Molecular determinants of arbovirus transmission by mosquitoes

Giel P. Göertz

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Propositions

1. Subgenomic flavivirus RNA is required for the transmission of flaviviruses by mosquitoes.
(*this thesis*)
2. Arboviruses need to establish intracellular interactions with specific mosquito host factors to facilitate virus transmission.
(*this thesis*)
3. Cell lines should be examined by next-generation sequencing to detect persistent virus infections that may impact the replication of viruses under study.
4. Dogmas in the scientific community impose a blind spot for both the interpretation of experimental results and the formulation of novel hypotheses.
5. Non-coding RNAs can be decrypted.
6. There is less pressure on your work when working under biosafety level 3 conditions.
7. Accurate S.W.A.G. (scientifically wild-ass guess) is important for success in science.
8. Artificial intelligence will be able to reproduce what human beings refer to as 'consciousness'.

Propositions belonging to the thesis entitled:

'Molecular determinants of arbovirus transmission by mosquitoes'

Giel P. Göertz

Wageningen, 29 March 2019

Molecular determinants of arbovirus transmission by mosquitoes

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Molecular determinants of arbovirus transmission by mosquitoes

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Thesis

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Chapter I

General introduction

The epidemic potential of arboviruses

While most people consider snakes or sharks as one of the most dangerous animals, mosquitoes are by far the deadliest animals on the planet [1]. Besides causing nuisance in the form of bumps and itches, mosquitoes can spread parasites such as *Plasmodium*, causing malaria, and pathogenic viruses such as Zika virus (ZIKV) and dengue virus (DENV). This poses a serious threat to human health, as in 2017 alone vector-borne diseases caused over 500,000 estimated deaths [2]. While malaria is still the major vector-borne disease considering the number of infections and casualties, arthropod-borne (arbo)viruses are becoming increasingly important in terms of their global spread, number of recorded cases and associated deaths. Currently, arboviruses are collectively responsible for roughly 400 million estimated human cases of infection each year [2–4]. Additionally, many arboviruses infect livestock, resulting in a disease burden for animals and associated economic losses in agriculture [5].

Arboviruses are transmitted from one human or vertebrate animal host to another via blood-sucking arthropods, such as mosquitoes and ticks, which serve as intermediate hosts, also called vector (Fig. 1.1). In both host types (vertebrate and arthropod) virus replication takes place [6]. 'Arbovirus' is not a true taxonomic term, but is used to designate a collection of viruses from different families with similar transmission cycles involving blood-sucking arthropods. Arboviruses of clinical importance typically belong to the order *Bunyavirales* (families *Phenuiviridae*, *Peribunyaviridae*, *Nairoviridae*) or the families *Flaviviridae* (genus *Flavivirus*) and *Togaviridae* (genus *Alphavirus*). The most significant mosquito-borne arboviruses for human health include DENV, ZIKV, yellow fever virus (YFV), Rift Valley fever virus, Japanese encephalitis virus (JEV), chikungunya virus (CHIKV) and West Nile virus (WNV). Most mosquito-borne arboviruses have originally been isolated from tropical forests on the African continent. However, over the last centuries many arboviruses have spread to reach a more worldwide distribution and currently threaten human health at a global scale (Fig. 1.2) [7]. The geographic distribution of a specific arbovirus is restricted to areas that contain both competent mosquito vectors and vertebrate hosts. Consequently, the rapid expansion of various arboviruses across the globe is largely explained by the introduction of vectors to new areas due to international trade, global travel and increasing temperatures that expand the territories of many mosquito species [8–10]. Furthermore, arboviruses have been introduced to new areas that are already inhabited by competent vectors and vertebrate hosts, yet had not been previously exposed to the virus [11]. In these areas, so called naïve host populations have not acquired protective immunity against the introduced disease, and are consequently more susceptible to infection. Additionally, human urbanization and the associated

Fig. 1.1 The arbovirus infection cycle. Arboviruses are transmitted between vertebrate hosts such as humans, primates, birds, and livestock, by arthropod vectors such as mosquitoes and ticks. Arboviruses infect and replicate in both the vertebrate host and the invertebrate vector.

domestication of competent mosquito vectors such as *Aedes aegypti* increases the potential for human arbovirus infections [12,13]. As factors such as global warming, international trade and global travel are expected to further increase in magnitude, the burden of arboviral disease is likely to intensify in the future. ZIKV, CHIKV and WNV are examples of arboviruses that have drastically expanded their geographical distribution over the last two decades and now impose a burden to human health at a global scale (Fig. 1.2). This thesis focuses on understanding the factors and mechanisms that allow these three emerging arboviruses to be successfully transmitted by their mosquito vectors.

Fig. 1.2 The geographic distribution of Zika, chikungunya and West Nile virus. Based on data from: Zika virus (ZIKV) [14], chikungunya virus (CHIKV) [15], West Nile virus (WNV) [16]. The inset represent the global distribution of ZIKV, CHIKV and WNV in 1995 based on data from: ZIKV [17], CHIKV [18], WNV [19–21].

Global impact, transmission cycle & symptoms of infection

Chikungunya virus

CHIKV belongs to the genus *Alphavirus* from the *Togaviridae* family. CHIKV was first discovered on the Makonde Plateau in Tanzania in 1955, where it was named chikungunya which means “*that which bends up*” in the language of Makonde people [22]. This original name refers to CHIKV-infected patients that suffer from severe joint pain and are therefore unable to stand straight. Outbreaks of CHIKV were rare before the year 2000 and were mostly reported in Africa and parts of Asia [18]. Between 2000 and 2010 CHIKV re-emerged in Africa and Asia and caused several severe outbreaks with over 1 million estimated cases of infection [18]. In 2005 CHIKV emerged on several islands in the Indian Ocean, and caused over 250,000 cases on La Reunion island alone. This outbreak was remarkable, as *Ae. albopictus* mosquitoes were responsible for the transmission instead of the primary CHIKV vector species *Ae. aegypti* [23,24]. It was later discovered that the virus acquired an Alanine to Valine mutation in the envelope protein (E1) that facilitated the switch from *Ae. aegypti* to *Ae. albopictus* [25]. In 2013 the first case of local CHIKV transmission in the Caribbean islands was reported. Since then CHIKV has caused over 1.4 million estimated cases of infection in the Americas and is currently endemic in Latin America [26,27]. On the European continent CHIKV caused outbreaks in Italy in 2007, and in France in 2010 and 2014 [28]. Currently, sporadic autochthonous transmission, whereby the virus is acquired and transmitted in the same region, is observed in both France and Italy, but CHIKV is not (yet) endemic in Europe [29].

