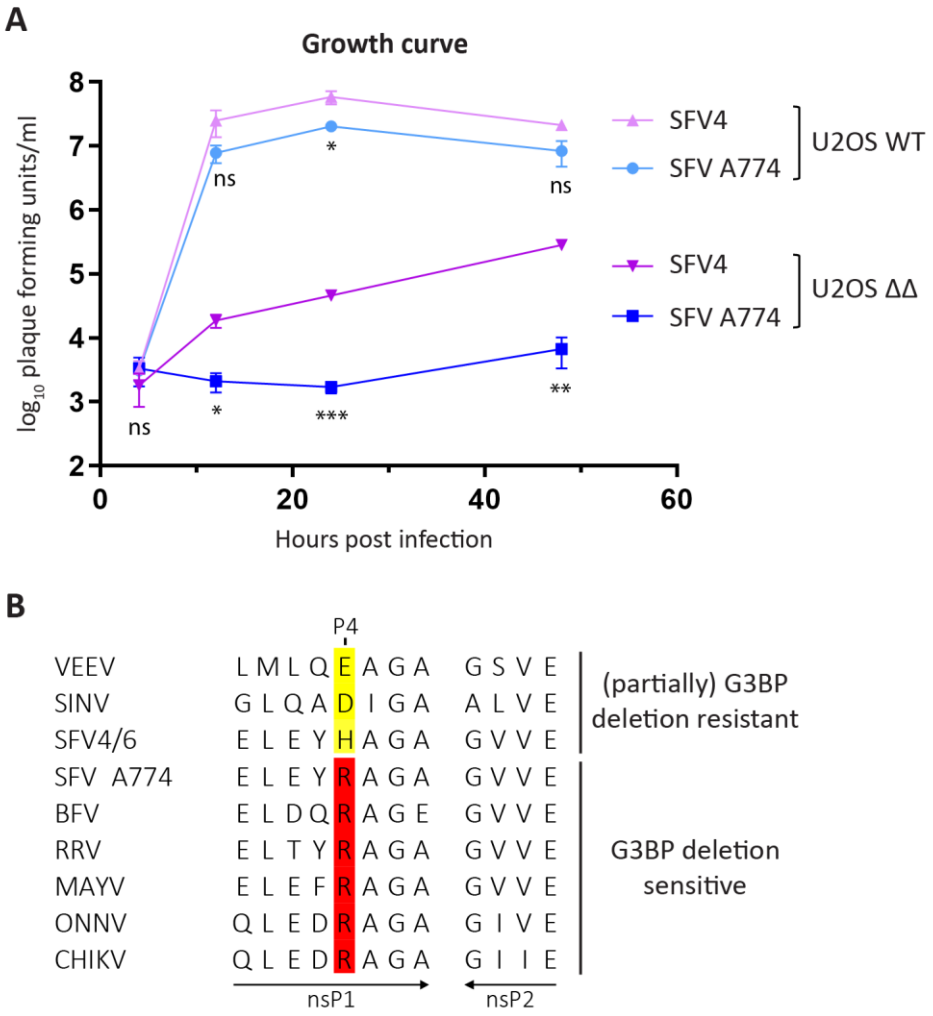

Figure 12: G3BP deletion sensitive alphaviruses have an Arg at the P4 position of the 1/2 site. (A) U2OS WT or U2OS ΔΔ cells were infected with SFV4 or SFV A774 at MOI 0.1 and viral titres quantified by plaque assay. Error bars represent standard error of mean (SEM). For statistical analysis SFV A774 titres were compared to SFV4 titres for each timepoint and cell line. ns P>0.05, *P≤0.05, **P≤0.01, ***P≤0.001. **(B)** Comparison of sequences adjacent to the 1/2 site for different alphaviruses. The P4 position is highlighted. All trans-replicases that were tested G3BP deletion sensitive (paper III, Figure 1C) have an Arg residue at the P4 position. In the resistant replicases this residue is always altered.

Next, we investigated if the identity of the P4 residue influences G3BP-dependence. Previous studies identified the P4 residue of the 1/2 site as a determinant for polyprotein processing and neurovirulence (Saul et al., 2015). Substitution of this residue in SFV4 was found to result in a compensatory change of amino acid residue 515 of nsP2 (Lulla et al., 2018) indicating a functional link between the P4 residue of the 1/2 site (position 534 of nsP1) and residue 515 of nsP2 (position 1052 of the non-structural polyprotein). For SFV4, residues 534 and 1052 of the non-structural polyprotein are His and Val, respectively. In SFV A774 these residues are Arg at position 534 and Glu at position 1052. We swapped the coding sequences for these two

residues between SFV4 and SFV A774, generating recombinant viruses, designated SFV4-HR+VE (His534Arg plus Val1052Glu) and SFV A774-RH+EV (Arg534His plus Glu1052Val). The WT and mutant sequences are illustrated in Figure 13A. For SFV4 the substitutions did not negatively affect viral growth in U2OS WT cells but SFV4-HR+VE was significantly more attenuated in U2OS ΔΔ cells than WT SFV4 (Figure 13B). For SFV A774 the substitution had the opposite effect. In U2OS WT cells SFV A774-RH+EV was slightly attenuated compared to WT SFV A774, however, the RH+EV substitutions provided a clear fitness advantage in U2OS ΔΔ cells, resulting in increased viral titres compared to WT SFV A774. In summary, these results indicated that the SFV non-structural polyprotein residues Arg534 and Glu1052 are associated with increased sensitivity towards G3BP-sensitivity, whereas His534 and Val1052 provide some resistance.

