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Role of alphavirus replicase in viral RNA synthesis, virus-induced cytotoxicity and recognition of viral infections in host cells

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Role of alphavirus replicase in viral RNA synthesis, virus-induced cytotoxicity and recognition of viral infections in host cells

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I. Pohjala, L., **Utt, A.,** Varjak, M., Lulla, A., Merits, A., Ahola, T. and Tammela, P. (2011). Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays. *PLoS ONE*, **6,** e28923. doi: 10.1371/journal.pone.0028923.

II. **Utt, A.,** Das, P.K., Varjak, M., Lulla, V., Lulla, A. and Merits A. (2015). Mutations conferring a noncytotoxic phenotype on Chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2. *Journal of Virology*, **89**, 3145–3162. doi: 10.1128/JVI.03213-14.

III. **Utt, A.,** Quirin, T., Saul, S., Hellström, K., Ahola, T. and Merits, A. (2016). Versatile *trans*-replication systems for chikungunya virus allow functional analysis and tagging of every replicase protein. *PLoS ONE,* **11,** e0151616. doi: 10.1371/journal.pone.0151616.

IV. Nikonov, A., Mölder, T., Sikut, R., Kiiver, K., Männik, A., Toots, U., Lulla, A., Lulla, V., **Utt, A.,** Merits, A. and Ustav, M. (2013). RIG-I and MDA-5 detection of viral RNA-dependent RNA polymerase activity restricts positive-strand RNA virus replication. *PLoS Pathogens*, **9**, e1003610. doi: 10.1371/journal.ppat.1003610.

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This thesis also contains unpublished data.

Author's contribution:

I. I participated in experimental design, performed selection of noncytotoxic replicons and stable cell lines, identified and confirmed cytotoxicity-reducing mutations, performed analyses of selected inhibitors using infectious CHIKV and analyzed the data.
II. I participated in experimental design and performed all experiments except the biochemical analysis of nsP2. I analyzed the data and wrote most of the manuscript.
III. I participated in experimental design, performed the cell culture experiments (except for CLEM and the immunofluorescence study), analyzed the data and prepared the manuscript.
IV. I performed western blot analysis and northern blot experiments using probes covering the entire SFV genome. I also assessed the infectivity of fractionated RNAs and analyzed the corresponding data.

LIST OF ABBREVIATIONS

aa	amino acid (residue)
BSL	biosafety level
CHIKV	Chikungunya virus
CMV	human cytomegalovirus
CPV-I	type I cytopathic vacuole (= replication organelle)
CSE	conserved sequence element
ds	double-stranded (for RNA or DNA)
ECSA	East/Central/South African (CHIKV genotype)
ER	endoplasmic reticulum
Fluc	*Firefly* luciferase
Gluc	*Gaussia* luciferase
GT	guanylyl transferase
HCV	hepatitis C virus
HVD	hyper variable domain (of nsP3)
IFN	interferon
IOL	Indian Ocean Lineage
IRF	interferon regulatory factor
kb	kilobase
kDa	kilodalton
MAVS	mitochondrial antiviral signaling protein (also known as IPS-1, VISA and Cardif)
MDA-5	melanoma differentiation-associated protein 5
MT	guanine-7-methyltransferase
MTL	methyltransferase-like domain (of nsP2)
mTOR	mammalian target of rapamycin
NES	nuclear export signal
NLS	nuclear localization signal
ns	nonstructural
nsP	nonstructural protein
nt	nucleotide (residue)
NTPase	nucleoside triphosphatase
ONNV	O'nyong'nyong virus
ORF	open reading frame
Pac	puromycin acetyltransferase
PAMP	pathogen-associated molecular pattern
PKR	protein kinase R
PRR	pattern recognition receptor
p.t.	post transfection
RC	replication complex
RdRp	RNA-dependent RNA polymerase
RIG-I	retinoic acid-inducible gene 1
Rluc	*Renilla* luciferase

RRV	Ross River virus
RTPase	RNA triphosphatase
SFV	Semliki Forest virus
SG	subgenomic
SINV	Sindbis virus
ss	single-stranded (for RNA or DNA)
STAT	signal transducer and activator of transcription
TF	TransFrame (protein)
UPR	unfolded protein response
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
VRP	virus replicon particle
WA	West African (for CHIKV genotype)
WEEV	Western equine encephalitis virus
wt	wild type
ZBD	zinc binding domain of nsP3 (also known as alphavirus unique domain, AUD)

1. INTRODUCTION

Viruses are subcellular infectious agents that are obligate intracellular parasites. Different viruses infect different hosts and, to a variable extent, take over host cells to replicate and produce mature progeny, namely, virus particles called virions. For these reasons, most viral infections are harmful and may eventually result in the death of the host cell. However, such instances of cell death are not always due to the specific actions of a virus: in multicellular organisms, for example, the death of virus-infected cells is commonly employed as a defense against viral infection. Hence, many viruses potently suppress cell death, especially during the early stages of viral infection. This is one of the reasons why a complex network of regulated interactions lies at the interface of viral infection and host antiviral response.

Alphaviruses are a group of globally distributed arthropod-borne positive-strand RNA viruses. This group includes several important human pathogens, including the re-emerging Chikungunya virus (CHIKV). Recent outbreaks of CHIKV infection have been responsible for millions of cases of acute illness and frequently result in long-term complications. There is no specific antiviral drug treatment or licensed vaccine available for the clinical management of CHIKV infection. One of the obstacles in the identification of new therapeutic agents and strategies against CHIKV has been the lack of suitable technical tools for CHIKV research. Thus, the study of the molecular biology of CHIKV is an important field of ongoing investigation. At the present time, numerous questions related to the molecular, cellular and organism levels of CHIKV infection remain unanswered.

CHIKV nonstructural protein 2 (nsP2) is a multifunctional protein, which, in addition to its functions as part of the virus replicase complex, counteracts interferon (IFN) expression and signaling. Furthermore, nsP2 induces cytopathic effects and the general shutdown of transcription and translation in vertebrate cells. Such shutdown is essential for limiting the production of antiviral proteins, mainly type I IFNs, which play a role in early innate immune response and are a first-line defense mechanism against viral infection. This shutdown also benefits the virus by reducing the capacity of an infected cell to signal surrounding cells *via* type I IFNs and other cytokines, thus expediting viral propagation. Depending on the balance of virus-induced cytotoxicity and cellular defense mechanisms, infection can end in either death of the host, persistent infection, or elimination of the virus. In the case of CHIKV, the exact outcome of infection is highly dependent on the activities of nsP2. Some mutations in nsP2 are involved in establishing a persistent infection and have been shown to prolong the survival of infected vertebrate cells. Such mutations can be used to adapt the alphavirus replicons for noncytotoxic growth in vertebrate cells or, possibly, to generate attenuated viruses that can be used as vaccines.

The central aim of the studies that form the basis of the current thesis was to develop and apply novel tools to study the molecular biology of CHIKV and to

gain insight into different aspects of virus-host interactions. This resulted in the creation of a large set of useful tools, the properties of which are highlighted below. The use of these tools led to several expected and unexpected findings. Interestingly and importantly, studies dedicated to seemingly different aspects of CHIKV molecular biology came together to produce a cohesive understand of the virus, indicating that they were not actually focused on different characteristics of viral infection but rather on different facets of the same process. Realization of this fact allowed better understanding of CHIKV replication and provided novel insights into the interactions that exist between alphaviruses and innate immune response pathways.

2. REVIEW OF LITERATURE

2.1 Alphaviruses

Viruses are the most numerous propagating biological objects on Earth, outnumbering living cells by at least an order of magnitude. They are also the only biological objects that may have genetic material other than double-stranded (ds) DNA. In fact, the largest group of viruses currently known possess single-stranded (ss) positive-strand RNA genomes. This group is also extremely diverse and consists of many recognized orders, families and genera as well as many unclassified viruses (and, by all likelihood, even larger numbers of unknown viruses). Each of these viruses has many unique properties as well as characteristics shared with several, many or even all positive-strand RNA viruses.

The *Togaviridae* family, a relatively small family of positive-strand RNA viruses, is divided into two genera: *Alphavirus* and *Rubivirus*. Their genomic organization is rather similar, despite that phylogenetic analyses have indicated that they are only distantly related (1). The genus *Rubivirus* contains a single member (rubella virus), while there are over 30 currently recognized members of the genus *Alphavirus* (2). Most alphaviruses are transmitted by arthropod vectors (usually mosquitoes) to a wide range of vertebrate species (most commonly birds or mammals). Infection of insect vectors is persistent and lifelong, with minimal effect on the viability of the vector; conversely, in vertebrates, infections are mainly acute and self-limiting, ending with the death of the host or clearance of the pathogen by the immune system. Thus, most alphaviruses are classical arboviruses (3). The few exceptions include fish-infecting alphaviruses, which lacks known arthropod vector, and the recently discovered Eilat virus, which infects only mosquitoes and cannot replicate in vertebrate cells (4, 5).

Alphaviruses that infect birds and mammals can been divided into New World and Old World viruses, depending on their geographic distribution (6). New World alphaviruses, including Eastern equine encephalitis virus, Venezuelan equine encephalitis virus (VEEV) and Western equine encephalitis virus (WEEV), are found in North and South America and typically cause encephalitis in humans and other mammals. Old World alphaviruses, such as Chikungunya virus (CHIKV), O'nyong'nyong virus (ONNV), Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus (SINV), are found in Europe, Africa, Asia and Australia (6, 7). Old World alphaviruses generally do not cause encephalitis (though it can occur, especially in experimental models); instead, they cause illness characterized by fever, rash, chills, headache, myalgia, vomiting and arthralgia. The disease is commonly acute; however, for some of these viruses (most notably CHIKV, see below), debilitating joint pain can persist for months to years after infection. Chronic disease has been linked to persistent viral replication in target cells and/or the establishment of a self-sustained inflammatory mechanism that leads to tissue damage (8–10).

The genus *Alphavirus* is endemic on all six continents. Historically, the range of individual species of alphavirus was confined to certain regions by environmental barriers and/or the geographical distribution of suitable vectors. However, climate change, globalization and the ease of overseas travel have increased the spread of alphaviruses to other regions habituated by competent mosquito vectors. Typically, the human populations inhabiting such regions lack pre-existing immunity. Thus far, only one alphavirus, CHIKV, has managed to achieve a large-scale spread in this manner (11, 12); however, in the future, other alphaviruses may also spread. CHIKV has historically caused small outbreaks in confined regions within Africa and Asia (13). However, it is possible that some outbreaks that have not been recognized as Chikungunya fever have occurred outside of this region. The 2004–2007 epidemic in the Indian Ocean region and India demonstrated the potential of CHIKV to rapidly spread and establish itself in previously unaffected areas (11, 14). This was further emphasized in late 2013, when a locally transmitted CHIKV infection was detected on St. Martin Island in the Caribbean, and the virus subsequently became established in Central and South America, Mexico and the mainland United States (12). To date, CHIKV has been identified in over 60 countries in Asia, Africa, Europe and the Americas (Fig. 1).

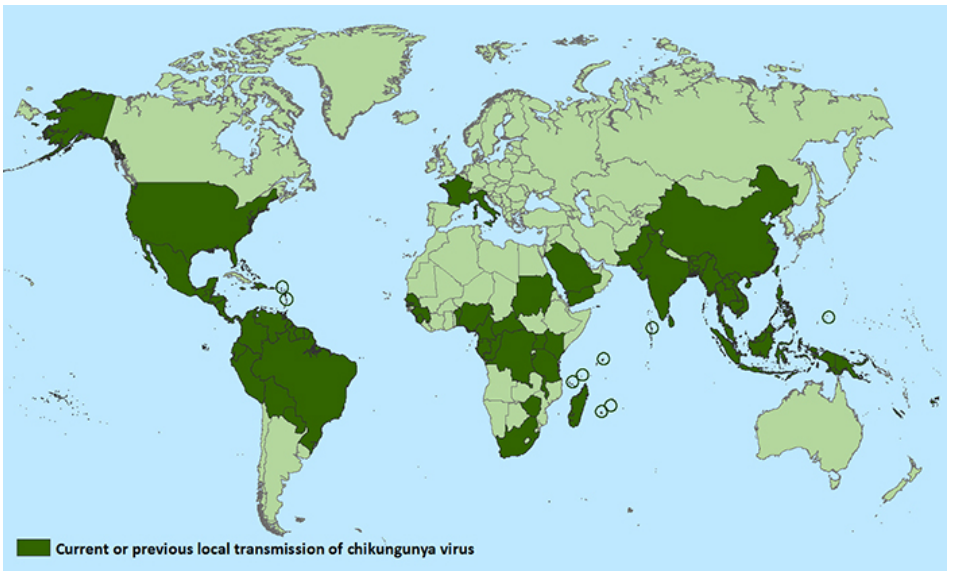

Figure 1. Global distribution of CHIKV as of October 2015. Countries and territories with local transmission are shown in dark green (http://www.cdc.gov/chikungunya/geo/index.html, permission obtained from the Centers for Disease Control and Prevention).

Many well-studied alphaviruses have several different strains and genotypes that are found in different areas and/or associated with different diseases/ symptoms. It should be noted that the classification of alphaviruses into strains, genotypes and lineages is not firmly established, and different terms are often used to designate the same viruses. Three CHIKV genotypes have been identified since its discovery in Tanzania in 1952: the West African (WA) genotype, East/Central/Southern African (ECSA) genotype and Asian genotype (15). Viruses belonging to the ECSA and WA genotypes have approximately 5% differences in the amino acid (aa) sequences of their encoded proteins. These differences are not evenly distributed and are most common in the C-terminal region of nsP3 (see below). The most variable part of the CHIKV genome (also other alphaviruses), however, is the 3' untranslated (UTR) region, which displays considerable variations in length, sequence and other properties (16, 17). These differences create the possibility that at least some functions of alphavirus-encoded proteins or of the *cis* elements of alphavirus genomes may differ between different CHIKV genotypes.

The Indian Ocean lineage (IOL) of CHIKV is a variant of the ESCA genotype that arose in 2004 (18, 19). Several IOL strains have adapted to a new vector, *Aedes albopictus*, without significantly compromising viral fitness in the initial vector, *Aedes aegypti*, thereby increasing the epidemic potential of the virus. Genomic sequencing of CHIKV isolates with increased infectivity for *Aedes albopictus* revealed one single aa change, in the virus envelope glycoprotein E1 (Ala226 to Val), that is responsible for the above phenotype, demonstrating how easily alphaviruses can switch to new vectors or increase the number of vector species they can infect (20).

2.1.1 Alphavirus virion

Alphaviruses have enveloped virions of approximately 70 nm in diameter. A single copy of a positive-strand RNA genome is assembled with 240 copies of capsid protein into a T=4 icosahedral nucleocapsid with a diameter of 40 nm (21). The nucleocapsid is surrounded by a host-derived lipid bilayer in which two transmembrane envelope glycoproteins, E1 and E2, are embedded. These two proteins are organized as 80 trimers of heterodimers (thus, the structure is 3×(E1+E2)), which are referred to as spikes (22, 23) (Fig. 2). The glycoprotein spikes and the nucleocapsid core are linked through interactions that occur between the capsid proteins and the cytoplasmic tails of E2; this interaction ensures 1:1 ratio of capsid protein and E1+E2 dimers in virions (24–27). This, together with size and structure of glycoproteins, ensures that alphavirus virions have very regular structure and that the membrane layer is fully covered by an outer glycoprotein layer. The regular structure of the alphavirus virion and the stability of its major glycoproteins have facilitated analyses of the molecular details of virion structure. For CHIKV, the 3D structure of E1-E2 dimers (both in mature form and as precursors) has been resolved and, in combination with high quality cryo-electron microscopy data, it has resulted in a high-resolution structure of CHIKV virions (28).

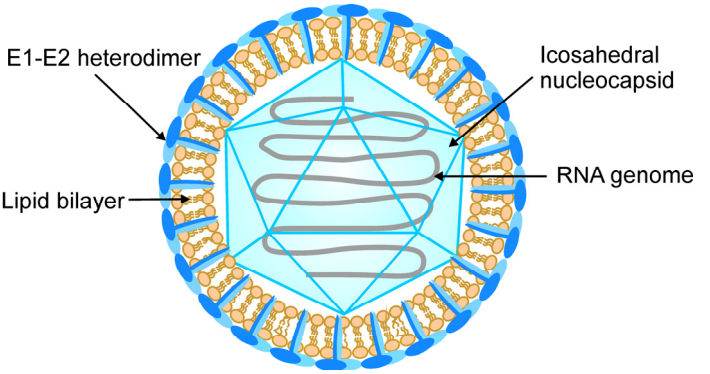

Figure 2. Schematic representation of an alphavirus virion. The alphavirus virion consists of a nucleocapsid surrounded by a lipid bilayer. The nucleocapsid is formed by a positive-strand RNA genome (grey) and 240 copies of capsid protein (lightest blue). The lipid bilayer (orange) is embedded with 240 heterodimers of the viral glycoproteins E1 and E2 (blue and light blue), which are arranged into 80 spike structures.

The E2 protein is responsible for receptor binding (29, 30). It is also the major antigen of the alphavirus virion and a target for broadly neutralizing antibodies that block both the entry and the egress of alphavirus virions (31). The E1 protein includes a fusion peptide that becomes exposed under low pH conditions, such as in endosomes. This initiates the fusion of the viral envelope and the endosomal membrane and ensures the release of the nucleocapsid into the host cell cytoplasm (32, 33). Moreover, alphavirus virions contain two small membrane-association proteins known as 6K and transframe (TF) (34, 35). The virions of some alphaviruses (such as SFV) also contain a third small glycoprotein, known as E3; however, this protein is not incorporated into CHIKV, SINV or WEEV virions (36). The 6K, TF and E3 proteins are important for regulating spike assembly and are necessary for efficient budding of the virus (37–39). E3 also has a role in protecting the E1 protein against the low pH conditions found in the secretory pathway and thus prevents premature exposure of the fusion peptide (40). The precise roles of the 6K and TF proteins are not known; however, both proteins affect the ability of virus to replicate (most likely influencing the release of virions from infected cells) and have impact on *in vivo* pathogenesis (41, 39, 38).

2.1.2 Genome organization

The alphavirus genome is a positive-stranded RNA molecule of approximately 12 kilobases (kb) in length. As in all positive-strand RNA viruses, the naked RNA genome of alphaviruses is sufficient to initiate the complete replication cycle (42).

The coding sequence of the genome consists of two large open reading frames (ORFs), which encode 10 proteins in total (Fig. 3). The first ORF,

covering approximately two-thirds of the genome, is translated directly from the genomic RNA and encodes the nonstructural (ns) polyprotein or polyproteins, depending on the presence or absence of a stop codon at the end of the nsP3 coding region. The ns polyprotein and nsPs are virus-specific components of the alphavirus replicase complex. The second ORF, corresponding roughly to one-third of the genome, encodes the structural proteins that function in the assembly of new virus particles and may also have other functions in the virus replication cycle. This ORF is translated from a subgenomic (SG) mRNA using an internal SG promoter located on the negative strand of a dsRNA replicative intermediate (42, 43). Both the genomic and SG RNA have a 5′ terminal cap structure and poly(A) tail on the 3′ end similar to eukaryotic mRNA (7). These elements are required for the stability and translation of the viral genome, as well as for its efficient replication and transcription. Interestingly, it was recently described that noncapped versions of these RNAs are also produced at all stages of infection and that this may be essential for virus infection (44).