For CHIKV two transmission cycles can be discriminated; a sylvatic cycle in which the virus circulates between non-human primates, and an urban cycle in which humans serve as the main vertebrate host (Fig. 1.3A). Vector species responsible for CHIKV transmission in the sylvatic cycle include *Ae. fuscifer*, *Ae. luteocephalus*, *Ae. dalzieli* and *Ae. africanus* [30]. In the urban transmission cycle CHIKV is predominantly transmitted by *Ae. aegypti* and *Ae. albopictus* mosquitoes [31–33]. Initially, the urban transmission of CHIKV was mainly caused by *Ae. aegypti* [34], however, as mentioned above, some CHIKV isolates have an amino acid substitution in the viral E1 protein (A226V) that allows these strains to be preferentially vectored by *Ae. albopictus* [25].

Human infection with CHIKV can lead to chikungunya fever which typically presents high fever, headache, myalgia and arthralgia. CHIKV-induced arthralgia can be severe and symptoms may last from weeks to several months [35,36]. Only 15 percent of patients with serological evidence of CHIKV infection are asymptomatic [37], emphasizing the severe burden of chikungunya fever. To date, no specific drugs or licensed vaccines are available for CHIKV, and treatments of patients that suffer from CHIKV disease usually consist of anti-inflammatory and broad-range antiviral drugs [35]. However, several promising antiviral drugs and vaccines are under development, of which some already faced phase I clinical trials [38].

Fig. 1.3 The transmission cycles of Zika, chikungunya and West Nile virus (A) Sylvatic and urban transmission cycles of Zika virus (ZIKV) and chikungunya virus (CHIKV). (B) Transmission cycle of West Nile virus (WNV) and occasional infection of dead-end hosts.

West Nile virus

WNV belongs to the genus *Flavivirus* within the *Flaviviridae* family. WNV was first isolated in 1937 from a febrile patient in the West Nile district of Uganda [39]. WNV gained relatively little attention for several decades, although various outbreaks were reported, such as in Egypt and Israel in the 1950s and in South Africa in the 1970s. In 1999, the concern for WNV disease was raised when it was introduced in New York City, representing the first known occurrence of WNV in the Americas. Within four years WNV spread all across the United States (US), and caused over 46,000 human cases of WNV disease and well over 2,000 human deaths in the US until today [40,41]. In western Europe, until the year 2010, cases of WNV were mostly sporadic, although evidence of WNV circulation was reported in southern European countries and the United Kingdom. However, outbreaks occurred mostly in Russia and eastern European countries [42]. WNV neuroinvasive disease was reported in Italy in 2008 [43] and in Spain in 2010 [44]. Also, in 2010, a large WNV outbreak was reported in Greece [45]. Since then, WNV has caused annual human outbreaks in Europe. In 2018 alone, over 1,400 confirmed human cases and over 170 associated deaths were reported [46]. Currently, WNV is endemic in southern Europe, Africa, Australia, North-America and South America (Fig 1.2).

WNV is maintained in a passerine bird reservoir and is transmitted between birds by *Culex spp.* mosquitoes (Fig. 1.3B). Occasionally, an infected mosquito may feed on alternate hosts, including horses and humans, that may thereby become infected. However, horses and humans develop too low WNV titers in the blood for subsequent transmission and are therefore considered as dead-end hosts. The main vectors for the transmission of WNV are mosquitoes of the *Culex pipiens* complex, such as *Cx. pipiens* and *Cx. quinquefasciatus*, although other *Culex* species such as *Cx. tarsalis* and *Cx. modestus* act as secondary vectors for WNV in the US [47].

Among WNV infected individuals, most cases are asymptomatic, 25% develop West Nile fever and less than 1% develop neuroinvasive disease. Symptoms of West Nile fever are usually mild and include headache, fever, myalgia, and general weakness and last from days to weeks [48]. The development of WNV neuroinvasive disease can cause meningitis (inflammation of the membranes surrounding the brain and spinal cord), encephalitis (inflammation of the brain), and acute flaccid paralysis [48]. While WNV vaccines are available for horses, no licensed vaccine is currently available for humans despite promising results from several phase I and II clinical trials [48].

Zika virus

ZIKV also belongs to the genus *Flavivirus* of the *Flaviviridae* family and was first isolated in 1947 in Uganda from an *Aedes* mosquito captured in the Zika forest [49]. The first significant ZIKV outbreak occurred on the Yap island of Micronesia in 2007. In 2013, ZIKV was introduced in French Polynesia, where it caused a large outbreak with roughly 28,000 reported cases [17]. ZIKV was first introduced into South America via Brazil in 2015, where it caused over 200,000 cases of infection [50]. Already in 2016, ZIKV had spread to most countries in South- and Central-America and to the US in North-America. Currently ZIKV is endemic in parts of Africa, Asia and Latin America [51,52]. No active ZIKV transmission is ongoing in Europe, although infections occasionally occur as a result of returning travellers from ZIKV endemic areas.

For ZIKV also two transmission cycles can be discriminated: an epizootic or sylvatic cycle where the virus is transmitted between non-human primates or other vertebrates by *Aedes* mosquitoes, and an urban cycle where humans serve as primary hosts (Fig. 1.3A). In the urban cycle, transmission occurs predominantly by *Ae. aegypti* [53]. *Ae. albopictus* mosquitoes have been suggested as potential secondary vector for ZIKV and have been implicated as a competent ZIKV vector in the laboratory [54–56]. However, it remains uncertain to which extent *Ae. albopictus* contributes to ZIKV transmission in the field.

