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William Bakhache

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie Santé

École Doctorale Sciences Chimiques et Biologiques pour la Santé

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Interactions de la protéine nsP1 du virus Chikungunya avec les membranes de l'hôte et conséquences fonctionnelles

Présentée par William Bakhache

Le 27 Mars 2020

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List of abbreviations

(-)RNA negative-sense RNA

(+)RNA positive-sense RNA

3' UTR 3' untranslated regions

5' UTR 5' untranslated regions

Ae. *Aedes*

Arboviruses arthropod-borne viruses

ATP adenosine triphosphate

AUD/ZBD Alphavirus unique/zinc finger binding domain

BAR Bin/amphiphysin/Rvs

BF Barmah Forest

BMV Brome mosaic virus

CHIKF Chikungunya fever

CHIKV Chikungunya virus

CPVI cytopathic vacuoles of type I

CPVII cytopathic vacuoles of type II

CSE conserved sequence element

DAG diacylglycerol

DENV Dengue virus

DNA deoxyribonucleic acid

dsRNA double-stranded RNA

E1 envelope 1

E2 envelope 2

ECSA East/Central/South African

EEE Eastern Equine Encephalitis

EEEV Eastern Equine Encephalitis virus

eIF2 α eukaryotic initiation factor 2 α

ELOVL fatty acid elongase

ER endoplasmic reticulum

ESCRT endosomal sorting complexes required for transport

FASN fatty acid synthase
FDS fatty acid desaturase
FHL1 Four-and-a-half LIM domain protein
FHV Flock house virus
FXR Fragile X syndrome
G3BP1 Ras GTPase-activating protein-binding protein 1
GAPs GTPase activating proteins
GDIs guanine nucleotide dissociation inhibitors
GDP guanosine diphosphate
GEFs guanine nucleotide exchange factors
GTase guanylyltransferase
GTP guanosine triphosphate
HCV Hepatitis C virus
HMG-CoA reductase 3-hydroxy-3-methylglutaryl-coenzyme A
hnRNPs heterogeneous nuclear ribonucleoproteins
HVD hypervariable domain
ICTV International Committee on Taxonomy of Viruses
IFN α type I interferon
IOL Indian Ocean lineage
LDLs low-density lipoproteins
LE/Ls late endosomes/lysosomes
MAYV Mayaro virus
MD macro domain
MID Middelburg complex
Mnks MAP kinase-activated protein kinases
mRNA messenger RNA
MTase methyltransferase
MUFAs monounsaturated fatty acids
MVBs multivesicular bodies
Mxra8 Matrix remodeling associated 8 protein

NC nucleocapsid
NDU Ndumu
NIF nsP1-induced filopodia
nsP1 non-structural protein 1
nsP2 non-structural protein 2
nsP3 non-structural protein 3
nsP4 non-structural protein 4
nsPs non-structural proteins
NTPs nucleoside triphosphatase
NW New World
ONNV O'nyong-nyong virus
OW Old World
PA phosphatidic acid
PARP Poly (ADP-ribose) polymerase
PM plasma membrane
PtdCho phosphatidylcholine
PtdEtn phosphatidylethanolamine
PtdIns phosphatidylinositol
PtdSer phosphatidylserine
PUFAs polyunsaturated fatty acids
RdRp RNA-dependent RNA polymerase
RER rough endoplasmic reticulum
RNA ribonucleic acid
RRV Ross River virus
RTPase RNA triphosphatase
SAM S-Adenosyl methionine
SAV Salmonid *Alphaviruses*
SCD1 Stearoyl-CoA desaturase
SCRIB Scribble
SFAs saturated fatty acids

SFV Semliki Forest virus

SHAPE selective 2'-hydroxyl acylation analyzed by primer extension

SINV Sindbis virus

sPs structural proteins

ssRNA single-stranded RNA

STAT signal transducer and activator of transcription

TBSV Tomato bushy stunt virus

TF trans-frame

TMD transmembrane domain

tRNA transfer RNA

UFAs unsaturated fatty acids

VEE Venezuelan Equine Encephalitis

VEEV Venezuelan Equine Encephalitis virus

WEE Western Equine Encephalitis

WEEV Western Equine Encephalitis virus

WHO World Health Organization

WNV West Nile virus

ZDHHCs DHHC domain containing palmitoyltransferases

ZIKV Zika virus

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Introduction

Chapter 1 Introduction

1 RNA viruses: A relic to the RNA world

The discovery that ribonucleic acid (RNA) can have certain enzymatic functions led to the emergence of the RNA world hypothesis. In this concept, it is proposed that RNA was an important player in early evolution as one of the first self-replicating molecules. It would have functioned as the main storage device, but also as a catalyst. Then, later in evolution, deoxyribonucleic acid (DNA) would replace RNA as the storage device and proteins would evolve as the working-horses in living cells (Bernhardt, 2012; Koonin et al., 2006). In the context of this hypothesis, it is interesting to imagine viruses that carry RNA as their genome without the involvement of DNA (RNA viruses) as a flashback to the primordial RNA world.

RNA viruses can either have as their genome: single-stranded RNA (ssRNA), either positive-sense RNA ((+)RNA) or negative-sense RNA ((-)RNA), or double-stranded RNA (dsRNA). Recent studies of the evolutionary relationships between these three classes revealed that (+)RNA viruses are possibly at the origin of RNA viruses. It also showed that dsRNA viruses evolved from (+)RNA viruses in at least two independent events, and that (-)RNA viruses evolved from a distinct group of dsRNA viruses. A (+)RNA to dsRNA to (-)RNA virus evolutionary pathway is coherent with the molecular mechanisms used for viral protein expression and genome replication. Indeed, dsRNA and (-)RNA viruses need to package their replication machinery in viral particles in order to start a new viral cycle (Fig. 1). In contrast, (+)RNA viruses use the most basic strategy for expression and replication of their genome as the positive sense of their RNA allows for direct translation of viral proteins and then amplification of the viral genome (Wolf et al., 2018).

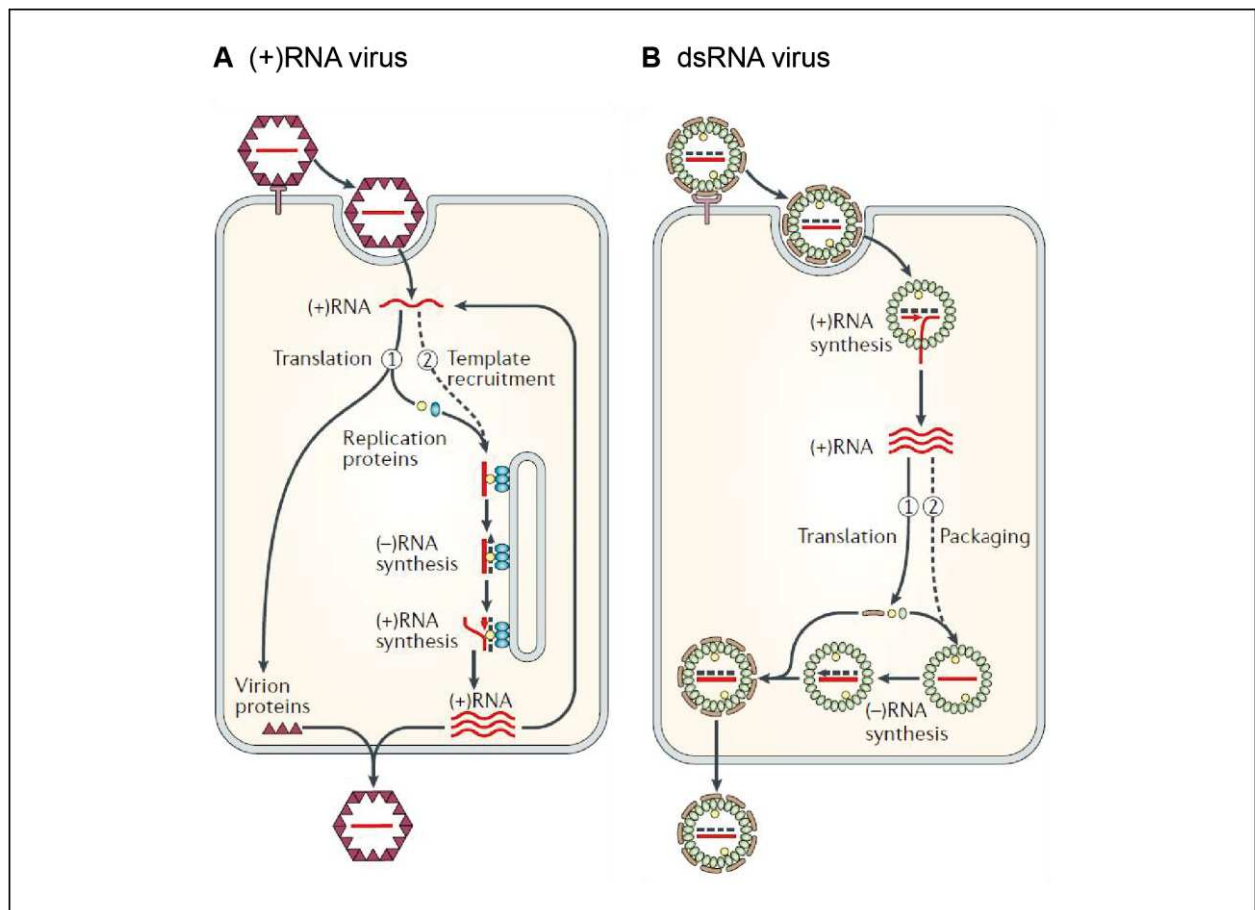


Fig. 1 Replication strategies of RNA viruses

(A) (+)RNA virus replication strategy: Endocytosed virions release their messenger-sense genomic RNA (straight red line) into the cytoplasm which will be directly translated to produce viral proteins. Viral replication proteins (blue) will then recruit the genomic RNA into membrane-associated replication compartments. In these sites, new (+)RNA will be synthesized and then packaged into new progeny virions. (B) dsRNA virus replication strategy: After endocytosis, the viral core of dsRNA viruses remains intact. In this core, the viral RNA polymerase (yellow) will be present and will produce new (+)RNA that will then be released into the cytoplasm. This RNA will be translated and packaged into the newly produced virion cores. Virion core maturation occurs after the synthesis of (-)RNA (dotted strand) leading to dsRNA formation (adapted from Ahlquist 2006).

2 Positive sense single-stranded RNA viruses

This ancient evolutionary past of (+)RNA viruses is mirrored by their abundance throughout the living world. They represent one of the largest classes of viruses and are present in all forms of life from bacteria to plants and animals (Strauss and Strauss, 2008).

All (+)RNA viruses encode an RNA-dependent RNA polymerase (RdRp). This enzyme will use the viral RNA as a template for the amplification of the viral genome, an essential process in the production of new virus progeny. In addition, (+)RNA viruses

will code for other types of enzymes (helicases and proteases) that will also be important in this process (Koonin et al., 2015).

Given the diversity and abundance of these viruses, comparison and alignment of viral sequences have given limited amount of information on their evolution and phylogeny. Therefore, here came the role of RdRp based phylogeny in combination with comparisons of viral enzymes modularity and arrangements for the large-scale division of (+)RNA viruses into three different superfamilies: picornavirus-like, flavivirus-like and alphavirus-like superfamilies (Koonin, 1991; Koonin and Dolja, 1993). The picornavirus-like superfamily is by far is the largest and most diverse superfamily. It is widely distributed in eukaryotic hosts. It is then followed by the alphavirus-like and flavivirus-like superfamilies. This led to suggestions of a scenario where ancestral picornavirus-like superfamily acted as an evolutionary origin for the other superfamilies (Koonin et al., 2015).

A common feature of all (+)RNA viruses is that the replication of the viral genome will pass by the synthesis of (-)RNA which will then be used as a template for (+)RNA production. This shared process will generate a dsRNA intermediate which is a hallmark element of (+)RNA viruses (Paul and Bartenschlager, 2013). Another fundamental aspect of (+)RNA replication machinery is that it will function in tight association with membranes which will be modified to produce replication organelles. However, the architecture and biogenesis of these virus-induced organelles can vary substantially within a certain virus superfamily and even family (Paul and Bartenschlager, 2013). I have described in a detailed review, the different type of replication organelles formed by (+)RNA viruses and the virus host-interactions involved in their creation (Bakhache et al., 2019). This review is presented in annex 1. These two prior described features universal to (+)RNA viruses might be intertwined since membrane-associated replication has been suggested to help with the evasion of dsRNA immune sensing (Scutigliani and Kikkert, 2017).

3 Alphaviruses

Among (+)RNA viruses, those belonging to the *Alphavirus* genus are impressive in their ability to cause important diseases in vertebrate hosts. Salmonid *Alphaviruses* (SAV) are a major economic problem for salmon and trout farmers. Losses due to infection by these viruses have been estimated at €35 million loss of turnover and €12 million

loss of profit during the 2003-2004 production period. Attesting to their economic downsides, measures have been put in place by the European Union to refuse import of salmon products from areas that have not been declared free of SAV (Jansen et al., 2017). A medicinally important *Alphavirus* causing encephalitis in horses, but also in humans is Venezuelan Equine Encephalitis virus (VEEV). In some cases, infection is fatal. Its devastating effects were seen during the 1992 epidemic with 100,000 reported human cases and 300 associated deaths. This outbreak also resulted in 4000 equine deaths (Weaver et al., 2004). Finally, the most impactful *Alphavirus* in recent history, Chikungunya virus (CHIKV) infection, in humans, leads to incapacitating muscle and joint pain, fever, and rash. CHIKV has caused devastating socio-economic impact on the communities where it spread. This has recently been observed in the Réunion outbreak where 255,000 cases were recorded with 260 resultant deaths. The incapacitating nature of infection by CHIKV also had consequential outcomes on public health care and presence of workers. During the 2015 CHIKV outbreak in the United States Virgin Islands, the economic figures were estimated to range from \$14.8 to \$33.4 million (Feldstein et al., 2019; Josseran et al., 2006). Finally, recently the “Centers for Disease Control and Prevention” federal agency has received multiple reports of infection with the Eastern Equine Encephalitis virus (EEEV), including 15 reported deaths. This raises the alarm on the re-emergence of yet another impactful *Alphavirus*.

In this section, the geographical distribution, classification, mode of transmission, evolution, and life-cycle of *Alphaviruses* will be discussed, with a special focus on CHIKV, the main *Alphavirus* studied in this thesis.

A- Taxonomy and phylogeny

According to the International Committee on Taxonomy of Viruses (ICTV) classification, the *Alphavirus* genus belongs to the *Togaviridae* family which also includes the *Rubivirus* genus with the Rubella virus as the only member (Strauss and Strauss, 1994). The *Togaviridae* family of viruses itself resides in the alphavirus-like superfamily which also contains various animal (Hepatitis E virus) and plant viruses (Brome Mosaic virus) as well. A characteristic of this superfamily is the presence of three modules, always in the same order, methyltransferase-helicase-RdRp. Finally, all viruses in this superfamily are capped at the 5' end and have a poly(A) tail or transfer RNA (tRNA) structure at the 3' end of their (+)RNA (Koonin and Dolja, 1993).

Traditionally, the classification of viruses inside the *Alphavirus* genus was mainly based on serological methods such as hemagglutination inhibition and complement fixation tests. If a newly identified virus strongly cross-reacted with antibodies against a recognized virus, then it was justified to include these two viruses into the same complex. This divided the *Alphavirus* genus which contains more than 30 different viruses into seven main antigenic complexes: Eastern Equine Encephalitis (EEE), Venezuelan Equine Encephalitis (VEE), Western Equine Encephalitis (WEE), Semliki Forest (SFV), Ndumu (NDU), Barmah Forest (BF), and Middelburg complex (MID) (Calisher et al., 1980).

Furthermore, with the advent of next-generation sequencing methods and availability of viral sequences, the natural evolution for classification was to compare partial or complete sequences of different *Alphaviruses*. In general, these studies have confirmed relationships drawn from antigenic based approaches. As it was shown that members belonging to the same antigenic complex were more genetically similar (nucleotide divergence less than 43%) than viruses with different antigenic complexes (nucleotide divergence usually exceeded 38%). Different subtypes of a given *Alphavirus* had as little as 3% nucleotide divergence and 2% amino acid divergence in some cases such as with Sindbis virus (SINV), and as high as 25% and 13% respectively in other cases such as with Ross River virus (RRV). Impressively, the maximum divergence was observed between different subtypes of VEE where a given subtype differed by at least 22% in nucleotide sequence and 18% in amino acid sequence. Indeed, VEE is one of the most genetically diverse complexes due to its history of circulation in hosts and vectors with limited dispersal potential. Finally, the two fish viruses (Salmon Pancreas Disease virus and Sleeping Disease virus) were the most distinct when compared to all other *Alphaviruses* with a nucleotide divergence of 49% and an amino acid divergence of 59%. However, these two aquatic viruses had minimal divergence between them (Powers et al., 2001; Weaver et al., 2012). A comparison of sequences also led to the re-construction of precise phylogenetic trees with high confidence (Fig. 2) (Forrester et al., 2012; Lavergne et al., 2006).

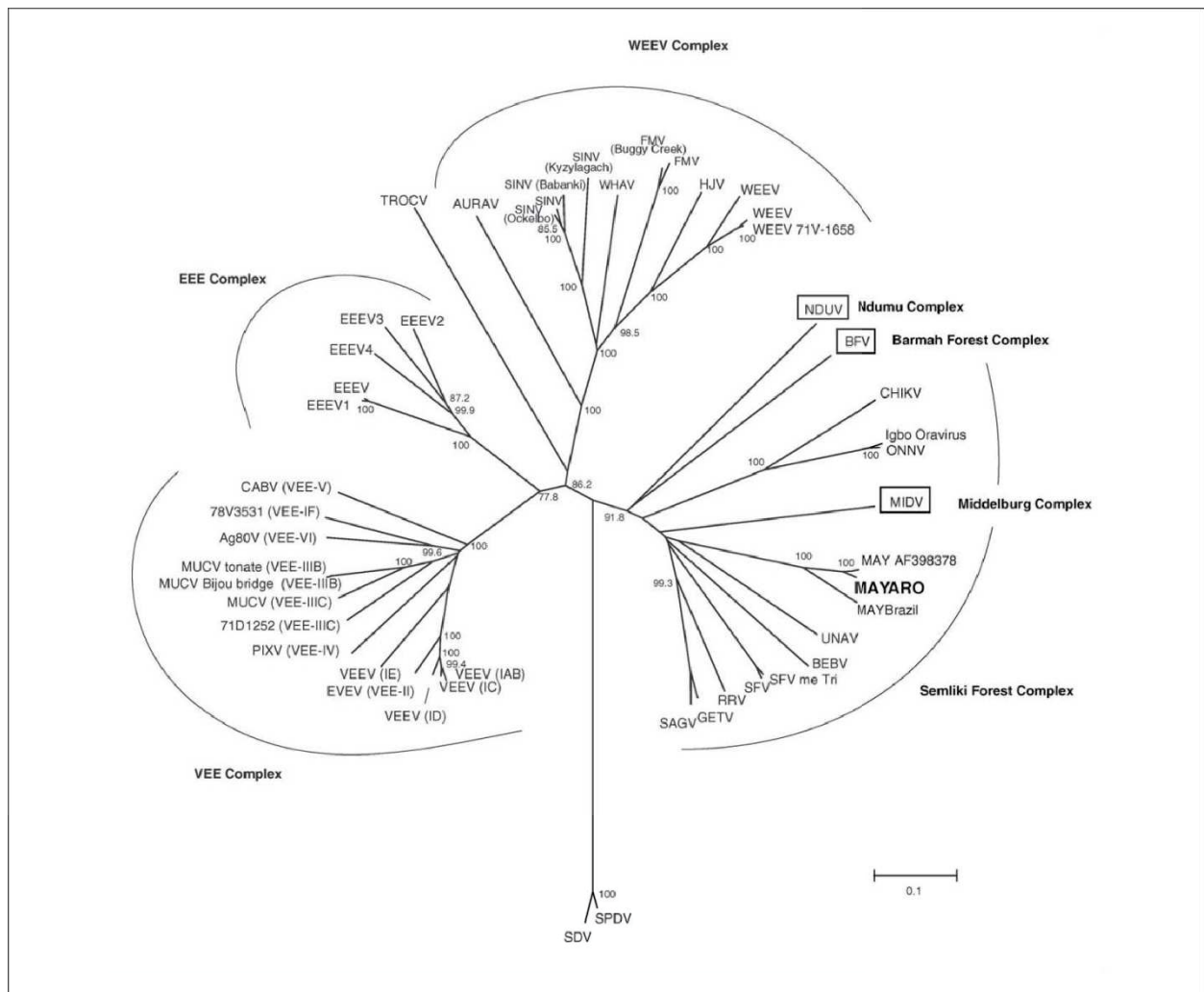


Fig. 2 Unrooted phylogenetic tree of *Alphavirus* species

Phylogenetic tree generated from partial E1 envelope glycoprotein gene sequences by using the Neighbor-Joining method with Kimura 2 parameter model. Virus abbreviations: MUCV, Mucambo virus; TONV, Tonate virus; PIXV, Pixuna virus; CABV, Cabassou virus; FMV, Fort Morgan virus; HJV, Highlands virus; WHATV, Whataroa virus; SINV, Sindbis virus; MIDV, Middelburg virus; SFV, Semliki Forest virus; RRV, Ross River virus; CHICK, Chikungunya virus; ONNV, O'nyong nyong virus; BFV, Barmah Forest virus; SAGV, Sagiya virus; SPDV, Salmon pancreas disease virus; SDV, Sleeping disease virus; GETV, Getah virus; NDUV, Ndumu virus; BEBV, Bebaru virus; TROCV, Troca virus; BCV, Buggy Creek virus; BABV, Babanki virus; OCKV, Ockelbo virus; AURAV, Aura virus; WEEV, Western Equine Encephalitis virus; VEEV, Venezuelan Equine Encephalitis virus; EEEV, Eastern Equine Encephalitis virus (adapted from Lavergne et al. 2006).

B- Geographical distribution and evolutionary history

Alphaviruses are widely spread all around the world, inhabiting all continents except the Antarctica. They can be classified according to their geographical origin and distribution. This split *Alphaviruses* into two main classes: New World (NW) and Old World (OW) *Alphaviruses*. NW and OW *Alphaviruses*, in most cases, differ in the type of symptoms induced upon infection (Strauss and Strauss, 1994).

NW *Alphaviruses* cause encephalitic symptoms and circulate in the Americas. They include viruses such as VEEV, EEEV, Western Equine Encephalitis virus (WEEV) (Strauss and Strauss, 1994).

On the other hand, OW *Alphaviruses* which lead to arthrogenic symptoms circulate in Europe, Asia, Australia and parts of Africa and include viruses such as CHIKV, SINV, Mayaro (MAYV), and O'nyong-nyong virus (ONNV) (Strauss and Strauss, 1994).

However, this geographical distribution is not as static as this classification suggests. The use of phylogenetic methods has allowed the revelation of exciting discoveries about the evolutionary history of the *Alphavirus* genus. One of the first eluding observations was that SINV, an *Alphavirus* which mainly circulates in the OW, is present in the NW WEE antigenic complex (Calisher et al., 1980). This was later reconciled by the finding that a recombination event occurred between the combined Envelope 2 and 1 (E2 and E1) genes of an ancestral form of SINV and the other viral genes of an EEEV-like ancestor. This episode led to the generation of the NW WEE complex (Weaver et al., 1997). Another intriguing remark was that *Alphavirus* distribution in NW and OW was not always associated with the respective symptoms mentioned above. This has been observed with certain *Alphaviruses* that share similar symptoms, but exist in diverse ecological conditions. A typical comparison is MAYV and ONNV which share almost indistinguishable clinical symptoms but circulate in different regions of the world. Unlike most OW *Alphaviruses*, MAYV circulation is mainly limited to South America. While, ONNV is only present in Africa. Reconstruction of the evolutionary history of this genus conciliated this remark and suggested that NW and OW *Alphaviruses* have circulated to opposite hemispheres multiple times probably by migratory birds and sailing ships.

This raised the question about the evolutionary origins of *Alphaviruses*. The reconstructed evolutionary history was consistent with either a NW or OW origin but required at least three transoceanic introductions between the hemispheres (Powers et al., 2001). However, there is still no substantial evidence favoring *Alphavirus* origin being from the NW or OW.

Interestingly, through the use of an even more robust and comprehensive phylogenetic analysis, a recent study has implicated a marine origin of the *Alphavirus* genus. In this scenario, *Alphaviruses* would have originated in the Pacific Ocean and then moved

from marine to terrestrial vertebrate hosts and then to mosquito vectors. Then, this ancestral *Alphavirus* of marine origin would have spread east and west leading to the creation of the ancestral NW and OW *Alphaviruses* of today. This evolutionary origin hypothesis would also require several re-introductions of NW and OW *Alphaviruses* across the hemispheres (Fig. 3). The argument for a marine origin has been supported by evidence showing that aquatic *Alphaviruses* were present at the basal root of the phylogenetic tree (Forrester et al., 2012). Additional support was that some NW *Alphaviruses* retained the ability to replicate in fish cells (Wolf and Mann, 1980).

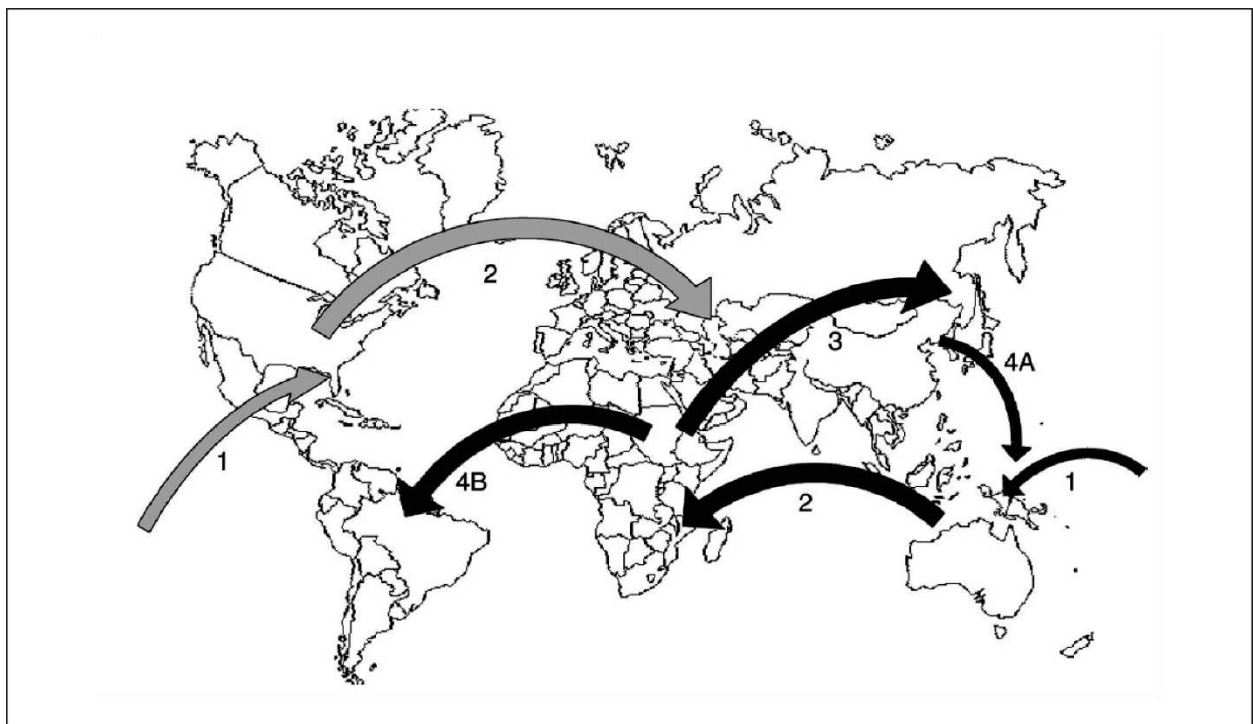


Fig. 3 Hypothetical evolutionary origin of *Alphaviruses*

Grey and black arrows represent New World *Alphaviruses*. Arrow 1: Introduction of ancestral marine origin *Alphavirus* from the Pacific Ocean to the New World; arrow 2: secondary introduction into the Old World. Black arrows represent Old World *Alphaviruses*. Arrow 1: introduction from the Pacific Ocean to Australia and New Zealand; arrow 2: secondary introduction into south Africa; arrow 3: tertiary introduction to Eurasia; arrow 4a, secondary introduction of Ross River virus to Australasia; and arrow 4b, secondary introduction of Mayaro virus to the New World (adapted from Forrester et al. 2012).

C- Distinct geographical distribution: Distinct evolutionary lineages

The emergence of these *Alphaviruses* in different geographical regions implied distinct evolutionary lineages with different adaptations to hosts. Indeed, striking differences in the molecular pathways have been described by several groups. For example, upon

infection, *Alphaviruses* are capable of inducing host-cell transcriptional shut-off as a mean to limit innate immune response detection. However, the *Alphavirus* protein responsible for this function varies between NW and OW *Alphaviruses*. For SINV and SFV, representatives of the OW *Alphaviruses*, mapping of the viral proteins responsible for this function revealed non-structural protein 2 (nsP2) as the determinant. The twist came when it was shown that the structural capsid protein is responsible for this function for VEEV and EEEV, representatives of the NW *Alphaviruses* (Garmashova et al., 2007). Another difference between NW and OW *Alphaviruses* relates to the nature of host-cofactors recruited for assisting viral replication. The usurping of such factors has been specially documented in studies dealing with interactants of nsP3.

nsP3 contains a long intrinsically disordered domain called the hypervariable domain (HVD) which has a low sequence identity between different *Alphaviruses*. The HVD domain is essential for viral replication and is capable of recruiting proviral cofactors. Interestingly, it was shown that the HVD of OW *Alphaviruses* recruits the stress granule assembly factor, Ras GTPase-activating protein-binding protein 1 (G3BP1) and that these interactions are essential for viral replication. However, NW *Alphaviruses* do not require HVD-G3BP1 interaction, and will instead recruit a functionally similar family of proteins named Fragile X syndrome (FXR). Interestingly, replacement of the NW *Alphavirus* HVD with its OW form did not have any deleterious effect on NW *Alphavirus* replication. However, the opposite manipulation led to deleterious effects on OW *Alphavirus* replication suggesting that interaction with FXR proteins came later on in *Alphavirus* evolution. Impressively, a follow-up study has found that the NW *Alphavirus* EEEV has a unique level of redundancy in its use for these two cofactors. This suggested that this virus acted as an evolutionary stepping stone for the interaction with these host proteins (Frolov et al., 2017; Kim et al., 2016). In conclusion, these different molecular pathways illustrated the distinct evolutionary past of OW and NW *Alphaviruses*. The functional importance of these interactions in the *Alphavirus* life cycle will be further discussed later in the introduction in section “Virus-Host interactions in replication complex assembly and functioning”.

D- Transmission: Host organisms and vectors

Viruses can be transmitted by a variety of different ways such as aerosol, sexual, and vector-mediated transmission.

For *Alphaviruses*, the mode of transmission is through insect vectors, mainly arthropods. They are, therefore, known as arthropod-borne viruses (arboviruses).

i. Arbovirus definition and history

In 1878, the first hint that arthropods could act as vectors for human pathogens was described by Sir Patrick Manson. He showed that mosquitoes were able to transmit filarial worms to humans and dogs (Manson, 1878). A few years later, Carlos Finlay suggested that the yellow fever disease was transmitted by mosquitoes (Chaves-Carballo, 2005). Yellow fever disease, at that time, was epidemic in many tropical and subtropical regions of Africa and the Americas. This led to significant attempts by the United States to study this disease in an endeavor to eradicate it. However, it was not until the year 1900 where the theory of Carlos Finlay was proven right in an expedition led by the team of Walter Reed. The breakthrough came from an experiment performed by Jesse Lazear, a researcher on this mission who was mainly charged with handling mosquitoes. In this experiment, Lazear allowed mosquitoes that had fed on patients with the yellow fever disease to bite him. Quickly after, Lazear died of this disease establishing these mosquitoes as the vector (Vasilakis et al., 2019).

This series of events led to coining the term “arthropod-borne viruses”. Arboviruses, according to the World Health Organization (WHO) are “viruses that share the characteristic of being naturally maintained through biological transmission between susceptible vertebrate hosts by hematophagous arthropods or transovarial transmission from infected female arthropods to her progeny” (WHO, 1985). Arboviruses include a wide range of RNA viruses encompassing the three main classes: (-)RNA, (+)RNA and dsRNA viruses.

ii. Mechanisms of arbovirus emergence

In nature, arboviruses are maintained in a complex transmission cycle with most arboviruses being zoonotic. Zoonotic arboviruses are defined by their adoption of rodents, birds and non-human primates as reservoir hosts. This transmission cycle between wild animals and arthropod vectors is defined as an enzootic transmission cycle. Epidemics in human populations occur when the enzootic virus is introduced into urban areas. This event is termed “spillover” and can occur in different scenarios. In some situations, the virus will cycle between domesticated animals and primary or accessory vectors in what is known as an epizootic cycle. This situation can cause an

outbreak of this viral disease in the domesticated animal population leading to virus amplification. Then, because of the proximity between domesticated animals and humans, this may extend the outbreak to human populations with the implication of anthropophilic vectors such as *Aedes (Ae.) aegypti* mosquitoes. In other cases, humans crossing sylvatic habitats which are incidentally bitten by infected zoonotic vectors might contribute to the spread of this virus upon return to urban habitats (Fig. 4). This capacity of arboviruses to emerge into urban areas has led to several outbreaks of high magnitude in the last decades (Weaver and Barrett, 2004).

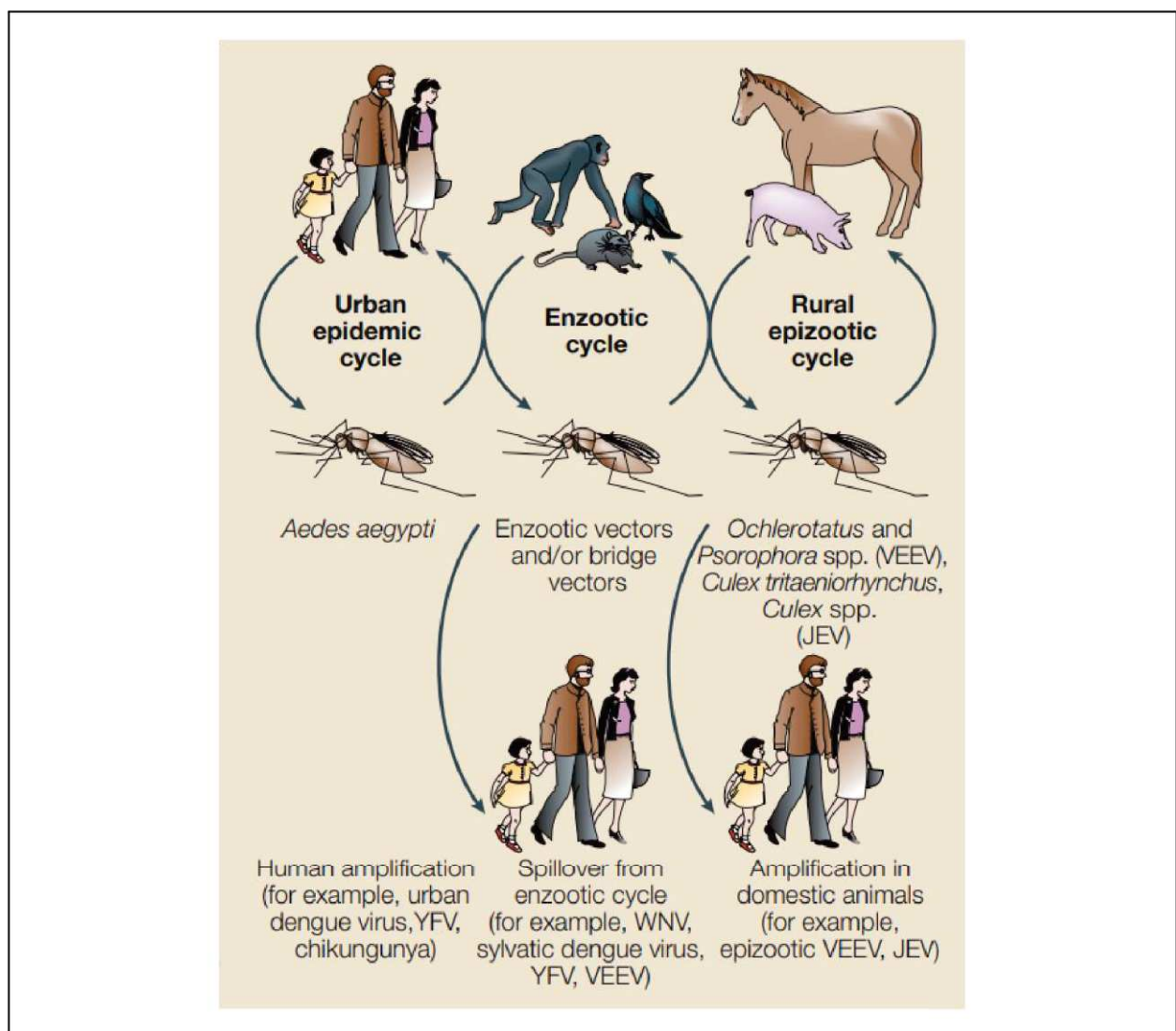


Fig. 4 Mechanisms of arbovirus emergence

The typical mechanism of arbovirus emergence and the mosquito vectors implicated are shown above. The different cycles of arbovirus transmission are also illustrated (adapted from Scott C. Weaver and Barrett 2004).

Whatever the scenario of the spillover event, it is important to consider each arbovirus emergence event as a result of an aggregation of complex factors:

(1) Climatic conditions are one of the deciding factors that play a role in arbovirus emergence. A prototypical example of the effect of the weather was devastatingly seen in the previously discussed 1878 yellow fever epidemic in Memphis. This event was recorded as one of the worst epidemics in the 19th century American history with a loss of around 16,000 human lives. In the years prior to this epidemic, rain days and intensity were particularly high. This led to a bloom in *Ae. aegypti* mosquitoes breeding in these regions which contributed to the destructive effects and rapid spread of this epidemic (Reed and Carroll, 1901). This established weather as an important driver in establishing populations of mosquitoes and therefore contributing to the extent of an epidemic. Given that climate change is a reality in today's world, several groups have predicted that temperature change might lead to an increase in arbovirus emergence in previously untouched areas of the world. In the case of Dengue virus (DENV), for example, an increase in temperature has been predicted to boost the epidemic potential of this virus in temperate northern hemisphere regions (Liu-Helmersson et al., 2014).

(2) Human activities can also affect this arboviral emergence. This has been evident with the growth of the used-tire industry where car tires were found to provide the optimal humidity and temperature needed for the rapid breeding of mosquitoes. Transport of car tires also contribute to the spread of *Alphaviruses* into new ecosystems. In addition, during drought periods, water storage tanks provide another excellent breeding spot for mosquitoes (Benedict et al., 2007; Kamal et al., 2018).

(3) The capacity of an arbovirus to adapt to new vectors and therefore invade new environments can also be critical in epidemic events. Additionally, arboviruses with the ability to evolve into more virulent strains can also contribute to a switch from an enzootic to epizootic/epidemic transmission cycle. Examples of *Alphavirus* that have emerged through these mechanisms will be described later on in the introduction in section "Evolution and dissemination".

iii. *Alphavirus* transmission

Coming back to *Alphaviruses*, one of the main hosts are birds. In certain *Alphaviruses*, such as EEEV, humans and equids are considered as dead-end hosts. Dead-end hosts

are defined by their incapability to act as a reservoir for vector re-infection. However, this is not the case for all *Alphaviruses*. Indeed, in the CHIKV transmission cycle, humans are not considered as dead-end hosts and will act as an important reservoir for re-transmission of the virus back to its vector (Weaver et al., 2012).

For *Alphaviruses*, the main vectors are mosquitos: either *Ae. aegypti*, *Ae. Albopictus* and *Ae. taeniorhynchus* for the urban transmission cycle or *Ae. furcifer*, *Ae. africanus* and *Culex melanoconion* for the enzootic transmission cycle (Weaver et al., 2004, 2012). Mites and ticks have also been shown to be infected with *Alphaviruses* (SINV and EEEV). However, the frequency of this event and the transmission efficiency is not high enough to consider them as primary vectors (Strauss and Strauss, 1994). For SAV, transmission appears to be mainly horizontal via water contact. However, it has been recently shown that these viruses could replicate in arthropod cells suggesting that their transmission could pass by invertebrate hosts. If true, this is mainly thought to be carried out by the haematophagous salmon louse *Lepeophtheirus salmonis* which has already been found to harbor SAV (Deperasińska et al., 2018; Hikke et al., 2014).

Furthermore, there exists regulators for mosquito mediated *Alphavirus* transmission. Parasites are one of the main players in this process where they can exert their effects by either enhancing or blocking the establishment of *Alphavirus* mosquito infection (Tan et al., 2017; Vaughan and Turell, 1996). Indeed, mosquitoes are frequently infected with parasites during blood meal acquisition from hosts. Furthermore, certain parasites can release what are known as microfilariae, an early developmental stage of parasites, into their host's bloodstream. Consequently, these microfilariae are then ingested by mosquitoes taking a blood meal from a vertebrate host (Vaughan and Turell, 1996). Interestingly, the microfilariae of a parasite called *Brugia malayi* has been shown to lead to a dose-dependent increase in EEEV and VEEV infection in mosquitoes (Vaughan and Turell, 1996). This was later observed to be mediated by binding of the *Brugia malayi* microfilariae to the virion facilitating the penetration of the mosquito midgut epithelium barrier and contributing to the establishment of *Alphavirus* infection. This phenomenon has been shown to be important for various arboviruses and has been termed "microfilarial enhancement of arboviral transmission" (Vaughan and Turell, 2017). Conversely, a parasite that has been studied for its capacity to negatively affect *Alphavirus* infection in mosquitoes is the endosymbiotic bacterium *Wolbachia*. *Wolbachia* is an intracellular maternally inherited bacteria that is present in more than

20% of insects. Recently, through comparisons between wild type and *Wolbachia* infected *Ae. Aegypti* mosquitoes, a study demonstrated the ability of this bacteria to efficiently block CHIKV infection. Interestingly, *Wolbachia* was also able to block Zika virus (ZIKV) and DENV infection demonstrating its ability to act on different arboviruses (Geoghegan et al., 2017; Tan et al., 2017). *Wolbachia* has been suggested to block CHIKV mosquito infection by either immune activation or competition with the virus for mosquito metabolic resources (Terradas and McGraw, 2017). With respect to *Wolbachia*'s mechanism of action for *Flaviviruses*, this bacteria has been shown to perturb intra-cellular trafficking of cholesterol, a sterol which is important for various steps of *Flavivirus* infection (Osuna-Ramos et al., 2018). It would be interesting to investigate whether this mechanism of action might occur for *Alphaviruses* as well. This virus blocking function of *Wolbachia* in mosquitoes has led to a high interest in utilizing this bacterium to limit arbovirus transmission (Hoffmann et al., 2011).

iv. Transmission cycle through mosquito vectors

The *Alphavirus* mosquito transmission cycle begins with a female mosquito blood-feeding off a viremic vertebrate host through its buccal apparatus. After blood-feeding, the virus will migrate to the midgut where viral replication occurs. However, during the blood digestion process, the midgut epithelium releases a peritrophic matrix which can block virus entry. Therefore, in the midgut epithelium, the virus will encounter its first barrier. Crossing of this barrier, known as the midgut infection barrier, will be essential to establish efficient infection and transmission. Once past this barrier, the virus will be present in the midgut where it will replicate and then escape into an open body cavity called the haemocoel. The haemocoel will be important for the dissemination and infection of the virus into other secondary tissues. The last step in the transmission cycle involves infection and replication in the salivary glands to sufficient virus titers for infection of the vertebrate host during the next blood meal. All of these steps, from crossing the midgut epithelium to replication in the salivary glands, will determine the vector competence for a particular virus (Lim et al., 2018).

In the next part, the evolution of *Alphaviruses* and their consequences on pathogenesis and dissemination will be reviewed. Specifically, the vector adaption of CHIKV from *Ae. aegypti* to *Ae. albopictus* mosquitoes will be discussed. This impressive evolution demonstrates the flexibility of *Alphaviruses* to adapt to new vectors and therefore to conquer new environments.

E- Evolution and consequences on pathogenesis and dissemination

RNA viruses are under a constant state of evolution. Evolution is caused by mutations committed during the copying process of the RdRp. Impressively, mutation rates for RNA viruses are high ranging between 10^{-6} and 10^{-4} substitutions per nucleotide site. This can be appreciated when compared to DNA viruses that have mutation rates in the order of 10^{-8} to 10^{-6} substitutions per nucleotide site (Duffy, 2018; Peck and Lauring, 2018). Mutations in the viral genome can lead to either beneficial or detrimental effects on virus fitness. By the process of natural selection, mutations that enhance virus fitness are positively selected during evolutionary time, and mutations having a negative effect on fitness are quickly diluted from the total virus growth pool.

This phenomenon is especially interesting in the case of arboviruses since selection needs to maintain fitness in both vertebrate and invertebrate hosts. In this context, mutations and evolution rates have been studied using the CHIKV model. In this model, mutation rates were shown to reach around $10^{-3.3}$ substitutions per nucleotide site. Interestingly, isolation of a mutagen resistant variant of CHIKV has led to the identification of a single amino acid change (C483Y) in the RdRp, nsP4, which increased replication fidelity by 1.5-fold. Analysis of virus fitness established that this variant with a higher copying fidelity replicated much less efficiently in both vertebrate and invertebrate hosts (Coffey et al., 2011). This possibly explains the high mutation rates detected for RNA viruses and arboviruses specifically.

In this scenario of high mutation rates for RNA viruses, the conservation of sequences will probably require high selective pressures and implies that these conserved residues are of high importance for virus infection. This has highly interested evolutionary biologists that aligned sequences of different *Alphaviruses* and checked for the conservation of nucleotides, amino acids and in some cases secondary structures (Ahola and Karlin, 2015; Kutchko et al., 2018).

i. Evolution and dissemination

A striking example of the evolution of an *Alphavirus* with consequences on virus dissemination is the case of the NW *Alphavirus* VEEV. VEEV belongs to the VEE antigenic complex which is comprised of 6 different antigenic subtypes denoted as I to VI. The I antigenic complex contains five different varieties AB, C, D, E and F (Powers et al., 2001). The antigenic subtypes II to VI and subtype I with varieties D and E have

not have been reported to cause any major outbreaks. They have no amplification potential due to their incapability to induce viremia in hosts and were therefore classed as enzootic strains. However, the antigenic subtype I with varieties AB and C, since their discovery in the 1930s, have caused sporadic cases of outbreaks with high rates of mortality in humans and equines. Interestingly, these VEEV subtypes disappeared for 19 years and were thought to either have gone extinct or are circulating in an enzootic cycle. It was not until 1992, in Venezuela, where VEEV re-emerged causing explosive epidemics (Weaver et al., 2004). An emerging hypothesis was that VEEV could have reappeared from enzootic strains that have acquired mutations leading to an increase in their amplification potential in equine hosts. The simplest explanation for an increase in amplification is an increase in the capacity of a virus to induce high viremia in hosts. Indeed, phylogenetic analysis of the VEEV strain of 1992, led to predictions that a mutation at the residue 213 of the E2 glycoprotein could have caused this switch from enzootic to epizootic/epidemic strain. Reverse genetics studies confirmed the ability of a T₂₁₃R in the E2 glycoprotein to transform an avirulent strain of VEE subtype I-D to a virulent strain. The exact role of this residue in VEEV infection remains to be determined. This recapitulated the evolutionary events leading to the 1992 VEEV outbreak and demonstrated the ability of *Alphaviruses* to re-emerge after long periods of absence (Anishchenko et al., 2006).

Another illustration of the capacity of *Alphaviruses* to evolve genetically and disseminate into urban areas is the OW *Alphavirus* CHIKV. A large-scale epidemic of CHIKV began in Kenya in 2004 and then spread to several Indian Ocean islands such as the Réunion island. *Ae. aegypti* mosquitos are scarce in these regions, and it was found that the main mosquito vector responsible for the transmission to humans was *Ae. albopictus*. This was the first reported case in which these mosquitoes acted as the main vector for CHIKV (Delatte et al., 2010). Analysis of CHIKV genome evolution in this epidemic led to the detection of several amino acid changes in the non-structural proteins (nsPs), mainly in nsP2 and nsP4. This analysis also revealed changes in the structural proteins (sPs) region, notably an alanine to valine mutation in position 226 in the membrane fusion E1 protein (Schuffenecker et al., 2006; Tsetsarkin et al., 2007). Reverse genetics studies confirmed particularly the role of this E1-A₂₂₆V mutation in increasing infectivity and transmission by *Ae. albopictus* mosquitoes. Further, this mutation was associated with an increase in cholesterol dependence for CHIKV in

these vectors (Tsetsarkin et al., 2007). Certain *Alphaviruses* (SINV and SFV) have been shown to be dependent on cholesterol in the early entry steps of the viral life cycle where E1 plays a role in the fusion of the viral and endosomal membranes leading to release of the viral genome (Li et al., 2010). Interestingly, in these studies, cholesterol dependence was mapped to the 226 residue present in the fusion loop of the E1 protein, the same residue which was important for CHIKV adaptation to *Ae. albopictus* mosquitoes (Chatterjee et al., 2000; Lu et al., 1999). It is therefore tempting to speculate that CHIKV infection in *Ae. albopictus* mosquitoes occurs more efficiently if the E1 mediated fusion process is cholesterol-dependent. On the other hand, for *Ae. aegypti* mosquitoes, CHIKV infection can occur without the need for cholesterol explaining the conservation of E1 226 alanine residue in CHIKV strains which efficiently infect these mosquitoes.

ii. Evolution and pathogenesis

On the other hand, an example of *Alphavirus* evolution and its implication in pathogenesis is the role of crucial residues in nsP1, nsP3, and the E2 protein which were found to regulate *Alphavirus* induced neurovirulence (Atkins and Sheahan, 2016). This led to several investigations in order to decrypt the molecular determinants involved in this virulence (Tucker et al., 1993).

A prominent *Alphavirus* used for these neurovirulence studies was SINV due to the vast knowledge of its molecular biology and pathogenesis. SINV is neurovirulent in neonatal, but not in weanling mice. However, the passage of SINV in mouse brains allowed the generation of laboratory-derived strains which were neurovirulent also in weanling mice. These different strains of SINV which differed in their capacity to induce neurovirulence in mice have been sequenced and compared revealing crucial residues implicated in this phenotype. The most reported mutation was a glutamine to histidine change at amino acid residue 55 in the E2 protein. This mutation was described to increase the capacity of the viral particle to bind to neurons (Lee et al., 2002) and to induce apoptosis (Ubol et al., 1994). Regulation of these two processes contributed to a greater neurovirulence for SINV strain with this mutation (Lee et al., 2002). This phenomenon demonstrated the capacity of *Alphaviruses* to mutate in order to efficiently replicate and spread in different organs.

Interestingly, SINV strains with high neurovirulence have been reported to infect poorly *Ae. aegypti* midgut epithelial cells indicating a possible loss of binding to receptors (Pierro et al., 2003). As mentioned earlier, these strains are laboratory-derived and might have therefore adapted to replicate in certain types of tissues while losing the capacity to replicate in invertebrate hosts. Therefore, in this artificial scenario, the adaptation to increase neurovirulence negatively affected virus dissemination.

In the next part, the biology of CHIKV will be detailed including its emergence, phylogeny, symptoms, and pathogenesis.

F- Chikungunya virus, an *Alphavirus*, always on the lurk

i. History and emergence

In 1952, a massive febrile illness with unknown cause spread in Mawia, Makonde and Rondo of present-day Southern Tanzania. This epidemic reached its peak in 1953 with 49 localities involved. At that point in history, the society of this region was of primitive structure. However, the distinctive features of this illness made it easy to track (Lumsden, 1955). Due to the porous nature of the soil in this region, the people often stored their water in their home which in occurrence were infested by mosquitoes mainly of the *Ae. aegypti* type thought to be the main culprit for the spread of this illness (Lumsden, 1955). Analysis of *Ae. aegypti* mosquitoes isolated from these houses pointed towards strong evidence that the causative agent is a virus. The word Chikungunya was then given to this virus which originates from the Makonde dialect meaning “disease that bends up the joints”. This reflected the symptoms that the Tanzanian people observed upon infection with CHIKV. Infected individuals were rendered paralyzed with extreme joint and muscular pain upon movement and therefore had this characteristic bend up appearance (Ross, 1956). Although the virus originating from this epidemic was first thought to be a strain of DENV due to the resemblance of symptoms, CHIKV was later shown to belong to the *Alphavirus* genus (Weaver and Forrester, 2015). Shortly after the 1952 epidemic, CHIKV was reported in Uganda. Then, the later part of the 20th century saw recognition of CHIKV in sub-Saharan Africa with sporadic outbreaks in Asia and Africa (Weaver and Forrester, 2015). However, it was not until 2004 that CHIKV spread took a striking turn. Reports of epidemics first began in Kenya and then spread to India, Southeast Asia and to the Indian Ocean islands. The best-described epidemic being in the French island of the

Réunion in 2005, where an estimated third of the Réunionese population were infected with CHIKV. This had a disastrous socio-economic impact in this island (Zeller et al., 2016). During this period, India also experienced a re-emergence of CHIKV after a gap of 32 years. This epidemic touched over 1.3 million people with an attack rate of 45% (Arankalle et al., 2007). Furthermore, the rest of Asia exhibited several outbreaks in countries such as Malaysia, Southern Thailand and Sri Lanka (Zeller et al., 2016). Infected travelers from India and the Indian Ocean islands led to imported cases of CHIKV in all areas of the world. In some instances, these imported cases caused small outbreaks in Europe such as the ones reported in northern Italy and Southern France (Grandadam et al., 2011; Rezza et al., 2007). CHIKV has recently emerged in the pacific regions spreading to countries such as New Caledonia and French Polynesia (Aubry et al., 2015). More recently reported cases in the Caribbean island of St Martin, indicate that CHIKV could also emerge in the western hemisphere and invade the Americas (Fig. 5) (Leparc-Goffart et al., 2014).

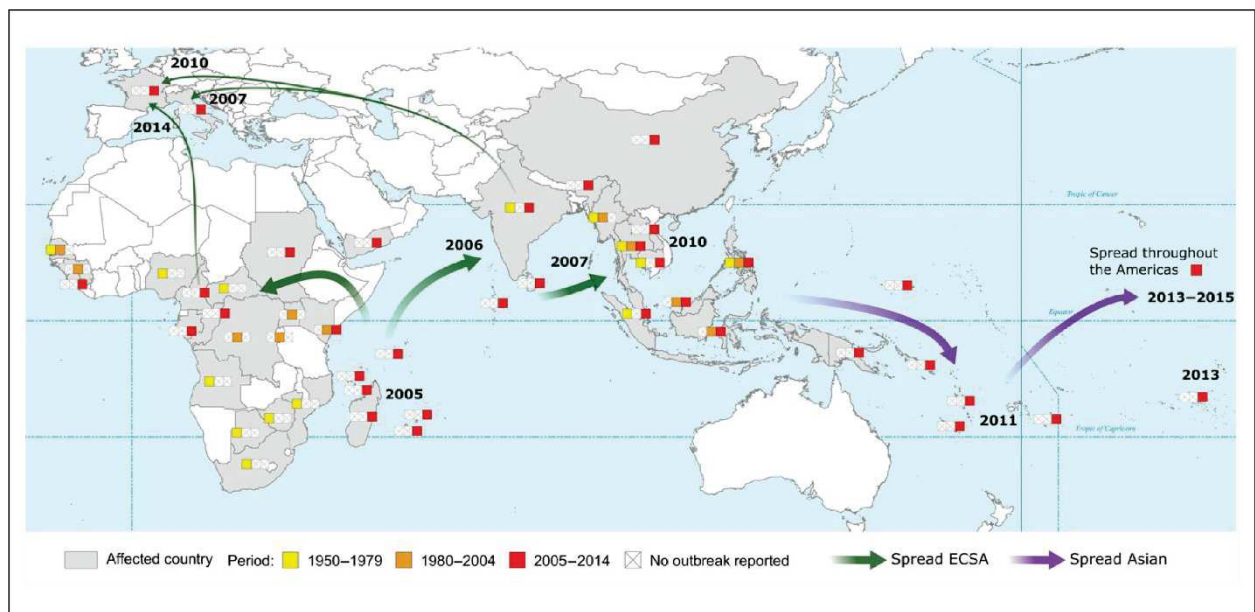


Fig. 5 Chikungunya virus outbreak events

The geographical spread of Chikungunya virus from Africa to new regions from 2005 to 2014 is illustrated. ECSA: East/Central/South African (adapted from Zeller, Van Bortel, and Sudre 2016).

ii. Geographical distribution and behavior of urban vectors

The capacity of CHIKV to cause epidemics in different parts of the world is reflected by the broad prevalence of urban mosquito vectors that carry strains of this virus. Indeed, *Ae. aegypti* and *Ae. albopictus* mosquitoes are present in various regions of the world. Recent occurrence studies have demonstrated that these vectors are mainly present in Asia and the Americas. More than 50% of *Ae. aegypti* mosquitoes are present in Brazil, India, Thailand, Mexico and United States. However, *Ae. Albopictus* mosquitoes are mainly localized in Taiwan, United States and Indonesia. The distribution of these two mosquito species overlapped in some areas in Asia and West Africa and differed remarkably in Europe, United States and East Africa. This distribution of *Ae. aegypti* and *Ae. Albopictus* mosquitoes was heavily affected by climatic conditions with *Ae. aegypti* being adapted to tropical and sub-tropical regions, and *Ae. albopictus* acclimated to temperate regions (Fig. 6) (Kamal et al., 2018).

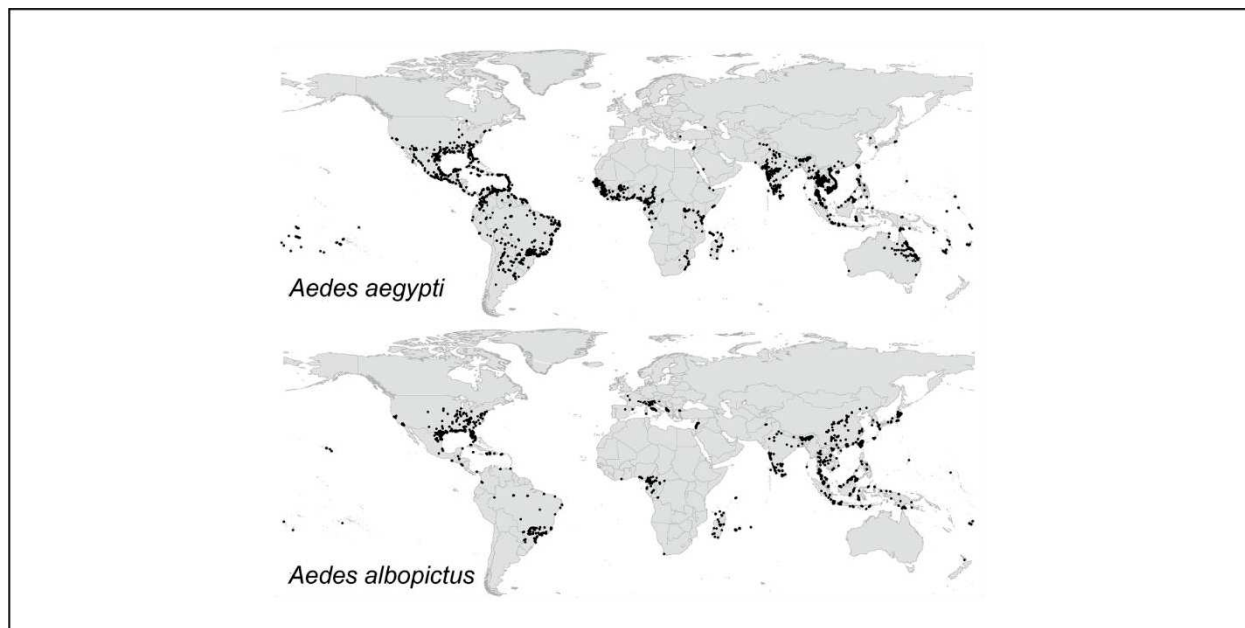


Fig. 6 *Ae. aegypti* and *Ae. Albopictus* occurrence records (adapted from Kamal et al. 2018).

The ability of arthropods to spread the virus throughout a community can be calculated by a mathematical epidemiological model called the basic reproduction rate (R_0). R_0 is calculated by a mathematical formula and will depend on multiple variables. One of

the most significant variables is the human-biting rate. Simply, the higher the human-biting rate, the higher the capacity to spread the virus in human communities, therefore a higher R_0 . Conversely, zoophilic mosquitoes that favor biting of non-human primates, birds and reptiles will have a lower R_0 (Roddam, 2001). *Ae. aegypti* are widely accepted as main vectors for various arboviruses and are considered to be anthropophilic mosquitoes with a high R_0 . This means that given the choice between humans and other mammals, these mosquitoes will preferentially bite humans (Crawford et al., 2017). However, conflicting results exist for *Ae. albopictus* mosquitoes that were thought to be opportunistic mosquitoes. This translates to a situation where these mosquitoes will bite humans if the opportunity arises, but would prefer other type of mammals. This is supported by the fact that *Ae. albopictus* mosquitoes are secondary vectors in most cases for arboviruses (Gould et al., 1970; Savage et al., 1993). Surprisingly, a pertinent study on *Ae. albopictus* mosquitoes in the Réunion found that these mosquitoes behaved as anthropophilic vectors. In these regions, *Ae. albopictus* mosquitoes had two peaks of activity for feeding. The first peak was in the early morning, and the second in the late afternoon. Nonetheless, these mosquitoes had a constant basal activity throughout the whole day. This study suggests that *Ae. Albopictus* mosquitoes are turning into dangerous primary vectors for CHIKV transmission (Delatte et al., 2010).

iii. Phylogeny and evolutionary origin

As is evident with the information presented earlier, CHIKV has spread to different parts of the world. This has led to efforts in performing phylogenetic analysis mainly revealing three main distinct geographical CHIKV lineages: Asian endemic/epidemic, West African and East/Central/South African (ECSA) enzootic lineages. These three CHIKV genotypes are highly conserved with 95.2% to 97% identity at the amino acid level (Am et al., 2000). The ECSA lineage has been the dominant lineage in the recent CHIKV outbreaks. This lineage is responsible for the emergence events of CHIKV from Kenya to the Indian Ocean islands and India. This recent emergence of CHIKV led to the establishment of an ECSA descendent strain known as the Indian Ocean lineage (IOL) strain. The ECSA lineage is also responsible for the recent outbreaks in the Americas (Haiti and Brazil) (White et al., 2018). The Asian lineage of CHIKV has also been associated with several outbreaks notably in Malaysia, New Caledonia, and French Polynesia. Further, the recently reported cases of isolated CHIKV in St Martin Island

were also attributed to this lineage (Gay et al., 2016; Leparc-Goffart et al., 2014). Finally, the Western African lineage of CHIKV is totally isolated and has not caused any outbreaks yet (de Bernardi Schneider et al., 2019).

CHIKV is thought to have originated from Africa (Chen et al., 2016). Indeed, in this region, CHIKV has been circulating for a long time in a sylvatic cycle between non-human primates and forest-dwelling mosquitoes such as *Ae. furcifer*, *Ae. africanus*, and *Ae. luteocephalus*. In some cases, incidental spill-overs have led to small outbreaks in this continent. However, the major CHIKV African outbreaks were caused by the ECSA CHIKV lineage seeding in regions having anthropophilic mosquitoes (mainly *Ae. Aegypti*) which then led to a human–mosquito cycle in urban areas of Africa. The Asian endemic/epidemic lineage resulted from an expansion of the CHIKV ECSA strain to South East Asia and its persistence in an urban cycle in these regions (Weaver, 2014).

iv. Clinical symptoms

The data accumulated to date show that CHIKV is an OW *Alphavirus* with mainly arthrogenic symptoms.

CHIKV will cause what is known as Chikungunya fever (CHIKF). Impressively, CHIKV will induce CHIKF in the majority (50–97%) of infected individuals (Yactayo et al., 2016). Characteristic symptoms of CHIKF include muscle and joint pain, fever, fatigue, and nausea. In rare cases, the infected individual will have neurological symptoms and submit to the Guillain–Barré syndrome (Balavoine et al., 2017). Fatal cases have been reported for CHIKV where the majority of attributed deaths were related with elderly people. Conformingly, being over the age of 65 increases the risk of CHIKV caused fatality by five times (Neto et al., 2019).

CHIKV infection is divided into an acute and chronic stage. In acute infection, the virus is usually cleared after one week mainly because of triggering of the innate immune. Innate immune response is mainly mediated through the production of various cytokines and chemokines which attract immune cells such as macrophages, neutrophils, natural killer cells, CD4⁺ and CD8⁺ T cells limiting viral infection. An important cytokine mediating CHIKV anti-viral response is type I interferon (IFN α). The importance of this cytokine for limiting viral infection has been strikingly observed in mice with impaired IFN α pathway. CHIKV infection in these mice developed severe

symptoms which was correlated with the inability to control viral infection (Couderc et al., 2008; Sourisseau et al., 2007). The chronic stage of CHIKV can last from months to years and is an important health problem in the countries afflicted with CHIKV epidemics. Chronic infection is present in around 30% of infected individuals and characteristic symptoms include rheumatism and fatigue (Ganesan et al., 2017). This has been in some cases attributed to innate immune response due to the finding that macrophages infiltrating muscle and joint tissues promote local inflammation (Gardner et al., 2010). Indeed, macrophages are thought to be a reservoir for CHIKV RNA during chronic stages of infection (Labadie et al., 2010) and are therefore perhaps the mediator of chronic arthritis symptoms. This has been further reinforced by the finding that macrophages not only infiltrate affected tissues, but will also transform into osteoclasts possibly in this manner mediating the chronic symptoms observed (Phuklia et al., 2013). A recent study has detected a rare subset of skin and muscle fibroblasts cells that survived chronic CHIKV infection and harbored CHIKV RNA long after infection. However, similarly to macrophages, no active virus replication has been detected in these cells and how this subset of cells contributes to pathogenesis remains to be elucidated (Young et al., 2019).

v. Natural history of *Alphavirus* infection in vertebrate hosts

Following intradermal inoculation by an infected mosquito bite, CHIKV is capable of actively replicating in skin fibroblasts (Couderc et al., 2008). This virus will then, in certain cases, escape with the help of mosquito saliva which contains some immunomodulators, through the lymph nodes to other tissues such as the liver, muscle, joint and brain (Schneider et al., 2004). Conformingly, virus dissemination throughout the body has been detected in the liver, brain, joints, and muscles of experimentally infected macaques (Labadie et al., 2010). The final step of CHIKV infection consists of replication in peripheral tissues such as the muscle and joints leading to high blood viremia reaching viral loads of 3.3×10^9 copies/ml during the first week of infection (Parola et al., 2006). At this stage, an infected individual represents a perfect target for a mosquito blood meal leading to subsequent transmission and amplification of the virus (Fig. 7).

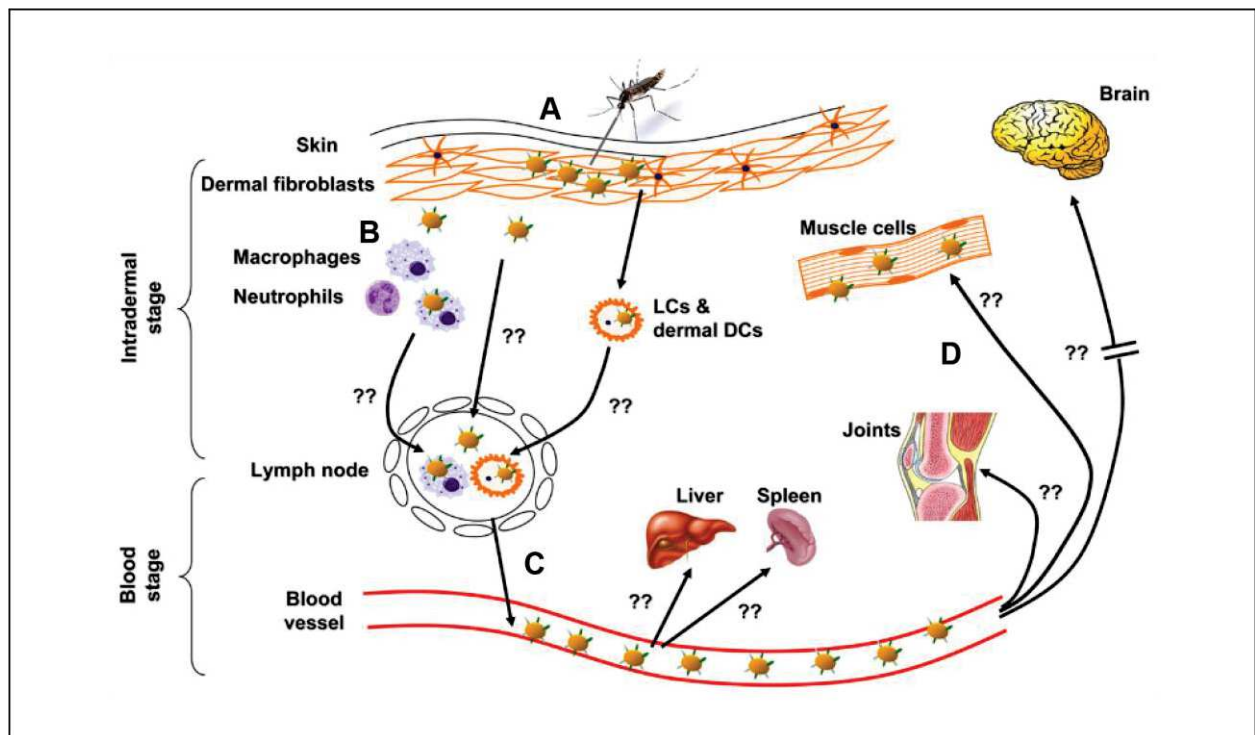


Fig. 7 CHIKV dissemination after a mosquito bite

(A) After a mosquito bite, CHIKV actively replicates in skin fibroblasts. (B) Replication in fibroblasts attracts immune cells such as macrophages and neutrophils. (C) CHIKV then migrates through the lymph node to the blood circulation (D) CHIKV is then capable of disseminating to various different organs such as the joints and muscle cells (adapted from Kam et al. 2009).