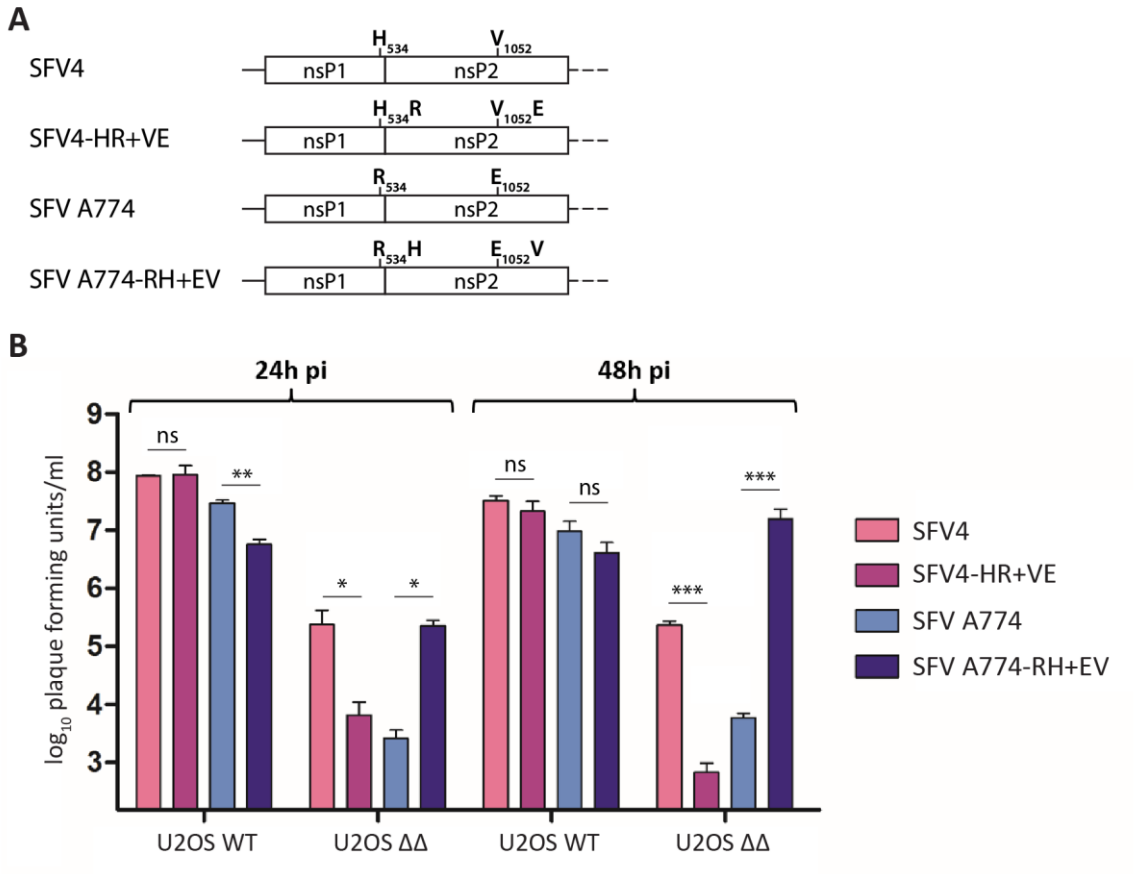

Figure 13: Substitutions linked to the processing of the 1/2 site also alter the sensitivity towards G3BP deletion. (A) Illustration of amino acid residues 534 and 1052 of the SFV non-structural polyprotein in wild-type and recombinant viruses. **(B)** U2OS WT or U2OS ΔΔ cells were infected with indicated viruses at MOI 0.1 and viral titres quantified by plaque assay. Error bars represent standard error of mean (SEM). For statistical analysis recombinant viruses were compared to the respective wild-type viruses for each timepoint and cell line. ns $P>0.05$, *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$.