Most ZIKV infections are asymptomatic and an estimated 20–50% of infected patients develop Zika fever. Symptoms of Zika fever include rash, fatigue, fever, arthritis, conjunctivitis and headache [57]. In severe cases, ZIKV infection of the peripheral nervous system can lead to the Guillain-Barré syndrome, an auto-immune disease that leads to muscle weakness [52]. ZIKV infection is most problematic in pregnant women, where infection can lead to congenital disease in the unborn child that may result in developmental defects and microcephaly, which is presented by an unusually small head size [52,58,59]. In February 2016, microcephaly and other neurological disorders, and their possible association with ZIKV were declared as a Public Health Emergency of International Concern (PHEIC). The PHEIC was ended in November 2016, because advances had already been made to understand the association between ZIKV and neurological disorders [60]. Moreover, in infected males ZIKV infection can result in defects in the testes that may negatively affect fertility [61,62]. Furthermore, persistently infected males may present infectious ZIKV in the semen, which facilitates the sexual transmission of ZIKV [52,63]. Several ZIKV vaccines are currently in Phase I or Phase II clinical trials and a number of drugs have been identified with anti-ZIKV activity [64,65]. However, no licensed vaccines or antiviral drugs are currently available.

Genome organisation and replication cycle

Chikungunya virus

CHIKV virions are spherical enveloped particles with quasi-icosahedral symmetry and a diameter of roughly 70 nm. The envelope surrounds the capsid, which contains the viral genomic (vg)RNA [66]. CHIKV has a single stranded (ss) positive sense (+) RNA genome with a 5' type-0 cap structure ($m^7G_{ppp}N_p$) and a poly-adenine (A)-tail at the 3' end (Fig. 1.4A). The vgRNA is infectious upon release into the cell cytoplasm and can directly be translated into the viral non-structural polyprotein. This non-structural polyprotein is cleaved by host- and viral proteases into the four non-structural proteins nsP1–4 [67]. nsP1 is a guanylyl transferase responsible for capping of the vgRNA and acts as a membrane anchor for the viral replication complex [68,69]. nsP2 possesses protease activity necessary for cleavage of the viral polyproteins, is a

helicase required for virus replication, and causes host-transcriptional shut-off [70,71]. Nsp2 also acts as an antagonist of the type-I interferon response in mammalian cells [72,73]. The functions of nsP3 are less well described, although this protein is essential for virus replication, possesses RNA binding, phosphatase and zinc-binding activities, and contains a highly-phosphorylated C-terminal domain [71]. nsP4 is the viral RNA-dependent RNA polymerase (RdRp) that is required for replication of the vgRNA and the transcription of the subgenomic (sg)RNA from the minus strand of the vgRNA. The sgRNA encodes the structural polyprotein, which is cleaved into the viral structural proteins Capsid (C), Envelope (E) 1, E2, E3 and 6K [74].

CHIKV entry occurs mostly via receptor-mediated, clathrin-dependent endocytosis. This delivers the virus particles into endosomal vesicles. Due to the relatively acidic environment of the endosomes, the viral glycoproteins undergo a conformational change. This change allows fusion of the viral envelope with the endosomal membrane and the subsequent release of the nucleocapsid into the cytoplasm [75]. In the cytoplasm, the nucleocapsid disassembles and the vgRNA is released into the cytosol [76], where it is ready to be translated and replicated. CHIKV replication takes place in so called replication spherules, which are connected with the plasma membrane [77,78]. After sufficient vgRNA has been synthesized, nucleocapsids either assemble at the plasma membrane or first assemble in the cytoplasm and then diffuse towards the plasma membrane [79]. Heterodimers of E1-E2 are synthesized in the ER and transported via the Golgi to the plasma membrane, where they interact with newly formed capsids and virion assembly proceeds. Finally, virions exit the cell by budding from the plasma membrane [80].

Fig. 1.4 A schematic overview of the alphavirus and flavivirus virion structure and genome organisation. (A) Alphavirus virion and genome organisation. Alphaviruses produce two polyproteins; a non-structural polyprotein, that is produced early after entry, and a structural polyprotein, that is produced from the subgenomic RNA at a later stage. (B) Flavivirus virion and genome organisation. Flaviviruses produce a single polyprotein, that is processed into both the structural and non-structural proteins. Flaviviruses produce a noncoding subgenomic flavivirus RNA (sfRNA), due to partial degradation of the viral genomic RNA by host 5'→3' exoribonucleases. Images of virion structures are a courtesy of Jean-Yves Sgro.

West Nile & Zika virus

WNV and ZIKV virions are approximately 50 nm in diameter and consist of a spherical envelope with quasi-icosahedral symmetry that surrounds the nucleocapsid (Fig 1.4B). Similar to the alphavirus genome, the flaviviral gRNA is a ss (+) RNA molecule. In contrast, it contains a 5' type-I cap structure ($m^7G_{ppp}Nm_p$), and instead of carrying a poly (A)-tail, the 3' end is highly structured and terminates in a conserved RNA stem-loop that has a 3' CU_{OH} group (Fig. 1.4B) [81]. The vgRNA contains a single open reading frame (ORF) that codes for the viral polyprotein. This ORF is flanked by 5' and 3' untranslated regions (UTRs) that contain various stem-loop and dumbbell secondary RNA structures, in addition to the 3' terminal stem-loop [82]. These RNA structures in the 5' and 3'UTR of flaviviruses are important for the recruitment of host RNA-binding proteins that are involved in virus replication and translation [83]. Importantly, the 5' and 3'UTR can form a long-range base pair interaction that results in genome cyclisation, which is required for replication of the vgRNA [84]. The circular form of the vgRNA is used as template for initiation of minus strand (-) synthesis, while the non-circularized form is used for translation [85]. The vgRNA translates into the viral polyprotein that is subsequently cleaved into the structural proteins C, precursor-membrane (prM) and E, as well as the seven non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [81]. NS1 is a glycoprotein that is essential for virus replication and initiation of RNA synthesis. NS1 also acts as an antagonist of the complement system of vertebrate hosts [86,87]. Additionally, NS1 is secreted from mammalian cells, and possibly mosquito cells [88,89]. The presence of secreted NS1 in the blood of infected vertebrate hosts, increases the infectivity of ZIKV, and possibly other flaviviruses, in mosquitoes and thereby contributes to virus transmission [89,90]. NS3 possesses nucleoside triphosphatase activity required for capping of the viral genomic RNA and ATPase/helicase activity to unwind the complex double stranded RNA structures during replication [91]. A complex of NS2B and NS3 acts as the viral protease responsible for processing of the polyprotein into the individual structural and non-structural proteins [92]. NS5 is the viral RdRp responsible for replication of the viral RNA, and also contains the methyltransferase activity required for methylation of the 5' cap of the vgRNA [93]. The functions of NS2A, NS4A and NS4B are largely unknown, although they have been implicated as potential interferon antagonists. Furthermore, NS2A has a role in genome encapsidation and NS4B may be involved in membrane proliferation [82,94].