All this information points towards CHIKV being an important disease that emerged first in the 1950s and re-emerged recently in the 21st century. The capacity of this virus to adapt to new vectors and spread world-wide raises alarms of its never-ending danger. CHIKV is a virus that is always on the lurk waiting for the perfect opportunity to remerge.

G- Virus structure and genome organization

i. Virion structure

An *Alphavirus* is composed of multiple organized layers which serve for the protection of viral RNA and its proper delivery into the host cell. The structure and organization of the viral particle have been resolved by cryo-electron microscopy by several groups (Fig. 8) (L. Chen et al. 2018; Mancini et al. 2000; Basore et al. 2019; Cheng et al. 1995). The innermost layer is an assembly of 240 copies of capsid proteins organized as 12 pentamers and 30 hexamers forming an icosahedral shell. This layer, together with the viral RNA will compose the nucleocapsid (NC). The NC is enveloped by a host-derived

lipid bilayer. The lipid bilayer itself is embedded with 80 glycoprotein spikes with an icosahedral symmetry as well. Each spike is a trimer of heterodimers composed of one copy of the E1 and E2 proteins (Chen et al., 2018). In the *Alphavirus* virion, a hydrophobic pocket in the C-terminus of the capsid protein will bind the cytoplasmic tail of E2 linking the inner core proteins with the glycoprotein surface proteins (Lee et al., 1996). The diameter of a mature virion is around 700 Å. As mentioned earlier, these layers will serve to protect the viral RNA which codes for the genetic information of the virus (Jose et al., 2009).

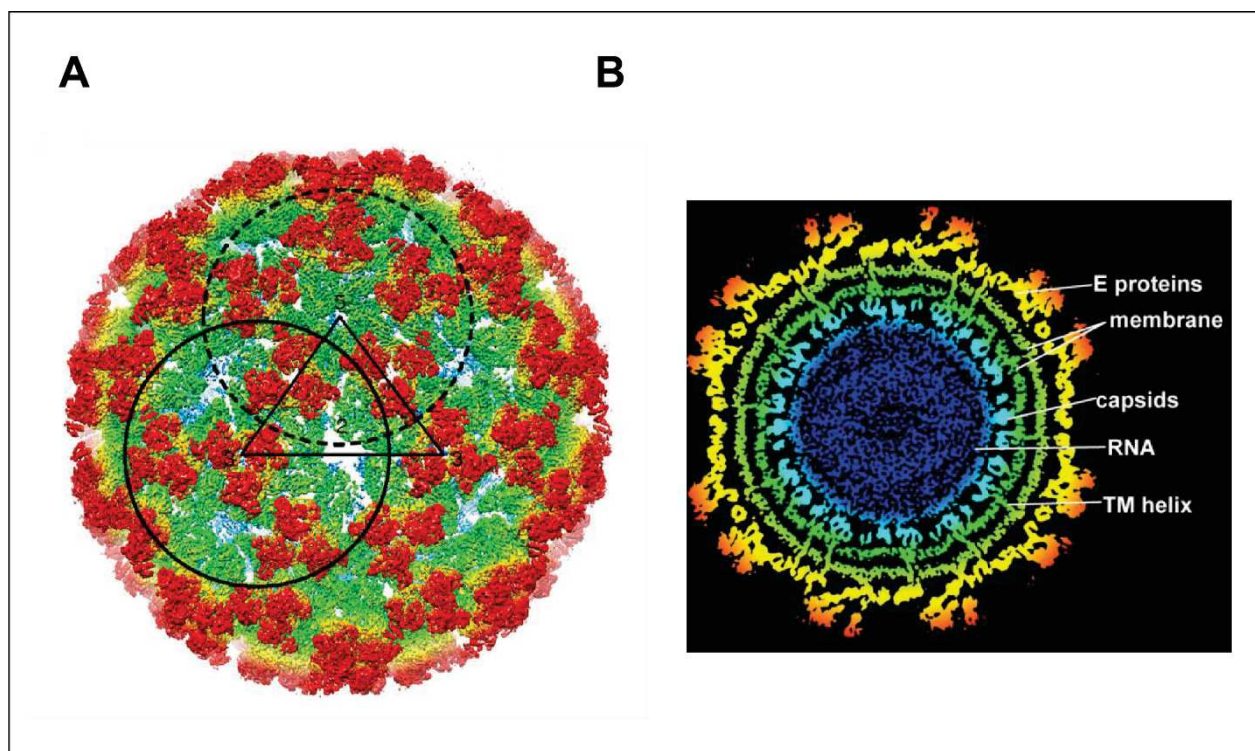


Fig. 8 *Alphavirus* structure

(A) 3D reconstruction of an *Alphavirus* particle with a 3.5Å resolution (B) Central cross-section of an *Alphavirus* particle (adapted from L. Chen et al. 2018; Sun et al. 2013).

ii. Viral genome organization and cis-acting elements

The *Alphavirus* genome is composed of a non-segmented single-stranded RNA of positive polarity and is around 12 kb in size (Fig. 9). This genome has a similar architecture to cellular messenger RNA (mRNA) as it includes a cap at the 5' terminus and a poly(A) tail at the 3' terminus. Unlike mRNA, the 5' cap is only methylated at the

guanosine residue and is therefore named Cap (0) (Wengler et al., 1979). The *Alphavirus* genome codes for two cistrons with their respective genomic and sub-genomic promoters. The first cistron at the 5' end codes for the viral nsPs (nsP1, nsP2, nsP3, and nsP4) which form the replication machinery. The second cistron will code for the viral sPs (Capsid, E3, E2, 6K, TF, and E1) that will form the new *Alphavirus* particles (Strauss and Strauss, 1994).

Other than the coding regions, there exists untranslated regions at the 5' and 3' end (5' UTR and 3' UTR) of the viral RNA which are variable in nucleotide length between different *Alphaviruses*. The 5' UTR range in length from 27 (SAV) to 85 (SFV) nucleotides, and the 3' UTR range in length from 87 (SAV) to 723 (CHIKV) nucleotides (Hyde et al., 2015).

These represent part of the cis-acting elements of the viral genome that have been described to play a role in RNA synthesis and immune evasion. Starting from the 5' end, the first cis-acting element, the 5' UTR, is a component of the promoter for viral RNA synthesis and has been described to form stem-loop structures (Frolov et al., 2001). These stem-loop structures were also identified to be important for the evasion of immune sensing, specifically IFIT1 mediated anti-viral activity (Reynaud et al., 2015). A recent study using biochemical "selective 2'-hydroxyl acylation analyzed by primer extension" (SHAPE) analysis, has identified a new stem-loop structure in the 5' UTR denoted as SL47 and found it important for efficient CHIKV genome replication. SL47 was also present in phylogenetically close *Alphaviruses* such as ONNV (Kendall et al., 2019).

Another cis-acting element is the 5' conserved sequence element (CSE) present at the start of the nsP1 sequence which can form up to 5 stem-loops (SL85, SL102, SL165, SL194, and SL246) (Kendall et al., 2019). Moving to the 3' end of the viral genome, there also exists two 3' CSEs, the first one is present in the sub-genomic RNA promoter and the second is localized directly upstream of the poly(A) tail (Ou et al., 1981). Modification of both 5' and 3' CSEs without changing the coding of their respective viral proteins has elucidated their importance in regulating viral RNA synthesis (Fayzulin and Frolov, 2004; Frolov et al., 2001).

The function of the 3' UTR is not well understood, but it has been implicated in mosquito vector adaptation. This has been specially investigated in studies dealing with the role

of the 3' UTR in vertebrate and invertebrate host infection. Interestingly, deletions in the 3' UTR do not completely abolish replication in vertebrate cells. However, these modifications decrease fitness in mosquito cells suggesting that the 3' UTR plays an important role in interacting with mosquito cell factors. The relevance of the 3' UTR for mosquito infection can be appreciated when comparing 3' UTRs of different *Alphaviruses*. Indeed, Eilat, an *Alphavirus* which is restricted to insect cells has a large 3' UTR (520 nt). While, as mentioned earlier, SAV which do not replicate efficiently in insect cells have very short 3' UTRs (Chen et al., 2013).

An interesting finding was that modifications in the 5' end of the viral genome affected (-)RNA synthesis. This implied that the 5' and 3' end need to interact in order to facilitate (-)RNA synthesis leading to circularization of the viral RNA (Frolov et al., 2001). However, no conclusive evidence exists for this interaction, and the role of host factors in this circularization has not yet been shown.

Finally, the genomic RNA contains packaging signals allowing specific packaging of the viral RNA into newly synthesized virions. Studies in SINV revealed the packaging signal to be localized in nsP1 (Frolova et al., 1997). However, for SFV, the packaging signal was shown to be in nsP2 (White et al., 1998).

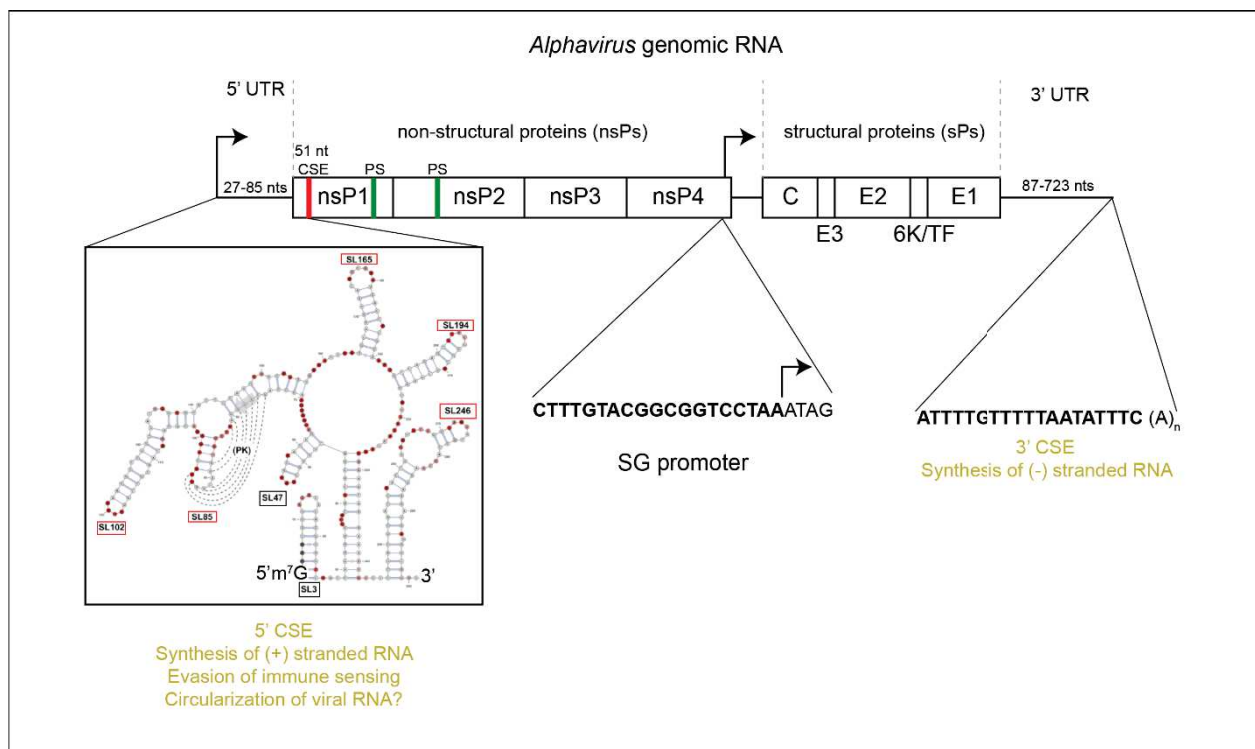


Fig. 9 *Alphavirus* genome and cis-acting elements

Schematic representation of the *Alphavirus* genome and cis-acting elements. CSE: conserved sequence element; nts: nucleotides; PS: packaging signal; SG: sub-genomic; SL: stem loop; UTR: untranslated region (adapted from Kendall et al. 2019; Hyde et al. 2015; Frolov, Hardy, and Rice 2001).

H- Life cycle of *Alphaviruses*: What we know / What we don't know

In this part, the knowledge on the *Alphavirus* life cycle will be detailed. It consists of over 30 years of elaborate studies on mainly SINV and SFV models. However, this knowledge mainly accumulated in these two models can also be transposed to understand CHIKV which remains less investigated. *Alphaviruses* are interesting in their capacity to replicate in vertebrate and invertebrate cells. In invertebrate cells, *Alphaviruses* will replicate without fitness downsides on the host cell. However, *Alphavirus* infection in vertebrate cells engenders highly cytopathic effects that will be described in details later on. Here, the life cycle of *Alphaviruses* will be given with respect to the knowledge mainly accumulated in vertebrate (mainly human) cells (Fig. 10).

i. Virus entry

Viruses enter the cell through the presence of entry factors at the plasma membrane of host cells. There are two types of entry factors: (1) receptors and (2) attachment factors. Receptors are defined by their specificity due to direct interactions with the envelope protein of the virus. However, attachment factors do not exhibit such specific interactions.

For *Alphaviruses*, interactions with receptors occur mainly through the E2 viral protein. Studies on SINV have revealed NRAMP2, an iron metabolic transporter also named DNMT1 or SLC11A2, as a receptor and SEC61A/VCP as regulators of this viral entry (Panda et al., 2013; Rose et al., 2011). However, recently, by the use of a CRISPR/Cas9-based screen, Mxra8 (also called DICAM, ASP3, or limitrin) was identified as a receptor for CHIKV. Mxra8, for matrix remodeling associated 8 protein, is an adhesion molecule containing immunoglobulin-like domains and is found in mammals where it is mainly expressed on epithelial and mesenchymal cells. Intricate studies have been performed on the complex formed between the E2 protein and the newly discovered CHIKV receptor. Interaction occurs at the “valley” between two protomers of the envelope spikes. Interestingly, this interaction will involve not only E2

but also the E1 and E3 protein where a unique 3:3 binding interaction will be adapted with this receptor (Basore et al., 2019; Song et al., 2019). The requirement of Mxra8 for viral entry is conserved for closely related OW *Alphaviruses* such as MAYV and ONNV. However, SINV and NW *Alphaviruses* show little dependence on this receptor (Zhang et al., 2018). This is of interest since SINV has a complex evolutionary past resulting from a recombination event which is consistent with the lack of Mxra8 requirement for this virus (Weaver et al., 1997; Zhang et al., 2018). It has to be mentioned that some cell lines lacking cell surface expression of Mxra8 are highly infected in vitro by CHIKV raising questions about the presence of other receptors for this virus.

Attachment factors play an important role in promoting virion binding to their target cell. *Alphavirus* attachment factors include cell surface heparan sulfate, DC-SIGN and L-SIGN which have been shown to help virion attachment to the host cell (Klimstra et al., 1998, 2003). Additionally, TIM1 and Axl, belonging to the TIM and TAM family of transmembrane proteins that participate in the phosphatidylserine (PtdSer) dependent phagocytic removal of apoptotic cells, have been shown to promote *Alphavirus* entry by their ability to bind PtdSer accessible between the envelope spikes of the virions (Jemielity et al., 2013; Morizono et al., 2011). Interestingly, attachment factors could also play roles in signaling as it has been shown for ZIKV, for example, that binding to Axl activates its kinase activity and downmodulates interferon signaling facilitating infection (Meertens et al., 2017). As *Alphaviruses* also use this attachment factor, it would be interesting to study whether a similar mechanism is hijacked by these viruses.

Finally, a dynamic for *Alphavirus* entry can be proposed from the previously mentioned information (1) non-envelope mediated binding through attachment factors that have affinity to certain lipids at the surface of virions allows close proximity to receptors at the plasma membrane that will then (2) bind specifically the E2 protein and promote viral entry.

ii. Endocytosis and fusion

After binding, the virion will enter the cell by clathrin-mediated endocytosis. Clathrin coating of endocytic vesicles allows rapid traversing of the membranes and delivery of the viral particles into the cell (Helenius, 1980). Yet, this is not the only possible entry

pathway since it has been shown that *Alphaviruses* are capable of entering the cell in a clathrin-independent manner (Bernard et al., 2010). A recent study has added another layer to the knowledge of *Alphavirus* entry showing the involvement of the macropinocytosis process. Macropinocytosis mediated virus entry involves the activation of receptor tyrosine kinases which will induce bleb or ruffle formation. Then, the collapse of these structures will lead to the delivery of viruses inside the cell (Lee et al., 2019). Surprisingly, it has been observed that *Alphavirus* entry could occur directly at the plasma membrane through the formation of a pore by the virus, and perhaps with the involvement of host proteins. However, this excludes the delivery of the viral particle into endocytic vesicles and comes in contradiction with the dogma of *Alphavirus* entry (Vancini et al., 2013).

After internalization by clathrin or the other endocytic pathways, viral particles will be delivered to early endosomes. Inhibiting the activity of Rab5, a Rab GTPase responsible for endosome biogenesis and transport has elucidated the importance of this delivery for the establishment of efficient *Alphavirus* infection (Bernard et al., 2010). Once inside the early endosomal compartment, the fusion of viral and endocytic membranes will depend on an acidic pH (lower than 6) environment of the vesicle. Acidification of endosomal vesicles is mediated through vacuolar ATPase pumps which will pump H⁺ into the vesicle leading to a decrease in pH. The use of inhibitors targeting endosomal acidification and vacuolar ATPase pump activities has confirmed the need for low pH for the fusion process (Bernard et al., 2010; Irurzun et al., 1997). So, why is low pH essential for viral fusion? Elaborate structural and functional studies have involved the E1 viral protein. In this model, acidification of endosomes will lead to dissociation of the E1 viral protein from its complex with E2 and its self-assembly into a hairpin-like homotrimer (Wahlberg et al., 1992). This homotrimer is capable of bringing the viral and endosomal membranes to proximity and in consequence merging the outer membranes which will lead to NC release (Gibbons et al., 2004). Controversially, it is important to note that several studies have found that fusion can occur independently of low pH as well (Cassell et al., 1984; Hernandez et al., 2001). These studies were carried out by the same group that proposed entry by fusion directly at the plasma membrane.

Certain types of lipids also play an important role in this process as it has been shown that cholesterol is required for fusion (Lu et al., 1999). However, this is controversial as

lipid rafts, membrane microdomains rich in cholesterol, were not required for fusion to occur (Waarts et al., 2002). This study used large unilamellar vesicles (LUVs) which are artificial in nature and might explain the discordance with the previous study.

iii. Translation

Once the viral particle is delivered in the cytosol, NC disassembly will occur quickly (5 minutes) by unknown mechanisms leading to the release of the viral RNA genome into the cytoplasm of the infected cell (Helenius, 1984). In this step, ribosomes recruitment for protein translation might occur through the capsid protein which has been shown to bind the large ribosomal subunit (Ulmanen et al., 1976). Additional evidence supporting this hypothesis is a recent study that demonstrated the importance of capsid/viral genomic RNA interactions after disassembly for efficient translation (Sokoloski et al., 2017).

The genomic RNA coding for the nsPs will be synthesized early in infection, and the sub-genomic RNA translation will be favored late in infection. nsPs are translated directly from the incoming RNA genome. The translation of nsPs begins at the AUG initiation codon present in nsP1. Translation will arrest at the stop codon (UGA) producing two different polyproteins: P123 and P1234. This selection is determined by an opal codon present at the C terminus of nsP3 which leads to the production of P123 in 90% of the cases (Carrasco et al., 2018; Strauss et al., 1983).

The viral RNA genome has a similar architecture to cellular mRNA and therefore has been suggested to utilize canonical cap-dependent translation machinery. This was confirmed by a study showing that a loss of expression or activity of the translation initiation factor eIF4E led to a significant decrease in *Alphavirus* infection (Joubert et al., 2015). eIF4E plays an important role in the assembly of the translational machinery where it is involved in directing ribosomes to the cap structure of mRNAs. Its activity is generally regulated by the mTOR pathway that controls host protein synthesis through phosphorylation/de-activation of the eIF4E inhibitor, 4E-BP1, leading to the release of translational repression (Joubert et al., 2015). However, surprisingly, mTORC1 inhibition has been shown to boost *Alphavirus* infection. Indeed, mTOR activity is inhibited during viral infection in order to limit cellular translational machinery (Martin et al., 2012). The final piece of the puzzle came with the finding that *Alphaviruses* regulate eIF4E through an mTORC1-independent manner through activation of the MAP kinase-

activated protein kinases (Mnks). In infected cells, Mnks directly phosphorylate and therefore activate eIF4E allowing the assembly of the translational machinery for viral protein production despite mTOR inhibition limiting general protein translation (Joubert et al., 2015).

iv. Replication complex formation

After nsP polyprotein translation, it will then be sequentially cleaved via the protease activity of nsP2. This sequential processing is essential for proper replication complex assembly (Lulla et al., 2018). The first cleavage occurs in cis at the level of the junction between nsP3^nsP4 leading to the release of nsP4. NsP4 together with P123 and viral RNA will associate at the plasma membrane via membrane interaction motifs in nsP1. This complex is short-lived and will be specialized in the production of (-)RNA leading to the formation of dsRNA intermediates. Further cleavage *in trans* of two different P123 precursors will lead to the release of nsP1 and then the final cleavage occurs in the junction between nsP2^nsP3. This final step will restructure the replication complex to its mature form and will lead to the synthesis of genomic and sub-genomic (+)RNA (Jose et al., 2009; Reynaud et al., 2015). In the section, “Focus on the replication complex”, the *Alphavirus* replication process will be further detailed.

v. Assembly and budding

Late in infection, the viral sPs will be produced from the sub-genomic RNA in the form of a polyprotein Capsid-E3-E2-6K-E1. The capsid, through its protease activity present in the C-terminus, will quickly be cleaved off the structural polyprotein and will then assemble into a multimer of 240 capsid copies which can interact with the viral RNA to form the NC. Capsid cleavage will reveal a signaling peptide in E3 which re-localizes the structural polyprotein to the endoplasmic reticulum (ER) where it will further be processed to yield E3-E2 (pE2), 6K and E1. After this processing, pE2 will form a heterodimer with E1. Then, the oligomerization of three such heterodimers will form the immature, non-fusogenic spike. Further modification will occur in the trans-Golgi network where the cellular enzyme furin will release E3 from pE2. This renders the spike fusogenic. Finally, the mature spike will be transported to the plasma membrane. Lateral spike-spike interactions will lead to the formation of hexagonal arrays which will promote E2/NC interactions. Where does this E2/NC interaction occur? The answer to this question remains unanswered, however, it has been suggested that E2/NC could

be co-transported in cytopathic vacuoles of type II (CPVII) (Soonsawad et al., 2010). This transport to the cell surface has been proposed to involve an actin-dependent transport through Arf1 and Rac1 trafficking-related proteins (Radoshitzky et al., 2016). Another hypothesis is that the NC could be pre-assembled at the plasma membrane and that E2 interactions will occur there. In any case, no matter where these interactions occur, the E2/NC complex will promote assembly and budding of the mature virion from the plasma membrane or from virus-induced intercellular extensions (Martinez and Kielian, 2016; Mendes and Kuhn, 2018; Snyder and Mukhopadhyay, 2012). The cellular machinery involved in the scission of the viral particles from membranes has not been elucidated yet for *Alphaviruses*. However, it is clear that this scission is independent of the endosomal sorting complexes required for transport (ESCRT) pathway which mediates the budding of virus particles of a plethora of enveloped viruses (Taylor et al., 2007). Furthermore, in the case of *Alphaviruses*, it has been suggested that the extensive protein interactions between the envelope and the NC proteins might be sufficient to drive this process.

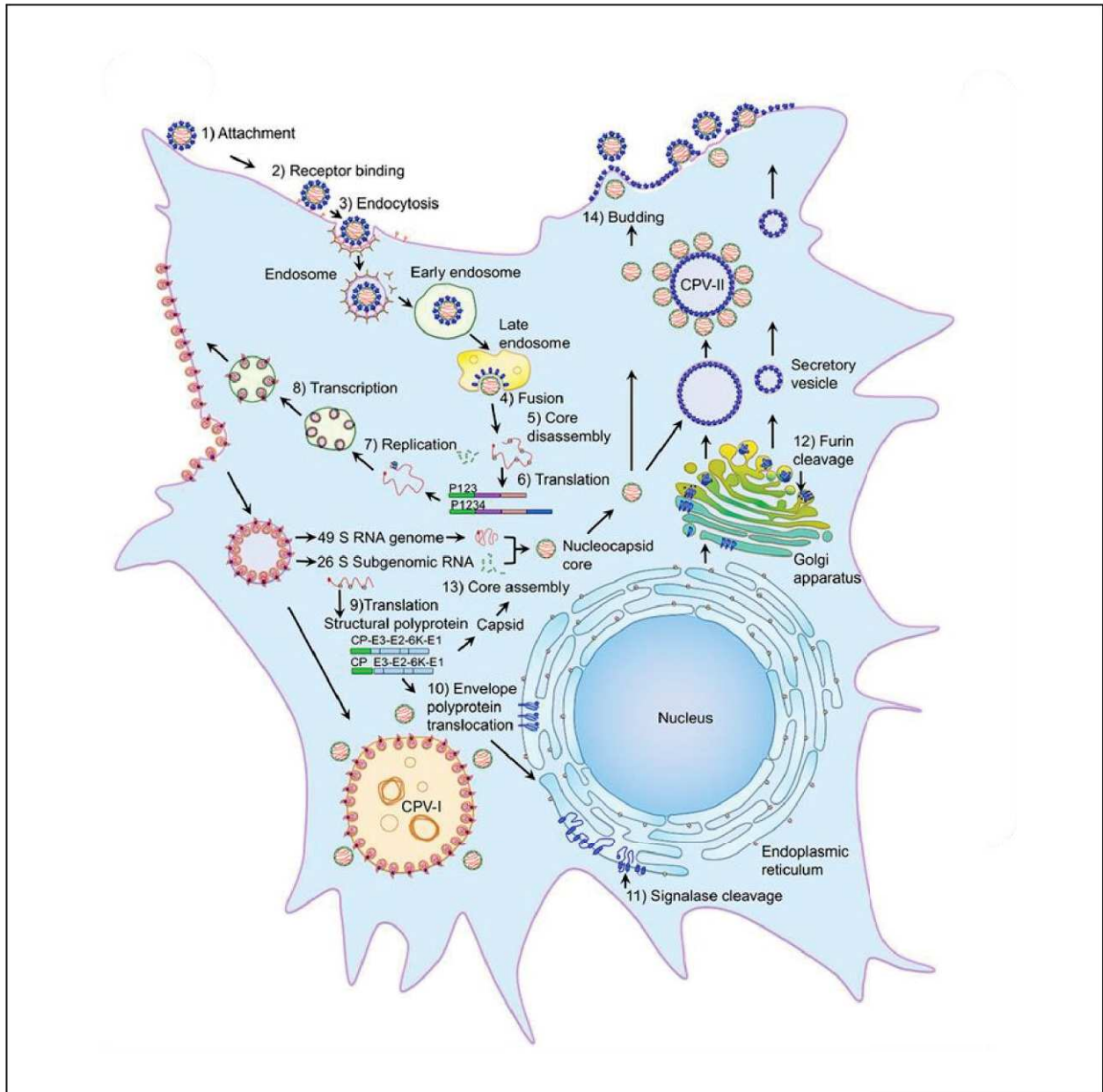


Fig. 10 Model of the *Alphavirus* life cycle

Model of the Alphavirus life cycle: After attachment and binding to receptors (step 1 and 2), an Alphavirus virion is internalized by clathrin-mediated endocytosis (step 3). Low PH mediated fusion will occur in late endosomes (step 4). This will then lead to nucleocapsid release and disassembly (step 5) discharging the viral genome which will then be translated producing the nonstructural polyproteins (step 6). Replication and host proteins along with the viral RNA will form replication complexes (step 7). These complexes form in association with the plasma membrane (PM) leading to the formation of spherule structures and are sites of transcription and replication of viral RNA (step 8). Spherules can then, in some cases, be internalized from the plasma membrane into vesicles that by fusion of with lysosomes will generate CPV-I structures. Subgenomic RNA will act as a template for production of the structural polyprotein (step 9) which will then be processed into the capsid protein (CP) and envelope polyproteins that are translocated (step 10) to the ER, processed by signalase (step 11) and glycosylated and transported through the Golgi complex, where furin cleavage removes E3 from E2 (step 12) to the PM via the secretory pathway. CP will bind viral RNA for nucleocapsid formation (step 13) which will then bind to the glycoprotein spikes present on the PM, and the virus will bud from the PM (step 14) (adapted from Jose, Taylor, and Kuhn 2017).

I- Viral proteins

Structural proteins

The viral sPs, as their name indicates, will participate in the formation of the structure of the mature virion which will then be capable of infecting another host cell.

In this sub-section, the role of each protein in the formation of an *Alphavirus* particle will be discussed. In addition, some atypical roles of these proteins will be mentioned as well.

i. Capsid

Monomeric capsid is a cytoplasmic protein with a molecular weight of 35 kDa.

The capsid protein has a serine protease activity that has been mapped to its C-terminus and involves a catalytic triad composed of Gly-Asp-Ser(219)-Gly. This activity allows this protein to cleave itself out of the newly produced structural polyprotein (Melancon and Garoff, 1987).

During the assembly process, the monomeric capsid is capable of forming multimers composed of 240 capsid proteins with a T=4 arrangement (Cheng et al., 1995). This multimerization involves coiled-coil interactions through a secondary structure known as the helix I in the N-terminus of the capsid protein (Perera et al., 2001). Binding of the capsid protein to genomic viral RNA to form the NC is mediated by the first 100 amino acids which are of very basic nature (Coombs and Brown, 1989).

Finally, for NW *Alphaviruses*, the capsid protein causes toxic effects on host cells. This property mapped to the N-terminus of this protein, will exert its effects through the inhibition of the expression of RNA polymerase II transcribed genes. The capsid protein can also phosphorylate the eukaryotic initiation factor 2 α (eIF2 α) subunit suggesting an effect on translation as well (Aguilar et al., 2007). However, this property is not conserved for OW *Alphaviruses* where it has been shown that nsP2 exerts this function (Garmashova et al., 2007).

Envelope proteins

The envelope proteins are glycosylated and palmitoylated transmembrane proteins which will insert into the host-derived lipid bilayer forming the final shell of the virus (the envelope) during the budding process. The viral envelope consists of 240 copies of the

E1 and E2 glycoproteins in a one to one ratio. The E2/E1 heterodimer will appear on the surface as a trimer of heterodimers giving the viral particle its characteristic spiky appearance as resolved by cryo-electron microscopy (Chen et al., 2018; Cheng et al., 1995).

ii. E1

The E1 protein (47 kDa) is an elongated molecule composed of three β -sheet-rich domains (DI-DIII). It is responsible for the fusion of the viral and endosomal membranes after virus entry. This function is induced after acidification of endosomes containing the viral particles which will lead to disassociation of E2/E1 heterodimers. This will expose the fusion loop present in the domain II of the E1 protein leading to its insertion into endosomal membranes. Then, the formation of E1 homotrimers will lead to the creation of a pore fusing the two membranes and allowing the release of the viral NC into the cytosol (Li et al., 2010).

iii. E2

E2 (47 kDa) has a long leaf-like structure and is composed of an ectodomain, stem region, and a transmembrane alpha helix. It is the envelope protein that will lead to virion recognition of host cell receptors and subsequent virus entry through receptor-mediated endocytosis.

Interestingly, E2 has a fascinating capacity to modify the host cell cytoskeleton leading to the formation of long intercellular extensions that contain actin and tubulin. It can exert this function either when expressed alone or in the context of an *Alphavirus* infection. These extensions were additionally shown to play a role in *Alphavirus* mediated cell to cell transmission (Martinez and Kielian, 2016).

iv. E3

Conversely to E1 and E2, the role of E3 is not clearly known. The emerging theory is that E3 acts as a chaperone for E2 folding during spike assembly. It also may associate with the E2/E1 heterodimers. This phenomenon, however, is not present in all *Alphaviruses* and is clade-specific (Snyder and Mukhopadhyay, 2012).

v. 6K

6K is a small (6 kDa) heavily acylated protein that is incorporated into virus particles in little amounts. (Gaedigk-Nitschko and Schlesinger, 1990). The exact functions of this

protein are poorly known. However, it is presumed to play an important role in the structure of the *Alphavirus* particle. This was supported by findings that mutations in 6K lead to a substantial decrease in virus yield. 6K possibly exerts its structural functions by interactions with E2 since mutations in 6K were shown to be suppressed by compensatory mutations in E2 (Ivanova et al., 1995).

Finally, 6K can form cation-selective ion channels in lipid bilayers and hence was characterized as a “viroporin” which is reminiscent of the HIV accessory protein, Vpu. This function might be important during the virus budding steps as ion strength has been shown to play a regulatory role in this process probably through conformational rearrangement of the E2 protein upon its release from the precursor pE2 (Melton et al., 2002; Strauss et al., 1980).

vi. TF

The discovery of a slippery codon motif in the 6K gene suggested the presence of an additional structural protein. This protein was called trans-frame (TF) because of its production due to a ribosomal frameshifting into a -1 open reading frame. This frameshifting occurs with an estimated efficiency of approximately 10-18%. The TF protein shares the N-terminus containing the transmembrane domain with ion channel activity with the 6K protein but has a unique C-terminus (Firth et al., 2008). The TF protein has been shown to be palmitoylated at its N-terminus promoting plasma membrane localization and incorporation into virions (Ramsey et al., 2017). Only one study has examined TF’s functions. In this study, TF was shown to be dispensable for genome synthesis and transport of envelope proteins to the cell surface. However, it was suggested that TF might be involved in virus particle assembly and release (Snyder et al., 2013). Palmitoylation of TF might be involved with this function since mutating palmitoylated cysteines led to abnormal virus particle morphologies (Ramsey et al., 2017). The discovery of TF impacts decades of *Alphavirus* research and new functions of this protein will probably be unveiled in the near future.

Non-structural proteins: In and out

The viral nsPs are responsible for the formation of the functional replication machinery. This will lead to the multiplication of the viral genome. In addition to their functions inside the replication complex, nsPs also exist in significant fractions elsewhere in the

cell exerting diverse effects. In this part, the knowledge accumulated on viral nsPs in and out of the replication complex will be described.

i. NsP1

NsP1 is composed of 535 amino acids and has a molecular weight of 60 kDa.

In:

NsP1 as the main capping enzyme

One of nsP1's prime functions inside the replication complex is to cap viral RNA. The mechanism behind nsP1-mediated viral capping has been well documented (Fig. 11). NsP1 methyltransferase activity (MTase) is able to transfer a methyl group from S-Adenosyl methionine (SAM) to guanosine triphosphate (GTP) (Laakkonen et al., 1994). This will then lead to the formation of an nsP1-m⁷GMP complex. The formation of this covalent complex is dependent on the presence of divalent ions such as Mg²⁺ or Mn²⁺ (Li et al., 2015). Finally, nsP1 guanylyltransferase (GTase) activity will transfer the methylated guanosine to the 5' end of the viral RNA previously processed thanks to nsP2's RNA triphosphatase activity (RTPase) (Ahola and Kääriäinen, 1995). This capping mechanism is unconventional when compared to the capping of cellular mRNA. Since the guanosine residue is transferred to the 5' end of mRNA and only then is this residue methylated (Decroly et al., 2011). Critical amino acids in the N-terminus of nsP1 have been identified for its corresponding GTase and MTase activity. These include D64, D90, R93 and C135 which upon their mutagenesis led to the abolishment of both GTase and MTase activities. The residue H38 is interesting in its relevance for only GTase activity and has been suggested to mediate nsP1 binding to m⁷GMP through a phosphoramidate bond (Ahola et al., 1997; Wang et al., 1996). In addition, these critical residues for MTase and GTase activities have been shown to also be vital for viral infection. This is coherent with the important role of capping in the protection of newly synthesized viral RNA from degradation. This has led to important efforts to develop inhibitors blocking these activities since such molecules could be used as anti-viral drugs against *Alphaviruses*. Furthermore, the attractiveness of anti-viral molecules targeting nsP1 capping activity is their specificity since, as mentioned earlier, nsP1 capping differs from the cellular capping mechanisms. In these investigations, one of the compounds examined was a natural molecule called lobaric acid that was capable of inhibiting MTase and GTase activity in-vitro and viral in-vivo infection (Delang et al.,

2016). Additionally, a new class of small molecules called ([1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones) were recently found to also act on nsP1 enzymatic activity. Interestingly, these molecules were only specifically active to the GTase activity. *Alphavirus* infected cells treated with the drug led to the generation of a mutation in the proline residue 34 in nsP1 indicating the possible binding region of these small molecules (Feibelman et al., 2018). Surprisingly, it has recently been shown that viral genomic RNAs of *Alphaviruses* are not all universally capped. A significant portion of viral particles encapsidated non-capped RNA which was in turn inversely related to viral particle infectivity (Sokoloski et al., 2015). A follow-up paper attempted to explain the function of these non-capped RNAs in viral infection by modifying certain residues in nsP1 that caused an increase in its capping efficiency. It was notably found that increase in capping activity negatively affected viral infection by perturbing proper particle assembly (LaPointe et al., 2018). This established, for the first time, an important role of non-capped RNAs in viral infection, and implied an importance in the balance of nsP1 capping activity for viral replication.

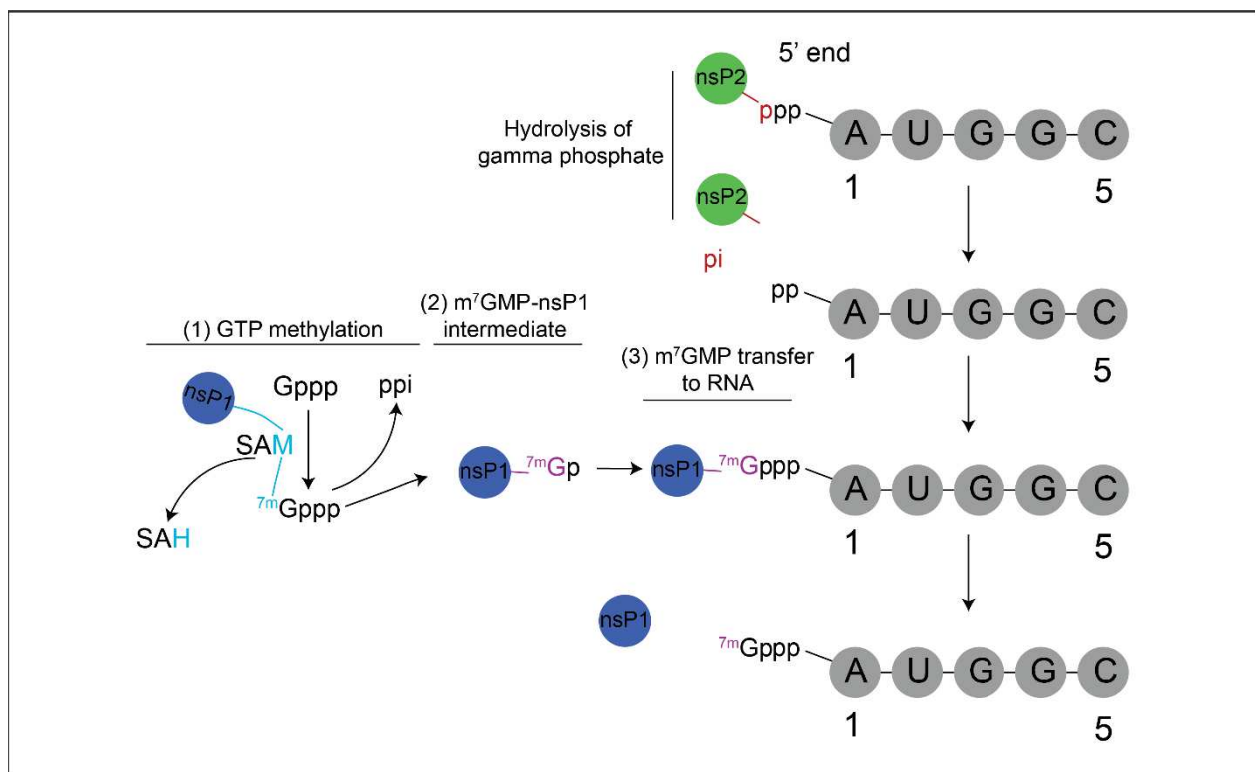


Fig. 11 *Alphavirus* nsP1 capping mechanism

Viral RNA capping starts with nsP2 mediated hydrolysis of gamma phosphate at the 5' end of viral RNA. (1) nsP1, through its methyltransferase activity, transfers a methyl group from S-Adenosyl methionine

(SAM) to guanosine triphosphate (GTP) leading to the (2) formation of a nsP1-guanosine monophosphate methylated at position 7 intermediate ($m^7\text{GMP-nsP1}$) (3) nsP1, through its guanylyltransferase activity, transfers $m^7\text{GMP}$ to the 5' end of viral RNA leading to the formation of the cap.

NsP1 as a membrane anchor

Another important function of nsP1 is its role in replication complex membrane anchoring. Indeed, nsP1 is the only nsP capable of interacting with cellular membranes. In imaging studies, this protein has been mainly observed at the plasma membrane but also in some instances at endosomal membranes (Peränen et al., 1995). The model is that nsP1 will guide the other nsPs to the plasma membrane for replication complex assembly. Historically, nsP1 membrane association was first attributed to the presence of one to three palmitoylated cysteine residues present at the C terminal region of the protein (418-420 in CHIKV sequence). Indeed, mutations in this region led to a partial loss of nsP1 membrane affinity (Laakkonen et al., 1996). Recently, the two zinc finger DHHC domain containing palmitoyltransferases (ZDHHCs), ZDHHC2 and ZDHHC19 were shown to be responsible for nsP1 palmitoylation (Zhang et al., 2019a). The importance of nsP1 palmitoylation for *Alphavirus* replication has been reported by several groups using different *Alphavirus* models. These studies have established that nsP1 palmitoylation can range from being unessential to absolutely vital for virus replication. Indeed, SINV with mutation in nsP1 palmitoylated cysteine is viable and replicate to high titers (Ahola et al., 2000). A moderate importance of nsP1 palmitoylation has been described for SFV where an initial delay in virus growth is detected but which is then counteracted by second site mutations in nsP1 (Žusinaite et al., 2007). These mutations did not restore nsP1 palmitoylation, but recovered nsP1-nsP4 interaction. The exact mechanism by which these mutations restore interaction between nsP1 and nsP4 remain to be described, but it has been suggested that these changes can affect the 3D conformation of nsP1 inside the replication complex. To the other end of the spectrum, there exists *Alphaviruses* which absolutely require nsP1 palmitoylation for replication. This is the case of CHIKV where mutations in palmitoylated cysteines of nsP1 have been shown in two separate studies to completely abolish viral replication. Additionally, silencing of ZDHHC2 and ZDHHC19, the palmitoyltransferases responsible for nsP1 palmitoylation, also lead to complete abrogation of CHIKV replication further demonstrating the importance of this

modification. These studies either employed a complete infectious system or a replicon-based system which recapitulated only the replication steps of *Alphavirus* infection (Utt et al., 2019; Zhang et al., 2019a). So, why does nsP1 palmitoylation requirement differs between these *Alphaviruses*? All nsP1 of these corresponding *Alphaviruses* demonstrate partial loss of membrane affinity upon mutation of nsP1 palmitoylated cysteines. Therefore, this membrane binding function does not completely explain the differences in palmitoylation requirement. It is therefore clear that in order to answer this question, more details on the exact role of nsP1 palmitoylation should be provided.

Since non-palmitoylated nsP1 mutant still retained partial membrane binding, and SFV with this mutation inserted into the viral genome replicated to high titers (Ahola et al., 2000). Intensive studies, through the use of deletion mutants, were done to reveal if there exists another membrane binding determinant. This led to the mapping of a central region in nsP1 (245-268) (Ahola et al., 1999) which was later shown to be amphipathic, in nature, and to structure as an α -helix upon binding to negatively charged lipids. In this α -helix, an important role has been attributed to the tryptophan residue at position 259 which was shown to intercalate in the lipid bilayer to the depth of the ninth and tenth carbons of the lipid acyl chains (Lampio et al., 2000). It has to be mentioned that this α -helix has been only produced in the form of a synthetic peptide and its presence in the complete nsP1 protein remains an open question due to the lack of structural information on nsP1. An interesting finding was that nsP1 with tryptophan to alanine mutation at position 259 was found to be poorly palmitoylated. This suggested that palmitoylation of nsP1 might be dependent on proper membrane targeting through the α -helix peptide (Spuul et al., 2007). The current model for nsP1 membrane binding involves initial membrane interactions through its α -helix. This step is followed by nsP1 palmitoylation which will tighten membrane interactions (Fig. 12).

The two functions of nsP1 inside the replication complex are possibly intertwined. Since it was shown that nsP1's capping activity is regulated by the presence of anionic membrane phospholipids (Ahola et al., 1999). This hints that nsP1 requires association with specific lipid species at the membrane for its proper enzymatic activity. But this might not be true for all *Alphavirus* nsP1. For example, detection of SINV nsP1 enzymatic activities do not require attachment to membranes. However, SFV enzymatic function absolutely depends on the presence of membranes (Ahola et al.,

1999). This might also be the case for CHIKV since attempts to purify an enzymatically active CHIKV nsP1 hasn't been successful to this date (Delang et al., 2016).

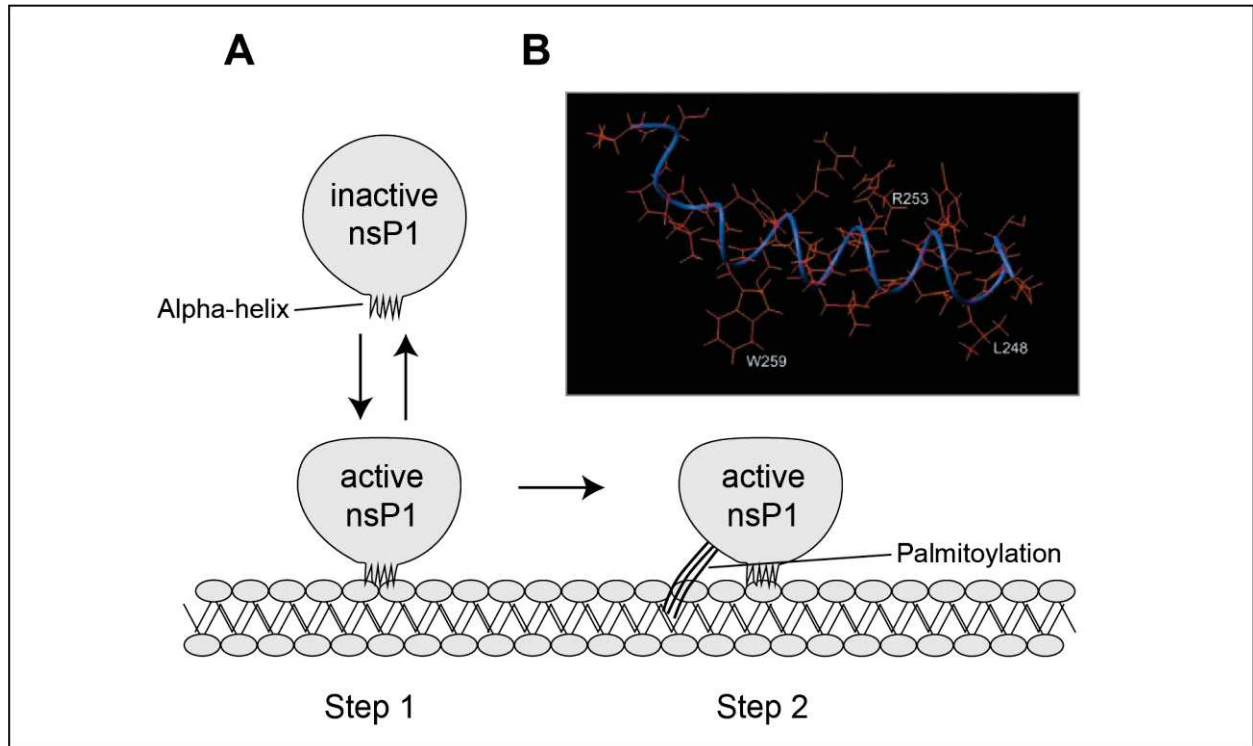


Fig. 12 Alphavirus nsP1 membrane binding mechanism

(A) Step 1: nsP1 associates with the plasma membrane through its central amphipathic alpha-helix motif. Association with anionic phospholipids at the plasma membrane will then activate nsP1's capping activity. Step 2: palmitoylation of nsP1 will further tighten membrane interaction. (B) Nuclear magnetic resonance structure of the binding peptide (aa 245-264) of nsP1 (adapted from Lampio et al. 2000; Kääriäinen and Ahola 2002).

Out:

Interaction between nsP1 and the innate immune response

Outside the replication complex, nsP1 has been described to exert functions with consequences on innate immunity. The first hint for such a function was demonstrated when nsP1 was identified as a critical determinant for RRV induced musculoskeletal inflammation (Jupille et al., 2011). It was later shown that this process was controlled by the production of IFN α by monocytes and that nsP1 counteracted this response (Haist et al., 2017). It is interesting to note that one of the determinants in nsP1 that have been associated with these functions is the palmitoylated cysteine residue at

position 416 which has been already implicated in *Alphavirus* neurovirulence (Ahola et al., 2000).

The exact mechanism behind nsP1/innate immunity interaction is yet to be elucidated. However, a glimpse for this role was discerned when nsP1 was shown to be capable of counter-acting tetherin mediated anti-viral activity. Tetherin is an interferon-stimulated gene that acts on the budding step of the *Alphavirus* lifecycle where it tethers the viral particle to the infected cell preventing its release from the cell surface. The exact mechanism behind nsP1-mediated downregulation of tetherin remains to be determined (Jones et al., 2013). Controversially, a recent paper has contested this nsP1 role as it was observed that no *Alphavirus* proteins encoded an antagonist for tetherin (Wan et al., 2019).

Membrane deformation ability of nsP1

Another intriguing role of nsP1 outside of the replication complex, when expressed alone or in the context of infection, is its capacity to remodel cellular membranes. Isolated expression of nsP1 was shown to induce the formation of filopodia-like structures containing actin filaments at the base and middle of these structures (Fig. 13). However, this function was proposed to be independent of actin due the growth of these filopodia structures in the presence of the actin polymerization inhibitor, cytochalasin D. Interestingly, nsP1 also led to the disappearance of actin stress fibers. This suggested that nsP1 actions on cellular membranes might possibly be linked to its ability to remodel the actin cytoskeleton. Finally, this membrane deformation activity has been shown to be dependent on palmitoylated cysteines suggesting a regulatory role of nsP1 palmitoylation in this function (Laakkonen et al., 1998). To date, there is no data on the mechanism by which nsP1 can deform membranes. Also, the fitness benefit behind this nsP1 function is still not understood.

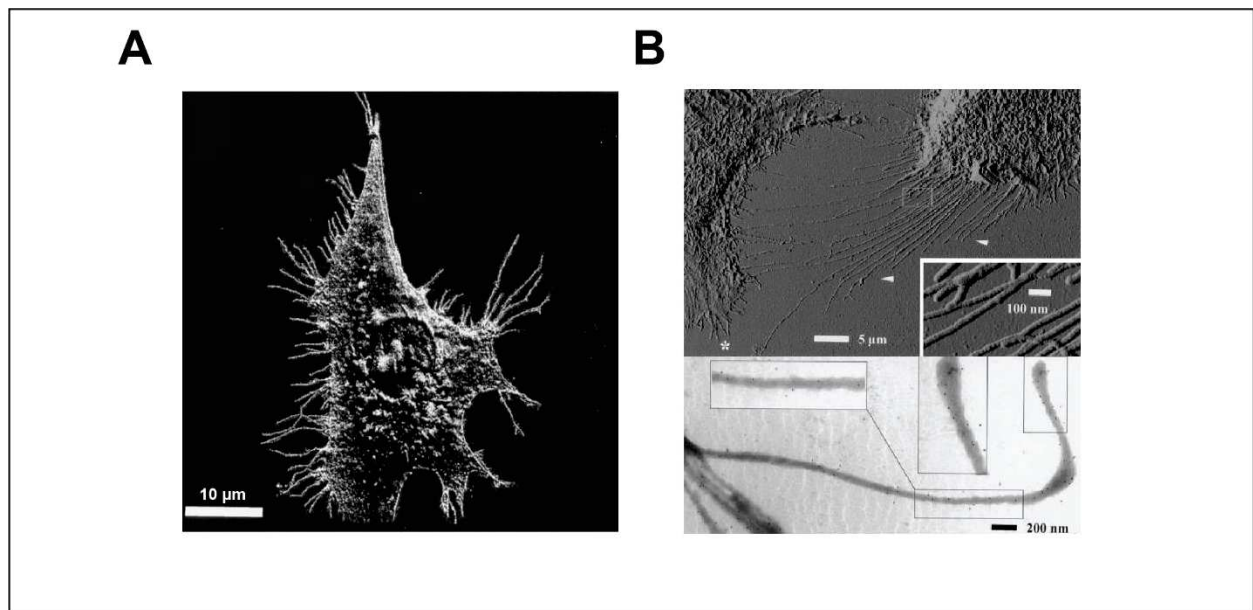


Fig. 13 *Alphavirus* nsP1 deforms the plasma membrane

(A) Scanning electron microscopy image of BHK cells infected with SFV. (B) nsP1 deforms the plasma membrane and is present along the whole length of these membrane deformations. Upper panel: Electron microscopy images of HeLa cells transfected with nsP1. Lower panel: HeLa cells were stained with an anti-nsP1 followed by immunogold labelling. Arrowheads indicate retraction fibers; asterisks indicate filopodia (adapted from P. Laakkonen et al. 1998).

ii. NsP2

In:

NsP2 is the viral nsP with the highest molecular weight of 90 kDa. This nsP is found mainly in (1) cellular membranes as part of the replication complex and (2) in the nucleus where it exerts various effects which will be explained later on. Like other nsPs, it is a multifunctional enzyme with an N terminal domain with helicase, nucleoside triphosphatase (NTPs) and RTPase activities and a C-terminal domain with protease activity and a methyltransferase-like domain (Russo et al., 2006).

nsP2's protease domain is similar to papain-like proteinases with the active sites mapped to cysteine (C481) and histidine (H558). By the means of this activity, nsP2 is capable of processing the produced nsP polyprotein precursor. nsP2 processes the P1234 and P12 polyprotein precursors in *cis* conformation, while the P123 precursor is processed in *trans*. Since timely cleavage and processing of the polyprotein is essential for correct replication complex assembly and functioning, nsP2 protease activity is

essential for viral replication. Indeed, mutations affecting this activity were shown to abolish viral replication (Balistreri et al., 2007; Lulla et al., 2018).

Bioinformatics analysis has classified *Alphavirus* nsP2 to the helicase superfamily 1. Experiments on recombinant nsP2 has confirmed its capacity to unwind double-stranded RNA from the 5' to 3' direction. This function has been proposed to play a role in unwinding secondary structures in the viral RNA enhancing promoter recognition by nsP4 (Cedrón et al., 1999; Das et al., 2014). Recently, the crystal structure of the nsP2 helicase domain in association with the 3' end of genomic RNA has been revealed. This illustrated the organization of this domain and demonstrated that the viral RNA stabilized the helicase region through hydrophobic interactions (Law et al., 2019).

Helicase activity is an active process which requires energy. This energy is mainly provided by the hydrolysis of adenosine triphosphate (ATP). Interestingly, nsP2 also possesses an ATPase activity. Conformingly, this function has been shown to be indispensable for its helicase functions (Das et al., 2014). Therefore, through its own ATPase activity, nsP2 is capable of fueling its helicase function.

Finally, NsP2's RTPase activity is capable of removing the 5' gamma phosphate from the nascent viral RNA before addition of the m⁷GMP mediated by nsP1. Therefore, nsP2 initiates capping at the 5' end of the newly synthesized viral RNA. Mutation of the lysine at position 192 in the N-terminal of nsP2 led to complete abolishment of RTPase activity and virus infectivity demonstrating the importance of this function (Rikkonen, 1996; Vasiljeva et al., 2000).

Out:

Outside of the replication complex, nsP2 plays an important role in controlling innate immune response by diverse mechanisms: (1) nsP2 has been shown to directly block phosphorylation of the protein STAT1 (Fros et al., 2010). Signal transducer and activator of transcription (STAT) proteins upon phosphorylation will re-localize to the nucleus where they will activate the expression of numerous interferon-stimulated genes and induce anti-viral responses. Therefore, nsP2 impact on STAT1 contributes to the shutting down of antiviral responses of the host cell. (2) NsP2 can translocate via a nuclear localization signal pentapeptide ⁶⁴⁸PRRRV⁶⁵² where the middle arginine plays a central role in this localization (Rikkonen et al., 1994). This nuclear localization of nsP2 is responsible for inducing transcriptional shut off by degradation of Rpb1, a

catalytic subunit of the RNA polymerase II subunit (Gorchakov et al., 2005). The inhibition of transcription is another critical mechanism employed to suppress the expression of cellular viral stress-inducible genes (IFN α/β) (Frolova et al., 2002; Gorchakov et al., 2005). (3) Finally, also nsP2 in the nucleus through its methyltransferase-like domain can reduce the levels of nuclear phospho-STAT1 through CRM1-mediated nuclear export again inhibiting this antiviral pathway (Göertz et al., 2018).

NsP2's ability to degrade Rbp1 and induce transcriptional shut-off leads to cytotoxicity and eventually programmed cell death by apoptosis. Mutations in nsP2 regions blocking its transcriptional shutoff activity will lead to loss of *Alphavirus* mediated cytotoxicity. However, this function of nsP2 is limited to OW *Alphaviruses* (Akhrymuk et al., 2018; Fros et al., 2010; Gorchakov et al., 2005).

iii. NsP3

In:

nsP3 (60 kDa) is a modular phosphoprotein separated into three domains, the N-terminal macro domain (MD), the central *Alphavirus* unique/zinc finger binding domain (AUD/ZBD), and the C-terminal HVD. In *Alphavirus* infected cells, nsP3 is concentrated in two main regions: (1) cytoplasmic aggregates with the stress granule protein G3BP1 and (2) at the plasma or intracellular membranes where the replication complex composed of the other nsPs and viral RNA is present.

The MD is a conserved domain among *Alphaviruses*. MD is an ancient protein domain family which can be present in other (+)RNA viruses (e.g. Hepatitis E virus nsP3), but also in eukaryotic systems such as in the human Poly (ADP-ribose) polymerase family (PARP) family of proteins (Rack et al., 2016). The role of this domain will be discussed in the "Out" part. AUD is highly conserved among *Alphavirus* where around 50% of amino acid residues are almost identical in comparisons performed between CHIKV and SINV (Gao et al., 2019). This domain has a distinct structure with a unique protein fold that coordinates, through four cysteines central in the domain, the binding of a zinc ion. Mutations of these cysteines have illustrated the importance of this function in viral replication (Shin et al., 2012). HVD, as its name indicates, is a domain that shows low sequence identity among different *Alphaviruses*. Although, this domain can have some homology between certain *Alphaviruses* belonging to either OW or NW, but it can also

vary significantly in sequence and size even between closely related members. Partial deletions in HVD demonstrate slight effect on viral replication making this domain an ideal location for insertion of fluorescent tags (Foy et al., 2013). This domain is predicted to have an intrinsically disordered structure and is heavily phosphorylated at residues 319 to 368. This post-translational modification has been involved in productive RNA synthesis (Vihinen and Saarinen, 2000; Vihinen et al., 2001).

In contrast to other nsPs, nsP3's role in the replication complex remained enigmatic for a long time due to the lack of associated enzymatic activity. The breakthrough for its role in the replication complex came from proteomics analysis performed on this protein. This led to the finding that G3BP1 and 14-3-3 proteins interacted with nsPs (Cristea et al., 2006). More importantly, these interactions had functional implications on *Alphavirus* replication which will be further detailed in the section "Virus-Host interactions in replication complex assembly and functioning". It has to be stressed that G3BP1 is part of the replication complex, but will also be recruited by nsP3 in other situations that will be mentioned in the "Out" part. Several nsP3 interactors have been further found by extensive proteomic analysis on the HVD of nsP3. This list includes host proteins such as FHL1, CD2AP, FXR and DHX9 (Meshram et al., 2018; Mutso et al., 2018). Today, the emerging view is that nsP3 is a hub for host-cofactors usurping with HVD as the main platform for this recruitment.

Finally, nsP3 is capable of activating the PI3K pathway. This function has been attributed to nsP3's HVD where it was shown that the YXXM motif will mimic cellular growth factors and activate this pathway. This activation was then shown to be responsible for the internalization of replication complexes from the plasma membrane to intracellular organelles. However, this function is not conserved among *Alphaviruses*. Since, for example, CHIKV moderately activates the PI3K pathway with no internalization of replication complexes from the plasma membrane detected. This is, in contrast, with SFV which can strongly activate the PI3K pathway and will lead to internalization of replication complexes (Thaa et al., 2015). The exact consequences of this internalization are not known; however, it has been proposed to draw closer the replication products to the cellular translation machinery. Since the PI3K signaling pathway is central to many cellular processes, the action of nsP3 on this pathway has been suggested to have a wide range of consequences on *Alphavirus* infection.

Indeed, this activation has also been associated with an increase in glucose metabolism towards the synthesis of fatty acids suggesting that nsP3 could reprogram lipid metabolism in favor of viral replication (Mazzon et al., 2018).

Out:

A significant part of nsP3 in infected cells, is present in intracytoplasmic aggregates. These intra-cellular aggregates were shown to be positive for the protein G3BP1, a marker for stress granules. In mammalian cells, translational attenuation via the phosphorylation of eIF2 α results in the formation of stress granules. Inside these granules, stalled translation initiation complexes are present. Stress granules form upon cell stress storing proteins involved in translation for recovery. These granules are often formed during viral infection and several observations have indicated that stress granules could be a mechanism of innate immune response activation (Fros and Pijlman, 2016). Therefore, in response, viruses have evolved to encode viral proteins that are able to disrupt stress granule formation, and also take advantage of its components. In the case of *Alphaviruses*, nsP3 through its two tandem FGDF motifs close to the C-terminus of the HVD disrupts stress granule formation by interacting and usurping G3BP1 at the level of its NTF2-like domain motif (Fros et al., 2012; Panas et al., 2015).

nsP3 also plays a role in the recruitment of the translational initiation machinery. Interestingly, this is also done through an interaction with G3BP1. However, this time, the interaction occurs at the level of the RGG domain which recruits the 40S ribosomal subunit to the vicinity of replication complexes leading to the efficient translation of viral mRNAs (Götte et al., 2019).

A final function for nsP3 outside the replication complex has been mapped to nsP3's MD. MDs are structural modules which have been shown to bind ADP ribosyl residues. ADP ribosylation is a post-translational modification that is implicated in various key cellular processes such as DNA repair, apoptosis, gene regulation and protein degradation. In eukaryotic cells, ADP ribosylation is added through the action of the PARP family of proteins. This signaling process is reversible, and is regulated by readers and erasers which can detect ribosylation on target proteins and can then erase this mark. A recent study on nsP3 MD has confirmed not only its ability to bind ADP ribosyl (reader), but also to hydrolyze the bond between ADP ribosyl and the

corresponding amino acid chain with this modification (eraser). This activity of nsP3 is essential for viral replication (Abraham et al., 2018; McPherson et al., 2017). This function has been proposed to be important for immune evasion since anti-viral host factors are known to be activated upon ADP ribosylation.

iv. NsP4

In:

nsP4 (70 kDa) is the most highly conserved protein in *Alphaviruses* (Khan et al., 2002; Weston et al., 2002). This corresponds well with its vital role as the work-horse of the replication complex. Indeed, nsP4 is responsible for the *de-novo* synthesis of the viral RNA genome. The first hint for this nsP4 activity came after sequence homology comparisons with other RdRps (Kamer and Argos, 1984). This was followed by experimental evidence mapping residues of nsP4 as regulators of *Alphavirus* (-)RNA synthesis (Sawicki et al., 1990). More evidence and mechanistic data emerged after the purification of nsP4 in the *E. coli* system. Purified nsP4 was shown to be able to synthesize (-)RNA from (+)RNA. However, this system required the addition of derived membrane fractions containing uncleaved polyprotein precursor P123 (Rubach et al., 2009). The core catalytic domain has been mapped to 97 residues in the N-terminal of nsP4. This region is predicted to be disordered and is speculated to play a role in cellular/viral-nsP4 protein interactions which can then modify nsP4 activities such as RNA synthesis direction (Kamer and Argos, 1984). Finally, nsP4, *in vitro*, was also shown to possess terminal adenosyltransferase activity. (Rubach et al., 2009). However, it remains to be seen whether it is the enzyme responsible for polyadenylation of *Alphavirus* genome *in vivo*.

Out:

Until today, no function of nsP4 outside the replication complex has been described. This viral protein is produced at low levels due to the presence of the opal codon between nsP3 and nsP4 (Strauss et al., 1983). It also has a short half-life and is thought to be rapidly degraded outside the replication complex by the N-end rule pathway (Groot et al., 1991).

J- Focus on the replication complex

The *Alphavirus* replication complex is composed principally of the viral nsPs, viral RNA and host-cofactors. As explained previously, in this complex each nsP will exert various

roles serving for the efficient production of new viral RNA. However, there exists no information on the exact arrangement and stoichiometry of the viral nsPs inside the replication complex. This is due to the lack of structural data on this complex. Though, it has to be mentioned that various methods have provided basic information on the interactions between the different nsPs. These methods include: (1) Expression of different combinations of the nsPs and then performing coimmunoprecipitation or yeast-double hybrid assays. This revealed nsP1 as a common interactant with all the other nsPs. Additionally, interaction between nsP2 and nsP4 was detected (Kumar et al., 2018; Rana et al., 2014; Salonen et al., 2003; Sreejith et al., 2012). (2) Another method mainly focused on interactions with the nsP4 protein. This approach consisted of mutating the N-terminal region of nsP4 predicted to mediate interactions with the other nsPs in a viral context, and then to detect compensatory mutations in the other nsPs that can rescue this phenotype. This led to the identification of the exact interaction sites between nsP4 and all the other nsPs (Rupp et al., 2011). These interactions as well as the modular organization of the nsPs are presented in Fig. 14.

In this section, the mechanism and features behind the replication of viral RNA will be discussed on the fundamental level. The second part will consist of discussing the membrane platforms where this process occurs. The third and final part will detail the host proteins that play a role in the replication process.