CHIKV and SFV A774 are both drastically affected by G3BP deletion. Based on the observation that SFV A774-RH+EV is partially G3BP-resistant, we aimed to determine if CHIKV replication can also be rescued by these substitutions. SFV residues 534 and 1052 correspond to CHIKV residues 532 and 1050, respectively. The replicase activity of CHIKV Arg532His in mammalian cells is very similar to the activity in the context of the Arg532His plus Glu1050Val double substitution (Bartholomeeusen et al., 2018). We tested the Arg532His substitution alone in the context of the CHIKV G3BP-binding mutant F3A$_{NC}$, generating CHIKV F3A$_{NC}$-RH. BHK-21 cells were then electroporated with infectious DNA of WT CHIKV or recombinants and virus rescue was detected by plaque assay (Figure 14). WT CHIKV produced high titres and strong cytopathic effects within 24 h post electroporation. In contrast, CHIKV F3A$_{NC}$ did not produce infectious particles up to 60 h post transection. This defect was partially overcome by the Arg532His substitution (CHIKV F3A$_{NC}$-RH). Thus, the P4 Arg-to-His substitution partially rescues CHIKV replication in the absence of G3BP. Similar observations were also made with a CHIKV trans-replication system in mammalian and mosquito cells (paper III, Figure 3).

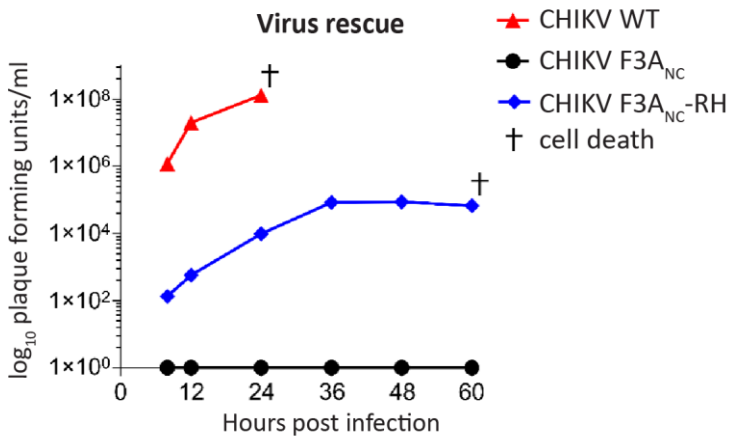

Figure 14: The Arg-to-His substitution of the P4 residue rescues CHIKV replication in absence of G3BP-binding. cDNA of indicated viruses was transcribed *in vitro* and electroporated into BHK-21 cells. Viral titres from indicated timepoints were measured by plaque assay. †, timepoint of cell death.

The P4 residue of the 1/2 site is part of the nsP2 protease recognition sequence and mutations affect the processing efficiency at this site (Saul et al., 2015). An Arg residue at this position is associated with a fast cleavage rate, whereas a His residue decreases this rate. Based on our observations, it can be concluded that a fast cleavage rate of the 1/2 site correlates with a high sensitivity towards G3BP deletion and a slower rate with partial resistance. This correlation

urged us to investigate if the nsP3:G3BP interaction leads to altered processing of the CHIKV non-structural polyprotein, which we assessed by pulse-chase labelling. We transfected U2OS WT cells with the non-structural polyprotein of WT CHIKV or CHIKV-RH (Figure 15A) and compared the processing of the polyprotein to that of G3BP-binding mutant CHIKV F3A$_{NC}$ or CHIKV F3A$_{NC}$-RH (Figure 15B), respectively. 12 h post transfection, cells were exposed to a [^{35}S]methionine-cysteine mixture for 15 min (pulse), followed by a 45 min incubation in presence of excess unlabeled methionine and cysteine (chase). Pulse-labelled and chased samples were then subjected to immunoprecipitation using nsP1 and nsP2 or nsP3 antibodies for pull-down of the non-structural polyprotein, its cleavage intermediates and the mature nsPs, followed by separation of proteins using SDS-PAGE. Polyprotein processing was then assessed by calculating the ratios of the amount of uncleaved P123 polyprotein to the amounts of mature nsPs. Processing of WT CHIKV compared to CHIKV F3A$_{NC}$ occurred at similar efficiencies. The reduced processing efficiency, caused by the Arg532His substitution, notably increased the P123/nsP ratios for CHIKV-RH and CHIKV F3A$_{NC}$-RH. Again, comparing the processing of CHIKV-RH to CHIKV F3A$_{NC}$-RH revealed similar processing efficiencies. Together, the results of the pulse-chase experiments suggest that G3BP proteins do not have a prominent effect on the processing of the non-structural polyprotein of CHIKV.