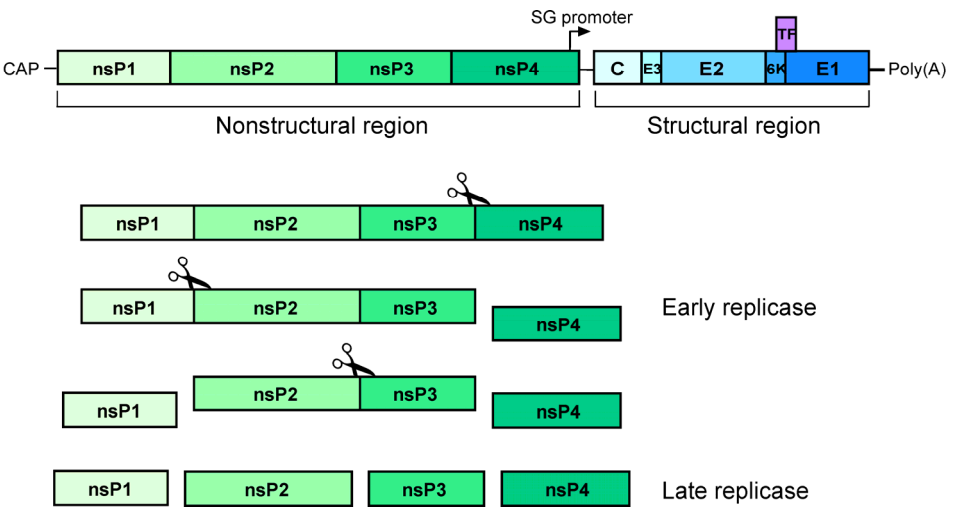

Figure 3. Alphavirus genome organization (above) and order of nonstructural polyprotein processing at early stages of infection (below). The alphavirus genome contains two ORFs that encode both ns and structural proteins. The precursor of the ns proteins (nsP1, nsP2, nsP3, and nsP4) is translated directly from the genomic RNA (green region). The precursors of the structural proteins (C, E3, E2, 6K, TF and E1) are translated from the SG RNA (blue region). The TF protein (violet) is produced by a ribosomal frame-shift that occurs in the region encoding the 6K protein. At early stages of infection, the protease activity of nsP2 cleaves the P1234 polyprotein into P123 and nsP4 (early replicase), which are subsequently processed into the short-lived nsP1+P23+nsP4 complex and finally into the late replicase, which consists of mature nsPs. This is the only expression and processing pathway leading to formation of functional replicase complexes.

In addition to its coding sequences, the alphavirus genome contains a short 5' UTR, a 3' UTR of variable length and a short intergenic region between the ns- and structural ORFs (7). All these regions contain important *cis*-acting elements. The most important *cis*-acting elements are conserved between different alphaviruses and are therefore called conserved sequence elements (CSE). In total, there are four CSEs in the alphavirus genome, and all are necessary for the replication and transcription of the virus RNA (2, 7):

- CSE1 approximately corresponds to the 44 first nucleotides (nt) located at the 5' end of the genome. This region forms a stem-loop structure that is important for the initiation of negative-strand RNA synthesis (45). In the context of negative-strand RNA, CSE1 functions as a promoter for positive-strand RNA synthesis (46). Interestingly, the secondary structure of this region was identified as a determinant of alphavirus pathogenicity. The structure was shown to mask the viral cap0 structure, which differs from the cellular cap1 structure and should therefore be recognized by the host IFN-induced protein with tetratricopeptide repeats (IFIT1) (47, 48). Thus, the presence of the specific RNA structure prevents the detection of viral RNA by the host and the subsequent activation of the innate immune system, resulting in enhanced translation and replication of the alphaviral genome in vertebrate cells.
- CSE2 (51 nt in length) is located near the 5′ end of the genome within the sequence that encodes nsP1; its secondary structure is represented by two stem-loop structures (46). This element enhances both negative- and positive-strand RNA synthesis (45). However, it has been shown that CSE2 is required only in insect cells and not in vertebrate cells, suggesting its possible recognition by host cell type specific factors (49).
- CSE3 overlaps with the sequence that encodes the C-terminus of nsP4 and, for some alphaviruses, extends to the intergenic region (43). CSE3, which is 24 nt in length, is also referred to as the minimal SG promoter because it is essential for the synthesis of SG mRNA. The SG promoter (in its full-length form) is exceptionally efficient. It is frequently used in alphavirus-based expression systems, where a duplicated SG promoter drives the expression of a gene of interest concurrent with virus replication (50–52).
- CSE4 is a 19-nt element located immediately before the poly(A) tail. It acts as a part of the promoter required for the synthesis of positive-strand RNA, probably *via* interaction with the 5′ and 3′ ends of the full-length genomic RNA. It also contains the start site for negative-stand RNA synthesis (45, 53). To be functional, CSE4 must be followed by at least 11 adenosine residues (54).

In addition to the CSEs needed for RNA replication or transcription, the alphavirus genome contains the following structured RNA elements that are also important for alphavirus infection:

- The packaging signal for the alphavirus genome is located in ns region of the genome. The position of this signal varies between different alphavirus

species (55, 56). For most alphaviruses, it is located in the region encoding nsP1; however, for members of the SFV group (including CHIKV), the packaging signal is located in the region encoding the protease domain of nsP2 (57).

- Many alphaviruses, including VEEV and SINV, contain a specific signal (stem-loop RNA structure) that enhances read-through of the in-frame termination codon, which is present at the end of the sequence encoding nsP3 (58).
- In some alphaviruses (SFV, SINV), the 5' end of the sequence encoding the capsid protein contains a translational enhancer, which is needed for efficient translation of the viral structural proteins in an infected cell. Recombinant viral vectors that lack this enhancer produce approximately 10–fold less protein from their SG RNA than those that contain the enhancer (59, 60). However, the function of this region as a capsid enhancer is not conserved among the alphaviruses; it is apparently absent from the genomes of several members of the genus, including VEEV and CHIKV.
- Another interesting feature of the SG RNA is that the reading frame for the structural proteins contains a −1 ribosomal frameshift signal located in the sequence encoding the 6K protein. This frameshift event leads to the expression of the structural TF protein (35).
- Many *cis*-acting sequences are located in the 3' UTR. These elements are often present as repeated sequence elements, and their copy numbers (and thus the length of the 3' UTR) show considerable variation. These sequences are needed for host-specific adaptation and are also important for stability of alphavirus RNA (16, 17, 61, 62).

2.2 Alphavirus infection cycle

The currently available information about the alphavirus infection cycle is fragmented: some stages of the infection process have been studied using one virus, while other stages have been studied using another virus. These pieces of information have been combined to obtain a general picture. The problem with this approach is that several studies have shown that not all key processes are executed in the same manner by all alphaviruses. Furthermore, two of the most well studied alphaviruses, SFV and SINV, are not really closely related; hence, the data obtained from studying these viruses are sometimes contradictory. Other alphaviruses that have also been studied in more detail include VEEV (a New World alphavirus) and CHIKV (phylogenetically relatively close to SFV). The resulting picture presents a puzzle where some parts are missing and some fragments are probably incorrectly placed. However, as replication is the most conserved process in viral infection, it is reasonable to assume that the picture that has been generated reflects an "average" situation relatively well and can be applied (with some modifications) to each individual alphavirus.

In vivo alphavirus infection starts when an infected mosquito feeds on a vertebrate host. The initial stages of *in vivo* infection are relatively poorly understood. Infection begins in the tissues surrounding the bite or in regional lymph nodes and then spreads to other organs. The landmark of alphavirus infection is the presence of a very high amount of virus in blood (viremia) that is required to infect mosquitoes and ensures viral transmission. However, as the infection is usually self-limited, an infected vertebrate is only capable of infecting mosquitos for a relatively brief period of time, namely, after viremia is established but before the immune response limits the amount of circulating virus (63).

During mosquito feeding, alphavirus virions are thought to be released within the dermis and into the subcutaneous capillaries of the skin. The virus delivered by mosquito (possibly around 100 plaque forming units/bite) is more infectious than the same amount of virus delivered by injection. This indicates that mosquito saliva has a role in the early stages of *in vivo* infection, although the molecular basis of this phenomenon has only resently started to emerge (64). Depending on the host and virus, alphaviruses reach the blood within 2–4 days and then disseminate to other parts of the body. The sites of subsequent virus replication vary with the virus and the host. The target organs of CHIKV include joints, muscles, skin, and, less frequently, liver, kidneys, eyes and the central nervous system. Infection of these organs is frequently associated with a marked infiltration of mononuclear cells such as monocytes/macrophages (65–67). During the 7- to 12-day-long acute viremic period, CHIKV load can reach 10^9–10^{12} viral particles per milliliter of blood. Viral RNA can persist in synovial macrophages for many weeks (and sometimes month or years) after clearance of the virus from blood (68). The significance of this phenomenon for the virus transmission cycle is not known. It should also be noted that the basic steps of alphavirus infection in mosquitoes are quite different from those in vertebrate hosts; in mosquitoes, infection beings in the midgut, and the virus disseminates to different organs, including the salivary glands. In case of both vertebrate and mosquito hosts, the replication cycle of alphavirus, especially the molecular details, are better known for *in vitro* system. Hence, most of the data reviewed below originated from different (mostly vertebrate) *in vitro* models.

2.2.1 Binding and entry

At the beginning of infection, alphavirus virions bind to receptors on the surface of host cells (Fig. 4). Generally these viruses are able to infect a large variety of cell types as well as cells from very different species, such as vertebrate hosts and arthropod vectors. The wide host range of alphaviruses may in part result from the ability of these viruses to bind to different receptors. Many proteins and polysaccharides have been implicated as being part of the receptor complexes used by alphaviruses. However, understanding the full details of virus-receptor interaction is challenging, as a single alphavirus can use different types

of receptors and, conversely, some receptors are shared between different alphaviruses. As a consequence, our knowledge about this step of alphavirus infection is fragmented. The high-affinity laminin receptor has been identified as a receptor for SINV in mammalian cells and for VEEV in mosquito cells (69, 70). In addition, heparan sulfate has been also demonstrated to act as a binding receptor for SINV (29, 71). The natural resistance-associated macrophage protein (NRAMP; a divalent metal ion transporter) was shown to mediate SINV, but not RRV, entry into both mammalian and insect cells (72). Recently, many new receptor candidates for CHIKV have been found. For example, prohibitin, phosphatidylserine receptor TIM-1 and glycosaminoglycans have all been suggested as CHIKV receptors in mammalian cells (73–75), and ATP synthase β subunit serves as a receptor in mosquito cells (76).

Alphaviruses are generally internalized *via* clathrin-mediated endocytosis. Curiously, a mechanism involving direct fusion of the viral membrane with the plasma membrane and subsequent entry of the viral genome into cells has been described for SINV (77, 78). The majority of classical and novel studies, however, support viral entry by endocytosis. According to these studies, as endocytosis proceeds, endosomal vesicles containing virions mature, and the pH in these vesicles becomes acidic. This change in pH subsequently induces conformational changes within the virion envelope. Specifically, the low pH of endocytic vesicles destabilizes E1-E2 heterodimers and leads to the exposure of the fusion peptide of E1, which was previously shielded by E2 (33). Subsequently, the fusion peptide inserts into the endosomal membrane, where it leads to the trimerization of E1 proteins (79, 80). This triggers fusion between the viral envelope and the endosomal membrane and results in the release of the nucleocapsid into the host cell cytoplasm. Finally, the disassembly of the nucleocapsid is facilitated by ribosomes, which actively remove the capsid proteins, thus releasing the genomic RNA and enabling the initiation of ns polyprotein synthesis (81, 82) (Fig. 4).

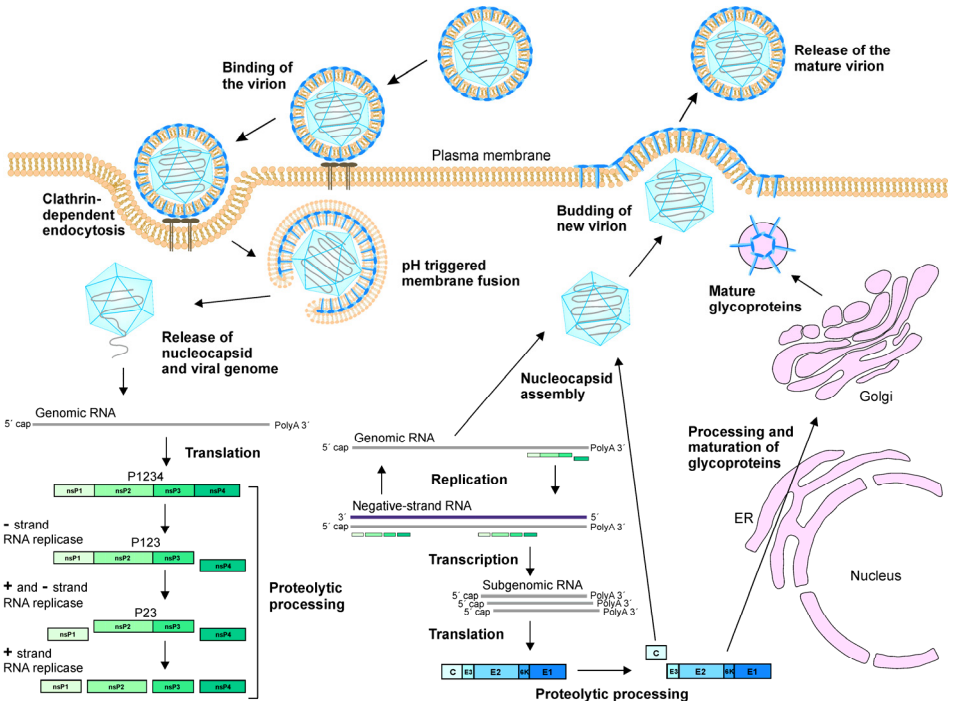

Figure 4. Alphavirus infection cycle. Alphavirus infection starts when a virion binds to a cellular receptor and then enters into the cell by clathrin-dependent endocytosis. The fusion of endosomal and virion membranes is triggered by low pH and results in the release of nucleocapsids, and subsequently viral genomic RNA, into the cytoplasm. The genomic RNA is immediately translated to yield the P1234 polyprotein (green), which is converted into the viral replicase through well-ordered proteolytic processing. The early replicase synthesizes a complementary (negative) RNA strand, leading to the formation of a dsRNA replication intermediate, which is subsequently used by the late replicase to create new genomic and subgenomic RNA molecules. The subgenomic RNA drives the expression of the structural polyprotein, which is co- and post-translationally processed. The C protein self-releases and associates with newly synthesized genomic RNA to form the nucleocapsid. The processing and maturation of the viral glycoproteins occur in the ER and Golgi. Mature glycoproteins are then transported to the plasma membrane. The nucleocapsid associates with glycoproteins and this binding event triggers the budding and the release of the virion.

2.2.2 Replicase expression and viral RNA replication

Almost all of what is known about alphavirus replicase expression and RNA replication has originated from studies of SFV and SINV. It is commonly assumed (though almost never directly proven) that these findings apply to other alphaviruses as well.

Once inside a cell, the positive-strand viral RNA genome directly serves as mRNA for ns polyprotein synthesis (83). For most alphaviruses (e.g. SINV and

VEEV), the prominent ns polyprotein is P123, as there is an opal termination codon in the end of the region encoding nsP3. A translational read-through, occurring with 10–20% efficiency, results in the synthesis of the P1234 polyprotein, which also contains the RNA polymerase (nsP4) region (84). In some strains of SFV, ONNV and CHIKV, the opal terminator is absent, and the only translational product of the virus genome is the P1234 polyprotein (85, 86).

The alphavirus RNA genome serves as a template both for the expression of replicase proteins and for the synthesis of negative-strand RNA. These functions are, however, mutually exclusive (they cannot occur on the same RNA at the same time). Hence, a mechanism(s) that enables a switch between ns polyprotein translation and RNA replication must exist. It has been hypothesized that this switch may be mediated by the actions of host cell proteins, such as G3BPs (87). Alternatively (or additionally), the RNA may become inaccessible to ribosomes through the formation of membrane-bound replication complex (RC) structures (see 2.2.3 for details). Indeed, it has been shown that alphavirus ns polyproteins localize to the plasma membrane and bind to the inner surface of this membrane; these processes are crucial for SFV infection (88). As ns polyproteins also bind to CSEs of the genome it is obvious that the viral RNA also becomes localized to the same region. However, the order of events that follows membrane binding has not been revealed.

The full-length P1234 polyprotein is thought not to be capable of performing RNA replication. Similarly, the individual alphavirus nsPs are unable to assemble into functional replicase complexes (89, 90). This indicates that alphavirus RNA synthesis requires the presence of cleavage intermediates of the P1234 polyprotein. The processing of P1234 is mediated by specific protease activity of nsP2 or the corresponding region of the ns polyprotein (90, 91). Both the timing and the order of cleavage events are important: only one processing pathway is known to lead to the assembly of a functional replicase (Fig. 3). Furthermore, the activities of the replicase are controlled by changes in the ns polyprotein. Namely, at early stages of infection (generally up to 3–4 h post-infection, p.i.), the P1234 is first cleaved between nsP3 and nsP4 to yield P123 and nsP4, which form an unstable early RC (92) (Fig. 3 and 4). This early replicase (also called the negative-strand replicase) binds to the genomic RNA and uses it as a template for the synthesis of one full-length negative-strand RNA, which forms dsRNA with its template (92–94). Next, a second cleavage event occurs between the nsP1 and nsP2 regions of P123, yielding nsP1, P23 and nsP4. This is known as the intermediate replicase complex, which theoretically is able to produce both positive- and negative-strand RNAs (92, 95, 96). In reality, however, it is unlikely that this replicase produces any of these RNAs due to the extremely short half-life of the P23 polyprotein (97). Thus, a third and final cleavage (P23 into nsP2 and nsP3) follows almost immediately and leads to the formation of a stable complex consisting of individual nsP1, nsP2, nsP3 and nsP4 proteins (Fig. 3 and 4). Under normal circumstances, this complex is unable to synthesize negative-strand RNA; instead, it produces both genomic and SG RNA, and this process continues until the cell dies (92, 94).

It should be noted that some of these rules are not absolute. For instance, it has been shown that certain mutations in nsP2 result in instability of the late replicase and enable consistent synthesis of the negative-strand RNA (98). Furthermore, some temperature-sensitive mutants of SINV can re-activate the synthesis of negative-strand RNAs, even in the absence of protein synthesis (99). Finally, a SINV mutant that is unable to process P123 due to mutations in the cleavage sites is capable of synthesizing both negative- and positive-strand RNAs (95). These facts indicate that the processing of P123 does not change the specificity of the viral replicase; rather, changes in the strand specificity of the virus replicase control P123 processing (97).

The produced genomic RNA can interact with newly synthesized capsid proteins (translated from SG RNA), resulting in the formation of nucleocapsids (100). However, at early stages of infection, the concentration of capsid protein in cells is low, and newly synthesized viral genomes re-enter into the RNA replication process to produce new ns polyproteins, which subsequently form early and late replicase complexes. However, at later stages of infection, the formation of new replicase complexes and the synthesis of negative-strand RNAs stops (101, 102). Two mechanisms contribute to this effect. First, the processing pattern of the P1234 polyprotein changes. This is caused by the accumulation of free nsP2, which cleaves new P1234 polyprotein molecules (possibly before their translation is completed) at the cleavage site located between the nsP2 and nsP3 regions. This results in the formation of P12 and P34 polyproteins that cannot form new replicase complexes (90) and are processed into individual nsPs that have also functions other than participation in RNA replication (see 2.3). Second, the inhibition of negative-strand RNA synthesis can also result from the shutdown of viral nsP expression that occurs at late stages of infection (7).