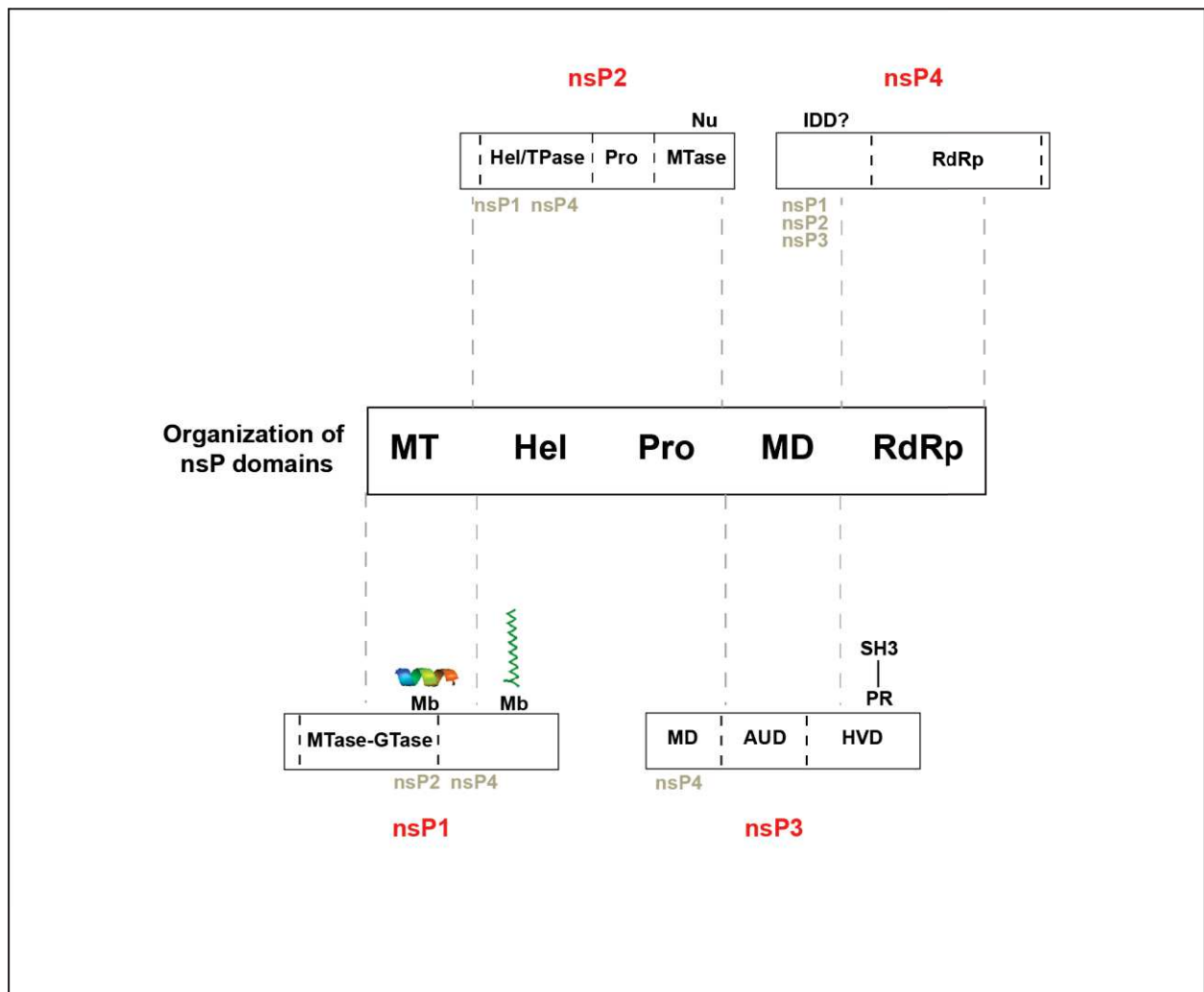


Fig. 14 Modular organization of *Alphavirus* non-structural proteins

The general organization of the *Alphavirus* replication proteins is illustrated. The modules of the different non-structural proteins are detailed as well. Non-structural proteins colored in gold indicate an interaction at the respective site. Nsp: non-structural protein; MT: methyltransferase; Hel: helicase; Pro: protease; MD: macrodomain; RdRp: RNA-dependent RNA polymerase; MTase-GTase: methyltransferase and guanylyltransferase; Mb: membrane binding motif; AUD: *Alphavirus* unique/zinc finger binding domain; HVD: hypervariable domain; PR: proline and arginine rich element; SH3: SRC Homology 3 Domain; Hel/ATPase: helicase and ATPase; Nu: Nuclear localization signal; IDD: intrinsically disordered domain.

i. RNA replication

As mentioned before, RNA replication is mediated by the viral encoded RdRp, nsP4. However, it is not the only element needed. It is important to mention that nsP4 interaction with the other nsPs will play a role in the regulation of this process. Here, RNA replication will be discussed in a step-wise fashion:

Formation of an early replication complex

After the release of the viral genome into the cytoplasm, it will rapidly be engaged with host cell translation machinery leading to the production of the non-structural polyprotein, P1234. The P1234 polyprotein is not able to synthesize viral RNA. It is only after the first cleavage leading to the release of nsP4 that the “early replication complex” will form (Shirako and Strauss, 1994).

Minus strand synthesis

Initiation of (-)RNA synthesis from (+)RNA occurs upon binding of the copying enzyme to a promoter region at the 3' end of the viral RNA genome (Fig. 15).

For *Alphaviruses*, this region has been mapped to the 3' CSE where specifically 13 residues (UUUUUAACAUUUC) were identified as part of the promoter for (-)RNA synthesis. In these residues, initiation occurs on the cytidylate in the 3' CSE region present at the -1 position with respect to the poly(A) tail. Modification of the previous 12 residues was shown to significantly decrease (-)RNA synthesis, and might, therefore, play a role in promoter recognition and initiation to elongation transition. In addition, in this initiation process, the presence of at least 12 poly (A) residues close to the 3' CSE region is essential (Hardy, 2006; Hardy and Rice, 2005). Finally, the interaction between the N-terminus of nsP4 and the region encompassing residues A348-T349 in nsP1 is important for promoter recognition and initiation of (-)RNA synthesis (Shirako et al., 2000).

After binding to the cytidylate residue, nsP4 will begin copying the viral RNA from the 3' to 5' direction leading to the synthesis of a complementary (-)RNA. This will lead to the formation of a dsRNA complex.

Positive strand synthesis

(-)RNA is an intermediate form of the replication process. However, the final product of replication is the production of viral RNA of positive polarity. This viral RNA will be packaged into newly formed virus particles, and can also serve as a template for the production of new viral proteins.

The shift of RNA synthesis activity of nsP4 towards (+)RNA begins with cleavage of nsP1 from P123. nsP1, P23 and nsP4 complex is capable of producing (-)RNA and (+)RNA. However, this complex is extremely short-lived and can only be detected

following mutation of the 2³ cleavage site. The final cleavage between nsP2 and nsP3 will lead to an irreversible transformation of the replication complex towards the production of genomic and sub-genomic RNA of positive polarity. This replication complex is denoted as the “late replication complex” (Shirako and Strauss, 1994).

How does cleavage of other nsPs affect the polarity of nsP4 mediated viral synthesis? No significant knowledge is present at the moment as structural information on the replication complex does not exist. It is presumed that cleavage will lead to a change in the conformation of nsP4 and therefore switching of viral RNA synthesis. Indeed, as mentioned earlier, nsP4 has been shown to interact with nsP1. In addition, studies showed that virus mutants deficient in minus-strand synthesis can be suppressed by second-site mutations in nsP1. This all points towards a hypothesis where cleavage of the polyprotein precursor can alter nsP1/nsP4 interactions and change its RNA synthesis activities. Second-site mutations have been also detected in nsP2 and nsP3 indicating a potential role in the modification of nsP4 synthesis activities as well. These data hint to the importance of interaction with other nsPs for nsP4 structuring, especially at the disordered region present at the N-terminal of nsP4 (Rupp et al., 2011).

The initiation site for (+)RNA synthesis, for genomic and sub-genomic regions, always starts with the AU residues. The initiation site for the genomic RNA synthesis is present at the 3' end of the *de novo* synthesized (-)RNA which corresponds to the 5' UTR of the viral genome. The key residues have been mapped to the positions 2-5 from the 3' end of the (-)RNA. It was shown that the residues 531-538 in nsP4 will mediate binding to this region. For the sub-genomic RNA synthesis, the -19 to +5 residues from the initiation site have been shown to be crucial. nsP4 residues 329-334, predicted to form a beta-strand, will mediate binding to the sub-genomic promoter. These two regions in nsP4, residues 531-538 and 329-334, mediate independently genomic and sub-genomic promoter binding respectively (Li and Stollar, 2007; Wielgosz et al., 2001).

Finally, this step will lead to the generation of the genomic (49S) and the sub-genomic (26S) viral RNA. The sub-genomic promoter is highly active leading to an abundance of 26S viral RNA. This shifts protein synthesis towards sPs late in replication. The genomic viral RNA represents a new copy of the viral genome that will be packaged leading to the formation of new viral particles.

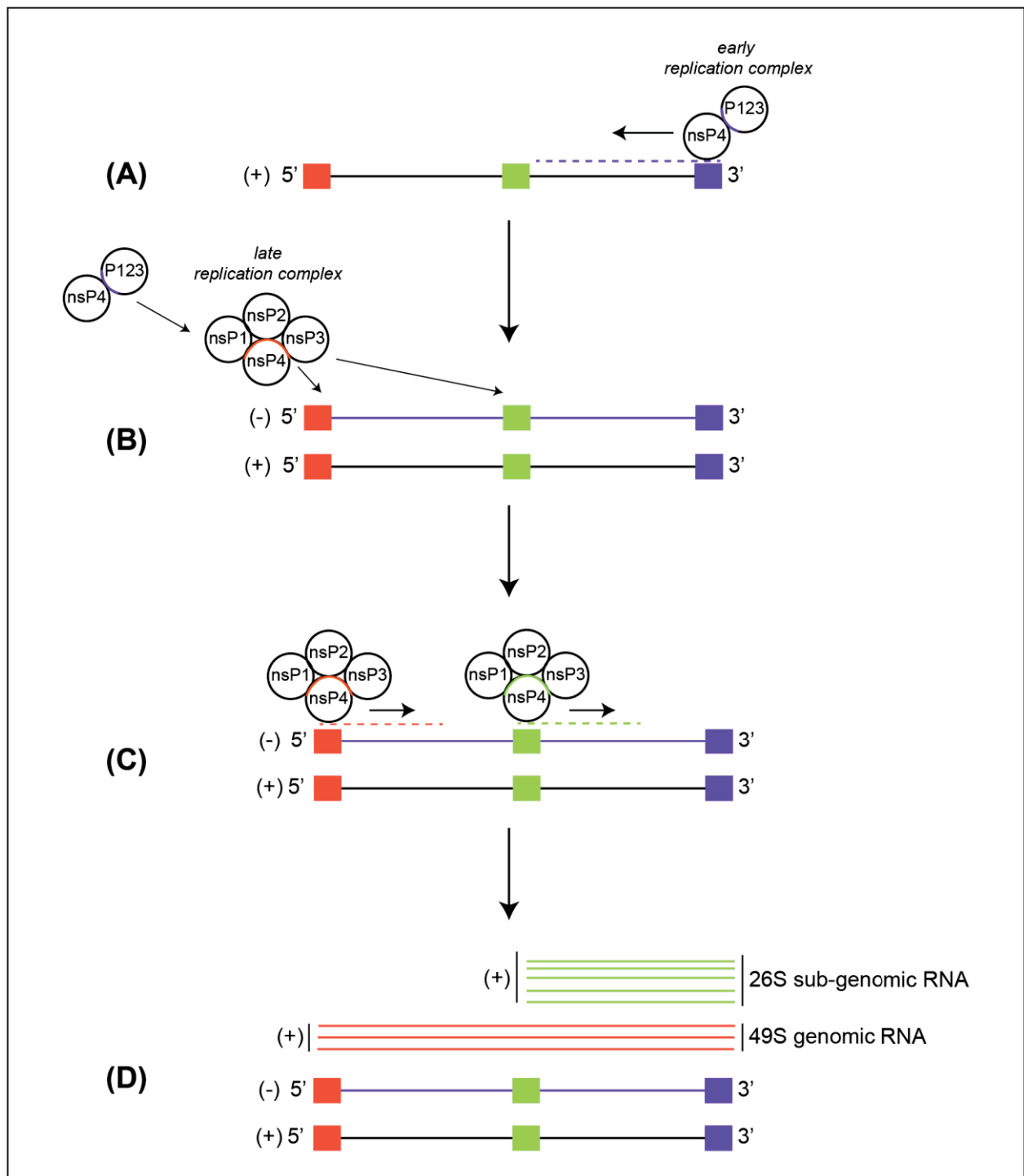


Fig. 15 Schematic representation of the *Alphavirus* replication process

(A) Early replication composed of P123 and nsP4 will synthesize negative stranded RNA. (B) Cleavage of P123 to nsP1, nsP2 and nsP3 will lead conformational changes in nsP4 and binding to the genomic and sub-genomic promoters. (C) This change in conformation will cause a shift in RNA polarity synthesis towards positive stranded RNA. (D) Genomic and sub-genomic RNA will be the final products of the *Alphavirus* replication process. Orange box: Positive strand genomic promoter; Green box: Positive strand sub-genomic promoter; Purple box: Negative strand genomic promoter.

ii. Spherule formation

The *Alphavirus* RNA replication process generates dsRNA as a replication intermediate. This intermediate is a possible activator of the innate immune system. As a way to evade an anti-viral response, *Alphavirus* RNA replication occurs in association with membranes.

Historically, the *Alphavirus* RNA replication complex was presumed to form in association with modified endo/lysosomal membranes (Fig. 16). This hypothesis arose from the observation that *Alphavirus* infected cells formed vacuolar structures that have been since denoted as cytopathic vacuoles of type I or CPVI having a diameter of 0.6-2 μm . CPVI structures have been established to be of endo/lysosomal nature since they stained positively for different endosomal (cationic ferritin and horseradish peroxidase) and lysosomal (lgpl20, lgpl10, lgp96, and cathepsin L) markers. Also, these structures were frequently found connected to the rough endoplasmic reticulum (RER) suggesting that they could also be sites of translation of viral proteins. In CPVI structures, invaginations could be detected at the limiting membrane. These corresponded to spherules which are invaginations of cellular membranes of 50-60 nm in size characterized by having at the base a highly curved narrow neck with an internal diameter of 8 nm (Froshauer et al., 1988).

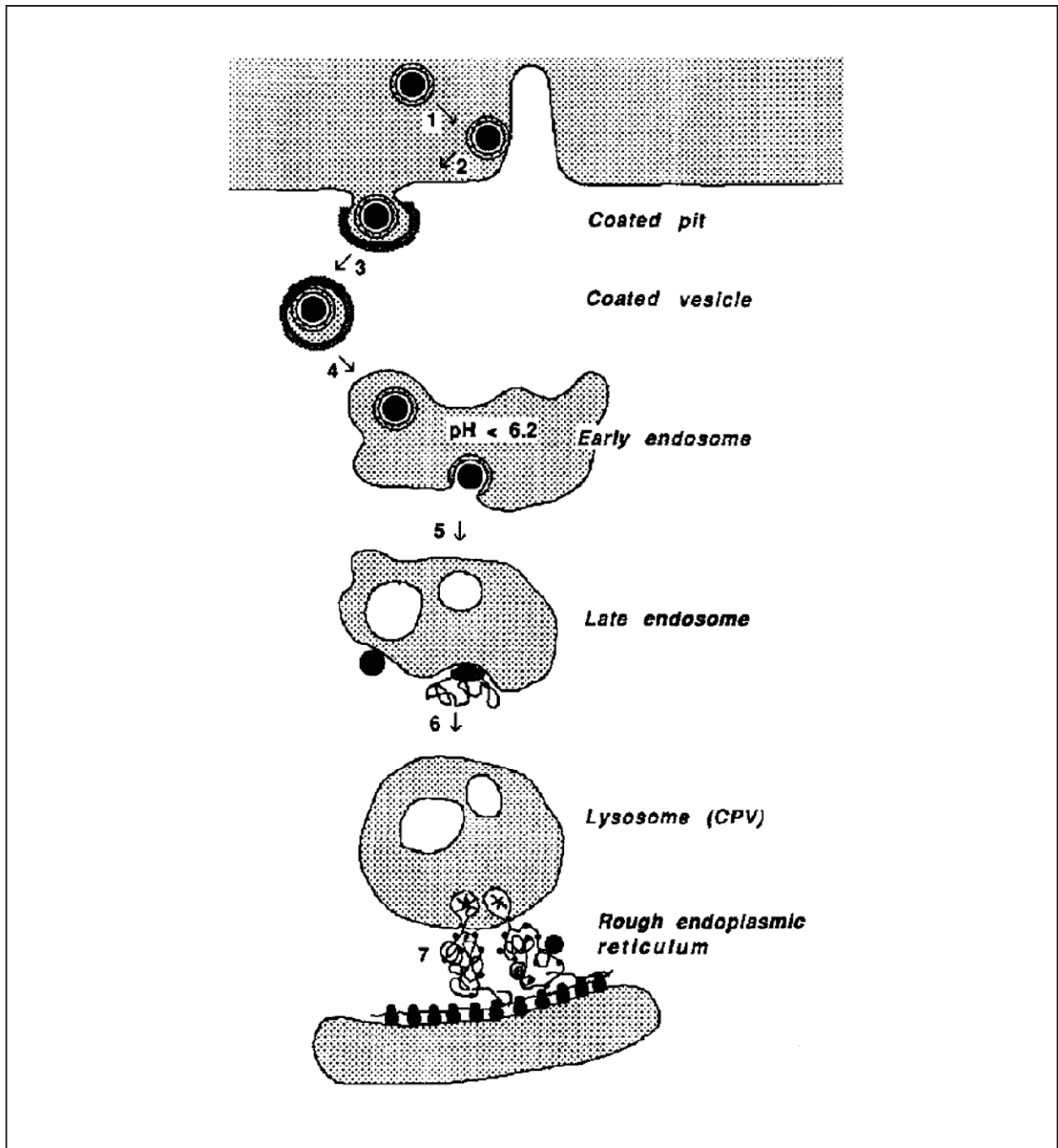


Fig. 16 Outdated model of *Alphavirus* RNA replicase in association with the cytoplasmic surface of endosomes and lysosomes

(1) An *Alphavirus* virion will bind to receptors at the plasma membrane, (2) move laterally and (3) will then be endocytosed into coated vesicles. (4) Coated vesicles will be delivered to endosomes where low pH will induce membrane fusion and nucleocapsid exposure to the cytosol. (5) The nucleocapsid remains associated with the endosomal membrane where it will uncoat and initial translation of non-structural proteins will occur. (6) The late endosome will fuse with a lysosome leading to the formation of a cytopathic vacuole with spherule invaginations formed by the replication proteins. Spherules may be connected to the rough endoplasmic reticulum favoring the translation of structural proteins and nucleocapsid assembly for completion of the late steps of the *Alphavirus* life cycle (Froshauer et al., 1988).

However, later studies showed that the replication complex forms initially at the plasma membrane and will then be endocytosed to form CPVIs, in some *Alphavirus* (Fig. 17). This was supported by evidence showing that early in infection (2 to 4 hrs post-infection), most spherules are present at the plasma membrane of the cell, with little CPVI structures detected. This has also been supported by the detection of dsRNA signal exclusively at the plasma membrane at these timepoints. Furthermore, the use of wortmannin, an inhibitor of the PI3K pathway, led to an accumulation of spherules at the plasma membrane. This further reinforced the argument that spherules formed at the plasma membrane and were then internalized by the PI3K signaling pathway. Another final strong evidence is the data accumulated on the profound ability of nsP1 to accumulate at the plasma membrane (Frolova et al., 2010; Pietilä et al., 2018; Spuul et al., 2011).

It is important to note that assembly of the *Alphavirus* complex at the plasma membrane is a phenomenon that has been only detected in mammalian cells. Since, in mosquito cells, no localization of the complex at the plasma membrane early in infection has been observed. This raises questions about differences in the assembly of the replication complex between mammalian and insect cells (Frolova et al., 2010). Indeed, a recent study has examined the differences in the *Alphavirus* life cycle between mammalian and mosquito cells. Differing with replication in mammalian cells, no significant amounts of nsPs (with the exception of nsP1) and dsRNA were detected at the plasma membrane of infected insect cells (Jose et al., 2017).

Therefore, the updated model for creation of *Alphavirus* spherules is as follows:

The early replication complex (P123, nsP4, and viral RNA) is addressed to the plasma membrane through membrane binding domains in nsP1 (Hellström et al., 2017). Upon minus-strand synthesis and generation of dsRNA intermediate of this early replication complex, plasma membrane invagination towards the extra-cellular medium will lead to the formation of spherule structures. Studies using partially uncleaved precursors of the non-structural polyprotein has demonstrated that the combination of P123 and nsP4 forms spherules most efficiently (Hellström et al., 2017). The newly synthesized RNA is relatively stable in the cytoplasm and therefore might be protected by encapsidation. This RNA will be engaged after for either virus assembly or used as a template for neighboring replication complexes (Pietilä et al., 2018).

Finally, it has to be mentioned that the length of the viral RNA template is a key determinant for the size of *Alphavirus* spherules. This has been beautifully demonstrated through the artificial modification of the template length. Long templates yielded spherules of around 45 nm, while short templates generated spherules of 40 nm emphasizing that the fact that RNA size played a major role in defining the size of a spherule (Kallio et al., 2013).

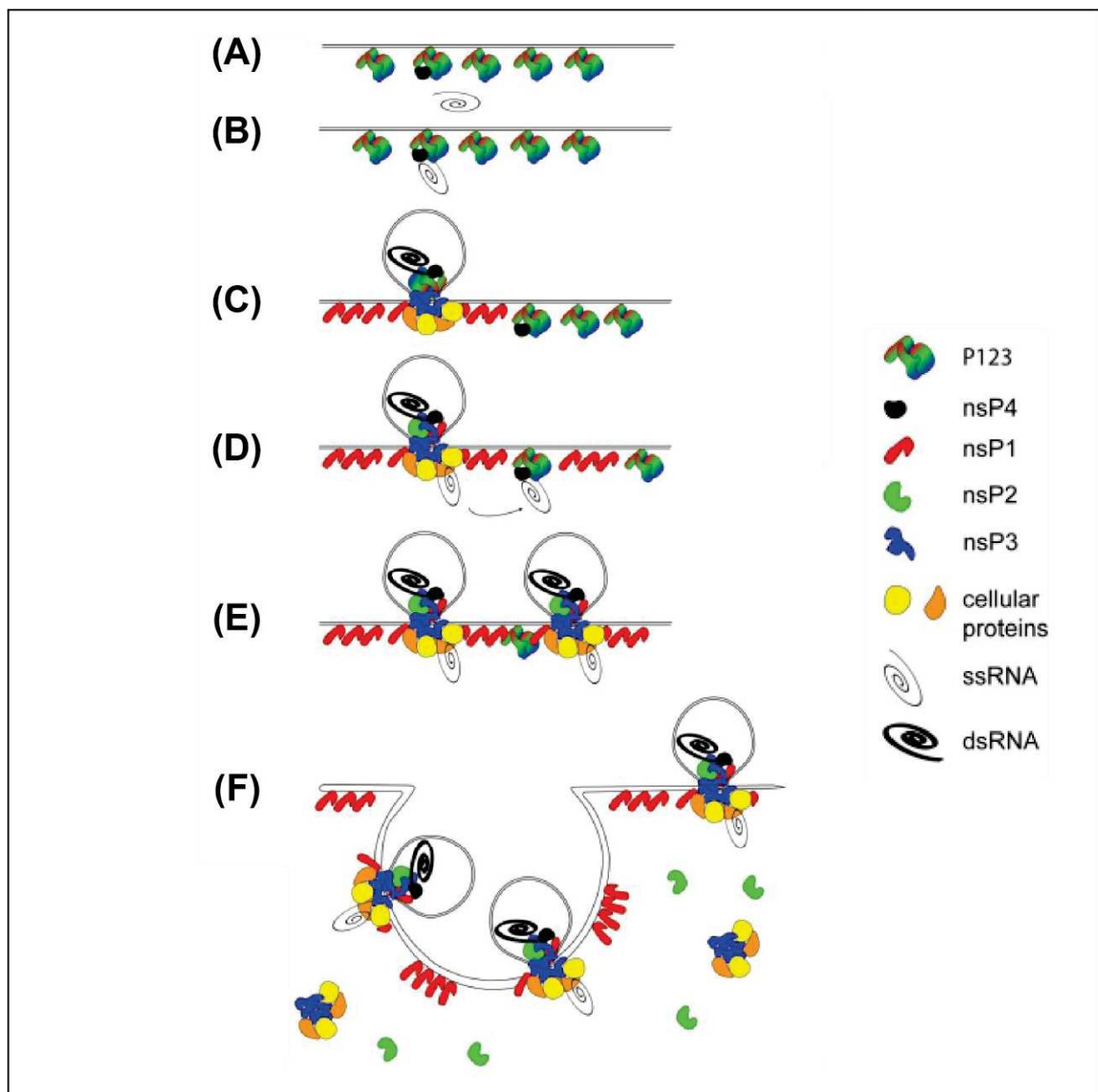


Fig. 17 Schematic representation of the updated model for *Alphavirus* spherule formation at the plasma membrane

(A) P123 complex will be anchored to the plasma membrane through motifs in nsP1. Some of the nsP4 molecules will bind to the P123 complex at the plasma membrane (B) Complex containing P123 and nsP4 will bind the viral genome. (C) Synthesis of dsRNA intermediate will lead to formation of membrane-bound spherules. (D and E) Further processing of P123 will lead to synthesis of genomic and subgenomic RNAs which will be released from spherules and might serve as templates for neighboring replication complexes. (F) In some Alphaviruses, spherules are endocytosed into cytopathic vacuoles of type I (adapted from Frolova et al., 2010).

iii. Virus-Host interactions in replication complex assembly and functioning

A virus, by definition, is a non-living organism. This definition originates from the incapacity of viruses to multiply without their host cells. This insinuates that viruses will use the host cell's machinery in order to replicate. In this part, the virus-host interactions established in the *Alphavirus* replication step will be detailed.

The *Alphavirus* replication complex consists minimally of the four nsPs and viral RNA. In addition, host-cofactors have been described to be recruited to the replication complex leading to regulation of the replication process.

The knowledge accumulated on *Alphavirus* cofactors arose mainly from four main approaches:

(A) Proteomic analysis of replication complexes isolated from membranes of infected cells (Varjak et al., 2013).

(B) Analysis of interactants of individual nsPs using proteomics or yeast-two hybrid approaches (Bouraï et al., 2012; Cristea et al., 2006; Mutso et al., 2018).

(C) Genome-wide screens identifying cofactors important for *Alphavirus* infection (Meertens et al., 2019; Tanaka et al., 2017).

(D) Comparative RNA-interactome capture technique that employs oligo(dT) to capture viral RNA followed by quantitative proteomics allowing the identification of viral RNA binding partners (Garcia-Moreno et al., 2019).

Approach (A) revealed a plethora of host-cofactors that played a role in different cellular processes such as protein folding, cytoskeleton reorganization, stress granule formation, RNA unwinding and translation (Varjak et al., 2013).

Approach (B) has also been proven to be useful. Since it first allowed the precision of the exact nsP that will recruit a specific cofactor. Second, this approach led to the

detection of new cellular cofactors that might have been missed due to sensitivity (Cristea et al., 2006). This has revealed nsP2 and nsP3 as the hubs for protein interactions. nsP4 seemed to interact the least with host cofactors, probably due to its specialized role in RNA replication. Interestingly, nsP2, nsP3 and nsP4 interactants overlapped revealing a redundancy in the cellular factor recruitment and possibly establishing importance to these interactions. While abundant data is present on the interactants of nsP2, nsP3 and nsP4 but information on nsP1 cofactors is lacking, probably due to its high affinity to membranes explaining why attempts on performing yeast-two hybrid studies on this protein have failed to identify any interactant (Bourai et al., 2012). The only cellular protein known to interact with nsP1 is RpS6 (Montgomery et al., 2006). However, this protein also interacts with nsP2 and therefore this interaction could be indirect. It has to be mentioned that interactions detected with individual nsPs are probably modulated in the context of a complete infectious system.

Approach (C) allowed the identification of cofactors which are functionally essential for *Alphavirus* infection. A subset of these cofactors was then later on shown to play an essential role in the replication step specifically.

Finally, Approach (D) allowed for the first time to detect host-cofactors directly binding to the viral RNA. These cofactors are possibly recruited by viral nsPs, but might also recognize specific sequences in the viral RNA. This revolutionary approach is recent, and will allow the uncovering of new modulators of *Alphavirus* replication (Garcia-Moreno et al., 2019; LaPointe et al., 2018).

All four approaches have proven to be informative and complementary. The robustness of these proteomics and screening approaches were validated by later work that was done on these cellular cofactors. Here, several examples of these cofactors and their mechanistic role will be discussed.

RNA binding proteins

hnRNPs

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a family of RNA binding proteins with various functions in cellular nucleic acid metabolism. They play a role in the maturation of newly produced RNA, but also stabilization of mRNA and transport to the cytoplasm for translation. Due to their function in RNA processing, these proteins

have been heavily investigated for their roles in RNA virus replication (Geuens et al., 2016).

The first evidence of their involvement in *Alphavirus* replication came from mass spectrometry experiments comparing proteomes of membranes of mock- and *Alphavirus*-infected cells. This revealed an enrichment of several hnRNPs namely hnRNP M, hnRNP C, and hnRNP K and PCBP1 in the *Alphavirus* infected condition (Varjak et al., 2013). Interestingly these proteins are mainly localized in the nucleus of a cell. However, upon infection, hnRNPs were found to be re-localized at the vicinity of *Alphavirus* replication complexes indicating a possible role in *Alphavirus* replication.

Functional studies utilizing replicons that mimic *Alphavirus* replication steps revealed differential effects depending on the hnRNP studied. For example, hnRNP M and hnRNP C inhibited viral replication. However, the protein PCBP1 was shown to be important for this step (Varjak et al., 2013). hnRNP K was interesting in the fact that its requirement was different among *Alphaviruses*. Deficiency in hnRNP K expression had little to no effect on SFV replication, but for SINV and CHIKV, hnRNP K deficiency significantly decreased viral replication. These differences in the need for hnRNPs can be explained by a difference in the concentration required of these proteins. In certain concentrations, an hnRNP could either act as an inhibitor or activator of replication. Finally, binding of these cellular factors to different regions of the viral RNA might additionally explain differences in the phenotypes observed (Varjak et al., 2013).

The mechanism by which hnRNPs function in SINV replication has been recently re-investigated. In this study, it was found that hnRNPs (hnRNP K, hnRNP I, and hnRNP M) were directly bound to the viral RNA and the exact localization of this binding was also defined. hnRNP K and hnRNP M mapped to regions within the structural coding part, while hnRNP I was found to interact with the 3' UTR region of the viral RNA. The knowledge acquired on the interaction sites within the viral RNA allowed through modification of these regions to elucidate whether these interactions are functionally important. Indeed, mutations at the interaction sites of these hnRNPs decreased viral titers. Furthermore, this study explored how hnRNPs exert their effect on *Alphavirus* infection. Interestingly, it was found that binding of these proteins decrease expression of sPs and might subsequently modulate *Alphavirus* induced host translation shutoff (LaPointe et al., 2018).

Another RNA binding protein that has been further mechanistically characterized for its role in *Alphavirus* replication is DHX9.

DHX9

DExH-box RNA helicase 9 as its name indicates is an RNA, but also DNA, helicase whose function is to unwind DNA and RNA structures. This enzyme shuttles between the cytoplasm and nucleus where it regulates several cellular processes such as transcription, transport, and translation of mRNA (Zhang and Grosse, 2004). It has been identified by several approaches as a cofactor of the *Alphavirus* replication complex. Similarly, to hnRNPs, DHX9 is re-localized to *Alphavirus* replication complex upon infection. Recently, a mechanism on the mode of action of DHX9 in the replication complex has been described. The model is that DHX9 is recruited to the replication complex via nsP3 early in infection where it will promote translation and inhibit replication. The exact mechanism of action is unknown, but it has been suggested that DHX9 through its RNA helicase enzymatic activity can lead to the unwinding of RNA structures favoring the sliding of ribosomes. Interestingly, similar importance of DHX9 for HIV RNA unwinding and subsequent translation has been described (Bolinger et al., 2010). Finally, late in infection, DHX9 is degraded leading to a translation to replication switch (Matkovic et al., 2019).

Cytoskeletal and membrane curvature proteins

Cytoskeletal and membrane curvature proteins are of high interest as partners for *Alphavirus* replication complex. Since they are presumed to play an essential role in spherule formation by membrane curvature induction. While the information on these partners remains scarce, here three different cofactors will be discussed.

Amphiphysin

Amphiphysin belongs to the family of Bin/amphiphysin/Rvs (BAR) proteins which are capable of sensing and inducing membrane curvature. BAR family of proteins can be divided into two different groups, ones that induce positive membrane curvature (N- and F-BAR), and ones that induce negative curvature (I-BAR). Positive membrane curvature is defined by a curve forming towards a relative compartment, while a negative membrane curvature will form away from a membrane compartment leading to the formation of membrane protrusions. BAR proteins are capable of exerting this

function by the adhesion of the BAR domain, insertion of amphipathic helices and, in certain conditions, oligomerization (Simunovic et al., 2015). Special attention has been given to this family of proteins since they can possibly induce the membrane curvature required for the formation of spherules.

The main proof for an implication of BAR proteins in *Alphavirus* replication comes from a study showing that SFV nsP3 is capable of recruiting both amphiphysin-1 and 2 (N-BAR proteins) in an SH3-dependent manner to replication complexes. This recruitment was essential for the viral replication since mutations in nsP3 SH3 binding motifs lead to replication-deficient viruses in infected cells and attenuated neurological symptoms in mice. In addition, the silencing of amphiphysin-2 decreased viral replication (Neuvonen et al., 2011). Furthermore, the recruitment of amphiphysin-1 and 2 appear to be limited to only to OW *Alphaviruses*. This was suggested due to the failure to detect these proteins in proteomics analysis using the HVD of VEEV and EEEV. However, other BAR domain-containing host factors such as SNX9 and SNX33 were detected for NW *Alphaviruses* suggesting a difference in the use of BAR proteins depending on the *Alphavirus* (Frolov et al., 2017; Meshram et al., 2018).

This designates BAR proteins as interesting candidates contributing to the membrane curvature required for spherule formation, but the exact function and mechanism remain to be shown. Additionally, the fact that no modification of spherule formation was observed in knockout cells for these proteins raises further questions on their implication or redundancy in their requirement (refer to the CD2AP part).

CD2AP

CD2AP is a scaffolding protein that regulates the actin cytoskeleton. It has been described to perform this function by binding to actin filaments through its actin-binding sites and capping them by recruiting the actin capping proteins (CAPZA1, CAPZA2, CAPZB). This recruitment has been reported to facilitate the formation of lamellipodia structures at the cell periphery (Zhao et al., 2013). Recently, through elaborate proteomics studies on nsP3, CD2AP was found to be recruited to replication complexes of SFV and CHIKV via nsP3's HVD. Mutations in the interaction motif denoted M2 (⁴²³PMASVR⁴²⁸) in nsP3 lead to a decrease in RNA infectivity and genome replication. However, this did not lead to a change in replication complex localization (Mutso et al. 2018). It has been recently suggested that CD2AP and BIN1 play redundant roles in

the initiation of CHIKV replication (Agback et al., 2019). However, the common function played by these two proteins in this process remains uninvestigated.

FHL1

Four-and-a-half LIM domain protein or FHL1 is a protein highly expressed in heart and muscle tissues and plays a role in several cellular processes. FHL1 was first found in mass spectrometry analysis that aimed to find binding partners for nsP3's HVD domain. (Meshram et al., 2018; Mutso et al., 2018) Interestingly, it was then identified as a top hit in a genome-wide screen searching for cofactors of CHIKV infection performed in HAP1 cells. This cofactor was of special interest since its high expression in muscle tissues correlates with CHIKV tropism. Knockout cells for FHL1 were not permissive by CHIKV. In addition, mice genetically engineered to not express FHL1 were not infected and did not generate any CHIKV-like symptoms. Further analysis on this cofactor showed that it played a direct role in the CHIKV replication step. Although, no direct co-localization between dsRNA and FHL1 were provided in this study. The requirement for this cofactor was surprisingly not found to be conserved for MAYV which belongs to the same antigenic complex as CHIKV. This could indicate that MAYV is taking on a separate evolutionary branch during its circulation in South America (Meertens et al. 2019).

While the emphasis in the previous section was on host-cofactors playing a role in RNA binding and cytoskeleton organization, there remains an exhaustive list of factors whose roles remain to be investigated in the replication of *Alphaviruses*.

iv. Knowledge acquired from other (+)RNA viruses on the biogenesis of spherules

Evidence that the previously mentioned host/virus interactions participate in the biogenesis of *Alphavirus* spherules remain scarce. Indeed, the mechanisms for the formation of these compartments have not been elucidated. On the other hand, there exists comprehensive information for phylogenetically neighboring viruses that generate morphologically similar replication compartments. This could aid in developing our understanding for *Alphavirus* replication complex biogenesis.

In this context, a recent re-classification of (+)RNA viruses based on metagenomic data has divided them into three phylogenetic groups in an original way. One of these new

phylogenetic branches grouped the alphavirus-like superfamily with the flavivirus-like superfamily and nodaviruses as well. Remarkably, all these viruses form spherule structures at membranes for their viral replication (Wolf et al., 2018).

However, it must be noted that the nature of membranes usurped as well as the mechanism behind membrane association differs between viruses of this branch. For example, *Alphaviruses* bind to the plasma membrane through an α -helix and palmitoylated cysteine(s), while *Flaviviruses* associate to the ER membrane through transmembrane domains. In addition, the number of replicase proteins varies substantially. *Bromoviruses* have two replicase protein, meanwhile, *Alphaviruses* have four replicase proteins. But, the conservation of spherule structures throughout evolution in eukaryotic hosts suggests the employment of common cellular mechanisms (Ahola, 2019).

In this section, a compelling way to understand how *Alphavirus* spherules are formed is presented by looking at the literature of other (+)RNA viruses that form the same type of structures. In this investigation, we can find two viruses, Brome mosaic virus (BMV) and Flock house virus (FHV), that form remarkably very similar structures to *Alphavirus* spherules. Therefore, the mechanisms behind spherule formation of these viruses will be detailed with comparisons with *Alphaviruses* when possible.

Brome mosaic virus spherules

BMV is a plant virus, belonging to the *Bromovirus* genus and to the *Alphavirus*-like superfamily, which infects many crop cereals without causing any economically important losses in crop production. However, due to several features that make it simple to investigate such as its capacity to replicate in yeast, it has been used as a model for (+)RNA virus replication studies. BMV has a segmented RNA genome composed of three capped RNAs. Only the two monocistronic RNAs coding for the viral proteins 1a and 2a^{pol} are required for BMV replication. The 1a contains the capping and helicase activity, and 2a^{pol} has a polymerase activity. Impressively, 1a is capable of forming spherules at the ER without the need for the 2a^{pol} protein or an RNA template (Noueiry and Ahlquist, 2003). BMV spherules (50–70 nm in diameter) have been observed by electron microscopy and are composed of a single lipid bilayer where electron-dense material could be observed inside these structures. Interestingly, the structure of BMV induced spherules has been paralleled to structures formed upon

retrovirus budding suggesting a common ancestor or shared mechanisms. Impressively, certain Gag mutations can block pinching off of budding virions leading to the formation of spherule-like structures in HIV infected cells (Schwartz et al., 2002).

So, how does BMV through the 1a replicase protein lead to spherule formation? And what are the host-cofactors required?

The first step of (+)RNA virus replication is the assembly of a replication complex at membranes. 1a is the master orchestrator of this process. The first step consists of self-interaction of 1a which will form a coat on the interior side of spherules and then the interaction with the 2a^{pol} protein occurs. After this step, 1a through membrane interaction domains mapped to residues 368–478, is capable of recruiting 2a^{pol} and the RNA template to the membrane of the ER. An 18 amino acid sequence in this domain is capable of forming an amphipathic helix with a hydrophobic face consisting of three leucine shown to be important for not only membrane association but also spherule induction. In these spherule structures, RNA templates will be protected from degradation and active replication can occur (Liu et al., 2009).

Due to their capacity to induce structures similar to BMV spherules, the ESCRT family of proteins has been investigated for their implication in BMV replication organelle biogenesis. Indeed, ESCRT proteins lead to invagination away from membranes inducing negative membrane curvature causing the formation of structures called multivesicular bodies (MVBs). The only distinguishable difference with BMV spherule formation is that the ESCRT structures finish by pinching off the membranes. This implies that if recruited for BMV spherule formation, this pathway must be tightly controlled. Additionally, there exist 4 complexes of the ESCRT machinery ESCRT-0 to III where their sequential recruitment will be responsible for the formation of MVB structures. Interestingly, BMV spherule formation was shown to require only the ESCRT III complex and especially the late ESCRT protein called Snf7p. Snf7p interacted with 1a, and its knockout lead to the complete abolishment of BMV spherule formation. A model was then drawn for ESCRT involvement in BMV spherule formation. The first step involves 1a self-interaction. Next, 1a associates and curves membranes of the ER. Here comes the role of the ESCRT complex III proteins, specially Snf7p which are recruited and will contribute to constriction of the wide membrane rim formed by 1a multimers. Usually, at this step, MVB necks will be cleaved off. However, as is evident BMV evades this step. It has been proposed that reticulons, a family of

membrane reshaping proteins, contribute to the stabilization of spherules and therefore inhibit cleavage by the ESCRT complex (Diaz et al., 2015) (Fig. 18).

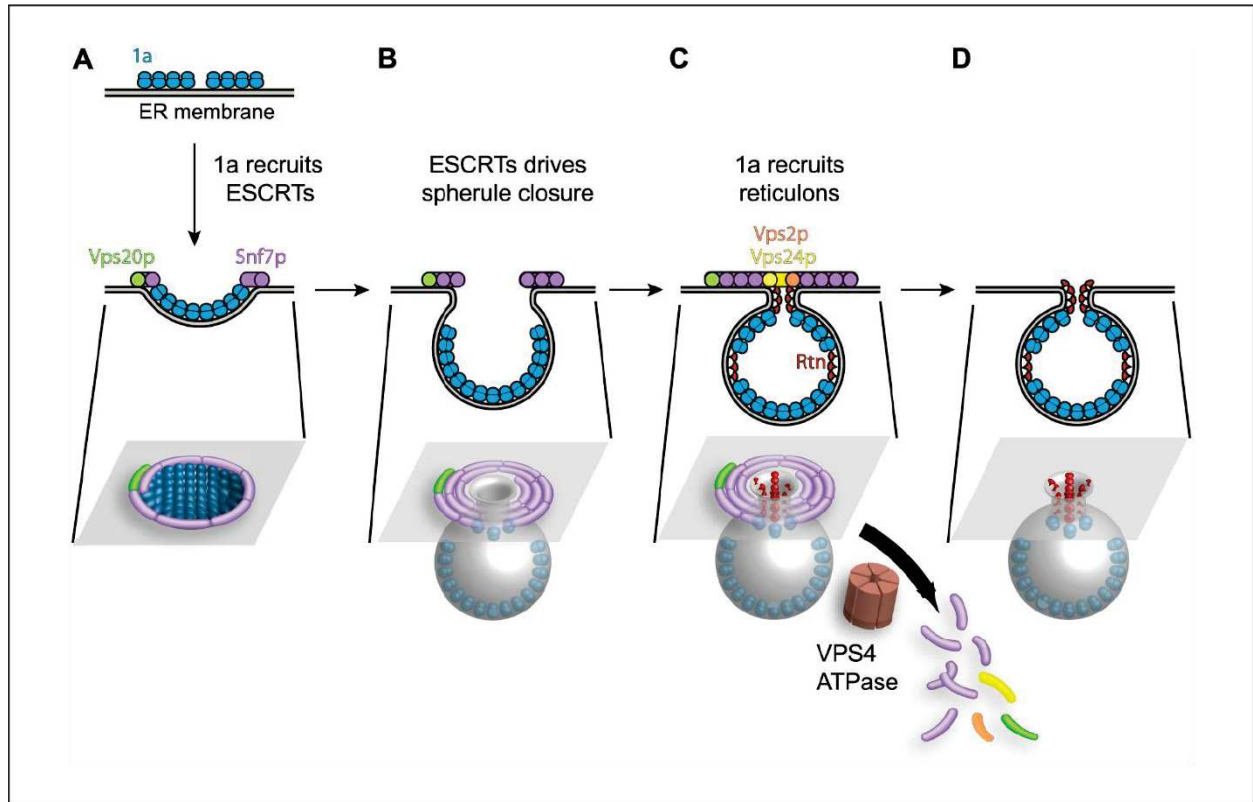


Fig. 18 Model for the contribution of the ESCRT complex in BMV spherule formation

(A) Multimeric BMV 1a interacts with the endoplasmic reticulum (ER) membrane initiating invagination away from the cytoplasm. At this step, 1a will recruit ESCRT proteins (B) ESCRT proteins will form concentric spirals driving the closure of the wide membrane rim induced by 1a leading to the formation of the narrow neck characteristic of spherules. (C) 1a might then recruit the reticulon proteins which can disassemble the ESCRT machinery inhibiting pinching off of the spherules and (D) stabilizing the BMV spherule structures (adapted from Diaz et al. 2015).

All this evidence points towards BMV induced spherules being a two-step process. The first step, that occurs without the need for host-cofactors, but through molecularly encoded determinants in the 1a protein (α -helix). The second step implicates proper replication complex assembly in these spherules which requires ESCRT proteins for establishing membrane curvature.

Comparisons with *Alphavirus* proteins have shown that the BMV 1a protein is a distant homologue to nsP1 and nsP2 proteins (Fig. 19). As mentioned earlier, 1a is capable of

forming spherules without the need for the other replicase protein. This is in contrast to nsP1 which upon expression alone can deform the plasma membrane but does not form spherule structures (Hellström et al., 2017; Kallio et al., 2013; Noueir and Ahlquist, 2003). This implies that the evolution of spherule formation could have started from being performed by one protein into a complex system where several viral proteins are involved. This does not exclude the hypothesis that nsP1 since it is capable of deforming membranes does participate in inducing the membrane curvature required for spherule formation, but that, in contrast to BMV, includes other nsPs for the regulation of this process. Another scenario would be that spherule formation has evolved two distinct times in RNA virus history. However, recent sequence analysis has revealed conserved secondary structures between these two genera indicating a possible common origin (Ahola, 2019; Ahola and Karlin, 2015).

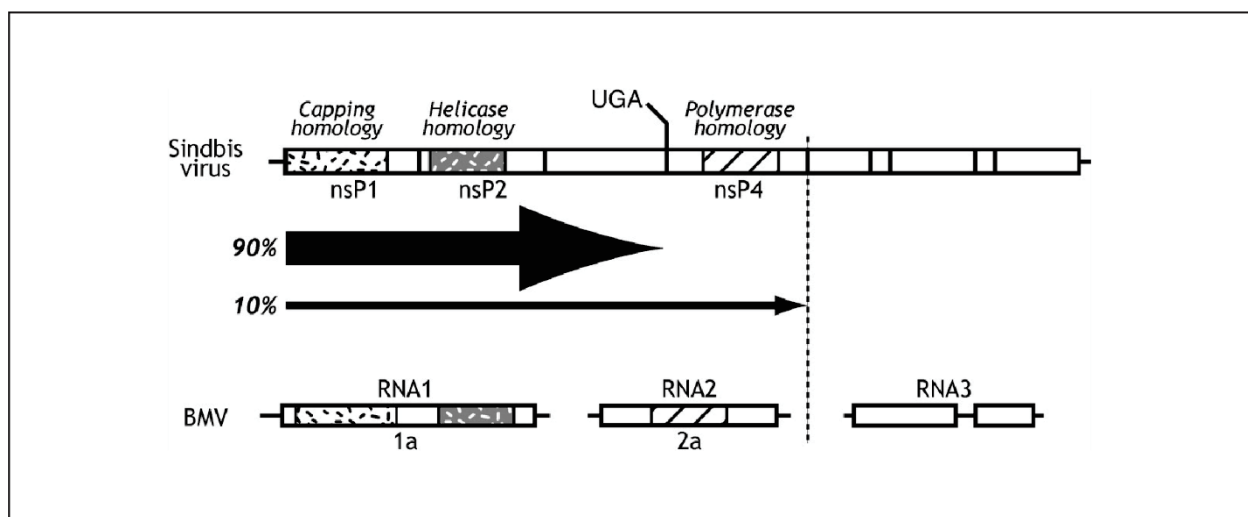


Fig. 19 Conservation regions between SINV and BMV genome

The viral genomes of Sindbis virus (SINV) and Brome Mosaic virus (BMV) are presented with similar fill patterns indicating conserved regions between the two genomes. The dashed line represents delimits the SINV and BMV genomes for the proteins coding for the replication proteins. The thick and thin arrows represent the normal and leaky opal reading frame respectively (adapted from Noueir and Ahlquist 2003).

Flock house virus spherules

FHV is an insect virus which belongs to the family *Nodaviridae*. FHV has a bipartite genome that will code for protein A and the capsid protein. The protein A is the sole

replicase protein and contains an N-terminal mitochondrial membrane association domain, RdRp domain and an RNA capping domain (Kopek et al., 2010).

Parallels between *Nodaviridae* and *Alphaviruses* have been drawn due to comparisons between their RNA capping proteins which have been aligned, and shown to contain conserved residues. This is was performed due to the limited information existing on the RNA capping activity residues of FHV protein A, and the similarity in the structures produced upon viral replication. Therefore, an interesting study using alignments between FHV and SFV RNA capping domains found that three residues (H93, R100 and D141) (Fig. 20) are highly conserved between *Alphaviruses* and *Nodaviridae*. Mutations in these residues led to the complete abolishment of capping activity of the protein A demonstrating a conservation for the importance of these residues between *Nodaviridae* and *Alphaviruses*. This suggested that these two replication proteins share similar mechanisms (Quirin et al., 2018).

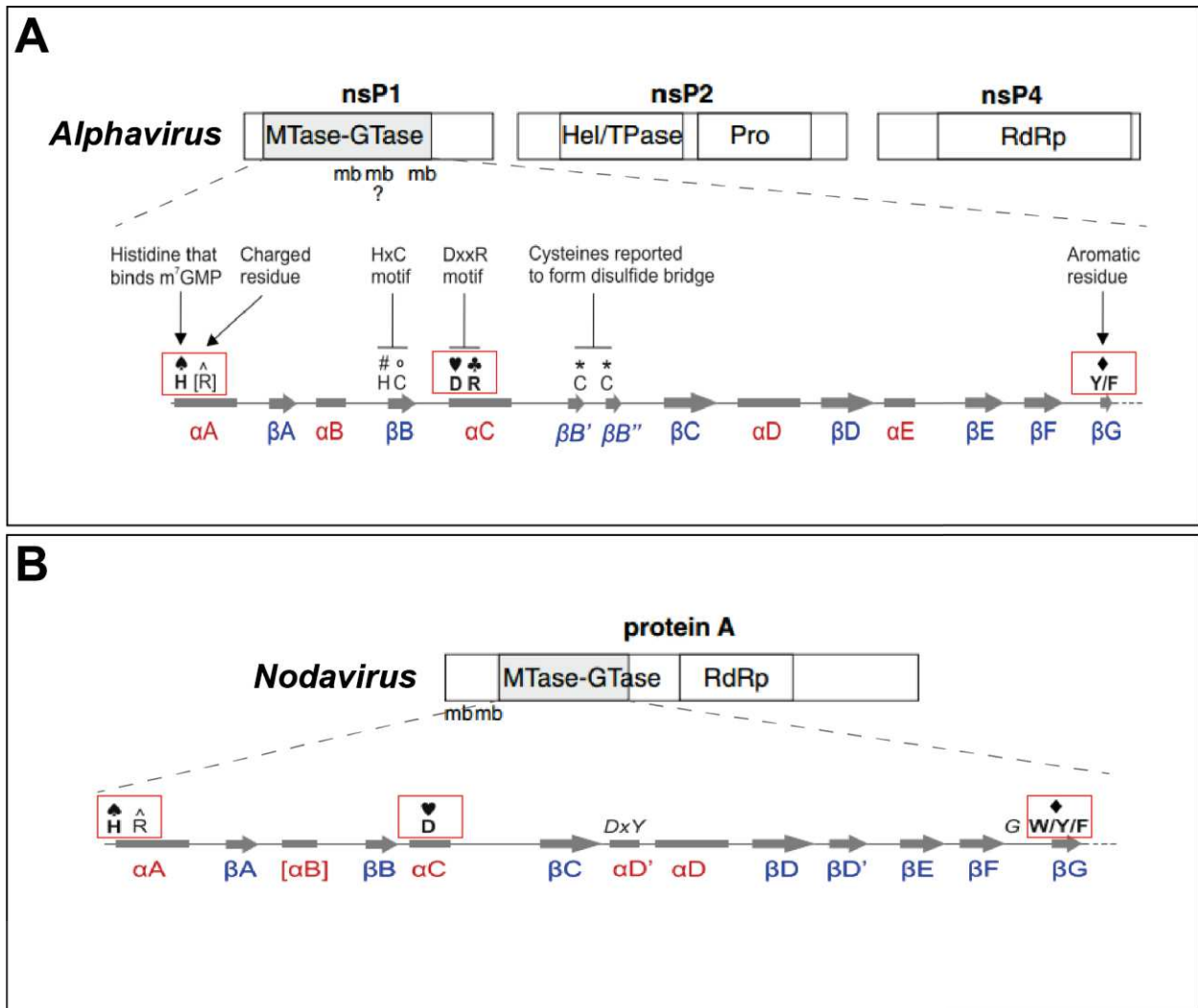


Fig. 20 Organization of the replicase domains and core region of the capping domain

Organization of *Alphavirus* (A) or *Nodavirus* (B) replicase domains with a zoom-in on the conserved residues in the core region of the capping domain. The red box represents conserved residues shown to be important for capping activity of both Alpha- and Noda-viruses. Mtase-Gtase: Methyl-transferase-Gaunyltransferase domain mb: membrane-associated region; Hel/TPase: Helicase/Triphosphatase; Pro: Protease; RdRp: RNA-dependent RNA polymerase domain. α -helices and β -strands are indicated by rectangles and arrows respectively (adapted from Tero Ahola and Karlin 2015).

Going back to mechanisms of spherule formation, upon infection, FHV protein A through mitochondrial membrane interaction domains will assemble the replication complex for *de-novo* RNA synthesis. This process will generate spherules between the outer and inner mitochondrial membranes of an infected cell. Interestingly, contrary to BMV and similarly to *Alphaviruses*, protein A requires a functional RNA template for

the induction of spherule formation. However, in contrast to *Alphaviruses*, the length of the template does not affect spherule size (Kallio et al., 2013; Kopek et al., 2010).

Extensive studies do not exist on the mechanisms of FHV induced membrane curvature for spherule formation. However, FHV has probably the most resolved spherule structure for a (+)RNA virus to date. The high-resolution structure of these FHV replication compartments was resolved by cryo-electron tomography studies.

Cryo-electron tomography combines multiple electron microscopy techniques to generate three-dimensional structures of the object of interest. Advantages include cryo-freezing which will allow conservation of structures in their native conditions that otherwise might be disturbed by chemical fixation. By this technique, electron-dense structures were localized directly above the necks of the FHV spherules. This revealed a novel feature present at the neck of spherules which consisted of cupped ring structures of twelve-fold symmetry with a central ~19 nm diameter ‘turret’ projection and was referred to as a “crown” structure (Ertel et al., 2017). In search of the viral factors that can contribute to this feature, protein A was a likely candidate since it is capable of multimerizing mainly through sequences present in its N-terminal region (Dye et al., 2005). Indeed, this crown structure was shown to consist of protein A multimers (Fig. 21).

The crown structure is thought to play a role in spherule formation by promoting injection of newly synthesized (-)RNA causing mitochondrial membrane deformation and hence spherule formation. The crown structure is also hypothesized to be the active site of FHV RNA capping. As mentioned earlier, *Alphavirus* nsP1 and protein A are potentially evolutionarily linked (Ahola and Karlin, 2015). Therefore, it is possible that a similar structure containing nsP1 multimers exists at the neck of the *Alphavirus* spherules. However, elaborate cryo-electron microscopy studies on *Alphavirus* spherules are still lacking. Proofing the existence of such structures for *Alphaviruses* may reflect common ancestry if an evolutionary pathway connecting the different structures can be reconstructed.

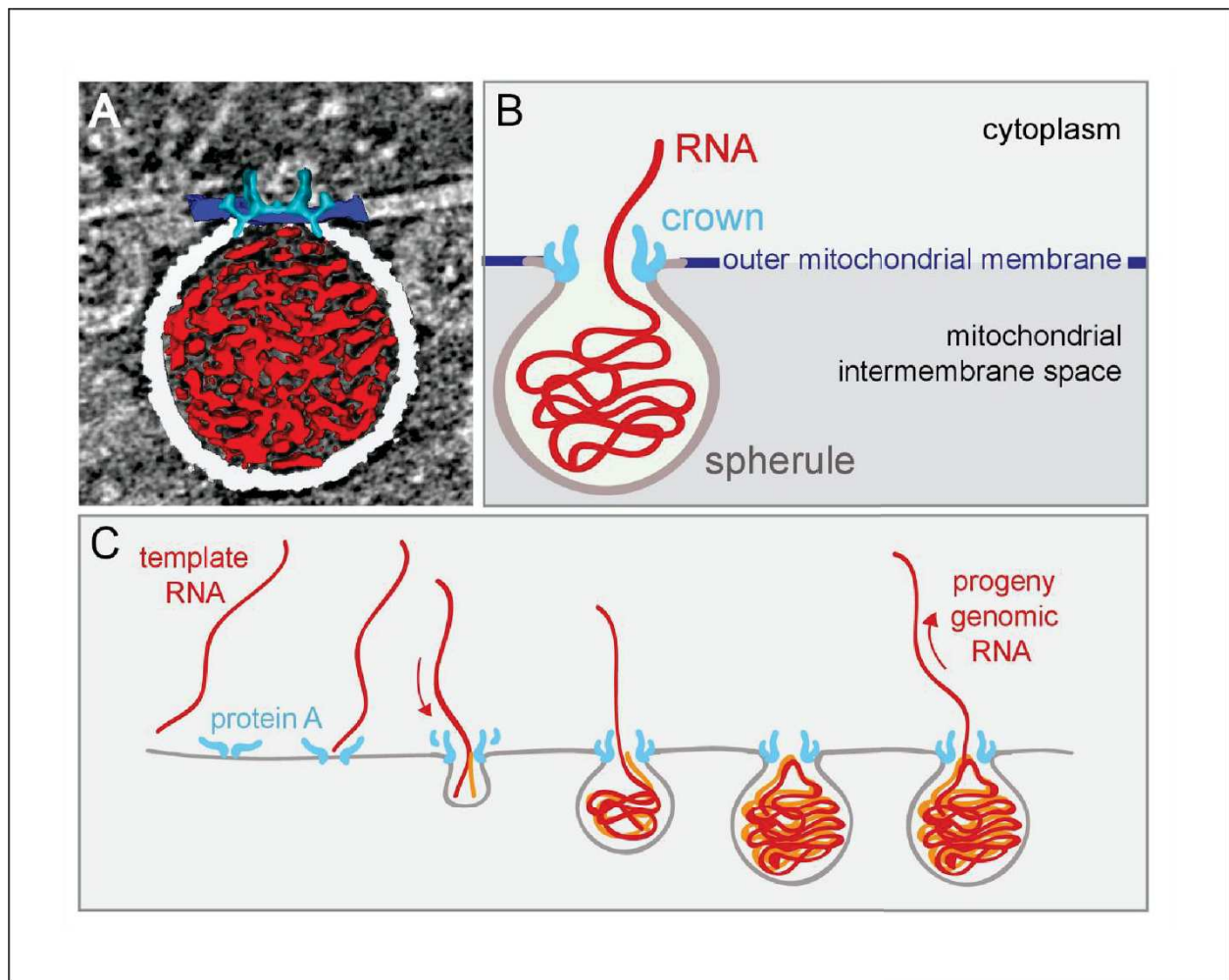


Fig. 21 Model of FHV replication complex structure and function

(A) Cryo-EM image 3D segmented of a single flock house virus (FHV) spherule formed at the mitochondrial membranes (dark blue). The spherule (white) membrane encloses viral RNA (red). The crown structure (light blue) composed of FHV protein A multimers is present at the neck of spherules and anchors the complex at the mitochondrial membrane. (B) Schematic representation of the FHV spherule presented in (A). (C) Model of FHV RNA complex and RNA synthesis. Positive stranded RNA (red) associates with protein A at mitochondrial membranes launching negative strand RNA synthesis (orange). Progeny positive-strand RNA are accommodated by increasing the volume of the spherule as if blowing up a balloon (adapted from Ertel et al. 2017).

4 Cellular membranes as a playground for *Alphavirus* replication complex

In the previous section, the *Alphavirus* life cycle was discussed with special attention to the process of replication. In that part, it was clear that the formation of replication organelles occurred in close association with membranes and required induction of membrane curvature. Additionally, it was mentioned that the MTase/GTase activity of nsP1 inside the replication complex was regulated by the presence of specific lipids. Therefore, in this subpart, the lipid composition of cellular membranes and their

organization, with special attention to microdomains, and the cellular machineries involved will be discussed.

A- Cellular membranes and their lipid composition

Cellular membranes are primary constituents of a cell. Not only do they delimit the cell defining its boundary and concentrating its constituents, but they also encircle intracellular organelles leading to segregation of chemical processes in defined areas inside the cell. Cellular membranes also function as transporters of signaling molecules through the release of extracellular vesicles such as exosomes. Finally, membranes are also central for signaling networks and transmission of extra-cellular cues. Conformingly, a significant portion of cellular proteins coded by the human genome (one third of the human genome) spend their life-time in contact with membranes.

At the most basic view, membranes are composed of two phospholipid layers opposing each other. This lipid bilayer is constituted of polar lipids having different affinity to aqueous solutions giving it an amphipathic nature. The hydrophobic “water-fearing” part is composed of the fatty acid tails directed towards the interior, and the hydrophilic “water-loving” part is composed of the phosphate head and is directed towards the intra and extra cellular aqueous medium. Minimizing the hydrophobic surface in contact with water to a low-energy conformation leads to the spontaneous assembly of this lipid bilayer (Simons and Sampaio, 2011).

Membranes are composed of a pool of different lipids. Recent advances in lipidomics, the large-scale study of cellular lipids in biological systems, has elucidated the existence of thousands of lipids in eukaryotic cells. These studies also demonstrated a correlation between membrane architecture complexity and lipid diversity. This is exemplified by the comparisons between prokaryotic and eukaryotic cells, the latter having membrane-bound organelles. Even if a subsequent portion of these lipids remain uninvestigated with respect to their role in cellular membranes, there exists significant knowledge on the basic lipid composition of membranes. As mentioned above, membranes are composed due to the self-assembly of polar lipids. One of the most abundant membrane polar lipids are the glycerophospholipids. Glycerophospholipids are composed of a diacylglycerol (DAG) where the glycerol head group can be further modified by addition of an amino alcohol group such as choline, serine or ethanolamine. Furthermore, fatty acyl chains can be of varying length and

have different degrees of unsaturation. All of these combinations will result in a diversity of glycerophospholipids which give different conformations to the membranes. The most abundant glycerophospholipids in cellular membranes include phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), PtdSer, phosphatidylinositol (PtdIns) and phosphatidic acid (PA) where PtdCho is responsible for more than 50% of the phospholipids in eukaryotic membranes. Another important polar lipids are sphingolipids which are composed of a sphingosine backbone that can be modified by the addition of different groups yielding a diversity of different lipids such as sphingomyelin, cerebroside and ganglioside. These lipids not only play an important role as a structural component of membranes, but can function as signaling molecules in response to external signals. Membranes also contain sterols where cholesterol constitutes the main sterol in mammalian cells. This lipid has a unique structure composed of four linked hydrocarbon rings forming a bulky steroid structure. A hydrocarbon tail is linked to the steroid at one end, and a hydroxyl group is attached at the other end.

An important thing to note is that lipid composition of membranes is different depending on the nature of organelles. Even, the lipid composition between the inner leaflet and outer membrane leaflet within the same organelle can vary substantially. In some cases, this has a functional cellular role. PtdSer, for example, is mainly localized in the inner leaflet of the plasma membrane. However, during a form of programmed cell death, apoptosis, this lipid is exposed through the action flippase proteins to the extra-cellular surface functioning as an “eat me” signal for phagocytes. Going back to the lipid composition of organelles, the plasma membrane is abundant in sterols and sphingolipids which are packed at a higher density than glycerophospholipids. This gives the plasma membrane a certain rigidity and stability. As seen in Fig. 22, the plasma membrane has the highest cholesterol to phospholipid ratio (1.0). On the other hand, other organelle membranes such as the ER and mitochondrial membranes, are relatively poor in this particular lipid (van Meer et al., 2008).

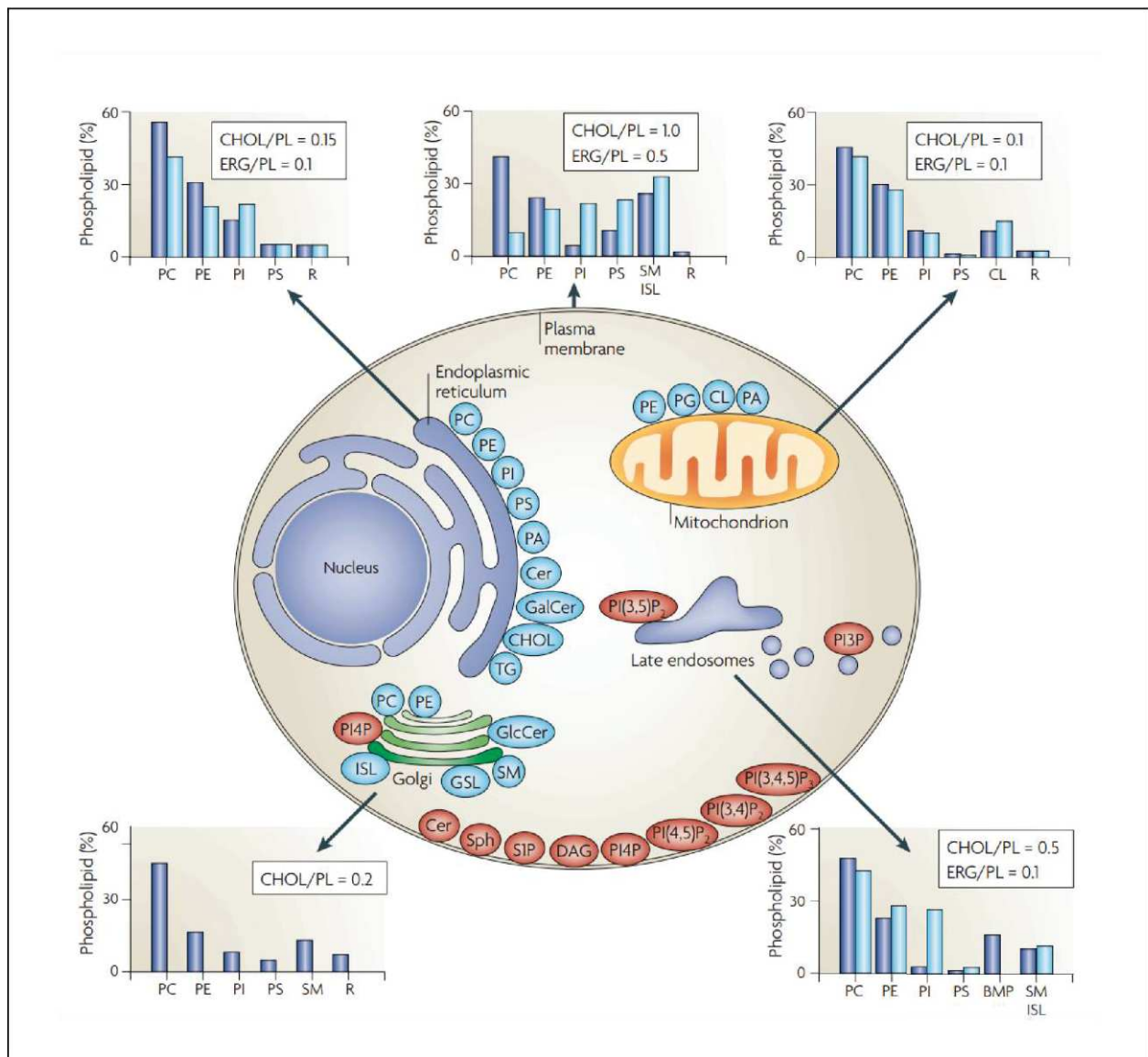


Fig. 22 Distribution of lipids in different membrane organelles

Lipid composition is expressed as a percentage of the total phospholipid (PL) content and is represented for yeast in light blue and mammals in blue. This is similarly represented for sterol content with cholesterol (CHOL) for mammals and ergosterol (ERG) for yeast. Inside the cell, the site of synthesis of the major phospholipids is represented by the blue circles and other lipids involved in signaling pathways are represented by the red circles. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI phosphatidylinositol; PS: phosphatidylserine; PA: phosphatidic acid; Cer: ceramide; GalCer: Galactosylceramide; SM: sphingomyelin; TG: triacylglycerol; GSLs: glycosphingolipids; ISL: yeast inositol sphingolipid; DAG: diacylglycerol; CL: cardiolipin; PI4P: phosphatidylinositol-4-phosphate; PI(3,5)P2: phosphatidylinositol-(3,5)-bisphosphate; PI(4,5)P2: phosphatidylinositol-(4,5)-bisphosphate; PI(3,4,5)P3: phosphatidylinositol-(3,4,5)-trisphosphate; BMP: bis(monoacylglycerol)phosphate; S1P: sphingosine-1-phosphate; R: remaining lipids (adapted from van Meer et al., 2008).

In this context, it has to be mentioned that a significant portion of a membrane's biomass is also composed of proteins which bind through hydrophobic domains or post-translation modifications present in these proteins.

B- Regulatory role of lipid composition on membrane organization and physical properties

The difference in membrane composition delimiting different organelles have important implication on the organization and physical properties of membranes. In biological membranes, two type of phases can be described: liquid disordered (Ld) and liquid-ordered (Lo) phases. Lo phases are described as highly organized thick layers with low membrane fluidity. On the other hand, Ld phases are more disordered allowing more place for diffusion and therefore an increase in fluidity. In this discussion, the lipid composition of membranes will play an important regulatory role. Saturated fatty acids (SFAs) allow tight packing of lipids increasing the thickness and rigidity of membranes. Conversely, unsaturated fatty acids (UFAs) will contain kinks allowing for a “looser” spacing of membrane lipids and increasing membrane fluidity. The fatty acid tail length also contributes to fluidity where the shorter the fatty acyl tail, the more fluid a membrane is. Cholesterol is also an essential player in this process where to it contributes to an increase in membrane order by intercalating between fatty acids (Fig. 23) (Sáenz et al., 2012).