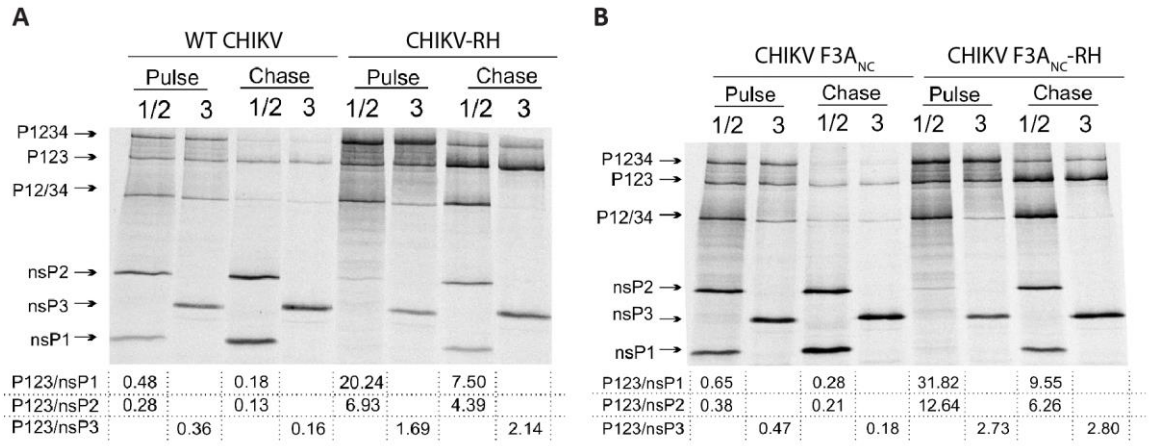

Figure 15: G3BP proteins do not influence the processing of the CHIKV non-structural polyprotein. (A) U2OS WT cells were transfected with the non-structural polyprotein P1234 of wild-type (WT) CHIKV or CHIKV-RH. **(B)** For comparison, U2OS WT cells were transfected with the non-structural polyprotein of G3BP-binding mutant CHIKV F3A$_{NC}$ or CHIKV F3A$_{NC}$-RH. 12 h post transfection, cells were labelled with a mixture of [^{35}S]methionine-[^{35}S]cysteine for 15 min (pulse), followed by 45 min incubation in presence of excess unlabeled methionine and cysteine (chase). Viral non-structural proteins and cleavage intermediates were isolated using nsP1 and nsP2 or nsP3 antibodies, resolved by SDS-PAGE and visualized using a Typhoon imager. Band intensities were quantified and ratios of P123 to mature nsP1, nsP2, or nsP3 calculated.

Experiments with a CHIKV trans-replicase system showed, that the RNA replication activity is severely affected by G3BP knockout, despite the presence of vast amounts of replicase proteins, which are produced from a separate plasmid (paper III, Figure 3). This indicates a possible role for G3BP during viral RNA replication. Active RNA replication involves the formation of dsRNA replication intermediates, which can be detected by immunofluorescence using specific antibodies. We transfected U2OS WT or U2OS ΔΔ cells with plasmids expressing CHIKV trans-replicase and corresponding template and stained for dsRNA and CHIKV nsP3 (Figure 16). In U2OS WT cells, strong dsRNA signals were detectable in cells that also stained positive for CHIKV nsP3. However, dsRNA signals were completely absent in nsP3-positive U2OS ΔΔ cells. RNA replication is initiated by cleavage at the 3/4 site and the corresponding P123-nsP4 replicase synthesizes predominantly negative-strand RNA (Kim et al., 2004). We generated a cleavage-deficient CHIKV trans-replicase by mutating key residues in the 1/2 and 2/3 sites (CHIKV 1*2*34). This prevents the early replicase P123-nsP4 from further processing. Whereas CHIKV 1*2*34 produced strong dsRNA signals in U2OS WT cells, no such signals could be detected in U2OS ΔΔ cells. These results suggest that G3BP is crucial already at the very early stages of CHIKV RNA replication.

Altogether, the observations of **paper III** highlight marked differences among alphaviruses regarding their sensitivity towards G3BP deletion. We provide evidence that the extent of G3BP-dependence is linked to the P4 residue of the 1/2 site and its functional role during non-structural polyprotein processing. However, our data suggest that G3BP proteins do not influence the processing of the non-structural polyprotein but are required for the initiation of viral RNA replication.