2.2.3 Sites of alphavirus RNA replication

The replication of alphaviral RNA occurs on cellular membranes. This is certainly not a unique feature, as all positive-strand RNA viruses of eucaryotes use and rearrange cellular membranes to create intracellular vesicles (or membranous webs) as an efficient way of isolating, protecting and concentrating viral components and to coordinate viral replication (103, 104). Alphavirus RCs, also called spherules, have the appearance of bulb-shaped membrane invaginations. Each spherule has an inner diameter of approximately 50 nm, and the interior of the invagination is always connected to the cytoplasm by a narrow neck-like structure (105). Spherules are formed during the early stages of infection, as their formation requires a specific pathway of P1234 polyprotein processing (90). In SFV-infected cells, spherules are located on the membranes of modified endosomes and lysosomes; such structures are called type I cytopathic vacuoles (CPV-I) and represent virus replication organelles (Fig. 5). However, it was recently shown that spherules are initially formed at the host

cell plasma membrane. Spherule formation is coupled with the synthesis of negative-strand RNA: in its absence no spherule formation can be observed (106, 107). In contrast, the synthesis of positive-strand RNAs is likely not required (108). Spherules contain dsRNA replication intermediates inside the cavities (109). Electron microscopy images have revealed that the length of the replication template plays a decisive role in defining the size of a spherule: shorter templates generate much smaller spherules than the full-length viral template. This is concordant with idea that each spherule contains a single dsRNA and also indicates that there are many determinants involved in spherule assembly and formation (110). Clearly, spherules also contain nsPs. However, the exact amounts, stoichiometries and localization patterns of the nsPs that are contained in spherules are not known. It has been hypothesized that nsPs are most likely located at the vesicle necks, which act as open channels for nucleotide import and newly synthesized positive-strand RNA export (105). Existing data also suggest that only a relatively small amount of nsPs exists in alphavirus spherules (106). In addition, each spherule also contains an unknown number of different host proteins.

After spherules are formed, they remain on the plasma membrane for only a short period of time. Later, the spherules are internalized through endocytosis and, in the case of SFV, are transported along microtubules to the perinuclear region (111). During this transport, they fuse with endosomes and lysosomes, which leads to the formation of large CPV-I structures. Surprisingly, interruption of this process by different inhibitors has no significant effect on viral RNA synthesis (106, 111). Recently, RC internalization was shown to be associated with the ability of the nsP3 of SFV to hyper-activate the phosphatidylinositol-3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway (112). This pathway has been previously shown to be involved in RC internalization (111). It was also found that mutations in SFV nsP3 that abolished this hyper-activation PI3K-Akt-mTOR pathway also interfered with RC-internalization – the RCs of the mutant viruses remained localized close to the plasma membrane, and large CPV-I structures were not formed. Moreover, clear differences between closely related alphaviruses have been documented; for example, CHIKV has a phenotype similar to those of mutant forms of SFV. Thus, CHIKV infection fails to hyper-activate the PI3K-Akt-mTOR pathway, and internalization of its RCs is rather inefficient, with spherules located close to the plasma membrane and the absence of prominent CPV-Is (112). Domain-swapping experiments have demonstrated that the differences in these phenotypes are determined by the C-terminal hypervariable domain (HVD) of nsP3; experiments with mutant viruses have revealed that the crucial determinants are localized at the beginning of the HVD. This finding is consistent with a previous observation that the nsP3 of SFV is crucial for the localization of nsPs into endo- and lysosomal compartments (89). In addition, it has been demonstrated that similarly located regions in the SFV and CHIKV nsP3 proteins have clearly different functions (112).

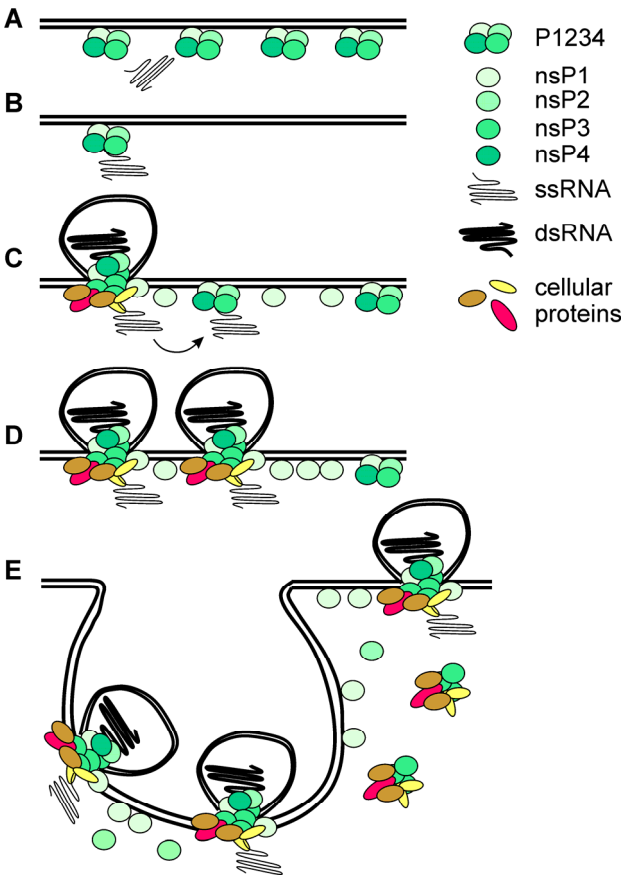

Figure 5. Formation of spherules during viral genome replication. (A) P1234 polyproteins are bound to the plasma membrane *via* the nsP1 region. (B) P1234 is processed into P123 and nsP4, which bind to the viral genome and initiate negative-strand RNA synthesis, leading to the formation of a dsRNA replication intermediate. (C) This process coincides with (and possibly induces) the formation of membrane-bound spherules on the outer surface of the plasma membrane and is followed by the processing of P123. These events result in the formation of late (also called positive-strand RNA) replicase complexes and the synthesis of genomic and SG RNAs, which are released from the spherules into cell cytoplasm (D). These newly created RNA genomes can bind to other available ns polyproteins (or serve as templates to produce more P1234), resulting in an increase in the number of early (and subsequently late) replicase complexes at the plasma membrane. (E) Continuing endocytosis results in the formation of vesicles containing multiple spherules (CPVs). (Figure is adapted from (106)).

2.2.4 Synthesis of structural proteins, virion assembly and budding

During alphavirus infection, large amounts of SG RNA are produced. This SG RNA serves as mRNA for the production of viral structural proteins. SG RNA is produced by the same RCs as viral genomes but tends to be 2- to 5-fold more abundant than genomic RNA. This is most likely because the SG promoter region in the negative-strand RNA template is more efficiently used by the replicase than the genomic promoter region. The structural proteins are translated in the form of a C-E3-E2-6K-E1 polyprotein (or a C-E3-E2-TF polyprotein if there is a frameshift event during translation), which is processed by a combination of viral and cellular enzymes (35, 113). The capsid protein contains a serine-protease domain in its C-terminal region and cleaves itself off from the rest of the structural polyprotein. This autocatalytic cleavage exposes a signal peptide in E3, which inserts the glycoprotein part of structural polyprotein into the endoplasmic reticulum (ER), where its translation continues using membrane-bound ribosomes (114, 115). Next, in the ER, the structural polyprotein is modified (glycosylated and palmitoylated) and then cleaved into the p62 (precursor of E3 and E2), 6K and E1 proteins by cellular proteases (116, 117). Then, p62 and E1 quickly form a heterodimer, which is transported from the ER to the Golgi complex. In the *trans*-Golgi compartment, p62 is processed by another cellular enzyme (furin) to form E2 and E3 (117). This cleavage completes the formation of the mature (fusion competent) E2-E1 heterodimers (118), which are transported to the plasma membrane, where they are incorporated onto the virion surface as trimeric spikes (40, 117). The fate of the E3 glycoprotein depends on the virus: it is a component of some alphavirus virions, such as SFV (119), but absent in others including SINV and CHIKV (36). The small 6K protein has also been found to be incorporated into virions. Although this protein is important for efficient virus budding, it is not absolutely required for particle release (34, 39, 37). The TF protein is also incorporated into virions, but its functions are unclear (38).

The assembly of alphavirus virions starts with the formation of the nucleocapsid. This occurs in the cytoplasm near the replication sites and is triggered by the recognition of an RNA encapsidation signal by the capsid protein (120). As the packaging signal is always localized to the ns region of the RNA genome, only genomic RNA is packed (121). Interaction of the capsid protein with the packaging signal leads to the multimerization of the capsid protein and results in the formation of the icosahedral nucleocapsid, which is then transported to the plasma membrane. Finally, the budding of the virus is triggered by an interaction between the capsid protein and the E2 glycoprotein (122, 123).

2.3 Functions of alphavirus nsPs

Alphaviruses encode four nsPs, all of which are required for the replication of the viral genome and the transcription of SG RNA. In addition to their functions as part of the viral replicase, each nsP has other important functions in the virus infection cycle. Moreover, all nsPs are also involved in interactions with host components.

2.3.1 NsP1

NsP1 (~60 kilodalton (kDa)) has multiple functions during alphaviral RNA synthesis. NsP1 is the only membrane anchor for the RC (89), it has guanine-7-methyltransferase (MT) and guanylyl transferase (GT) activities (124, 125), and it participates in the synthesis of negative-strand RNA (126). More specifically, it has been shown that nsP1 regulates negative-strand synthesis *via* interactions with the N-terminal region of nsP4 (127, 128).

In infected or transfected cells, free (not bound to RCs) nsP1 is found at the inner surface of the plasma membrane (105, 129), suggesting that it is specifically targeted there. NsP1 is tightly bound to membranes in the context of the ns polyproteins, in mature RCs, and as an individual protein (89). An amphipathic alpha helix in the central part of the protein is responsible for this membrane association. Interactions with membrane phospholipids mediated by the amphipathic helix are essential for the enzymatic activities of nsP1 of SFV. Thus, the substitution of a one single critical aa residue in this area that abolishes nsP1 membrane binding is also lethal for SFV (88). However, it has been shown that an association with membranes is not universally required for the enzymatic activities of the nsP1 for all alphaviruses (130). As described below (see 2.3.4), the same applies to the functional connection between nsP1 membrane association and the ability of the viral replicase to perform RNA synthesis. Palmitoylation in the C-terminal region of the protein strengthens its membrane binding and renders nsP1 similar to integral membrane proteins (129). Nevertheless, palmitoylation is not needed for the enzymatic activities of nsP1. Mutations preventing the palmitoylation of nsP1 have been reported to render the virus nonpathogenic for mice (131). However, it is not clear what exactly causes this defect. Namely, it was shown that mutation in the palmitoylation site drastically diminishes SFV infectivity, most probably because it interrupts the interaction between nsP1 and nsP4. This interaction could be restored by the emergence of secondary compensatory mutations. Thus, the palmitoylation-negative SFV used in animal experiments most likely represented a mixture of different pseudo-reverted viruses (132).

The N-terminal region of nsP1 is responsible for MT/GT activities involved in the capping of newly synthesized viral genomic and SG RNAs (124). This structure is essential for mRNA translation and prevents viral mRNA from degradation by cellular 5’ exonucleases. In addition, a point mutation that specifi-

cally destroys the GT activity of nsP1 was reported to be lethal for the virus (133). However, when the replicase of SFV harbors mutations in the catalytic site of nsP1, it is clearly capable of negative-strand RNA synthesis and can initiate spherule formation (108). The most likely explanation for this finding is that the negative-strand RNA of alphaviruses is not capped and hence its synthesis is not affected by mutations inhibiting cap synthesis.

The synthesis of alphavirus cap occurs through a unique mechanism. To initiate capping, the RNA triphosphatase (RTPase) activity of nsP2 removes the gamma-phosphate from the 5' end of the nascent RNA (134). Subsequently, GTP is methylated, forms a covalent m^7GMP-nsP1 intermediate and only then gets transferred to the mRNA to create the cap0 structure. Thus, the three last steps of the capping reaction are carried out by nsP1 (124, 135). In contrast, reactions used for the synthesis of cap structures of cellular mRNAs occur in a different order: GMP is first covalently bound to RNA and only then becomes methylated (124).

The functions of free (non-RC-associated) nsP1 are poorly understood. It has been shown that this protein localizes at the plasma membrane, initiates the disruption of the actin cytoskeleton and induces the formation of filopodia-like structures on the cell surface (136, 137). Only recently it has been shown that free nsP1 facilitates the release of virus particles by antagonizing the effects of the cellular antiviral protein tetherin (138). NsP1 is also the most stable alphavirus nsP and contributes to the stabilization of other nsPs in infected cells, which most likely occurs *via* the interactions that take place in the RC (139). It is likely that nsP1 also interacts with a number of cellular proteins, although their identities have not been revealed, in part because no alphavirus vectors expressing tagged but functional nsP1 have been constructed.

2.3.2 NsP2

NsP2 (~90 kDa) is the largest alphavirus nsP. It has multiple known enzymatic activities as well as many non-enzymatic functions. The N-terminal region (aa residues 1–470) of nsP2 has functions important for viral RNA synthesis and modifications, including RTPase and nucleoside triphosphatase (NTPase) activities (134, 140, 141). The ability of nsP2 to function as an RNA helicase has also been predicted (142). However, truncated versions of nsP2 lack this activity (140, 143), and only the full-length nsP2 of SFV (144) or CHIKV (143) are capable of unwinding dsRNA in 5'–3' direction. In addition, nsP2 was shown to have RNA matchmaker activity; again, only full-length nsP2 has this property. Thus, interaction(s) between different regions of nsP2 is absolutely required for some of its activities (RNA helicase, matchmaker) and greatly stimulates other (NTPase) activities (143). However, the significance of RNA helicase activity is not known. It is assumed that this activity may be required to unwind RNA secondary structures or dsRNAs formed during viral RNA replication. It has been proposed that nsP4, which synthesizes RNA in the 5'–3'

direction, and nsP2, which unwinds dsRNA in the same direction, likely act in a coordinated manner (143). RNA helicase activity is fueled by the NTPase hydrolysis activity of the same protein (141, 143, 144), and the same active site is also needed for RTPase activity (145). As a result, it is currently not possible to study the significance of these reactions for alphavirus separately from each other. Recent data suggest that these activities are absolutely required for RNA replication and that no RNA synthesis or RC formation takes place in their absence (108). Very recently, it was also shown that nsP2 and nsP4 together regulate replication fidelity of alphaviruses and that viruses can alter their RC fidelity to overcome intracellular nucleotide-depleting conditions (146).

A papain-like protease domain as well as an enzymatically nonfunctional methyltransferase-like domain (MTL) can be found in the C-terminal region of nsP2, which is the only part of nsP2 with resolved 3D structure (147). The protease activity of nsP2 is essential for the correct processing of the virus ns polyproteins, and it therefore coordinates the replication of the viral genome (148). The cleavage of the P1234 is very well regulated and involves both recognition of short cleavage-site sequences and presentation of these sites to the protease (149). The catalytic site of the protease is able to cleave the nsP3/nsP4 junction (hereafter 3/4 site) and the 1/2 site *in cis* (90, 148). In addition, the 3/4 site of SFV can be efficiently cleaved *in trans*. In contrast, the 2/3 site can be processed only *in trans* (90) because the nsP2 molecule sterically cannot cleave its own C-terminus (147). It has also been shown that, unlike other cleavage events, the processing of the 2/3 site of SFV requires a full-length nsP2 protease with an authentic N-terminus as well as a precise assembly of the cleavage complex. Even small changes in the N-terminus of the enzyme are poorly (or not at all) tolerated. Moreover, for cleavage of the 2/3 site, the region located ~165 aa downstream of the cleavage site (at the end of the N-terminal macro domain of nsP3) is absolutely required (97). Thus, other domains and structural configurations of nsP2 as well as other nsPs modulate the protease activity of the protein.

As noted above, the C-terminus of nsP2 also contains an MTL domain that is apparently nonfunctional as a methyltransferase because of the absence of a number of crucial structural elements (147). However, this region is essential for alphavirus replication: it has been reported to have roles in the regulation of negative-strand RNA synthesis and in the induction of cytopathic effects, which differentially modulate host defense mechanisms (150) (for more details, see 2.4 and 2.5 and publications **I, II, III**). Indirect data indicate that this region likely interacts with the N-terminal region of nsP2; clearly, its presence is crucial for the RNA helicase activity of nsP2 (143).

NsP2 proteins of Old World alphaviruses also have nuclear functions, including the shutdown of cellular transcription and the inhibition of type I IFN signaling (see 2.4 for details and references). For this, part of the nsP2 molecules localize to the nucleus of an infected cell. For SFV, the nuclear localization of nsP2 was originally proposed to be mediated by a canonical nuclear localization signal (NLS) located in the C-terminal region of the protein (151).

Mutation in this element blocks nsP2 translocation to the nucleus and remarkably reduces the ability of the virus to counteract the induction of type I IFN (152, 153). Mutations of this site also reduce the pathogenicity of SFV infection for mice (154). However, the mutations in the proposed NLS interrupt the nuclear transport of nsP2 only at +37°C and not at +28°C (153); concordantly, at reduced temperature this SFV mutant is fully capable of counteracting the type I IFN response (publication **IV**). Furthermore, none of the NLSes that have been predicted for the nsP2 of SINV have any role in the nuclear transport of the protein (155), and no classical NLS has been predicted in nsP2 of CHIKV. These data are more supportive of the idea that there is no true NLS in alphavirus nsP2. If this is the case, then mutations in the "NLS" of SFV most likely act by disturbing the conformation of the protein under normal (but not low) temperature. In short, it is not clear how the nuclear transport of nsP2 actually occurs.

NsP2 is also central for induction of shutdown of host cell translation. This process is different and independent from induction of shutdown of host cell transcription. However, the molecular details of this process are poorly understood because shutdown of host-cell translation is caused by several different mechanisms (156, 157).

Given its roles in the inhibition of host cell transcription and translation, it is not surprising that nsP2 is the most important factor causing the cytotoxicity associated with Old World alphavirus infection in vertebrate cells. Even the expression of nsP2 as an individual protein leads to shutdown of cellular transcription and translation (157, 158). Mutations in the C-terminus of nsP2 are often responsible for persistent alphavirus infection or for prolonged survival of infected vertebrate cells (159–161). A key feature of these mutants is their inability to inhibit host cell transcription (158, 162). The best-characterized mutation responsible for imparting a noncytotoxic phenotype to Old World alphavirus replicons has been mapped to the MTL domain. The residue affected is proline 726 (Pro726) in the case of SINV (158–160) or Pro718 in the case of SFV or CHIKV (153, 163). For SINV, this mutation reduces viral RNA replication levels and renders nsP2 unable to shut off host-cell transcription and translation. In the context of SFV replicons, substitution of Pro718 with a Gly residue reduces but does not completely eliminate their cytotoxic properties (153). In the case of CHIKV of the WA genotype, the effect of the Pro718 to Gly mutation is similar to that observed for SFV (163); however, as shown in the *Results and Discussion* section, this does not fully apply to CHIKV of the ECSA genotype (see 4.1). In addition, such cytotoxicity-reducing mutations in nsP2 result in reduced stability of late RCs as well as moderate to severe reduction of viral RNA synthesis and continuous negative-strand RNA synthesis in alphavirus-infected cells (98).

2.3.3 NsP3

NsP3 (~60 kDa) can be divided into three domains of roughly similar length: the N-terminal macro domain, the central zinc-binding domain (ZBD; also known as AUD – alphavirus unique domain) and the C-terminal hypervariable domain (HVD), which is predicted to be intrinsically unstructured (112). Historically, the role of nsP3 in alphavirus infection has been less obvious than the roles of the other nsPs. Surely, nsP3 is needed for RNA replication; furthermore, it has been shown to be the main determinant of SFV neurovirulence (164, 165). However, this protein has no relevant enzymatic activity, and with exception of its N-terminal macro domain, it shows no similarity with proteins from other viruses or organisms.

Macro domains are widely distributed throughout all eukaryotic organisms as well as bacteria and archaea, indicating an important basic biological function (166). Such domains are also found in the nsPs of several positive-strand RNA viruses, including hepatitis E virus, rubella virus and coronaviruses (1). The crystal structures of the macro domains of CHIKV, VEEV and SINV have been determined (167, 168). The alphavirus macro domain can bind RNA, poly(ADP-ribose) and ADP-ribose. It has been assumed that RNA binding might be the main function of the nsP3 macro domain in viral genome replication (167, 169, 170). In addition, macro domains of several alphaviruses exhibit very weak ADP 1'-phosphate phosphatase activity (167). This activity is not universal (the macro domain of SFV lacks this activity) (169) and cannot be logically connected with any known process occurring during alphavirus infection. This has led to the suggestion that this activity is not important for the viral lifecycle and most likely represents a consequence (or side effect) of another, currently unknown, enzymatic activity of the macro domain. It may be linked to the ability of nsP3 to bind poly(ADP-ribose) – a molecule added to numerous proteins by poly(ADP-ribose) polymerases. IFN-induced poly(ADP-ribose) polymerases have been shown to act as potent inhibitors of alphavirus infection (171); hence, it is logical to assume that alphaviruses may have a mechanism that counteracts the antiviral activity of these proteins. It is also clear that the macro domain interacts with other nsPs (97, 168). These interactions are important for the viral life cycle; for example, it has been shown that the C-terminal region of the macro domain has a role in ns polyprotein processing. Currently available data suggest that the macro domain is needed for the precise positioning of the cleavage site in the P23 precursor relative to the active site of the nsP2 protease to provide access (and cleavage) to the previously unexposed site (97).