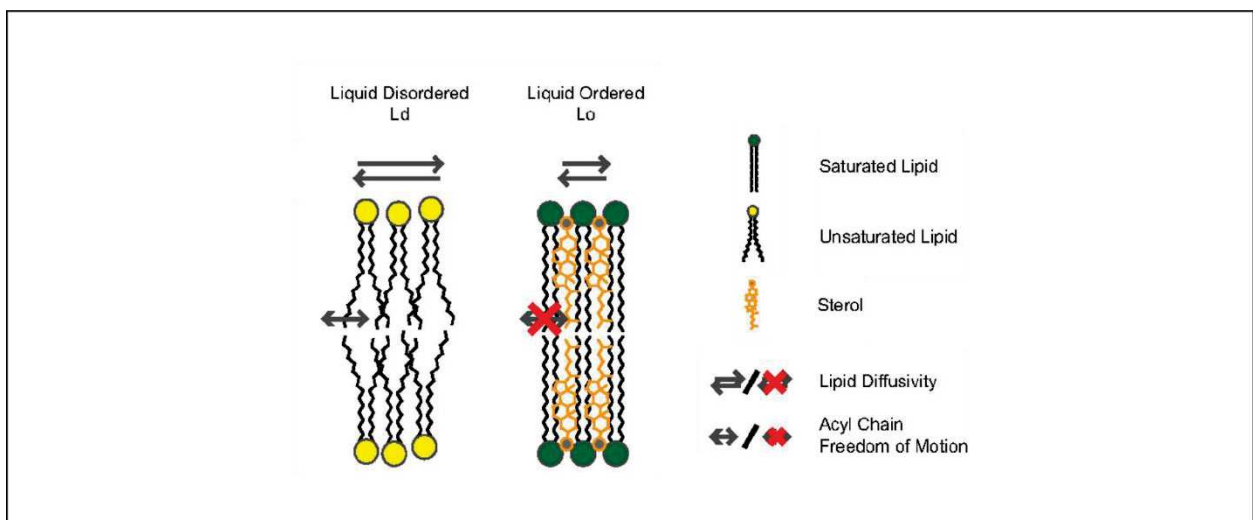


Fig. 23 Lipid bilayer phase behavior and membrane lipid composition

Membranes depending on their lipid composition can have different phase behavior. Liquid disordered phases (Ld) are rich in unsaturated lipids allowing high lipid diffusivity and acyl chain freedom of motion. While, liquid ordered phases (Lo) have low lipid diffusivity and acyl chain freedom of motion due to the presence of tightly packed saturated fatty acids intercalated with cholesterol (adapted from Sáenz et al. 2012).

C- Lipid metabolism

Most lipids are synthesized in the ER. The starting point for *de-novo* production of lipids is the glucose derived acetyl-CoA molecule produced from the Krebs cycle. The acetyl-CoA molecule can then be used for the production of cholesterol or fatty acids (Fig. 24).

i. Fatty acid synthesis and unsaturated fatty acids

Fatty acids are basic building blocks for the majority of cellular lipids and are therefore a source of components necessary for increased membrane production. The core reaction of fatty acid synthesis is catalyzed by the fatty acid synthase (FASN) that condensates one molecule of acetyl-CoA with seven molecules of malonyl CoA to generate the 16-carbon SFA palmitate. Starting from free fatty acids, stearoyl-CoA desaturase (SCD1) also known as Δ -9-desaturase, catalyzes the biosynthesis of monounsaturated fatty acids (MUFAs) used as precursors for the synthesis of various lipids including phospholipids, triglycerides and cholesteryl esters. Elongation and further desaturation through the mammalian fatty acid elongase (ELOVL) and fatty acid desaturase (FDS) enzymes will yield a repertoire of fatty acids with different saturation levels known as polyunsaturated fatty acids (PUFAs). PUFAs are building blocks for the production of eicosanoids and sphingolipids. With respect to palmitate, this fatty acid can be added to proteins leading the post-translational modification named palmitoylation. This modification is important in targeting of proteins to membranes (Baenke et al., 2013).

ii. Cholesterol synthesis

Cholesterol *de novo* synthesis occurs in the ER with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase being the rate-limiting enzyme. Starting from an HMG-CoA molecule, this enzyme produces mevalonate which then through a series of reactions will finally lead to the production of cholesterol. Cholesterol can also be obtained through receptor-mediated intake from the extracellular medium in the form of low-density lipoproteins (LDLs). In this scenario, LDLs are delivered to and hydrolyzed in late endosomes/lysosomes (LE/Ls) where free cholesterol is released. Then, cholesterol requires proper intracellular transport to exit the LE/Ls and reach its final destination mainly at the plasma membrane, in the Golgi apparatus and the ER. This is ensured by Niemann-Pick C 1 and 2 (NPC1 and NPC2) proteins localized at the

limiting membrane of lysosomes and in the lysosomal lumen respectively (Baenke et al., 2013).

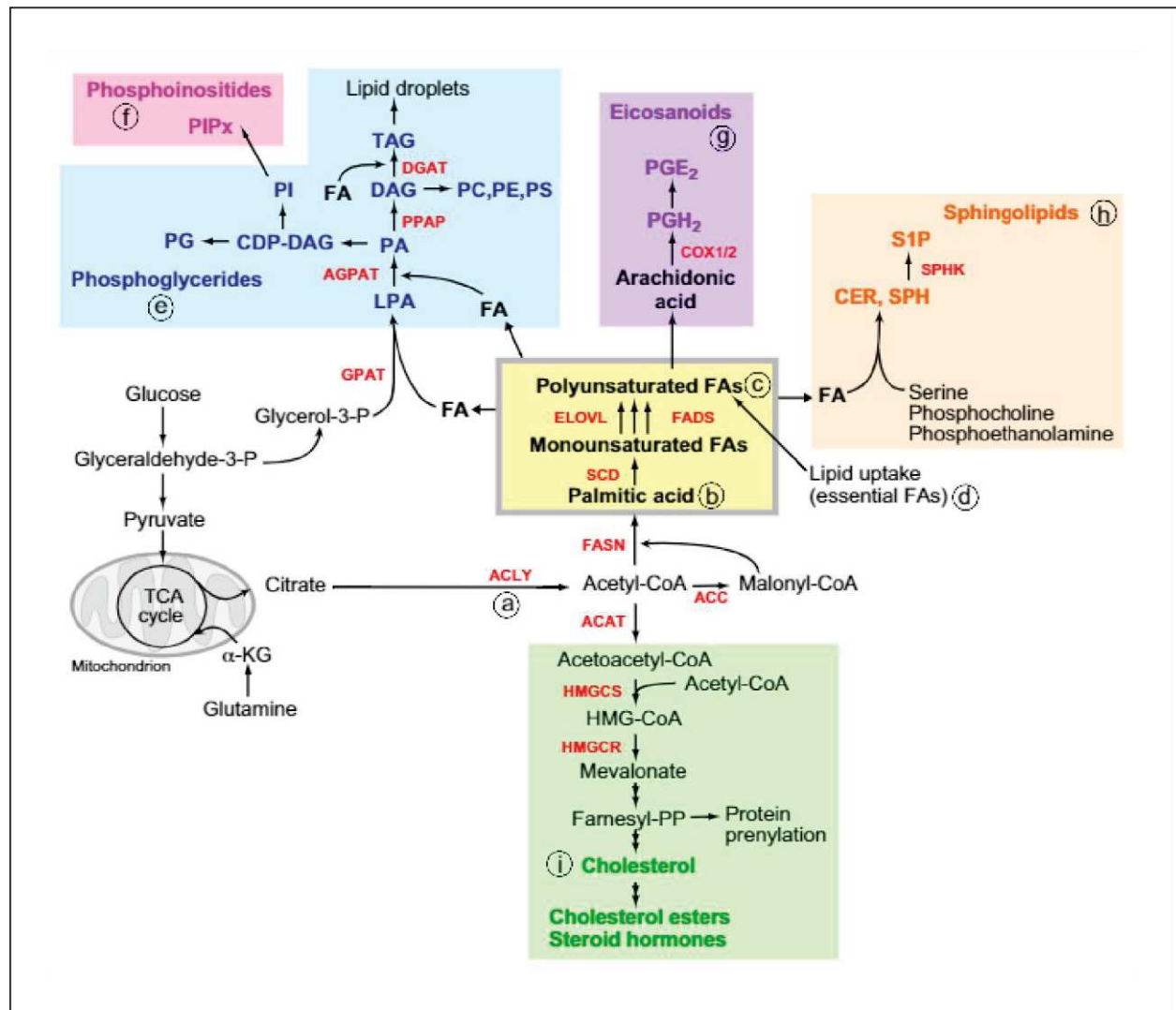


Fig. 24 Lipid biosynthesis pathway

Schematic of the pathways involved in the synthesis of fatty acids (FAs), cholesterol, phosphoglycerides, eicosanoids and sphingolipids. The enzymes involved in these pathways are indicated in red. (a) Glucose-derived citrate is converted to acetyl-CoA by ACLY. (b) For production of FAs, the acetyl-CoA is converted into malonyl-CoA. The repeated condensation of these molecules by FASN will generate palmitic acid. Then, the SCD enzyme can generate a double in the $\Delta 9$ position of the acyl chain that will lead to the production of mono-unsaturated FAs. (c) Further elongation and desaturation will produce a plethora of FAs with different saturation levels. (d) Essential FAs cannot be synthesized and are obtained by dietary sources. (e,f) Combination of FAs with glycerol-3-phosphate (glycerol-3-P) will generate (e) phosphoglycerides and (f) phosphoinositides. (g) Eicosanoids are produced by from arachidonic acid, a polyunsaturated FA. (h) Sphingolipids contain acyl chains and polar head groups deriving from serine, phosphocholine or phosphoethanolamine (i) Conversion of acetyl-CoA to acetoacetyl-CoA will initiate cholesterol synthesis. The addition of another acyl group by HMGCS will produces 3-methylglutaryl-3-hydroxy-CoA that is converted to mevalonate by HMGCR. Several reactions later will lead to the synthesis of cholesterol which also forms the structural backbone for steroid hormone biosynthesis. Enzyme abbreviations: ACAT: acetyl-CoA acetyltransferase; ACC: acetyl-CoA carboxylase; ACLY: ATP

citrate lyase; AGPAT: 1-acylglycerol-3-phosphate Oacyltransferase; PTGS: prostaglandin-endoperoxide synthase ; DGAT: diacylglycerol O-acyltransferase; ELOVL: fatty acid elongase; FADS: fatty acid desaturase; FASN: fatty acid synthase; GPAT: glycerol-3-phosphate acyltransferase; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase; PPAP: phosphatidic acid phosphatase; SCD: stearoyl-CoA desaturase; SPHK: sphingosine-1-kinase. Metabolite abbreviations: α -KG: α -ketoglutarate; CDP-DAG: cytidine diphosphate-diacylglycerol; CER: ceramide; DAG: diacylglycerol; FA: fatty acid; LPA: lysophosphatidic acid; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PGE2: prostaglandin E2; PGH2: prostaglandin H2 PI: phosphatidylinositol; PIPx: phosphatidylinositol phosphate; PS: phosphatidylserine; S1P: sphingosine-1-phosphate; SPH: sphingosine; TAG: triacylglyceride (adapted from Baenke et al. 2013).

D- Lipid rafts

The discovery that certain lipids could be sorted through the Golgi network to be transferred to different regions of the cell raised questions about the capacity of cells to create domains with certain lipid composition. One of the first evidence for this lipid sorting was clear in epithelial cells. These cells are polarized having an apical and basolateral domain where the former domain is enriched in sphingolipids and cholesterol. This implied that in membranes, domains could be found with specific lipid composition and membrane organization. These domains have been termed lipid rafts (Simons and Ikonen, 1997). Different type of rafts exists, but these domains are generally defined by having a highly ordered membrane organization or an L_0 lipid phase as defined earlier. The highly ordered membrane organization of rafts can be explained when looking at the lipid composition of these domains. They have been shown to be highly enriched with cholesterol and sphingolipids. Lipid rafts are purified by to their property of resistance to certain non-ionic detergents such as Triton X-100 at cold temperatures. These methods have been also combined with the use of molecules such as methyl-beta-cyclodextrin that can selectively deplete cholesterol from membranes. The use of such molecules permitted to check whether raft association and localization were dependent on cholesterol, a main component of lipid rafts. Additionally, if such molecules perturbed the physiological or viral machineries studied, then it was presumed that raft formation is associated with the mechanism studied (Simons and Sampaio, 2011). Recent intensive proteomics studies on purified lipid rafts have revealed these microdomains as centers for signaling networks such as immune signaling (Foster et al., 2003). So, how are proteins implicated in these signaling pathways addressed to rafts? One of the most well described targeting signals to lipid rafts is palmitoylation, a post-translation modification that will add palmitate to cysteines (Fig. 25). It was also seen that in palmitoylation dependent raft

affinity was further enhanced protein oligomerization. Some examples of proteins which are addressed to rafts through palmitoylation include the Rho GTPase Rac1 (Navarro-Lérida et al., 2012) and the Influenza hemagglutinin protein (Takeda et al., 2003). In this discussion, it is interesting to address the studies performed on Rac1. The wild type form of this Rho GTPase protein was shown to be addressed to lipid rafts through palmitoylation. This addressing was also essential for the activation of this Rho GTPase. Interestingly, oligomerization also participated in this process. This targeting of Rac1 to lipid rafts has important implications on actin cytoskeleton remodeling regulating membrane organization. Going back to target signals for lipid rafts, for transmembrane proteins, a critical modifier for lipid raft association is the length of the transmembrane domain (TMD) where shorter TMD was correlated with a lower raft affinity.

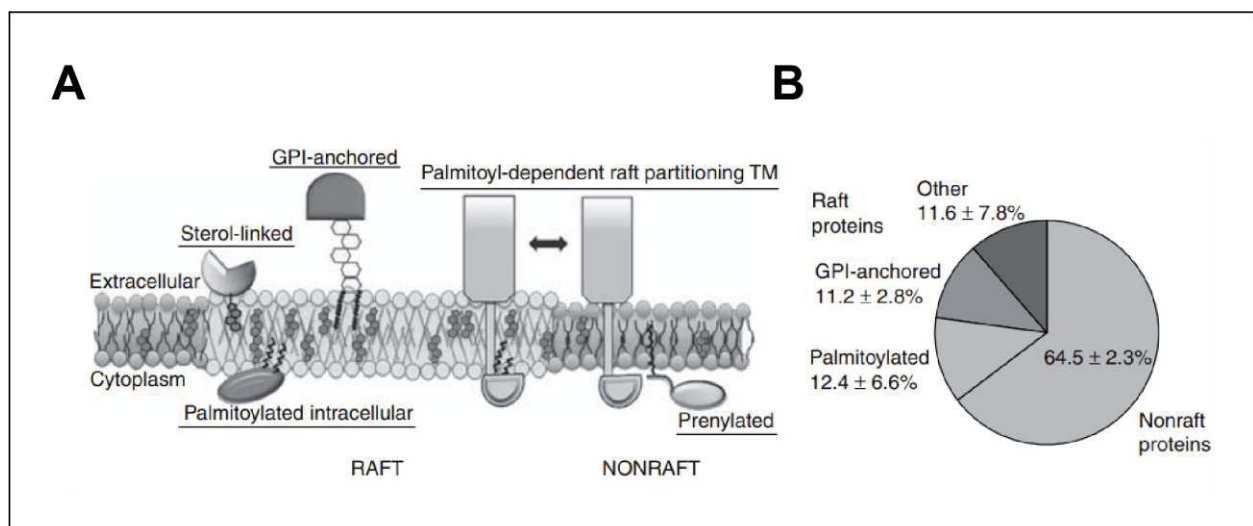


Fig. 25 Lipid rafts and associated proteins (adapted from Simons and Sampaio 2011).

E- Lipid requirements for (+)RNA virus replication

As (+)RNA viruses form their replication complexes in association with membranes, the implication of membrane lipids in this process has been a wide area of investigation. This has revealed remarkable mechanisms by which these viruses can usurp lipid metabolism to attain the correct environment required for the creation of replication organelles. In this part, the requirement of membrane lipids for (+)RNA virus replication is illustrated by focusing on the knowledge accumulated on the Tombusviridae and

Flaviviridae family of viruses. The role of membrane lipids for *Alphavirus* replication represents one of the main objectives of this thesis, and will therefore be detailed later in the objectives and results part.

i. *Tombusviridae* replication and membrane lipids

Tombusviridae is a family of plant viruses with Tomato bushy stunt virus (TBSV) being the most studied. TBSV has been used as a model for investigations on virus-host interactions and recombination in yeast models. This virus has a small (+)RNA genome (4.8 kb) which codes for its viral proteins. In this context, the viral encoded integral membrane proteins p33 and p92^{pol} with the viral RNA and host-cofactors will form the viral replication complex in association with peroxisomal membranes, and occasionally with ER membranes. At these sites, TBSV will form spherule structures similar to *Alphaviruses*. Therefore, it is interesting to discuss the membrane lipid requirements for replication complex formation of this virus.

In this bibliography, sterols have been shown to be essential for replication of TBSV. Since peroxisomes and ER membranes are poor in sterol content, TBSV has evolved mechanisms to enrich sterols at replication site assembly. This is ensured by the direct binding of p33 to the oxysterol-binding machinery, a non-vesicular pathway for trafficking of sterols. Furthermore, viral replication sites have been shown to be present in detergent-resistant membrane microdomains rich in sterols. It should be mentioned that sterol binding sites have been identified in p33 and p92^{pol} establishing further evidence for the implication of sterols in TBSV replication complex formation (Barajas et al., 2014; Xu and Nagy, 2017). So, why are sterols required for TBSV replication? The most direct explanation is that this sterol could ensure the correct oligomerization of replicase proteins. This has been supported by evidence that sterol play a role in the stability of p33 (Barajas et al., 2014). Additionally, sterols allow tighter packing of phospholipids which could be important for stabilizing spherule structures.

Phospholipids are another membrane lipid component show to be important for TBSV replication. This has been demonstrated through knockdown of genes (*INO2/INO4*) that regulate the synthesis of phospholipids. In this scenario, phospholipids are not re-directed to viral replication sites, but are upregulated by the interaction of p33 with repressors of phospholipid biosynthesis genes. This requirement for phospholipids is important for the attachment of viral replicase proteins to membrane replication sites.

Recently, the phospholipid, PtdEtn, was described to play a particular role in TBSV replication. In this study, artificially-produced vesicles containing PtdEtn were sufficient to harbor TBSV replication. Interestingly, other phospholipids did not exert this property demonstrating the specificity for the importance of PtdEtn for TBSV replication. The conical structure imposed by this phospholipid has been proposed to contribute to the negative membrane curvature present at the constricted neck of spherules (Sharma et al., 2011; Xu and Nagy, 2015).

ii. *Flaviviridae* replication and membrane lipids

Membrane lipids have also been investigated for their implication in the replication step of various other (+)RNA viruses. In this scenario, the most well studied model remains the *Flaviviridae* family of viruses. Even if the replication compartments formed by *Flaviviridae* can be different, the knowledge acquired on this model can inspire future studies on *Alphaviruses*.

The *Flaviviridae* family constitutes a main branch of (+)RNA viruses. This family is subdivided to different genera with the *Flavi-* and *Hepacivirus* containing critically important human pathogens such as DENV, ZIKV, West Nile virus (WNV) and Hepatitis C virus (HCV). The viral genome of *Flaviviridae* encodes seven nsPs (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) which are responsible for the formation of the *Flaviviridae* replication complex. This complex in association with the ER membranes will form either single membrane or double membrane vesicles (SMVs or DMVs) where active genome replication will take place. In this context, the NS2B, NS4A and NS4B proteins through their transmembrane domains anchor the other nsPs to the ER membrane (Yu et al., 2013).

Given that ER membranes have specific lipid composition, important efforts have been exerted on elucidating the necessity of specific lipids for *Flaviviridae* replication revealing impressive mechanisms employed by these viruses to usurp lipid metabolism machinery.

***Flaviviridae* replication and cholesterol**

One of the most studied lipids shown to be important for *Flaviviridae* replication is cholesterol. This sterol has been demonstrated by several groups to be vital for *Flaviviridae* replication through the use of a plethora of drugs that act on cholesterol

biosynthesis and transport such as U18666A, and lovastatin. siRNA mediated silencing of proteins involved in this pathway have further confirmed the requirement for proper cholesterol metabolism for *Flaviviridae* replication (Aizaki et al., 2004; Mackenzie et al., 2007; Stoeck et al., 2017).

However, the first real hint of cholesterol association at replication sites was the finding that the HCV replication complex associates with cholesterol-rich lipid raft membranes. (-)RNA and (+)RNA were also detected in these domains designating these sites of active replication (Aizaki et al., 2004). Accumulation of unesterified cholesterol at replication sites has been further characterized through electron microscopy studies on affinity-purified DMVs formed by HCV (Paul et al., 2013). So, how can these viruses accumulate cholesterol at their replication sites given that they form in association with ER membranes which have relatively poor sterol content. Impressively, these viruses have developed mechanisms to redistribute intracellular cholesterol to sites of viral replication. The up-regulation of the activity of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthesis pathway, has been proposed to participate in this accumulation. This upregulation occurs through reduction of this enzyme's phosphorylation levels through inhibition of AMPK activity (Soto-Acosta et al., 2017). Additionally, this enzyme has been observed to be re-localized to sites of viral replication contributing to a more restricted accumulation (Mackenzie et al., 2007). But, the massive accumulation of this lipid at ER membranes suggests that an active transport of cholesterol to viral replication complexes occurs. Recently, a study on the HCV has implicated the recruitment of the endosomal and lysosomal lipid transfer protein machinery at the ER membrane replication sites for the fulfillment of cholesterol recruitment. Knockdown of such proteins (STARD3 and NPC1) residing at late endosome and lysosome membranes and required for efficient HCV has demonstrated their necessity for cholesterol transport to viral replication sites. Surprisingly, this mechanism is not conserved for *Flaviviruses* suggesting that accumulation of cholesterol occurs through a different mechanism (Stoeck et al., 2017).

The requirement of *Flaviviridae* for cholesterol at viral replication is presumed to be important for the stability of the membrane invaginations of these replication complexes. Although, direct evidence for this association has not been established yet. Nonetheless, HCV replication complex/cholesterol association has been linked with resistance to RNase and protease digestion (Aizaki et al., 2004). Interestingly, the need

for cholesterol has been also linked to a perturbation of the cellular immune response. Indeed, the previously described redistribution of cholesterol to ER membranes disrupts cholesterol-rich microdomain formation at the plasma membrane impairing the ability of cells to respond to IFN-stimulated JAK-STAT antiviral signaling response (Mackenzie et al., 2007).

An important thing to note is that *Alphavirus* replication complexes form at the plasma membrane which are incidentally rich in cholesterol. This differentiates *Alphaviruses* from *Flaviviridae* which replicate in association to membranes with poor cholesterol content forcing them to invest in means to recuperate this sterol. This suggests that if *Alphavirus* replication requires cholesterol that it may occur in association with cholesterol-rich lipid raft microdomains.

De-novo fatty acid synthesis for creation of Flaviviridae replication organelles

Flaviviridae induction of extensive membrane curvature on ER membranes suggests the need for increased lipids at these sites for sufficient membrane surface area to harbor these invaginations. Therefore, the requirement of FASN, a key enzyme in the *de-novo* fatty acid synthesis pathway, for *Flavivirus* replication came as no surprise. This enzyme was shown to be recruited to viral replication sites where it had increased activity via an interaction with the N-terminal domain of the NS3 protein. Conformingly with the need of fatty acids for *Flavivirus replication*, ACACA, another enzyme involved the fatty acid biosynthetic pathway, has also been shown to play a role in this process. However, the exact molecular details of its mode of action remains to be determined. Increasing fatty acid synthesis at these sites and their incorporation to ER membranes allows significant membrane expansion to harbor *Flavivirus* replication organelles (Heaton et al., 2010).

Requirement of unsaturated fatty acids for *Flaviviridae* replication

Fatty acids can vary in length and saturation degree. This consequently can cause important effects on membrane fluidity. Specifically, the role of UFAs in the modulation of the physical properties of membranes is clearly established. In contrast with SFAs, UFAs have more distance between the tails and thus fewer intermolecular interactions and more membrane fluidity. The knockdown of the key de-saturating enzyme SCD1 has demonstrated the absolute requirement of UFAs for *Flavivirus* replication. Of high interest, inhibitors blocking SCD1 activity inhibited *Flavivirus* infection designating

these molecules of high interest for treatment strategies. No molecular details exist on the implication of SCD1 in *Flavivirus* replication. This enzyme is present at the ER, therefore, proof of re-localization to viral replication sites might prove challenging. However, the requirement of SCD1 for viral replication suggests that *Flaviviruses* require ER membranes rich in UFAs for the formation of replication organelles (Hishiki et al., 2019).

The membrane lipids implicated in the replication of the *Flaviviridae* replication complex formation are summarized in Fig. 26.

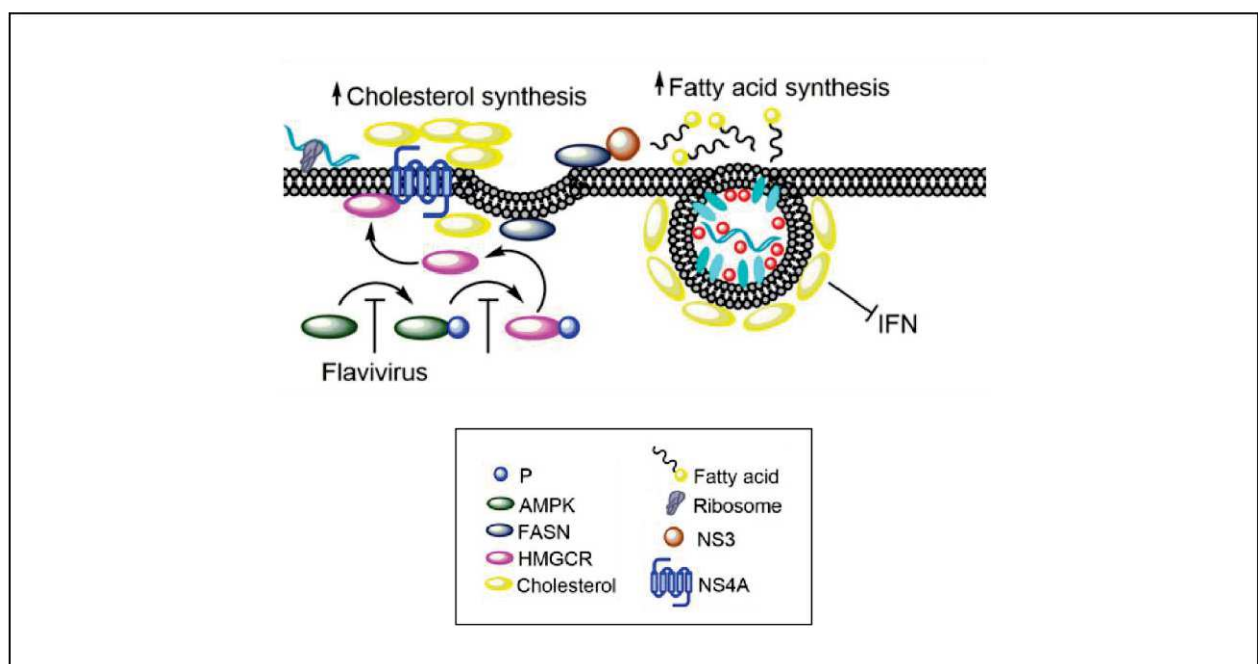


Fig. 26 Model for *Flaviviridae* replication complex formation and implication of membrane lipids

Cholesterol biosynthesis is locally increased at viral replication sites through the recruitment of the HMG-CoA reductase enzyme. Reduction of HMG-CoA reductase phosphorylation levels through inhibition of AMPK will lead to an increase in this enzyme's activity. Fatty acid synthesis is stimulated through NS3-mediated recruitment of FASN to the ER membrane. The increase in cholesterol and fatty acids will participate in the formation of membrane organelles conducive of viral replication (Osuna-Ramos et al., 2018).

In conclusion, it is clear that (+)RNA viruses manipulate the lipid metabolism in order to create a niche with specific lipid composition suitable for the establishment of replication organelles. Although the mechanisms for the recruitment of cholesterol and

fatty acids may vary among (+)RNA viruses, their requirement for optimal viral replication is conserved in this class of viruses.

Objectives of the thesis

Objectives of the thesis

In the section “Focus on the replication complex”, the mechanism behind *Alphavirus* replication was discussed. In that part, it was clear that *Alphavirus* replication complexes formed in association with the plasma membrane where the *Alphavirus* nsPs and their timely processing played an essential role in this process. In this context, nsP1 anchored the replication complex to the lipid bilayer, and interestingly could induce membrane curvature when expressed alone. Finally, nsP1 capping activity was shown to be modulated by the presence of specific lipids in-vitro.

A missing element to this discussion is the role of the lipid composition of the membrane platforms where *Alphavirus* replication complexes form. Additionally, even if certain host co-factors have been identified, the exact molecular mechanism for the establishment of membrane curvature for the creation of such structures remain largely uninvestigated.

For this reason and to address these gaps in *Alphavirus* literature, the objectives of this thesis are

- I. to define the membrane lipid requirements for efficient *Alphavirus* replication
- II. to elucidate the mechanism behind *Alphavirus*-induced membrane curvature

In all of these investigations, nsP1 will be at the centerpiece. This is because, as mentioned earlier, nsP1 anchors the replication complex to the membrane and is also capable of inducing membrane curvature alone. The *Alphavirus* model primarily used will be CHIKV due to its medicinal relevance as is clear in recent epidemics. However, comparisons with other *Alphaviruses* will be performed, when possible, in order to study if findings on CHIKV can be extended to other *Alphaviruses*.

Results

Chapter 2 Results

Part I *Alphavirus* lipid requirements

Project A The role of fatty acid synthase and stearoyl-CoA desaturase-1 in Old World *Alphavirus* genome replication

i. Article summary

As mentioned in the introduction, the lipid composition of membranes has important consequences on a membrane's organization and physical properties. In that discussion, it was clear that fatty acids especially UFAs contributed to membrane fluidity. Fatty acid synthesis is primarily carried out by the multi-enzyme protein FASN which catalyzes the conversion of acetyl-CoA to the fatty acid palmitate. In addition, fatty acids can be further modified by introducing double bonds into the acyl chain, a process which is mainly carried out by the activity of the desaturase enzyme SCD1.

Because *Alphavirus* replication complexes form in close association with membranes, studying the implication of lipid metabolism in viral replication is of high relevance. Here came the design of this study, which aimed specifically to investigate the role of the two membrane lipid components, fatty acids and UFAs in *Alphavirus* replication. The primary *Alphavirus* model used was CHIKV. Nevertheless, this research was also applied to MAYV, a poorly studied *Alphavirus* endemic in South America, in order to observe whether the necessity for these lipid components was retained in OW *Alphaviruses*.

The aggregation of these examinations demonstrated an absolute requirement for fatty acid and UFAs for proper CHIKV and MAYV replication. Furthermore, mechanistic data on the requirement of fatty acids for *Alphavirus* replication was provided. This identified FASN products as important for nsP1 membrane affinity possibly through participation in the nsP1 palmitoylation process. However, the role of UFAs in *Alphavirus* replication and nsP1 functions remains to be determined.

ii. Article 1 “Fatty acid synthase and stearyl-CoA desaturase-1 are conserved druggable cofactors of Old World Alphavirus genome replication”



Fatty acid synthase and stearoyl-CoA desaturase-1 are conserved druggable cofactors of Old World *Alphavirus* genome replication

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ABSTRACT

Chikungunya virus (CHIKV) is a rapidly emerging mosquito-borne RNA virus that causes epidemics of debilitating disease in tropical and sub-tropical regions with autochthonous transmission in regions with temperate climate. Currently, there is no licensed vaccine or specific antiviral drug available against CHIKV infection. In this study, we examine the role, in the CHIKV viral cycle, of fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD1), two key lipogenic enzymes required for fatty acid production and early desaturation. We show that both enzymes and their upstream regulator PI3K are required for optimal CHIKV infection. We demonstrate that pharmacologic manipulation of FASN or SCD1 enzymatic activity by non-toxic concentrations of cerulenin or CAY10566 decreases CHIKV genome replication. Interestingly, a similar inhibitory effect was also obtained with Orlistat, an FDA-approved anti-obesity drug that targets FASN activity. These drugs were also effective against Mayaro virus (MAYV), an under-studied arthritogenic Old world *Alphavirus* endemic in South American countries with potential risk of emergence, urbanization and dispersion to other regions. Altogether, our results identify FASN and SCD1 as conserved druggable cofactors of *Alphavirus* genome replication and support the broad-spectrum activity of drugs targeting the host fatty acids metabolism.

1. Introduction

Alphaviruses are widespread arthropod-borne viruses responsible for several medically important emerging diseases. Their impact on human health has been illustrated in the last decade by the rapid spread of the Chikungunya virus (CHIKV) that causes an acute transient febrile arthralgic illness, and may lead to chronic incapacitating articular disease (Burt et al., 2017). At the beginning of the 21st century, CHIKV re-emerged in East Africa and spread to countries neighboring the Indian Ocean before invading India, South-Eastern Asia, Caribbean Islands and South America, with autochthonous transmission episodes also reported in Europe (Pezzi et al., 2019). Over the last 15 years, CHIKV has caused at least 5 million autochthonous cases in > 40 countries. CHIKV belongs to the Semliki Forest virus sero-complex in Old World *Alphaviruses*. In this phylogenetic clade, CHIKV genome displays up to 40% nucleotide divergence with Mayaro virus (MAYV) (Lavergne et al., 2006). First isolated in 1954 in Trinidad and Tobago, MAYV generates a

self-limiting acute viral disease that may evolve towards chronic arthralgia very similar to CHIKV (Anderson and French, 1957; Causey and Maroja, 1957). While responsible for sporadic and small outbreaks in south and central American countries around the Amazon basin (Azevedo et al., 2009; Izurieta et al., 2011; LeDuc et al., 1981; Neumayr et al., 2012; Schmidt et al., 1959; Talarmin et al., 1998), the recent detection of MAYV in Brazil and Haiti raises growing concerns about its potential to spread to other regions similar to CHIKV in the past (Lednicky et al., 2016; Mackay and Arden, 2016). Currently, there is no vaccine against any of these *Alphaviruses* and no specific FDA-licensed drug for routine use is available. The limited efficacy of therapies tested so far for the management of CHIKV disease, including ribavirin (Ravichandran and Manian, 2008), the anti-malaria drug chloroquine (Roques et al., 2018) and corticosteroids (Javelle et al., 2015) points at the urgent need for basic antiviral research to identify the first therapeutic targets and molecules effective against *Alphaviruses*.

Cell membranes are critical for almost all steps of (+)RNA virus life

Abbreviations: CHIKV, Chikungunya virus; MAYV, Mayaro virus; FASN, fatty acid synthase; SCD1, stearoyl-CoA desaturase; PI3K, phosphatidylinositol 3-kinase; nsP3, non structural protein 3

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cycle. The exploitation of membranes for initial interactions of the virion with the host cell, penetration and fusion of the viral particle, or for assembly and budding of enveloped progeny virions has long been demonstrated (Altan-Bonnet, 2017). More recently, a key role has been assigned to cell membranes during the replication phase of (+)RNA viruses. This step generally occurs in host membrane-derived replication organelles whose creation and appropriate composition is pivotal for genome replication (Zhang et al., 2019). Remodeling the cell membranes to create autophagosomes, despite being reported as having either pro- or antiviral function according to the viral species, has also found critical for virus replication (Jackson, 2015). Being the major components of membranes, lipids therefore play active roles in the virus life cycle. As such, lipids and associated metabolism are attractive targets for the development of antiviral therapeutics.

Fatty acids are basic building blocks for the majority of cellular lipids and are therefore a source of components necessary for increased membrane production. The core reaction of fatty acid synthesis is catalyzed by the fatty acid synthase (FASN), a homodimeric enzyme that condensates one molecule of acetyl-CoA with seven molecules of malonyl-CoA to generate the 16-carbon saturated fatty acid palmitate. Starting from free fatty acids, stearyl-CoA desaturase (SCD1) also known as Δ -9-desaturase, catalyzes the biosynthesis of mono-unsaturated fatty acids (MUFAs) used as precursors for the synthesis of various lipids including phospholipids, triglycerides and cholesterol esters. Once incorporated in membranes, fatty acids, and its derivatives, depending on the length and saturation degree of their hydrocarbon tail, exert a structural effect on membrane fluidity, permeability, and flip-flop dynamics among others, and also alter the function of integral membrane proteins (Bond et al., 2016). Both FASN and SCD1 lipogenic enzymes are controlled by the sterol regulatory element-binding protein (SREBP) family members, the master transcriptional factors placed under control of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling axis (Furuta et al., 2008). Recently, *Alphavirus* were reported to manipulate the PI3K/AKT/mTOR signaling pathway through the presence of conserved sequences in virus-encoded nonstructural protein 3 (nsP3), with consequences on their host nucleotide, amino acid and lipid metabolisms (Mazzon et al., 2018). According to this information, this study was therefore conceived to explore the functional role of FASN and SCD1 during Old World *Alphavirus* infection and to investigate their relevance as therapeutic targets for antiviral purpose. Using RNA interference and pharmacological drugs, we demonstrate that FASN and SCD1 expression and enzymatic activity are required for optimal CHIKV genome replication. We demonstrate that their proviral function is conserved for MAYV, thereby reporting among the very first host co-factors for this poorly studied pathogen. Finally, we provide evidence that the US Food and Drug Administration (FDA)-approved anti-obesity drug Orlistat, an inhibitor of FASN activity is active against CHIKV and MAYV infection. Altogether, these data point at the intricate relationship existing between arthritogenic Old World *Alphaviruses* and fatty acid metabolism in their vertebrate host and highlight the therapeutic potential of targeting fatty acid biosynthesis and desaturation to limit the multiplication of a broad range of *Alphaviruses* in human cells.

2. Material and methods

2.1. Cells

HEK293T cells (ATCC number ACS-4500) used for infection experiments and Vero cells (ATCC number CCL-81) used for titration of the CHIKV and MAYV strains were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fischer Scientific) supplemented with penicillin and 10% fetal calf serum (FCS, Lonza) and grown at 37 °C in a 5% CO₂ atmosphere. The C6/36 mosquito cell line, derived from *A. albopictus*, was grown at 28 °C and 5% CO₂ in Minimal Essential Medium (MEM) (Sigma) complemented with 10% inactivated FCS and

1% antibiotics. Cell viability was measured using the Cell Titer 96 Aqueous one solution cell proliferation assay kit (Promega) according to the manufacturer's protocol.

2.2. Reagents and antibodies

Quercetin, cerulenin and Orlistat were purchased from Sigma Aldrich. CAY10566 was from Abcam. Drugs were diluted in DMSO. Appropriate concentration of DMSO was used in control experiments. Antibodies against FASN, SCD1 and GAPDH were purchased from Santa Cruz Biotechnologies, Inc. Anti-tubulin and anti-actin mAbs were from Sigma-Aldrich and Na⁺/K⁺ ATPase mAbs were purchased from Abcam. Rabbit polyclonal serum against nsP1 was a generous gift from Pr. Andres Merits (Tartu University, Estonia). Alexa-647-conjugated wheat germ hemagglutinin (WGA) and secondary antibodies were from Thermo Fisher Scientific.

2.3. Viruses

The CHIKV reporter virus was produced from the pICRES1 full-length CHIKV genomic clone (LR2006.OPY1 strain) containing the renilla luciferase marker inserted into the region encoding nsP3 (Pohjala et al., 2011). The CHIKV-mCherry-377 was previously described (Kummerer et al., 2012). CHIKV RNA was transcribed *in vitro* from the SP6 or T7 promoter using the mMACHINE kit (Ambion-Life Technologies). RNA (1 µg) was then transfected with lipofectamine 2000 (Thermo Fisher Scientific) into 10⁵ HEK293T cells. The MAYV reporter virus was obtained from an infectious clone derived from the complete sequence of the TRVL 4675 strain with a nano-luciferase coding sequence inserted in the nsP3 gene and cloned under the control of the CMV promoter (Chuang et al., 2019). The virus was produced by transfection of the infectious clone in Vero cells. Forty-8 h after transfection, the supernatants were collected, filtered through a 0.45 µm membrane, and titered using plaque assay as previously reported (Bernard et al., 2010).

2.4. Plasmids and transfection

The nsP1 sequence was amplified by PCR using pCHIKV-LR-5'GFP (LR-OPY strain) as a template and cloned into the pEGFP-C1 plasmid as previously reported (Matkovic et al., 2018). CHIKV reporter transreplication system (kindly provided by Andres Merits) was composed of the CMV-P1234 plasmid encoding a CMV-driven CHIKV nonstructural ORF and of the HSPoll-Fluc-Gluc pseudogenome template containing a Firefly luciferase and a Gaussia luciferase reporter genes under control of CHIKV genomic and subgenomic promoters respectively (Utt et al., 2019). Plasmids were transfected using JetPei reagent (Polyplus Transfection). Twenty-four hrs post-transfection, replication was monitored using the Dual-Glo luciferase assay kit (Promega) and a Spark luminometer (Tecan). Values were normalized according to protein content in the sample determined using the BCA Assay (Pierce).

2.5. RNA interference

siRNA targeting FASN or SCD1 mRNA were purchased from Integrated DNA technologies and Sigma-Aldrich, respectively. Non-targeting siRNA were from Dharmacon. Transfection was achieved using Interferin (Polyplus Transfection). At 48 h post-transfection, an aliquot of the cells was harvested to determine the silencing efficiency by Western blotting. The remaining cells were infected with CHIKV.

2.6. Cell fractionation and membrane flotation assays

Cells were incubated in hypotonic buffer (10 mM Tris/HCl [pH 7.4], 10 mM NaCl supplemented with protease inhibitors) for 10 min on ice and then lysed with a Dounce homogenizer (30–40 strokes). The lysates

were clarified by low-speed centrifugation at 1000g for 10 min. Post-nuclear supernatants were then adjusted to a final concentration of 500 mM NaCl and incubated for 30 min on ice. Cytosolic fraction (supernatant, S25) and membrane fraction (pellet, P25) were obtained by ultracentrifugation at 25,000 g for 20 min in an MLA-150 rotor (Beckman Coulter). P25 samples were solubilized in lysis buffer composed of 1% Brij 96 in 20 mM Tris-Cl pH 7.5 before analysis.

2.7. Immunoblotting

Samples were separated by SDS-PAGE, then transferred to a PVDF membrane (Hybond, Amersham). Membranes were blocked against nonspecific binding by using 5% non-fat milk in PBS, 0.1% Tween 20, and probed with appropriate primary antibodies. After washings in PBS containing 0.1% Tween 20, the membranes were incubated with HRP-conjugated secondary antibodies. After final washes, the detection was performed by incubating the membranes with Luminata Forte (Merck) and then image acquisition was made using a Chemidoc (Bio-Rad). Band intensity has been determined utilizing ImageJ software.

2.8. Immunofluorescence microscopy and image analysis

Cells grown on glass coverslips were washed with PBS and then fixed with 4% paraformaldehyde in PBS (Sigma Aldrich) for 10 min. Then, the cells were permeabilized with 0.1% Triton-X100 in PBS and blocked for 30 min with PBS containing 0.2% bovine serum albumin. Incubation with primary antibody was performed at 37 °C for 1 h at room temperature and secondary reagents were added for 30 min at 37 °C. DAPI (Sigma-Aldrich) was used to stain the nucleus. Staining with WGA-Alexa 647 was performed by incubation at room temperature for 10 min. After final washes, coverslips were mounted with Prolong Gold antifade mounting media (Thermo Fisher Scientific). Images were acquired using a Leica SP5-SMD scanning confocal microscope equipped with a 63 ×, 1.4 numerical aperture Leica Aplanochromat oil lens at the Montpellier Resources Imaging platform.

2.9. Statistical analysis

All of the analyses (unpaired Student's t-test) were performed using GraphPad Prism version 6 (GraphPad Software Inc.). * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$; **** $p < 0.0001$.

3. Results

3.1. PI3K activity is essential for CHIKV infection

The PI3K/AKT/mTOR signaling axis is essential for the control of cell proliferation, protein translation and anabolic activities within the cell. Under nutrient abundance, mTOR activation stimulates aerobic glycolysis, and *de novo* lipid synthesis especially through the control of FASN expression (Fig. 1) (Furuta et al., 2008). Starting from the recent observation that Ross River virus (RRV) and Semliki Forest virus (SFV), both grouped with Old World arthritogenic *Alphaviruses*, enhance glycolysis and biosynthesis of C16:0 (palmitate) and C18:0 (stearate) fatty acids in their host by activating the PI3K/AKT/mTOR pathway (Mazzon et al., 2018), we investigated the functional importance of this signaling axis in CHIKV infection. For this purpose, we took advantage of the plant flavonoid quercetin (3,3',4',5,7-pentahydroxyflavone), a well-characterized pharmacological PI3K inhibitor (Agullo et al., 1997). Quercetin, used in a concentration range that was controlled to have no toxic effect (Supplementary Fig. 1A), was added to HEK293T cells 30 min before challenge with a CHIKV reporter virus encoding a luciferase gene inserted in nsP3 sequence (Fig. 2A; upper panel) (multiplicity of infection (MOI) = 1). After 24 h in culture, the infection was monitored by quantification of luciferase expression in cell extracts. In these conditions, quercetin showed a concentration-dependent

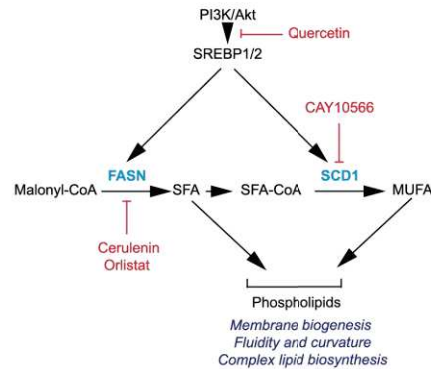


Fig. 1. Fatty acid biosynthesis pathway and inhibitory drugs used in this study.

inhibitory effect on CHIKV infection (Fig. 2A; lower panel). Host lipid metabolism is expected to be pivotal in the various steps of enveloped virus's life cycle. To discriminate between inhibition of viral entry/fusion or subsequent steps in the CHIKV replication cycle, we next used a CHIKV transreplication system (depicted in Fig. 2B; upper panel). Cells were transfected for 2 h with equal amounts of CMV-P1234 and HSPoII-Fluc-Gluc plasmid to reproduce CHIKV post-entry replication (Utt et al., 2019), before the addition of increasing concentrations of quercetin to the culture medium. In these conditions, quercetin displayed a dose-dependent inhibitory effect on virus-encoded luciferase activities (Fig. 2 B, C), supporting that inhibition occurred at the genome replication step. These results were consistent with post-infection experiments performed using a replicating CHIKV (Supplementary Fig. 2A). Altogether, these results, therefore, designate the PI3K/AKT/mTOR signaling axis as essential for CHIKV post-entry events.

3.2. Fatty acid synthase regulates CHIKV genome replication

The PI3K/AKT/mTORC1 signaling has been previously reported to be molecularly connected with FASN expression (Sande et al., 2002). Indeed, in our hands, quercetin-mediated PI3K targeting was able to reduce FASN protein levels in HEK293T cells (Fig. 2D). According to this information and to quercetin anti-CHIKV activity reported above, we explored the direct contribution of FASN in CHIKV genome replication. HEK293T cells were transfected with siRNA specific for FASN or with a non-targeting control duplex. After 48 h, FASN expression was monitored by immunoblotting, showing that FASN protein level was reduced by over 70% by targeting FASN siRNA (Fig. 3A). Then, the cells were transfected with CMV-P1234 and HSPoII-Fluc-Gluc plasmids. In these conditions, reduction of FASN protein level correlated with a 3-fold reduced luciferase activities (Fig. 3B), attesting that FASN expression is required for CHIKV genome replication. Mammalian FASN is a multifunctional enzyme with 7 different catalytic activities that catalyzes the reaction leading to the production of palmitate, a 16-carbon fatty acid. Activity of the N-terminal β -ketoacyl-synthase and C-terminal thio-esterase in FASN can be inhibited using cerulenin antibiotic ((2R)(3S)-2,3-epoxy-4-oxo-7,10-dodecadienolamide) and the FDA-approved anti-obesity drug Orlistat (tetrahydrolipstatin), respectively (Schcolnik-Cabrera et al., 2018) (Fig. 1). Using these drugs, and the CHIKV transreplication system, we next explored whether FASN enzymatic activities are required for genome replication. CHIKV-encoded luciferase activities were dramatically inhibited by cerulenin at concentrations above 15 μ M (Fig. 3) that were non-toxic (Supplementary Fig. 1). A similar effect (> 80% inhibition) was also observed in the presence of 50 or 100 μ M Orlistat (Fig. 3E and F). Despite reducing cell viability by around 20%, this dramatic effect clearly attests of

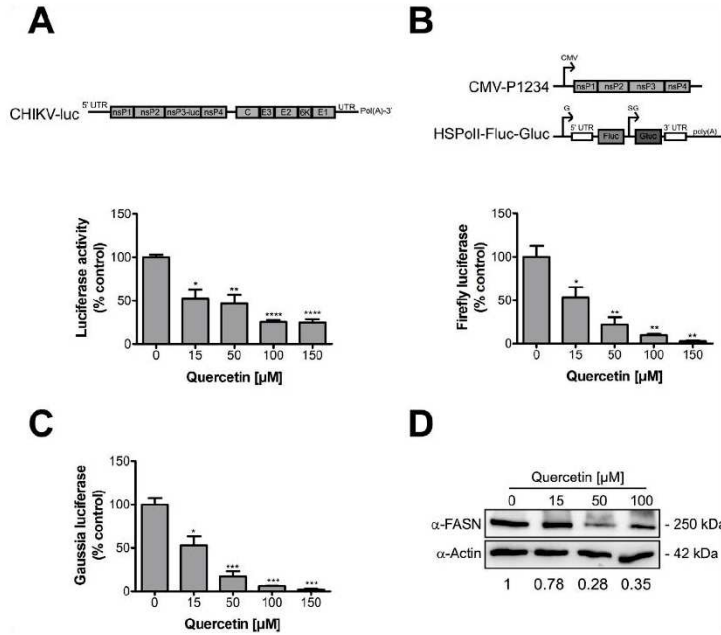


Fig. 2. Pharmacological inhibition of the PI3K/Akt/mTOR/FASN axis and consequences on CHIKV infection. (A) HEK293T cells were pre-incubated with the indicated concentrations of quercetin for 30 min before challenge with a CHIKV-Luc reporter virus (MOI = 1). After 24 h, infection was monitored by quantification of virus-encoded luciferase activity in the cell lysate. (B) (C) Cells were transfected with plasmids depicted in (B) and treated 2 h post-transfection with the indicated concentrations of quercetin. After 24 h, viral genome replication was determined by quantification of Firefly and Gaussia luciferase activities in the cell lysates. Experiments were performed in triplicate and values are expressed as a percentage of the control condition. (D) FASN protein level was monitored in cells incubated for 24 h in the presence of the indicated concentration of quercetin. Actin expression is shown as loading control. Molecular sizes are indicated on the right and relative FASN to actin band intensity is shown below each lane.

Orlistat potent anti-CHIKV activity. Interestingly, both drugs were also effective at inhibiting CHIKV infection in C6/36 cells (Supplementary Fig. 3). Altogether these results support that expression and enzymatic activity of FASN are required for optimal CHIKV genome replication. This result was further validated using CHIKV-infected cells treated with cerulenin or Orlistat after virus exposure (Supplementary Figs. 2B and C).

3.3. Stearoyl-CoA desaturase 1 expression and enzymatic activity are required for CHIKV genome replication

Starting from saturated fatty acids, preferentially palmitoyl-CoA and stearoyl-CoA, the reaction product of FASN, SCD1 introduces a double bond between carbons 9 and 10, to yield palmitoleoyl-CoA and oleoyl-CoA, respectively. To evaluate the impact of fatty acid desaturation on the CHIKV viral cycle, SCD1 was knocked down using siRNA, leading to the almost complete inhibition of SCD1 expression (Fig. 4A). When these cells were transfected with the CHIKV plasmids, expression of the luciferase reporter genes was dramatically reduced as compared to cells transfected with non-targeting control siRNAs (Fig. 4B). SCD1 activity can be manipulated by CAY10566 (3-[4-(2-chloro-5-fluorophenoxy)-1-piperidinyl]-6-(5-methyl-1,3,4-oxadiazol-2-yl)-pyridazine), a potent inhibitor of fatty acyl-CoA conversion to monounsaturated long-chain fatty acids (Liu et al., 2007). Taking advantage of this property, we questioned the contribution of SCD1 enzymatic activity in CHIKV replication. In the presence of increasing concentrations of CAY10566, CHIKV-encoded luciferase activities were significantly decreased up to 50%, indicating that SCD1 activity is required for optimal CHIKV genome replication (Fig. 5C). Altogether, these results identify SCD1 as an essential novel cofactor of CHIKV whose enzymatic activity is required for genome replication events and therefore demonstrate that desaturation of fatty acids is pivotal to post-entry events.

3.4. FASN and SCD1 are conserved cofactors of Old World Alphavirus replication

While reported at risk of spreading to new environments, the biology of MAYV is almost unknown and no antiviral molecule has been identified against this virus. We therefore investigated the inhibitory effect of lipid metabolism inhibitors on MAYV *in vitro*. Because this virus remains understudied, we first established that it actively replicates in human epithelial HEK293T cells (Supplementary Fig. 4). A parallel infection with a MAYV-Luc reporter virus (Fig. 5A) and a CHIKV-Luc (MOI of 1) demonstrated that after 24 h in culture, they generated a comparable luciferase activity in the cell lysate. Using this cell model, MAYV infection was investigated in the presence of quercetin, cerulenin, Orlistat and CAY10566 to determine its requirement for PI3K, FASN and SCD1 activity respectively. Inhibitors were added either 1.5 h before (Fig. 5B–E) or 1.5 h after the viral challenge (Fig. 5F–I). As previously observed for CHIKV, each inhibitor induced a dose-dependent inhibitory effect on MAYV-directed luciferase reporter gene expression independent of the fact that it was added before or after viral challenge. These results, therefore, parallel those obtained with CHIKV, indicating that PI3K, FASN and SCD1 have conserved proviral activity during genome replication of distantly related Old World Alphaviruses.

3.5. Outcome of FASN and SCD1 in Alphavirus-replicating cells

We next investigated whether FASN and SCD1 protein level are manipulated in Alphavirus-infected cells. Since antibodies are lacking for MAYV, we used CHIKV as a model for subsequent studies. First, FASN and SCD1 expression was analyzed overtime upon CHIKV infection using immunoblotting experiments. As shown in Fig. 6A, FASN and SCD1 protein levels remained almost unchanged until 16 h of infection. At this time, a slight decrease was observed reflecting CHIKV toxicity. FASN was previously reported to be recruited to replication foci formed CHIKV-infected (Karlas et al., 2016), a result that was confirmed by us

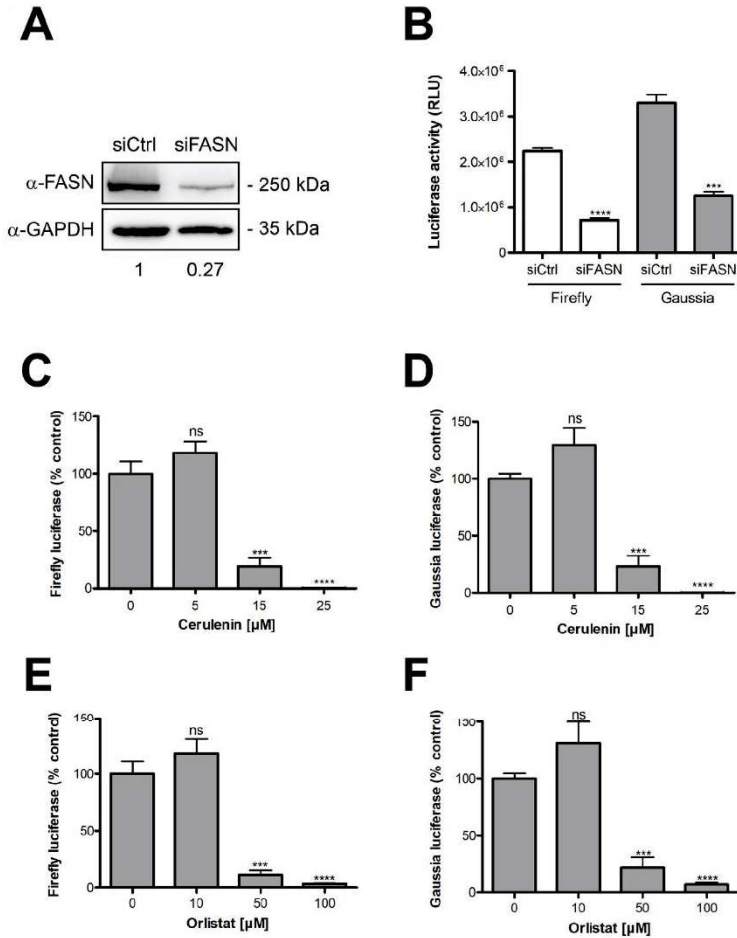


Fig. 3. FASN expression and enzymatic activity are required for CHIKV genome replication. (A) HEK293T cells transfected with siRNA against FASN (siFASN) or non-targeting siRNA (siCtrl) were processed for immunoblot analysis using anti-FASN antibodies. Anti-GAPDH antibodies were used as a loading control. Molecular sizes are indicated on the right. Relative FASN to GAPDH band intensity is indicated below each lane. (B) Cells from (A) were transfected with CHIKV transreplication plasmids. Firefly and Gaussia luciferase activities in the cell lysates were monitored after 24 h in culture. (C–F) Increasing concentrations of cerulenin (C, D) or Orlistat (E, F) were added to cells transfected with CHIKV transreplication plasmids. CHIKV genome replication was determined after 24 h by quantification of virus-encoded Firefly and Gaussia luciferase activities in the cell lysates. Values, expressed as a percentage of the control condition, are means of triplicate experiments \pm SEM.

(data not shown). We therefore investigated the consequences of CHIKV infection on SCD1 subcellular localization. In control cells, SCD1 staining accumulated in the cytoplasm (Fig. 6B) with a distribution coherent with the previously reported endoplasmic reticulum localization of this protein (Nguyen et al., 2014). This pattern persisted in infected cells throughout the time of the infection experiment. No clear colocalization could be observed with the CHIKV non-structural proteins (nsPs) taking part to the replication complex. According to these results, FASN and SCD1 behave differently upon CHIKV infection, with FASN possibly recruited to replication complexes as previously reported (Karlus et al., 2016) while this could not be evidenced for SCD1. These differences may reflect difference in the proviral function of these enzymes in *Alphavirus* replication cycle.

3.6. Consequences of fatty acid metabolism manipulation on CHIKV nsP1 binding to host membranes

Alphavirus genome replication is ensured by the four nsPs complexed with the RNA genome in bulb-shaped membranous organelles derived from the host plasma membrane (Spuul et al., 2011). In these compartments, the replication machinery is tightly anchored to the

lipid bilayer thanks to nsP1, the viral methyl/guanylyltransferase. Membrane anchoring is mediated through a central α -helix (position 244–263 in the CHIKV protein) and reinforced by the palmitoylation of conserved cysteines at the C-terminus (position 417–419 in CHIKV nsP1) (Peranen et al., 1995; Utt et al., 2019). Both nsP1 palmitoylation and the host-encoded ZDHHC2 and ZDHHC19 palmitoyl transferases involved in nsP1 acylation are required for CHIKV infectivity (Utt et al., 2019; N. Zhang et al., 2019). Given the pivotal role of FASN in palmitate synthesis, we investigated the consequences of FASN inhibition on nsP1 membrane binding capacity. HeLa cells expressing a CHIKV GFP-nsP1 protein were left untreated or maintained in the presence of cerulenin or Orlistat at concentrations that were effective against CHIKV replication. In control cells, confocal microscopy imaging detected the GFP-nsP1 fluorescence colocalized with Alexa647-conjugated wheat germ hemagglutinin (WGA) that stains the cell membranes (Fig. 7A). Cell fractionation, clearly evidenced the presence of the GFP-nsP1 protein in the membranous (P25) fraction that also stained positive for the Na⁺/K⁺ ATPase membrane-associated protein. GFP-nsP1 was in contrast detected at low level in the cytosolic (S25) fraction (Fig. 7B). In the presence of cerulenin, the GFP-nsP1 protein detected by confocal microscopy was more diffuse in the cytoplasm

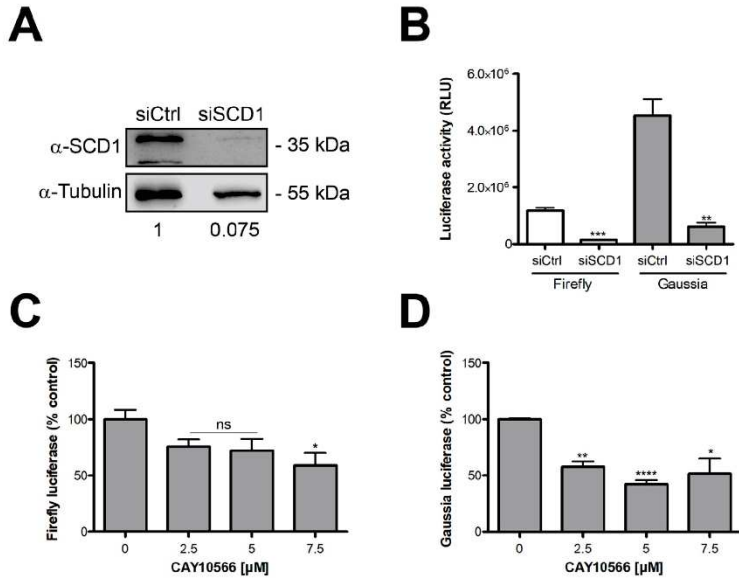


Fig. 4. Consequences of SCD1 inhibition on CHIKV genome replication. (A) HEK293T cells transfected with siRNA against SCD1 (siSCD1) or non-targeting siRNA (siCtrl) were processed for immunoblot analysis using anti-SCD1 antibodies. Anti-tubulin antibodies were used as a loading control. Molecular sizes are indicated on the right. Relative SCD1 to tubulin band intensity is indicated below each lane. (B) Cells from (A) were transfected with CHIKV transreplication plasmids. Firefly and Gaussia luciferase activities were monitored after 24 h in culture. (C-D) Increasing concentrations of CAY10566 were added to HEK293T cells transfected with CHIKV transreplication plasmids. After 24 h, CHIKV-encoded Firefly or Gaussia luciferase activities were determined in the cell lysates. Values are percentages of the DMSO control condition. Means of triplicate experiments \pm SEM are shown.

(Fig. 7A). After cell fractionation, an increased proportion of this protein was also detected in the S25 sample and concomitantly slightly reduced in the P25 sample (Fig. 7B). This phenotype was further exacerbated starting from cells cultured in the presence of Orlistat. Therefore, cerulenin- and Orlistat-mediated inhibition of FASN activity and the expected associated decrease in availability of fatty acids in the

cell reduce nsP1 membrane association. According to these results, the reduced CHIKV genome replication generated by FASN inhibitors may partly reflect a decrease in nsP1 anchoring to membranes, a capacity required for optimal activity of CHIKV replication machinery as recently reported (Utt et al., 2019).

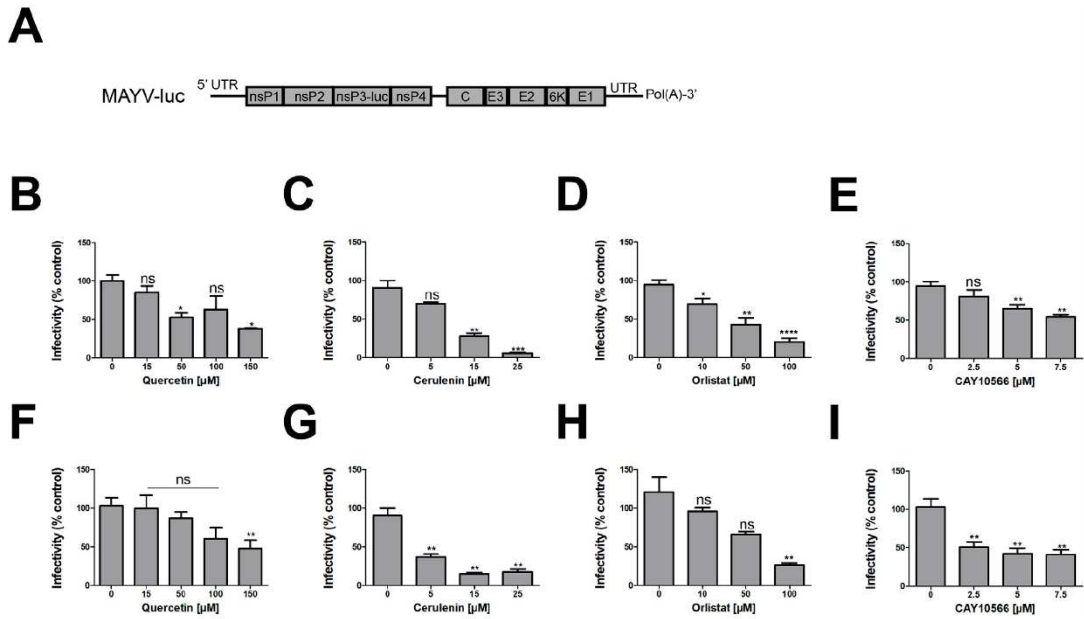


Fig. 5. FASN and SCD1 have proviral activity for MAYV replication. HEK293T cells were infected with the MAYV-luc reporter virus depicted in (A) (MOI = 1). The indicated concentrations of quercetin, cerulenin, Orlistat or CAY10566 were added to the cells either 1.5 h before (B-E) or 1.5 h after (F-I) viral challenge. Virus replication was determined by quantification of luciferase activity in cell extracts. Values (triplicates \pm SEM) are expressed as a percentage of the control condition.

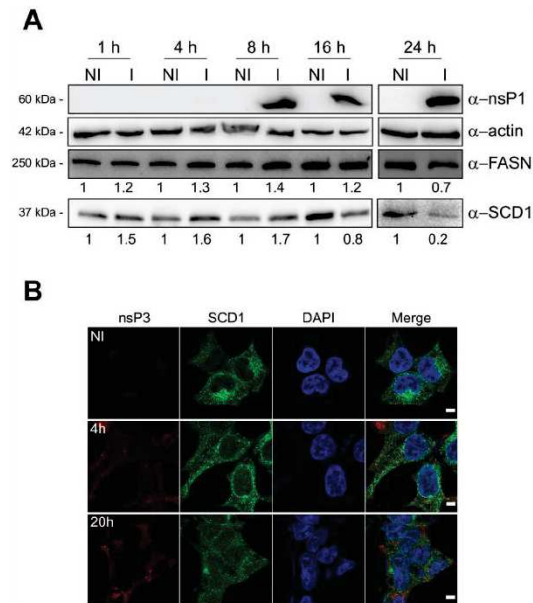


Fig. 6. Consequences of CHIKV infection on FASN and SCD1 expression and localization. (A) Cells infected for the indicated time with CHIKV (I) were subjected to immunoblotting with antibodies against FASN or SCD1. Viral replication in the culture was controlled using anti-nsP1 antibodies and anti-actin antibodies were used as a loading control. Lysate from non-infected cells (NI) cultured in the same conditions were run in parallel. Relative FASN and SCD1 expression level normalized to actin level in the sample is indicated for each condition. (B) Cells were infected with the CHIKV-mCherry-377 virus for the indicated time and labelled with anti-SCD1 antibodies and Alexa-488-conjugated secondary antibodies. Nuclei were stained with DAPI and the cells were analyzed by confocal microscopy. Non-infected cells (NI) are shown as control. Bars: 5 μ m.