Flaviviruses also enter the cell via clathrin-dependent, receptor-mediated endocytosis [95]. Acidification of the endosome results in a conformational change of the viral E proteins that, similar to entry of alphaviruses, induces membrane fusion and eventually release of the vgRNA into the cytoplasm [96]. Incoming capsid proteins may stay associated with the vgRNA to shield it from degradation by cellular nucleases and prevent signalling by innate immune receptors [82]. Flavivirus replication takes place in invaginated endoplasmic reticulum (ER)-derived replication organelles [97]. During the phase of exponential viral genome replication, progeny vgRNA molecules escape from these replication organelles through a pore and interact with ER membrane-associated capsid dimers in E- and prM-rich areas in the ER lumen. This eventually results in the assembly of immature virions that bud into the ER lumen [82]. These immature virions are transported through the Golgi system and subsequently, furin-cleavage of the prM molecules occurs in the trans-Golgi compartment [98]. This cleavage results in a conformational change in the viral E proteins, leading to maturation of the virions. Mature virions move towards the plasma membrane and are released from the infected cell via exocytosis [82].

Determinants of alphavirus and flavivirus transmission

From the >3,600 known mosquito species [99], only a few are able to transmit human-infecting arboviruses. This is caused by the low vectorial capacity of most vectors, which is defined as “*the combined effect of all physiological, ecological and environmental factors that determine the ability of a mosquito species to serve as vector for a certain arbovirus*” [100]. For example, higher environmental temperatures usually result in higher vectorial capacity, while a shortened lifespan of the mosquito vector would result in a reduced vectorial capacity. A key parameter that determines the vectorial capacity is vector competence, which is described as “*the ability of a mosquito to become orally infected and transmit a certain arbovirus*” [100]. Vector competence is influenced by many factors including the ability of the virus to overcome mosquito infection barriers and evade invertebrate innate immune responses as discussed in more detail in Chapter II. As the fitness of arboviruses largely depends on the efficiency of transmission by their mosquito vector, arboviral genomes likely contain factors that aid in overcoming the physical barriers and innate immune responses of the mosquito vector. Indeed, antagonists of innate immune responses are commonly found in genomes of insect pathogenic viruses, however few have so far been described for arboviruses [101,102].

The RNA interference (RNAi) response [103] is considered the most potent antiviral response in insects [104] and is described in more detail in Chapters II and III. Briefly, viral double-stranded (ds)RNA molecules that occur as replication intermediates during the viral life cycle are processed into small RNAs, and loaded into RNA-induced silencing complexes (RISC). These RISCs then target RNA molecules with complementarity to the loaded small RNA, resulting in degradation of the target RNA. In the case of viral dsRNA as input, the target will be vgRNA, hence RNAi acts as an antiviral mechanism. For flaviviruses, the NS4B protein has been identified as an antagonist of the RNAi response [103]. Furthermore, flaviviruses produce a highly abundant, noncoding viral RNA that has been implicated as a suppressor of innate immunity [105]. During flavivirus infection the vgRNA is prone to degradation by cellular nucleases. Exoribonuclease (XRN)1 and other 5'→3' XRNs degrade most of the vgRNA, but stall on RNA secondary and tertiary structures in the flaviviral 3'UTR [106–108]. The stalling of XRNs on these resistant RNA structures results in the formation of a noncoding RNA molecule referred to as subgenomic flavivirus RNA (sfRNA; Fig. 1.5 centre) [106]. A detailed description of sfRNA formation and its functions is presented in Chapter VII. In mammalian cells, sfRNA antagonises the host innate interferon response through interaction with TRIM25 [109], stress granule proteins [110], and via additional, currently unidentified mechanisms [111,112]. As the formation of sfRNA is conserved across the *Flavivirus* genus, *i.e.* in the mosquito-borne, but also the tick-borne, no-known vector and insect-specific flaviviruses [105,113,114], sfRNA is likely to play an important role not only during infection of the mammalian host, but also during infection of the invertebrate vector. In fact, sfRNA has been identified as a potential inhibitor of RNAi [115–117], which may affect the infection of mosquitoes by flaviviruses.

Fig. 1.5 Inhibition of innate immune pathways by subgenomic flavivirus RNA. Subgenomic flavivirus RNA (sfRNA) is formed after *de novo* (+) vgRNA synthesis by stalling of the host 5'→3' exoribonuclease XRN1. SfrRNA inhibits multiple innate immune pathways including the interferon response, stress granule-initiated interferon stimulated gene expression in vertebrate cells, and the RNAi pathway in invertebrates. Adopted from [118].