The central domain of nsP3 is conserved among alphaviruses (7). This region was crystallized as part of a fragment of P23 and has been shown to bind zinc ions, which led to its name (ZBD). Mutational studies have revealed that this function is crucial for viral infectivity. In addition, its 3D structure suggests that this region participates in RNA binding and may assist the macro domain in this process (168). Beyond this, very little is known about the role of the ZBD.

A recent study found that a defect caused by the swapping of regions downstream of the ZBDs of SFV and CHIKV, was rescued by a second-site mutation located in the ZBD (112). These data indicate that the ZBD and HVD of nsP3 interact with each other and that this interaction is crucial for functional RC formation. It has also been recently shown that nsP3 is involved in the stabilization of nsP4 (164).

The C-terminal HVD is highly variable in length and sequence. However, it does contain some conserved motifs that are shared between few, many or all alphaviruses. As this region is intrinsically unstructured, it is also tolerant to different deletions as well as marker-protein insertions (41, 172). Based on the unstructured regions of other viruses, it can be assumed that the HVD interacts with multiple cellular proteins. Thus far, the most studied interaction is the binding of the nsP3 of Old World (but not New World) alphaviruses to G3BP proteins (or to Rasputin, a G3BP homolog in mosquito cells). This event inhibits the formation of stress granules during SFV, CHIKV and SINV infection (173–177, 87). Cellular stress granules are induced by many types of environmental stressors and act as a translational silent storage unit for mRNA. They are also induced by viruses and possibly function in cellular antiviral defense (178). Indeed, SFV lacking ability to inhibit stress granule formation is attenuated (173). Interestingly, however, the depletion of G3BP proteins from a cell also inhibits alphavirus replication (87), indicating that these proteins also have some proviral functions. In addition, a proline-rich motif within the HVD has been demonstrated to interact with amphiphysins. These interactions were shown to promote viral replication; however, the mechanism was not described (179). Clearly, the list of cellular proteins capable of interacting with HVD will continue to expand.

The N-terminal region of HVD is phosphorylated at multiple serine and threonine residues. It has been shown that SFV mutants that are totally defective in nsP3 phosphorylation remain viable but exhibit a decreased rate of RNA synthesis and reduced pathogenicity in mice (180). The significance of nsP3 phosphorylation for RNA replication has also been demonstrated for SINV (181). In SINV, the phosphorylation of the HVD plays a role in negative-strand synthesis (182). In the case of VEEV, the phosphorylation of this region is important for virus replication in insect cells but not in vertebrate cells (172). It has been assumed, but not directly demonstrated, that HVDs of other alphaviruses are similarly phosphorylated. Very recently, it was demonstrated that SFV, but not CHIKV, infection causes hyper-activation of the PI3K–Akt–mTOR pathway. The functional determinant for this activation was the HVD of SFV nsP3. Furthermore, the sequence elements required for the hyper-activation of the PI3K–Akt–mTOR pathway seemed to overlap with the phosphorylation region; however, nsP3 phosphorylation itself was not required. Interestingly, the ability to hyper-activate the PI3K–Akt–mTOR pathway correlated perfectly with the ability of viruses and their mutants to drive RC internalization (112).

2.3.4 NsP4

The RNA-dependent RNA polymerase (RdRp) activity of alphavirus replicase is located in the nsP4 (~70 kDa). The large C-terminal region of this protein contains a conserved GDD motif that is characteristic of viral RNA polymerases (183) and most probably has a 3D structure that is similar to other RdRps. The N-terminal region of nsP4 (~100 aa residues) is unique (conserved only among alphaviruses) and may lack a fixed 3D structure (184).

Compared to other nsPs, the amounts of nsP4 present in alphavirus-infected cells are relatively low. One reason for this is the presence of an opal termination codon located upstream of the nsP4-encoding region. This codon is present in most alphaviruses, and terminator read-through of this region occurs with only 10–20% efficiency (7). Another reason for the low abundance is an absolutely conserved N-terminal tyrosine residue that, according to the N-end rule, acts as a destabilizing aa residue (185). This results in rapid degradation of free nsP4; however, nsP4 proteins included in RCs are protected from degradation (186). Although nsP4 is directly responsible for the synthesis of viral RNAs, other nsPs are also required for this activity (187). The N-terminal Tyr residue in nsP4 (and possibly N-terminal domain of nsP4 as well) is involved in interactions with nsP1. These interactions are crucial for negative-strand RNA synthesis and are interrupted if the Tyr is replaced with a nonaromatic aa residue (188). The interaction between nsP4 and nsP1 is believed to be important for the formation of RCs (128, 189). Mutations introduced into the presumably unstructured N-terminal region of nsP4 have resulted in defects in negative- or positive-strand RNA synthesis (184). Additionally, nsP4 has also been suggested to interact with host protein components that modulate viral RNA replication (184, 190).

The expression of functional full-length nsP4 as a soluble recombinant protein has been extremely difficult to achieve. However, the expression and isolation of SINV nsP4 was achieved by truncating the N-terminal portion of the protein (Δ97nsP4). *In vitro* studies using Δ97nsP4 showed a lack of RdRp activity. However, the recombinant protein was shown to possess terminal adenyltransferase activity. This function is apparently needed for the synthesis of the poly(A) tail on positive-strand RNAs (54, 191). RdRp activity was detected only for the full-length recombinant nsP4 of SINV (187), indicating that the presence of the N-terminal region is crucial for this activity. To perform RNA replication, nsP4 should be able to bind CSEs in the viral genome; this function may be assisted by other nsPs. The requirements for the recognition and binding of different CSEs differ from each other. Thus, purified nsP4 is capable of performing synthesis of full-length negative- and positive-strand RNAs but not SG RNAs (192). This can partially be explained by the fact that nsP4 recognizes different CSEs using different aa motifs. Thus, motif required for recognition of the genomic promoter in the negative-strand RNA spans from nsP4 aa residue 531 to aa residue 538. In contrast, a peptide fragment that contained a sequence corresponding to aa residues 329–334 of nsP4 (presumably

corresponding to the fingers domain of RdRp) was found to be essential for binding to the SG promoter. In both cases, however, the binding of nsP4 to promoter sequences required the presence of other nsPs (193, 194).

2.4 Recognition of alphavirus infection by cell and viral counteraction to cellular defense mechanisms

Mammals have evolved elaborate defenses to fight infections by viruses. A large number of gene products capable of suppressing alphavirus infection can be expressed in response to a viral infection. However, the products of such genes are often rather harmful to host cells (195), preventing their continuous expression. Conversely, the window of opportunity for a host cell to counteract alphavirus infection is quite small – within a few hours, an alphavirus will overtake a host cell, which then becomes unable to respond to subsequent IFN treatment (196). Thus, the success or failure of a cell to respond to a viral infection depends on early recognition of the infection.

Cells are capable of recognizing pathogen-associated molecular patterns (PAMP) using various pattern recognition receptors (PRRs). The most common PAMPs associated with infection of RNA viruses are abnormal (from the cellular standpoint) RNA molecules, such as dsRNAs and RNAs lacking cap-structures at their 5' ends. Such molecules can be recognized by membrane-bound receptors such as Toll-like receptors and cytosolic RNA helicases as well as by different effector proteins such as protein kinase R (PKR). All these molecules have been shown to have some role in limiting alphavirus infection (197, 198). However, recent data clearly indicate the leading roles of cytosolic helicases, retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) in this process (199). Interestingly, there is considerable discrepancy regarding which of these proteins is the key factor for the detection of alphavirus infection (199–201). There are also important differences in the PAMPs that are recognized by these receptors. MDA-5 recognizes long (>4 kbp) dsRNAs (202), such as the dsRNA replication intermediates of alphaviruses. RIG-I can also recognize long dsRNA molecules; however, it also recognizes ssRNA molecules that contain a triphosphate structure at the 5' end (203) as well as short dsRNAs in which at least one RNA strand has a 5' triphosphate structure (204). While the recognition of short dsRNA duplexes by RIG-I requires the presence of a 5' triphosphate, this element is not needed for the recognition of dsRNAs with a length >200 bp (205). Alphaviruses have been shown to produce all of the above types of PAMPs: they produce long dsRNAs during the replication of their RNA, uncapped ssRNAs during the infection (44) and partially double-stranded RNAs harboring 5' triphosphates due to the ability of their replicases to bind and copy cellular RNAs (publication **IV**).

Activated RIG-I and MDA-5 both signal *via* mitochondrial antiviral signaling protein (MAVS). The activation of MAVS in turn activates several

downstream effectors, including interferon regulatory factors (IRFs) 3 and 7 (206, 207), which enter the nucleus and activate the expression of several cellular defense molecules, including type I IFNs (Fig. 6). It has been shown that the expression of antiviral genes in response to RIG-I activation is sufficient for the suppression of CHIKV replication, without the need for further amplification of the response (208). However, the secretion of type I IFN activates auto- and paracrine IFN signaling, resulting in the amplification and spread of the antiviral response (Fig. 6).

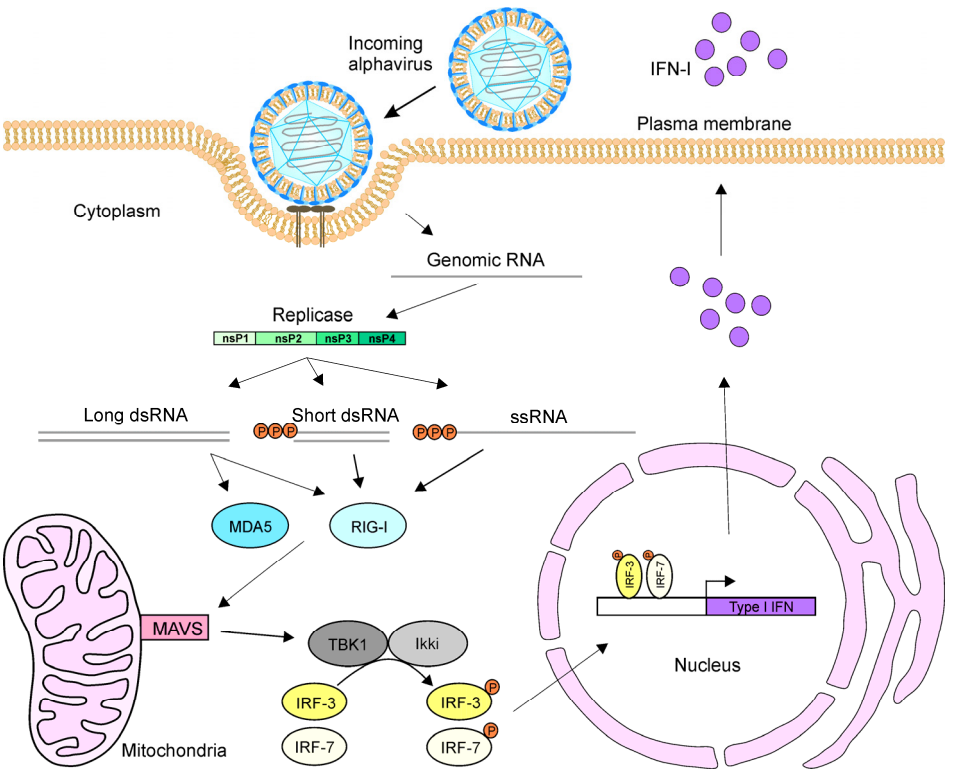

Figure 6. Simplified scheme of the induction of type I IFNs during alphavirus infection. Upon alphavirus infection, the viral replicase is responsible for the synthesis of dsRNA replication intermediates and, depending on virus, short partially dsRNAs (at least one RNA strand has a 5′ ppp structure) and viral ssRNAs with 5′ ppp structures. In immunocompetent cells, these PAMP molecules are recognized by the cytoplasmic pattern recognition receptors RIG-I and MDA5 (blue). Activated receptors initiate signaling through MAVS (an adaptor protein on the mitochondrial membrane), resulting in the activation of the transcription factors IRF3 and IRF7 (yellow). The activated transcription factors translocate into the nucleus, where they activate the expression of type I IFN genes, resulting in the production and secretion of IFN (violet). IRF3 and IRF7 also activate the transcription of other antiviral genes; similarly, the binding of type I IFN to its receptor on the cell surface results in additional signaling and leads to the amplification of the antiviral response (not shown).

As alphaviruses produce large amounts of PAMPs, they must be easily recognized and thus vulnerable to host defenses. However, viruses in turn have acquired counter-defenses that allow them to exist and continue to infect mammalian hosts. The simplest and most robust counter-defense is to rapidly shut down a host cell after infection and to produce progeny virus very quickly (7). The suppression of host cell gene expression also allows alphaviruses to use all cellular resources and metabolic pathways for their replication. Thus, it is not surprising that alphaviruses can effectively inhibit both host cell transcription and translation through separate mechanisms (157).

Transcriptional shutdown is essential to limiting the production of antiviral proteins, including type I IFNs, that play a role in the early innate immune response and represent a first-line defense mechanism against viral infection (152, 209). The virus also benefits from the shutdown because the capacity of the infected cell to signal to surrounding cells *via* type I IFNs and other cytokines is reduced, thus expediting virus propagation (157, 209). Old World alphaviruses use nsP2 to inhibit cellular transcription (158, 210). During the early phases of infection, nsP2 inhibits cellular transcription by inducing the rapid degradation of Rpb1, which is the catalytic subunit of the RNA polymerase II complex. Unlike picornaviruses, where viral proteases directly cleave their cellular targets, the protease activity of nsP2 does not play a major (or maybe any) role in this degradation; instead, nsP2 somehow induces the ubiquitination of Rpb1. Because Rpb1 is the subunit that catalyzes the polymerase reaction during RNA transcription, its degradation prevents both ongoing transcription and activation of cellular genes. Moreover, complete degradation of Rpb1 occurs before any other virus-induced change; therefore, it has been proposed that the degradation of Rpb1 is the first and most critical step in the down-regulation of the cellular antiviral response (210). Interestingly, New World alphaviruses use very a different mechanism to down-regulate cellular transcription. Their capsid proteins have both NLSs and nuclear export signals (NESs), respectively allowing them to bind to importin α/β and the nuclear export receptor RCM1. Thus, the capsid protein of these viruses forms complexes that accumulate at the nuclear pores and inhibit nuclear-cytoplasmic traffic, causing transcriptional shutoff (211). Interestingly, the capsid proteins of Old World alphaviruses, such as CHIKV, also contain both NLS and NES elements and traffic between the cytoplasm and the nucleus (212). The role and significance of this process for CHIKV infection are currently unknown.

In addition to the repression of overall gene expression, it has been shown that Old World alphavirus infection can also specifically inhibit the induction and expression of antiviral genes. This may be achieved by specific suppression of type I IFN expression (152) and/or by inhibition of IFN signaling. Indeed, some alphavirus strains, including those of CHIKV and SINV, have been shown to inhibit type I IFN-mediated signaling by interfering with the JAK/STAT1 (signal transducer and activator of transcription) pathway (213, 214). Inhibition of STAT1 phosphorylation and/or nuclear translocation is also an important determinant of alphavirus virulence (214, 215). Interestingly, the main factor

responsible for both of these effects is nsP2. In SINV mutants, the determinant of virulence was mapped to the 1/2 site of P123, suggesting that the critical factor determining a nonvirulent/virulent phenotype may be the release of nsP2 from the ns polyprotein. The significance of this event was highlighted by using a SINV mutant that was unable to process the P123 polyprotein (95). As studies describing the general shutdown of transcription, the specific shutdown of IFN expression, and the inhibition of IFN signaling mediated by nsP2 have been performed in different laboratories using different viruses and different cell lines, it is difficult to conclude whether these are all independent mechanisms or whether they represent different facets of the same process. However, as the elements of nsP2 that are required for the induction of the general shutdown of transcription and for the inhibition of STAT1 signaling are different (158, 163), it is likely that these processes (though mediated by the same protein) are different from each other. Thus, multiple mechanisms (including both general and more specific mechanisms) may be involved in the inhibition of type I IFN expression/signaling.

NsP2 also induces the shutdown of host cell translation. The molecular details of this event are not well understood because the shutdown of host cell translation is caused by several different mechanisms (156, 157). However, it is clear that alphaviruses use, at least in part, cell's own defense mechanisms to achieve this shutdown. The inhibition of translation in virus-infected cells is probably an ancient host defense mechanism that aims to block viral multiplication. In vertebrate cells, dsRNA-activated PKR, the expression of which is activated by type I IFN, is one of the key regulators of protein translation. Viral dsRNA induces strong PKR activation, which leads to the phosphorylation of the translation initiation factor eIF2α and to the inhibition of translation initiation in alphavirus-infected cells (156, 157). Therefore, similar to many other viruses, alphaviruses have developed ways to circumvent this mechanism and/or to use it for their own benefit. For example, the translation of SINV SG RNA does not depend on eIF2α, as it (similar to the SG RNAs of some other alphaviruses) contains a highly stable RNA hairpin loop (a translational enhancer) located downstream of the initiation codon. This structure can stall the ribosome on the correct site to initiate the translation of SG RNA, thereby bypassing the requirement for a functional eIF2α and facilitating continuous viral protein synthesis despite the presence of PKR activity (216, 217).

Another pathway leading to eIF2α phosphorylation and translation shutdown in alphavirus-infected cells is the unfolded protein response (UPR) in the ER (197, 218). The UPR offers a defense against high concentrations of misfolded proteins in the ER. The alphavirus glycoproteins E1 and E2 are expressed, processed and modified in the ER, where their quantities likely exceed folding capacity, leading to the accumulation of their unfolded forms. This is sensed by protein kinase R-like endoplasmic reticulum kinase, which then becomes activated and phosphorylates eIF2α, resulting in the repression of protein translation (218, 219). At least two different proteins have been shown to inhibit the UPR in CHIKV-infected cells: nsP4 (220) and nsP2 (221). Thus, alphaviruses

have multiple ways of triggering host defenses and also possess multiple means to counteract them or even to use them to their own advantage.

2.5 Alphavirus-based vector systems

The alphavirus genome is extremely amenable to genetic manipulation due to its small size and the existence of full-length infectious viral cDNA clones. Most vectors based on alphaviruses have been constructed for one of the following purposes: 1) for basic research efforts investigating the virus itself or 2) for use as biotechnological tools, in vaccine development or in gene therapy. There are different ways to classify alphavirus-based vectors. One possibility is to divide them into two basic categories, namely, replicon vectors and full-length replication-competent vectors, which are respectively based on the absence or presence of the structural region in the genome of the vector. In both of these systems, the viral replicase is produced from a replicating RNA template. Recently, a completely different approach has been applied: the replicase has been expressed from non-replicating mRNA transcribed from a standard (plasmid) expression vector, while replication-competent template RNA is produced from other plasmid vector (e.g., it is provided *in trans*). Thus far, such systems, called *trans*-replicase systems, have been mostly used to study alphavirus replication (107, 108, 110), but they can also potentially be applied as genetic vaccines and/or as tools to study virus-host interactions (publication **IV**).