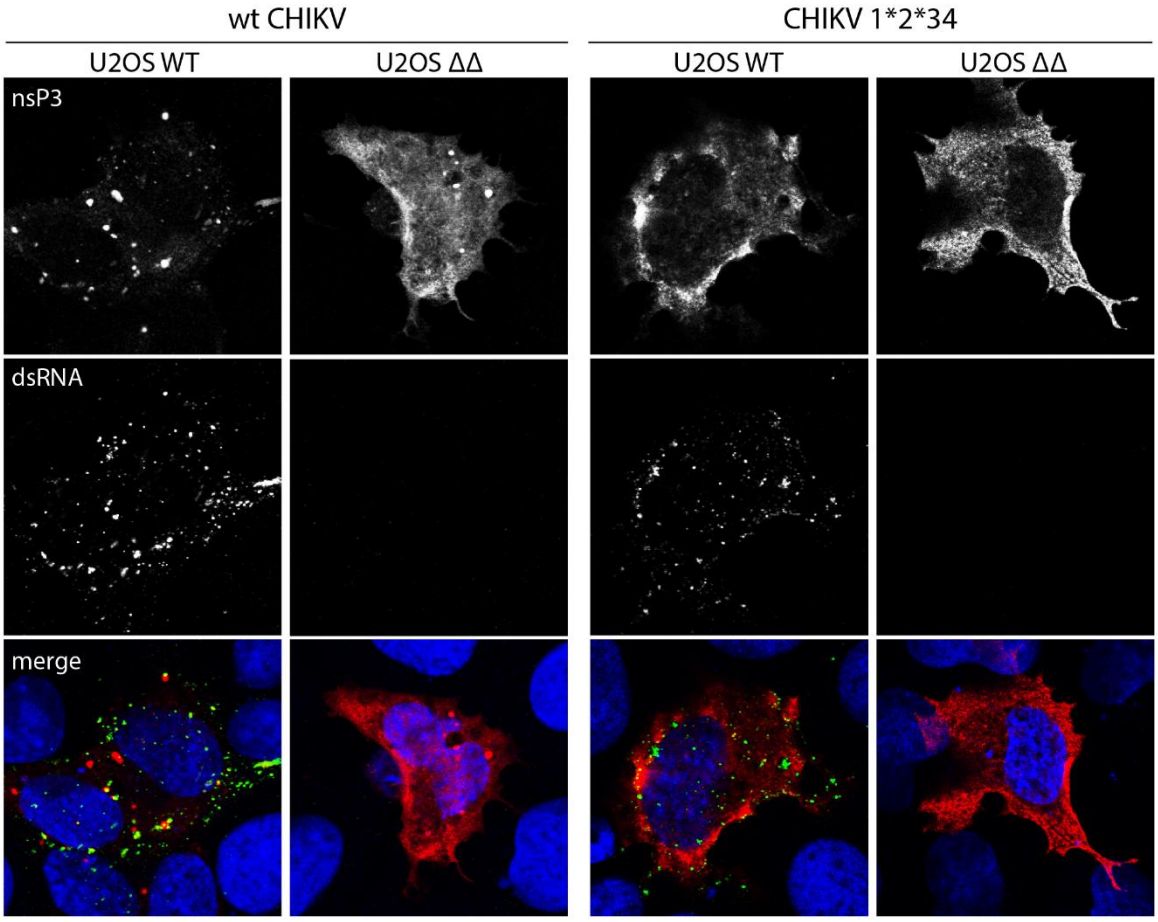

Figure 16: CHIKV trans-replicase fails to produce dsRNA in absence of G3BP. U2OS WT or U2OS ΔΔ cells were co-transfected with either WT CHIKV or processing-deficient CHIKV 1*2*34 trans-replicase and corresponding template. 24 h post transfection, cells were fixed and stained for nsP3 (grey/red) and double-stranded RNA (dsRNA) (grey/green). Nuclei in blue.

4 DISCUSSION

The long-standing co-evolution of viruses and their hosts has stimulated the development of a versatile host immune repertoire and specific viral countermeasures. Phosphorylation of eIF2α and subsequent inhibition of host translation by reduced availability of eIF2-GTP-$tRNA_i^{Met}$ ternary complex and SG formation is a common stress response of a virus infected cell. Viruses have developed a diverse set of strategies to interfere with SG formation, reviewed in (Garcia et al., 2006; Poblete-Duran et al., 2016; Reineke and Lloyd, 2013). For Old World alphaviruses sequestration of the host protein G3BP, which is essential for the formation of SGs in response to eIF2α phosphorylation, disassembles SGs and blocks their reformation (Panas et al., 2015; Panas et al., 2012). Previous work from our group has established that the nsP3:G3BP interaction is critical for the efficient disassembly of SGs. In this early work, the disassembly of SGs has been thought of as the main purpose of the nsP3:G3BP interaction. However, the work presented in this thesis provides evidence that G3BP also exerts SG-independent proviral activities.

Utilization of genetically modified cell lines, expressing various GFP-fused G3BP1 mutants in absence of endogenous G3BP, allowed us to investigate the functional role of particular G3BP1 domains during infection with SFV and CHIKV (paper II). Importantly, we found that the NTF2-like domain of G3BP1 (GFP-G3BP1 1-135) is sufficient to promote efficient replication of SFV (Figure 9B). GFP-G3BP1 1-135 expressing cells do not form SGs in response to viral infection, but neither do cells expressing only GFP in which, however, SFV growth was attenuated. In paper I, we showed that the two FGDF motifs of SFV, present in the nsP3 HVD, can crosslink dimers of the NTF2-like domain into an nsP3:G3BP oligomer (Figure 7). Both motifs bind G3BP, but mutation of a single motif impairs SFV replication to the same level as when both motifs are mutated (Figure 8C). Naturally the nsP3:G3BP oligomer can only form if both FGDF motifs are functional, highlighting the importance of this oligomeric structure for SFV replication. Based on the observations of paper I we had hypothesized that nsP3:G3BP complexes could tether and stabilize multiple viral replication complexes. The formation of patches of spherules and engagement of newly synthesized replicase proteins to the sites of viral RNA production could increase the replication efficiency. These considerations were supported by the results presented in paper II, showing that expression of GFP-G3BP1 1-135 efficiently rescues SFV replication, which is associated with the appearance of clusters of viral replication complexes (Figure 10). To our knowledge, the NTF2-like domain does not possess any enzymatic activity and in conclusion we propose a solely structural role of this domain.