4. Discussion

This study was conceived to question the interplay between CHIKV infection and the host fatty acid metabolism in human cells. Using RNA interference and pharmacological inhibitors, we show that FASN and SCD1 expression and activity are required for optimal CHIKV genome replication. FASN is pivotal for various RNA viruses including Respiratory Syncytial Virus (Ohol et al., 2015), West Nile Virus (Martin-Acebes et al., 2011), Astrovirus (Murillo et al., 2015), hepatitis C virus (HCV) (Tongluan et al., 2017; Yang et al., 2008) and dengue virus (DENV) (Huang et al., 2013; Tongluan et al., 2017). Its enzymatic activity regulates important metabolic processes. First, palmitate, the byproduct of FASN is required for post-translational protein acylation. Here, we show that cerulenin and Orlistat, two well known FASN inhibitors that reduce palmitate availability, decrease nsP1 membrane binding capacity. The role of nsP1 acylation in membrane anchoring of the *Alphavirus* replication complex has been long reported (Laakkonen et al., 1996). While dispensable for Sindbis virus infectious cycle (Ahola et al., 2000; Zusinaite et al., 2007), the conserved nsP1 acylated cysteines have been recently found critical for CHIKV replication (Utt et al., 2019). Moreover, FASN inhibition by C75 was previously reported to reduce CHIKV nsP1 palmitoylation (Zhang et al., 2019). Accordingly, cerulenin and Orlistat anti-CHIKV activity reported herein may partly result from the decreased nsP1 membrane binding capacity. Besides, it may also directly result from a reduced availability of FASN

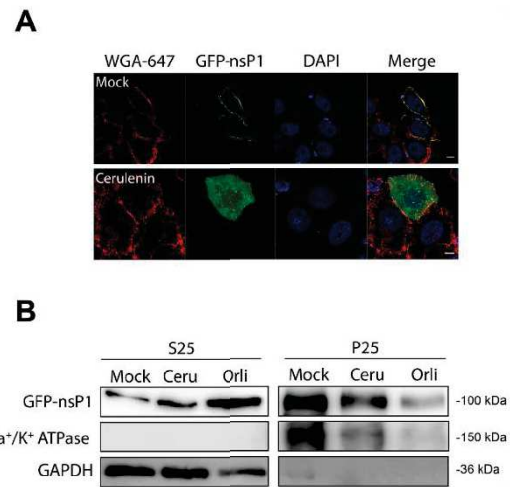


Fig. 7. FASN inhibitors decrease nsP1 membrane binding capacity. (A) HEK293T cells expressing the GFP-nsP1 protein, cultured in the presence of DMSO (Mock) or 15 μ M cerulenin were labelled with Alexa647-conjugated WGA, DAPI and processed for confocal imaging. Bars: 5 μ m. (B) GFP-nsP1 expressing cells cultured in the presence of DMSO (Mock) or 15 μ M cerulenin or 50 μ M Orlistat were subjected to cell fractionation. Cytosolic (S25) and membrane (P25) fractions were separated on SDS-PAGE and probed with anti-nsP1, anti-Na⁺/K⁺ ATPase and anti-GAPDH antibodies. Molecular sizes are indicated on the right.

reaction products, palmitate (16:0) and stearate (18:0), for complex lipid biosynthesis. By catalyzing their conversion into palmitoleate (16:1n-7) and oleate (18:1n-9), SCD1 regulates the saturated fatty acids to MUFA balance with consequences for ATP production via β -oxidation and energy storage in the form of triglycerides. It also regulates for the biosynthesis of phospholipid fatty-acyl chains, triglycerides and cholesterol esters pivotal for membrane extension, fluidity and curvature (Ralston and Mutch, 2015). Here, by using siRNA and taking advantage of the inhibitor CAY10566, we demonstrate that SCD1 expression and desaturase activity are required for CHIKV post-entry events thereby identifying SCD1 as a new CHIKV proviral factor. SCD1 has already been identified as an important cofactor for the replication of *Flaviviruses*, namely DENV (Hishiki et al., 2019), Zika virus and yellow fever virus (Gullberg et al., 2018) in mammalian cells. A screen performed to identify cellular factors and functions required for Brome mosaic virus (BMV) RNA replication in yeast also designated OLE1 the yeast homolog of SCD1 as a proviral factor for viral RNA synthesis. (Lee et al., 2001). For HCV, genome replication defect observed upon SCD-1 blockade was directly correlated with an alteration virus-induced membranous replication organelles as a consequence of modifications in membrane composition (Lyn et al., 2014; Nguyen et al., 2014). The exact function of SCD1 and MUFA in CHIKV post-entry replication events remains to be determined. Nevertheless, requirement for a functional SCD1 suggests that cerulenin and Orlistat antiviral effect not only results from the above discussed reduced nsP1 membrane binding capacity, but may also reflect a decreased fatty acids availability for MUFA biosynthesis. This result therefore prompts the investigation of MUFA contribution in CHIKV life cycle. Interestingly, we observed a similar requirement for an enzymatically active FASN and SCD1 for MAYV genome replication as found for CHIKV. Despite a generally good conservation of virus-host interactions among Old world *Alphaviruses*, some differences have been reported (Thaa et al., 2015). Our results provide among the very first information on an understudied *Alphavirus* with high risk of emergence and suggest the possibility to transpose

some knowledge acquired from CHIKV.

Altogether these results provide evidence that Old World *Alphavirus* genome replication is tightly interconnected with host lipid metabolism. This observation may open novel avenues for the elaboration of antiviral strategies with broad spectrum application in this group of viruses. *De novo* lipogenesis is currently considered an emerging therapeutic target (Escribá, 2017). More specifically, FASN activity can be modulated through pharmacological drugs that are well tolerated in humans. Mainly, Orlistat, a derivative of lipstatin, approved by the FDA (Ballinger and Peikin, 2002), active against CHIKV and MAYV in our hands, displays antiviral activity against Cocksackievirus B3 (Ammer et al., 2015), HCV (Nasheri et al., 2013) and DENV (Tongluan et al., 2017). The wide spectrum antiviral properties of this anti-obesity drug suggests its possible repurposing for anti-infectious applications. The recent development of liver-targeted SCD1 inhibitors (Lachance et al., 2012) and of the oral SCD1 inhibitor Aramchol, evaluated in patients with primary nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (Ajmera et al., n.d.; Safadi et al., 2014) further supports the possibility to target lipogenic enzymes as a promising avenue for the elaboration of antiviral strategy applicable to medically important *Alphaviruses*.

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Declaration of competing interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

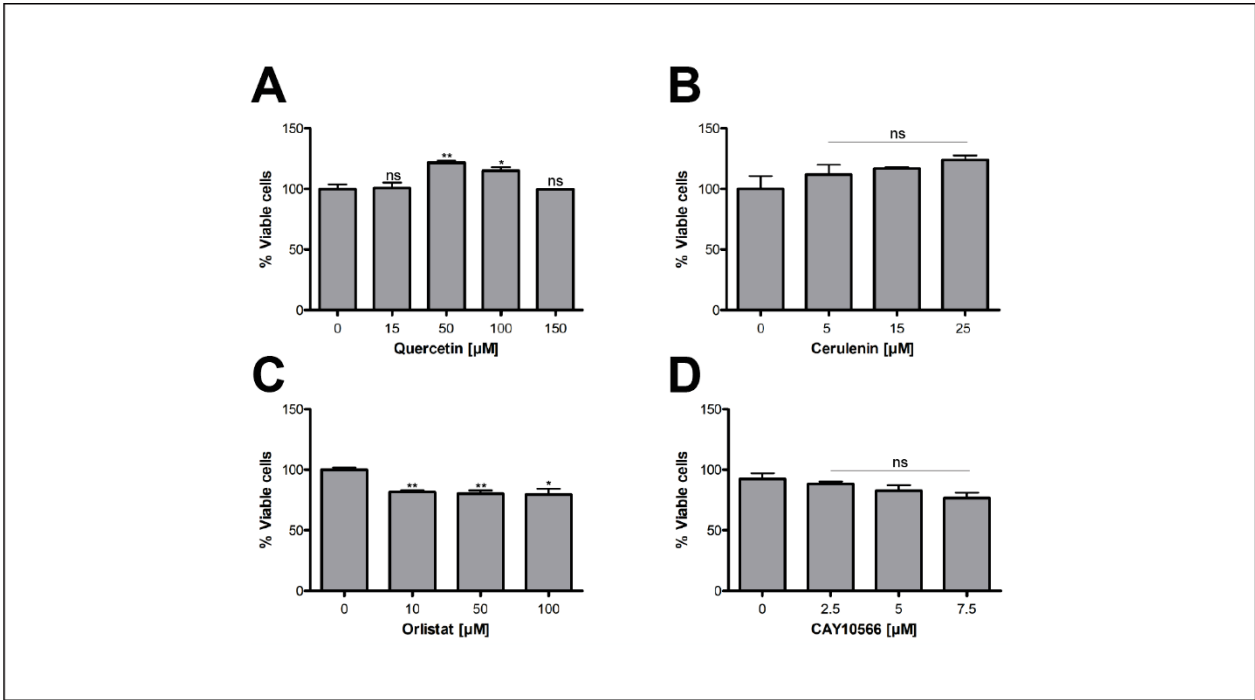
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2019.104642>.

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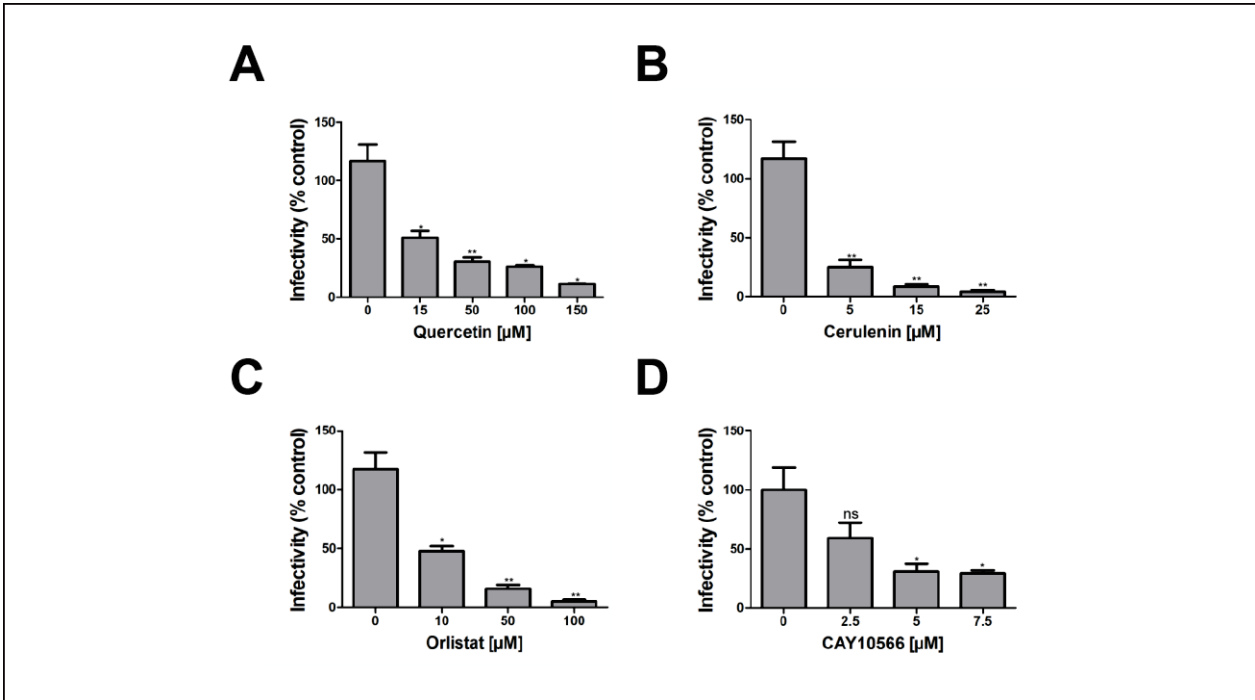
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iii. Supplementary data



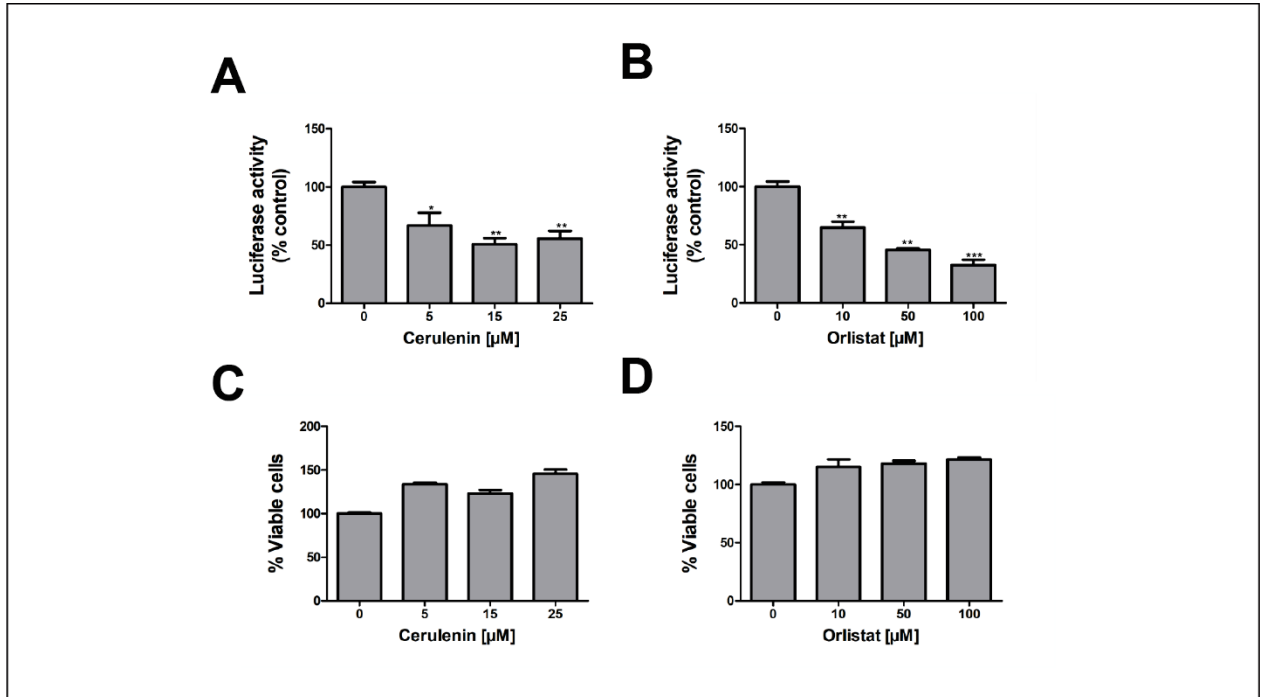
Supp. Fig. 1

Viability of HEK293T cells incubated with increasing concentrations of quercetin (A), cerulenin (B), Orlistat (C), CAY10566 (D) was determined after 24 h. Values are expressed as a percentage of the DMSO-treated control condition and are mean of triplicate ± SEM.



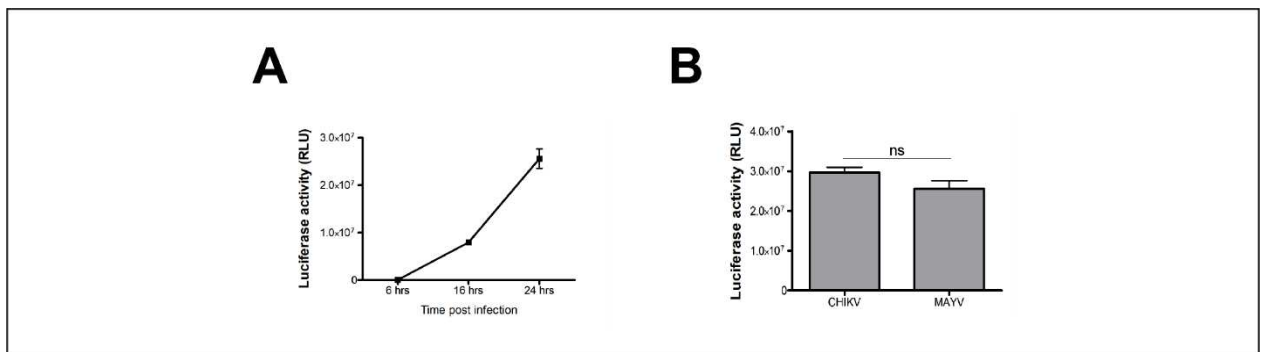
Supp. Fig. 2

Increasing concentrations of (A) quercetin, (B) cerulenin, (C) Orlistat, (D) CAY10566 were added to HEK293T cells infected with CHIKV-luc for 1.5 h. After 24 h, luciferase activities were quantified in the cell lysates. Values are expressed as a percentage of the DMSO condition.



Supp. Fig. 3

Aedes albopictus C6/36 cells were infected with CHIKV-luc for 1.5 h and then treated with increasing concentrations of cerulenin (A) or (B) Orlistat. After 24 h in culture, virus replication was monitored by quantification of luciferase activities in the cell lysates. (C–D) Cell viability was determined for each drug concentration. Values are expressed as a percentage of the control condition.



Supp. Fig. 4

(A) HEK293T cells were infected with the MAYV-luc reporter virus (MOI = 1). Replication was monitored over time by quantification of virus-encoded nanoluciferase gene expression in the cell lysate. (B) Cells were infected with CHIKV-luc or MAYV-luc virus (MOI = 1). Luciferase activity was monitored after 24 h. Values were normalized according to protein content in the sample.

Project B Functional role of cholesterol in Chikungunya virus replication

i. Article summary

Another main membrane lipid component with important implications on the fluidity and organization of membranes is cholesterol. This sterol, by inserting between fatty acids, contributes to the increased thickness and rigidity of biological membranes. This membrane lipid is also an essential component of the highly ordered plasma membrane microdomains named lipid rafts.

As mentioned earlier, this sterol has been shown to be important for the replication of other (+)RNA viruses (Flaviviridae). Recently, a paper has suggested that this membrane lipid plays a role in the replication step of CHIKV (Wichit et al., 2017). This was demonstrated using drugs (imipramine and U18666A) that sequester cholesterol in intra-cellular aggregates depleting it from the plasma membrane. However, the exact role of this sterol in the CHIKV replication step was not provided. Indeed, observation of cholesterol relocation to CHIKV replication complexes is challenging since these organelles form in association with the plasma membrane which is rich in cholesterol. This is in contrast with Flaviviridae that assemble their replication complex with the ER membranes which are poor in cholesterol facilitating the observation of relocation events. Therefore, we took advantage of cholesterol transport inhibitors to understand the role of this lipid in CHIKV replication. The focus was put on nsP1 since it is the nsP that targets the *Alphavirus* replication complex to membranes. Interestingly, cholesterol trafficking inhibitors relocated nsP1 into intra-cellular cholesterol-rich aggregates. This tempted the speculation that nsP1 could be localized to lipid rafts. Indeed, experiments confirmed nsP1 lipid raft affinity. Furthermore, in a replicon or infectious based systems, nsP1 was able to recruit the other components of the replication complex to these microdomains. The search for molecular determinants in nsP1 identified palmitoylation as the main player in this protein's targeting to lipid rafts. This came in perfect agreement with recent studies showing that CHIKV nsP1 palmitoylation is absolutely essential for CHIKV replication, and indicates that this newly discovered lipid raft and cholesterol association of nsP1 is functionally relevant. This association also uncovers the poorly understood role of palmitoylation in *Alphavirus* replication.

In summary, this study showed that cholesterol is required for *Alphavirus* replication. This requirement for cholesterol was then associated with the affinity of palmitoylated

nsP1 to cholesterol-rich microdomains. However, why *Alphavirus* replication complexes will assemble at lipid rafts remains an open area for investigation.

ii. Article 2 “Anchoring of nsP1 methyl-guanylyltransferase to cholesterol-enriched plasma membrane microdomains and functional consequences on Chikungunya virus replication”

Palmitoylated cysteines in chikungunya virus nsP1 are critical for targeting to cholesterol-rich plasma membrane microdomains with functional consequences for viral genome replication

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Running head: CHIKV nsP1 affinity for cholesterol enriched membranes

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Key words: Chikungunya virus, Methyl/guanylyltransferase, Cholesterol, Plasma membrane, Replication complexes, Alphavirus,

Abstract

In mammalian cells, alphavirus replication complexes are anchored to the plasma membrane. Interaction with lipid bilayers is mediated through the viral methyl/guanylyltransferase nsP1 and reinforced by palmitoylation of cysteine residue(s) in the C-terminal region of the protein. Here, we explore the membrane binding capacity of nsP1 with regards to cholesterol. Using the medically important chikungunya virus (CHIKV) as a model, we report that nsP1 co-segregates with cholesterol-rich detergent-resistant membrane microdomains (DRMs), also called lipid rafts. We identify nsP1 palmitoylation as a critical factor for cholesterol partitioning. In cells infected with CHIKV or transfected with CHIKV trans-replicase plasmids, nsP1 together with other nonstructural proteins are detected in DRMs. While the functional importance of CHIKV nsP1 preference for cholesterol-rich membrane domains remains to be determined, we observed that U18666A- and imipramine-induced sequestration of cholesterol in late endosomes redirected nsP1 to these compartments and simultaneously dramatically decreased CHIKV genome replication. A parallel study of Sindbis virus (SINV) revealed that nsP1 from this divergent alphavirus displays a low affinity for cholesterol and only moderately segregates with DRMs. Thus, behaviors of CHIKV and SINV with regards to cholesterol match with the previously reported differences in requirement for nsP1 palmitoylation that is dispensable for SINV but strictly required for CHIKV replication. Altogether, this study highlights the functional importance of nsP1 segregation with DRMs and provides new insight into the functional role of nsP1 palmitoylation during alphavirus replication.

Importance

Functional alphavirus replication complexes are anchored to the host cell membranes through the interaction of nsP1 with the lipid bilayers. In this work, we investigate the importance of cholesterol for such association. We show that nsP1 has affinity for cholesterol-rich membrane microdomains formed at the plasma membrane and identify conserved palmitoylated cysteine(s) in nsP1 as the key determinant for cholesterol affinity. We demonstrate that drug-induced cholesterol sequestration in late endosomes not only redirects nsP1 to this compartment but also dramatically decreases genome replication, suggesting the functional importance of nsP1 targeting to cholesterol-rich plasma membrane microdomains. Finally, we evidence that nsP1

from chikungunya and Sindbis viruses display different sensitivity to cholesterol sequestering agents, that parallel with their difference in the requirement for nsP1 palmitoylation for replication. This research, therefore, gives new insight into the functional role of palmitoylation in nsP1 for the assembly of functional alphavirus replication complexes in their mammalian host.

Introduction

In the last decade, evidence has pointed toward the intricate relationship between host lipid metabolism and the replication of viral pathogens. Indeed, viruses can co-opt or reprogram lipid signaling, synthesis and metabolism either to generate ATP, to extend cellular membranes or to remodel membrane lipid content. These modifications will serve to create an environment that is optimal for viral replication. This need is dictated by the pivotal role played by membranes in almost all steps of virus life cycle. Indeed, the importance of cellular lipids during the binding/entry process and assembly/budding of new infectious progeny into the extracellular space has long been appreciated (for review see (1)). However, the discovery that viruses with a positive-strand RNA genome ((+)RNA viruses) replicate in association with host cell membranes has expanded the regulatory function of lipids to viral replication. It is now well established that such viruses create membranous compartments, also called virus replication organelles, originating from the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, peroxisomes, endosomes/lysosomes or from the plasma membrane (PM) (2). In these compartments, viral replication proteins bind cell membranes with an affinity for determined lipid species (3, 4).

Cell membranes are composed of phospholipids, glycolipids, and cholesterol. Among other lipids, cholesterol constitutes a unique type of cellular membrane building block. It is responsible for regulating fluidity and impermeability to lipid bilayers. In vertebrate cells, cholesterol homeostasis is maintained through *de novo* synthesis in the ER with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase being the rate-limiting enzyme, and through receptor-mediated intake from the extracellular medium in the form of low-density lipoproteins (LDLs) (5). In this scenario, LDLs are delivered to and hydrolyzed in late endosomes/lysosomes (LE/Ls) where free cholesterol is released. Then, cholesterol requires proper intracellular transport to exit the LE/Ls and reach its final destination mainly at the PM, in the Golgi apparatus and the ER. This is ensured by Niemann-Pick C 1 and 2 (NPC1 and NPC2) proteins localized at the limiting membrane of lysosomes and in the lysosomal lumen respectively (6). Once at the PM, cholesterol together with glycosphingolipids, glycosphosphatidylinositol (GPI)-anchored proteins and

transmembrane proteins can cluster into discrete domains. These cholesterol-enriched detergent-resistant membrane microdomains referred to as DRMs or lipid rafts have been identified as platforms for both endocytosis of penetrating viral particles and for progeny assembly and budding (7). More recently, cholesterol regulatory function was extended to the replication step of (+)RNA viruses. The local accumulation of cholesterol was proposed to contribute to the creation of a membrane microenvironment conducive to assembly and optimal function of replication complexes formed by members of the *Flaviviridae* family, including hepatitis C virus (HCV) (8–10) and West Nile virus (11), by members of *Picornaviridae* family, including Coxsackievirus and poliovirus (12) and by plant viruses from the *Bromoviridae* family (Brome mosaic virus; BMV) (13). Consistent with the idea that cholesterol accumulation may be required for optimal activity of viral replication machinery, manipulation of cholesterol metabolism was found to impair genome replication of taxonomically divergent (+)RNA viruses (8, 12, 14–18).

Alphaviruses are (+)RNA viruses, which are predominantly transmitted to vertebrates by mosquito vectors. Chikungunya virus (CHIKV) is an Old World alphavirus causing millions of infections in tropical and subtropical geographical areas with a potential risk of spreading to regions with a temperate climate. It has recently received significant attention as a consequence of its re-emergence in the Indian Ocean and Caribbean Islands before spreading worldwide (19). CHIKV, like other alphaviruses, replicates its genome in membranous niches derived from the host PM (20–22). The replication complex confined in these organelles is termed as spherule and contains four non-structural proteins nsP1, nsP2, nsP3 and nsP4 encoded by the 5' ORF in the viral genome and expressed in form of P123 and P1234 polyprotein precursors. In this complex, membrane binding is mediated by nsP1, the viral methyltransferase (MTase) and guanylyltransferase (GTase), which catalyzes the formation of the cap structure at the 5' end of nascent positive strand viral RNAs (23, 24). Interaction with membranes is strictly required for Semliki Forest virus (SFV) nsP1 enzymatic activity (25) and genome (26) replication while it is dispensable for enzymatic activity of nsP1 of Sindbis virus (SINV)(27). S-acylated cysteines located in the C-terminal region of nsP1 play a crucial role for membrane attachment. These residues were proposed to mediate and stabilize interaction with lipid bilayers (28). However, the functional requirement for nsP1 palmitoylation for genome replication varies

among alphaviruses. Especially, alanine substitution of palmitoylated cysteines completely abolishes the replication of CHIKV (29, 30). It leads to the acquisition of compensatory mutations for SFV (31, 32), and has only a marginal inhibitory effect on SINV replication (31, 33).

In vitro, SFV nsP1 has affinity for anionic phospholipids especially phosphatidylserine, phosphatidylglycerol, and cardiolipin; these lipid species significantly improve its capping activity (25, 34). In cells, however, nsP1 affinity for specific lipids remains almost uninvestigated. In recent years, cholesterol metabolism was reported to be critical for the replication of alphavirus genome. Indeed, SINV RNA replication and protein synthesis is significantly decreased in fibroblasts from patients with type A Niemann-Pick disease (NPD-A) which induces cholesterol and sphingolipid storage in LE/Ls (35). More recently, we reported that U18666A, a class II cationic amphipathic steroid 3- β -[2-(diethylamino)ethoxy]androst-5-en-17-one, and the anti-depressant drug imipramine, which both phenocopy NPD-A, are CHIKV inhibitors with potential activity against RNA replication steps (16). Here, we further question the importance of cholesterol metabolism in alphavirus life cycle. We especially explore the outcome of cholesterol manipulation on nsP1 subcellular distribution and membrane anchoring. We show that U18666A or imipramine redirect CHIKV nsP1 to Lamp2-positive endosomal compartments where unesterified cholesterol accumulates. nsP1 affinity for DRMs was confirmed by membrane flotation assays. Investigating the molecular basis of nsP1 targeting to DRMs revealed that the palmitoylated cysteines are the main determinants for association with these domains. Interestingly, when expressed transiently in the context of a P1234 polyprotein precursor or by an infectious CHIKV, nsP2, nsP3, and nsP4 were found to co-segregate with nsP1 in cholesterol-rich membrane fractions, a property that was abolished when nsP1 palmitoylated cysteines were mutated. In a parallel study we found that nsP1 association with cholesterol containing membranes through palmitoylation is conserved for SINV despite being less marked than for CHIKV. Moreover, SINV nsP1 was also less sensitive to U18666A-induced cholesterol sequestration. This phenotype with regard to nsP1 cholesterol partitioning parallels the reduced requirement for cysteine palmitoylation previously reported for SINV replication (31, 33). Altogether this study provides clues on the proviral role of cholesterol in alphavirus replication suggesting its regulatory function

in nsPs association with the PM and presumably in formation of functional replication complexes.

Results

Cholesterol is pivotal for alphavirus genome replication.

First, we set out to study the involvement of cholesterol homeostasis in CHIKV replication. Cholesterologenesis was inhibited using lovastatin, an FDA-approved drug that inhibits the activity of HMG-CoA reductase, that catalyzes the conversion of HMG-CoA to mevalonate in the cholesterol biosynthesis pathway (Fig 1A) (36). Cholesterol availability at the PM was reduced using U18666A or imipramine. By targeting NPC1 transporter, both drugs block the transfer of endocytosed cholesterol from late endosomes to different organelles, including the PM or ER, without significant effect on other lipid species (37). Additionally, U18666A inhibits enzymes of the cholesterol synthesis pathway (38). Each drug, used in a concentration range that was controlled to have limited toxicity (Fig 1B-b,d,f), was added to HEK293T cells 30 mins before infection with CHIKV-LR-5'GFP at a multiplicity of infection (MOI) of 0.5. After 24 hrs in culture, the infection was monitored measuring levels of GFP reporter expressed by recombinant virus. In each case, CHIKV infection was significantly reduced as compared with the mock-treated condition (Fig 1B-a,c,e).

We and others have demonstrated that the depletion of membrane cholesterol is deleterious for fusion of the virion and host membranes (39, 40). Because host membranes are also pivotal for replication of alphavirus RNAs through the creation of membranous replication organelles, we investigated whether cholesterol biosynthesis is also required for the post-entry step of CHIKV infection cycle. To this end, we performed experiments in which cells were treated with cholesterol metabolism and transport inhibitors 1 hr after CHIKV infection. In these conditions, lovastatin treatment, but also treatment with U18666A or imipramine, decreased CHIKV genome replication indicating that cholesterol biosynthesis inhibitors impair intracellular steps of CHIKV life cycle (Fig 2A). To definitively omit drug effects on viral entry, we finally took advantage of CHIKV trans-replication system that recapitulate CHIKV RNA replication (29). Plasmids CMV-P1234 and HSPoll-Fluc-Gluc, encoding the P1234 polyprotein and a replication-

competent template RNA containing firefly and *Gaussia* luciferase reporter genes under the control of genomic and subgenomic viral promoters respectively (Fig 2B), were cotransfected in HEK293T cells. Consequences of cholesterol transport inhibition were assayed by adding increasing concentrations of lovastatin, U18666A, and imipramine to transfected cells and quantification of reporter activities in cell lysates. As depicted in figures 2C and 2D, reporter expression directed by both genomic and subgenomic promoters was decreased by the drugs when compared to control conditions. Altogether, these results indicate that cholesterol homeostasis, including ongoing biosynthesis and transport of unesterified cholesterol to the host membranes, is pivotal for CHIKV genome replication.

Putative α -helix and palmitoylated cysteines cooperate for CHIKV nsP1 binding to host membranes.

Alphavirus replication complexes are anchored to the host membranes thanks to nsP1 membrane binding capacity. While extensively reported for nsP1 encoded by SFV and SINV (33, 34), this feature has not yet been studied functionally for CHIKV. To investigate CHIKV nsP1 behavior with regard to host cell membranes, a plasmid encoding a GFP-fused CHIKV nsP1 protein (GFP-nsP1; Fig 3A) was generated and used to transfect HEK293T cells. NsP1 membrane association was investigated by fractionation of transfected cells. Post-nuclear extract prepared from GFP-nsP1-expressing cells was separated into membranous (P25) and cytosolic (S25) samples by differential centrifugation as previously reported (41). Each fraction was resolved using SDS-PAGE and probed with anti-GFP antibodies and with antibodies against Na⁺/K⁺ ATPase or GAPDH, that respectively associate with membrane and cytosolic compartments. In these conditions, GFP alone was detected in the cytosolic sample together with GAPDH (Fig 3B and 3C). By contrast, GFP-nsP1 was detected in the membrane fraction also containing Na⁺/K⁺ ATPase. In parallel HeLa cells expressing these proteins were analyzed using confocal microscopy. As expected, the fluorescence of individual GFP was diffuse in the cytoplasm and nucleus. However, GFP-nsP1 fluorescence overlapped with PM stained using wheat germ hemagglutinin (WGA)-conjugated with Alexa Fluor 647. As previously reported for related alphaviruses (42), expression of GFP-nsP1 generated huge

membrane reshaping creating filopodia- and lamellipodia-like structures covering the entire cell surface, that stained positive for the green fluorescence. Of note, this profile was also observed for a C-terminally GFP-tagged nsP1-GFP protein as well as for an untagged nsP1 detected by mean of anti-nsP1 serum (Fig 3E), thereby supporting that GFP-nsP1 behaves as native nsP1 regarding localization and association with membranes. According to the focal plane chosen, a fraction of each of these proteins was also detected as small cytosolic aggregates as illustrated for on figure 3E.

Because alphavirus nsP1 was previously proposed to be trafficked to endosomes (43) and in light of our confocal microscopy analysis, we further questioned nsP1 subcellular distribution by performing fractionation assays that separated the PM from other cell membranes. The post-nuclear extract was prepared from GFP-nsP1 expressing cells and separated by isopycnic centrifugation in a self-forming linear 10-20-30% iodixanol density gradient. Twenty-four samples were collected from top to bottom and assayed by western blotting for GFP-nsP1 content. A roughly equal proportion of GFP-nsP1 was detected in the top fractions 1 and 2 and also in fractions 9 to 12 that all stained positive for the Na⁺/K⁺ ATPase membrane marker (Fig 3F). These fractions also contained flotillin-1 (FLOT1) that is known to localize predominantly to the PM and endosomal compartments, *i.e.* late endosomes and recycling endosomes (44). By contrast, individual GFP segregated with fractions 17 to 24 at the bottom of the gradient that corresponded to the cytosolic compartment. Altogether, these results show that CHIKV nsP1 is a membrane-associated protein, that cofractionates equally with the PM and some internal membranes, probably endosomal in nature, suggesting that this protein may traffic between the two compartments.

We next questioned whether the functions of membrane binding determinants previously identified in alphavirus nsP1 proteins are also conserved for CHIKV nsP1. Extensive analysis performed using SFV and SINV as models established that membrane association of nsP1 relies on a central conserved sequence that folds as an amphipathic α -helix when studied as a synthetic peptide in solution (34). In this sequence, a pivotal role in membrane anchoring was attributed to tryptophan at position 259 (W₂₅₉) that sinks into the phospholipid bilayer (34). This interaction was proposed to be reinforced by the

presence of cysteine residue at position 420 (C420) SINV or cysteine residues 418-420 in SFV (C418-420) which are covalently palmitoylated and render the protein highly hydrophobic (28, 33). These motifs are conserved in the CHIKV nsP1 sequence where W258 and C417-419 of residues correspond to W₂₅₉ and C₄₁₈₋₄₂₀ of SFV nsP1, respectively (Fig 3A). To study the contribution of each motif in CHIKV nsP1 membrane binding, we generated GFP-nsP1 mutants in which W258 (GFP-nsP1_{W258A}) or C₄₁₇₋₄₁₉ (GFP-nsP1_{3A}) residues in nsP1 sequence were replaced by alanine as well as a double nsP1 mutant bearing a combination of W_{258A} or C_{417-419A} mutations (GFP-nsP1_{DM}). Fluorescence microscopy of cells transfected with the corresponding plasmids evidenced a diffuse green fluorescence that predominated in the cytoplasm by contrast with cells expressing GFP-nsP1 (Fig 3D). Fractionation experiments confirmed that GFP-nsP1_{W258A} and GFP-nsP1_{3A} were more abundant in the cytosolic fraction than GFP-nsP1, with a significant amount of each mutant protein remaining associated with cell membranes (Fig 3B and 3C). The same cell extracts were then subjected to membrane flotation assay in iodixanol gradient to appreciate their capacity to associate with the internal/plasma membrane compartments. GFP-nsP1_{W258A} and GFP-nsP1_{3A} proteins were both detected in fractions 18-24 of the gradient thereby confirming that these proteins have a decreased membrane affinity (Fig 3F). Each mutant protein was also present at the top of the gradient in fractions 1-2 corresponding to the PM and in fractions 9-12 corresponding to internal membranes. Distribution of mutant proteins in these fractions generally mimicked that of GFP-nsP1, except that GFP-nsP1_{W258A} was somewhat more abundant in internal membrane fractions. This was also reflected in more diffuse localization of GFP fluorescence in cells expressing GFP-nsP1_{W258A} (compare panels b and d of Fig. 3D) indicating possible role of W258 for PM affinity of CHIKV nsP1. Nevertheless, the observed differences were small suggesting that W_{258A} and C_{417-419A} mutations despite decreasing membrane affinity do not prominently modify nsP1 distribution between intracellular and plasma membranes or nsP1 trafficking capacity. Finally, membrane association was further decreased when W_{258A} and C_{417-419A} mutations were combined in GFP-nsP1_{DM} (Fig 3B and 3D). Altogether these results establish that CHIKV nsP1 associates both with the plasma and endosomal membranes, an association dictated by the cooperation of motifs that contain C₄₁₇₋₄₁₉ and W258 amino acids in the protein.

Cholesterol storage defect redirects CHIKV nsP1 to late endosomes.

Based on the observation that cholesterol synthesis and transport is required for CHIKV RNA replication and on the intimate relationship of CHIKV nsP1 with membranes, we next wondered whether cholesterol homeostasis has an impact on nsP1 behavior. We first investigated nsP1 subcellular localization with respect to cholesterol distribution. Cells transfected with plasmid expressing GFP-nsP1 were incubated with U18666A or imipramine to generate a cholesterol entrapment in LE/Ls (45). This capacity was controlled using the antifungal antibiotic filipin III that forms a fluorescent complex upon association with unesterified cholesterol (46). In the presence of U18666A or imipramine, filipin III evidenced the formation of large fluorescent cytosolic aggregates that contrasted with the presence of cholesterol at the PM and more evenly distributed in the cytoplasm of mock-treated cells (Fig 4A and data not shown). For U18666A, these clusters colocalized with Lamp2 (Fig 4C), a LE/Ls marker, thereby confirming that this pharmacological agent stimulates the accumulation of unesterified cholesterol in late endosomes as previously reported (47). Using these experimental conditions, we next assessed the impact of cholesterol storage defect on GFP-nsP1 subcellular localization and membrane affinity. Fractionation assays established that drug treatment did not increase amount of cytosolic nsP1 (Fig 4B; S25 fraction) indicating that it had no consequence on nsP1 membrane affinity. However, microscopy imaging revealed that, in the presence of U18666A, a significant part of GFP-nsP1 fluorescence was redirected from the PM to cytosolic aggregates. This signal overlapped with filipin III staining as attested by cross-sectional analysis of the fluorescent signals (Fig 4D and 4E). In similar experimental conditions, no redistribution of individual GFP fluorescence was observed. Accordingly, upon cholesterol storage condition, GFP-nsP1 colocalized with unesterified cholesterol stored in late endosomes. This situation contrasted with that of mock-treated cells in which the GFP-nsP1 fluorescence colocalized with filipin III at the PM and did not overlap with Lamp 2 staining. Altogether, our results argue that the inhibition of NPC1-mediated cholesterol transport by U18666A redirects GFP-nsP1 to LE/Ls where unesterified cholesterol accumulates. Re-targeting GFP-nsP1 to these compartments has no significant impact on nsP1 membrane binding capacity.

Palmitoylated cysteines but not W258 residue determine CHIKV nsP1 sensitivity to cholesterol distribution.

Palmitoylation governs protein trafficking and association with membranes. A major focus of studies on protein palmitoylation has been the role of this modification in promoting interaction with gangliosides and cholesterol, leading at certain conditions, to protein translocation to raft/caveolae membrane domains (48). Having demonstrated that palmitoylated cysteines in CHIKV nsP1 are required for optimal membrane association, we investigated their contribution to GFP-nsP1 cholesterol affinity. The above described experiments were repeated using cells transfected with GFP-nsP1_{W258A} and GFP-nsP1_{3A} plasmids. In the presence of U18666A, GFP-nsP1_{W258A} fluorescence was concentrated in cytosolic foci colocalized with filipin III fluorescence (Fig 5A) as observed for GFP-nsP1. In contrast, GFP-nsP1_{3A} fluorescence remained diffuse in the cytoplasm whether the cells were maintained in the presence of U18666A or with an appropriate concentration of vehicle (Fig 5C). These phenotypes were confirmed by cross-sectional analysis of the fluorescent signals (Fig 5B and D). Accordingly, W258, even if required for strengthening nsP1 membrane affinity, is not critical for cholesterol affinity. In contrast, palmitoylated cysteine-to-alanine substitution in mutant GFP- nsP1_{3A} abolished sensitivity to U18666A, suggesting a critical role of palmitoylation in nsP1 cholesterol affinity.

To further explore GFP-nsP1_{3A} behavior with regard to cholesterol, we took advantage of CD81, a heavily palmitoylated tetraspanin, segregating mainly with DRMs, as a lipid raft biomarker (49). Indeed, in our hands, this protein was mainly detected at the PM of untreated U2OS cells (Fig 6A). It was redirected to cytosolic aggregates in cells cultured with U18666A or imipramine (Fig 6B) where it colocalized with GFP-nsP1 as attested by Mander's coefficient calculation (Fig 6D). CD81 therefore appears as a sensitive cholesterol sensor. Using this property, we explored the importance of nsP1 palmitoylation for cholesterol dependency. Cells transfected to express GFP-nsP1_{3A} were incubated in the presence of U18666A. Despite inducing the clustering of CD81 into intracellular compartments, this treatment did not affect GFP-nsP1_{3A} distribution (Fig 6C). Indeed, GFP-nsP1_{3A} remained detected as a diffuse cytoplasmic protein as observed in mock-treated cells thereby contrasting with the phenotype observed for GFP-nsP1. No

colocalization of CD81 and GFP-nsP1_{3A} fluorescence was observed (Fig 6D). Optical sectioning (z-stack) and three-dimensional (3D) volume reconstruction from image stacks confirmed that conversely to GFP-nsP1, GFP-nsP1_{3A} poorly colocalized with CD81 in drug treated cells (Fig 6E and 6F). These phenotypes were confirmed from cells cultured with imipramine. According to these results, nsP1 behaves similarly to CD81 with regard to cholesterol storage, a property that requires the presence of palmitoylated cysteines in nsP1 C-terminus.

CHIKV nsP1 partitions with DRMs.

Given that nsP1 subcellular localization is sensitive to cholesterol redistribution to the PM, we questioned its capacity to segregate with cholesterol-enriched membrane microdomains. Cholesterol is not uniformly distributed in membranes. In living cells, it concentrates in nanoscale assemblies, also enriched in sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins, referred to as lipid rafts. These compartments are characterized biochemically by their insolubility in non-ionic detergents, a property reflected in their name (DRM - detergent-resistant membrane microdomains), and by their light density on sucrose gradients. Therefore, they can be separated from non-raft membranes by centrifugation methods. Samples prepared from cells transfected with GFP-nsP1 expression plasmid were treated with Triton X-100 at 4°C, separated on a 10-80% sucrose density gradient, and then analyzed by western blot. Detergent-resistant fractions corresponding to DRMs were identified utilizing an antibody against FLOT1, a well-known raft-associated protein (50). In our experimental conditions, FLOT1 fractionated into light density fractions 1 and 2 at the top of the gradient thereby identifying DRMs (Fig 7A). In contrast, the non-raft marker Na⁺/K⁺ ATPase remained associated with fractions 7-9 of heavier density corresponding to non-raft membranes and cytosolic compartment (detergent sensitive; DS). Using this protocol, more than 85% of the GFP-nsP1 protein was detected in FLOT1-positive fractions at the top of the gradient supporting its capacity to associate with cholesterol-enriched DRMs (Fig 7A and 7B). To confirm GFP-nsP1 affinity for cholesterol-enriched microdomains, this experiment was repeated starting from cells cultured in the presence of the cholesterol depleting agent methyl-β-cyclodextrin (βMCD). Due to its ability to sequester cholesterol in its hydrophobic pocket,

β MCD extracts cholesterol from the lipid bilayer and disrupts DRMs (51). Under these cholesterol depletion conditions, FLOT1 was redistributed from DRMs to DS fractions and sedimented at the bottom of the density gradient (Fig 7C and 7D). Analysis of the same fractions with anti-nsP1 antibodies revealed that GFP-nsP1 was barely detectable in top fractions while it accumulated in fractions of heavier density together with FLOT1. Therefore, GFP-nsP1 fractionation with DRMs is sensitive to cholesterol extraction from cell membranes. Finally, this experiment was repeated starting from cells expressing GFP-nsP1_{W258A} or GFP-nsP1_{3A} mutant proteins that displayed different sensitivity to U18666A. As observed for GFP-nsP1, the GFP-nsP1_{W258A} mainly segregated with DRMs fractions attesting that raft association was not disrupted by mutation of W258 residue in the putative α -helix of nsP1 (Fig 7E and 7F). Analyzing the behavior of the GFP-nsP1_{3A} mutant protein revealed, in contrast, that most of this protein was absent from the top fraction and co-segregated with the DS fractions. These experiments demonstrate that the presence of GFP-nsP1 in DRMs is mainly dictated by acylated cysteines while mutation in the putative α -helical peptide has only marginal impact on this phenotype.

nsPs associate with DRM fractions in cells with CHIKV RNA replication.

Unlike SFV, CHIKV replication is completely abolished by nsP1_{3A} mutation regardless of the type and growth temperature of cells. In contrast, nsP1_{W258A} mutation allows virus to grow efficiently in insect cells or in mammalian cells cultivated at 28°C (29, 52). Combined with data from previous experiments it strongly indicates that presence of nsP1 in DRM is an absolute requirement for CHIKV genome replication. Therefore, we questioned whether nsP1 affinity for DRMs was conserved in cells containing functional CHIKV replicase complexes. To this end, HEK293T cells either transfected with a trans-replication system that reproduces CHIKV RNA replication (Fig 2B) or infected with the CHIKV-LR-5'GFP were used. DRM isolation followed by immunoblot analysis revealed that most of nsP1 expressed in transfected or infected cells (~85 and ~40%, respectively) was detected in DRMs (Fig 8A and 8B). NsP1 is the only alphavirus nonstructural protein with membrane affinity. In the replication complex, nsP1 co-localizes with nsP2, nsP3, and nsP4 (53–56). Thus, it is suspected to play a critical role in replication complex anchoring to the PM. Therefore, we investigated whether nsP1 capacity to segregate with

DRMs has an impact on the association of other nsPs with specialized membrane microdomains. Probing gradient fractions prepared from transfected and infected cells with antibodies against nsPs established that a significant part of each of them was detected in DS fractions thereby agreeing with the previously reported capacity of nsPs to be present in different cytosolic compartments (57). However, approximately 10% of total nsP3 and nsP4 levels were also present in raft fractions. In some, but not in all experiments, nsP2 was also detectable in DRMs; most probably this reflects weaker interaction of nsP2 with other components of replicase complexes. In order to assess the relevance of presence of nsP2, nsP3 and nsP4 in DRM fractions, these experiments were reproduced starting from cells transfected with a CHIKV trans-replicase system in which the nsP1 protein contained the C₄₁₈₋₄₂₀A mutation. As expected, this mutation prevented nsP1 association with DRMs (Fig 8C). Concomitantly, nsP2, nsP3 and nsP4 were also excluded from these fractions. Altogether these results confirm the capacity of nsP1, expressed in the context of trans-replicase or by infectious CHIKV, to associate with DRMs, an association that dictates targeting of other nsPs, albeit at low levels, to cholesterol-enriched membrane microdomain. Furthermore, the C₄₁₈₋₄₂₀A mutation that completely inactivates CHIKV trans-replicase (29) completely prevented association of CHIKV nsPs with DRM.

Conservation of nsP1-directed cholesterol affinity among divergent Old World alphaviruses.

The presence of palmitoylated cysteine residues in C-terminal region of nsP1 is a conserved feature of distantly related Old World alphaviruses (30, 33). Functional studies have highlighted differences with regard to cysteine requirement for genome replication: cysteine-to-alanine mutation are lethal for CHIKV (29, 30) but is well tolerated by SINV (33). In the light of such differences and of the herein evidenced role of the CHIKV nsP1 cysteines in lipid raft association, we next questioned whether SINV nsP1 behaves similarly with respect to cholesterol. To achieve this, a plasmid encoding a GFP-fused SINV nsP1 protein was generated (Fig 9A) and used for the transfection of HEK293T cells. Then, we tested SINV nsP1 partitioning to DRMs by membrane flotation assays. As previously observed for CHIKV nsP1, SINV nsP1 was also detected in the top fractions of

the density gradient supporting its ability to partition with cholesterol-rich membrane microdomains. However, contrasting with CHIKV for which almost 85% of nsP1 associated to DRM fractions, a roughly equal proportion of the SINV nsP1 was associated with DRMs and DS (Fig 9B). We further investigated SINV GFP-nsP1 affinity for cholesterol by testing its sensitivity to U18666A in confocal microscopy experiments. In control conditions, SINV GFP-nsP1 was detected at the PM, including in filopodia-like membrane protrusions that were abundantly observed as reported before for the untagged protein (42) and for CHIKV GFP-nsP1 (Fig 9C). In the presence of U18666A, SINV GFP-nsP1 was still mainly detected at the PM, while cholesterol stained with filipin was concentrated in intracellular storage compartments as expected. In these conditions, co-localization of GFP-nsP1 SINV with cholesterol-enriched endosomes was unfrequently detected. This is in contrast with the results obtained for CHIKV nsP1 (Fig 4D). Next, we investigated the role of palmitoylated cysteine by repeating these experiments starting from cells expressing a cysteine-to-alanine SINV nsP1 palmitoylation-negative mutant protein (GFP-nsP1_{C420A}). As shown in Figure 9B, the DRM association of GFP-nsP1_{C420A} was significantly reduced when compared with that of SINV GFP-nsP1 (Fig 8A). Analyzing SINV GFP-nsP1_{C420A} subcellular localization in U18666A treated cells confirmed that this mutant did not colocalize with filipin-labelled cholesterol (Fig 9D). Altogether these results suggest that SINV nsP1 is targeted to DRMs, a property that depends of palmitoylated cysteine as previously observed for CHIKV nsP1. However, compared with CHIKV counterpart, SINV nsP1 is equally abundant in DS compartments and displays only modest sensitivity to U18666A suggesting a reduced affinity for cholesterol.

Discussion

The present study identifies CHIKV nsP1 as a lipid-raft co-segregating protein with an affinity for cholesterol. We defined cysteine residues that can be palmitoylated as the molecular determinant important for this targeting. In the context of cells with ongoing CHIKV RNA replication, nsP1, together with a fraction of other nsPs, partitions with cholesterol-rich DRMs. Together with evidence that drugs reducing cholesterol availability at the PM impair CHIKV RNA replication, our results support that nsP1 targeting to

cholesterol-rich PM microdomains may have a functional importance for viral genome replication (Fig 10).

Cholesterol is a main component of membranes. Together with sphingolipids, it segregates into discrete microdomains, referred to as lipid rafts or DRMs, present both on the inner and the outer leaflet of the PM (58, 59). These membrane domains with a size on the nm scale are highly dynamic. They accumulate a subset of membrane proteins, mainly GPI-anchored proteins, transmembrane proteins, and acylated cell components (60, 61). Based on these properties, rafts were seen as platforms that compartmentalize cellular processes with an important function in receptor-ligand interaction, signal transduction and endocytosis (60). Herein, we establish that CHIKV nsP1 associates with the PM. In this compartment, a pool of nsP1 is targeted to cholesterol-containing DRMs, an association that was reversed by β MCD cholesterol-removal agent. Affinity for cholesterol was further supported by investigating nsP1 behavior with regard to U18666A- or imipramine-mediated cholesterol sequestration in LE/Ls. We found that intracellular cholesterol storage resulted in nsP1 accumulation in endosomes without consequence on overall nsP1 membrane binding ability. Altogether these results indicate that availability of cholesterol at the PM is required for appropriate targeting of CHIKV nsP1 to this compartment.

In the last decade, the biochemical or biophysical underpinnings that govern nsP1 association with membranes have been the focus of an intense attention. For SFV and SINV, the central α -helical motif in nsP1 spanning amino acid residues 245 to 264 was proposed as the main determinant for membrane anchoring to lipid bilayers with W₂₅₉ residue being critical for hydrophobic interactions with the phospholipid acyl chains (34). Conserved acylated cysteine(s) in nsP1 were proposed to tighten this membrane interaction (26, 28, 30, 33). Using cell fractionation assays, we show that both W₂₅₈A substitution in putative α -helix of CHIKV nsP1 and C₄₁₇₋₄₁₉A mutations indeed decrease nsP1 affinity for cell membranes. Interestingly, combining W₂₅₈A and C₄₁₇₋₄₁₉A mutations further reduced nsP1 membrane association thereby suggesting that in the CHIKV nsP1 the two domains may synergize for membrane interaction, a situation that was not described for other alphaviruses. Analyzing the contribution of these membrane

interaction determinants in nsP1 targeting to lipid rafts revealed that W₂₅₈A mutation had only a slight effect on nsP1 co-fractionation with cholesterol-rich domains. This mutation also slightly reduced PM association of nsP1 and facilitated its association with internal membranes. This may indicate that W258 residue is important for membrane/plasma membrane targeting of nsP1 but not for its palmitoylation, DRM association and enzymatic activity. How these properties correlate with the proposed role of W258 residue as one of membrane anchors of nsP1 is currently unclear. For the proper understanding of the somewhat controversial data regarding the importance of W258 residue, the structure of the membrane bound α -helical peptide of nsP1 (34) should be compared with the structure of membrane-bound enzymatically active nsP1 which, to the date, is yet available. In contrast to W₂₅₈A mutation the effects of C₄₁₇₋₄₁₉A mutation on CHIKV nsP1 were unambiguous. The mutation, previously reported to prevent CHIKV nsP1 palmitoylation and replication (29, 30), dramatically reduced DRM co-fractionation. Moreover, by contrast with wild-type nsP1, nsP1_{C417-419A} sequestration with Lamp2 in endosomes could not be observed under U18666A or imipramine treatment. According to these experiments, acylation appears as critical to direct nsP1 to cholesterol-enriched membrane microdomains. This result parallels previous evidence regarding the role of palmitoylation in cellular (p59^{fyn} and p60^{src}) (62) or viral (influenza hemagglutinin) (63) proteins association with rafts. This observation raised the question of the functional outcome of nsP1 association with cholesterol-rich DRMs.

Because other nonstructural proteins of alphaviruses cannot directly associate with membranes, nsP1 plays a decisive role in proper targeting and membrane binding of other nsPs involved in the formation and functioning of alphavirus replication complex (24). Starting from cells infected with CHIKV, we found that a fraction of each of the four nsPs was associated with DRMs. These results were confirmed in cells transfected with plasmids encoding for a CHIKV trans-replication system, in which other non-structural proteins co-sedimented with DRMs depending on the integrity of nsP1 C417-419 residues. Currently the functional importance of nsPs targeting to rafts is unknown. Nevertheless, we observed that in addition to the nsP1 palmitoylation-dependent sequestration into late endosomes, U18666A and imipramine also generated a significant

drop in CHIKV genome replication. This raises the question of the functional consequences of nsP1 mistargeting and of its impact on replication complexes assembly.

Over two decades, alphaviruses encoding nsP1 mutants with reduced membrane binding ability have been in the focus of different studies. They established that nsP1 palmitoylation had only a mild impact on SINV or SFV infectivity, highlighting the essential role of W259 in nsP1 central α -helix for both membrane anchoring and genome replication (31, 33). For CHIKV, W₂₅₈A mutant is viable, albeit having a temperature-sensitive phenotype (29). By contrast, C₄₁₇₋₄₁₉A mutation results in complete inactivation of the CHIKV replicase, leading to non-functional enzymes unable to synthesize any viral RNAs both in mammalian and insect cells (29, 30, 52). Here, direct comparison of SINV and CHIKV revealed that SINV nsP1 partitions with DRMs but to a lesser extent than observed for CHIKV nsP1. This phenotype was equally dependent upon palmitoylated cysteine in nsP1. Moreover, SINV nsP1 targeting to the PM was less sensitive to cholesterol manipulation by U18666A. These discrepancies therefore parallel the differences in nsP1 palmitoylation requirement reported for CHIKV and SINV replication (29). However, counterintuitively, SINV genome replication was also sensitive to U18666A and cholesterol sequestration (this study and (35)) albeit less than observed for CHIKV (data not shown). Conversely to CHIKV, this might not reflect dependency on PM rafts, but instead cholesterol-induced alteration of endosomes where SINV preferentially replicates as proposed by others (35, 64).

In the last decade, cholesterol-rich membrane microdomains, beyond their role in virus entry and exit, have also been identified as platforms for the assembly and anchoring of replication complexes produced by a broad range of RNA viruses including HCV, picornaviruses or flaviviruses (9, 10, 12, 18, 65). A dramatic reduction of viral RNA replication was observed upon membrane cholesterol extraction or in conditions reducing cholesterol availability (12, 14, 66, 67) establishing functional importance for this association. Besides changing membrane composition and fluidity, which may affect different interactions between virus-encoded replicase subunits, cholesterol partitioning in membranes also attract cellular proteins, with functions in cell signaling and intracellular trafficking. We can assume that targeting CHIKV nsP1 and possibly other nsPs at these

sites may favor interaction with raft-associated cellular factors indispensable for viral replication. The exact necessity for nsP1 partitioning to DRMs therefore will deserve further investigations.

Materials and Methods

Antibodies and reagents: The following antibodies/reagents and respective dilutions were used in this study: rabbit polyclonal antisera against CHIKV nsP1, nsP2, nsP3, nsP4 (all in-house, 1:1,000), monoclonal antibodies against GAPDH (1:1,000) (Santa Cruz Biotechnologies Inc.), Na⁺ K⁺ ATPase (1:50,000) (Ab76020, Abcam), GFP (1:1,000) (Chromotek), Lamp2 (1:1,000), CD81 (1:500) (Clone JS-81, BD Biosciences) and flotillin-1 (1:1,000) (BD Biosciences). Secondary antibodies conjugated to horseradish peroxidase or Alexa Fluor were purchased from Jackson ImmunoResearch and Thermo Fisher Scientific, respectively. Filipin III, U18666A, Lovastatin, methyl- β -cyclodextrin were purchased from Sigma-Aldrich. Imipramine was obtained from Abcam and WGA-647 from Thermo Fisher Scientific.

Cells: HEK293T cells (ATCC # ACS-4500), HeLa cells (ATCC # CRM-CCL2), BHK21 cells (ATCC # CCL-10) and U2OS cells (ATCC # HTB-96) used for propagation and Vero cells (ATCC # CCL-81) used for titration of the CHIKV were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fischer Scientific) supplemented with penicillin and 10% fetal calf serum (FCS, Lonza) and grown at 37°C in a 5% CO₂ atmosphere. Cell viability was measured using Cell Titer 96 Aqueous one solution cell proliferation assay (Promega) according to the manufacturer's protocol.

Viruses: The pCHIKV-LR-5'GFP, full-length molecular clone of CHIKV (LR2006OPY1 strain) with GFP reporter (68), was linearized and transcribed *in vitro* using the mMMESSAGE mMACHINE kit (Ambion-Life Technologies). 1 μ g of RNA was then transfected with lipofectamine 2000 (Thermo Fisher Scientific) into 10⁵ HEK293T cells and the cells were incubated at 37°C for 24 hrs. Culture medium was collected, and virus stock was amplified on BHK-21 cells. After 48 hrs at 37°C, the supernatant was collected, filtered through a 0.45 μ m membrane, aliquoted and stored at -80°C. Viral stocks were tittered using plaque assay as previously reported (39).

Infection with CHIKV-reporter viruses: The cells (70-80% confluence) were rinsed once with PBS before infection with CHIKV-LR-5'GFP diluted to achieve the desired MOI. For pre-infection experiments, the cells were pre-incubated with the virus for 1 hr or 2 hrs before drug addition. For post-infection experiments, the cells were incubated with drugs for 30 min before infection. After 24 hrs in culture, the cells were lysed with RIPA buffer. GFP reporter fluorescence was measured directly from the cell lysate using an Infinite F200PRO fluorometer (Tecan). Values were normalized to the protein content in the sample determined using the BCA Assay (Pierce).

Plasmids and transfection: Sequence encoding for CHIKV nsP1 was amplified by PCR using pCHIKV-LR-5'GFP as a template; obtained fragment was cloned into the pEGFP-C1 plasmid as previously described (69). Sequences encoding for a nsP1_{W258A}, nsP1_{C417-419A} and nsP1_{DM} were generated using the Quikchange site-directed mutagenesis kit by Agilent. SINV nsP1 and nsP1_{420A} were obtained using PCR and pTOTO1101 (70) or its derivative pSINV-C_{420A}1 (33) as templates. These inserts were cloned in frame with EGFP in the pEGFP-C1 plasmid. Cells were transfected with obtained plasmids using JetPei reagent (Polyplus Transfection) according to manufacturer recommendations.

Trans-replication assay: For trans-replication assays, CMV-P1234 (71) or CMV-SINV-P1234 (B. Götte, A. Utt, R. Fragkoudis, A. Merits, G.M. McInerney; submitted for publication (72)) plasmids encoding nonstructural polyprotein from CHIKV or SINV respectively were cotransfected together with the HSPoll-Fluc-Gluc (29) or HSPoll-SINV-tFluc-Gluc (72) template plasmids encoding for replication-competent template RNA of CHIKV or SINV containing firefly and *Gaussia* luciferase reporter sequences placed under control of genomic and subgenomic promoters respectively. Equal amounts of plasmids were transfected into HEK293T cells using JetPei transfection reagent. After 24 hrs in culture, cells were washed in PBS and lysed using Passive Lysis Buffer (Promega). Expression of firefly and *Gaussia* luciferase was determined using the Dual-Glo Luciferase Assay system (Promega) and a Spark luminometer (Tecan). Reporter activities were normalized to the protein content in the sample determined using the BCA Assay (Pierce).

Immunoblotting: Samples were separated by SDS-PAGE and then transferred to PVDF membrane (Hybond, Amersham). Membranes were blocked using 5% non-fat dry milk in PBS and probed with appropriate primary antibodies. After wash steps with PBS – 0.1% Tween 20, the membranes were probed with HRP-conjugated secondary antibodies. After final washes, the revelation was performed by incubating the membranes with either Luminata Forte (Merck) or Clarity Max (BioRad) and then image acquisition was done using a Chemidoc (Bio-Rad).

Cell fractionation and membrane flotation assays: Cells were incubated in hypotonic buffer (10mM Tris/HCl [pH 7.4], 10 mM NaCl supplemented with protease inhibitors) for 10 min on ice and then lysed with a Dounce homogenizer (30-40 strokes). The lysates were clarified by low-speed centrifugation at 1,000 g for 10 min. Obtained post-nuclear supernatants (PNS) were then adjusted to a final concentration of 500 mM NaCl and incubated for 30 min on ice. After ultracentrifugation at 25,000 g for 20 min, the cytosolic (supernatant, S25) and membrane fraction (pellet, P25) were collected. P25 samples were solubilized in lysis buffer composed of 1% Brij 96 in 20 mM Tris/HCl [pH 7.5] before analysis. For membrane flotation experiments, cells were resuspended in 250 mM sucrose in PBS supplemented with protease inhibitors and then lysed with a Dounce homogenizer (30-40 strokes). Cell lysates were spun at 1,000 g for 10 min to pellet the nuclei. The supernatant referred to as crude lysate (CL) was then adjusted to 30% iodixanol concentration by mixing Optiprep (Axis-Shield). CL (4 mL) was loaded at the bottom of a centrifuge tube, and then overlaid with 4 mL 20% iodixanol and then 4 mL 10% iodixanol. The gradient was then spun 200,000 g at 4°C for 16 hrs in a Beckman SW41 rotor. Finally, 24 fractions were collected from top to bottom.

Detergent-resistant membrane isolation: Cells were lysed on ice in TNE buffer (10 mM Tris/HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA) containing 0.5% Triton X-100 for 30 min. Lysates were then further treated with the Dounce homogenizer, and then clarified by low-speed centrifugation at 1,000 g for 10 min to obtain the PNS. PNS (0.5 mL) was adjusted to 60% sucrose by adding 1.5 mL of 80% sucrose TNE (w/v). The lysates were layered over 500 µL of 80% sucrose TNE, then covered with 2 mL of 50% sucrose TNE, 6 mL of 38% sucrose TNE, and 1.5 mL of 10% sucrose TNE. The sucrose gradients were

centrifuged at 100,000 g at 4°C for 18 hrs in a Beckman SW41 rotor (Beckman Coulter). Nine fractions were then collected and then analyzed by immunoblotting.

Immunofluorescence microscopy and image analysis: Cells grown on glass coverslips were washed with PBS and then fixed with 4% paraformaldehyde/PBS (Sigma Aldrich) for 10 min. For intracellular labeling, the cells were permeabilized with 0.1% Triton-X100 in PBS and blocked for 30 min with PBS containing 0.2% Bovine Serum Albumin. Incubation with primary antibody was performed for 1 hr. After washes with PBS, secondary reagents were added for 30 min. DAPI (Sigma-Aldrich) was used to stain the nuclei. Filipin or WGA-647 staining was performed by incubation at room temperature for either 1 hr or 10 min respectively. After final washes, coverslips were mounted with Prolong Gold antifade mounting media (Thermo Fisher Scientific). Images were acquired using a Leica SP5-SMD scanning confocal microscope equipped with a 63×, 1.4 numerical aperture Leica Aplanachromat oil lense at the Montpellier Resources Imaging platform. Image analysis was performed utilizing Fiji ImageJ and the JACoP plugin. 3D reconstruction was performed by Imaris software.

Statistical analysis: All of the analyses (unpaired Student's t-test) were performed using GraphPad Prism version 6 (GraphPad Software Inc.). A p-value of <0.05 was considered statistically significant. Following designations are used on figures: * p<0.05; ** p<0.001; ***p<0.0001; **** p<0.0001.

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Authors contribution

Conceptualization: LB, AM; *Methodology:* LB; *Funding acquisition:* LB; *Investigation-main experiments:* WB. *Investigations - additional experiments:* AN, EB; *Supervision:* LB; *Writing original draft:* LB, AM, WB.

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Competing interests

The authors have declared that no competing interests exist.

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Figure legends

Figure 1: Cholesterol homeostasis regulates CHIKV life cycle.

(A) Cholesterol metabolism and biosynthesis inhibitors. (B) Compounds affecting cholesterol metabolism inhibit CHIKV infection. HEK293T cells treated for 30 min with the indicated concentrations of (a) lovastatin, (c) U18666A, (e) imipramine were infected with CHIKV-LR-5'GFP at a MOI of 0.5. Infection was monitored after 24 hrs by quantification of GFP reporter in the cell lysates. For each condition cell viability was determined using Cell Titer Glo assay (b, d, f). Values are expressed as a percentage of control condition that was taken as 100%. Means of triplicate experiments \pm SEM are shown. p-values are calculated by comparing each treated-condition with the mock.

Figure 2: Cholesterol transport inhibitors regulate intracellular CHIKV replication.

(A) Post-infection addition of lovastatin, U18666A or imipramine reduces CHIKV replication. Increasing concentrations of drugs were added to HEK293T cells 1 hr after CHIKV infection at a MOI of 1. CHIKV replication was monitored by quantification of GFP fluorescence in the cell lysates. Values, normalized to the protein content in the samples, are expressed as a percentage of the non-infected (NI) condition. (B) Schematics of CMV-P1234 and HSPoll-Fluc-Gluc constructs, plasmid backbones are not shown. (C) HEK293T cells were transfected with plasmids depicted in (B) and treated with the indicated concentrations of lovastatin, U18666A or imipramine at 2 hrs post-transfection. Firefly and Gaussia luciferase activities were determined after 24 hrs in culture. Values are expressed as a percentage of the untreated condition and are means of triplicate experiments \pm SEM. NT designates non-transfected control cells. p-values are calculated by comparing each treated-condition with the mock.

Figure 3: CHIKV nsP1 associates with the PM through conserved sequence motifs.

(A) Organization of the CHIKV nsP1. Conserved amino-acids involved in the central α -helix (aa 244-263) and those required for nsP1 acylation (C417-419) are indicated. Mutants used in this study are depicted. (B) Membrane affinity of GFP-nsP1, GFP-nsP1_{3A},

GFP-nsP1_{W258A} and GFP-nsP1_{DM} was determined by cell fractionation assays. HEK293T cells transfected to express each GFP-fused protein were fractionated to produce cytosolic (S25) and membranous (P25) fractions. Equal amounts of fractions were analyzed by SDS-PAGE and western blotting with anti-nsP1 or anti-GFP antibodies. Antibodies against Na⁺/K⁺ ATPase and GAPDH were used to control the separation of membranes and cytosolic compartments respectively. For each experiment a sample of the unfractionated cell lysate (CL) was run in parallel to determine the relative amount of each nsP1 variant expressed. Cells expressing the GFP protein alone are shown as a control. Molecular mass markers are shown on the left. (C) The intensity of bands in S25 and P25 samples from panel B was determined using Image J software and plotted as a histogram. (D) Subcellular localization of GFP-nsP1 and its mutant variants was determined by confocal imaging of transfected HeLa cells. Cell membranes are labeled with Alexa Fluor 647-conjugated wheat germ hemagglutinin (WGA) and nuclei are stained with DAPI. Scale bars are 5 μm. (E) Confocal microscopy imaging of HeLa cells transfected with expression plasmids for nsP1-GFP or for an untagged nsP1. The untagged nsP1 protein was detected with rabbit antiserum and Alexa-647-conjugated secondary reagents. Nuclei were stained with DAPI before analysis by confocal imaging. GFP-nsP1 at the plasma membrane and filopodia-like structures formed at the surface of transfected cells are indicated by white arrows. Cytosolic aggregates are indicated by blue arrows. Bars: 5 μm. (F) Lysates of cells expressing GFP-nsP1, GFP-nsP1_{3A}, GFP-nsP1_{W258A}, GFP were subjected to membrane flotation assay in iodixanol gradient. Fractions collected from top to bottom were separated on SDS-PAGE and probed with antibodies specific for nsP1. Lysates from GFP expressing cells were probed with antibodies specific for GFP, flotillin-1 (FLOT1) or Na⁺/K⁺ ATPase. Data are representative of three separate experiments.

Figure 4: Cholesterol trafficking inhibitors redirect nsP1 to endo/lysosomal compartments.