2.5.1 Tagged alphavirus genomes

Full-length replication-competent alphavirus vectors have been designed to replicate in infected cells and to trigger formation of new virions. Thus, theoretically, such vectors can undergo unlimited rounds of infection. Although such vectors can be applied in gene therapy and biotechnology, they suffer from a limited capacity for the insertion of additional genetic material: due to the icosahedral capsid, the size of genome that can be packed into the virions is limited. Such vectors also suffer from genetic instability due to non-proofreading nature of the viral RNA polymerase and, perhaps more importantly, due to RNA recombination coupled with the growth advantage of shorter genomes that have lost inserted sequences (222). Thus, such vectors are typically designed for and used in studies of alphavirus infection, both *in vitro* and *in vivo*. For these purposes, virus genomes tagged with gene(s) that encode marker protein(s) are especially useful.

The simplest approach for marker gene insertion is to duplicate the sequence of the SG promoter and then place a sequence encoding a marker protein under the control of either a native or additional SG promoter (223, 224). Cells infected with such viruses can be easily recognized by the expression of the marker protein, enabling regions of virus replication to be identified *in vivo*. As

the quantification of marker protein expression is generally simpler than the quantification of viral proteins or the copy number of viral RNAs, the efficiency of virus replication (or an effect of an antiviral drug) is also easy to measure using markers (225, 226). However, in general, using such vectors provides little information about the virus replication process inside of the infected cell. The same applies for vectors where marker protein is expressed as a cleavable part of an alphavirus-encoded structural or ns polyprotein (212, 227).

To obtain additional information about replicase proteins and/or the replication process itself, a fluorescent tag must be fused to nsP such that the tagged protein remains functional (ideally, the insertion should cause no functional defect at all). Therefore, this insertion strategy requires either the screening of viral genomes using random insertion mutagenesis or educated guesses based on known or predicted 3D structures of nsPs. It has been shown that a marker protein can be inserted into many positions in the HVD of nsP3 as well as in some other regions of this protein. Similarly, there are at least three regions of nsP2 that can tolerate an EGFP insertion (228, 229). In contrast, no reports of successful tagging of functional nsP1 are available. This is also the case for nsP4, although this protein has been shown to tolerate short peptide tags at its C-terminus (87, 230). All such recombinant viruses represent important tools for the study of virus-host interactions and the tracking of viral nsP and RC localization in infected cells (228–231).

2.5.2 Alphavirus replicon vectors

Alphavirus replicon vectors contain modified viral genomes in which the region encoding the viral structural proteins has either been removed or replaced with a foreign sequence. Consequently, the replicons cannot make virions and are therefore restricted to undergoing only a single cycle of replication (232). When transfected into cells, replicon RNA serves as mRNA for the viral replicase, which then amplifies the replicon and transcribes the SG RNA. As many types of cells can be infected with alphaviruses but cannot be efficiently transfected with large RNA molecules, a virus replicon particle (VRP) system is commonly used. High-titer VRP stocks can be generated using replicon and helper (encoding viral structural proteins) RNAs that have been transcribed *in vitro* and then co-transfected into mammalian cells (232, 233). To prevent the formation of infectious genomes *via* RNA recombination, the structural region of alphaviruses can be split between different helper RNAs and/or the SG promoter can be removed from helper RNAs (234, 235).

Alphavirus-based replicon vectors are widely used. Replicons expressing tagged nsPs or individual marker proteins are used in much the same way as tagged viruses; they are especially useful in cases where a lack of spread of infection represents a benefit. When alphavirus replicon vectors are used for biotechnological applications, they are characterized by high-level expression of foreign genes and a broad range of susceptible host cells, including those of insect and mammalian origin. Other characteristics include high cytotoxicity,

rapid death of infected cells and, for cells with an intact type I IFN response, high levels of IFN induction (236, 237). Depending on the application, these properties of replicons can either represent benefits (such as in anti-cancer therapy approaches or in genetic vaccination) or obstacles.

Cell lines harboring continuously replicating viral replicon RNA can be used for several purposes. First, such cells can be used for the production of recombinant proteins. Second, they are useful for screening and analysis of inhibitors of virus replication. Much of the progress that has been made in the development of hepatitis C virus (HCV) inhibitors is owed to the development of HCV replicon cell lines (238, 239). Such cell lines can also be useful for alphaviruses that, unlike HCV, grow well in cell culture, as replicon cell lines can be used at lower biosafety level (BSL) conditions and/or to select specific inhibitors that affect virus RNA replication. New World alphavirus-based replicons lack sequences encoding the capsid protein, the major determinant of cytotoxicity in these viruses. Therefore, only minimal effort was needed to develop corresponding noncytotoxic vectors (240). However, wild type (wt) replicons of Old World alphaviruses are, similar to the corresponding viruses, highly cytotoxic (157). Thus, attempts to reduce the cytopathic effect of these vectors have been made.

Conflicting data have been obtained about the cytotoxicity of alphavirus replicon vectors that express anti-apoptotic proteins (139, 241), and no stable replicon cell line has been made using this approach. A more successful approach is based on the introduction of point mutations into nsP2, the main factor responsible for the cytotoxicity of Old World alphaviruses (see 2.3.2 for details). Alternatively (or sometimes additionally), such mutations or combinations of mutations have been obtained using the selection procedure that was applied in publication **I**. Using replicons with such mutations, it is possible to create stable cell lines containing persistently replicating replicon RNAs of SINV, SFV and CHIKV. Interestingly, in the case of SINV, a single mutation in nsP2 (aa residue 726) was found to be sufficient to render the corresponding replicon noncytotoxic and capable of generating stable cell lines (160). In SFV, mutations similar to those used in SINV vectors have resulted in replicons with cytostatic effect – infected cells can survive for a long time but are not capable of multiplying (153). It was later found that at least one additional mutation in nsP2 is needed to attenuate the SFV replicon to a level that allows for the permanent growth of replicon cell lines (242) and the production of high levels of human therapeutic proteins (243). In the case of CHIKV of the ECSA genotype, mutations analogous to those evaluated in SINV studies had almost no effect on the RNA replication and cytotoxicity of replicon vectors (see *Results and Discussion* and publications **I**, **II** and **III** for details). Thus, there is significant variation regarding the requirements for the noncytotoxic growth of replicons of different Old World alphaviruses. Most likely, this results from the different properties of the nsP2 proteins and/or the different properties of other replicase proteins that interact with nsP2. Furthermore, it is not known which activities of nsP2 are affected by mutations that allow the noncytotoxic growth of replicons of Old World alphaviruses.

3. AIMS OF THE STUDY

A few years ago, the World Health Organization (WHO) recognized two viruses (rabies and dengue) as causative agents of major neglected tropical diseases. Then, a third virus, CHIKV, was included due to its rapid spread and massive outbreaks. Although CHIKV has been recognized as a major human pathogen, there is still no licensed vaccine to prevent and no antiviral drug to treat CHIKV infection. The development of efficient vaccines and antivirals crucially depends on our knowledge about the molecular biology of the virus. However, in the case of CHIKV, our knowledge is limited and fragmented. The successful development of antivirals also depends on the availability of tools needed for the screening of drug candidates as well as tools for analysis of the mechanism(s) of drug candidate actions. Hence, the research described in this thesis was focused on the development of new tools essential for studies of CHIKV infection. Subsequently, these tools were used for analysis of the molecular biology of CHIKV infection and alphavirus-host interactions.

The studies included in this thesis had the following objectives:

1. To develop cell culture-adapted CHIKV replicon vectors that can be used to screen compounds with anti-CHIKV activity. In parallel, the molecular basis underlying the noncytotoxic phenotype of these vectors was analyzed.
2. To develop novel *trans*-replication system for CHIKV to use it for the development of CHIKV replicons expressing tagged replicase proteins and for functional analysis of the effects caused by mutations introduced into CHIKV replicase proteins.
3. To use previously developed SFV *trans*-replicase and newly developed CHIKV *trans*-replicase systems to analyze virus-host interactions. In particular, the aim was to analyze the mechanisms by which different alphaviruses activate and counteract host antiviral responses.

4. MATERIALS AND METHODS

The methods that were applied for the selection of CHIKV replicons with a noncytotoxic phenotype are provided in publications **I** and **II**. The methods used for the analysis of mutant CHIKV replicons in cell culture and for the analysis of recombinant CHIKV nsP2 proteins are described in publication **II.** Please note the same replicon is named differently in publication **I** (CHIKV-NCT) and publication **II** (ChikvRepRluc-5A-PG); for consistency, only the latter name is used in this thesis. Detailed descriptions of the materials and methods used for the construction of the CHIKV *trans*-replicase system and its subsequent analysis in cell culture experiments are provided in publication **III.** The methods used for the analysis of type I IFN induction by SFV and CHIKV *trans*-replicases in transfected cell cultures are described in publication **IV**. Plasmids encoding the replicases of different SFV strains and their mutant forms were obtained and analyzed together with Sirle Saul. These procedures were already described in the PhD thesis of Sirle Saul (244) and are therefore not repeated here.

The construction of CHIKV *trans*-replicase plasmids, which were used to generate unpublished data, was performed as follows. Plasmids for the expression of CHIKV replicases harboring a Gly534 to Val mutation (CHIKV-GV1), a Gly1332 to Val mutation (CHIKV-GV2), a combination of Gly534 to Val and Gly1332 to Val mutations (CHIKV-GV12), an Arg532 to His mutation (CHIKV-RH), a combination of Arg532 to His and Glu1030 to Val (residue 515 of nsP2) mutations (CHIKV-RH-EV) or a combination of Arg532 to His, Glu1030 to Val and Pro1253 to Gly mutations (CHIKV-RH-EV-PG) were obtained using a human cytomegalovirus (CMV) promoter-based CMV-P1234 vector (**III**) *via* site-specific mutagenesis and subcloning procedures. The sequences of the obtained vectors were confirmed using Sanger sequencing.

5. RESULTS AND DISCUSSION

5.1 Construction of noncytotoxic CHIKV replicon vectors and analysis of molecular defects in nsP2 associated with a noncytotoxic phenotype (I, II)

5.1.1 Development of stable BHK-21 CHIKV replicon cell lines

Although CHIKV is not transmitted by an air-borne route, its handling nevertheless requires significant safety measures and, in some countries, security measures as well. These measures are lacking in many institutes. Furthermore, BSL3 laboratories often lack essential high-cost equipment such as confocal microscopes, cell sorters etc. Hence, the development of experimental systems that allow CHIKV replication studies to be conducted at a lower BSL is clearly useful. When this study was initiated, our institute (and university) also lacked a laboratory suitable for working with infectious CHIKV. As such, the use of CHIKV replicons unable to produce infectious virions was the only available option.

CHIKV replicons can be easily packed into VRPs (245). VRPs mimic a viral infection, but they are only available in a limited supply because VRPs do not propagate and their production is expensive. This considerably limits their usage, especially in applications requiring the infection of large numbers of cells. For example, for massive screening projects, hundreds of thousands samples typically need to be analyzed. Even if a 384-well format is used, this is still a very large number of infected cells. This limitation could partially be solved by the development of stable replicon cell lines similar to those successfully used for HCV studies (238, 239). However, unlike HCV, vectors based on wt replicons of Old World alphaviruses (e.g., SFV, SINV and CHIKV) possess high cytotoxicity (246). To establish cell lines that persistently contain alphavirus replicon RNAs and express the corresponding replicase proteins, modifications to the virus replicative machinery that diminish virus-induced cytopathic effects must be introduced (161, 163). This can be achieved through rational design, through the introduction of random mutations or through the selection of spontaneously occurring mutations that result in a desired phenotype.

Thus far, all mutations known to reduce the cytotoxicity of Old World alphavirus replicons have a similar characteristic: they all affect the nsP2 protein, most commonly its C-terminal region (153, 161, 163, 242, 247). As the sequence (and especially the 3D structure) of this region is reasonably conserved, rational design can be used. Previous studies have shown that mutations of Pro726 of SINV, which is in a similar location to Pro718 of SFV, or to nearby residues in nsP2 alter the replicon phenotype, allowing for prolonged (SFV) or unlimited (SINV) survival of mammalian cells harboring replicons with such mutations (153, 160). Based on this knowledge, we replaced the Pro718 residue of CHIKV (in the LR-2006-OPY1 isolate, belonging to the

ECSA genotype) nsP2 with Gly. This substitution, which affected the MTL domain of nsP2 and is hereafter referred to as the PG mutation, was introduced into the CHIKV replicon. Disappointingly, but not totally unexpectedly, stable cell lines were not obtained using this replicon. In sharp contrast to SFV (153), the effect caused by the PG mutation was essentially undetectable (see below). As a motif that is similar to the so-called NLS of SFV nsP2 (another site where mutations reducing the cytotoxicity of SFV replicons are located) was not found in CHIKV nsP2 using NLS prediction software (https://www.predictprotein.org), further attempts to apply rational design were abandoned. Therefore, a method based on the selection of naturally generated mutations that reduce the cytotoxicity of alphavirus replicons (161, 247) was applied.

The principle of applied approach is simple: RdRp of CHIKV (like those of other RNA viruses) is an error-prone enzyme and is therefore constantly making random errors. In nature, this feature is used by the virus to evolve. This error-prone RdRp produces divergent viral progeny, from which variants harboring changes most beneficial to existing conditions have a growth advantage (248). This property can be used for the selection of mutants with desired properties by placing a virus (or, in our case, a replicon) into conditions in which only cells harboring noncytotoxic replicons would survive. Briefly, a puromycin acetyltransferase (Pac) coding sequence was cloned under the SG promoter of a CHIKV replicon harboring the PG mutation (ChikvRep-PG). To visualize replicon-containing cells, the Pac sequence was fused to an EGFP-coding sequence *via* the foot and mouth disease virus 2A autoprotease sequence (resulting in the release of free Pac into the cytoplasm of replicon-containing cells). When this replicon was used to transfect BHK-21 cells, the vast majority of the transfected cells died. However, after the removal of the nontransfected cells using puromycin, the formation of puromycin-resistant EGFP-positive colonies was observed (**I**, Fig. 1B). The selection and propagation of these colonies followed by the sequencing of the corresponding replicon genomes allowed for the identification of two different combinations of mutations that were apparently responsible for the noncytotoxic phenotype of the replicon (**II**, Fig. 1A). Not surprisingly, the adaptive mutations were identified in nsP2. To confirm the functional significance of these mutations, they were back-engineered into the ChikvRep-PG vector. Then, BHK-21 cells that were transfected with these mutant replicons were subjected to a cell survival assay. This assay confirmed that most of the BHK-21 cells that were transfected with ChikvRep-PG RNA died within 72 h post transfection (p.t.); no significant difference between ChikvRep-PG RNA-transfected cells and wt ChikvRep RNA-transfected cells was noticed (**II**, Fig. 1A). This confirmed the initial observation that the PG mutation alone was not able to render the CHIKV replicon as noncytotoxic. However, in the presence of either of the additional combinations of mutations, a noncytotoxic phenotype was achieved.

The first adaptation was represented by a combination of two mutations. Neither of these mutations was similar to any previously reported change associated with noncytotoxic phenotype of alphavirus replicons. However, one of

these mutations, the Gly620 to Glu (hereafter referred to as the GE mutation), was located at the beginning of the MTL domain (**II**, Fig. 1A) – a region that is commonly altered by mutations associated with a noncytotoxic phenotype. Another point mutation, the Glu116 to Lys (hereafter referred to as the EK mutation), was identified in the N-terminal domain of nsP2. A survival test showed that ChikvRep-EK, ChikvRep-GE, ChikvRep-EK-GE and ChikvRep-GE-PG were all cytotoxic. In contrast, cells transfected with ChikvRep-EK-PG or ChikvRep-EK-GE-PG survived and were dividing at 72 h p.t. (**II**, Fig. 1A). Thus, the noncytotoxic phenotypes of these replicons were caused by a combination of the EK and PG mutations. This result was surprising because other known mutations associated with the noncytotoxic phenotype of CHIKV replicon are located in the MTL domain (163). Thus, the unusual location of the EK mutation provides a hint that the mechanism responsible for how this mutation caused a noncytotoxic phenotype might be different from those of other mutations. Additional studies (see below, **III** and section **5.2**) demonstrated that this is indeed in the case.

A second adaptation found in selected replicons was an insertion of five aa residues (Gly-Glu-Glu-Gly-Ser; hereafter referred to as the 5A mutation) between residues 647 and 648 of nsP2. The localization of this mutation (in the region corresponding to the "NLS" of nsP2 of SFV) is similar to those of many other mutations shown to reduce the cytotoxicity of alphavirus replicons (153, 163, 242). However, in contrast to previously reported changes, this mutation is not a substitution but an insertion. Of note, we cannot explain how such an insertion occurred. Most likely, it is a product of recombination rather than a simple synthesis mistake by RdRp; however, we could not identify from where this sequence originates (except that it is not derived from the replicon itself). As the site (though not the nature) of this mutation is clearly associated with a noncytotoxic phenotype of alphavirus replicons, it was no surprise that the cell survival assay revealed that ChikvRep-5A-PG clearly lacked cytotoxic effect, although the 5A mutation alone was not sufficient to confer a noncytotoxic phenotype (**II**, Fig. 1A). It was also observed that cells containing ChikvRep-EK-GE-PG (or ChikvRep-EK-PG) exhibited much slower growth compared to cells containing ChikvRep-5A-PG. Consistent with these results, the fluorescence produced by a marker protein was also much more prominent in cells containing ChikvRep-5A-PG (**II**). These observations are concordant with the hypothesis that the mechanisms underlying the noncytotoxic phenotype caused by the EK and 5A mutations (each combined with the same PG mutation) are different.

Taken together, this part of our study revealed that noncytotoxic CHIKV replicons clearly differ from those of SINV, wherein individual mutations are sufficient to suppress replicon cytopathogenicity (155, 160). Thus, the requirements needed to achieve a noncytotoxic phenotype for replicons of different Old World alphaviruses are not identical. Furthermore, studies performed using replicons from CHIKV of the WA genotype revealed that when these replicons contained a Pro718 substitution they permitted the formation of a stable

noncytotoxic cell line, but only when used in combination with additional mutations (KR649AA or D711G) (163). Thus, more than one attenuating mutation is needed to obtain stably replicating noncytotoxic CHIKV replicons.

The presence of an easily detectable marker that is expressed from replicon RNA greatly simplifies the analysis of viral replication. For quantitative purposes, various luciferase-encoding genes are generally the most useful in this regard (due to their very low background levels and the large range that is available for measurements). However, the incorporation of a sequence encoding such a reporter into the replicase coding region is a major mutation on its own. Furthermore, such an insertion may modulate the effects of other mutations in an unpredictable manner. Based on these considerations, all identified mutations were also incorporated into ChikvRep-PG together with a *Renilla* luciferase (Rluc) marker fused to the C-terminal HVD of nsP3, a preferable region for marker insertion in the alphavirus genome (**I**, Fig. 1A; **II**, Fig. 1B) The BHK-21 cell line, containing persistent CHIKV replicon expressing Pac and EGFP (or ZsGreen) from SG promoter and containing Rluc (in optimal position) in nsP3 grew almost as fast as the parental BHK-21 cell line. Both markers were present at high and (importantly) constant levels, demonstrating the suitability of these cells as a safe surrogate model that could be used to screen anti-CHIKV compounds (**I,** Table 1). To date, this model has been used in several studies (249, 250).

Nevertheless, there is also an obvious restriction: analyses performed using this cell lines are mostly limited to direct-acting antivirals that affect CHIKV RNA replication. At the same time, in order to be active, antiviral compounds do not strictly have to influence the virus directly; they can also act through host cell factors that are necessary for virus infection. Although many of these factors may be sufficiently similar between hamster (the source of BHK-21 cells) and human cells, there would also be factors that are obviously different. Hence, as CHIKV is a human pathogen, we also wanted to create a cell line of human origin containing the noncytotoxic CHIKV replicon. This cell line would be a more relevant tool not only for the screening of host-targeting antivirals but also (and perhaps even more importantly) for studies of host factors involved in CHIKV replication using genome-wide siRNA screens. Actually, our BHK cell line has recently been used for such purpose (251); however the differences in human/hamster sequences do clearly limit efficiency for its use. Therefore, whether the generated replicons were capable of persistent replication in human cells was also analyzed.