In contrast to SFV, replication of CHIKV is not supported by the NTF2-like domain alone and production of infectious virus is not detectable in the absence of the nsP3:G3BP interaction (Figure 9B). This suggests that the dominant functional role of G3BP differs between SFV and CHIKV, even though both share the same binding mechanism with G3BP, facilitated through two FGDF motifs. Nevertheless, in the context of CHIKV, a single functional motif provides a clear benefit for viral growth, which is a profound difference to SFV, for which a single motif does not enhance virus production and efficient replication requires both motifs. Already in paper I we reported that BHK-21 cells transfected with infectious clones of CHIKV $F3A_N$ or $F3A_C$ showed a strong cytopathic effect within 60 h post transfection, which was not observed for the double mutant CHIKV $F3A_{NC}$. The $F3A_N$ and $F3A_C$ substitutions were also tested in the context of a CHIKV trans-replication system, showing that a single motif is sufficient to allow for replication in mammalian and mosquito cells (data not shown). The observation that a single FGDF motif is sufficient to support CHIKV replication has also been made by another group (Kim et al., 2016). nsP3:G3BP oligomers cannot form if one of the two FGDF motifs is mutated. The fact that a single motif promotes replication, but mutation of both motifs is lethal, indicates that formation of a nsP3:G3BP poly-complex is not a critical requirement for CHIKV. Accordingly, GFP-G3BP1 1-135 was not enough to rescue CHIKV growth. Only in very few cases did we detect CHIKV nsP3-positive signals in GFP-G3BP1 1-135 cells. In those cells, CHIKV nsP3 associated with GFP-G3BP1 1-135 predominantly in puncta (paper II, S2 Figure), similar to observations made with SFV (Figure 10A; paper II, Figure 2A). It should be noted that replication of CHIKV with only a single functional FGDF motif is, however, slightly less efficient than WT CHIKV. This could be due to the lower amount of bound G3BP, but could also imply a partial role for nsP3:G3BP oligomeric structures.

In addition to the NTF2-like domain of G3BP, CHIKV also depended on the presence of the C-terminal RGG domain (paper II, Figure 1). This domain is required for SG formation and facilitates binding to 40S ribosomal subunits (Kedersha et al., 2016). In paper II we show that in SFV-infected cells, the RGG domain is required for the formation of electron-dense material (Figure 10B) and the enrichment of translation initiation factors around viral replication complexes (paper II, Figure 4). In addition, we demonstrate that nsP3:G3BP1 complexes also bind 40S ribosomal subunits, depending on the RGG domain (Figure 11A; paper II, Figure 5). The electron-dense appearance and presence of components of the translation machinery is also characteristic for SGs (Kedersha and Anderson, 2007; Kedersha et al., 2016; Souquere et al., 2009). By immuno-electron microscopy we could show that the localization of G3BP is indeed not restricted to the boundaries of viral replication complexes, but instead, G3BP is distributed together with nsP3 over a large area, surrounding several CPVs (paper II, Figure 4A). The

similarities to SGs suggest that the high concentration of G3BP molecules in close proximity to viral replication complexes nucleates a phase separation and is associated with the recruitment of the translation initiation machinery. In contrast to bona fide SGs, which are sites of translational repression, the condensates around viral replication complexes are sites of enhanced translational activity (Figure 11B; paper II, Figure 6). Based on these observations we proposed that the recruitment of the translation initiation machinery to the sites of viral RNA replication is critical for the synthesis of CHIKV proteins and hence, viral growth.

Under conditions of phosphorylated eIF2α and SG formation, canonical cap-dependent translation is inhibited. Nevertheless, the subgenomic RNA of some alphaviruses, including SFV, is still efficiently translated, due to a translation enhancing stem-loop structure (McInerney et al., 2005; Ventoso et al., 2006). This structural element has been predicted for several alphaviruses, but not for CHIKV sgRNA (Toribio et al., 2016), which might explain the more stringent requirement of active recruitment of translation initiation components for CHIKV. However, this difference only regards the structural proteins, which are expressed from the sgRNA, and cannot explain why production of CHIKV non-structural proteins is also not detectable in absence of G3BP (non-published observation). Moreover, in paper III we utilized a CHIKV trans-replication system, in which expression of the replicase is independent of viral RNA replication, and found that CHIKV trans-replicase activity was severely impaired in the absence of G3BP-binding (paper III, Figure 3). Accordingly, U2OS ΔΔ cells transfected with trans-replicase components did not produce dsRNA replication intermediates to detectable levels, as assessed by immunofluorescence (Figure 16). Together these results describe G3BP as a critical host factor for CHIKV RNA replication, independent of its potential role during translation of viral proteins. An important role of G3BP1 for viral genome amplification has also been demonstrated for HCV (Yi et al., 2011).