(A) HeLa cells cultured in the presence of 12μM U18666A were stained with filipin III to visualize cholesterol distribution. Cells cultured in medium containing an appropriate amount of vehicle are shown as control (Mock). (B) Consequences of U18666A treatment

on GFP-nsP1 membrane binding capacity was determined by cell fractionation assays. Crude lysates (CL) and cytosolic fractions (S25) prepared from mock-treated or U18666A-treated HEK293T cells were separated on SDS-PAGE and probed with antibodies against GFP or GAPDH. (C) Cells expressing the GFP-nsP1 protein were maintained for 24 hrs in the presence of 12 μ M U18666A and then co-labeled with filipin III and anti-Lamp2 antibodies to visualize cholesterol and endo/lysosomes respectively. Cells maintained with vehicle alone are shown as controls (Mock). (D) HeLa cells transfected with GFP or GFP-nsP1 expression plasmid and maintained with 5 μ g/mL U18666A or in medium alone (Mock) for 24 hrs were stained with filipin III and DAPI and then processed for confocal imaging. (E) Histograms indicate the signal intensity profile of filipin III and green fluorescence along the white lines in (D). Bars: 5 μ m.

Figure 5: GFP-nsP1_{W258A} and GFP-nsP1_{3A} display different sensitivity to U18666A-induced cholesterol storage defect.

HeLa cells expressing either GFP-nsP1_{3A} (A) or GFP-nsP1_{W258A} (C) were incubated with 12 μ M U18666A for 24 hrs. Unesterified cholesterol was stained with filipin-III and cells were processed for confocal microscopy. (B) and (D) Histograms showing filipin III and GFP fluorescence intensity along the white lines indicated in (A) and (B) respectively. Bars: 5 μ m.

Figure 6: Palmitoylated cysteines are key determinants for CHIKV nsP1 targeting to CD81-positive cholesterol-rich compartments.

U2OS cells expressing (A) the GFP, (B) GFP-nsP1, (C) GFP-nsP1_{3A} were cultured in control condition or in medium supplemented with 12 μ M U18666A or 75 μ M imipramine for 24 hrs. Then, the cells were probed with anti-CD81 mAbs and Alexa Fluor-594-conjugated secondary antibodies. Nuclei were stained with DAPI and the cells were processed for confocal microscopy. Scale bars are 5 μ m. (D) Quantification of the degrees of colocalization of GFP fluorescence and CD81 staining in treated cells was determined by calculation of Mander's overlap coefficients. The total number of cells per condition was 10. (E and F) 3D image reconstruction was performed from z-stacks acquired from treated cells expressing either GFP-nsP1 or GFP-nsP1_{3A}.

Figure 7: CHIKV nsP1 partitioning with lipid rafts in human cells requires palmitoylated cysteines

(A) HEK293T cells were transfected with plasmids encoding GFP-nsP1 and processed for raft flotation. Nine fractions of the density gradients, numbered from top to bottom, were collected and analyzed using SDS-PAGE. Proteins were probed with antibodies against nsP1, FLOT1 and Na⁺/K⁺ ATPase. Fractions containing detergent-resistant membranes (DRM) and detergent soluble membranes (DSM) are indicated. (B) The amount of GFP-nsP1 and FLOT1 associated with each fraction was determined by densitometry scanning of immunoblots and expressed as a percentage of total protein expression level. The diagram is representative of three experiments. (C, D) A similar experiment was performed starting from cells incubated for 30 minutes in the presence of 10 mM β MCD. (E) Cells expressing the GFP-nsP1_{3A} or GFP-nsP1_{W258A} mutants were processed as in (A). (F) Amounts of GFP-nsP1, GFP-nsP1_{3A} or GFP-nsP1_{W258A} in DRM and DS fractions were determined by densitometry scanning of the immunoblots shown in (A) and (E) and expressed as a percentage of total protein signal.

Figure 8: nsPs expressed by a CHIKV trans-replicase or an infectious CHIKV co-segregate with DRMs depending of nsP1 palmitoylated cysteines.

DRM were isolated from (A) HEK293T cells transfected with CMV-P1234 and HSPoll-Fluc-Gluc plasmids (depicted in Fig 2B); (B) Cells infected with CHIKV-LR-5'GFP (MOI 0.1); (C) cells transfected with CMV-P1_{3A}234 and HSPoll-Fluc-Gluc plasmids. Fractions were prepared and analyze as described for Fig 7A, immunoblots were probed with antibodies against each CHIKV nonstructural protein as indicated.

Figure 9: SINV nsP1 affinity for cholesterol.

(A) Organization of the SINV nsP1 and mutant studied. Conserved amino-acids involved in the central α -helix (aa 245-264) the conserved palmitoylated cysteine (C420) are indicated. Mutant used in this study is depicted. (B) HEK293T cells transfected with either SINV GFP-nsP1 or GFP-nsP1_{C420A} plasmids were processed for DRMs isolation as

described for Fig 7. Fractions were probed in immunoblot with antibodies against GFP. Fractions are numbered from top to bottom. (C) HeLa cells were transfected with a plasmid encoding a SINV GFP-nsP1 protein for 4 hrs and cultured for an additional 24 hrs in the presence of 12 μ M U18666A. Cells were labeled with filipin III to visualize cholesterol. Controls cells (Mock) were maintained with vehicle. (D) HeLa cells transfected to express the GFP-nsP1_{C420A} mutant were processed as in (C). Bars: 5 μ m.

Figure 10: Model for nsP1/cholesterol interplay in transfected and CHIKV-infected cells.

a) Palmitoylation of wild type nsP1 is critical for targeting to cholesterol-rich membranes microdomains. In infected cells, the preferential association of nsP1 with DRMs increases its local concentration which may facilitate its oligomerization and dictates the localization of a fraction of other nsPs to lipid rafts. In this scenario CHIKV efficiently replicates its genome. **b)** nsP1_{3A} that weakly interacts with the plasma membrane is not targeted to DRM microdomains. In this situation CHIKV genomes replication is impaired. **c)** Imipramine and U18666A, by inhibiting cholesterol trafficking to the PM, generate nsP1 accumulation in Lamp2-positive late endosomes/lysosomes. Association of nsP2, nsP3 and nsP4 to cholesterol rich microdomains is no more observed. Under these conditions, CHIKV genome replication is abolished by a mechanism that remains to be fully elucidated. For simplicity, only mature nsPs are shown for this model; in infection the membrane association begins by modification and membrane targeting of polyprotein precursors.

Fig. 1 Cholesterol homeostasis regulates CHIKV life cycle

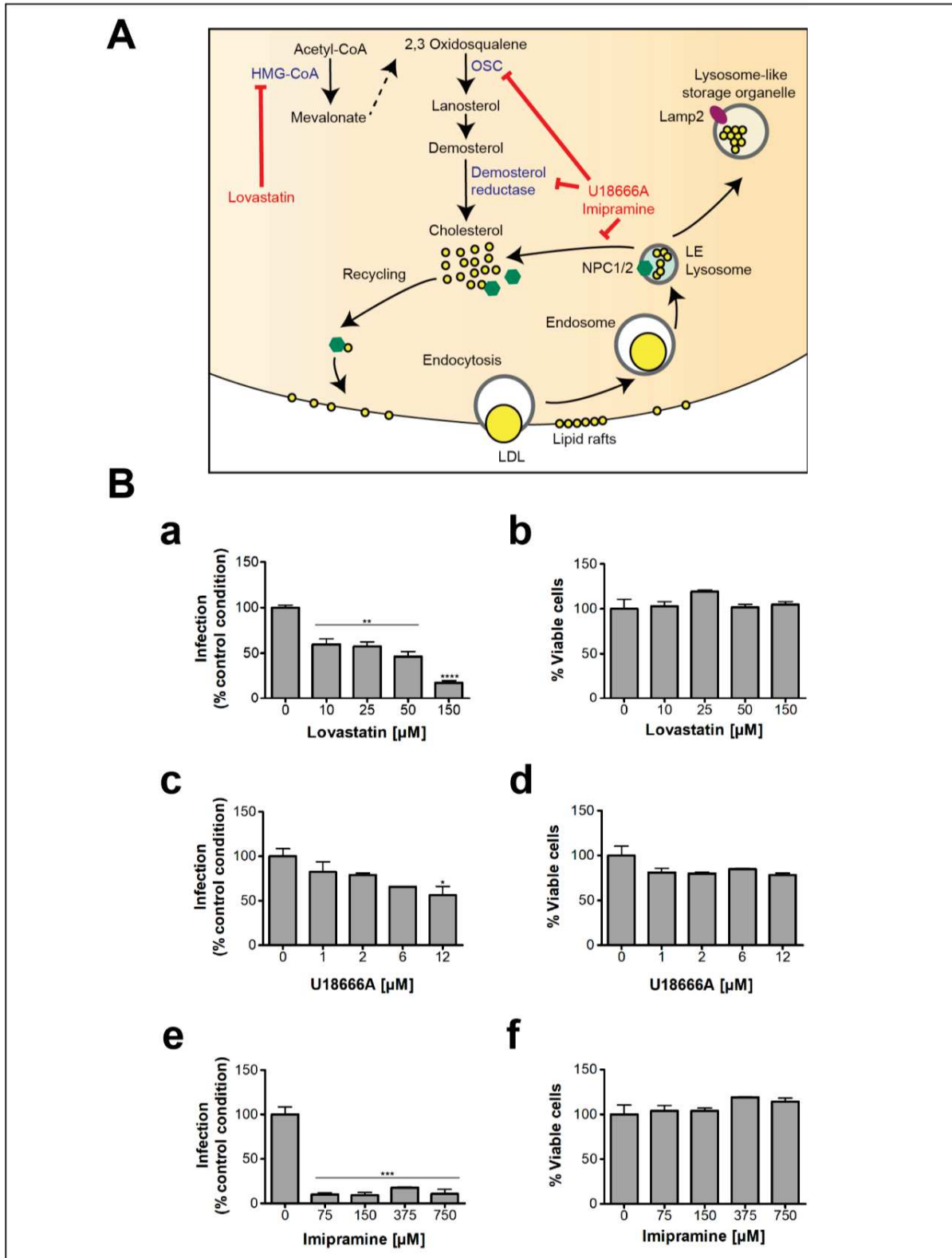


Fig. 2 Cholesterol transport inhibitors regulate intracellular CHIKV replication

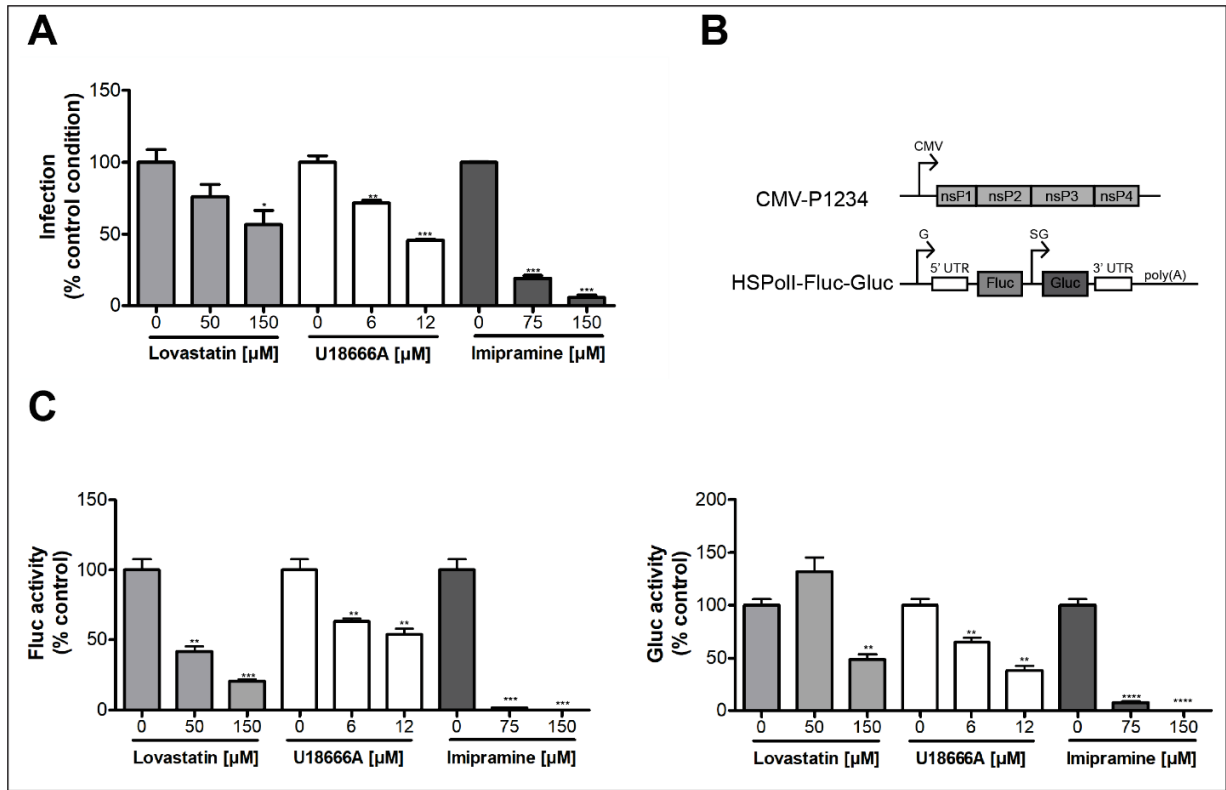


Fig. 3 CHIKV nsP1 associates with the PM through conserved sequence motifs

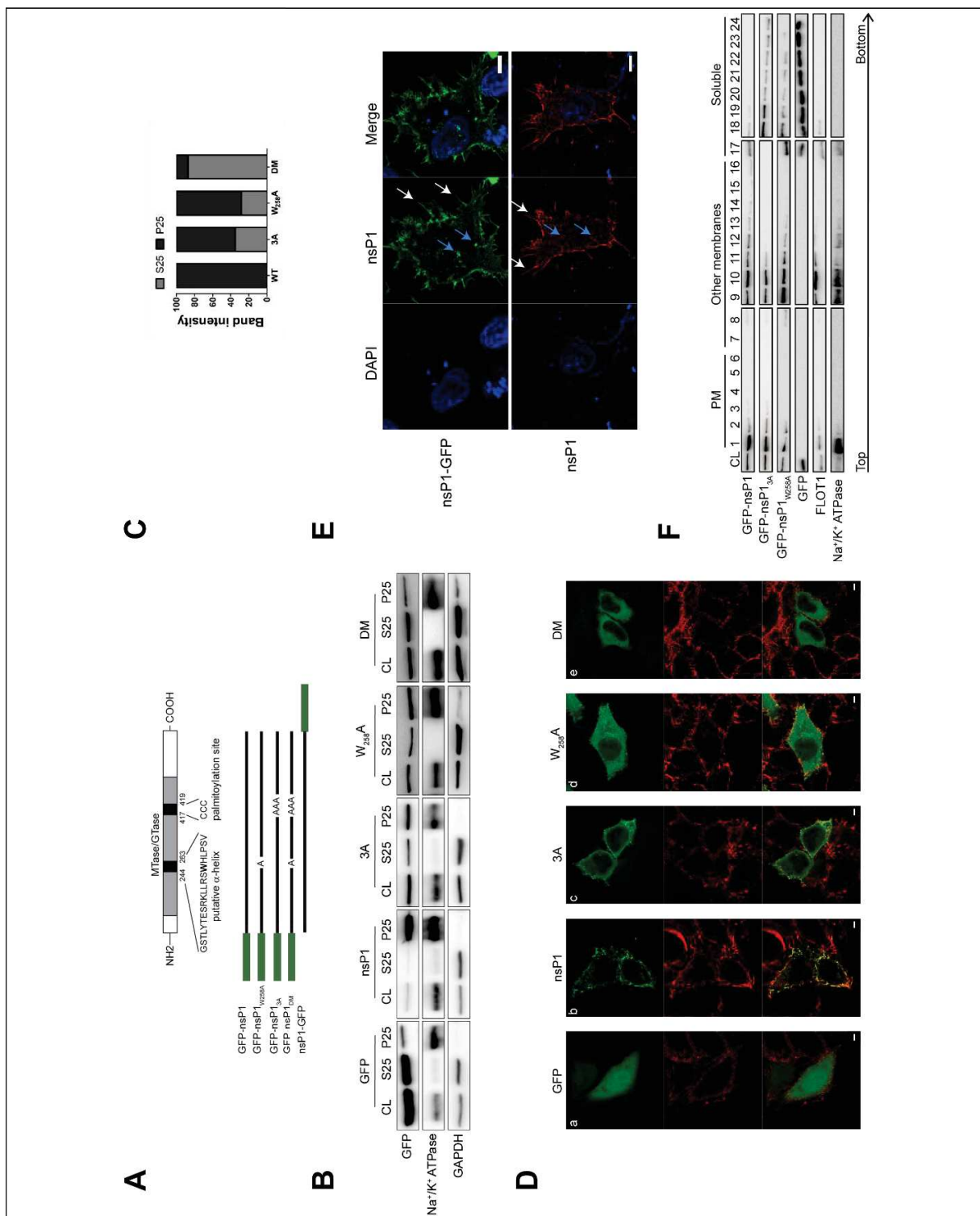


Fig. 4 Cholesterol trafficking inhibitors redirect nsP1 to endo/lysosomal compartments

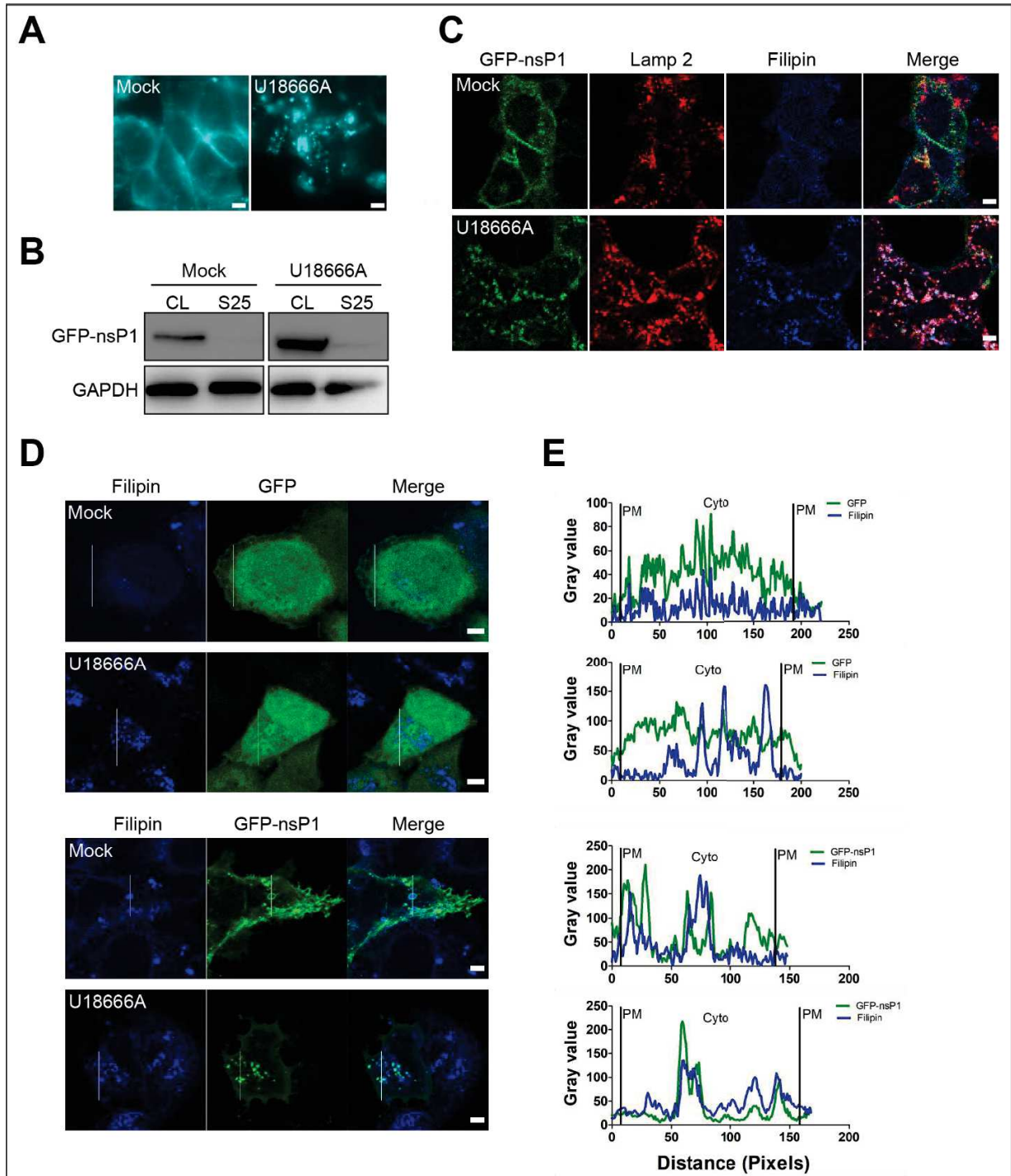


Fig. 5 GFP-nsP1_{W258A} and GFP-nsP1_{3A} display different sensitivity to U18666A-induced cholesterol storage defect

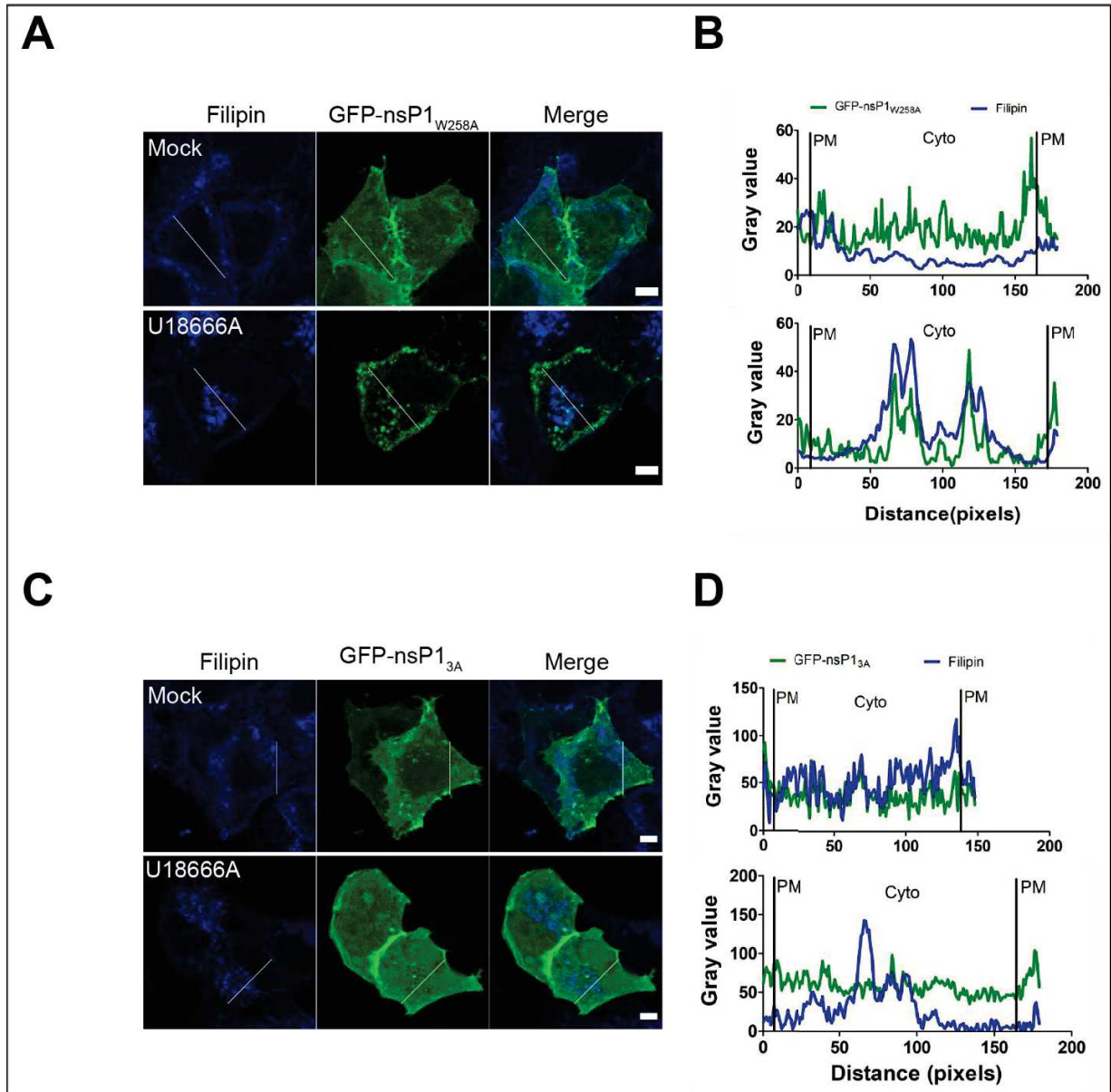


Fig. 6 Palmitoylated cysteines are key determinants for CHIKV nsP1 targeting to CD81-positive cholesterol-rich compartments

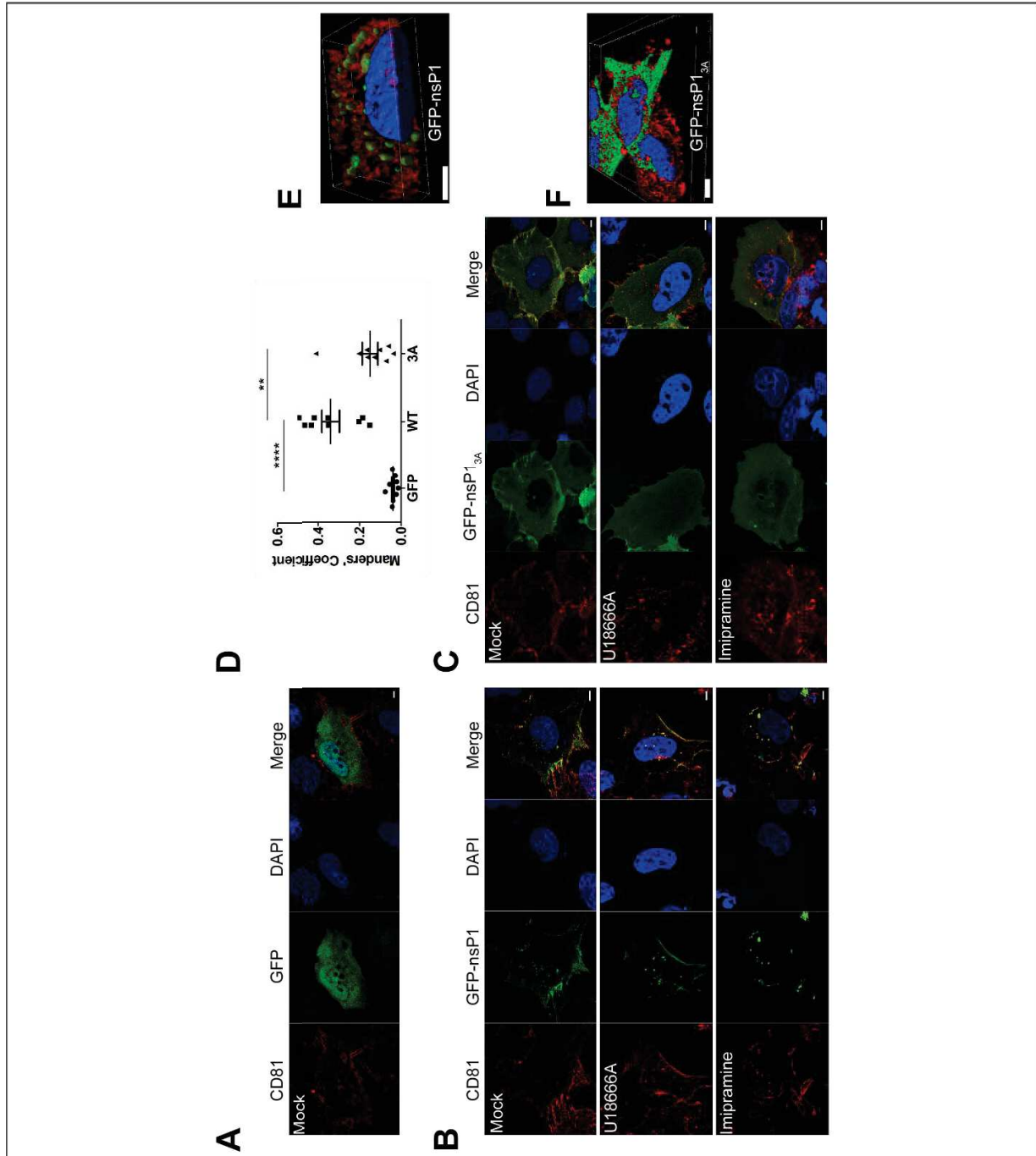


Fig. 7 CHIKV nsP1 partitioning with lipid rafts in human cells requires palmitoylated cysteins

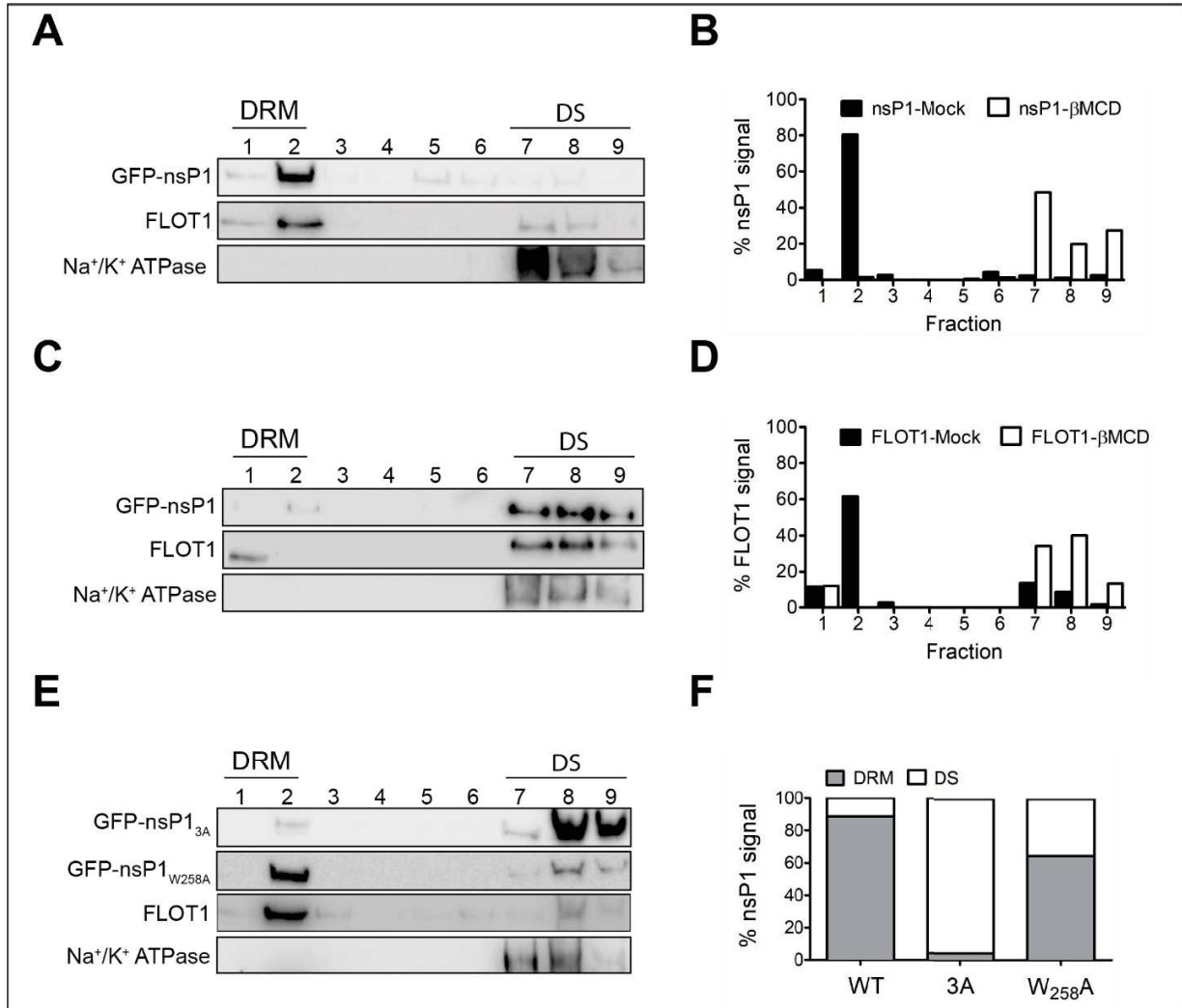


Fig. 8 nsPs expressed by a CHIKV trans-replicase or an infectious CHIKV co-segregate with DRMs depending on nsP1 palmitoylated cysteines

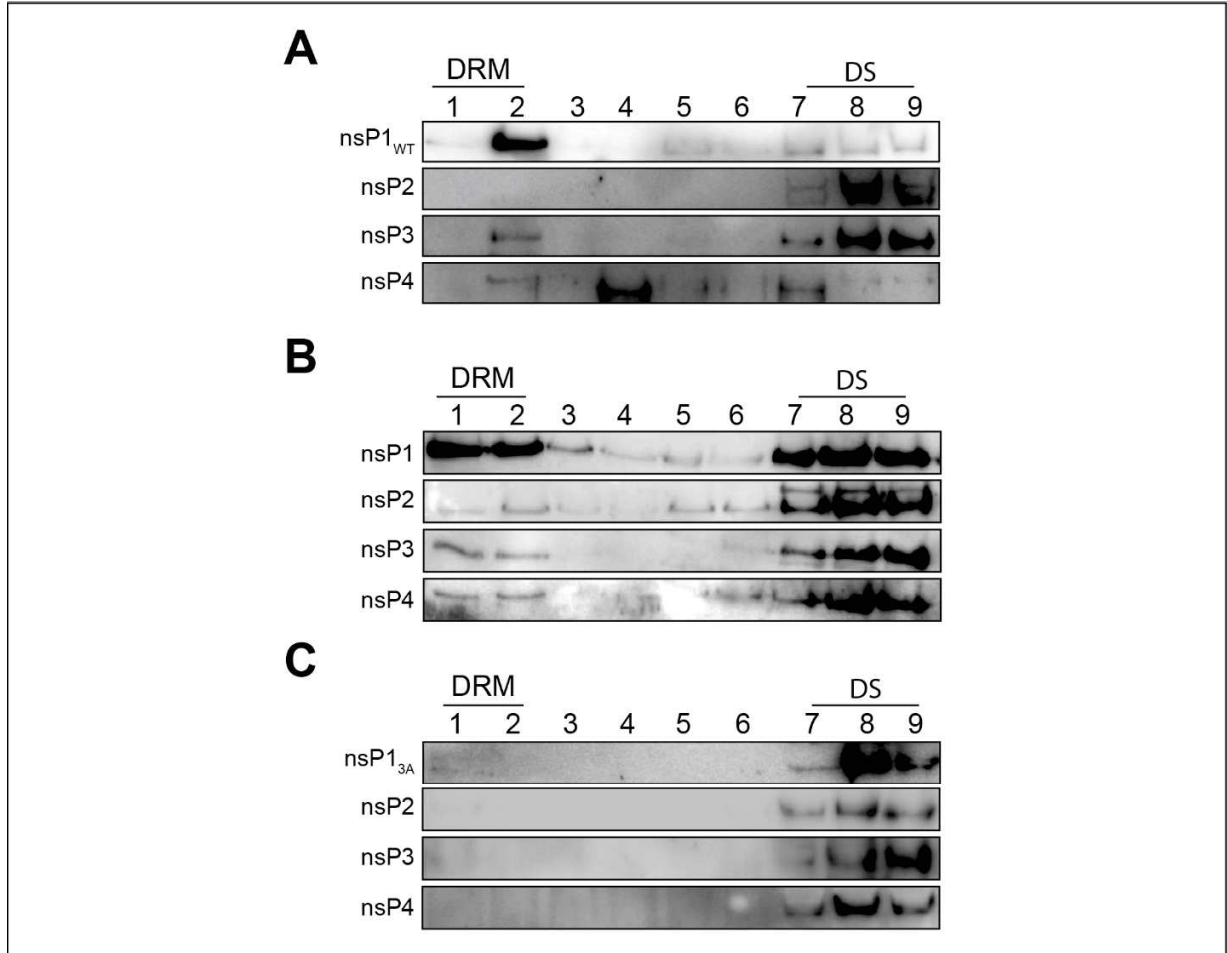


Fig. 9 SINV nsP1 affinity for cholesterol

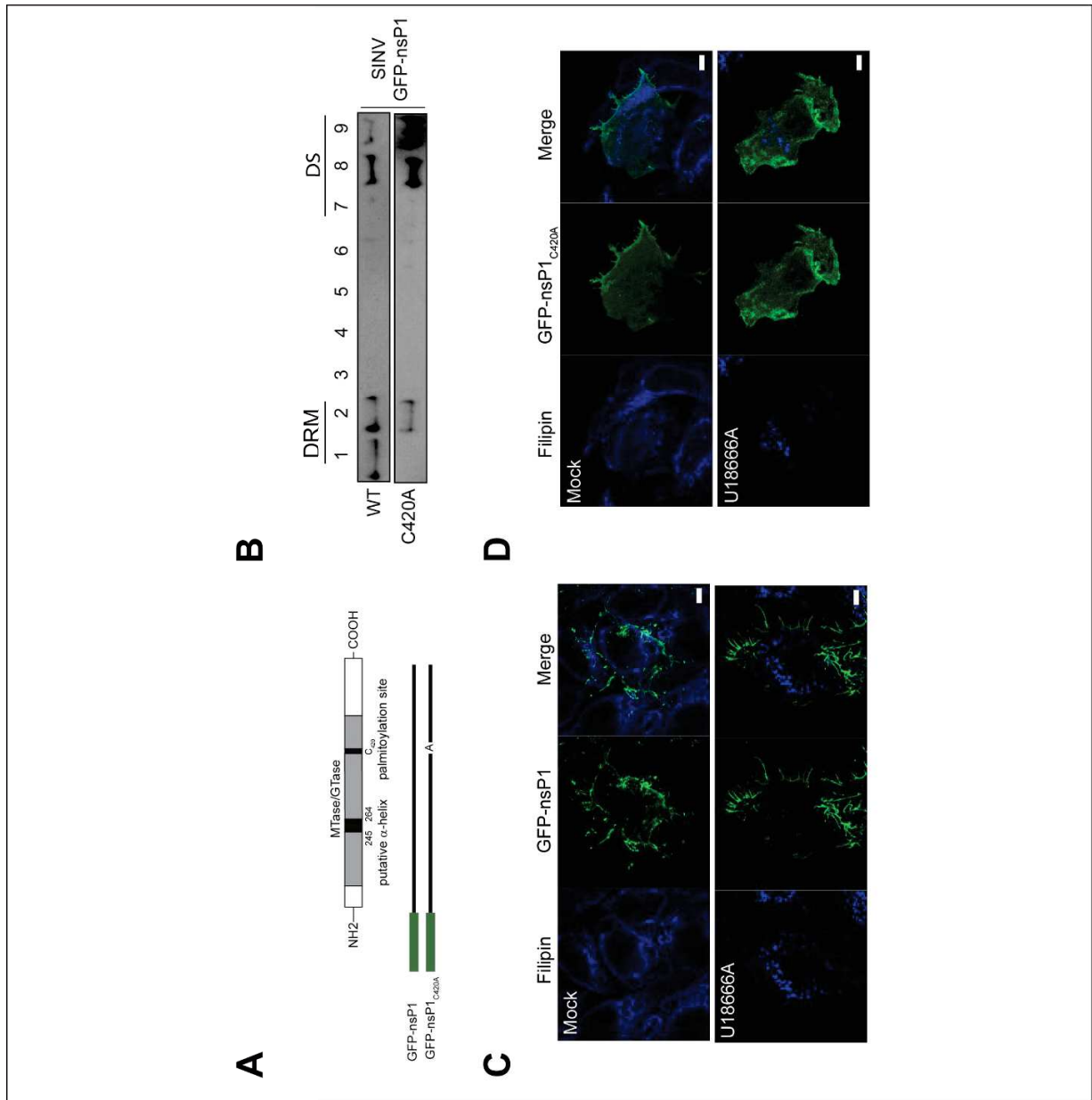
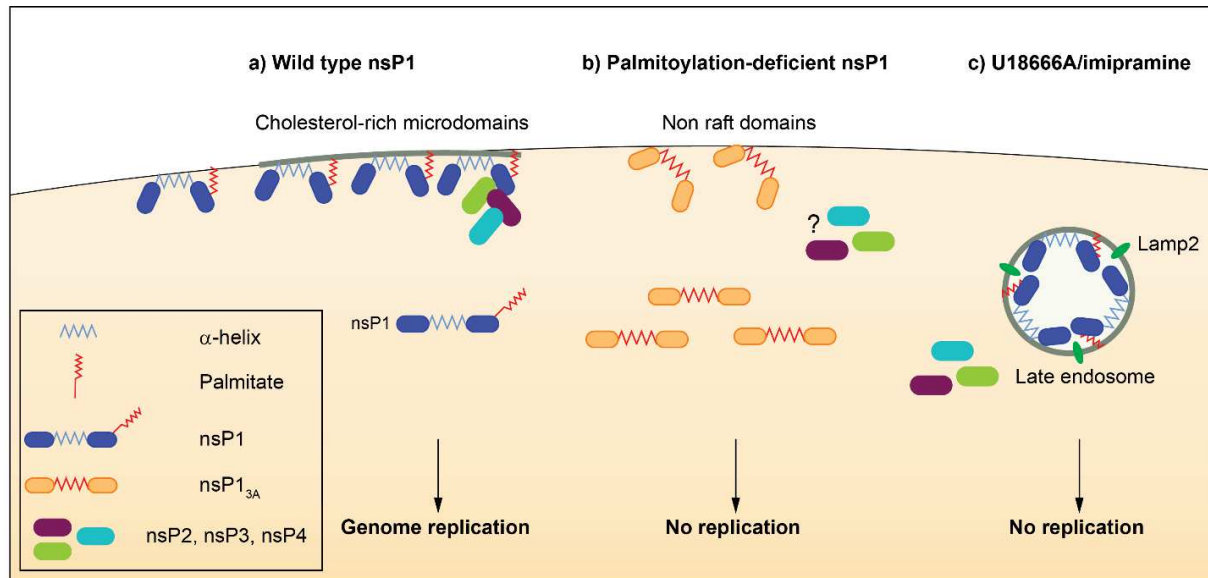
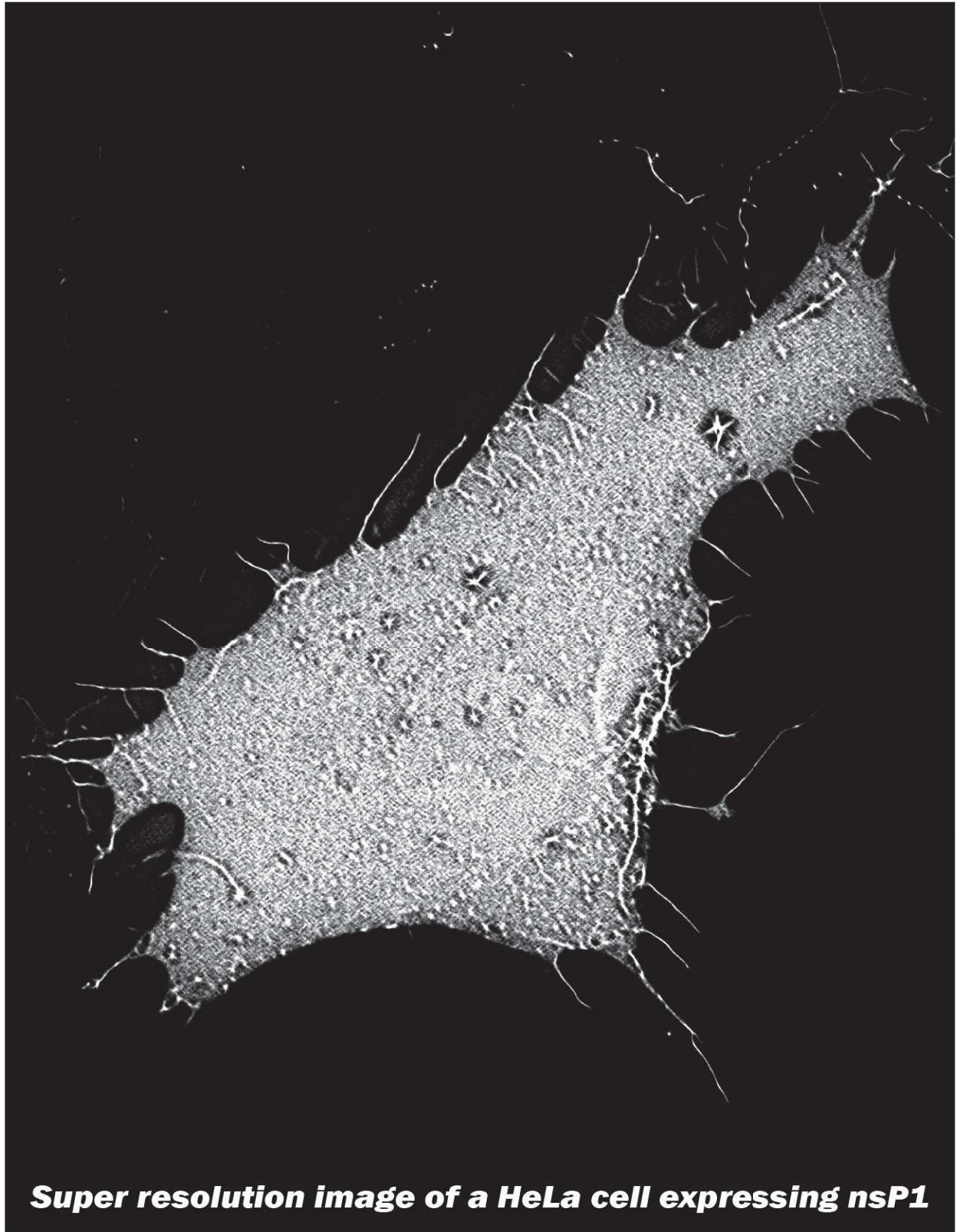


Fig. 10 Model for nsP1/cholesterol interplay in transfected and CHIKV-infected cells





Super resolution image of a HeLa cell expressing nsP1

Part II Studying the mechanism behind nsP1-induced membrane deformation

Project C Implication of host-cofactors in Chikungunya virus nsP1-induced membrane protrusions

i. Introduction and objectives

Infection with *Alphaviruses* leads to the formation of membranous structures called spherules which are sites of active genome replication. Spherules are 50-70 nm electron translucent bulb-shaped organelles that result from negative curvature of the plasma membrane. They are in continuity with the lipid bilayer and stay connected to the cytosol through a narrow opening estimated at about 8-10 nm.

Although elaborate information exists on the formation of spherule structures by other (+)RNA viruses, the host machinery for the formation of *Alphavirus* spherules remains a mystery. This motivated the design of this study with the strategy of focusing on CHIKV nsP1 which, when expressed alone, has the capacity to deform membranes forming filopodia-like structures. This nsP1 function, designated this nsP as an important actor for formation of these structures. And, thus, the idea is that by studying the mechanism behind nsP1 membrane protrusions, this information can then be later expanded to understanding the process of formation of *Alphavirus* spherules.

To introduce this study, the concept of actin-driven membrane protrusions will be detailed with a special emphasis on the molecular machinery and cytoskeletal components involved in the formation of filopodia structures.

Actin-driven membrane protrusions

Cells maintain their structure through components forming the cell's "skeleton" otherwise known as cytoskeleton. The cytoskeleton consists mainly of three different elements (actin, microtubules and intermediate filaments) which differ in function and protein composition. These components not only ensure that the cell conserves its shape, but will also play a central role in the movement of a cell. One of the most well described machineries involved in cellular movement are actin-driven membrane protrusions. In this

case, the three main type of projections described in the literature are filopodia, lamellipodia and stress fibers. Filopodia are thin extensions of the plasma membrane while lamellipodia are sheet-like protrusions that form at the edge of cells. Finally, stress fibers appear as filaments or fibers inside the cell (Hall, 1998) (Fig. 27). Actin is central in the formation of all these types of structures. However, in order to fulfill its functions, actin initially produced in a monomeric form named globular actin (G-actin) will polymerize into filaments denoted as F-actin allowing the generation of the forces needed to form such cellular protrusions. While actin is the central player in this process, molecular motors and regulatory machineries decide the type of protrusions formed. In this context, Rho GTPases are the master regulators of these processes.

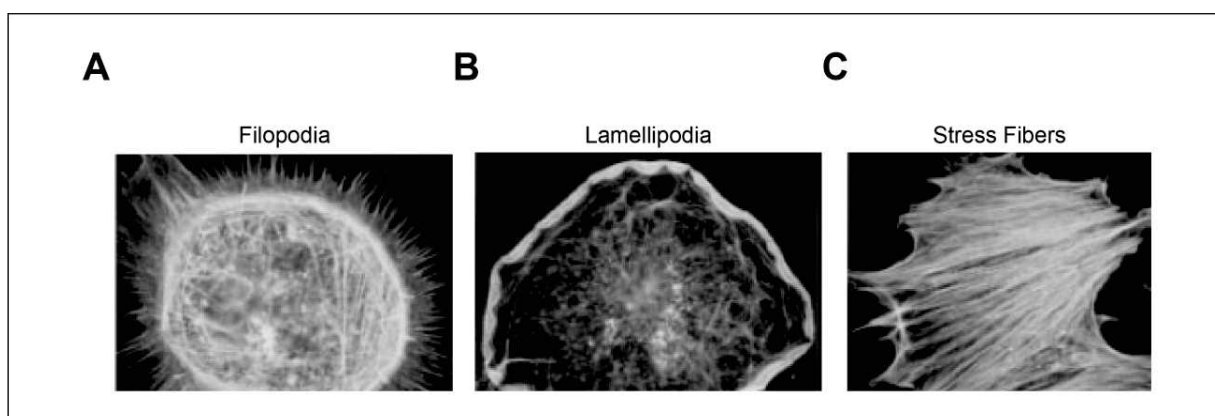


Fig. 27 Actin-driven membrane protrusions

Swiss 3T3 fibroblasts were either microinjected with (A) FGD1 leading to activation of Cdc42 (B) constitutively active Rac1 or (C) treated with lysophosphatidic acid leading to Rho activation. Actin filaments were visualized through rhodamine phalloidin staining (adapted from Hall, 1998).

Rho GTPases and the actin cytoskeleton

The Rho family of GTPases is a family of small (~21 kDa) signaling G proteins which sense and mediate extra-cellular signals by regulating numerous biological processes. This family of proteins, as all G proteins, are molecular switches having two different conformations (1) inactive conformation: Rho GTPase bound to guanosine diphosphate (GDP) and (2) active conformation: Rho GTPase bound to GTP (Fig. 28). These molecular

switches are under the control of two types of antagonistic regulators: the GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GEFs stimulate the activation of Rho GTPases by their ability to exchange GDP to GTP while GAPs stimulate the intrinsic ability of Rho GTPases to hydrolyze GTP leading to a switch to an inactive conformation. There exists also another layer of regulation of Rho GTPases through the action of guanine nucleotide dissociation inhibitors (GDIs). GDIs sequester Rho GTPases in their GDP-bound state modifying their intracellular localization. The most well studied Rho GTPases for their role in actin-based membrane protrusions are Cdc42, Rac1, and Rho where each has been described to lead to the formation of different types of structures. Cdc42 has been implicated in the formation of filopodia structures, Rac1 for lamellipodia formation, and Rho for stress fiber formation. It is important to note that such pathways could intersect as it has been observed, for example, that Cdc42 can activate Rac1 (Moon and Zheng, 2003).

Activation of these GTPases will lead to binding to their downstream effector proteins which can then exert their effect on the actin cytoskeleton. In the next part, the knowledge accumulated on filopodia formation through this pathway will be introduced.

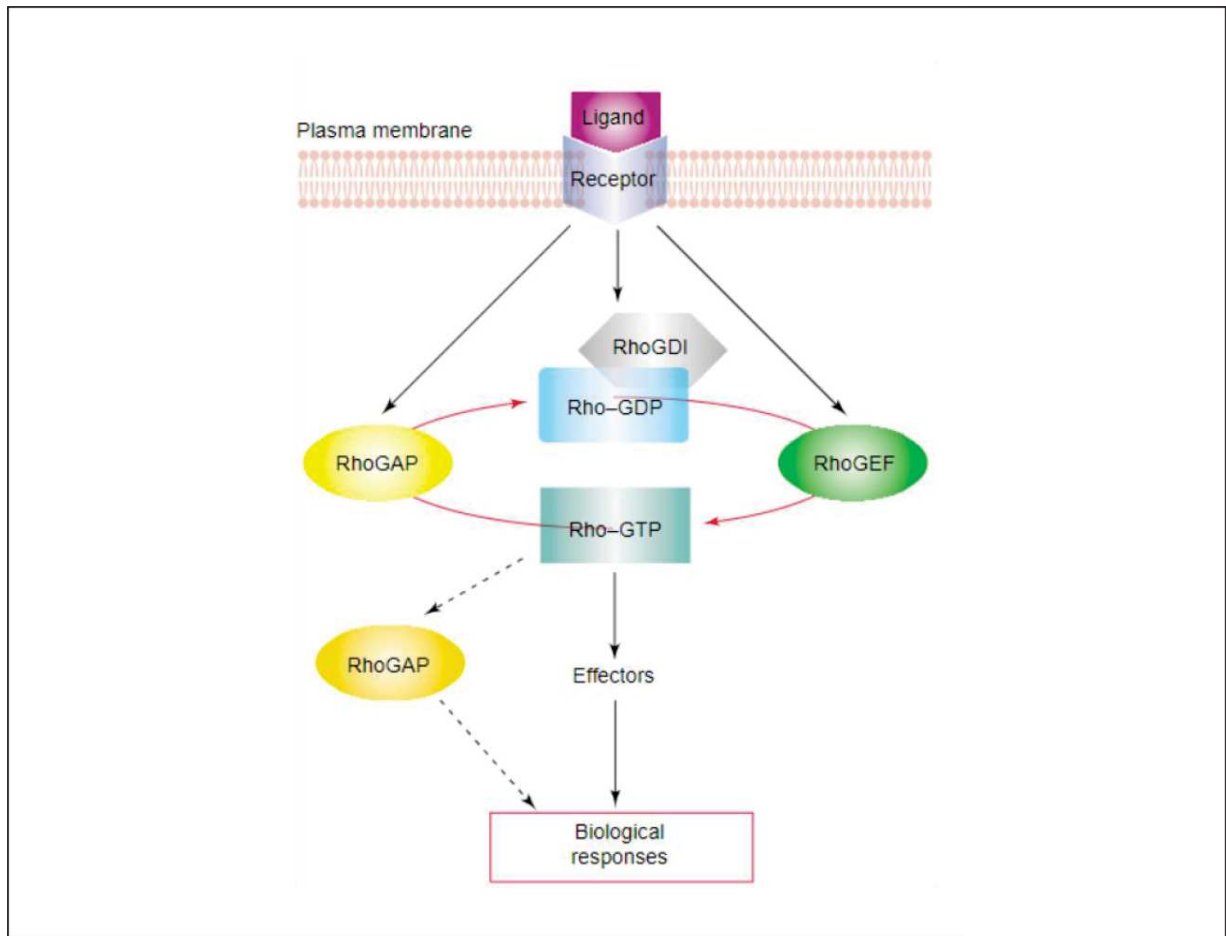


Fig. 28 Regulation of Rho GTPase activity

Rho GTPases are regulated by three different class of regulators: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide disassociation inhibitors (GDIs). The activity of these three class of regulators are under the control of extra-cellular signals. GEFs are responsible for the activation of Rho GTPases by stimulating the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP). On the other hand, GAPs will stimulate the hydrolysis of GTP to GDP leading to Rho GTPase inactivation. The final class of regulator proteins, GDIs will sequester Rho GTPases in their inactive form. Activation of Rho GTPases will stimulate effectors that will then exert different biological outcomes (adapted from Moon and Zheng, 2003).

Filopodia formation

As mentioned earlier, filopodia are thin membrane protrusions with 100–200 nm in diameter and reaching up to 10 μ m or more in length. Structurally, a filopodia is composed of a base, shaft and a tip. Inside these protrusions, filaments of actin are packed in parallel arrays. These extensions are implicated in the sensing of the extra-cellular environment,

and subsequently transmitting information to the cell about its surroundings. This is supported by the small surface area present at the tip of the filopodia making them ideal as sensitive “finger-tips” of a cell. Additionally, filopodia have been implicated in cell to cell transmission in the case of viral infections.

Much of the knowledge accumulated on filopodia formation is based on studies dealing with the contribution of these organelles to the morphology of neural growth cones. As mentioned previously, the formation of filopodia are under the action of the Rho GTPase Cdc42. Upon activation, this Rho GTPase will recruit the cellular machinery required for the formation of these structures. One of these types of effector proteins are elongation factors (Fig. 29). These proteins are actin nucleators that will be responsible for the elongation of actin filaments generating the force required for the creation of filopodia structures. Formins and Ena/VASP proteins form the integral part of this type of effector proteins. Another effector protein essential, this time, for the stability of filopodia is the protein Fascin. Fascin is capable of cross-linking actin filaments into a bundle giving a certain rigidity and stability for filopodia. Finally, filopodia structures at the tip require highly curved membranes. Here comes the role of the final type of effector protein which are BAR domains. These type of proteins with IRSp53 being the most studied will be recruited at the tip of filopodia conferring the membrane curvature present at the tip of these structures. IRSp53 together with elongation factors are highly enriched at the tip of filopodia and will form what is called the “tip complex” (Mellor, 2010; Yang and Svitkina, 2011).

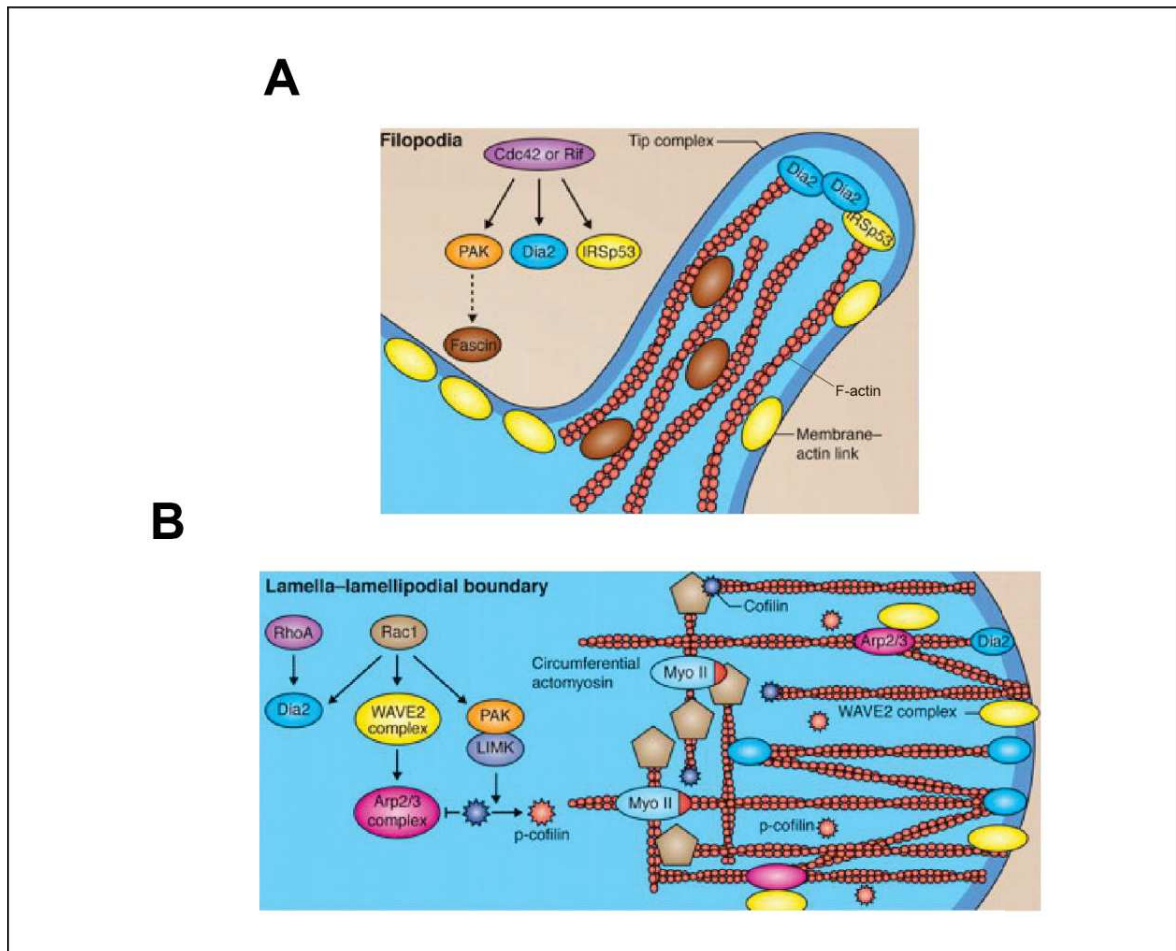


Fig. 29 Rho GTPase signaling and actin organization

(A) After activation, Cdc42 can bind and activate numerous downstream effector proteins to promote the formation of filopodia. Three type of effector proteins are mainly recruited for filopodia formation (1) Actin crosslinkers such as Fascin, (2) Actin nucleators or elongation factors such formins and (3) membrane curvature proteins such IRSp53. (B) Rac1 is master regulator for lamellipodia formation. This Rho GTPase is responsible for recruiting the Arp2/3 complex which is capable of branching actin by creating new nucleation cores. Another effector family of proteins for lamellipodia formation are LIM kinases (LIMK). These proteins phosphorylate and inactivate cofilin, a protein that is responsible for actin filament severing and depolymerization (adapted from Sit and Manser, 2011).

Although, the effectors studied for the formation of filopodia are well established, there still exists controversy on the initiation of these structures where mainly two models exist: (1) the tip nucleation model and the (2) convergent elongation model (Fig. 30). The first model suggests that actin nucleators cluster at a specific region at the plasma membrane that will then elongate filaments of actin and therefore generate the force required for

these membrane protrusions. The convergent elongation model suggests that pre-existing branched actin will act as a base for filopodia formation. Branched-actin is mainly present during lamellipodia formation and is therefore under the control of Rac1. A main effector protein for the formation of lamellipodia is the Arp 2/3 complex which is responsible for the branching of actin. Going back to the convergent elongation model, the barbed ends of branched actin will serve as binding spots for actin nucleators allowing then the elongation of parallel filaments of actin and filopodia formation (Yang and Svitkina, 2011). This model has gained popularity due to the unexpected finding that the Arp 2/3 complex and hence branched actin play an important role in filopodia formation (Korobova and Svitkina, 2008).

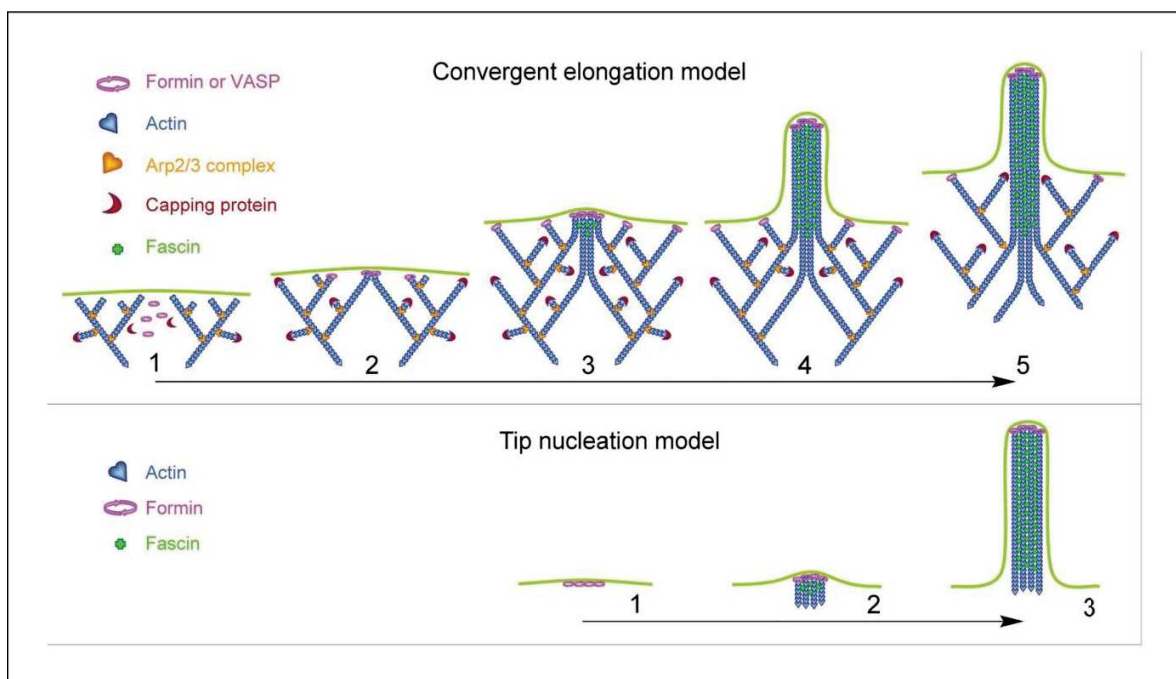


Fig. 30: Model for filopodia initiation

(Top) *Convergent elongation model: (1) Branched actin previously formed through the Arp2/3 complex will serve as a base for (2) binding of actin nucleators such as formins and VASP at barbed ends (3) These nucleators will promote the parallel elongation of converged actin filaments which are then (4) crosslinked by the action of Fascin. (5) With time, the Arp2/3 complex will dissociate leaving free ends of actin filaments.* (Bottom) *Tip nucleation model: (1) Activated formins will cluster at the plasma membrane and will (2) promote actin polymerization. (3) Actin filaments are crosslinked by the action of Fascin leading to the formation of an actin bundle (adapted from Yang and Svitkina, 2011).*

Finally, filopodia are highly dynamic structures which can typically grow at a speed of 0.2 $\mu\text{m/s}$ until reaching its critical length. The elements regulating filopodia's indefinite growth are named capping proteins. These proteins will cap actin at the tip complex and therefore stop filopodia extension promoting retraction of these structures (Mellor, 2010).

Our investigations on the machinery behind nsP1 induced filopodia-like structures will now be presented.

ii. Results

nsP1 induces the formation of dynamic filopodia-like extensions in a palmitoylation-dependent manner

As mentioned in the introduction, SINV and SFV nsP1 have been previously described to form filopodia-like extensions upon ectopic expression. This function has been reported to be dependent on nsP1 palmitoylation (Laakkonen et al., 1998). However, this membrane deformation capability has not yet been demonstrated in the CHIKV model.

In order to extend this function to CHIKV nsP1, HeLa cells were transfected with plasmid encoding the GFP-fused CHIKV nsP1 protein (GFP-nsP1) and were then processed for z-stack confocal imaging. Abundant filopodia-like extensions of variable length were observed at the bottom stacks of GFP-nsP1 transfected cells (Fig. 31A). For simplicity, these structures will be referred to as nsP1 induced filopodia (NIF). nsP1 signal was present all along these NIF structures. Interestingly, in some cases, nsP1 was highly concentrated at the tip of NIF. Furthermore, in the top stacks, nsP1 was localized at the plasma membrane. To investigate whether nsP1 palmitoylation played a role in this function, the same experiment was performed using a mutant form of nsP1, where the three palmitoylated cysteines were replaced by alanine residues (GFP-nsP1_{3A}). In these conditions, few extensions were detected at the bottom stacks, and the top stacks showed a cytosolic localization of this mutant.