Surprisingly, it was found that mutations resulting in noncytotoxic phenotype of CHIKV replicon in BHK-21 cells failed to produce the same phenotype in Huh7 (human hepatocellular carcinoma) and HeLa (human cervical cancer) cells. Although ChikvRep-EK-GE-PG and ChikvRep-5A-PG have somewhat different phenotypes, the general findings were the same: when puromycin selection was applied, the transfected cells stopped growing, and in a couple of weeks no viable cells remained (**II**). Originally (and mistakenly), we thought that the tested replicons were lethal to these cells because they were insuffi-

ciently attenuated. This promoted further selection/adaptation experiments. For some unknown reason, cells transfected with ChikvRep-5A-PG RNA (without Rluc fused to nsP3) always died after a week or two of selection. In contrast, when Huh7 cells were transfected with the ChikvRepRluc-5A-PG replicon, puromycin-resistant colonies were obtained. Sequence analysis revealed two additional point mutations in the adapted ChikvRepRluc-5A-PG RNA. The first was found in the region encoding the C-terminal half of nsP1 (Phe391 to Leu, hereafter referred to as the FL mutation), while the second was found in the region corresponding to the beginning of the ZBD of nsP3 (Ile175 to Leu, hereafter referred to as the IL mutation) (**II**, Fig. 1B). These mutations have two properties in common: neither is located in nsP2, and both are located in the presumably unstructured regions that lie between the predicted domains of their corresponding nsPs. Thus, it became obvious that these mutations are unlikely classical cytotoxicity reducing mutations. BHK-21 cells are known to be extremely permissive to alphaviruses, allowing active viral replication and viral gene expression. In most other cell lines, alphavirus replication and gene expression occur at considerably (and often much) lower levels. Therefore, it seemed plausible that Pac expression by ChikvRep-5A-PG was sufficient to protect BHK-21 cells from the effects of puromycin but was insufficient to protect Huh7 cells (**II**, Fig. 2B, D) or other cells of human origin. Concordantly, a subsequent cell survival assay showed that the FL and IL mutations (either alone or in combination) were unable to reduce the cytotoxicity of the ChikvRepRluc replicon (**II**, Fig. 1B). In contrast, the Huh7 cell line containing the ChikvRepRluc-FL-5A-PG-IL replicon showed rapid growth and high levels of expression of the ZsGreen and Rluc reporters (**II**, Fig. 1B). Thus, instead of protecting cells from the cytotoxic effect of CHIKV replication (and/or that of the CHIKV replicase proteins), these mutations (FL and IL) were apparently needed to provide protection against puromycin and did so by activating the replication and transcription of noncytotoxic CHIKV replicon RNA (and thus the expression of Pac) in Huh7 cells (see also **5.1.2**). A comparison of the obtained human cell line with the BHK-21 cells containing the ChikvRepRluc-5A-PG replicon revealed that, under puromycin selection, both cell lines maintained similarly high levels of marker protein expression. The same was observed for Huh7 cells harboring the ChikvRepRluc-FL-5A-PG-IL replicon in the absence of puromycin selection, even after 10 passages. In contrast, in the absence of puromycin, reductions in ZsGreen fluorescence and Rluc activity were observed in the BHK-21 cell line containing ChikvRepRluc-5A-PG (**II**). This difference can also be explained by the enhanced replication of the ChikvRepRluc-FL-5A-PG-IL replicon, which produces more replicon RNAs (and replicase complexes), making their loss in the process of cell division less likely. Thus, a human cell line harboring a persistently replicating CHIKV replicon has the potential to be a convenient and safe tool for the identification of cellular factors involved in CHIKV replication as well as for the analysis of inhibitors that act through such host factors.

5.1.2 How do mutations in nsP2 cause a noncytotoxic phenotype?

Despite intensive studies, the reasons for the noncytotoxic phenotypes of the alphavirus replicons have largely remained enigmatic. On the one hand, it has been shown that mutations associated with a noncytotoxic phenotype always reduce (sometimes severely) the synthesis of alphavirus positive-strand RNAs (153, 160, 163, 242). As nsPs are translated from these RNAs, their synthesis is generally also reduced (153, 252). On the other hand, it is assumed (though not always directly demonstrated) that mutations associated with a noncytotoxic phenotype alter some properties of nsP2, such as by modulating its enzymatic activity and/or altering its subcellular location. As replicase proteins (such as nsP2) and the synthesis of replicon RNA are coupled, it is unclear how the phenotype evolves: is the compromised activity of nsP2 the primary reason for the lack of cytotoxicity, or is the noncytotoxic phenotype caused by reduced amounts of nsP2 (as a consequence of reduced RNA replication)? To some extent, we were able to address these questions by developing a CHIKV *trans*-replication system (see 5.2, **III**). However, initially classical approaches were used to analyze effect of nsP2 mutations on replicons, viruses and on nsP2 itself.

First, BHK-21 and Huh7 cells were transfected with mutant replicon RNAs that were synthesized *in vitro*, and the levels of positive-strand RNAs and nsP2 were analyzed. It was confirmed that cells transfected with the noncytotoxic CHIKV replicons contained reduced amounts of nsP2 and replicon RNAs (**II**, Fig. 2). Similar findings have previously been shown for SFV and SINV: cells containing replicons with mutations enabling persistent infection synthesize replicon RNAs of positive polarity at significantly (sometimes >10-fold) lower levels (153, 160). Hence, it could be concluded that, in this regard, CHIKV is very similar to other Old World alphaviruses. Importantly, the results obtained from this analysis supported and complemented those produced in the cell survival tests. Thus, the PG mutation led to only a minor reduction in the accumulation of positive-strand RNAs in BHK-21 and Huh7 cells (**I**, Fig. 2; **II**, Fig. 2A, B). This clearly differs from findings made using SINV (160, 252), SFV (153) and even CHIKV of the WA genotype (163). The replicon containing only the 5A mutation displayed considerably reduced levels of RNA synthesis (**II**, Fig. 2A, 2B), and the replicon containing 5A-PG, the main cytotoxicity-reducing combination of mutations, displayed significantly reduced RNA and nsP2 levels (**II**, Fig. 2A, B). The effects of the EK and the EK-PG mutations on RNA replication were even more notable: neither the corresponding positive-strand RNA nor nsP2 could be detected on normally exposed filters (**II**, Fig. 2A, 2C), indicating that they were present at very low levels. Finally, the introduction of the FL, IL or FL-IL mutations into ChikvRepRluc or ChikvRepRluc-5A-PG resulted in slightly increased levels of nsP2 and SG RNAs (**II,** Fig. 2B, D). Thus, as was already hypothesized above, the FL and IL mutations act in an additive manner and boost SG RNA (and possibly also genomic RNA) synthesis in Huh7 cells, which in turn allows synthesis of Pac at levels sufficient to

provide resistance to puromycin. The mechanisms by which these mutations boost CHIKV replicon replication in Huh7 cells (**II**, Fig. 2B, D) remain unclear. It could be hypothesized that these mutations somehow rearrange the interactions that exist between the virus and the host cell (as shown below, these adaptations seem to be specific for Huh7 cells). There are several explanations for how this may occur, including changes in RC formation, changes in interprotein interactions and/or changes in interactions between viral proteins and host cell proteins.

Second, the effects of the mutations described above were analyzed in the context of a complete CHIKV genome. Concordant with previous observations, the results from an infectious center assay performed in BHK-21 cells showed that all RNAs that contained the EK mutation had very low infectivity. Moreover, it was found that in the genomes of rescued viruses the EK mutation had always been reverted (**II**, Fig. 3). Thus, the low level of RNA replication was sufficient for the noncytotoxic growth of the corresponding replicon in BHK-21 cells; however, it was not sufficient in the context of infectious virus. As expected, the RNA of CHIKV-PG was highly infectious, and the same was the case for RNAs harboring the FL, GE, IL and FL-IL mutations (**II,** Fig. 3). More interestingly, while the 5A and 5A-PG mutations resulted in similar (~10-fold) reduction in rescue efficiency, we were able to detect clear cytopathic effects only for CHIKV-5A infected cells (**II**, Fig. 3). This finding is consistent with the noncytotoxic growth of ChikvRep-5A-PG in BHK-21 cells (**II**, Fig. 1A). The combination of FL-5A-PG-IL mutations caused a major (>2000-fold) reduction in recombinant RNA infectivity. Thus, as the FL and IL mutations boosted the replication of replicon RNA in Huh7 cells (**II,** Fig. 2B, D), they may represent cell type-specific adaptations. However, this does not mean that they necessarily cause a reduction in RNA replication in BKH-21 cells; this defect may arise from a different mechanism. Concordant with this assumption, CHIKV-FL-5A-PG-IL was able to produce cytopathic effects, while the more infectious CHIKV-5A-PG was not (**II,** Fig. 3). Thus, it is plausible that the FL and IL mutations inhibited the rescue but not the RNA replication of CHIKV-FL-5A-PG-IL. Whether this is the case could be easily revealed using the CHIKV *trans*-replication system developed in this work (described in **III**); however, such experiments have not been performed to date.

Third, the effects of mutations on the nuclear localization of CHIKV nsP2 were analyzed. Although the nsP2 of CHIKV lacks a putative NLS at the beginning of its MTL domain, it is still capable of entering the nucleus. This is highly important as, regardless of the exact mechanism of entry, the nuclear localization of nsP2 is required to turn off cellular transcription (210) and to counteract the interferon response (152, 209). Thus, it is also a prerequisite for the cytotoxic properties of Old World alphavirus replicons (155, 213). Interestingly, the 5A mutation in nsP2 of CHIKV is located in a position corresponding to the predicted NLS of SFV nsP2 (151), clearly indicating the importance of this region for the noncytotoxic phenotype of the ChikvRep-5A-PG replicon. Initially, the effects of the introduced mutations on the subcellular

localization of nsP2 were studied using BHK-21 cells transfected with CHIKV replicons and immunofluorescence analysis. The obtained results showed that the presence of the PG mutation alone resulted in more prominent nuclear localization of nsP2 (**I**, Fig. 2B), whereas in cells transfected with ChikvRep-5A-PG nsP2 was largely, but not completely, excluded from nuclei (**I**, Fig. 2B). It should be noted that these very preliminary data were included in publication **I** only upon the demand of one of the reviewers; otherwise, we would have preferred to omit these findings. The reason for our hesitation is that direct comparison of the phenotypes of wt and mutant replicons is difficult (actually almost impossible) due to the very different replication kinetics and nsP2 levels that they produce in transfected cells (**I**, Fig. 2A; **II**, Fig. 2). In addition, prior analysis performed using wt CHIKV (full virus) had already shown that the nuclear localization of CHIKV nsP2 is not uniform or constant over time. Briefly, in every preparation that was analyzed, there were infected cells where nsP2 was present in nuclei as well as cells where it was almost exclusively detected in cytoplasm. The general tendency that we observed was that the nuclear localization of nsP2 was more dominant at earlier timepoints of infection and less so at later timepoints. Therefore, for the next study, we repeated the experiments in a more systemic manner. The results obtained from these experiments showed that there is actually no difference between the localization patterns of nsP2 in cells infected with wt CHIKV or CHIKV-5A-PG (**II**, Fig. 4A). We consider these data more reliable (compared to the results presented in **I**), as the replication kinetics of wt CHIKV and CHIKV-5A-PG (both are full viruses) are more similar to each other than those of the corresponding replicons. Furthermore, for both viruses, we again observed different nsP2 localization patterns, including cells where nsP2 was predominantly located in the nucleus, cells where it was mainly located in the cytoplasm, and cells were it was found equally in both of these compartments (**II**, Fig. 4A). To clarify the matter, the localization of nsP2 was also analyzed through the fractionation of infected cells into nuclear and cytoplasmic fractions. This analysis revealed that nsP2 of wt CHIKV localized almost equally between the nucleus and the cytoplasm (**II**, Fig. 4B), similar to the nsP2 of other Old World alphaviruses (151, 253). CHIKV-PG nsP2 behaved similarly; however, its nuclear localization was more extensive, as was also previously observed with SFV (153). The nsP2 of CHIKV-5A-PG was shown to be localized in a pattern similar to that of the nsP2 of CHIKV-PG (**II**, Fig. 4B). The analysis also confirmed that our initial observations from immunofluorescence analysis were correct: nsP2 was indeed always more abundant in nuclei at early stages of infection (**II**, Fig. 4B). This raised an interesting question: what happens to the nsP2 that is transported to the nucleus at early timepoints of infection? One possibility is that it is later transported out of the nucleus. Another possibility is that nsP2 in the nucleus is degraded faster than that in the cytoplasm, causing the nuclear pool of the protein to rapidly diminish once synthesis of nsP2 stops. This would represent an interesting topic for future studies, as we currently lack data supporting (or opposing) either of these possibilities. Despite this uncertainty, our results

clearly indicate that mutations associated with the noncytotoxic phenotype of the CHIKV replicon of the ECSA genotype do not hamper the nuclear localization of nsP2. Thus, the noncytotoxic effect must originate from some other functional defect(s) in the mutant nsP2 proteins.

Fourth, we took advantage of a system previously developed for the production of functional recombinant nsP2 and cell-free assays to analyze the multiple enzymatic activities of this protein (143). As recombinant nsP2 protein is rather difficult to handle (and, as it turned out, the purification and use of its mutant forms is even more challenging), such assays have not been performed in a systematic manner in any previous study. Somewhat surprisingly, it was found that of the two mutations that resulted in a noncytotoxic phenotype when combined, the 5A mutation had only a limited effect on the protease, RNA helicase and NTPase activities of nsP2 (**II**, Fig. 5B, 7, 8). In contrast, the PG mutation had a prominent effect on the protease and NTPase activities of the protein (**II**, Fig. 5B, 7, 8). Such behavior would have been logical for nsP2 of SINV or SFV harboring the PG mutation (this mutation is sufficient for or is the major contributor to the noncytotoxic phenotypes of the corresponding replicons), but not for the nsP2 of CHIKV of the ECSA genotype, where the PG mutation had very limited effect on RNA replication (**II**, Fig. 2) and RNA infectivity (**II**, Fig. 3). This discrepancy can most likely be explained by the observation that in the context of P1234 polyprotein the effect of the PG mutation on the protease activity of nsP2 was smaller and that no negative effects resulting from this mutation could be detected in cells infected with the corresponding virus (**II**, Fig. 5). Thus, the defects caused by the PG mutation are most likely masked (or compensated for) when other viral (and possibly host) components are present. Somewhat disappointingly, no functional defect was exclusively found for the combinations of mutations (EK-PG or 5A-PG) that resulted in the noncytotoxic phenotype. Indeed, these combinations only resulted in a prominent (but not statistically significant) reduction in enzymatic activity in the case of the RNA helicase activity (**II**, Fig. 8C, D). One reason why we were unable to identify a common defect caused by these mutations may be very simple: such a defect may not exist. That possibility was not actually considered when studies **I** and **II** were performed; however, with the accumulation of new data (and a more critical review of older data), the possibility that the mechanisms responsible for the noncytotoxic phenotypes of CHIKV-EK-PG and CHIKV-5A-PG are different began to appear more likely. Thus, the defects that cause different replicons to adopt similar phenotypes may actually be different. This is one of the reasons why we returned to this topic in study **III**.

5.2 Construction and use of the CHIKV *trans*-replication system (III)

For positive-strand RNA viruses, the viral genomic RNA also acts as an mRNA directing the translation of the viral replicase proteins. Therefore, the expression of the replicase proteins is intrinsically coupled with the synthesis of viral genomes. Often, notably also in the studies described in the previous section, this limits the functional analysis of viral replicase proteins. Thus, as mutations in nsP2 associated with noncytotoxic phenotype of alphavirus replicon reduce RNA replication, the expression levels of the mutant forms of nsP2 are also reduced (**II,** Fig. 2). As already said above, this leads to the question of whether the noncytotoxic phenotype of such a replicon is a direct result of alterations in the cytotoxic properties of nsP2 and/or whether it is caused by reduced amounts of the protein (which is an indirect consequence because this reduction results from the alteration of the properties of the mutant replicase). It is not easy to distinguish between these possibilities. Similarly, it is not easy to compare the mutant replicon to the wt replicon. For this reason, our comparison led to the incorrect conclusion that the nuclear localization of nsP2 was reduced in the presence of the 5A-PG mutation (**I**), and a substantial amount of work was required to settle this issue in a later study (**II**). Notably, many mutations that are introduced into ns proteins cause genetic instability in the viral genome/ replicon. Such mutations cannot be studied in the context of replicating RNA genomes, as they always revert, pseudorevert and/or become compensated by secondary mutations (88, 97, 149). We have observed such phenomena when studying the EK and EK-PG mutations in the context of the CHIKV genome (**II**, Fig. 2A, C; **II**, Table 1). Thus, the development of a system in which the reduction (or increase) of RNA replication does not cause changes in the levels of replicase protein production and where mutations introduced into the replicase proteins cannot revert would have great value for alphavirus studies.

It has been shown that alphavirus replicases are capable of replicating any suitable RNA templates provided in *trans*; for example, helper RNAs that are used for the generation of VRPs are effiently replicated and transcribed (235). In the replicon/helper RNA system, the viral replicase is, however, still expressed from self-replicating replicon RNA. As a next step toward uncoupling replicase expression and replicase-mediated template RNA replication/ transcription, we designed, constructed, tested and used a *trans*-replication system for CHIKV. This system is based on a previously described *trans*-replicase system for SFV (107) but contains several improvements, such as the use of a codon-optimized replicase coding sequence, strong promoters to drive the synthesis of the template RNA used by the replicase and mRNA encoding the replicase itself. The basic idea of this system is simple: the expression of the replicase proteins must be independent of any of their enzymatic activities (although it may still to some degree be affected by changes in the cytotoxic properties of the replicase proteins, **III,** Fig. 8B). Only then could the effects of mutations affecting the ability of the viral replicase to perform RNA synthesis

be analyzed directly. In our system, the expression of replicase mRNA and corresponding template RNA is carried out by either bacteriophage T7 RNA polymerase or cellular RNA polymerase II. The design strategies used to create cassettes for T7 and CMV promoter-based expression of CHIKV replicase mRNA and to create the plasmids used for the expression of the corresponding template RNAs are shown in **III** (Fig. 1A and Fig. 1B, respectively). In transfected cells, the CHIKV replicase is translated from its own mRNA but, due to the lack of essential *cis* sequences, is unable to replicate it. Instead, once synthesized, the replicase finds suitable template RNA and then binds to it to trigger RC (spherule) formation. This is accompanied by the synthesis of negative-strand RNA and followed by the amplification of template RNA as well as by the synthesis of large amounts of SG RNA. The introduction of reporters into the genomic (corresponding to the ns region) and SG (corresponding to the structural region) regions of the template RNA (**III,** Fig. 1B) was hypothesized to provide an easy readout for replication/transcription efficiencies.

We started our analysis by making a head-to-head comparison of CHIKV *trans*-replicases based on different promoters. The CMV promoter-based *trans*-replicase system was tested in two human (Huh7, U2OS) and three rodent (COP-5, BHK-21, BSR) cell lines. For comparison, BSR cells (BHK-21 cells stably expressing T7 RNA polymerase) were co-transfected with the T7 promoter-based replicase and template expression plasmids. *Gaussia* luciferase (Gluc) activity (note that the Gluc was encoded by a second ORF in the template RNA and thus is almost exclusively translated from the replicase-generated SG RNAs) and *Firefly* luciferase (Fluc) activity were high in all cell lines, although the highest levels were detected in BHK-21 and U2OS cells (**III**, Fig. 2A). However, as Fluc is encoded by the first ORF of the template RNA, it can also be efficiently expressed in the absence of an active CHIKV replicase. Therefore, we saw only a small increase in Fluc activity for the CMV promoter-based system, for which the inactive polymerase was used as a control (**III,** Fig. 2C and 2D). Co-transfection of BSR cells with the T7 promoter-based wt replicase and template RNA expression constructs resulted in levels of Fluc expression that were similar to those measured for the CMV promoter-based system. In contrast, however, when the construct expressing inactive replicase was used in T7 promoter-based system, the Fluc expression levels were low (**III**, Fig. 2C). This may be due to the poor translation of the T7 RNA polymerase-generated template RNA transcripts, which lack 5' cap structures (in contrast to CMV promoter-derived transcripts that have 5'cap and are efficiently translated). In this regard, it is interesting to mention that the cap0 structure at the 5' end of the alphavirus genome is also thought to be essential for replicase binding and functioning. While we cannot exclude the role of cap0 in this process, it is evident that the presence of the 5' cap0 structure could not be an absolute requirement for template recognition, recruitment and replication, as RNA replication was clearly observed in the T7 promoter-based system (**III**, Fig. 3B). Comparison of the RNA synthesis and expression of the reporter proteins revealed very good correlation between Gluc activity and SG RNA levels in transfected cells (**III**, Fig. 2A;

Fig. 3B). Therefore, Gluc activity indeed serves as a good indicator of the efficiency of SG RNA transcription for both the CMV and T7 promoter-based *trans*-replication systems. At the same time, it was concluded that Fluc activity could serve as a good indicator of template RNA replication only for the T7 promoter-based *trans*-replication system. The template RNA replication in the CMV promoter-based system was at least as powerful as that in the T7 promoter-based system (**III**, Fig. 3B); however, due to high background, this was not clearly reflected by increased Fluc activity (**III,** Fig. 2).