Remarkably, in paper III we found that the P4 residue of the 1/2 site is linked to the sensitivity of G3BP deletion (Figure 13 & 14; paper III, Figure 3). A His residue at the P4 position confers relatively slow processing of the 1/2 site (Saul et al., 2015) and is associated with partial resistance towards G3BP deletion. On the contrary, an Arg at this position results in increased processing efficiency, which is associated with higher sensitivity towards G3BP deletion. If cleavage of the 1/2 site is blocked, substitution of the P4 residue loses its effect on G3BP-dependence, confirming that the processing rate is linked to G3BP-dependence (paper III, Figure 6). We hypothesized that the effect of the P4 residue on polyprotein processing might mimic a function of G3BP. Nonetheless, pulse-chase experiments showed that the processing

of the non-structural polyprotein occurs at a very similar rate for both WT CHIKV and CHIKV F3A$_{NC}$, which suggests that G3BP proteins do not alter the processing (Figure 15).

Based on the observations presented in paper III, we can assume that in the absence of G3BP, initiation of negative-strand RNA is inefficient. A slower processing at the 1/2 site stabilizes the P123-nsP4 negative-strand replicase and could thereby increase the probability that synthesis of the negative-strand RNA is initiated. For CHIKV and SFV A774, the faster polyprotein processing results in a shorter half-life of the negative-strand replicase, and negative-strand RNA synthesis is therefore restricted to a shorter time. Previous work has shown that the temporal regulation of the 1/2 site is crucial and too efficient processing drastically reduces infectivity (Lulla et al., 2018). Accordingly, sequences at the 1/2 site that are recognized too efficiently can be compensated for by adaptive changes in the cleavage site itself or in the nsP2 protease domain, which normalize the cleavage rate. Similarly, growth defects caused by a hyperactive nsP2 protease can be rescued by compensatory changes in the 1/2 site, which result in sequences that are recognized less efficiently.

However, it should be noted that for CHIKV the P4 Arg-to-His substitution cannot entirely compensate for the loss of the G3BP-interaction. It is likely that G3BP fulfills multiple proviral functions at different stages of the viral life cycle. Various Old World alphaviruses interact with G3BP proteins but differ regarding their requirement for G3BP (paper III, Figure 1B) and utilize different functions (paper II). The work presented in this thesis contributes to our understanding of the proviral activities of G3BP during Old World alphavirus infection and paves the way for future investigations.

5 FUTURE PERSPECTIVES

Further work is required to explore and understand the versatile activities of G3BP during virus infection. This will also broaden our understanding of the viral replication cycle itself and potential cellular functions of G3BP.

In paper I and II we proposed a potential role for the formation nsP3:G3BP oligomeric structures in clustering of viral replication complexes, which efficiently promote SFV replication. Indeed, a noticeably patchy distribution of SFV spherules at the plasma membrane has been observed by scanning electron microscopy (Hellström et al., 2017). Based on our model, it can be hypothesized that in the absence of G3BP-binding the distribution of spherules should be more diffuse, which could be validated by scanning electron microscopy. However, we still lack a mechanistic understanding of the functional relevance of these clusters. We proposed that clusters of spherules at the plasma membrane would ensure a more efficient internalization and formation of CPVs in the perinuclear region, close to the endoplasmic reticulum. However, preliminary data with mutant SFV, which does not internalize its replication complexes (Mazzon et al., 2018), suggest that the proviral effect of GFP-G3BP1 1-135 for SFV is independent of spherule internalization (non-published observation). The SFV-based trans-replication system, utilized in paper II, could be a useful tool to investigate the functional relevance of the NTF2-like domain of G3BP. In this system, expression of the replicase is maintained in a replication-independent manner. Consequently, the influence of the NTF2-like domain specifically on viral RNA replication can be determined. In addition to the two FGDF motifs in nsP3, formation of nsP3:G3BP poly-complexes also depends on the ability of the NTF2-like domain to form dimers. Based on structural data of G3BP proteins (Kristensen, 2015; Vognsen et al., 2013), substitution of residues that make close contact with each other in the dimer interface might disrupt the dimer formation and hence, the oligomeric nsP3:G3BP structures, without disrupting the actual nsP3:G3BP interaction.