For alphaviruses, nsP3 has similarly been implicated as a determinant of mosquito infection. A chimeric CHIKV equipped with the nsP3 of O'nyong nyong virus, which is predominantly transmitted by *Anopheles* spp. mosquitoes, changed the CHIKV vector preference from *Ae. aegypti* to *An. gambiae*, suggesting that nsP3 determines the infectivity of alphaviruses in mosquitoes [119]. Also, nsP3 may antagonize the mosquito RNAi response [120], resulting in more efficient virus transmission. Furthermore, nsP3 interacts with proteins of stress granules (SGs). SGs are non-membranous cytoplasmic foci that are formed upon cellular stress. SGs store large amounts of mRNAs, thereby halting host mRNA translation, and regulate several innate immune responses [121]. Alphavirus nsP3 specifically interacts with Ras GTPase-activating protein-binding protein 1 (G3BP1) to prevent stress granule assembly and this process facilitates the efficient replication of alphaviruses in mammalian cells (Fig. 1.6) [122–124]. Conserved duplicate "FGDF" amino acid motifs in the hypervariable C-terminal domain of nsP3 are responsible for the interaction with the nuclear transport factor 2 (NTF2)-like domain of G3BP. Consequently, mutation of these FGDF motifs disrupts the interaction with G3BP and attenuates alphaviruses in mammalian cells [123,125–127]. In mosquito cells, alphavirus nsP3 interacts with the mosquito G3BP ortholog Rasputin [128,129]. Interestingly, Rasputin is required for the successful infection of CHIKV in the mosquito vector *Ae. albopictus* (Fig. 1.6) [129]. This suggests that a direct interaction between nsP3 and Rasputin may be essential for the transmission of alphaviruses by mosquitoes. However, it remains to be determined whether or not the interaction between the FGDF

Fig 1.6 Overview of alphavirus nsP3 interaction with G3BP/Rasputin. (1) NsP3 interacts with G3BP in mammalian cells, and Rasputin (Rin) in mosquito cells, via conserved FGDF motifs in the C-terminal hypervariable domain (HVD). Hereby, nsP3 sequesters G3BP/Rin into nsP3-G3BP/Rin foci, which prevents the assembly of *bona fide* stress granules. (2) NsP3-bound G3BP/Rin may shield viral replication complexes (vRC) from antiviral responses. Hence, disrupting the ability of nsP3 to interact with G3BP generally leads to the attenuation of alphaviruses. (3) The presence of Rin in mosquitoes is essential for the efficient infection and transmission of CHIKV by *Ae. albopictus* mosquitoes via an unknown mechanism. A direct interaction between nsP3 and Rin may be essential to establish an infection in the mosquito vector. Alternatively, the inhibition of stress granules, or protection of replication complexes from antiviral responses may contribute to enhanced infection and transmission efficiencies.

motifs in CHIKV nsP3 and the NTF2-like domain of Rasputin are essential for CHIKV transmission by mosquitoes.

Scope of the thesis

As the incidence of disease caused by known arboviruses increases and novel arboviruses continue to emerge [3,11], it is important to better understand the complex interplay between arboviruses and their vector(s). Few arbovirus-vector interactions have been described in detail and there is an apparent lack of mechanistic understanding of these interactions. The aim of this thesis is to **identify key factors that determine the transmission of human-infecting arboviruses by mosquitoes**. The identification of such factors will contribute to the development of novel arboviral prevention strategies, which may limit the further spread and global burden of arboviral disease.

The first section of this thesis focuses on factors that influence the vector competence of mosquitoes for arboviruses. Chapter II reviews the relevant literature on this topic and includes a description of the physical barriers for virus infection and transmission, the current knowledge on the molecular responses against arbovirus infection in mosquitoes and the possible role of microbiota present in the mosquito gut in determining vector competence. The most potent antiviral response in mosquitoes, RNAi, is mediated by small RNAs. In Chapter III, the small RNA responses triggered to arbovirus infection are studied in two mosquito cell lines via a deep sequencing

approach. Furthermore, the deep sequencing data will indicate the presence of replicating insect-specific viruses in these mosquito cell lines. By analysing the small RNA profiles of these insect-specific viruses and WNV, the small RNA responses of mosquitoes are compared across different viral families and cell lines. In the field, co-infections of mosquitoes with multiple arboviruses may occur, and potentially affects the efficiency of virus transmission. Chapter IV investigates if ZIKV and CHIKV interfere with or assist each other during co-infection in *Ae. aegypti* mosquitoes. Furthermore, the ability of a single *Ae. aegypti* mosquito to simultaneously transmit two arboviruses is explored.

The second section of this thesis concentrates on two potential determinants of arbovirus transmission. Chapter V investigates how CHIKV nsP3 might determine the infection and transmission of CHIKV by mosquitoes. Specifically, the importance of a direct interaction between CHIKV nsP3 and mosquito Rasputin for the infection of and the subsequent transmission by *Ae. aegypti* mosquitoes is investigated. Chapter VI focuses on sfRNA as a potential determinant of WNV infection of and transmission by *Culex* mosquitoes. Via a small RNA deep sequencing approach the effect of sfRNA on the magnitude of the RNAi response is investigated *in vivo* in mosquitoes. By comparing the infection and transmission rates of wild type and sfRNA-deficient WNV via infectious blood meals and intrathoracic injections, the role of sfRNA as a determinant of mosquito transmission is assessed. Chapter VII compiles the current knowledge on RNA modifications, RNA structures, and coding strategies during flavivirus infection, with a focus on ZIKV. Chapter VIII follows up on Chapter VI and studies whether the function of sfRNA as a determinant of transmission is conserved between *Aedes*- and *Culex*-transmitted flaviviruses. Specifically, the effect of sfRNA on ZIKV infection and transmission by *Ae. aegypti* mosquitoes is investigated. Furthermore, this chapter aims to identify mosquito proteins associated with sfRNA to ultimately understand the molecular mechanism that underlies sfRNA-mediated infection and transmission of flaviviruses by mosquitoes.

Finally, Chapter IX discusses the findings presented in this thesis in the broader context of the existing literature. Furthermore, a perspective is given on future research that may lead to novel strategies to prevent the further spread of arboviruses by mosquitoes.