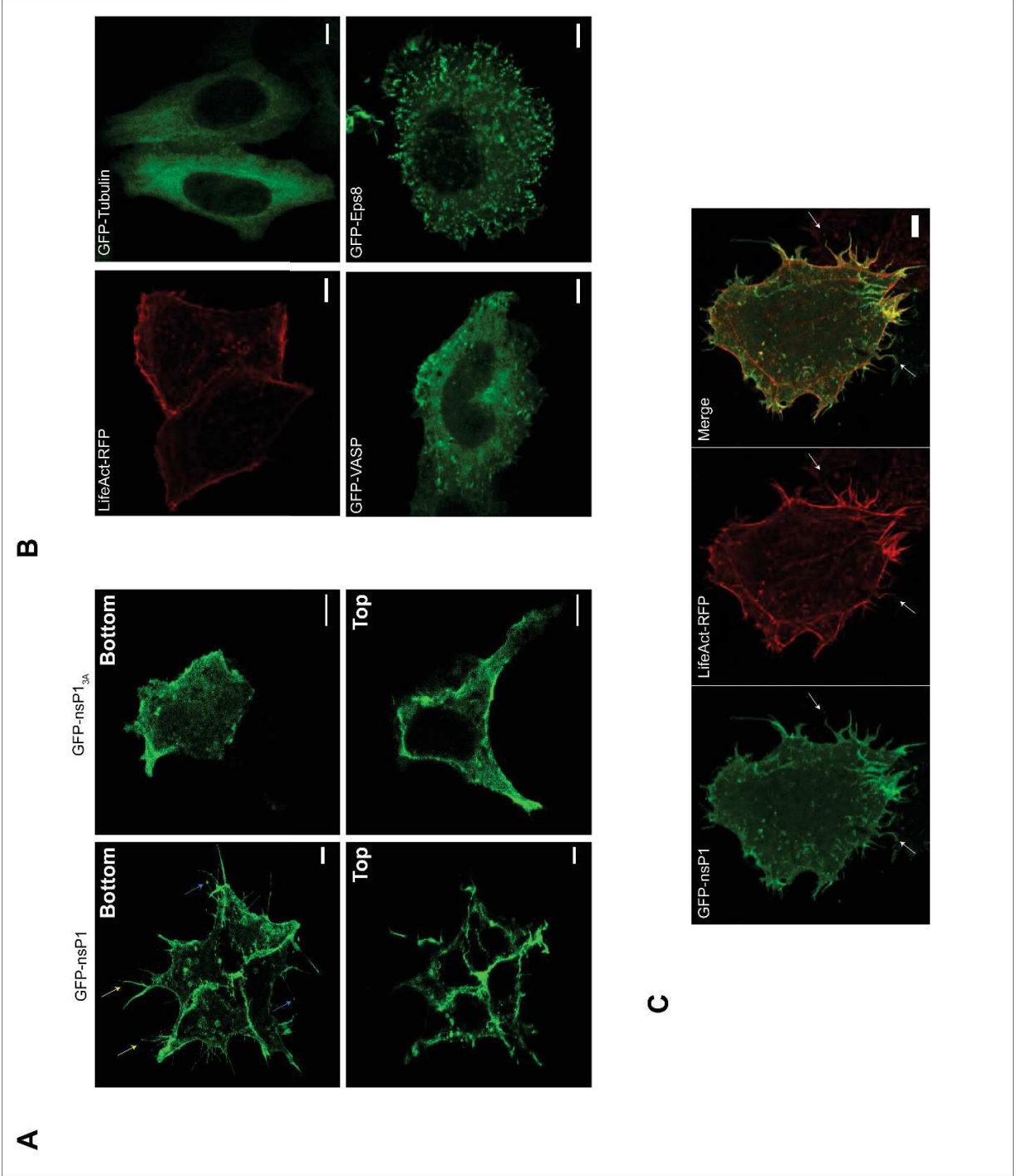
Then, to observe the dynamics of NIF structures, live cell confocal imaging was performed on GFP-nsP1 expressing cells. This established NIF as highly dynamic since it was capable of forming and retracting in videos lasting less than 5 minutes. Dynamics of membrane deformations permitted to hypothesize how these structures can form. In most

cases, NIF formed through the retraction of previously formed membrane ruffles (Supp. Vid.1).

Physiological filopodia extensions implicate the polymerization of monomeric actin into filament bundles organized in a parallel array. In order to observe if nsP1 protrusions contained actin filaments, the plasmid LifeAct fused to RFP was co-transfected with the plasmid GFP-nsP1. The LifeAct-RFP plasmid encodes a 17-amino-acid peptide which specifically stains filaments of actin (Fig. 31B). Imaging of co-transfected cells demonstrated the presence of actin filaments in NIF (Fig. 31C). Additionally, live cell imaging in the same conditions confirmed the existence of actin filaments during the formation of nsP1 extensions (Supp. Vid. 2). On the other hand, tubulin, another cytoskeleton component, was not found to be as highly abundant as actin in nsP1 protrusions (Fig. 31D).

NsP1's concentration at the tip of these membrane extensions and the presence of actin was reminiscent of classical filopodial machinery. Therefore, we set out to investigate whether certain filopodia markers co-localized with nsP1 in these structures through the co-transfection of cells with a non-tagged nsP1 expression plasmid and GFP-fused filopodia markers. Interestingly, we could detect co-localization events with the protein Eps8 at the base of NIF indicating a possible classical filopodia machinery pathway. Another protein of interest was VASP which has been shown to be heavily concentrated at the tip of filopodia exerting an anti-capping activity at that position. VASP was present at the tip of some NIF. However, this was not consistently observed for all NIF structures (Fig. 31E and F).

These findings extend the capacity of nsP1 of *Alphaviruses* to form filopodia-like structures to CHIKV, and confirmed the importance of nsP1 palmitoylation in this function. Elaborate characterization of NIF showed that they are dynamic, actin-rich and contain certain classical filopodia markers.



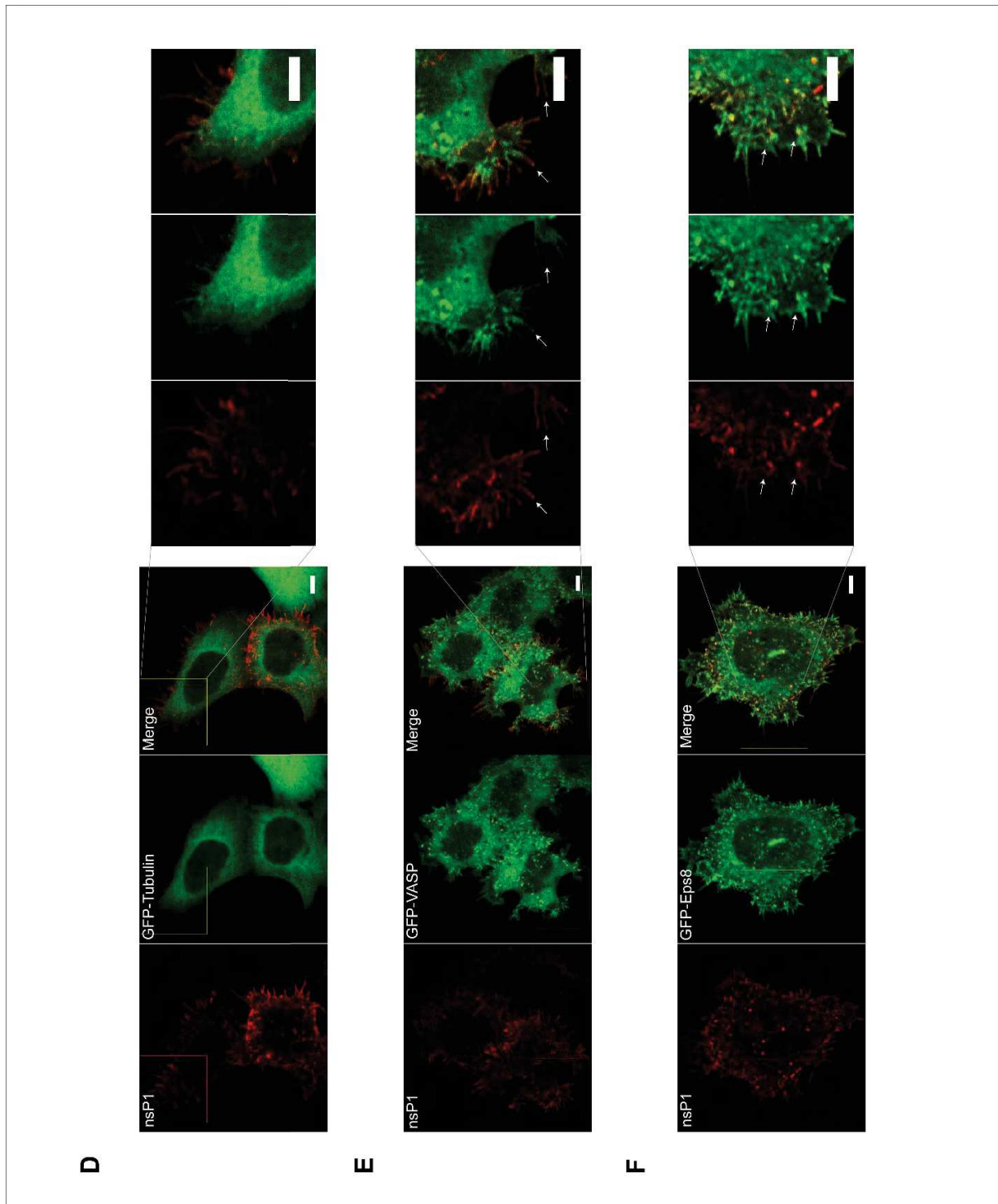


Fig. 31 nsP1 forms filopodia-like structures in a palmitoylation dependent manner

(A) HeLa cells were transfected with either GFP-nsP1 or GFP-nsP1_{3A} and then processed for z-stack confocal imaging. Yellow arrows indicate NIF, and blue arrows indicate nsP1 concentration at the tip of NIF. (B) Cells were transfected with constructs that mark different cytoskeleton and filopodia markers and were

imaged by confocal microscopy (C) Cells were co-transfected with GFP-nsP1 and LifeAct-RFP constructs and then imaged as in (B) (D-F) Confocal imaging was performed on cells co-transfected with an nsP1 expressing plasmid with either GFP-Tubulin, GFP-VASP or GFP-Eps8 constructs. White arrows indicate NIF structures containing different cytoskeleton or filopodia markers. Scale bar length is 5 μ M.

nsP1 requires active form of Rac1 for filopodia formation

In order to study if activation of Rho GTPases is essential for NIF formation, wild type or dominant negative versions of these two Rho GTPases fused to GFP were co-transfected with nsP1. The dominant negative constructs of these Rho GTPases have a mutation at the residue 17 which abolishes the protein's affinity for GTP. Immunofluorescence images in these conditions revealed that both wild type Cdc42 and Rac1 co-localized with nsP1 in NIF (Fig. 32A and B). On numerous occasions, this co-localization was concentrated at the tip of filopodia. Interestingly, the dominant negative form of Cdc42 did not have any significant effect on NIF formation. However, Rac1 dominant negative mutant led to a decrease in the formation of NIF structures. The importance of Rac1 activity for NIF formation was further confirmed using a selective inhibitor of Rac1 activity named NSC23766. Treatment with 100 μ M of NSC23766 led to a significant decrease in extensions formed by nsP1 while treatment with 20 μ M of the Cdc42 inhibitor ML141 had a marginal effect (Fig. 32C). These observations were consolidated with quantification of filopodia count and length (Fig. 32D and E). Live cell imaging experiments on GFP-nsP1 transfected cells treated with NSC23766 established that this Rac1 inhibitor blocks nsP1 NIF dynamics (Supp. Vid. 3).

To investigate whether nsP1 could activate Rac1 directly we took advantage of a FRET probe, consisting of the Rho GTPase flanked with two different fluorophores, YFP and CFP. Upon activation of a given Rho GTPase due to GTP loading, a conformational change will lead to an increase in proximity of these two fluorophores and detection of a FRET signal. The ratio of FRET can then be calculated and is directly proportional to the activity of the Rho GTPase. This allows the visualization of Rho GTPase activity in living cells. Additionally, for this experiment, an nsP1 fused to dsRed was used to avoid overlapping of signals with the YFP/CFP fluorophores. In control conditions, Raichu probes were co-transfected with a dsRed plasmid. In this situation, Rho GTPase activity

in cells varied substantially, but no significant amount of structures resembling NIF were detected (data not shown). On the other hand, upon expression of dsRed-nsP1, significant amounts of NIF structures were observed. Interestingly, we were able to detect active forms of Rac1 in these structures (Fig. 32F).

Finally, in order to establish if Rac1 activity is essential for CHIKV replication, we took advantage of the CHIKV replicon system used previously. This established that NSC23766 had an inhibitory effect on CHIKV replication demonstrating the importance of Rac1 activity for this process (Fig. 32G).

These findings hint at the possible implication of Rac1 in NIF formation, and possibly replication complex formation.

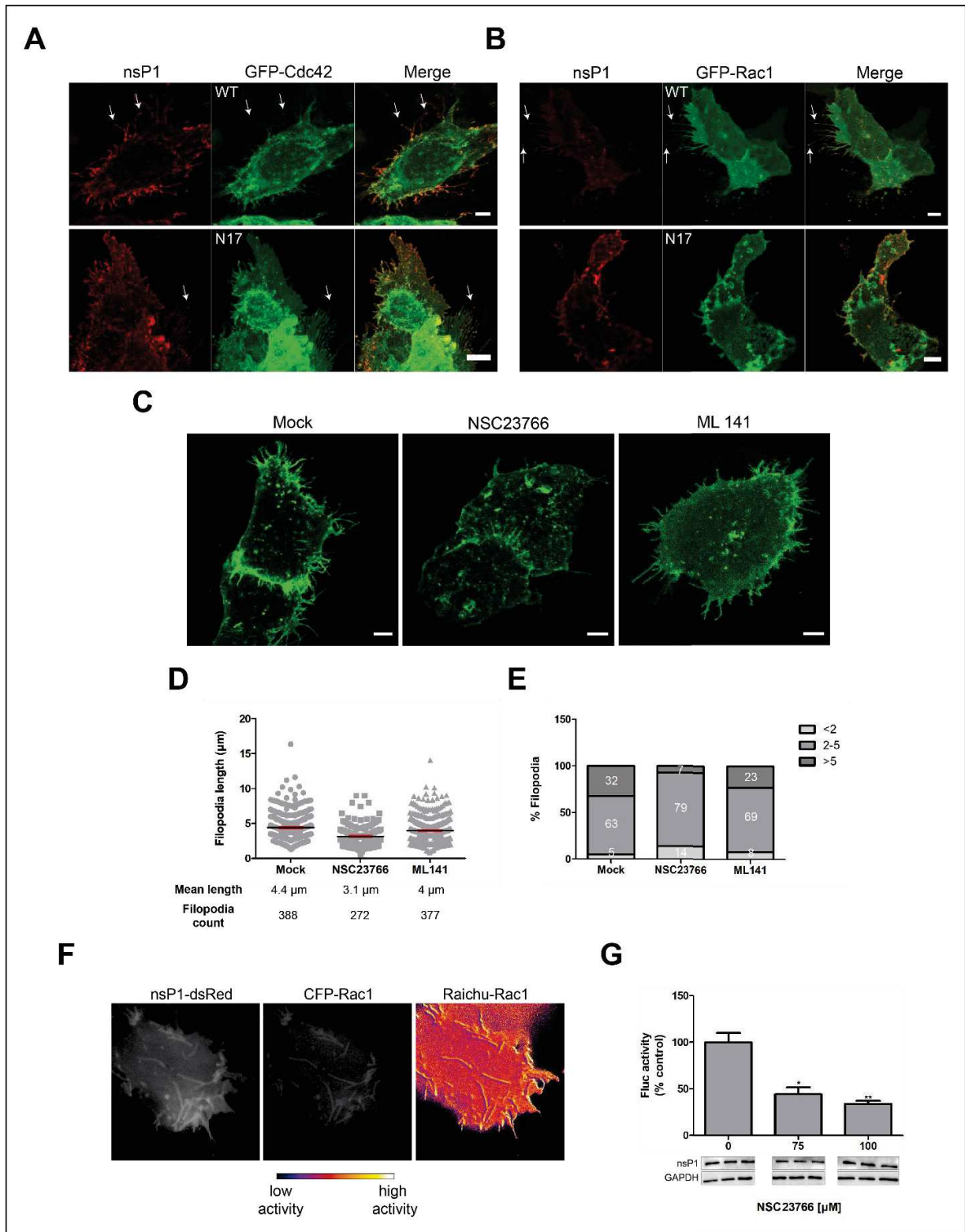


Fig. 32 nsP1 forms filopodia through a Rac1 dependent machinery

Non-tagged nsP1 was co-transfected with either WT or N17 versions of Cdc42 (A) or Rac1 (B) and were then imaged by confocal microscopy. (C) Cells were transfected with GFP-nsP1 plasmid and were then treated with 20 μ M of the Cdc42 inhibitor ML141 or 100 μ M of the Rac1 inhibitor NSC23766 for 24 hrs and imaged as in A and B. (D) Quantification of filopodia length in the conditions presented in C. 15 images were analyzed. (E) Grouping of structures quantified in D into three groups according to filopodia length. Numbers on the stacks represent % of filopodia structures belonging to this category. (F) Co-transfected cells with nsP1-dsRed and Rac1 raichu probes were processed for FRET imaging. (G) Increasing concentrations of NSC23766 were added to HEK-293T cells were transfected with CHIKV transreplication plasmids. Firefly luciferase activities were monitored after 24 hrs in culture. Values are percentages of the DMSO control condition. Cells in the same conditions were processed for immunoblot analysis using anti-nsP1 and anti-GAPDH antibodies.

nsP1 proteomics methodology

In order to fully decrypt nsP1's filopodia machinery, a proteomics approach was designed to establish nsP1's interactome. The strategy consisted of transiently transfecting HEK-293T cells with GFP, GFP-nsP1 or GFP-nsP1_{3A} constructs, and then performing immunoprecipitation using GFP-trap magnetic agarose beads (Fig. 33A). Cell lysis was optimized in order to efficiently solubilize nsP1 from membranes. After screening different detergents, Brij96 was selected as the optimal detergent for cell lysis (Fig. 33B). Coomassie staining of immunoprecipitated GFP-fused proteins validated the technique as a band was observed at ~36 kDa in the GFP lane, and ~100 kDa in the nsP1 conditions (Fig. 33C). Mass spectrometry detection of trypsin-digested immunoprecipitated nsP1 showed that around 80 % of nsP1's amino acid sequence was retained. A unique peptide corresponding to the location of nsP1 palmitoylated cysteines was also detected. In wild type nsP1, this peptide contained the three palmitoylated cysteines residues (Fig. 33D). Conformingly in the GFP-nsP1_{3A} condition, three alanine residues were detected.

Altogether, we have developed a proteomics methodology adapted for efficient solubilization and immunoprecipitation of nsP1.

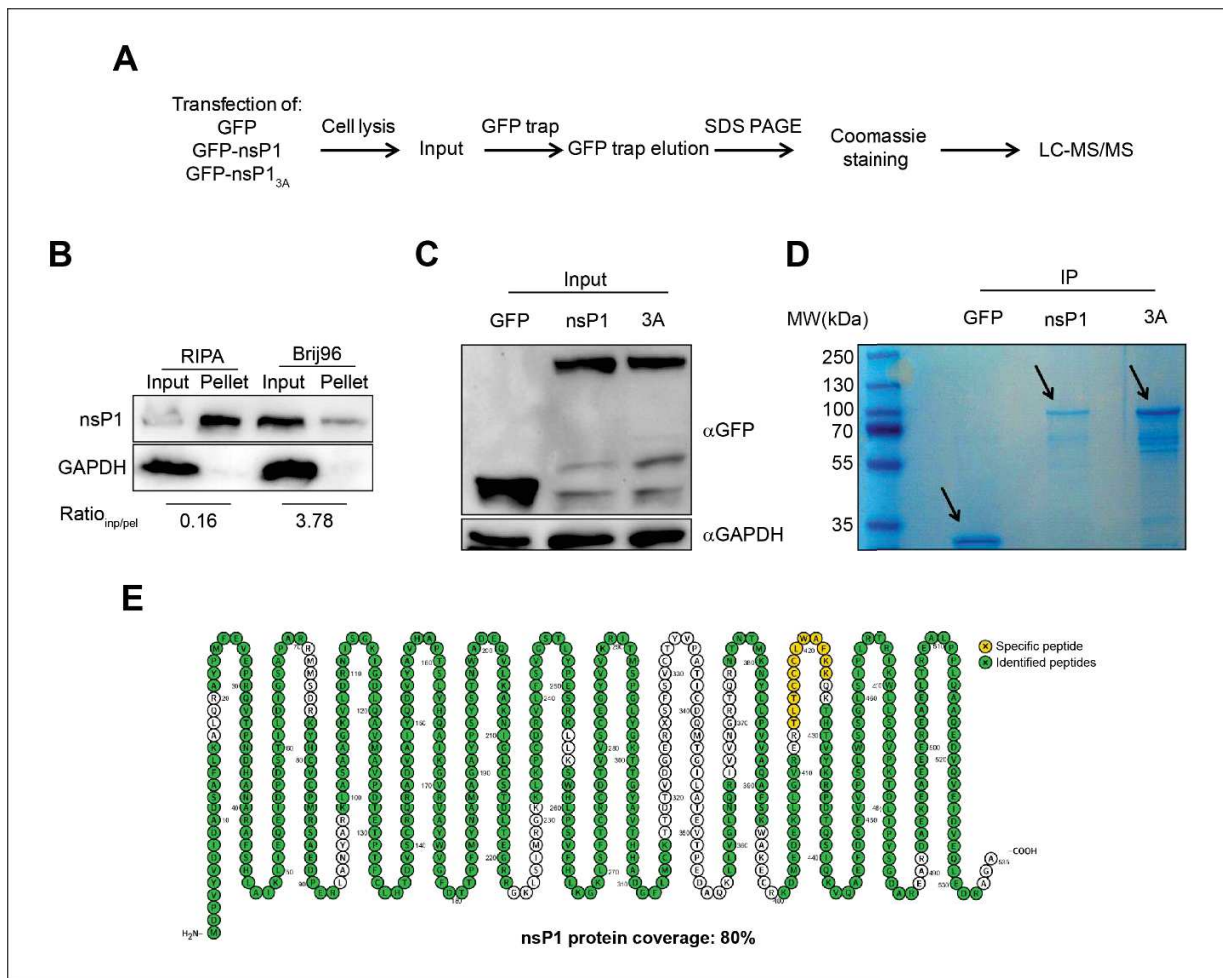


Fig. 33 Proteomics methodology for nsP1

(A) Experimental scheme for sample preparation prior to MS/MS analysis (B) HEK-293T cells transfected with GFP-nsP1 were lysed either with RIPA or Brij96 buffer. Then, samples were centrifuged at 16,000 g to yield a pellet which consists of cellular membranes. The input corresponds to the supernatant. (C) Cells were transfected with either GFP, GFP-nsP1 or GFP-nsP1_{3A} constructs and then processed for western blot. (D) Lysates from (C) were incubated with GFP trap beads to immunoprecipitate GFP-fused protein and were then loaded in an SDS-PAGE gel. The gel was stained with Coomassie blue to detect proteins. Black arrows indicate GFP-fused immunoprecipitated proteins (E) nsP1 amino acid sequence is illustrated using the Protter software. The yellow highlighted amino acids belong to a specific peptide and the green highlighted amino acids correspond to the identified peptides in the MS/MS analysis.

nsP1 interactome established

Proteomics analysis, in the conditions mentioned above, allowed the establishment of nsP1 interactome. This proteomics work was performed in collaboration with the functional proteomics platform of Montpellier (FPP).

Several types of analysis were performed in order to establish nsP1 specific interactants. An initial analysis was performed comparing the GFP condition, the negative control, with the GFP-nsP1 condition. This consisted of first determining proteins which were significantly enriched in the nsP1 sample. Furthermore, GFP-nsP1 and GFP-nsP1_{3A} conditions were compared to check whether the set of proteins retained as nsP1 specific interactants in previous analysis were lost in the GFP-nsP1_{3A} condition (Fig. 34A and B).

Gene ontology analysis of the nsP1 interactome web revealed proteins belonging to different cellular components and having different functional roles (Fig. 34C). One of the most enriched pathways included the cytoskeleton proteins. These set of proteins are of high interest since they could contribute to NIF formation. Surprisingly, nsP1 interactants also clustered into sets of mitochondrial and ER proteins. This might be a guide to the discovery of new nsP1 related functions.

Previous to establishing nsP1 interactome, we had employed a machine-learning framework called DeNovo in order to predict nsP1-human host protein interactions. This work was performed in collaboration with Fatma Elzahraa Eid (Virginia Tech) which was responsible for the development of this framework (Eid et al., 2016). This approach produced significant amounts of data with human host proteins given a score for their probability of interacting with nsP1. We used our nsP1 proteomics data to establish if this framework is capable of successfully predicting nsP1-host protein interactions. Plotting LFQ (Label-free quantification) against DeNovo predicted score of nsP1 interactants established a moderate positive relationship between these two scores with a correlation coefficient of around 0.35 (Fig. 34D). This data hints that the DeNovo framework can be a useful tool for nsP1-host interaction specifically, and virus-host interactions in general. The data and prediction scores for nsP1 predicted interactants will be provided in supplementary table 1.

In conclusion, for the first time in *Alphavirus* research, we were able to establish nsP1 interactants in mammalian host cells and showed the regulatory role of palmitoylated cysteines in these interactions.

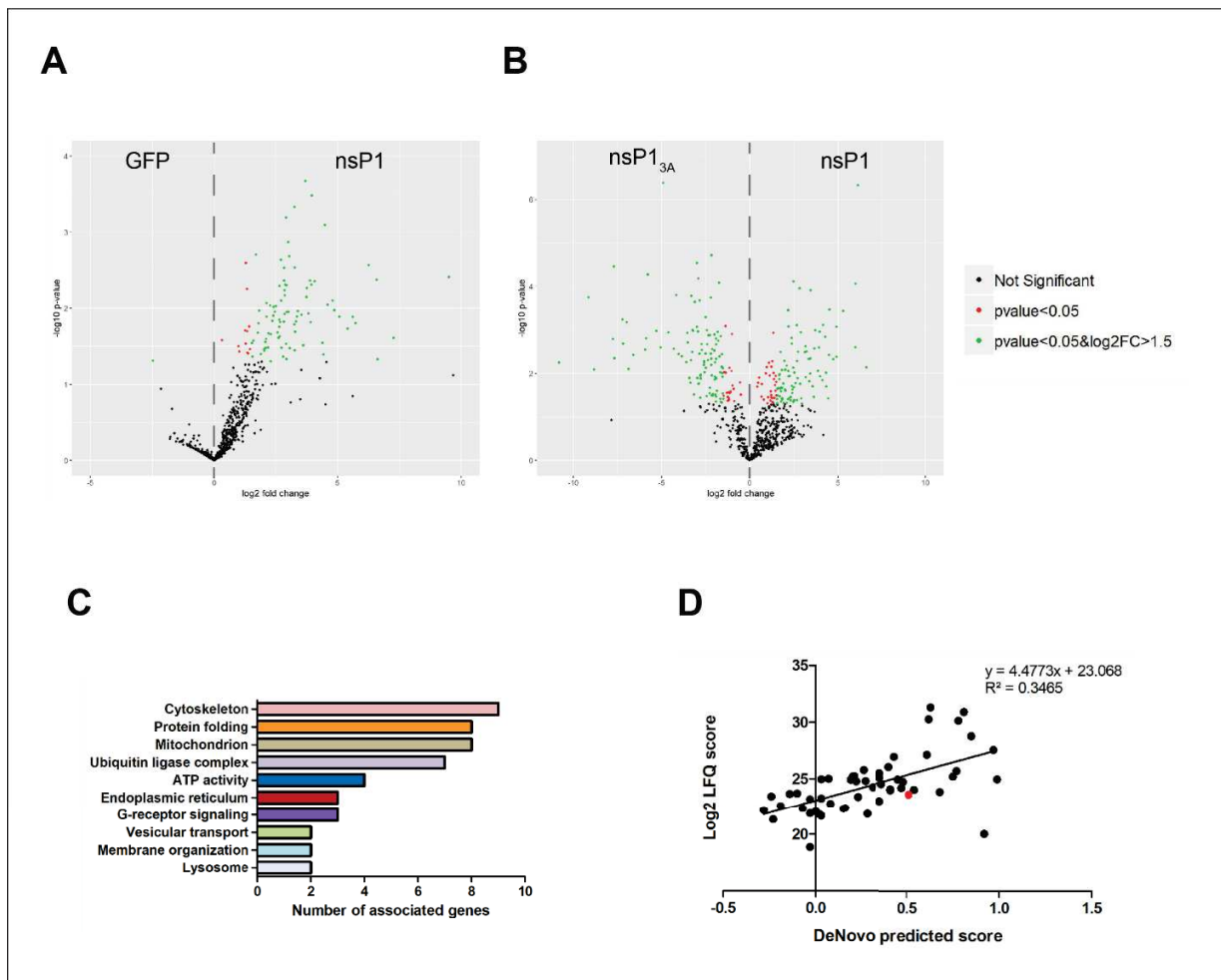


Fig. 34 nsP1 interactome

Volcano plots representing comparisons between the conditions (A) GFP vs. nsP1 and (B) nsP1 vs nsP1_{3A} (C) Proteins were clustered into functional modules using enriched gene ontology terms as a guideline and manual mining of literature. (D) DeNovo framework prediction scores of nsP1 interactants was plotted against proteomics Log₂ LFC score in nsP1 conditions. A linear regression line with the corresponding formula was plotted on the graph. The red circle represents the candidate Scribble.

Scribble interacts with CHIKV nsP1

Proteomics methods generate important amount of data, but can lead to false-positives and be labile to certain methodology bias. This is why it is essential to validate interactants using biochemistry-based techniques. Attesting to the success of our GFP-trap based methodology, we were able to confirm the specific interaction between nsP1 and three different hits in this proteomics screen (data not shown). We then focused specifically on

one of the top hits in our screen named Scribble (SCRIB). SCRIB is a master regulator of cell polarity, cytoskeleton protrusions and has been shown to activate the Rho GTPase Rac1. Therefore, the link with NIF was evident. The proteomics results detected SCRIB only in the GFP-nsP1 condition and not in the GFP or GFP-nsP1_{3A} samples. Co-immunoprecipitation experiments in cells co-transfected with SCRIB with an HA tag (SCRIB-HA) and the corresponding GFP-fused proteins confirmed that SCRIB interacted specifically with nsP1, and that this interaction was dictated by nsP1 palmitoylated cysteines (Fig. 35A). In order to explore whether nsP1 was the only nsP capable of interacting with SCRIB, the same experiment was repeated, but this time GFP-fused nsP2, nsP3 and nsP4 proteins were also co-transfected with SCRIB-HA. This established nsP1 as the only nsP capable of interacting with SCRIB (Fig. 35B).

Finally, to determine whether nsP1 co-localizes with SCRIB, HeLa cells co-transfected with GFP, GFP-nsP1 or GFP-nsP1_{3A} were processed for confocal microscopy. In GFP conditions, SCRIB had a punctuate staining throughout the cell (Fig. 35C). Impressively, upon GFP-nsP1 expression, SCRIB was re-localized into intra-cellular aggregates positive for GFP-nsP1 signal. SCRIB was not abundant in NIF. This phenotype was again dependent on palmitoylated cysteines in nsP1.

Altogether, through co-immunoprecipitation experiments, we have validated nsP1 proteomics data and have further characterized the functionally relevant hit SCRIB. The role of SCRIB in NIF remains to be determined.

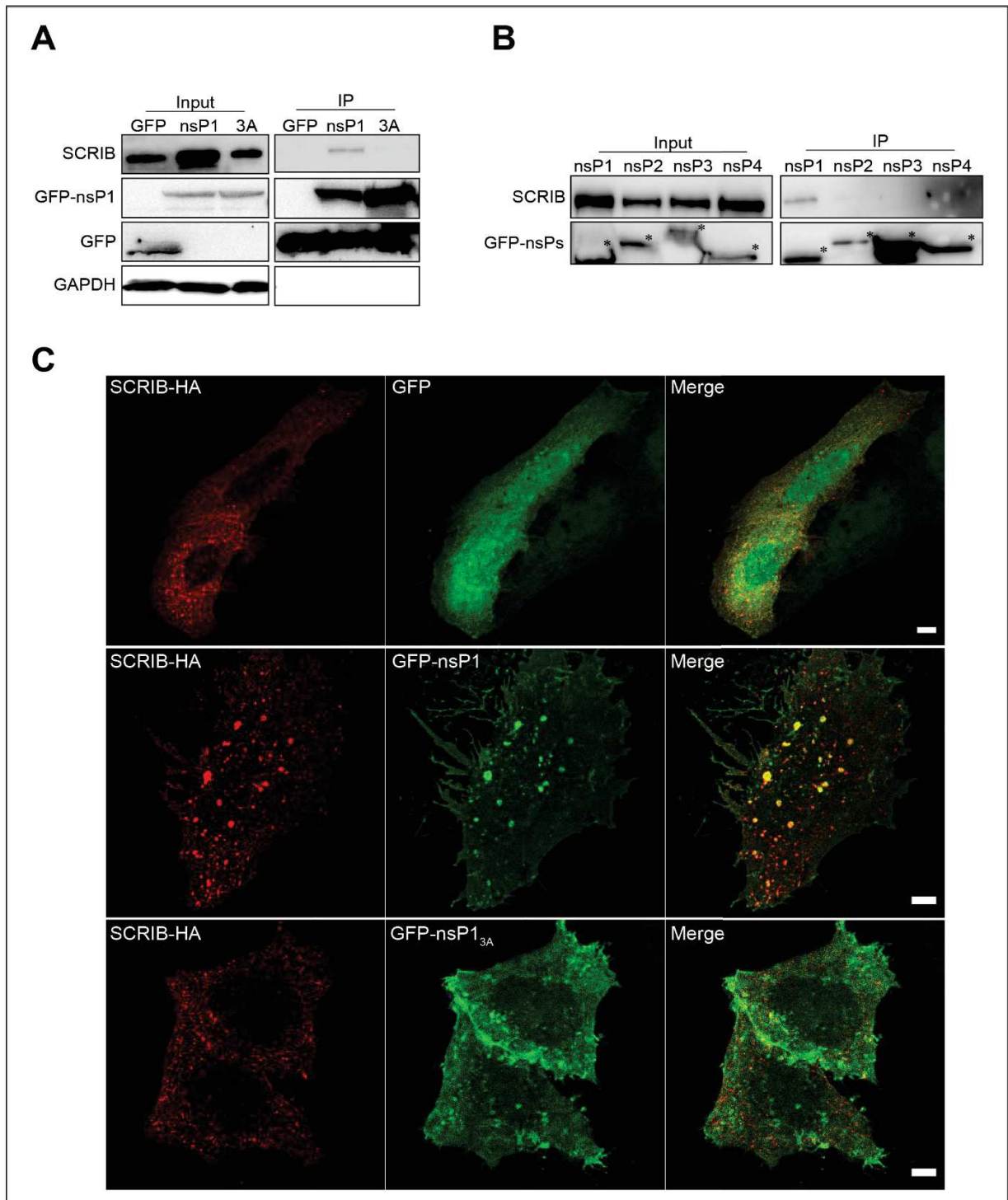


Fig. 35 SCRIB interacts with nsP1, an interaction dictated by nsP1 palmitoylated cysteines

(A) HEK-293T were co-transfected with GFP, GFP-nsP1 or GFP-nsP1_{3A} and SCRIB-HA and then processed for immunoprecipitation experiments using the GFP-trap technique. (B) Cells were co-transfected with

SCRIB-HA and GFP fused nsP1, nsP2, nsP3 or nsP4 proteins and processed as in (A). IP: Immunoprecipitation (C) HeLa cells were co-transfected as in (A), marked with an anti-HA antibody then processed for confocal microscopy. Scale bar length is 5 μ M.

iii. Materials and Methods

Antibodies and reagents: The following antibodies/reagents and respective dilutions were used in this study: Rabbit polyclonal antisera against CHIKV nsP1, nsP2, nsP3, nsP4 (all in-house, 1:1,000), monoclonal antibodies against GAPDH and HA (1:1,000) (Santa Cruz Biotechnologies Inc.), GFP (1:1,000) (Chromotek) and Rac1 (1:1,000) (BD Biosciences). Secondary antibodies conjugated to horseradish peroxidase or Alexa Fluor were purchased from Jackson ImmunoResearch and Thermo Fisher Scientific respectively. NSC23766 and ML141 were purchased from Sigma-Aldrich. Rac1 activation G-LISA kit was purchased from Cytoskeleton, Inc.

Cells: HEK293T cells (ATCC # ACS-4500) and HeLa cells (ATCC # CRM-CCL2) used for propagation were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fischer Scientific) supplemented with penicillin and 10% fetal calf serum (FCS, Lonza) and grown at 37 °C in a 5% CO₂ atmosphere. HEK293T cells were used for biochemistry and proteomics experiments and HeLa cells were mainly employed for imaging experiments.

Plasmids and transfection: Sequence encoding for CHIKV nsP1 was amplified by PCR using pCHIKV-LR-5'GFP as a template; obtained fragment was cloned into the pEGFP-C1 plasmid. Sequences encoding for nsP1C417-419A were generated using the Quikchange site-directed mutagenesis kit by Agilent. Cells were transfected with obtained plasmids using JetPei reagent (Polyplus Transfection) according to manufacturer recommendations. In co-transfection experiments, the two plasmids were co-transfected in a 1:1 ration ratio.

GFP trap assay: Cells were lysed in 200 μ l of the Brij96 based lysis buffer for 30 minutes on ice. After cell lysate clearing by centrifugation at 13,000 RPM, the supernatant was diluted with 300 μ l of dilution buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA). Then, 450 μ l of cell lysate was incubated with 15 μ l of magnetic agarose GFP-trap beads

at 4°C under rotation. 50 µl of cell lysate was kept as input for western blot experiments. After 4 hrs of incubation, three washes (2 times with 150mM NaCl and once with 300 mM NaCl wash buffer) were performed on these beads. Finally, 40 µl of Laemmli buffer were added to the beads that were then heated at 95 °C for 10 minutes to release attached proteins. The samples were then processed for immunoblotting.

Proteomics and MS/MS analysis: The GFP-trap samples were deposited on a 12 % SDS-PAGE gel and were migrated for a short period of time. Then, one band was cut and was resuspended in 10 µl of 0.1% formic acid and 2% ACN solution. This solution was then injected into an HPLC nano debi (RSLC U3000, ThermoScientific) coupled to a mass spectrometer having a nanoESI source (Q-Exactive Plus, ThermoScientific). Peptides were then separated on a capillary column (phase inverse C18, NanoViper, Dionex) having a gradient of 0-40% of 0.1% formic acid and 80% ACN for 80 min (run of 130 min) at a rate of 300nL/min. The spectra were saved using the Excalibur software (ThermoScientific). The raw spectral data were then analyzed using the MaxQuant v1.5.5.1 software. The database to match the spectral data was RefProteome_HUMAN-cano_2018_01.fasta (source UniProtKB) with the nsP1 sequence added.

GTP trap assay: Cells were lysed using an NP-40 based lysis buffer for 30 minutes. Then, lysates were cleared by centrifugation at 16,000 g for 10 minutes. Equal quantity of cell lysates was then incubated with agarose PAK-1 beads under constant mixing. A small fraction of lysate was retained as input control for subsequent western blot experiments. After 1 hr, beads were washed three times using the lysis buffer, and were then resuspended in Laemmli buffer and heated at 95°C for 10 minutes. Samples were then analyzed by western blot. Until the last step, all of this experiment was carried in a cold room set at 4°C.

G-LISA assay: After transfection or drug treatment, cells were lysed and processed according to manufacturer's conditions.

FRET imaging: For FRET imaging, cells were plated in a fluorodish and were then co-transfected with the Raichu probes and corresponding dsRed plasmids for 24 hrs. Image acquisition was then performed using the inverted widefield epifluorescence microscope Nikon with the incubation temperature set at 37°C and 5 % CO₂. CFP and FRET signal

was detected using the 420nm and 510nm filters respectively. Image analysis was performed using ImageJ. First, background noise signal was subtracted from CFP and FRET images. Then, masks were created to define structures of interest. The signal for the FRET image was then divided with the CFP image to yield an image which represents FRET/CFP signal. This image was then represented using a lookup table which will assign a certain color to a pixel depending on the FRET/CFP ratio.

Immunoblotting: Samples were separated by SDS-PAGE and then transferred to PVDF membrane (Hybond, Amersham). Membranes were blocked using 5% non-fat dry milk in PBS and probed with appropriate primary antibodies. After wash steps with PBS – 0.1% Tween 20, the membranes were probed with HRP-conjugated secondary antibodies. After final washes, the revelation was performed by incubating the membranes with either Luminata Forte (Merck) or Clarity Max (BioRad) and then image acquisition was done using a Chemidoc (Bio-Rad).

Immunofluorescence microscopy and image analysis: Cells grown on glass coverslips were washed with PBS and then fixed with 4% paraformaldehyde/PBS (Sigma Aldrich) for 10 min. For intracellular labeling, the cells were permeabilized with 0.1% Triton-X100 in PBS and blocked for 30 min with PBS containing 0.2% Bovine Serum Albumin. Incubation with primary antibody was performed for 1 hr. After washes with PBS, secondary reagents were added for 30 min. DAPI (Sigma-Aldrich) was used to stain the nuclei. After final washes, coverslips were mounted with Prolong Gold antifade mounting media (Thermo Fisher Scientific). For live cell imaging, cells were plated on a fluorodish in medium without phenol red. Imaging was performed under normal cell maintenance conditions (37 °C in a 5% CO₂ atmosphere). Images were acquired using a Leica SP5-SMD scanning confocal microscope equipped with a 63×, 1.4 numerical aperture Leica Apochromat oil lense at the Montpellier Resources Imaging platform.

Discussion

Chapter 3 Discussion

Viral infection, at the cellular level, is a multi-step process that begins with virus entry and ends with virus budding outside of a cell. Between these two steps, the virus will use all its prowess to ensure the replication of its own genetic material and the creation of a viral particle that will encapsidate the newly produced viral genome. During this race for multiplication, however, the virus will encounter multiple hurdles that will try to block its capacity to establish infection. This is why viruses have adopted multiple strategies to counteract cellular defense systems. In this thesis, the focus was shed on the replication step of (+)RNA viruses. It was evident from the bibliographic synthesis presented that these viruses have evolved to multiply their viral genome in association with cellular membranes partly in order to avoid detection by the immune system. This process generated different membranous structures (membranous webs, single or double membrane vesicles) depending on the virus. Naturally, an important part of the research on (+)RNA viruses focused on understanding the mechanism behind the formation of these structures. In this area of research, several aspects of (+)RNA virus replication complex formation were examined (1) the membrane platforms, their nature and exact lipid composition (2) the replication proteins and their corresponding functions inside the replication complex and (3) the cellular host-cofactors, their recruitment and impact on viral replication. These three aspects when combined created comprehensive molecular models on the formation and functioning of (+)RNA virus replication complexes. An englobing aspect that must not be forgotten is the structural information obtained on these organelles which can provide further information on their organization.

In this body of work, with the exception of studying the structural aspect, I attempted to cover all three aspects of (+)RNA virus replication complex formation, focusing specifically on the *Alphavirus* family with the medicinally important CHIKV as the main model and taking nsP1 at the core of these studies.

Indeed, the requirements for certain lipids at the CHIKV replication membrane platforms were first addressed. Additionally, the poorly understood function of nsP1 to deform membranes was investigated. Finally, the cellular host-cofactors implicated in this

capacity were also studied in an attempt to later expand the role of these factors for replication complex formation.

In the light of the results presented in this thesis, I will cover all these three aspects chronologically by employing the rather diverse information available on the formation of replication complexes of other (+)RNA viruses as the main subject of discussion.

A- Membrane lipid composition of *Alphavirus* replication platforms

In this study, our investigations established a requirement for fatty acids, UFAs and cholesterol in *Alphavirus* replication.

i. Fatty acids

Fatty acids are building blocks of biological membranes. The most common fatty acid found in animals and plants is palmitate. This fatty acid is produced by the action of the enzyme FASN, and serves as the skeleton for the production of other fatty acids (Baenke et al., 2013).

Palmitate has been shown to be essential for replication of numerous (+)RNA viruses such as DENV, WNV and HCV. For these viruses, requirement for palmitate has either been attributed to (1) availability of this fatty acid for palmitoylation of viral proteins or to (2) increase local membrane lipid biogenesis at replication sites. In order to induce these effects, some viruses (such as DENV) will recruit FASN to replication sites and stimulate an increase of its activity. However, this is not the case for all viruses, since HCV, for example, does not recruit FASN to its replication compartments, but can increase the expression of this enzyme promoting an increase in fatty acid synthesis (Zhang et al., 2019b).

Blocking FASN activity has demonstrated the requirement of fatty acids for *Alphavirus* replication. Since it has been shown that blocking FASN activity can decrease nsP1 palmitoylation (Zhang et al., 2019a), it is clear that our observed requirement for fatty acids is tightly linked to bioavailability of palmitate for nsP1 palmitoylation. This post-translational modification is essential for *Alphavirus* replication (specially CHIKV) further emphasizing the importance of the observed phenotype (Utt et al., 2019). However, it

cannot be excluded that this fatty acid can also play a role in the creation of an increased surface area for the production of new replication organelles.

With respect to the mechanism by which FASN is utilized in *Alphavirus* replication, in our study, we could not detect a significant increase in the expression of this enzyme. However, FASN has already been shown to be relocalized to CHIKV replication complexes by dsRNA staining (Karlis et al., 2016). In the lab, we have further attributed this recruitment to the nsP3 protein (data not shown). Finally, whether FASN activity is regulated after its recruitment to *Alphavirus* replication complexes remains to be investigated.

Another final possibility is that by inhibiting the production of palmitate, the generation of other fatty acids or more complex palmitate-derived lipids are also reduced. This has been clear in the case of BMV replication and its requirement for PtdCho, a membrane lipid with palmitate as the main component. In this context, 1a is capable of recruiting choline requiring 2 (Cho2p), a cellular enzyme involved in PC synthesis, to sites of viral replication. This feature seems to be common among (+)RNA viruses since BMV, HCV and poliovirus also enhanced accumulation of PtdCho (Zhang et al., 2016). However, this remains unexplored for *Alphaviruses* and will deserve further studies. The establishment of changes in membranes lipids during *Alphavirus* infection through lipidomics studies could help in this endeavor.

Another palmitate-derived lipids are UFAs which stem from desaturation of this fatty acid. UFAs are a diverse class of fatty acids which can be either mono- or poly-unsaturated. The rate limiting enzyme in the production of these MUFAs is the enzyme SCD1. Finally, further elongation and desaturation will yield PUFAs which involve the enzymes ELOVL and FDS (Baenke et al., 2013).

In our previously reported study, by targeting SCD1 activity or decreasing its expression, we were able to demonstrate an importance for UFAs in *Alphavirus* replication. Using this same approach, we could not detect any significant decrease in nsP1 membrane affinity. This suggested that these fatty acids did not play any critical role in anchoring of the *Alphavirus* replication complex. This came in agreement with a study performed on BMV where it was shown that UFAs were essential for replication of this virus but did not alter

the membrane association of the protein 1a (Lee et al., 2001). How UFAs contribute to (+)RNA virus replication still remains unexplored. However, with the information discussed above, it seems clear that these fatty acids are not essential for membrane interaction, but might play an intricate role contributing to the fluidity required for formation of membranous replication complexes.

Another continuity for this study would be to establish which type of UFAs are essential for viral replication. As it has been shown for HCV, for example, that certain MUFAs can promote viral replication, but some PUFAs inhibited viral replication (Kapadia and Chisari, 2005). This again suggests that (+)RNA viruses orchestrate their membrane lipid composition at viral replication organelles.

ii. Cholesterol

Cholesterol is perhaps the most studied lipid in the replication of (+)RNA viruses. This sterol has been implicated in the replication of a plethora of (+)RNA viruses such as HCV, WNV, DNV. This requirement for cholesterol has been shown by using various drugs that act on the synthesis and intra-cellular transport of this drug. Attesting to the importance of this lipid, (+)RNA viruses have evolved ways to subvert cholesterol into replication organelles. This is specially the case for (+)RNA viruses that replicate in association with organelles having poor cholesterol content such as *Flaviviridae* which replicate with the ER. For attraction of cholesterol to these replication organelles, some viruses re-direct the rate-limiting enzyme for cholesterol synthesis, HMG-CoA reductase, to these structures leading to increase in local cholesterol content paralleling the mechanisms used for FASN and fatty acid synthesis. In some viruses, active subversion of cholesterol has been observed by hijacking the lipid transfer machinery (Zhang et al., 2019b).

In the case of *Alphaviruses*, the fact that replication complex assembly occurs at the plasma membrane which is rich in cholesterol content suggested different mechanisms. Indeed, our initial observation that drugs re-directing cholesterol from the plasma membrane to endosomes abolished CHIKV replication demonstrated that plasma membrane cholesterol is important for proper functioning of the CHIKV replication complex. However, here, we propose another mechanism for acquiring high cholesterol content in replication organelles. Conversely to other viruses that recruit cholesterol to

replication organelles, our observations indicate that the *Alphavirus* replication machinery are capable of assembling at pre-existing cholesterol-rich plasma membrane microdomains. In comparison with other (+)RNA viruses, this seems to be the most resource efficient strategy to obtain high cholesterol content at replication organelles.

By focusing on the only nsP with membrane affinity, nsP1, we have also elucidated the mechanism for assembly of the replication complex to these regions. In this context, palmitoylated cysteines of CHIKV nsP1 were crucial for this function. In contrast, the membrane binding peptide of nsP1 was not involved in this association. These two domains have been both implicated with membrane attachment of this viral protein with differential importance on replication depending on the *Alphavirus*. Mutations in the membrane binding peptide of nsP1 abolishes SINV replication, but has a temperature sensitive phenotype for CHIKV replication. However, palmitoylation is vital for CHIKV replication while being highly dispensable for SINV and SFV (Ahola et al., 2000; Utt et al., 2019). Therefore, this study established for the first time a unique function for palmitoylation of *Alphavirus* nsP1. It would be interesting to attempt and establish if a unique function exists for the membrane binding peptide of *Alphavirus* nsP1. Furthermore, a special requirement for palmitoylation for CHIKV replication once again demonstrates that this virus has evolved to behave in a distinct manner than other OW *Alphaviruses*. This also further reinforces the importance of addressing to cholesterol-rich membrane microdomains for CHIKV. We hypothesize that these domains in addition to having the correct membrane lipid composition for replication complex formation might also contain certain co-factors essential for CHIKV replication as will be discussed later on.

In conclusion, our study on the lipid requirements for *Alphavirus* replication has established an importance for (1) palmitate which is likely to be linked with nsP1 palmitoylation, (2) cholesterol through the observation that palmitoylated nsP1 is targeted towards cholesterol-rich microdomains and (3) UFAs whose exact function remains to be investigated. These data provide strong evidence that CHIKV replication complex will assemble at membranes with specific lipid content. This emerging hypothesis is supported by the work of Tero Ahola showing that nsP1 enzymatic activity requires specific lipid species specially PtdSer (Ahola et al., 1999). While we have shown an importance for

UFA and cholesterol for CHIKV replication complex functioning, the use of lipid biosensors to study nsP1 lipid affinities will allow more detailed information on the exact lipid composition of CHIKV replication complexes.

The next part will deal with another aspect of *Alphavirus* replication organelle biogenesis by focusing on nsP1 and its ability to deform cellular membranes.

B- nsP1 and membrane deformation

In 1998, the team of Leevi Kääriäinen described that *Alphavirus* nsP1 was capable of inducing filopodia-like structures when expressed alone (Laakkonen et al., 1998). These extensions often had a thick root and were branched at their distal parts to thin filopodia and their formation depended on palmitoylation of nsP1. In this revolutionary paper, it was surprisingly stated that the formation of these structures was independent of actin filament formation due to the lack of effect of the molecule cytochalasin D, an inhibitor of actin polymerization. However, in the same paper, nsP1 was found to co-localize with the protein Ezrin, an important component of filopodia. Additionally, filaments of actin could be observed at the base and middle of these structures suggesting the possible implication of actin in nsP1 filopodia formation. Until now, no publications have attempted to re-investigate the mechanism of formation of these nsP1 induced structures.

In the work presented above, we have attempted to re-delve into understanding the mechanism behind the formation of nsP1 filopodia-like structures. We decided to take on this path because (1) these filopodia-like structures represent a poorly studied function of nsP1 when compared to other functions such as its RNA capping activity, and (2) we think that this membrane deformation ability is an indication of this protein's capacity to induce membrane curvature for replication complex formation. Therefore, by understanding the mechanism behind nsP1 filopodia formation, we might also acquire a glimpse of the mechanism for the formation of spherule structures. We are aware that this nsP1 membrane deformation function will have to be controlled by other nsPs or host-cofactors, since the two structures produced are different.

In this investigation, we have found that, in agreement with the paper of Leevi Kääriäinen, nsP1 induced the formation of filopodia-like structures that were dependent on this nsP's

palmitoylation. Actin filaments were present along imaged nsP1 filopodia and contrasting with the publication mentioned above, the formation and dynamics of these structures also depended on actin filament polymerization. Through access to live cell imaging technology, we were also able to acquire additional information on the dynamics of nsP1 induced structures. Such structures began with the formation of lamellipodia which then retracted leading to the generation of the nsP1 filopodia which are often observed in fixed cell microscopy.

Interestingly, nsP1 was also found concentrated at the tip of these filopodia structures. This is remarkably similar to the localization of proteins (formins, ENA/VASP, and IRSp53) present at the tip complex of physiological filopodia (Fig. 36). Additionally, in some cases, filopodia markers such as Eps8 and VASP co-localized with nsP1 inside these structures, suggesting a classical filopodia machinery with actin as the main cytoskeletal component. These two proteins belong to the Eps8/IRSp53/VASP network that can differentially control actin remodeling for filopodia formation (Vaggi et al. 2011) and it could therefore be interesting to further explore the interplay of these proteins in the formation of nsP1 filopodia.

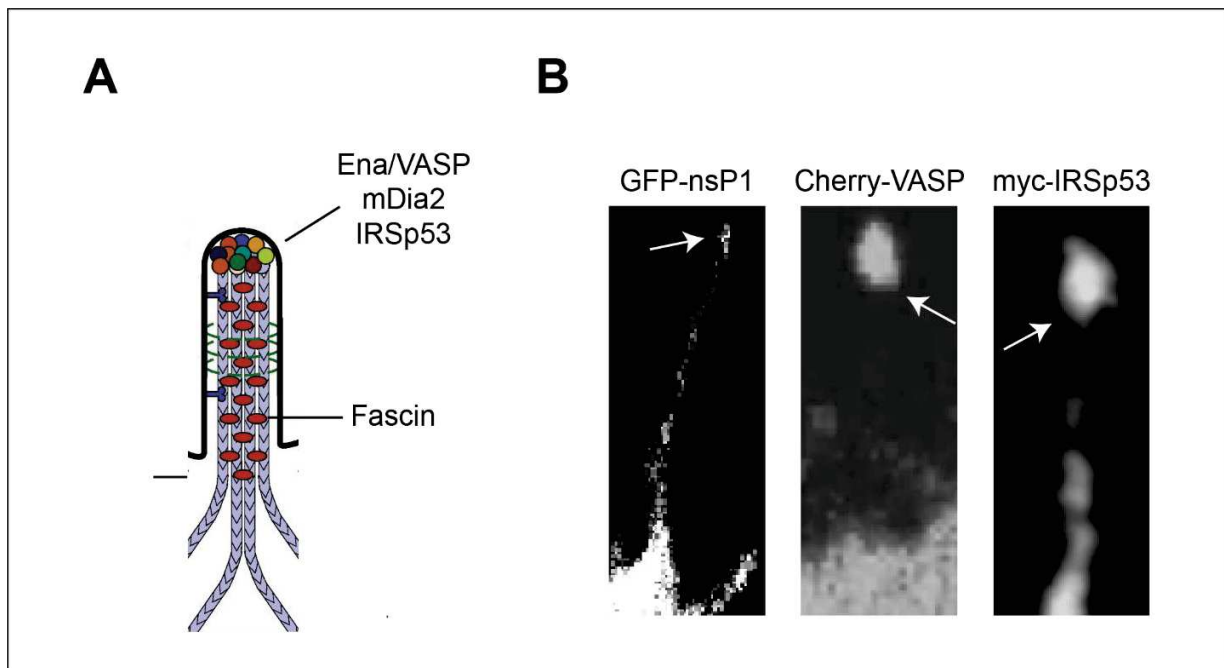


Fig. 36 nsP1 mimic the localization of proteins localized at the filopodia tip complex

(A) Schematic representation of the composition of filopodia at the tip complex. (B) Immunofluorescence images showing the localization of GFP-nsP1, Cherry-VASP and myc-IRSp53 in filopodia. White arrows indicate the filopodia tip (adapted from Alieva et al. 2019; Crespi et al. 2012; Mellor 2010).

A missing element of the previous study was the implication of Rho GTPases which are master regulators of actin-based membrane protrusions (Sit and Manser, 2011). Our experiments demonstrated an importance of Rac1 activity for the formation of nsP1 structures. This is surprising since the established regulator for filopodia formation is the Rho GTPase Cdc42 while Rac1 regulates the formation of lamellipodia. However, in our conditions, we could not detect any significant involvement of Cdc42. It should be mentioned that these pathways can intersect, and therefore might explain the observed phenotype. In addition, our findings can be explained by looking at the two main hypotheses for the initiation of filopodia formation (Yang and Svitkina, 2011). The first model named “tip nucleation model” implicates actin nucleators clustering at the plasma membrane generating parallel filaments of actin which will eventually lead to the production of filopodia. On the other hand, the convergent elongation model suggests that filopodia initiation begins with formation of branched actin networks by the Arp2/3 complex which then serve as the base for actin nucleators for formation of filopodia. Both the implication of Rac1 and our imaging of nsP1 dynamics favors the more relevant convergent elongation model for nsP1 filopodia formation.

The possible involvement of Rac1, but not Cdc42, is also of interest when looking at the affinity of these Rho GTPases for membrane microdomains. Rac1 has been described to associate, through palmitoylation, with pre-existing lipid order domains or lipid rafts. This association favors the activation of this Rho GTPase and will initiate spatially confined branched-actin polymerization (Navarro-Lérida et al., 2012). On the other hand, Cdc42 is not recruited to lipid rafts (Jaksits et al. 2004). This comes in agreement with the data obtained on nsP1's affinity to lipid rafts where we observed that nsP1 is targeted to these regions also by the same post-translational modification. Conformingly, nsP1 palmitoylation was also essential for formation of the filopodia-like structures mimicking the requirement of Rac1 association to these microdomains. A proposed model for

nsP1/Rac1 interplay would be that (1) nsP1 interacts directly or indirectly with Rac1 in the cytosol (2) palmitoylated nsP1 with Rac1 will then associate with lipid rafts favoring Rac1 activation due to close vicinity to activators of this Rho GTPase (3) binding to effectors such as PAK can then lead to initiation of spatially confined branched-actin polymerization leading to lamellipodia formation (4) through unknown mechanisms, retraction of lamellipodia forms nsP1 induced filopodia-like structures (Fig. 37). To validate this model, it would be essential to demonstrate whether nsP1 could recruit Rac1 to lipid rafts, and to check whether this recruitment would be dependent on nsP1 palmitoylation.

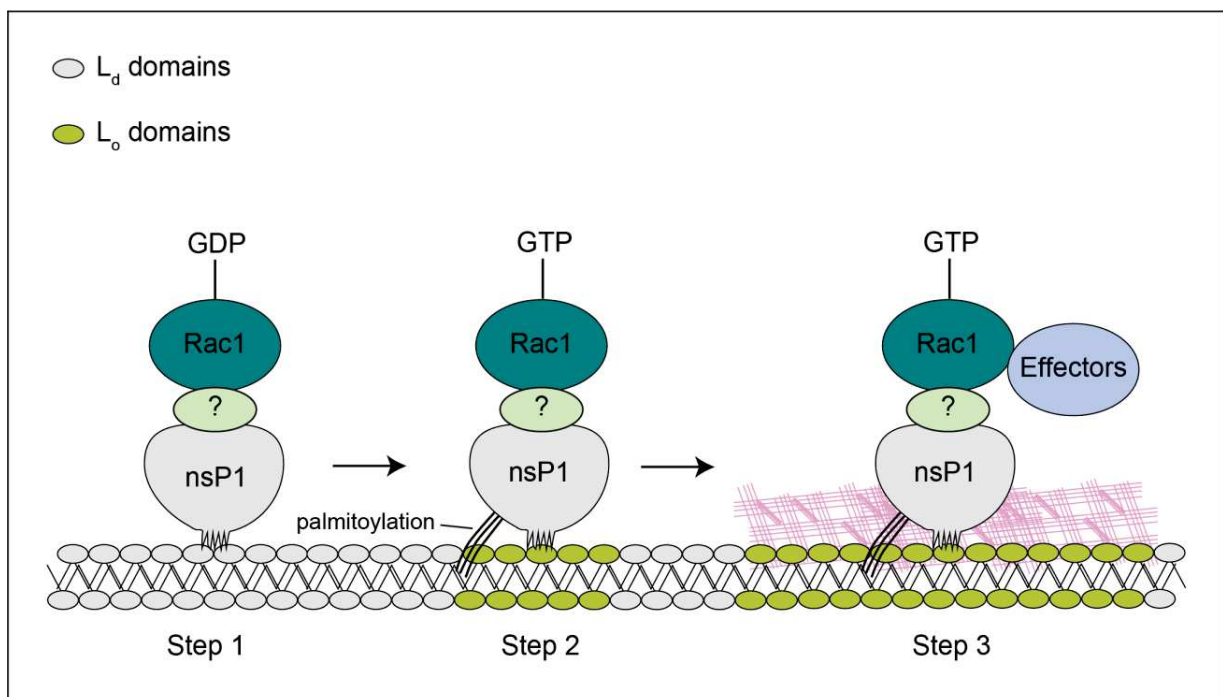


Fig. 37 Model for nsP1/Rac1 interplay

(Step 1) nsP1 interacting directly or indirectly with Rac1 binds to the plasma membrane through its α -helix. (Step 2) Palmitoylation of nsP1 will lead to its translocation from L_d to L_o domains favoring activation of Rac1 due to close vicinity to activators (not shown). (Step 3) Activated Rac1 will recruit effectors leading to the initiation of spatially confined branched-actin polymerization which eventually will initiate lamellipodia formation.

The capacity of nsP1 to deform membranes is reminiscent of the ability of replication proteins of other (+)RNA viruses to have the same function. BMV protein 1a and FHV

protein A are both capable when expressed alone to deform the membranes of host cells (Kopek et al., 2010; Schwartz et al., 2002). In this case, it has to be mentioned that the structures induced by these replication proteins highly resemble their corresponding (+)RNA virus replication complex. This insinuates that nsP1's capacity to deform membranes must be regulated by the other nsPs or the involvement of host-cofactors. In that scenario, it is interesting to draw a parallel with the regulation of nsP4 function in *Alphavirus* replication. nsP4's polymerase activity in the replication complex is highly regulated by interaction with the other viral nsPs. As mentioned before, nsP4 with the P123 polyprotein produces mainly (-)RNA, but nsP4 interacting with the mature nsPs will shift this synthesis towards positive-stranded RNA (Shirako and Strauss, 1994). A similar phenomenon might be occurring for nsP1 and its membrane deformation function (Fig. 38). A hint for this regulation has recently been evidenced in a creative paper by the team of Tero Ahola which investigated the different combination of nsP precursors required for spherule formation. In this investigation, the combination of the P123 polyprotein with nsP4 were capable of forming remarkably similar structures to spherules observed during *Alphavirus* infection (Hellström et al., 2017). Not surprisingly, this combination of proteins did not generate filopodia structures indicating that nsP1 inside the P123 polyprotein is regulated for curvature of membranes. Therefore, a natural continuity of this study will be to establish the regulatory effect of the other nsPs on this nsP1 function while integrating the newly found mechanisms described above.

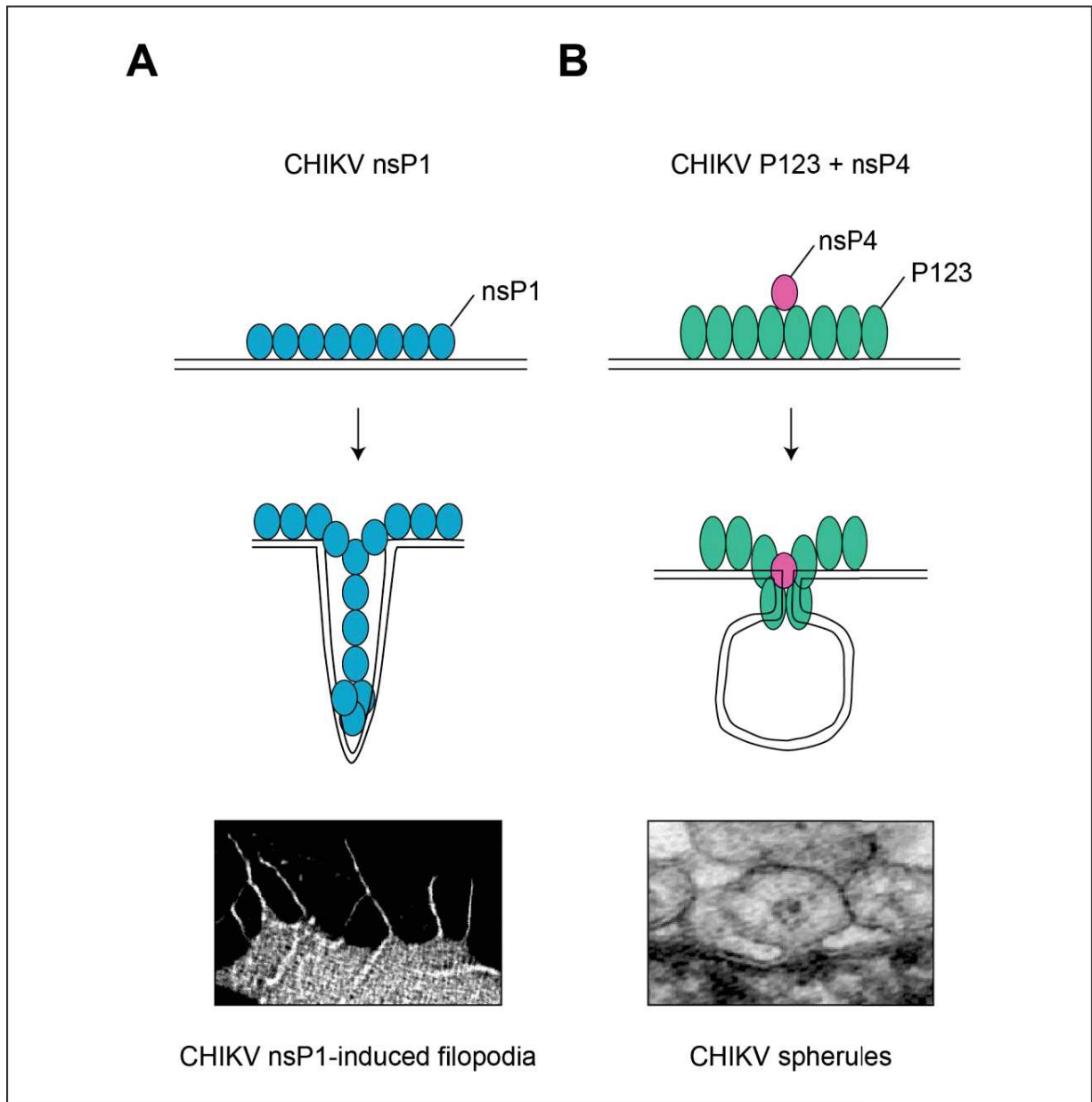


Fig. 38 Model for regulation of membrane deformation induced by CHIKV nsP1

(A) CHIKV nsP1, when expressed alone, leads to the formation of filopodia-like structures. (B) Expression of P123 in combination with nsP4 leads to the formation of spherule structures similar to that observed in CHIKV infection.

C- nsP1 and host-cofactors

The ability of nsP1 to deform membranes can be (1) intrinsic to this protein's structure similar to BAR proteins which can sense and curve membranes or (2) rely on the

recruitment of host-cofactors. While, the first mechanism for membrane deformation is certainly possible, the lack of structure for nsP1 does not allow the validation of this hypothesis. Furthermore, the regulatory role of palmitoylation as well as the implication of Rac1 for this function suggests the requirement of certain cofactors, probably present in plasma membrane microdomains.

This motivated the search for nsP1 host-cofactors and led, for the first time in *Alphavirus* research, the establishment of nsP1's interactome. Until now, nsP1's interactome has remained uninvestigated probably due to the high affinity of this protein to membranes making proteomics analysis challenging. Indeed, in our hands, nsP1 was hard to efficiently solubilize and more importantly to immunoprecipitate. In retrospect, this certainly had to do with the presence of this protein in microdomains resistant to the detergent Triton X-100. Conformingly with this hypothesis, the palmitoylation mutant form of nsP1 was easier to solubilize and immunoprecipitate. In our study, we screened for detergents that can most efficiently solubilize nsP1 from membranes and found Brij-96 as the best detergent for our purposes. Brij-96 or related detergents (since this exact version is no longer produced) could be useful in other biochemical studies on nsP1.

Going back to the findings on nsP1's interactome, no significant overlap could be found when comparing nsP1 interactants with proteomics analysis performed on the other nsPs or *Alphavirus* replication complexes (Bourai et al., 2012; Varjak et al., 2013). However, functionally related modules could be detected such as sets of proteins involved in cytoskeleton remodeling and protein folding. This suggests that the interactants obtained in this study are specific to nsP1 along with its corresponding functions and thus can provide new information on the *Alphavirus* lifecycle. It has to be mentioned that FASN has been previously studied by us and others where it was found to be essential for *Alphavirus* replication (Bakhache et al., 2019; Zhang et al., 2019a). Unfortunately, no proteins were found in common between nsP1 interactome and the genome-wide screens that identified host-cofactors important for *Alphavirus* infection (Meertens et al., 2019; Tanaka et al., 2017). However, such screens can have methodology-based biases and might favor the identification of cofactors implicated mainly in virus entry and cytotoxicity. Therefore, the nsP1 hits identified here could shed the light on previously unidentified cofactors

specifically involved in the *Alphavirus* replication step. Functional studies on candidates are being performed in the lab to study their role in *Alphavirus* infection.