Based on the results from paper II we know that CHIKV replication strictly depends on the presence of the RGG domain. We have shown that this domain facilitates the recruitment of the translation initiation machinery (paper II), but CHIKV trans-replicase experiments also suggest a crucial role for G3BP during RNA replication (paper III), for which the recruitment of translation components cannot provide an obvious explanation. RGG domains are known to mediate RNA-binding, but have also been described to mediate protein-protein interactions, reviewed in (Thandapani et al., 2013). Further experiments are required to clarify if the RGG

domain of G3BP is capable of interacting with (viral) RNA or if its proviral activity is linked to protein-protein interactions. Direct RNA-binding activity of G3BP has been confirmed in previous work by an *in vitro* binding assay and deletion of a C-terminal region abrogated this interaction (Tourriere et al., 2001). However, the deleted C-terminal region covered the whole RGG domain and partially the RRM domain and hence, no conclusion about an RGG domain-mediated RNA-binding can be drawn. Also, the interaction with 40S ribosomal subunits, mediated via the RGG domain of G3BP, is partially RNase labile and might therefore partially be facilitated through RNA-interactions (Kedersha et al., 2016). *In vitro* studies with purified proteins would be required to clarify this issue.

It is known that the Arg residues within the RGG domain of G3BP are post-translationally modified through methylation, which regulates SG formation (Tsai et al., 2016). Charge neutralizing Arg-to-Phe substitutions, which mimics the effect of methylation, suppresses SG formation. It would be interesting to test the effect of the Arg-to-Phe substitutions in the context of CHIKV replication. Moreover, these mutants could be useful to validate if the proviral activity of the RGG domain correlates with its ability to interact with 40S ribosomal subunits.

Deletion of the RRM domain of G3BP1 supported CHIKV replication, however, replication was significantly delayed compared to G3BP1 WT (Figure 9B), which suggests a functional role of the RRM domain for CHIKV. The RRM domain of G3BP contains two conserved ribonucleoprotein motifs, commonly found in RNA-binding proteins (Parker et al., 1996). Indeed, it has been demonstrated that the RRM domain of G3BP1 mediates binding to the 5' terminal region of the HCV negative-strand RNA; the functional significance, however, has not been described (Yi et al., 2011). Whether the RRM domain can facilitate direct interaction of G3BP with alphavirus RNA remains to be investigated.

Our systematic approach to identify proviral functions of G3BP was mainly focused on G3BP1 (paper II). Nevertheless, G3BP2 alone can also promote efficient CHIKV replication (Kim et al., 2016) and also binds 40S ribosomal subunits (Kedersha et al., 2016). It is not yet known if both these functions are also linked to the RGG domain. The RGG domains of G3BP1 and G3BP2 share 63% sequence identity and differ regarding the number and order of RG and RGG motifs. Comparing the properties and sequences of both domains could help to identify proviral characteristics of the RGG domain. Recent experiments from our group show that expression of mosquito Rasputin in U2OS ΔΔ cells can rescue CHIKV replication, depending on the RGG domain. Plant G3BPs also contain RGG domains. A systematic analysis of these domains could provide insights into common characteristics of proviral RGG domains and potentially allow the prediction of other RGG domain-containing host proteins with proviral

properties. Interestingly, G3BP1, but not G3BP2, is required for efficient replication of HCV (Yi et al., 2011) and murine norovirus (MNV) (Fritzlar et al., 2019). For MNV, the role of G3BP1 could be linked to the RRM and RGG domains, which suggests functional differences of these domains between G3BP1 and G3BP2.

The P4 Arg-to-His substitution partially rescues CHIKV replication in absence of G3BP (paper III). However, this single substitution is not sufficient to promote replication as efficient as in the presence of G3BP. Passaging of CHIKV-RH in U2OS ΔΔ cells could lead to the emergence of adaptive mutations that further increase viral fitness in absence of G3BP. Identification of such adaptive mutations could provide clues to the role of G3BP.

Studying the influence of G3BP on alphavirus replicase activity in an *in vitro* system through biochemical assays could be highly informative. However, this approach is limited based on the fact that the replicase activity strictly depends on the formation of replication complexes in association with host membranes (Spuul et al., 2007). Interestingly, it has been demonstrated that membranous replication complexes can be purified and remain capable of producing viral negative- and positive-strand RNA *in vitro* (Pietilä et al., 2018).

The successful purification of alphavirus replication complexes could also be of benefit for the acquisition of high-resolution structures of the complete viral replicase and the membrane-associated replication complex. Those structures are still largely lacking for alphaviruses. The most comprehensive replicase structure available covers a region of the SINV P23 precursor, including the protease and methyltransferase-like (MT-like) domains of nsP2 and the N-terminal macro and alphavirus-unique/zinc-binding domains of nsP3 (Shin et al., 2012). A structure of the alphavirus replicase in complex with G3BP would certainly be of great value to understand the mechanistic function of G3BP. Due to technical advances, cryo-electron microscopy can overcome previous limitations in determining high-resolution structures of macromolecular complexes and might be a suitable method.

In paper III we show that several Old World alphaviruses are sensitive towards G3BP deletion, including medically relevant members, such as CHIKV and RRV. This makes the nsP3:G3BP interaction an ideal target for the development of antivirals. The mechanism of this interaction has already been described at near-atomic resolution which provides a good basis for *in silico* drug design.

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