Chapter II

Vector competence of mosquitoes for arboviruses

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Abstract

Mosquito-borne arboviruses are a burden for global health, and the increasing incidence of mosquito-borne disease raises the need for mosquito and arbovirus control strategies. Mosquito-borne arboviruses are dependent on a competent mosquito vector for their transmission between vertebrate hosts. However, only a few mosquito species act as competent vectors for the transmission of human-infecting arboviruses. The ability of a given mosquito to transmit a specific arbovirus is referred to as vector competence. The vector competence is largely dependent on the presence of physical barriers to virus infection, anti-viral innate immune pathways, and competing microorganisms present in the mosquito vector. This chapter reviews the current knowledge on vector competence of mosquitoes for arboviruses, with a focus on flaviviruses and *Culex* mosquitoes. Furthermore, knowledge gaps regarding vector competence are identified that, once addressed, will provide important insights into arbovirus transmission and ultimately contribute to effective strategies to control arboviruses.

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Introduction

Mosquito-transmitted arthropod-borne (arbo)viruses depend on their mosquito vector for the transmission between vertebrate hosts. Most arboviruses are vectored by a narrow range of mosquito species, as the infection, dissemination and subsequent transmission of arboviruses is dependent on the vectorial capacity of the mosquito vector. Vectorial capacity can be defined as the efficiency of a vector to transmit a pathogen [130]. Vectorial capacity relies on several factors, such as the feeding behaviour of mosquitoes, mosquito abundance, mosquito survival, and environmental conditions. One of the key components of vectorial capacity is vector competence, which is defined as the ability of a vector to acquire, maintain, and transmit a pathogen [100]. Only competent vectors can contribute to arbovirus transmission, which makes vector competence an important determinant of vectorial capacity. However, a highly competent mosquito species may for example not efficiently contribute to arbovirus transmission if it only feeds on dead-end hosts. Nevertheless, vector competence provides important insights in species that should be considered as important contributors to arbovirus transmission. This is necessary for targeted control of highly competent mosquitoes to reduce the potential of arbovirus transmission. Here, we aim to provide a complete and detailed overview of factors that influence vector competence of mosquitoes for arboviruses, with a focus on flaviviruses and *Culex* mosquitoes.

Mosquito barriers to arbovirus infection and transmission

The outcome of the interaction between mosquito and flaviviruses is largely dependent on the specific combination of the mosquito species, mosquito origin, virus species, and virus strain. For a virus-exposed mosquito to become infectious, the virus has to overcome various barriers within the mosquito body: the peritrophic membrane, the midgut barrier and the salivary gland barrier. The midgut and salivary gland barriers are both further divided into an infection and an escape barrier (Fig. 2.1) [100]. These barriers can limit virus infection both mechanically and through a range of antiviral immune responses, thereby determining the vector competence of the mosquito to transmit a certain arbovirus.

After ingestion of an infectious blood meal, virus particles travel through the foregut, cardia (proventriculus; foregut-midgut junction), and eventually end up in the midgut. Although infection of the foregut and cardia has been described for some arboviruses [131], the majority of virus infections occur in the midgut epithelial cells (Fig. 2.1; right inset). The first potential mosquito barrier that arboviruses may encounter is the peritrophic membrane. The peritrophic membrane is a sac-like structure composed of chitin, proteins, and glycoproteins that form a filamentous matrix surrounding the blood meal in the midgut [132]. The peritrophic membrane is not constantly present in adult mosquitoes, but forms within a few hours after uptake of a blood meal. In *Culex* species, formation of the peritrophic membrane can be readily observed at 2-8 hours post blood feeding, and reaches its peak thickness of ~100 µm after 24 hours [133]. The ~20 nm pores of the peritrophic membrane [134], are smaller than the ~50 nm diameter of flavivirus virions [135]. This makes it necessary for flaviviruses to infect the midgut epithelial cells within 8 hours after ingestion of the viraemic blood meal [134,135]. Indeed, flaviviruses can infect the midgut epithelial cells within 8 hours after a blood meal, and knock-down of genes required for the formation of the peritrophic membrane does not affect flavivirus infection in mosquitoes [136,137].

Fig. 2.1 Schematic overview of the mosquito barriers to arbovirus infection. Schematic longitudinal cross-section of a mosquito. Arrows indicate the passage of virions through the midgut (MG) and salivary gland (SG) barriers. The dashed circle in the midgut represents the peritrophic membrane that is formed after ingestion of blood. Right inset: 1) Infection of midgut epithelial cells via binding to a putative receptor protein. 2) Virus replication in midgut epithelial cells. 3) Release of virus via budding from midgut epithelial cells and direct passage through the basal lamina into the haemolymph. 4) Direct virus passage into the haemolymph through a "leaky midgut". 5) Virus infection of trachea after budding from midgut epithelial cells. 6) Budding of virus from the trachea into the haemolymph. Left inset: 1) Infection of the salivary gland epithelial cells after passage through the basal lamina. 2) Virus replication in the salivary gland cells. 3) Virus release via budding from salivary gland cells into the salivary gland lumen. 4) Virus release from the salivary gland cells into the salivary gland lumen via apoptosis.

Thus, despite the limited literature available, the peritrophic membrane does not seem to pose a strong barrier against flaviviruses in mosquitoes.

Entry of flaviviruses into the midgut epithelial cells most likely occurs through interaction with cellular membrane-associated receptors (Fig. 2.1; right inset). Flavivirus receptors in mosquitoes have not yet been identified, although some studies have suggested the involvement of cellular proteins for dengue virus (DENV) entry in C6/36 mosquito cells [138,139]. These proteins are required for the binding and uptake of virions during DENV infection and are either membrane-bound or membrane-associated intracellular proteins [139]. Studies with WNV in *Culex* mosquitoes indicated that infection of the midgut can be observed by the formation of irregular virus positive clusters of midgut epithelial cells at 3 days post initial midgut infection (dpi) [140]. At

5 dpi the virus spreads more widely throughout the midgut and fully infects the midgut at 7 dpi. Studies with WNV virus-like particles showed that initial infections occur randomly across the luminal posterior midgut epithelium, suggesting that most cells in the posterior midgut are susceptible for virus entry [141].