Conversely with the lack of overlap with *Alphavirus* host-cofactors, a significant portion of interactants have been previously described to play various roles in viral infection. This was particularly clear with the set of proteins belonging to the ubiquitin ligase complex which have been reported to mediate degradation of several restriction factors. Specifically, with the presence of two proteins which both belong to the CUL4-DDB1 E3 ubiquitin-protein ligase complex. An example of such recruitment is the usurping of this E3 ligase complex by HIV-1 Vpr to induce degradation of MCM10 (Romani et al. 2015). Interestingly, this could help reveal the mechanism behind the previously described ability of nsP1 to mediate degradation of the restriction factor tetherin (Jones et al., 2013). Another set of interactants implicated in the viral life cycle, specifically at the virus entry and assembly steps, are the Rab proteins. The two identified Rab proteins which associate with early and late endosomes respectively have been implicated in the entry of numerous enveloped viruses, including *Alphaviruses* (Spearman, 2017). But, nsP1 acting on this step doesn't fit with the model of the *Alphavirus* lifecycle. However, the previously described finding that nsP1 has an affinity to late endosomes (Peränen et al., 1995), and our results demonstrating that nsP1 could circulate between the plasma and endosomal membranes affirms the interest to study these candidates. It is possible that nsP1 through interaction with the Rab proteins can direct endocytosis of the viral replication complexes formed initially at the plasma membrane. Indeed, studies investigating the replication proteins implicated in the endocytosis of replication complexes have identified nsP1 and nsP3 as key players in this process (Salonen et al., 2003). An interesting finding in the nsP1 interactome was the presence of a significant number of mitochondrial proteins. This is surprising since there is no evidence in the literature of an affinity of nsP1 to mitochondrial membranes. The only indication for such an association comes from the thesis of Pirjo Spuul who observed that tandem repeats of the nsP1 binding peptide (α -helix) fused to GFP was targeted to mitochondrial membranes. This association with mitochondrial proteins or membranes could possibly be interpreted as being a remnant property of ancestral *Alphaviruses* replicating in association with mitochondrial membranes. Another possibility is that nsP1 could be an

actor in the previously described alteration of mitochondrial dynamics upon *Alphavirus* infection (Keck et al., 2017).

An important remark on this interactome is the use of a non-palmitoylated form of nsP1 which allowed narrowing down of selected hits of interest due to two main reasonings (1) non-palmitoylated nsP1 does not deform membranes and (2) does not segregate to membrane microdomains. This gave a functional outlook to our proteomics approach. In this context, we have mainly focused on one hit of interest which is the protein SCRIB. SCRIB is a scaffold membrane protein which is involved in cell migration, cell polarity and cell proliferation. Furthermore, this protein has been shown to play a regulatory role on the activity of Rho GTPases. Particularly, SCRIB is capable of activation of Rac1 (Bonello and Peifer, 2019). This protein has also been identified to be a raft protein by an unbiased quantitative proteomics approach specific for lipid rafts (Foster et al., 2003). Finally, SCRIB has been implicated in the lifecycle of numerous viruses such as HIV and Influenza A virus where it has been shown to be degraded to protect cells from apoptosis. Whether this cofactor has a pro- or antiviral for CHIKV infection remains to be determined. The information obtained on this cofactor till now is that it interacts with only the palmitoylated form of nsP1. Additionally, other nsPs did not interact with this hit demonstrating that SCRIB recruitment is specific to nsP1. We hypothesize that SCRIB is possibly the direct mediator for nsP1 induced Rac1 activation. Current studies are going on in the lab to explore this scenario.

Finally, comparing host-cofactors implicated for spherule formation of other (+)RNA viruses is perhaps one of the most relevant strategies to identify the most relevant hits for nsP1 membrane deformation functions. Accordingly, the host cell machinery used by (+)RNA viruses replication proteins to deform membranes forming spherules have been perhaps the most deeply investigated in the case of BMV where it was found that a highly regulated form of the ESCRT pathway was involved in the membrane curvature for BMV spherule formation (Diaz et al., 2015). The protein 1a, a distant homologue of nsP1, regulated these interactions with the ESCRT pathway. Unfortunately, no components of this pathway could be detected in the nsP1 interactome, and preliminary tests in our lab did not find a positive association with this machinery for *Alphavirus* spherule formation.

However, a point in common between the nsP1 interactome and BMV replication is the implication of protein folding proteins such as heat shock proteins. Indeed, heat shock proteins have been found to be important for appropriate folding of replication components of BMV. One can imagine that the abundant presence of this family of proteins in nsP1 interactome could indicate that nsP1 has an important function in the appropriate folding of the replication components inside the replication complex. However, this function remains to be absolutely demonstrated and could be further elucidated with the eventual detailing of the 3D structure of the *Alphavirus* replication complex.

In conclusion, we have established the very first information on the host-cell machinery behind nsP1 membrane deformation. This established an important role for the Rho GTPase Rac1, possibly mediated by SCRIB, in these deformations. The discovered mechanisms will serve as a base for future studies on *Alphavirus* replication organelle biogenesis. Since these organelles are viral factories contributing to the amplification of viral infection, the discovery of new actors in this process could allow the development of anti-viral molecules targeting *Alphaviruses*, specially CHIKV.

A model combining the present data in this thesis with the previously described bibliography on *Alphavirus* replication organelle biogenesis is illustrated in Fig. 39.

Fig. 39 Model for *Alphavirus* replication organelle biogenesis

(A) The non-structural polyprotein P123 binds to the plasma membrane through the α -helix motif present in nsP1. (B) nsP3 recruits FASN promoting synthesis of fatty acids, specially palmitate. A palmitoyl group will be transferred on nsP1 cysteines through the action of the ZDHHC2 and ZDHHC19 proteins. (C) Palmitoylation of nsP1 in the P123 polyprotein will lead to its translocation to lipid ordered domains rich in cholesterol. In these regions, the *Alphavirus* replication complex will assemble together with nsP4, viral RNA and host-cofactors. (D) Spherule structures will form and will be stabilized by the presence of branched actin at their base. nsP4 will synthesize (-)RNA leading dsRNA formation (E) Cleavage of the non-structural polyprotein into individual non-structural proteins will shift nsP4 synthesis into genomic and subgenomic (+)RNA. Also, mature nsP1 can form filopodia structures in association with Rac1.

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Architecture et biogenèse des organelles de répllication des virus à ARN de polarité positive

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Résumé. La répllication des agents pathogènes viraux repose sur leur capacité à manipuler leur hôte à des fins provirales. De nombreuses étapes du cycle infectieux dépendent de l'usurpation des membranes cellulaires. Pour les virus possédant un génome composé d'un ARN de polarité positive (ARN(+)) et se répliquant dans le cytoplasme de leur hôte, la réorganisation et le détournement des membranes permettent en particulier la formation d'organelles membranaires hébergeant la répllication virale. La formation de ces compartiments constituant de réelles usines à virus donne lieu à des modifications morphologiques de l'hôte très variables selon le pathogène considéré. Les mécanismes moléculaires mis en jeu dans de tels remaniements restent le plus souvent incompris. Ils font cependant l'objet d'un intérêt intense puisque la formation de ces organelles de répllication, indispensables à la multiplication virale, représente une cible thérapeutique potentielle. Cette revue fait un état des lieux des connaissances relatives à l'architecture et aux interactions virus-hôte participant à la biogenèse des organelles de répllication des virus à ARN(+).

Mots clés : virus à ARN, organelles de répllication, membranes, interactions hôte-virus

Abstract. The replication of viral pathogens relies on their ability to manipulate their host. Several steps of the infectious cycle require the hijacking of cellular membranes. Positive stranded RNA viruses replicating in the cytoplasm of their host reorganize cellular membranes. This leads to the formation of organelles, which host viral replication. The formation of such compartments, which are genuine viral factories, induces morphological modifications of the host cell, which vary depending on the pathogen. However, the molecular mechanisms underlying such a remodeling remain unclear. These mechanisms are subject to intense research since their formation is indispensable to viral multiplication and therefore represent an attractive therapeutic target. In this review, we provide a bird's eye view on the current knowledge of the architecture and virus-host interactions involved in the biogenesis of positive stranded RNA virus replication organelles.

Key words: RNA virus, replication organelles, membranes, virus-host interactions

Introduction

Les virus à ARN de polarité positive (ARN(+)) constituent plus d'un tiers des virus connus. Cette classe d'agents infectieux regroupe des virus capables d'infecter les plantes ou les animaux et nombre d'entre eux sont responsables

d'épidémies humaines contemporaines, tels que le virus de l'hépatite C (HCV), le virus chikungunya (CHIKV), le virus de la dengue (DENV) ou le virus du syndrome respiratoire aigu sévère (SARS). L'impact de ces pathogènes sur la santé publique s'accompagne de façon générale d'une absence quasi totale de molécule antivirale et de stratégie vaccinale permettant de protéger les populations exposées. Les travaux engagés dans la recherche de cibles thérapeutiques pertinentes ont démontré que, malgré des caractéristiques parfois très distinctes, ces

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virus répliquent invariablement leur génome en association avec les membranes de leur hôte. L'infection entraîne en effet un remaniement significatif des membranes cellulaires, observable en microscopie électronique sous la forme de vésicules ou de réseaux membranaires complexes dérivés des membranes intracellulaires ou encore de la membrane plasmique. Ce type de structure est d'ailleurs indifféremment observé, qu'il s'agisse de virus d'animaux, d'insectes ou de virus de plantes. Il apparaît désormais que ces organelles renfermant l'ARN et les enzymes virales sont de véritables usines à virus permettant l'ancrage des complexes de réplication et favorisant la concentration de la machinerie enzymatique virale, des métabolites et des cofacteurs cellulaires nécessaires au fonctionnement optimal du complexe de réplication. Ces organelles membranaires constituent également des barrières physiques capables de protéger la machinerie de réplication, tout particulièrement les ARN viraux, vis-à-vis des senseurs et des effecteurs de la réponse immune de l'hôte. Enfin, la formation de ces compartiments constitue une stratégie unique qui permet de séparer spatialement et temporellement l'ARN engagé dans la réplication des processus concurrents tels que la traduction et l'encapsidation d'ARN, souvent localisés à proximité des sites de la réplication virale.

L'identification des mécanismes participant à la formation des compartiments de réplication des virus à ARN(+) constitue donc désormais un enjeu majeur pour l'identification de cibles thérapeutiques pertinentes et le développement de stratégies permettant d'empêcher la multiplication de pathogènes à fort impact sur la santé humaine. Les connaissances actuelles ont largement démontré la contribution des protéines virales non structurales dans les remaniements membranaires indispensables à la biogenèse de ces organelles de réplication. Il est également évident que la formation et le maintien de ces structures nécessitent la contribution de cofacteurs cellulaires dont un grand nombre sont des acteurs du métabolisme lipidique ou participent à la régulation des courbures membranaires ou du trafic vésiculaire. Dans cette revue, nous faisons état des connaissances relatives à l'organisation des organelles membranaires formées lors de la réplication des virus à ARN(+). Nous décrivons également les principales interactions virus-hôte contribuant à la biogenèse de ces compartiments membranaires qui séparent, coordonnent et protègent la réplication du génome viral.

Architecture des organelles membranaires hébergeant la réplication des virus à ARN(+)

La réplication des virus à ARN(+) est initiée, après l'étape de décapsidation, par la prise en charge directe

du génome viral par la machinerie traductionnelle cellulaire (*figure 1*). Ces événements précoces permettent invariablement la traduction d'une polyprotéine dont la maturation par les protéases virales, et parfois cellulaires, permet l'assemblage des enzymes virales (protéines non structurales) et la formation d'une machinerie de réplication fonctionnelle. Ce complexe s'associe rapidement à une copie de ce même ARN génomique et permet la synthèse de nouveaux ARN de polarité positive grâce aux activités polymérase, hélicase et méthyltransférase qu'il contient. Cette synthèse nécessite la production d'un ARN de polarité négative complémentaire au génome viral conduisant temporairement à la présence de duplex d'ARN (ARNdb) dans le cytoplasme des cellules infectées. De façon générale, ces événements se réalisent en étroite association avec les membranes cellulaires. La présence, dans le complexe de réplication, de protéines non structurales contenant un ou plusieurs domaines hydrophobes permet en effet de cibler la machinerie de réplication vers les membranes de divers organites cellulaires où l'assemblage du complexe de réplication actif induit des remaniements membranaires. Ces événements aboutissent à la formation d'un réseau vésiculaire plus ou moins complexe [1]. La détection dans ces compartiments de protéines virales non structurales et de l'ARNdb, servant d'intermédiaire à la transcription de nouveaux ARN, a définitivement désigné ces compartiments comme sites de réplication des virus à ARN(+).

Selon la famille virale, les membranes qui servent de plateforme pour l'assemblage de ces compartiments dérivent soit du réticulum endoplasmique (RE) (*Coronavirus*, *Picornavirus*, *Flavivirus*, *Hepacivirus*, *Arterivirus*), du Golgi (*Enterovirus*), des membranes mitochondriales (*Nodavirus*), de la membrane plasmique (*Alphavirus*) ou encore des chloroplastes dans les cellules de plante (*Potyvirus*, *Tymovirus*) [2] (*tableau 1*, page 163). Dans certains cas (virus Flock house [FHV], virus Tomato Bushy Stunt [TBSV]), l'adressage vers ces compartiments spécifiques peut être contourné sans affecter l'efficacité du processus de réplication [3], suggérant la capacité de certains virus à utiliser les membranes de différentes organelles cellulaires en fonction des conditions ou de l'espèce hôte.

Le développement des techniques de cryofixation à haute pression combinées à la microscopie électronique (*cryo-electron microscopy* [Cryo-EM]) ou à la tomographie électronique (*cryo-electron tomography* [Cryo-ET]) (*figure 2*) [4, 5] ainsi que la microscopie électronique à corrélation de fluorescence (*correlative light and electron microscopy* [CLEM]) [5], qui combine les avantages du marquage fluorescent et de la microscopie électronique, ont permis de résoudre l'architecture tridimensionnelle de ces compartiments à une résolution nanométrique, faisant ainsi progresser fondamentalement notre compréhension de leur organisation. Leur utilisation a permis de

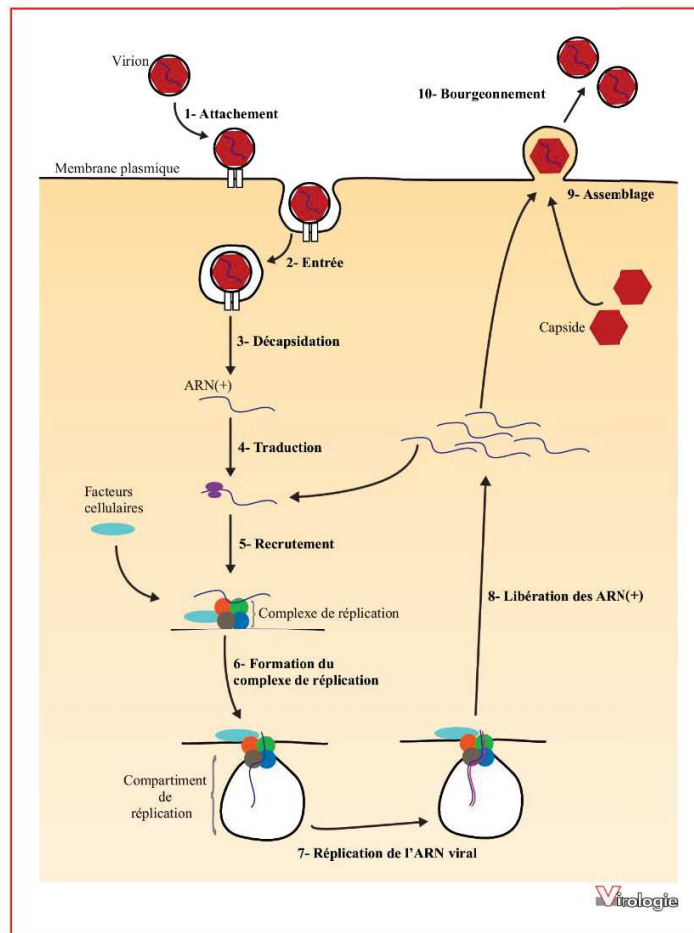


Figure 1. Cycle de réplication des virus à ARN de polarité positive. Après attachement à la membrane plasmique *via* des récepteurs membranaires (1), le virus pénètre dans la cellule hôte (2). S'ensuit une étape de décapsulation (3) qui permet la libération de l'ARN génomique viral dans le cytoplasme. Ces événements précoces mènent à la prise en charge du génome viral par la machinerie de traduction de la cellule hôte, permettant la synthèse de protéines virales (4). Les enzymes virales (protéines non structurales) synthétisées complexées à l'ARN génomique viral ainsi qu'à des facteurs cellulaires sont recrutées à la membrane (5), formant un complexe de réplication fonctionnel (6). La réplication de l'ARN viral est ensuite initiée dans ce compartiment de réplication (7), conduisant à la formation de duplex d'ARN, jusqu'à la libération de multiples copies d'ARN néosynthétisés dans le cytoplasme (8). De nouveaux virions sont finalement assemblés (9) puis libérés dans le milieu extracellulaire (10).

séparer ces compartiments en deux catégories morphologiques (figure 3) :

– les compartiments résultant d'une invagination positive des membranes cellulaires vers le cytoplasme. Il s'agit généralement de structures vésiculaires délimitées par une

double membrane (DMV) et connectées les unes aux autres, formant un compartiment de 150-300 nm identifié par exemple dans les cellules infectées par les *Coronavirus* (virus du syndrome respiratoire aigu sévère [SARS-CoV] [6], virus du syndrome respiratoire du Moyen-Orient

Tableau 1 Facteurs cellulaires impliqués dans la biogenèse des organelles de réplication des virus à ARN(+).

Famille	Virus	Hôte	Organelle de réplication	Compartiment	Facteur cellulaire	Références
Flavivirus	DENV				OSBP, FASN, vimentine, Arf1/GBF1, réticulon	[51, 60, 70, 85]
	WNV	Homme/oiseau/ primate/moustique		RE	HMGCR, réticulon	[49, 85]
	ZIKV		Vimentine, réticulon		[14, 85]	
	YFV				Arf1/GBF1	[68]
Alphavirus	SINV				BIN/amphiphysin	[86]
	SFV	Homme/moustique	SMV	MP/lysosomes	BIN/amphiphysin	[86]
	CHIKV			MP	BIN/amphiphysin	[86]
Nodavirus	FHV	Insecte		Mitochondrie	Inconnu	
Tombusvirus	TBSV	Plante/levure		Péroxyosome	ESCRT, actine	[77, 78]
Bromovirus	BMV	Plante/levure		RE	ESCRT, réticulon, Ole1	[76, 82, 113]
Picornavirus	CBV3	Homme		Golgi	OSBP, Arf1/GBF1, PI4KIII α	[44, 47]
	PV		Arf1/GBF1, LC3		[67, 114]	
Hepacivirus	HCV	Homme		RE	OSBP, FASN, PI4KIII α , PI4KIII β FAPP2, Arf1/GBF1, hVAP-A, PSTPIP2, réticulon	[43, 44, 48, 53, 58, 83, 84, 91, 115]
Coronavirus	MHV	Homme	DMV		Arf1/GBF1, LC3-I	
	SARS-CoV	Homme			Inconnu	
	MERS-CoV	Homme		RE	Inconnu	[72, 93, 111]
	IBV	Homme			Inconnu	
Arterivirus	PRRSV	Mammifère		RE	Inconnu	

BMV : virus de la mosaïque du Brome ; CBV3 : coxsackievirus B3 ; CHIKV : virus chikungunya ; DENV : virus de la dengue ; FHV : virus Flock House ; HCV : virus de l'hépatite C ; IBV : virus de la bronchite infectieuse ; MERS-CoV : Coronavirus du syndrome respiratoire du Moyen-Orient ; MHV : virus de l'hépatite murine ; PRRSV : virus du syndrome respiratoire et reproducteur porcin ; PV : poliovirus ; SARS-CoV : Coronavirus du syndrome respiratoire aigu sévère ; SFV : virus de la forêt de Semliki ; SINV : virus Sindbis ; TBSV : virus Tomato Bushy Stunt ; WNV : virus du Nil occidental ; YFV : virus de la fièvre jaune ; ZIKV : virus Zika ; DMV : vésicule à double membrane ; MP : membrane plasmique ; RE : réticulum endoplasmique ; SMV : vésicule à simple membrane.

[MERS-CoV] et virus de l'hépatite murine [MHV]) [7] ou de taille plus limitée (95-100 nm) dans le cas des *Arteriviridae* [3]. Ces structures à double membrane (DMV) sont également observées dans un contexte d'infection par le HCV (figure 2D) par opposition aux autres *Flaviviridae* [8]. La purification de ces compartiments a permis d'y détecter une activité réplicase, confirmant le rôle de ces structures dans la multiplication virale. Cependant, de façon surprenante, seulement 10 % de ces structures à double membrane apparaissent connectées au cytoplasme [9], suggérant soit

la possibilité que seul un petit nombre de ces structures sont engagées dans la réplication virale, soit l'existence de systèmes actifs de transport qui permettraient l'import, depuis le cytoplasme, des métabolites nécessaires à la réplication et l'export des ARN néosynthétisés depuis les organelles de réplication. La détection de nucléoporines (Nup358, Nup155, Nup53, Nup153, Nup98) dans les régions du cytoplasme avoisinant les organelles formées par le HCV [10] est en faveur de cette dernière hypothèse. Certaines études proposent enfin une localisation du complexe de

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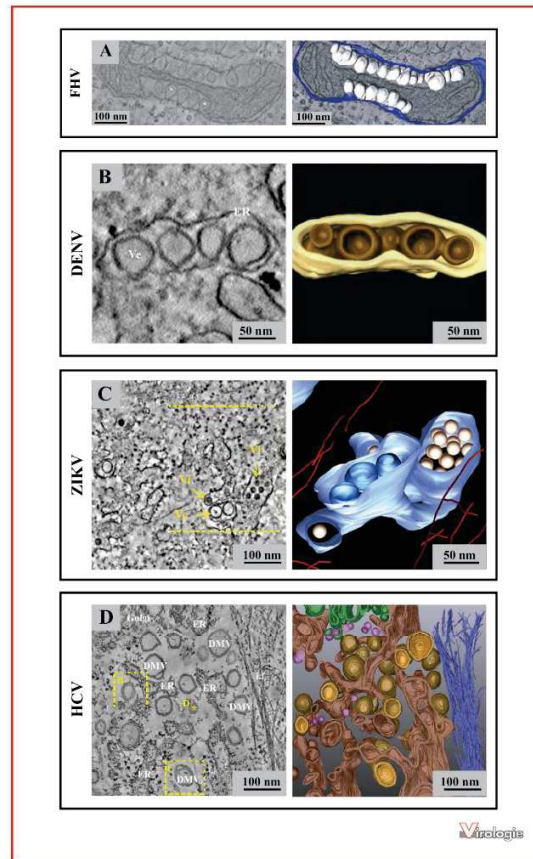
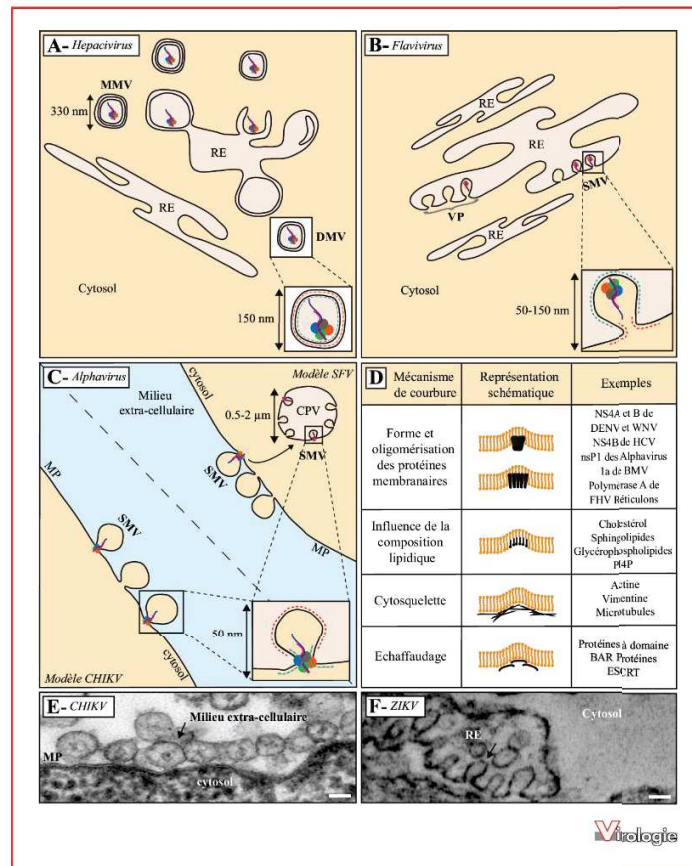


Figure 2. Observation microscopique et reconstruction tridimensionnelle des organelles de réplication de différents virus à ARN de polarité positive. A) Image de tomographie électronique d'une mitochondrie de cellule de drosophile infectée par le virus Flock House (FHV). Gauche : acquisition tomographique des sphérules induites par le FHV à la membrane mitochondriale. Les astérisques indiquent deux sphérules connectées à la membrane externe de la mitochondrie par un pore. Droite : reconstruction 3D de la même mitochondrie modifiée par le FHV superposée à l'image de microscopie électronique présentée à gauche. La membrane mitochondriale externe est colorisée en bleu, les sphérules induites par le FHV sont figurées en blanc [26]. **B) Image de tomographie électronique du réticulum endoplasmique d'une cellule infectée par le virus de la dengue (DENV).** Gauche : tomogramme montrant les vésicules de réplication (Ve) correspondant à des invaginations de la membrane du réticulum endoplasmique (ER) connectées au cytoplasme par un pore de 10 nm de diamètre. Droite : reconstruction 3D correspondante. Le réticulum endoplasmique est représenté en jaune, les organelles de réplication sont colorisées en marron [112]. **C) Image de tomographie électronique d'organelles de réplication induites par le virus Zika (ZIKV) dans des progéniteurs neuraux humains.** Gauche : tomogramme montrant des vésicules de réplication (Ve) induites par ZIKV dans le réticulum endoplasmique ainsi que des virions (Vi). Droite : reconstruction 3D d'une partie de la région encadrée en pointillés jaunes. La membrane du réticulum endoplasmique est indiquée en bleu clair, les vésicules de réplication sont représentées en bleu foncé, les virions en blanc et les filaments intermédiaires en rouge [14]. **D) Image de tomographie électronique d'organelles de réplication induites par le virus de l'hépatite C (HCV) dans des cellules Huh7.5.** Gauche : tomogramme montrant diverses altérations membranaires induites par HCV (ER : réticulum endoplasmique ; DMV : vésicules à double membrane ; i.f. : filaments intermédiaires). L'astérisque et les encadrés en pointillés jaunes indiquent des vésicules à double membrane. Droite : reconstruction 3D du tomogramme complet. Le réticulum endoplasmique est représenté en marron foncé, la membrane interne des vésicules à double membrane est indiquée en jaune ocre, leur membrane externe en marron clair. Les vésicules à simple membrane sont colorisées en rose, les filaments intermédiaires en bleu foncé et l'appareil de Golgi en vert [9].

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Figure 3. Compartiments cellulaires supportant la formation des organelles de réplication de différents virus à ARN de polarité positive. A-C) Représentation schématique des compartiments de réplication induits par différentes familles virales. A) Les *Hepadnavirus* induisent des compartiments de réplication de type vésicules à double membrane (DMV) dont la membrane externe dérive du réticulum endoplasmique (RE). Ces structures sont associées à la présence de vésicules multi-membranaires (MMV). B) Les *Flavivirus* forment des sphérules à simple membrane (SMV) ainsi que des ensembles de sphérules appelées *vesicle packets* (VP) par invagination du réticulum endoplasmique (RE). C) Les *Alphavirus* génèrent des sphérules à simple membrane (SMV) par évagination de la membrane plasmique (MP) pouvant être endocytés dans des vacuoles cytopathiques (CPV) dans le cas d'une infection par le virus de la forêt de Semliki (SFV) par exemple, ou persister à la membrane plasmique lors de l'infection par le virus chikungunya (CHIKV). Les zones de courbure négative (pointillé vert) et positive (pointillé rouge) de la membrane sont indiquées pour chaque type de compartiment. D) Mécanismes de courbure membranaire impliqués dans la formation des compartiments de réplication viraux. E, F) Observations en microscopie électronique des sphérules induites lors d'une infection de cellules HEK293T (E) et Vero (F) par le virus chikungunya (E) et le virus Zika (F). Flèche noire : sphérule ; barre d'échelle : 50 nm.

réplication sur la face externe de ces structures [9]. En l'absence de technique permettant la détection des ARN viraux dans les préparations observées en microscopie électronique, la localisation exacte du complexe de réplication associé aux DMV reste donc débattue. L'observation

des régions avoisinant les DMV produites par différents virus permet généralement de retrouver, à proximité de ces compartiments, une variété de structures membranaires plus ou moins complexes (vésicules à simple membrane, vésicules multi-membranaires, tubules, membranes

zippées) susceptibles de représenter des états intermédiaires de la biogenèse de ces organelles [11, 12]. Les étapes exactes qui conduisent à la production finale des DMV sont encore sujettes à spéculations. Enfin, dans ce modèle, les organelles de réplication jouxtent les événements de bourgeonnement supportés par les membranes en continuité avec les vésicules de réplication. Cela implique l'existence d'un continuum entre les différents événements du cycle infectieux [7] :

– les compartiments résultant d'une courbure négative des membranes correspondent à des projections de membranes préexistantes vers la lumière d'une organelle ou vers le milieu extracellulaire. Ces compartiments de réplication sont délimités par la continuité des membranes cellulaires et reliés au cytoplasme par un pore de 8 à 11 nm supposé permettre l'accès aux métabolites et aux cofacteurs cellulaires nécessaires à la réplication et l'export des ARN néosynthétisés. Ces structures, appelées sphérules et décrites pour la première fois dans les années 1960 à partir de cellules infectées par l'*Alphavirus* SFV [13], sont également observées dans un contexte d'infection par les *Tombusviridae*, les *Bromoviridae*, les *Nodaviridae* et les *Flaviviridae*. Pour cette dernière famille qui inclut notamment le DENV et le ZIKV, la reconstruction 3D de ces organelles étudiées par tomographie électronique a établi que ces compartiments à simple membrane, produits par déformation de la membrane du RE, s'organisent en un réseau complexe de vésicules interconnectées dont la taille peut atteindre 80 à 150 nm de diamètre (figure 2B-C) [12, 14]. Dans le cas des *Alphavirus* (SFV et SINV), ces compartiments, formés initialement à la membrane plasmique de la cellule infectée, sont rapidement internalisés suite à l'activation de la voie phosphatidylinositol-3-kinase (PI3K)-Akt-mTOR (*mammalian target of rapamycin*)-dépendante [15], donnant naissance aux vacuoles cytopathiques (CPV), visualisées sous la forme de larges compartiments cytoplasmiques (de diamètre de 600 à 2000 nm) portant des sphérules sur leurs membranes limitantes et dont la nature correspond à des endosomes/lysosomes modifiés [16]. L'importance fonctionnelle de cette internalisation reste mal comprise, dans la mesure où son inhibition n'a que peu d'impact sur la réplication virale et où ce processus d'internalisation n'est que très marginal pour certains *Alphavirus* comme CHIKV [17, 18]. Dans ce modèle de vésicules, une attention particulière a été portée à l'organisation des pores qui connectent l'organelle de réplication avec le cytosol. De récents travaux réalisés sur le modèle du FHV ont démontré que cette zone est composée d'un assemblage en couronne formé par 12 copies de la réplicase virale A, une structure qui semble assurer le maintien du pore de connexion avec le cytoplasme [19]. Comme pour les DMV, ces vésicules avoisinent généralement les sites d'assemblage de particules virales néoformées [14]. Ces

réseaux membranaires sont donc susceptibles de constituer des plateformes assurant la coordination des étapes de réplication de l'ARN, de traduction et d'assemblage viral.

Protéines virales, déformations membranaires et compartiments de réplication viraux

La formation de ces compartiments repose en tout premier lieu sur l'interaction des protéines non structurales des virus à ARN(+) avec les feuillettes lipidiques des membranes cellulaires. Un grand nombre de ces protéines, dotées de domaines transmembranaires ou d'hélices amphipathiques capables de s'insérer dans les feuillettes lipidiques, remodelent les membranes de la cellule hôte, y compris lorsqu'elles sont exprimées hors contexte d'infection. Cette situation est tout particulièrement illustrée par la protéine 1a du virus de la mosaïque du brome (BMV, appartenant à la famille des *Bromoviridae*), dont la surexpression dans la levure ou la cellule végétale génère des invaginations vers la lumière du RE très similaires aux sphérules observées dans un contexte d'infection [20]. Cette protéine, responsable du transfert de la coiffe des ARN viraux et assurant également les fonctions NTPase et hélicase essentielles à la réplication virale, se structure en hélice amphipathique dans sa partie centrale (a.a. 392-405) [21]. Son affinité membranaire permet l'adressage et l'ancrage du complexe de réplication aux membranes du RE grâce aux interactions qu'elle engage directement avec les ARN viraux et avec les autres protéines du complexe de réplication. Dans la cellule infectée, la protéine 1a se localise à la face interne des sphérules formées pour la réplication de ce virus. Les déformations cellulaires générées sont associées à la capacité de cette protéine à s'oligomériser. L'analyse par microscopie électronique combinée à l'immunomarquage a défini que chacun des compartiments membranaires formés par le BMV contient 200 à 400 protéines 1a pour seulement une ou deux copies d'ARN et dix à 20 copies de polymérase [22]. Ces caractéristiques signalent la capacité de la protéine 1a à former un manteau rigide tapissant la membrane du RE et essentiel à la déformation membranaire et au maintien de la morphologie des compartiments de réplication viraux. Un scénario identique a été établi pour la polymérase A des *Nodavirus*, dont plus de 100 copies sont retrouvées dans les sphérules produites par déformation des membranes mitochondriales [23]. La fonction de ces protéines virales serait ainsi à rapprocher de celle des capsides virales au cours du bourgeonnement.

Cette situation n'est cependant pas systématiquement retrouvée pour les virus produisant des compartiments à simple membrane. En effet, dans le cas des *Alphavirus*, l'ancrage membranaire du complexe de réplication

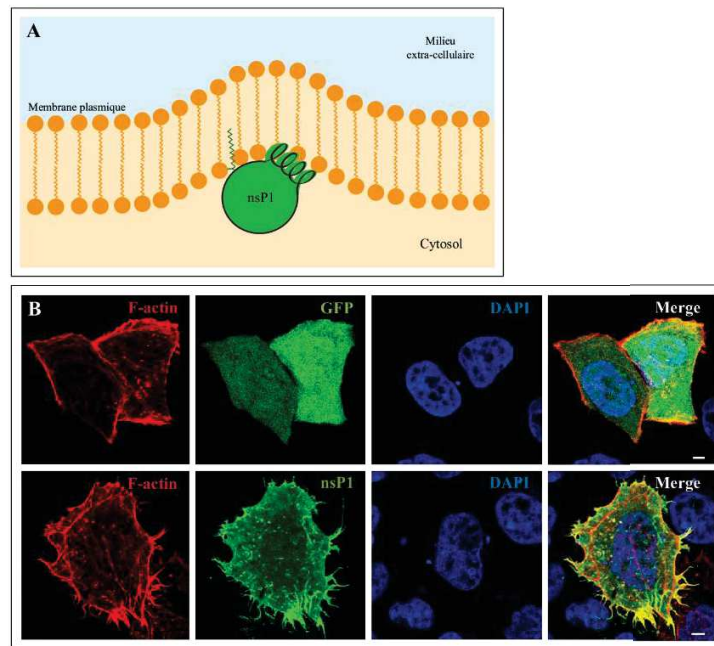


Figure 4. Protéines non structurales et déformations membranaires : exemple de la protéine nsP1 des *Alphavirus*. **A) Représentation schématisée de l'interaction de nsP1 avec la membrane plasmique.** Cette association est conditionnée notamment par l'interaction d'une hélice alpha amphipathique avec la bicouche lipidique et par la présence d'une ou de plusieurs cystéines palmitoylées [27, 29]. **B) Observations en immunofluorescence de cellules HeLa transfectées avec la protéine virale nsP1 du virus chikungunya fusionnée à la GFP.** L'expression isolée de la protéine de fusion GFP-nsP1 induit la formation de filopodes riches en filaments d'actine. Les filaments d'actine (F-actin) sont marqués par coexpression du plasmide LifeAct. Barre d'échelle : 5 μ m.

est assuré par la protéine méthyl/guanylyltransférase nsP1 [24]. Comme les protéines 1a du BMV [21] et poLA de FHV [25, 26], nsP1 contient une séquence centrale enrichie en acides aminés chargés positivement et hydrophobes qui se structure en hélice amphipathique au contact de membranes artificielles [27] (figure 4A). Les interactions polaires engagées avec les phospholipides membranaires gouvernent l'interaction de nsP1 avec les membranes cellulaires [28]. Celle-ci est renforcée par un motif de N-acétylation, également situé dans la partie centrale de la protéine [29]. Hors contexte d'infection, l'expression isolée de nsP1 induit des déformations significatives de la membrane plasmique et génère des structures de types filopodes et lamellipodes dynamiques au niveau desquelles la protéine nsP1 est détectée [30] (figure 4B). La morphologie de ces structures diffère cependant significativement de celle des sphérules qui hébergent la réplication des alphavirus [13], suggérant l'action coordonnée de nsP1 avec d'autres protéines non structurales et des cofacteurs cellulaires dans la production

des sphérules nécessaires à la réplication virale. Ce type de mécanisme complexe impliquant l'action synchronisée de plusieurs protéines non structurales semble également contribuer à la formation des vésicules à double membrane. Ainsi, dans le cas du HCV, chacune des protéines non structurales remanie les membranes auxquelles elles s'associent. Parmi celles-ci, NS4B, dotée de quatre domaines transmembranaires et d'hélices amphipathiques, génère une courbure positive des membranes dans lesquelles elle s'insère, aboutissant à la production de vésicules à simple membrane [31]. NS5A, quant à elle, suffit à produire des déformations à double membrane semblables à celles observées au cours d'une infection lorsqu'elle est exprimée isolément [9, 32]. Bien que cette dernière semble jouer un rôle prépondérant dans la formation des réseaux complexes qui hébergent la réplication de HCV, aucune de ces protéines ne suffit à elle seule à créer des compartiments membranaires identiques en taille ou en morphologie à ceux observés au cours d'une infection. Ceux-ci nécessitent, en revanche,

la coopération des protéines NS3, NS3-4A et NS4B-5B [8, 9, 31, 33]. Dans ce contexte, l'association de NS4B et de NSSA sous forme d'homodimères ou d'oligomères, notamment grâce à la présence de motifs de type « glycine zipper » dans les hélices transmembranaires de NS4B, serait requise pour induire des déformations membranaires similaires à celles observées dans les cellules infectées [34, 35].

Interactions hôte-pathogène impliquées dans la formation des organelles de réplication des virus à ARN(+)

Si la contribution des protéines virales est essentielle à la formation des compartiments de réplication des virus à ARN(+) à l'interface avec les membranes cellulaires, de nombreuses observations indiquent clairement la contribution de machineries cellulaires. La connaissance des cofacteurs nécessaires à la réplication des virus à ARN(+) a bénéficié de l'avènement des stratégies de criblage à l'échelle génomique (interférence ARN [RNAi], CRISPR/Cas9, mutagenèse insertionnelle) [36-39]. En combinaison avec les études biochimiques et fonctionnelles, le développement des techniques de génétique inverse et d'imagerie cellulaire, ces approches ont été appliquées avec succès à l'étude des *Flaviviridae* HCV, WNV (virus du Nil occidental), DENV et aux virus de plantes TBSV, BMV, permettant d'identifier les premiers facteurs cellulaires nécessaires à la biogenèse des compartiments de réplication de ces pathogènes. Ces résultats ont ainsi mis en évidence la nécessité, pour les virus à ARN(+), qu'ils se répliquent dans les cellules de vertébrés, chez l'insecte ou chez la plante, d'usurper des fonctions communes de leur hôte, ayant le plus souvent une fonction physiologique dans l'extension, le remaniement des membranes ou le transport vésiculaire. Ce paragraphe résume les connaissances relatives aux principaux acteurs cellulaires qui participent à la création des organelles de réplication chez différentes familles virales.

Lipides membranaires et organelles de réplication

La recherche des facteurs de l'hôte participant à la biogenèse des compartiments de réplication viraux a conduit à s'intéresser en tout premier lieu au métabolisme lipidique de l'hôte. La création de ces organelles repose en effet souvent sur l'expansion très significative des membranes cellulaires, suggérant une activation des voies qui contrôlent la lipogenèse. La caractérisation des profils lipidiques des cellules infectées a confirmé l'impact des virus à ARN(+) sur l'homéostasie lipidique de leur hôte. Celle-ci est le plus souvent signée par une augmentation de la synthèse de phospholipides, de sphingolipides et de stérols

[40, 41]. La ségrégation des ARNdb et des protéines non structurales avec des microdomaines membranaires enrichis en ces mêmes espèces lipidiques renforce l'hypothèse d'une accumulation spécifique de lipides sur les sites de réplication de nombreux virus [42]. Il apparaît désormais que les virus à ARN(+) modifient au moins localement la composition des membranes afin de créer un environnement favorable à l'assemblage et à l'activité de leur complexe de réplication [37, 43-45]. Ces modifications peuvent tout d'abord résulter d'une captation accrue de lipides extracellulaires [46] et de leur acheminement vers le site de réplication par des transporteurs spécifiques tels que l'*oxysterol-binding protein* (OSBP), responsable de l'enrichissement en cholestérol des organelles de réplication des *Enterovirus* et des *Hepacivirus* [47, 48]. Les modifications membranaires observées reposent également sur la stimulation et le détournement des enzymes du métabolisme lipidique. Ainsi, dans les cellules infectées par le *Flavivirus* WNV, la 3-hydroxy-méthylglutaryl-CoA réductase (HMGCR), enzyme clé de la synthèse du cholestérol, est surexprimée [49]. Sa relocalisation vers les réseaux membranaires dérivés du RE, où elle s'associe à la machinerie de réplication virale, permettrait l'enrichissement en cholestérol des membranes servant de plateforme à la réplication virale. Un scénario analogue a été décrit pour l'acide gras synthase (*fatty acid synthase* [FASN]) qui assure la synthèse du palmitate, précurseur des phospholipides membranaires, dont l'activité est indispensable à la réplication des *Flavivirus* [50, 51]. Dans le cas de HCV, l'expression de FASN est fortement stimulée par l'action de la protéine virale NS4B sur le facteur de transcription SREBP (*sterol-regulatory element-binding protein*) qui régule l'expression des enzymes de la lipogenèse [52]. Le recrutement de FASN vers les organelles de réplication des *Flaviviridae* par les protéines non structurales (NS5B [53] pour HCV, NS3 pour DENV [51, 54]) est supposé assurer l'accumulation locale d'acides gras nécessaire à l'expansion des membranes du RE.

Récemment, cette enzyme a également été détectée sur le site de réplication de l'*Alphavirus* CHIKV [55]. Bien que son activité soit essentielle à la réplication virale, son éventuelle implication dans la création des sphères membranaires formées par ce pathogène reste inconnue à cette heure. Les phosphatidylinositol-4-kinases de type III (PI4KIII) constituent un autre exemple d'enzyme lipogénique contribuant à la réplication de nombreux virus à ARN(+). Les PI4KIII existent sous la forme de deux isoformes (α et β) situées dans des organites distincts. Le produit de ces enzymes, le PI4P, joue un rôle de précurseur dans la synthèse d'autres espèces de phosphoinositides. L'isoforme PI4KIII α , présente dans le RE, constitue un facteur essentiel à la réplication de HCV recruté vers les sites de réplication et activé par la protéine virale NSSA [43].

L'inactivation de cette enzyme modifie significativement la morphologie des réseaux membranaires qui abritent les organelles de réplication de HCV, reliant ainsi directement la PI4KIII et la formation des compartiments de réplication de ce virus. De façon similaire, le recrutement de la CTP-phosphocholine cytidyl transférase α (CCT α) sur les sites de réplication des *Picornavirus* pourrait favoriser la production locale de phosphatidylcholine nécessaire à la production d'organelles de réplication fonctionnelles [56]. Outre leur rôle fondamental dans la production de nouvelles membranes intracellulaires, ces enzymes, en favorisant la concentration d'espèces lipidiques dont les caractéristiques biochimiques (composition de leur tête polaire, longueur ou saturation de la chaîne acyle) influent sur la plasticité membranaire, sont susceptibles de participer à la courbure des membranes nécessaire à la formation des compartiments de réplication des virus à ARN(+). Les conséquences des modifications lipidiques rencontrées au cours de ces infections dépassent cependant largement le simple remodelage membranaire. L'accumulation locale de lipides spécifiques est, en effet, indispensable à la localisation et à l'activité optimale des enzymes virales participant au complexe de réplication. À titre d'exemple, l'activité de la méthyl/guanylyltransférase nsP1 des *Alphavirus* est fortement dépendante de la présence de phospholipides anioniques, en particulier la phosphatidylsérine, dans les membranes cellulaires [28]. De façon similaire, l'accumulation membranaire de PI4P joue un rôle essentiel dans l'ancrage et l'activité de la polymérase 3D du PV au sein du complexe de réplication [44]. Enfin, la concentration locale de sphingolipides stimule également l'activité de la polymérase de HCV de manière spécifique au génotype [57]. Pour finir, ces variations lipidiques, comme la création de microdomaines membranaires riches en cholestérol, sont susceptibles de favoriser le recrutement local des cofacteurs cellulaires nécessaires à la réplication virale. L'accumulation locale de PI4P est en effet responsable de la relocalisation vers les sites de réplication des transporteurs de lipides FAPP2 (*four-phosphate adaptor protein 2*) et OSBP (*Oxysterol-binding protein 1*) permettant respectivement l'enrichissement local en glycosphingolipides et en cholestérol [48, 58]. Ainsi, le détournement du métabolisme lipidique constitue non seulement une stratégie visant à augmenter la surface des membranes disponibles pour la création d'organelles de réplication, mais permettrait aussi de créer un environnement favorable au fonctionnement optimal de la machinerie de réplication virale.

Le cytosquelette

La fonction physiologique du cytosquelette dans le transport intracellulaire et dans la dynamique des membranes

cellulaires laisse entrevoir son importance dans la formation des organelles membranaires de réplication virale. De nombreuses études ont permis de localiser les complexes de réplication des virus à ARN(+) à proximité immédiate des réseaux de microfilaments formés par l'actine, des microtubules ou des filaments intermédiaires riches en vimentine [17, 59]. Les structures caractéristiques composées de filaments intermédiaires et de microtubules, formant de véritables cages englobant les organelles de réplication du ZIKV, représentent l'exemple le plus caractéristique de cette association [14]. De façon générale, les virus à ARN(+) remanient le cytosquelette d'actine, les microtubules et les filaments intermédiaires de leur hôte à des fins de réplication [60-62]. Dans le cas du DENV, ce remaniement met en jeu l'interaction directe des protéines NS5A [63] et NS4A [60] avec la vimentine, conduisant à la concentration de cette dernière vers les sites de réplication virale où elle s'associe à la protéine NS1 [64]. Bien que la vimentine se soit révélée essentielle pour la localisation des complexes de réplication dans la cellule infectée [60], son rôle et celui des filaments intermédiaires dans la formation et/ou le maintien de l'organisation des complexes de réplication des *Flavivirus* reste encore mal compris. Dans le cas des *Alphavirus*, il est également clairement établi que la réplication virale nécessite l'intégrité du cytosquelette cellulaire [17]. Dans ce modèle, la capacité de la méthyl/guanylyltransférase nsP1 à stimuler la formation de filopodes et de lamellipodes suggère son action sur le cytosquelette. Ainsi l'expression de nsP1 génère le remaniement des fibres de stress sans qu'un effet de cette protéine sur les réseaux d'actine et les microtubules ait pu être observé [30]. Des interactions directes ont été décrites entre la protéine nsP3 de cette famille de virus et les protéines du cytosquelette (actine, tubuline, myosine) et des filaments intermédiaires (vimentine) [59, 65]. Les données les plus récentes acquises sur cette famille virale décrivent les interactions du domaine C-terminal de la protéine nsP3 des *Alphavirus* CHIKV et SFV avec plusieurs protéines du cytosquelette dont CD2AP (*CD2-associated protein*) et SH3KBP1, impliquées dans l'organisation des fibres d'actine lors de l'endocytose et qui colocalisent avec les compartiments de réplication viraux [66]. La contribution précise de chacun de ces phénomènes dans la formation des organelles de réplication reste donc à déterminer.

Les régulateurs du trafic vésiculaire

Les facteurs cellulaires naturellement impliqués dans les remaniements membranaires et jouant un rôle essentiel dans le transport vésiculaire apparaissent comme des partenaires privilégiés de la formation des organelles de réplication. En tout premier lieu, ce sont les composants de la voie de sécrétion COPI qui ont été décrits comme acteurs essen-

tiels de la formation des organelles de réplication des virus à ARN(+). Parmi ceux-ci, la GTPase Arf1 (*ADP Ribosylation Factor 1*) et son facteur d'échange nucléotidique GBF1 (*Golgi Brefeldin A Resistant Guanine Nucleotide Exchange Factor 1*), impliqués dans le bourgeonnement des vésicules de sécrétion précoce Golgi/ER, sont recrutés par les protéines non structurales 3A et 3CD du poliovirus et relocalisés à proximité des complexes de réplication de ce virus [67]. La sensibilité de la réplication virale à la bréfeldine A, un inhibiteur de GBF1, suggère l'importance fonctionnelle de ce recrutement. Il existe désormais de nombreuses preuves du rôle de cette voie dans la réplication des virus à ARN(+) (*Coronavirus/MHV*, *Enterovirus/CBV3*, *Hepacivirus/HCV*, *Flavivirus/DENV*, *YFV* [virus de la fièvre jaune]) [68-72]. Toutefois, bien que la structuration du domaine N-terminal de Arf1 en hélice amphipathique, capable de courber les membranes, soit compatible avec un rôle dans la formation des organelles membranaires de réplication, sa fonction directe dans la biogenèse des compartiments de réplication de ces virus reste très débattue. L'absence de modification morphologique des organelles de réplication en présence de Bréfeldine A ou lors d'une inactivation de GBF1 ou Arf1 [73] et l'incapacité du PV à stimuler Arf1 orientent désormais vers un rôle de ces protéines dans la maturation ou le fonctionnement des organelles de réplication virales plutôt que dans leur biogenèse précoce. Un rôle indirect de Arf1 et GBF1 basé sur le recrutement de la PI4KIII β et la régulation du contenu membranaire en PI4P a également été proposé [74, 75]. Les protéines du complexe ESCRT (*endosomal sorting complexes required for transport*) sont d'autres exemples de régulateurs du trafic vésiculaires participant à la biogenèse des compartiments de réplication des virus à ARN(+). Ces protéines capables d'induire des courbures négatives des membranes endosomales nécessaires au bourgeonnement des corps multivésiculaires dans la lumière des endosomes tardifs participent à la biogenèse des organelles de réplication générées par les virus de plante BMV [76] et TBSV [77, 78]. Dans le cas du TBSV, la protéine Vps23p (homologue de Tsg101 des mammifères) participant au complexe ESCRT-I, est recrutée par la protéine virale p33 ubiquitinyliée vers les membranes péroxysomales où elle initie les déformations nécessaires à la création des organelles de réplication [78]. Le recrutement successif des facteurs Snf7, Vps20p, Vps24p et de l'ATPase Vps4p composant le complexe ESCRT-III permet les déformations membranaires nécessaires à la biogenèse des sphères de réplication. Ce complexe participe aussi possiblement au maintien de ces structures par la stabilisation des pores de connexion de ces organelles. L'inactivation des protéines ESCRT a, en effet, comme conséquence d'inhiber la réplication de l'ARN viral en favorisant son accessibilité par les ribonucléases [77]. Ce partenariat lors de la biogenèse

des compartiments de réplication fait écho aux événements de bourgeonnement de certains virus dans lesquels les protéines ESCRT jouent un rôle prépondérant.

Les facteurs de remodelage membranaire

Les protéines de la famille réticulon ont également été identifiées comme partenaires privilégiés de la formation des compartiments de réplication des virus à ARN(+). Ces protéines naturellement impliquées dans la morphogénèse membranaire sont présentes dans les zones du RE de forte courbure et permettent la transition de la structure en feuillet vers les structures tubulaires de cet organelle [79]. Elles possèdent deux domaines hydrophobes qui s'organisent en épingle à cheveux. Leur insertion dans la membrane induit une asymétrie entre les deux feuillets de la bicouche lipidique, aboutissant à une courbure positive des membranes. Les réticulons ont initialement été identifiés comme cofacteurs de la réplication des *Enterovirus* et partenaires de la protéine virale 2C [80]. Ils interagissent également avec la protéine 1a du BMV [81] et sont recrutés vers les compartiments viraux [82]. Étant donné leur capacité à générer des courbures membranaires positives incompatibles avec la morphologie des organelles de réplication de ces virus, leur contribution a été essentiellement proposée dans la formation et le maintien des zones de connexion des compartiments de réplication au cytoplasme. L'incorporation de ces protéines dans les sphères produites par le BMV pourrait par ailleurs contrebalancer les courbures négatives générées par la protéine 1a et ainsi permettre l'expansion de ces compartiments. Dans tous les cas, la déplétion de ces protéines altère la morphologie des compartiments de réplication dont la taille est significativement réduite [81]. La contribution des réticulons à la réplication virale a ensuite été étendue aux *Flaviviridae*. Ils ont été identifiés comme des partenaires de la protéine NS4B du HCV [83, 84]. Leur inactivation réduit significativement les remodelages membranaires observés au cours de l'infection et rend la machinerie de réplication sensible à l'action des protéases cellulaires, avec pour effet de diminuer drastiquement la réplication de ces virus [85]. Ces interactions ont été récemment confirmées pour les *Flavivirus* WNV, DENV et ZIKV [85], soulignant la conservation des interactions virus-hôte requises pour la formation des compartiments de réplication au sein de cette famille virale. Chez les *Alphavirus*, la protéine BIN1/amphiphysine-2 est proposée comme partenaire lors de la formation des organelles de réplication [86]. Cette protéine, caractérisée par la présence d'un domaine N-BAR (BIN/amphiphysin/Rvs) structurellement courbé, s'associe par liaison électrostatique aux phospholipides chargés négativement. Elle s'insère dans les membranes grâce à sa structure en hélice amphipathique afin de favoriser et stabiliser les courbures

positives de la bicouche lipidique, notamment au cours du processus d'endocytose [87]. BIN1/amphiphysine-2 ainsi que son homologue BIN2/amphiphysine-1 sont recrutées vers les sites de réplication des *Alphavirus* SFV, CHIKV, SINV par une interaction avec le domaine C-terminal hypervariable de la protéine nsP3 [86, 88]. Cette interaction est essentielle à la synthèse de l'ARN(-) et de l'ARN(+) subgénomique, sans que la démonstration directe de son rôle dans la formation des sphérules des *Alphavirus* ait pu être faite. BIN1/amphiphysine-2 a, par ailleurs, été identifiée comme un partenaire de la protéine NS5A du HCV [89]. Dans ce cas, cependant, l'amphiphysine ne participe pas directement au remodelage des membranes du RE, mais régule la phosphorylation de NS5A nécessaire au recrutement de son partenaire cellulaire hVAP-A (*vesicle-associated-membrane protein A*) [90], lui-même impliqué dans le trafic vésiculaire. Ce même virus, par ailleurs, détourne la proline sérine thréonine phosphatase PST-PIP2, également membre de la superfamille des protéines à domaine BAR, pour la formation des compartiments membranaires de réplication [91]. Recrutée par les protéines virales NS4B et NS5A, PSTPIP2, dont la surexpression induit des structures membranaires tubulaires, participerait à l'induction et/ou à la stabilisation des compartiments de réplication de HCV.

La machinerie autophagique

La morphologie des compartiments de réplication à double membrane et leur connexion étroite au RE ont rapidement suggéré l'implication de la voie autophagique dans la formation de ces organelles. Dans un contexte physiologique, les compartiments à double membrane sont des événements rares, associés à la séquestration de grosses portions cytoplasmiques en vue de leur dégradation après fusion avec le compartiment lysosomal. Ce processus catabolique, appelé autophagie et permettant, en général, l'élimination d'organelles endommagées ou d'agrégats protéiques, est déclenché par des stress environnementaux incluant les stress trophiques et l'hypoxie. Il implique plus de 35 protéines d'autophagie (Atg) et se caractérise par la lipidation de la protéine LC3, un événement clé dans l'induction du processus autophagique [90]. Ce marqueur, parfois associé à des protéines lysosomales, est détecté à la surface des compartiments de réplication à double membrane induits par le HCV [92], les *Enterovirus* [93] ou encore les *Coronavirus* (MHV) [93]. Cette caractéristique, comme la capacité de ces virus à stimuler les voies autophagiques dans les cellules qu'ils infectent, ont suggéré une contribution de la machinerie autophagique dans la formation des compartiments de réplication de ces pathogènes [93, 94]. La capacité des protéines non structurales nsP6 des *Coronavirus* MHV, IBV (virus de la bronchite infectieuse), SARS

et de nsP5-7 de l'artérovirus PRRSV (virus du syndrome respiratoire et reproducteur porcin) à générer des structures vacuolaires Atg5⁺/LC3-II⁺ similaires aux autophagosomes précoces a renforcé l'hypothèse du rôle de la machinerie autophagique dans la formation des compartiments de réplication de ces virus [95]. Malgré ces observations et bien que l'autophagie puisse favoriser diverses étapes du cycle infectieux, l'inactivation du processus autophagique à l'aide d'inhibiteurs pharmacologiques ou *via* l'inactivation des gènes d'autophagie Atg5 et/ou Atg7 a produit des résultats contradictoires, voire négatifs, qui ne permettent pas de valider définitivement cette contribution [93, 96-98]. Les travaux récents d'Abernathy *et al.* démontrant la capacité de ces pathogènes à mobiliser des voies non canoniques de l'autophagie permettent d'expliquer certains résultats contradictoires obtenus jusqu'ici [99].

En résumé, le détournement de facteurs cellulaires naturellement impliqués dans le remodelage membranaire est un trait commun des virus à ARN(+), quelle que soit la morphologie des compartiments membranaires qu'ils produisent pour assurer la réplication de leur génome. La nature des facteurs recrutés diffère cependant. Ces différences pourraient être en relation avec la disponibilité de chacun de ces cofacteurs dans les différents compartiments membranaires usurpés lors de la réplication virale. L'usurpation des amphiphysines localisées à la membrane plasmique, des réticulons dans le réticulum endoplasmique, des protéines Arf/GBF1 dans le Golgi par les *Alphavirus*, *Enterovirus*, *Flaviviridae* est en effet en accord avec la localisation des compartiments de réplication créés par ces différentes familles virales.

Organelles de réplication et réponse immune

Si la fonction des réseaux et compartiments membranaires semble être de concentrer la machinerie de réplication, les cofacteurs et métabolites cellulaires nécessaires à son fonctionnement optimal, l'organisation de ces compartiments quasi clos permet aussi d'envisager un rôle de protection vis-à-vis du système immunitaire de l'hôte. Le système immunitaire inné constitue la première ligne de défense contre les agents infectieux. Sa fonction initiale est de reconnaître les déterminants moléculaires associés aux pathogènes (*pathogen-associated molecular patterns* [PAMP]) afin de stimuler la production d'effecteurs capables d'interférer efficacement avec la propagation de l'infection. Les ARN viraux, en particulier les duplex d'ARN servant d'intermédiaires à la réplication virale, sont des inducteurs puissants de la signalisation antivirale innée qui conduisent à la production rapide et

massive d'interférons et de cytokines pro-inflammatoires. Leur reconnaissance met en jeu les récepteurs cytosoliques de la famille des RLR (*RIG-I-like receptors*) et les récepteurs membranaires de la famille Toll (*Toll-like receptors* [TLR]) [100]. On retrouve tout particulièrement ces récepteurs sur les membranes péroxysomales, mitochondriales et du RE également impliquées dans la formation des organelles de réplication des virus à ARN(+). Bien qu'il n'y ait pas de démonstration formelle, la fonction des organelles de réplication virale dans la protection vis-à-vis des acteurs de l'immunité innée est notamment étayée par les expériences de déstabilisation membranaire. Ainsi, la synthèse des ARN des *Coronavirus* [101] et des *Flavivirus* WNV, JEV (virus de l'encéphalite japonaise) et DENV [102, 103] est rendue sensible à l'action des protéases et des nucléases par les détergents non ioniques déstabilisant les membranes. Cette hypothèse est également renforcée par la comparaison des réponses immunes générées par les virus JEV et DENV, qui démontre une corrélation entre la capacité de ces virus à ségréguer plus ou moins efficacement leurs ARN dans des organelles de réplication et l'induction d'interférons [104]. Enfin, la détection des RLRs dans un contexte d'infection par les virus HCV et HAV a clairement démontré que les senseurs RIG-I et MDA5 sont exclus des compartiments de réplication de ces virus [103]. En revanche, les mécanismes permettant de limiter l'accès à ces compartiments restent inconnus. La présence de nucléoporines et de facteurs d'import nucléaire dans les organelles de réplication du HCV pourrait cependant permettre de contrôler les échanges entre ces compartiments et le cytosol [10]. Enfin, comme l'ensemble des étapes de la réplication virale, la formation des organelles membranaires de réplication des virus à ARN(+) est la cible des effecteurs de la réponse immunitaire. La capacité des interférons à empêcher la formation des compartiments à double membrane dérivés du RE hébergeant la réplication des *Arterivirus* et à favoriser l'accumulation de structures membranaires aberrantes suggère fortement l'existence de mécanismes antiviraux ciblant spécifiquement la biogenèse de ces organelles [105]. Cette hypothèse est également étayée par les observations réalisées dans le contexte de l'infection par HCV qui démontrent que l'effet principal de la cholestérol 25-hydroxylase (CH25H), un facteur antiviral contrôlé par l'interféron, repose sur sa capacité à inhiber la formation du tissu vésiculaire contenant les organelles de réplication, indépendamment de tout effet sur la réplication de l'ARN [106]. Le constat additionnel que l'action antivirale de la vipérine, un autre facteur antiviral stimulé par l'interféron capable de limiter la réplication de HCV, repose sur son association avec les compartiments de réplication formés par ce virus sur la face cytosolique du RE est un argument supplémentaire en faveur de ce modèle [107].

Conclusions et perspectives

L'élucidation des mécanismes impliqués dans la biogenèse des compartiments membranaires de réplication est essentielle à la connaissance des virus à ARN(+). Au cours des dernières années, les avancées technologiques relevant du domaine de l'imagerie cellulaire et des cribles à l'échelle du génome entier ont permis non seulement d'accéder à l'architecture tridimensionnelle des compartiments de réplication de nombreux virus à ARN(+), mais également de définir les premières interactions virus-hôte nécessaires à leur biogenèse. De nombreuses questions demeurent cependant. La dynamique des interactions hôte-virus permettant la production de tels compartiments reste le plus souvent inconnue. Le rôle fonctionnel des différentes structures membranaires complexes (paquets vésiculaires, réseaux membranaires complexes...) observées en association avec les compartiments de réplication est incompris. De plus, les connexions spatiales et temporelles existant entre ces compartiments de réplication et les étapes ultérieures du cycle infectieux (assemblage et bourgeonnement viraux) restent encore à élucider. Ces questions pourraient bénéficier par exemple de l'application de techniques d'imagerie dynamique.

Les connaissances actuelles ont toutefois mis en lumière une communauté de mécanismes gouvernant la création des organelles de réplication de virus taxonomiquement éloignés. À titre d'exemple, les *Flaviviridae* comme le HCV, DENV, WNV et ZIKV sont sensibles aux inhibiteurs de la synthèse et du transport lipidiques tels que l'imipramine [37, 108], un antidépresseur tricyclique qui empêche la redistribution du cholestérol vers la membrane plasmique. Leur réplication est également sensible à la céruléine et l'orlistat [55, 109] qui inhibent l'activité de synthèse des acides gras de la FASN, ou encore à la tyrphostine AG1478, un anticancéreux bloquant l'activité de la PI4KIII [110]. Si ces molécules, interférant avec le métabolisme lipidique, ont fait la preuve de leur activité antivirale, leur capacité à inhiber la formation des organelles de réplication reste cependant à démontrer. La poursuite des efforts relatifs à l'étude de ces compartiments membranaires constitue donc une opportunité de définir des cibles thérapeutiques attractives et de développer des stratégies antivirales à large spectre d'efficacité.

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Abstract: Positive strand RNA ((+) RNA) viruses share the common capacity to rearrange cellular membranes into vesicular organelles. These membranous compartments referred to as replication organelles (ROs), are seen as providing an appropriate environment recruiting all viral components and cofactors required for replication. Because of their strict necessity for viral replication, these compartments and the molecular mechanisms required for their assembly have generated an intense interest in recent years. Contrasting with the consequential advances made in this field for other (+)RNA viruses, virtually no mechanistic data has been produced on the formation of ROs by *Alphaviruses* which in the last decade have proven to be medically paramount viruses, especially with the recent spread of Chikungunya virus (CHIKV). CHIKV is a re-emerging virus transmitted by mosquitoes that has caused outbreaks with devastating socio-economic impact in countries where it propagates. Symptoms include high fever and rash, with a significant percentage of patients suffering of long-term, often incapacitating, joint pain. Currently there is no vaccine or anti-viral treatment for this virus.

CHIKV ROs appear as 50-60 nm electron translucent bulb-shaped spherules resulting from negative curvature at the plasma membrane. Inside these compartments, the replication machinery is anchored to the membrane through the direct interaction of the non-structural protein 1 (nsP1) with the lipid bilayer. When expressed as an isolated protein nsP1 dramatically remodels cellular membranes into filopodia-like protrusions. Therefore, this designated nsP1 as a critical factor in cellular membrane reshaping observed during infection. In this context, the aim of this thesis, with nsP1 at its centerpiece, is to characterize nsP1 interactions with cellular membranes and to define their functional consequences on viral replication. In this investigation, we have demonstrated the role of host cell lipid metabolism in nsP1 membrane anchoring and viral infection. Our results indicate that fatty acid synthesis is required for viral life cycle and favors nsP1 interaction with membranes. We also provide the very first information on the role of unsaturated fatty acids in *Alphavirus* replication. In-depth studies on the role of cholesterol revealed that palmitoylated nsP1 anchored CHIKV non-structural proteins to cholesterol-rich microdomains with functional consequences on replication. Finally, we have identified nsP1 interactome in order to identify host-cofactors required for the membrane deformation induced by this viral protein. Taken together, this thesis provides new information on nsP1/membrane lipids and host cofactors interplay. This work will allow the further comprehension of the mechanisms behind membrane deformation observed during *Alphavirus* replication.

Résumé: Les virus à ARN de polarité positive (ARN(+)) partagent la capacité de réorganiser les membranes cellulaires en organelles vésiculaires. Ces compartiments, appelés organelles de réplication (OR), fournissent un environnement approprié permettant d'héberger la machinerie de réplication virale, ses cofacteurs cellulaires et les ARN viraux néo-synthétisés. En raison de leur rôle indispensable à la réplication virale, ces compartiments et les mécanismes moléculaires nécessaires à leur assemblage ont suscité un réel intérêt ces dernières années. Alors que des progrès significatifs ont été réalisés dans ce domaine pour d'autres virus à ARN(+), peu de données relatives au mécanisme de formation des ORs des *Alphavirus* ont été produites. Ces virus ont pourtant été associés à des enjeux majeurs de santé publique au cours de la dernière décennie, en particulier avec la propagation récente du virus Chikungunya (CHIKV). CHIKV est en effet un virus réémergent transmis par les moustiques et à l'origine d'épidémies ayant des conséquences socio-économiques dévastatrices dans les pays où il se propage. Les symptômes se caractérisent par une forte fièvre et une éruption cutanée, avec un pourcentage significatif de patients qui souffrent de douleurs articulaires à long terme, souvent invalidantes. À l'heure actuelle, il n'existe aucun vaccin ou traitement antiviral pour ce virus.

Les OR de CHIKV se présentent comme des sphérules de 50 à 60 nm résultant d'une courbure négative de la membrane plasmique. À l'intérieur de ces compartiments, la machinerie de réplication est ancrée à la membrane par l'interaction directe de la protéine non structurale 1 (nsP1) avec la bicouche lipidique. Cette protéine virale, exprimée de façon isolée, conduit à des déformations membranaires de type filopodes. Ainsi, nsP1 apparaît comme un acteur majeur du remodelage membranaire au cours de l'infection par les *Alphavirus*. Dans ce contexte, le but de cette thèse, centrée sur nsP1, est de caractériser les interactions de nsP1 avec les membranes cellulaires et de définir les conséquences fonctionnelles de ces interactions dans la réplication virale. Nous avons mis en évidence le rôle du métabolisme lipidique dans l'ancrage membranaire de nsP1 et dans l'infection virale. Nos résultats indiquent que la production d'acides gras est nécessaire au cycle infectieux et favorise l'interaction de nsP1 avec les membranes. Ils mettent en évidence le rôle complètement nouveau des acides gras insaturés dans l'étape de réplication des *Alphavirus*. Nous avons également démontré l'affinité de la forme palmitoylée de nsP1 pour les microdomaines lipidiques riches en cholestérol de la membrane plasmique. Nous avons établi les conséquences fonctionnelles de cette affinité sur la localisation des autres protéines non structurales et sur la réplication virale. Enfin, nous avons défini l'interactome fonctionnel de nsP1, de façon à identifier les cofacteurs cellulaires pouvant contribuer aux déformations membranaires induites par cette protéine virale. Ce travail permet de mieux comprendre les mécanismes de déformation membranaires observés au cours de l'infection par les *Alphavirus*.