Trans-replication systems have been used to study the biogenesis of SFV and SINV replicase complexes (110, 107, 106) and for analysis of the biological effects of different mutations introduced into SFV replicase proteins (108). Here, we applied our system to study the possibility of tagging CHIKV replicase proteins and to analyze the effects of mutations affecting the cytotoxicity of CHIKV replicons on the RNA replication. There are many studies that have used recombinant alphaviruses carrying tags in their nsPs to evaluate various aspects of the viral infection cycle (111, 228, 230, 231). This is a powerful approach; however, its use is limited by the fact that tagging alphavirus replicase proteins without compromising their activities is rather complicated. There are also multiple studies in which the functions of CHIKV nsPs have been analyzed using transient expression systems. While these studies are certainly very important, they suffer from two shortcomings. First, the functions of individual nsPs are different from those in the context of virus infection, where all nsPs act in coordinated manner. Second, expressed nsPs were often visualized using tags attached to their terminus. This is a major problem, as alphavirus nsPs do not tolerate well (and sometimes not at all) modifications to their N-terminus. For example, even the presence of very short tags (such as a hexahistidine tag) can result in major changes in nsP functions – some of its activities may be severely diminished while others may be significantly enhanced (143). This does not mean that tagging of nsPs should be avoided, but rather that the data originating from such studies must be interpreted very carefully. However, it would certainly be ideal if tags could be inserted into regions where they do not alter the activities of nsP. The best test for such a system would be the generation of modified (tagged) viral genomes, replicases and, when available, *trans*-replicases and the demonstration that they function normally.

Previously, our laboratory has generated multiple tagged versions of SFV and CHIKV genomes. Many of these have been very useful and have been applied in many published works (222, 225–227, 254). We have observed the same tendency as other researchers: only nsP2 and nsP3 allow large insertions in certain positions (not everywhere) (228, 229). Although successful tagging has been achieved, many attempts to construct tagged viruses have failed. Examples of the latter include the addition of EGFP to the C-terminus of SFV and CHIKV nsP4: such viruses could be rescued, but they were genetically very unstable and the inserted sequence was lost in a single passage (unpublished data from our laboratory). To the best of our knowledge, no group (including ours) has previously succeeded in tagging nsP1 in the context of functional viral genome or replicon. Therefore, we attempted to use our *trans*-replication

system to engineer an EGFP tag into all of the nsPs and to determine the reasons for the genetic instability observed in some of the tagged constructs. Predictably, tagging worked best in the case of nsP3. In nsP1 and nsP2, a marker sequence was inserted into different positions to identify an optimal location (**III**, Fig. 4A). The most suitable position (a total of 5 variants, including one that included a fusion to the N-terminus of nsP1, were tested) for the EGFP tag in nsP1 was following aa residue 516. For nsP2, the best insertion site was located between the NTPase and protease regions of the protein (**III**, Fig. 4B). Tagging nsP4 was the most difficult. No matter how EGFP was fused to the C-terminus of nsP4, the replicase activity was always severely compromised. This was in perfect agreement with data from experiments with corresponding viruses: their infectivity indicated that nsP4-EGFP is functional, while their genetic instability indicated that loss of the EGFP tag resulted in a huge growth advantage of resulting aberrant genomes. Based on this, attempts to use fluorescent or luciferase (we tested several) tags in nsP4 were abandoned, and shorter immunological tags were used instead. Even this approach was not simple, as the HA-tag (Hemagglutinin-tag) invariably compromised the activity of the CHIKV replicase, and only the SF-tag (consisting of Streptavidin and 3×FLAG tags) was well tolerated (**III,** Fig. 4). When the tagging was performed in the context of CHIKV replicons or genomes, it was observed that all tags that allowed efficient replication in the *trans*-replicase system were also well tolerated in the context of the replicons (**III**, Fig. 5B). In contrast, the tags that severely reduced replication in the *trans*-replicase system invariably caused instability in the corresponding replicating RNAs (**III**, Fig. 4, 5B). Thus, the *trans*-replicase can be used to estimate whether a specific tag would work in the context of the viral/replicon genome. Furthermore, our analysis of tagged *trans*-replicases and a matching set of replicon vectors revealed that the tagged nsPs maintained characteristic subcellular localizations (**III,** Fig. 6). Our *trans*-replicases also enabled efficient spherule formation near the plasma membrane, confirming that essential steps associated with RNA replication were correctly reproduced in this system. In all analyzed cases, no negative effect of correctly placed tags (e.g., those located in positions that permitted efficient RNA replication) on spherule formation was observed (**III,** Fig. 7).

As described in section 5.1.2, we were unable to complete functional analysis of the effects caused by cytotoxicity-reducing mutations using the tools that were available at the time when studies **I** and **II** were performed. Hence, the effects of these mutations were studied using the *trans*-replicase system. One additional aspect should be taken into account while analyzing these mutations: in contrast to most of nsP mutations, which have little to no effect on replicase polyprotein production in the *trans*-replicase system, the cytotoxicity-affecting mutations are expected to have such an effect. This is not related to RNA replication (as in the context of replicons or genomes); rather, the presence of cytotoxicity-reducing mutations should prevent the shutdown of cellular transcription/translation and allow prolonged (and thus more efficient) production of replicase proteins. Interestingly, an increase in nsP levels was clearly

observed for replicases harboring the PG mutation (**III**, Fig. 8B), which, on its own, had little effect on the cytotoxic properties of CHIKV replicons (**I, II**). The increase in nsP level was less evident for the replicase containing the 5A mutation, even when it was combined with the PG mutation (**III,** Fig. 8B). Interestingly, the amounts of nsP2 harboring both the 5A and PG mutations were clearly reduced (**III**, Fig. 8B), most likely because this combination of mutations destabilized the protein; this finding also indicates that mutations in nsP2 can modulate each other's effects. Overall, despite these discrepancies, the PG and 5A mutations indeed resulted in effects consistent with what could be expected for cytotoxicity-reducing mutations. However, for the EK mutation, this clearly was not the case: its presence did not have any detectable effect on nsP accumulation (**III**, Fig. 8B). Once again, this indicates that the mechanism through which this mutation produced a noncytotoxic phenotype for the CHIKV replicon must be different from those of classical cytotoxicity-reducing mutations. In this regard, it was highly informative that the EK and EK-PG mutations in the CHIKV replicase drastically reduced its ability to activate Gluc expression (Gluc levels were <0.5% compared to those measured at the presence of the wt replicase; **III,** Fig. 8C). This result is consistent with the very low levels of replication of the corresponding replicon RNAs (**II**, Fig. 2A). Taken together, these findings clearly indicate that the noncytotoxic phenotype of the replicons containing the EK mutation represents a consequence of a severe defect in RNA replication. This defect led to drastically reduced nsP2 levels, which, in the presence of the PG mutation, were insufficient to cause cytotoxic effects in cells containing the corresponding replicon (**II**, Fig. 1). As of now, we have no evidence that the EK mutation actually attenuates (or eliminates) the cytotoxic properties of nsP2. In sharp contrast, it was observed that the PG mutation had no negative effect on the activity of CHIKV *trans*-replication (**III**, Fig. 8C). Thus, at the cellular level, we could not detect any consequences of the defects with regard to the protease, NTPase and RNA helicase activities of nsP2 harboring PG mutations (**II**). The 5A mutation reduced the ability of the replicase to boost Gluc expression by only approximately 2-fold. However, the combination of the non-cytotoxic mutations (5A-PG) reduced Gluc expression (compared to that of the wt replicase) by approximately 5-fold. This finding demonstrates that severely compromised replicase activity is not an absolute pre-requisite for noncytotoxic phenotype of Old World alphavirus replicons. In addition, this result confirms that the PG and 5A mutations indeed affect each other: the phenotype caused by the combination of these mutations was different from those caused by either of them alone (**II**). Taken together, the effects of the mutations that were associated with a noncytotoxic phenotype in CHIKV as detected by the *trans*-replicase system were similar to those observed in the context of replicons and genomes. In the *trans*-replicase system, because RNA replication does not affect replicase production, additional details concerning the modes of action for the cytotoxicity-reducing mutations were revealed. Thus, the CHIKV *trans*-replication system represents a powerful tool that can be applied for functional studies of CHIKV nsPs, replicase complexes and virus-host interactions.

5.3 Early replicases of different alphaviruses synthesize novel types of PAMP RNAs using cellular templates (IV; unpublished)

5.3.1 The SFV replicase synthesizes novel types of PAMP RNAs using cellular templates

The T7 promoter-based SFV *trans*-replication system has been used to reveal new biological properties of the virus (108, 110, 111). However, it is not the first alphavirus *trans*-replicase that has been constructed. Back in 2007, when I was an undergraduate student, my very first project was to work with a CMV promoter-based SFV *trans*-replicase that was constructed and tested in collaboration between our laboratory and FIT Biotech Oy Plc (Finland). The aim of this project was to develop a new gene expression system. The constructed SFV *trans*-replicase worked well; however, it was less efficient than standard SFV replicon vectors. Therefore, the results of this project were only included in a patent application (255) and never published as an article. However, when experimenting with the system, researchers from FIT Biotech observed that the SFV *trans*-replicase was a very efficient inducer of type I IFN expression. As expected, this effect depended on the RdRp activity of nsP4; however, contrary to the existing paradigm, it did not depend on the presence of template RNA that could be replicated/transcribed by the SFV replicase. It took several years of hard work to reach the conclusion that RNAs of viral origin (such as dsRNA replication intermediates or non-capped ssRNAs) are not the only RNAs made by the SFV replicase that are recognized by cellular PRRs. It was shown that in addition to these "classical" PAMP RNAs the SFV replicase also makes large amounts of extremely potent type I IFN inducing RNAs using nonviral, and thus by definition cellular, RNAs as templates. This finding was clearly important, but it also contradicted the existing understanding of how alphaviruses (or other viruses) are recognized by an infected cell; as such, it was also very difficult to publish (at least in high-level journals). A repeated concern from such journals was regarding whether some sort of massive artifact was responsible for the above-mentioned observations. In other words, do these type I IFN-inducing RNAs also exist in cells infected with SFV? This was clearly a valid concern; however, it was not easy to provide a suitable answer. This was also the point when I (after a 5-year break) returned to the project.

The major problem was that while we were aware of the general properties of nonviral PAMP RNAs, most importantly that they are relatively short and not polyadenylated (**IV**, Fig. 3), we did not know their sequences. Furthermore, we assumed that the sequences of these RNAs are likely to be diverse. Therefore, the only feasible way to prove their existence in SFV-infected cells was to demonstrate that RNAs isolated from SFV-infected cells maintain the ability to induce type I IFN expression even when all known PAMP RNAs are removed. In the context of SFV (and RNA viruses in general), it was believed that the expression of type I IFN is triggered mainly, if not exclusively, by viral ssRNA

containing a 5' triphosphate and/or dsRNA produced by viral replicases during viral genome replication. To analyze whether this was also the case for SFV, mouse embryonic fibroblasts were infected with SFV4 or a noncytotoxic mutant (SFV4-RDR), and total RNA was purified and fractionated into polyA+ RNAs containing a large majority of known PAMP RNAs (including dsRNA with an unpaired poly(A) tail, **IV,** Fig. 7A) and polyA− RNAs presumably containing novel PAMP RNAs. Using northern blotting, it was demonstrated that the polyA− fraction indeed contained only trace amounts of viral negative-strand RNAs and no detectable negative strands of virus-derived defective interfering RNAs (**IV,** Fig. 7B). Thus, all known viral PAMP RNAs were depleted from this fraction. Furthermore, analysis of the infectivity of the different RNA fractions revealed that polyA− RNA was also depleted (by approximately 15-fold) from viral positive-strand genomes. To avoid subsequent infection, both RNA fractions were UV-inactivated and used to transfect type I IFN competent COP-5 cells (murine fibroblasts). As expected, the polyA+ RNA was a powerful inducer of type I IFN expression. However, the polyA− RNA, which lacked (or was strongly depleted from) known PAMP RNAs, was a quite comparable inducer of type I IFN expression (**IV**, Fig. 7C). This experiment unequivocally demonstrated that large amounts of previously unknown PAMP RNAs were indeed present in polyA− RNA fraction obtained from cells infected with SFV. In subsequent experiments, it was demonstrated that the properties of these RNAs were similar (possibly identical) to those of RNAs made in cells expressing SFV replicase (**IV**, Fig. 7D). Thus, it was conclusively proven that SFV replicase makes, both during transient expression and natural infection, large amounts of very potent nonviral PAMP RNAs.

The discovery of this novel function of the SFV replicase led to an obvious question: why does a virus activate the type I IFN response in the first place? Type I IFN is known to mediate a wide variety of antiviral effects and represents an important first-line defense against virus infection. Our initial idea was that SFV simply cannot avoid nonviral PAMP production, which is to say that this production is simply the cost associated with the ability of the viral polymerase to be active *in trans* and/or to carry out RNA recombination with different RNA molecules (**IV**). To compensate for excessive type I IFN induction, alphaviruses have developed an impressive diversity of tactics to avoid IFN responses and/or their consequences (see 2.4 for details). Furthermore, some poorly understood mechanisms make SFV4 (the virus strain used in study **IV**) highly resistant to the antiviral effects of type I IFN (256). Therefore, it seemed that virus has developed sufficient amount of countermeasures. However, is this the whole truth? Are novel replicase-generated RNAs actually important for the viral life cycle? Although we were happy with the provided explanation, we did not exclude such possibilities. Furthermore, unexpectedly and rather rapidly, we were able to produce strong evidence supporting the above.

Parallel studies conducted in our laboratory and summarized in the PhD thesis of Sirle Saul (ISSN 2228-0855; http://hdl.handle.net/10062/49532) revealed that SFV has two determinants of neurovirulence: the nsP3 region and,

interestingly, the speed of the maturation of the replicase complex (the efficiency of the processing of the P123 precursor) (164). It was assumed that one or both of these determinants may be associated with the induction of type I IFN or with counteracting its antiviral effects. However, it was difficult to analyze the production of type I IFN in cell culture infected with the corresponding virus strains, chimeras and mutants. In contrast to the SFV mutants that showed reduced cytotoxicity, all of these viruses were fully capable of shutting down type I IFN production in the initially infected cells. Therefore, only cells that were primed by primary infected cells (196) and then got infected with a next generation of virus were able to respond by inducing type I IFN expression. It was, however, nearly impossible to synchronize this secondary infection using a panel of viruses each of which had slightly different growth kinetics. Therefore, the SFV-based *trans*-replicase constructs were generated to analyze the abilities of different SFV replicases and their mutants (Fig. 7A) to induce type I IFN. First, it was observed that replicases from nonvirulent (A7(74)) and virulent (SFV6; almost identical to that of SFV4, which was used in study **IV**) viruses can replicate and transcribe provided template RNA with quite similar (differences $\leq$ 2-fold) efficiencies (Fig. 7B). This was expected, as the corresponding viruses replicated to similar titers in cell culture (164). It also indicates that the ability to induce type I IFN using classical (derived from viral RNA or, at the case of *trans*-replicases, from provided template RNA) PAMPs was similar between the viruses. Second, the induction of type I IFN using non-cellular PAMP RNAs (e.g. in the absence of replication competent template RNA) was analyzed. This analysis provided a clear answer that was completely opposite of what was expected. Namely, replicases from avirulent strain A7(74) and nonvirulent recombinant virus (SFV6-74-RE) were, in the absence of template RNA, poor inducers of type I IFN. In contrast, the replicase of the neurovirulent strain SFV6 and the replicases of all studied virulent recombinants (for example, A7(74)-6-HV) were much more efficient type I IFN inducers (Fig. 7C). Thus, the neurovirulence of SFV clearly correlates with the enhanced production of type I IFN, and, consequently, almost certainly represents a consequence of some kind of immune pathology. This phenomenon is not completely unique in the world of alphaviruses; similar behavior has also been observed for SINV (257). What is unique is that the over-induction of type I IFN and the resulting immune pathology were clearly caused by the production of PAMP RNAs made by use of cellular templates. To the best of our knowledge, this represents the first clear demonstration of the biological significance of novel replicase-generated PAMP RNAs and serves as a topic for further studies.

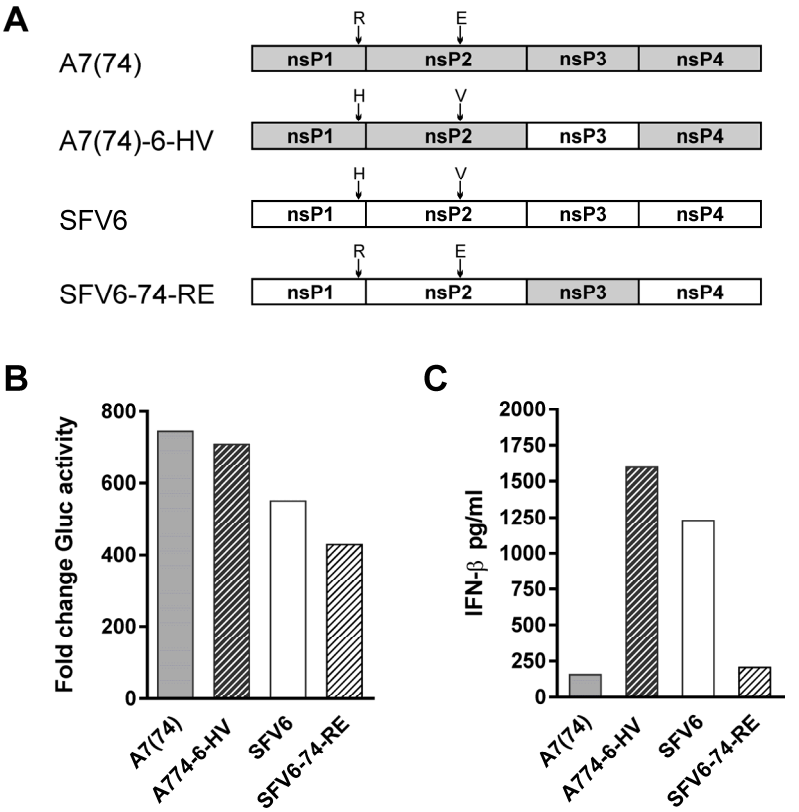

Figure 7. Replicases of neurovirulent variants of SFV are powerful inducers of type I IFN expression. (A) Schematic presentation of the analyzed SFV replicases. Sequences originating from avirulent A7(74) are shown in gray, while sequences from virulent SFV6 are shown in white (note the switch of the nsP3 regions in the recombinant viruses). The arrows above the drawings point to aa residues 534 and 1052 of P1234, which are responsible for the different processing speeds of the A7(74) and SFV6 ns polyproteins. (B, C) COP-5 cells were transfected with constructs expressing the indicated replicases or (B) co-transfected with the indicated replicase expression constructs and plasmid for the production of template RNA. The template RNA contains a Gluc-encoding region under a SG promoter, allowing Gluc expression to occur in response to replicase-mediated SG RNA synthesis. Control cells were transfected under the same conditions, except that a plasmid expressing an inactivated replicase (harboring a GDD to GAA mutation in nsP4) was used. At 48 h post-transfection, the culture medium was harvested. (B) Gluc activity in the growth medium were measured and normalized to that measured for the control cells. The activation of Gluc expression serves as a measure of RNA replication and transcription, as shown in **III.** (C) Levels of IFN-β in supernatant were measured by ELISA. Data from one out of three reproducible independent experiments are shown.