After infection and replication in the midgut epithelial cells, virus particles need to disseminate through the haemolymph in order to reach the salivary glands. The haemolymph can be reached by passing through the basal lamina with or without a virus replication cycle in the midgut epithelial cells, through infection of the midgut tracheae, or through direct budding into the haemolymph (Fig. 2.1). The basal lamina is a dense matrix of proteins that block large entities such as bacteria, fungi, and larger viruses. However, the basal lamina is permeable to small molecules, potentially forming a barrier for flavivirus dissemination to the haemolymph [142]. The thickness of the midgut basal lamina differs between mosquito species and strains, and can affect the ability of virions to pass through to the haemolymph. Differences in thickness of the basal lamina of *Aedes (Ae.) albopictus* did not affect the dissemination of DENV-1 [143]. Additionally, WNV particles can be observed within the intact basal lamina which suggests that WNV virions move freely within the lamina [135]. These observations suggest that the basal lamina does not pose a strong barrier to flaviviruses. An alternative way of reaching the haemolymph is via infection of the tracheae. These form a network of tubular structures that reach from the midgut epithelial cells to the outer layer of the basal lamina. This network can sustain virus passage through the basal lamina [144]. Indeed, DENV-2 can infect the tracheal system and infection of the tracheae is positively correlated with virus dissemination [145]. Furthermore, it has been shown that the midgut tracheae of *Cx. pipiens* can serve as a conduit for Rift Valley fever virus passage through the basal lamina [146]. Finally, it has been shown that a small proportion of mosquitoes can have a 'leaky' midgut, which refers to the direct passage of virions into the haemolymph immediately after an infectious blood meal, without a replication cycle in the midgut epithelial cells [133,147]. Although leaky midguts have not been shown as a characteristic of flavivirus infections, it is quite possible that in a small proportion of mosquitoes an infectious blood meal leads to direct infection of the haemolymph. To summarize, while most WNV infections pass through the midgut epithelial cells, some may pass this barrier by infection of the tracheae or directly through the 'leaky' midgut.

After infection of the haemolymph, virions disseminate towards and infect secondary tissues including the fat body, haemocytes, muscles and ultimately the salivary glands. After infection of the salivary glands, viral titers increase in the saliva, and eventually reach plateau levels [134]. Female *Culex* mosquitoes have two salivary glands that each consist of one median and two lateral lobes [148]. The salivary gland lobes are surrounded by the salivary gland basal lamina, which forms a physical barrier to virus infection of the salivary gland epithelial cells [142,146,148]. Although less profound than the midgut barrier, the presence of a salivary gland entry barrier has been reported for several flaviviruses in both *Aedes* and *Culex* mosquitoes [149–151]. Both the mechanical and the molecular nature of the salivary gland infection and escape barriers have not been defined completely. Presumably, the combination of basal lamina and organization of the tissue surrounding the salivary glands can prevent viruses from infecting the salivary glands, while antiviral immune pathways can restrict virus replication intracellularly [142,152]. Additionally, some studies have shown that the release of transmissible virus into the salivary gland lumen may require the induction of apoptosis [146,153].

Underlying mechanisms of vector competence of *Culex* mosquitoes for flaviviruses

The vector competence of a given mosquito vector depends on the ability of the virus to overcome the mosquito barriers to transmission. It is therefore important to understand the nature of these barriers and how they interfere with virus dissemination. One way to study the role of the midgut barrier is to compare infection rates between orally exposed and intrathoracically injected mosquitoes. Several studies have shown that intrathoracic injections of WNV into *Cx. pipiens* mosquitoes results in infection rates of ~100%, as compared to up to 63% after an infectious blood meal where the virus has to escape from the midgut lumen by infecting midgut epithelial cells [154–157]. This indicates that *Cx. pipiens* mosquitoes have a midgut infection barrier for WNV.

The same studies show that WNV transmission rates (WNV presence in saliva) of *Cx. pipiens* are lower compared to WNV infection rates (WNV presence in body) after oral exposure. This suggests the presence of either a midgut escape barrier, a salivary gland barrier, or both. The presence of a salivary gland barrier can be assessed by comparing transmission rates between orally exposed and injected mosquitoes. In the absence of a salivary gland barrier, mosquitoes intrathoracically injected with WNV would have transmission rates of ~100%. Indeed, it has been repeatedly shown that intrathoracic injections result in transmission rates of ~100% in *Cx. pipiens* mosquitoes, indicating the absence of a salivary gland barrier for WNV transmission [154–157]. However, studies on *Cx. modestus* suggest the presence of a salivary gland barrier, as they reach only 40–50% transmission rates whereas dissemination rates were 89–91% [158,159]. As the vector competence of mosquitoes is dependent on the ability of the virus to overcome the midgut and salivary gland barriers, it is important to understand the underlying mechanism of these barriers.

Innate immune responses

As a defence to incoming pathogens, mosquitoes have evolved several antiviral responses, such as the RNA interference (RNAi), Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), Toll, immune deficiency (IMD) and apoptosis pathways [160]. Additionally, experimental evidence suggests that heat shock proteins and reactive oxygen species also have antiviral properties (reviewed in [161]), but due to the limited knowledge available on these responses they will not be discussed here. The expression of genes that encode proteins and factors that function in the mosquito innate immune pathways can be upregulated or activated after ingestion of an infectious blood meal. In this way, these immune pathways play an important role in the midgut and possibly the salivary gland barriers that in turn contribute to the vector competence of a mosquito for certain arboviruses. Different mosquito species can have variation in the effectiveness of their antiviral immune responses against certain arboviruses. Such variation may explain differences in vector competence of different mosquito species for certain viral strains. Most knowledge on invertebrate immunity is derived from research on *Drosophila*, although there is an increasing number of studies on immunity against alphaviruses and flaviviruses, mostly conducted on *Aedes* mosquitoes. In this section, the literature concerning flavivirus infections and their role in triggering the mosquito antiviral immune responses in both *Aedes* and *Culex* species mosquitoes is reviewed.