5.3.2 CHIKV replicase differs from SFV replicase in the ability to use cellular templates for the synthesis of PAMP RNAs

The novel PAMP RNAs made by the SFV replicase turned out to be highly biologically relevant. This led to the important question of whether such RNAs are made by the replicases of other viruses. In study **IV**, it was demonstrated that the RdRp (NS5B) of HCV makes such RNAs, which has also been observed in other studies (258). Properties consistent with the production of nonviral PAMP RNAs have also been observed for replicases from noroviruses (259) and picornaviruses (260). Furthermore, quite recently, it was demonstrated that picornavirus RdRp produces molecules with biological effects very similar to those observed for SFV replicase-generated PAMP RNAs (261). Thus, the phenomenon is certainly not restricted to SFV. Therefore, it was reasonable to expect that replicases of other alphaviruses would also produce nonviral PAMP RNAs. Here, we investigated whether this new mechanism also applies in the context of CHIKV *trans*-replicase.

Interestingly, and very unexpectedly, it was found that the replicase of CHIKV is drastically different from that of SFV with regard to the above. As described in study **III**, wt CHIKV replicase was very efficient in template RNA replication/transcription in many cells, including type I IFN-competent COP-5 cells (Fig. 8B). Nevertheless, in COP-5 cells, the replicase failed to induce type I IFN production in a template-independent manner (Fig. 8C). Based on the lessons learned when conducting study **IV** and on data from several publications (163, 213), it was first assumed that nsP2 of CHIKV might be an extraordinarily efficient inhibitor of type I IFN production/signaling. Therefore, the experiment was repeated using a panel of CHIKV *trans*-replicases harboring previously described noncytotoxic mutations (**II,** Fig. 8A). As previously observed, these mutations had various effects on the ability of CHIKV replicase to replicate/transcribe template RNA (**III,** Fig. 8C). This was also true in COP-5 cells, except that the replicase harboring the PG and 5A mutations was strongly attenuated in these cells (Fig. 8B). However, even though replicases harboring PG or 5A mutations were efficient in RNA replication/transcription (Fig. 8B), they were still virtually unable to induce IFN-β synthesis in a template-independent manner (Fig. 8C). Thus, the problem was apparently not an efficient counteraction of type I IFN response. Instead, it was concluded that CHIKV replicase simply does not make non-viral PAMP RNAs that can trigger type I IFN production. However, very weak type I IFN induction (nowhere near that achieved by the SFV replicase; ~3000 pg/ml, Fig. 8E) was observed for the replicase harboring both the PG and 5A mutations (Fig. 8C). It was therefore hypothesized that weak but detectable type I IFN induction might originate from the compromised protease activity of mutant nsP2, leading to the stabilization of ns polyproteins that was observed in cell-free system (**II**, Fig. 5B). Stabilization of ns polyproteins, as we have shown for SFV, correlates with type I IFN induction (Fig. 7C). Could the same process, i.e., the stabilization of ns polyproteins, also create the new (for CHIKV replicase) function of non-viral PAMP RNA synthesis?

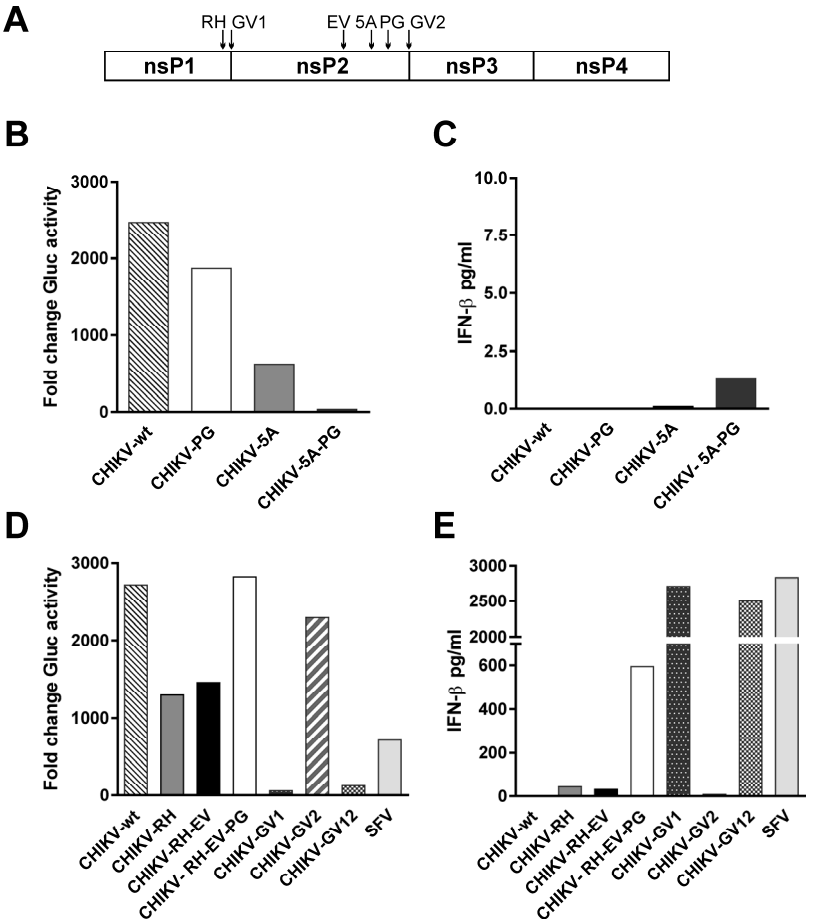

Figure 8. CHIKV replicase cannot synthesize non-viral PAMP RNAs; however, such activity can be generated by the introduction of mutations affecting the processing of the 1/2 site of P1234 (A) Locations of mutations affecting P1234 processing in the CHIKV ns polyprotein. (B, D) Abilities of CHIKV replicase and its mutant forms to replicate and transcribe template RNA and (C, E) to induce IFN-β production in a template-independent manner. (C, E) COP-5 cells were transfected with the indicated replicase expression constructs or (B, D) co-transfected with replicase expression constructs and a plasmid for template RNA expression (CMV-Fluc-Gluc, **III**, Fig. 1B). Control cells were transfected with a plasmid expressing an inactivated ns polyprotein (harboring a GDD to GAA mutation in nsP4). At 48 h post-transfection, culture media were harvested. (B, D) Gluc activity in the growth medium was measured and normalized to that measured for the control cells. (C, E) Levels of IFN-β in supernatant were measured by ELISA. Data from one out of three reproducible independent experiments are shown.

The processing determinants for CHIKV P1234 are similar to those for P1234 of avirulent SFV A7(74): position P4 in the 1/2 cleavage site is occupied by an Arg residue (His in virulent SFV6), while position 515 of nsP2 (1030 in P1234) is occupied by a Glu residue (Val in virulent SFV6). Mutations changing these residues (Arg532 to His: RH mutation; Glu515 to Val: EV mutation) were made and included, individually or in different combinations (including in combination with PG), into the CHIKV *trans*-replicase (Fig. 8A, D, E). In addition, mutants with inactivated cleavage sites between nsP1 and nsP2, nsP2 and nsP3 or both were created (Fig. 8A, D, E). When the corresponding constructs were analyzed for the ability to replicate/transcribe template RNA, all were found capable of doing so, albeit to different extents (Fig. 8D). The effect of the GV2 and RH-EV-PG mutations was minimal. The RH and RH-EV mutations resulted in no more than a 2.5-fold reduction in RNA replication/transcription (Fig. 8D). In contrast, a mutation in the 1/2 site prominently reduced RNA replication. Interestingly, however, the effect caused by the double mutation GV12 was reproducibly ~2-fold smaller than that caused by the GV1, affecting only 1/2 site (Fig. 8D). The most likely explanation for this observation is that GV12 completely prevents the cleavage of P123 into mature nsPs and thus locks the CHIKV replicase complex into a P123+nsP4 (early replicase) configuration. Compared to the late replicase, the early replicase is clearly less efficient in template RNA replication/transcription. However, it still displays significant ability synthesize SG RNA: Gluc activity is ~130-fold over background (measured in the presence of inactive $P1234^{GAA}$) (Fig. 8D). The GV1 mutation also prevents mature (late) replicase formation, but in this case P123+nsP4 complexes could be further converted into P12+nsP3+nsP4 complexes, which are either not active or have lower activity than P123+nsP4 complexes. Whether this is true represents a topic for further studies. When all of the above replicase constructs were analyzed for the ability to induce type I IFN production in template-independent manner, clear results were obtained. The RH mutation (which most likely slows the cleavage of P123) slightly elevated type I IFN production. The effect was somewhat smaller for the combination of RH-EV mutations (apparently because the EV mutation accelerates P123 processing) and much larger for the RH-EV-PG combination (Fig. 8E). The prominent boosting of the effect by the PG mutation may result from the further slow down of P123 processing and/or from the reduced cytotoxicity of nsP2 harboring this mutation. The effect of the GV2 mutation was very small but still reproducibly detected (Fig. 8E). Again, this effect may result from altered P123 processing (the GV2 mutation results in a nsP1+P23+nsP4 replicase that can synthesize both positive- and negative-strand RNAs) and/or from a reduction in the ability of nsP2 to counteract type I IFN signaling (P23 is not known to localize to the nucleus). Most drastic effects, however, were caused by the GV1 and GV12 mutations which locked the CHIKV replicase either permanently (GV12) or temporarily (GV1) into its early (P123+nsP4) conformation. Both of these replicases induced type I IFN production at a level comparable to that observed for the SFV replicase (Fig. 8E). While alone this observation may still

be explained by a reduction in the ability of nsP2 to counteract the type I IFN response (P12 and P123 are attached to the plasma membrane and do not enter the nucleus (89)), the whole obtained dataset clearly and unambiguously indicates that the production of nonviral PAMP RNAs is a property of the early, but not late, replicase of CHIKV. The same is almost certainly true for SFV as well, taking into account the data shown in Fig. 7 as well as the results obtained during the analysis of SFV replicases harboring numerous different mutations, including analogs of the GV1 and GV12 mutations (unpublished data from our laboratory). Thus, our results provide novel insights about the interactions that exist between alphaviruses and innate immune response pathways and demonstrate that two closely related alphaviruses (SFV and CHIKV) are recognized differently by host cell.

5.4 Future perspectives

When studies described in this thesis were initiated, there was little reason to think that all of the topics discussed above (noncytotoxic mutations in CHIKV replicons, viral RNA replication, SFV and CHIKV *trans*-replicases and novel type I IFN-inducing PAMP RNAs) would ultimately come together. Furthermore, there was certainly no indication that all of this would be linked to the topic of the *in vivo* pathogenesis of alphavirus infections. However, our findings coalesced, and a more general picture of alphavirus biology began to emerge. While preparing the final publication, used in this thesis, we were able to reveal a number of other unique insights into alphavirus biology. This includes the unexpected differences found in the abilities of SFV and CHIKV to activate the PI3K-Akt-mTOR pathway, which is associated with the internalization of replicase complexes (112) and may or may not be linked to the different abilities of CHIKV and SFV to induce the type I IFN response. It also includes the unexpected properties of CHIKV harboring EV and/or RH mutations, which may or may not be linked to the effects observed using the models described in this thesis. Although the above endeavors resulted in many unanswered inquiries, it is certain that a lot of interesting work lies ahead.

6. CONCLUSIONS

Based on the data obtained in the studies included in this thesis, the following conclusions can be made:

- CHIKV replicons capable of persistent replication in vertebrate cell lines were developed. This was achieved by the selection, identification and analysis of different mutations located in the nsP2 protein of CHIKV. The noncytotoxic phenotypes of these CHIKV replicons represent a consequence of the functional defects caused by these mutations. Furthermore, different combinations of mutations causing a noncytotoxic phenotype most likely cause the phenotype through different molecular mechanisms.
- Different cell lines had different requirements for supporting the noncytotoxic growth of the CHIKV replicon. CHIKV replicon with additional adaptive mutations in nsP1 and nsP3 that enabled its persistence in human cell line was constructed and analyzed. Cell lines harboring noncytotoxic replicons that also contained sequences encoding marker proteins possessed good stability and may represent safe surrogate models for the screening of compounds with antiviral activity.
- A novel CHIKV *trans*-replication system in which CHIKV replicase expression and template RNA replication are uncoupled was constructed. It was demonstrated that this system represents an excellent tool that can be used to analyze the effects of different mutations or tags introduced into replicase proteins. The unique properties of the *trans*-replicase make it especially useful for studies of mutations that cause instability in the context of replicating RNA genomes.
- Alphavirus *trans*-replicase systems were found to be useful tools for studies of virus-host interactions. The SFV *trans*-replicase system led to the discovery of a new function of the alphavirus replicase, namely, the synthesis of PAMP RNAs using cellular templates. The combined use of the SFV and CHIKV *trans*-replicases, their mutant variants and the corresponding viruses revealed important differences in how these viruses are recognized by host cells. It was also found that an early alphavirus replicase is responsible for the synthesis of PAMP RNAs from cellular templates. The same set of studies revealed a connection between the ability of the SFV replicase to synthesize new types of PAMP RNAs and SFV neurovirulence.

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SUMMARY IN ESTONIAN

Alfaviiruse replikaasi roll genoomi replikatsioonis, viirus-indutseeritud tsütotoksilisuses ja viirusinfektsiooni tuvastamises peremeesraku poolt

Alfaviirused on positiivse polaarsusega RNA genoomsed viirused, mis omavad laia peremeesteringi ning on võimelised paljunema erinevates rakutüüpides. Alfaviiruste levik selgroogsete peremeeste vahel toimub enamasti lülijalgsete vektorite vahendusel. Alfaviiruste perekonda kuulub ka suure meditsiinilise tähtsusega viiruseid nagu Chikungunya viirus (CHIKV). CHIKV infektsiooni iseloomustab kõrge palavik, lööve ja lihasevalu, eriti iseloomulik on aga liigesevalu, mis võib muutuda krooniliseks ning kesta kuid või isegi aastaid. Praeguseks hetkeks puudub selle viiruse vastane vaktsiin või spetsiifiline ravim, võimalik on leevendada vaid sümptomeid. Samas on kliima muutused ja ülemaailme reisimine võimaldanud CHIKV-l levida ka piirkondadesse, kus teda varem ei ole olnud. Näiteks jõudis CHIKV 2013. aasta lõpus Ameerikasse, kus tema levik on võtnud epideemia mõõtmed. Seetõttu on CHIKV uurimine küll intensiivistunud, kuid siiski on tema kohta käivad teadmised lünklikud ja ebapiisavad. Selleks, et välja töötada CHIKV vastased vaktsiinid või ravimid tuleb esmalt põhjalikult uurida tema molekulaarbioloogiat ja, mis veelgi olulisem, mõista kuidas CHIKV ja tema peremees teineteist vastastikku mõjutavad. Paraku on tegemist ohtliku patogeeniga, mille käsitlemine on raskendatud kõrgest (kolmas ohuklass) bioohutuse tasemest tulenevate töötingimuste tõttu. Seetõttu on CHIKV süvauuringuteks eriti vajalikud ohutud ja samas ka efektiivsed tööriistad.

CHIKV infektsioon pärsib selgroogsest peremehest pärinevate rakkude metabolismi põhjustades tsütopaatilisi efekte ning lõpuks ka raku surma. See on vähemalt osaliselt põhjustatud nsP2 valgu toimest. NsP2 on multifunktsionaalne mittestruktuurne valk, mis lisaks oma funktsioonidele viiruse replikaasi kompleksis on ka raku transkriptsiooni ja translatsiooni mahasuruja. Peale selle on CHIKV ja teiste Vana Maailma alfaviiruste nsP2 vastutav tüüp-1 interferooni tootmise ja selle toime mahasurumise eest. Kuna tüüp-1 interferoonid on esmasteks viirusvastase kaitse vahendajateks võimaldab nende toime blokeerimine viirusel kiiremini paljuneda ja levida. NsP2 valgu tähtsusele nende protsesside mõjutamisel viitab ka asjaolu, et teatud mutatsioonid nsP2 valgus võimaldavad persistentse infektsiooni kujunemist selgroogse peremehe rakkudes.

Väitekirja kuuluvate tööde esimeseks eesmärgiks oli konstrueerida mittetsütotoksilised CHIKV replikonid, mis võimaldavad neid sisaldavate imetaja rakkude paljunemist ning iseloomustada sellise fenotüübiga seonduvaid defekte nsP2 valgu funktsioonides ja/või viiruse RNA sünteesis. Töö käigus loodi vastavad BHK-21 rakkude põhised püsiliinid ja tehti kindlaks erinevad mutatsioonide kombinatsioonid nsP2 valgus, mis vähendasid CHIKV replikoni tsütotoksilisust. Iseloomustati ka mutatsioonid nsP1 ja nsP3 valkudes, mis

võimaldasid mittetsütotoksilise CHIKV replikoni püsimist inimese rakkudes. Läbiviidud analüüsid näitasid, et mutatsioonid nsP2 valgus mõjutavad selle ensümaatilisi aktiivsuseid ning vähenenud tsütotoksilisusega fenotüübid erinevad seda efekti põhjustavate molekulaarsete mehhanismide poolest. Ka leiti, et saadud püsivalt replitseeruvaid replikone sisaldavad ja nende vahendusel markervalke tootvad rakuliinid kujutavad endast efektiivseid ja ohutuid tööriistu, mis võimaldavad CHIKV vastaste inhibiitorite otsimist ja nende toime analüüsimist.

Siin kokku võetud tööde teiseks eesmärgiks oli CHIKV *trans*-replikaasi süsteemi väljatöötamine, iseloomustamine ja katsetamine. Selleks konstrueerisime süsteemi, kus replikaasi valkude tootmine ja nende poolt läbiviidav RNA replikatsioon on teiseteisest lahutatud. Näitasime, et selline süsteem võimaldab uurida erinevate mutatsioonide või mittestruktuursetele valkudele lisatud *tagide* otseseid efekte replikaasi võimele läbi viia RNA sünteesi. Võrrelduna infektsioonilise viirusega omab CHIKV *trans*-replikaas sellistes analüüsides selgeid eeliseid, sest viiruse kontekstis põhjustavad paljud mutatsioonid geneetilist ebastabiilsust.

Väitekirja kolmandaks eesmärgiks oli Semliki Forest viiruse (SFV) ja CHIKV *trans*-replikaaside kasutamine viirus-peremeesrakk vaheliste interaktsioonide uurimiseks. SFV *trans*-replikaasi kasutades avastasime alfaviiruste replikaasi uue funktsiooni – võime kasutada rakulisi RNAsid sünteesimaks kaasasündinud immuunvastust aktiveerivaid kaheahelalisi RNA molekule. CHIKV *trans*-replikaasi kasutamine näitas, et selle viiruse metsik-tüüpi replikaas ei ole võimeline meie poolt kirjeldatud viisil immuunsüsteemi aktiveerima. Erinevate *trans*-replikaaside võrdlemine võimaldas kindlaks teha peamised faktorid, mis vastutavad raku RNAde põhiste kaheahelaliste molekulide sünteesi eest.

Käesoleva uurimustöö käigus on loodud rida unikaalseid ja efektiivseid CHIKV molekulaarbioloogia uurimise tööriistu. Nende kasutamine on võimaldanud paremini mõista nii CHIKV molekulaarbioloogiat kui ka alfaviirus-peremees vahelisi interaktsioone. Läbiviidud töö näitas, et kahe sarnase alfaviiruse äratundmine peremeesraku poolt toimub väga erineval moel.

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