RNAi is one of the most important immune responses of invertebrate vectors [104]. The RNAi response is an antiviral response of insects that is induced upon recognition of non-self double-stranded (ds)RNA. Even though flaviviruses are

single stranded (ss)RNA viruses, dsRNA intermediates are produced during viral RNA replication that serve as substrates for the endoribonuclease Dicer-2 to activate the RNAi response. Additionally, the genomic RNA can fold into dsRNA secondary structures that can be recognized by the RNAi machinery of the mosquito. Activation of the RNAi response leads to degradation of viral genomic RNA through cleavage by Argonaute-2, resulting in decreased virus replication. Various studies have shown that viral small interfering RNAs are produced during infection of Usutu, DENV-2, and WNV in mosquitoes [155,157,162]. This indicates that the RNAi response is initiated upon infection of arboviruses in mosquitoes, and can influence the susceptibility of these mosquitoes for arbovirus infection. RNAi is capable of controlling DENV infection in *Ae. aegypti* mosquitoes and results in decreased viral titers and transmission rates [163]. Additionally, silencing of important effector proteins in the RNAi pathway increases DENV-2 infection rates and titers in *Ae. aegypti* [164]. Interestingly, in *Cx. quinquefasciatus* RNAi targets the WNV genomic RNA in the mosquito midgut [165]. As RNAi is a potent antiviral response that restricts flavivirus transmission, it is not surprising that several RNAi suppressors have been identified in flavivirus genomes. For example, yellow fever virus capsid proteins can bind to dsRNA and thereby could suppress RNA silencing, while for DENV the NS4B protein was identified as an RNAi suppressor [103,166]. WNV encodes for a putative RNAi suppressor in the form of a highly structured subgenomic RNA, that acts as a decoy substrate for Dicer and may thereby prevent the induction of the RNAi response [115,157].

The invertebrate **JAK/STAT** pathway is the insect innate immune pathway that resembles the mammalian interferon response [167]. The JAK/STAT pathway is initiated upon virus recognition by a transmembrane receptor that activates a signaling cascade which upregulates the expression of antiviral response genes. These antiviral genes act by recognizing virus infection, suppressing virus replication, and by triggering nearby cells to enter an antiviral state. Although strongly established as an antiviral defense system in *Drosophila* (reviewed in [168]), the influence of the JAK/STAT pathway on flavivirus infection in mosquitoes remains to be resolved. One study with DENV-2 in *Ae. aegypti* mosquitoes showed that silencing either components or effectors of the JAK/STAT pathway increases DENV-2 titers, while silencing of a JAK/STAT antagonist decreases DENV-2 titers [169]. This suggests that under natural conditions, the *Ae. aegypti* JAK/STAT pathway restricts DENV-2 and influences the vector competence. In *Ae. aegypti*, infection with WNV and DENV has been shown to modulate the expression of JAK/STAT-related genes [170,171]. WNV infection in *Cx. quinquefasciatus* activates the JAK/STAT transcription factor STAT1A, suggesting that the JAK/STAT pathway is involved in controlling WNV infection. However, this activation has not been shown to restrict WNV dissemination or transmission [172]. In *Cx. quinquefasciatus*, activation of the RNAi pathway increases expression of the cytokine Vago, which is secreted from infected cells, to subsequently activate the JAK/STAT signaling cascade in neighboring cells. Through this indirect mechanism, Vago thus decreases WNV titers in a JAK/STAT dependent manner [173], supporting the role of the JAK/STAT pathway in controlling flavivirus infections in *Culex* mosquitoes.

Another conserved immune response in mosquitoes is the **Toll** pathway. The Toll pathway is activated after recognition of virus infection by Toll-like receptors. This eventually results in the expression of antiviral effector genes (reviewed in [102]). DENV-2 infection of *Ae. aegypti* mosquitoes activates the main components of the Toll pathway by increasing their transcriptional level [171]. Depletion of Toll regulators results in increased DENV-2 and DENV-4 titers, whereas silencing of a Toll pathway inhibitor results in decreased DENV titers [171,174]. This indicates that the Toll pathway restricts flavivirus replication in mosquitoes and could influence the vector competence

of mosquitoes. DENV infection of salivary glands in *Ae. aegypti* results in upregulation of Toll pathway related genes in the salivary glands, indicating that the salivary glands can mount an antiviral response against flaviviruses [152]. Transcriptomic approaches have shown that components of the Toll pathway are also differentially expressed during yellow fever virus, but not WNV infection [170]. In another study, infection of *Cx. quinquefasciatus* with WNV alters the regulation of various genes, but Späetzle-like genes were the only modulated Toll-related genes [172]. Future studies should identify whether the Toll pathway truly restricts flavivirus transmission by mosquitoes and whether the Toll pathway is also involved in restriction of WNV by *Culex* mosquitoes.

The **IMD** pathway is activated upon recognition of virus infection by the adaptor IMD protein, subsequently resulting in the transcription of IMD effector genes (reviewed in [102]). Studies with DENV-2 have shown that infected *Ae. aegypti* have increased expression of IMD pathway components [171,175]. However, silencing of the IMD antagonist Caspar does not affect DENV-2 titers, suggesting that the IMD pathway is not involved in controlling DENV-2 infection in *Ae. aegypti* [171]. Interestingly, silencing of the IMD pathway in a DENV refractory *Ae. aegypti* population resulted in increased DENV-2 replication suggesting that the IMD pathway contributes to the refractoriness of this mosquito population [102]. WNV infection in *Cx. quinquefasciatus* does not affect the regulation of IMD related genes [172], although more studies are required to understand the role of the IMD pathway during WNV infections in *Culex*.

Apoptosis (programmed cell death) is considered an antiviral response by the host to eliminate viral infection by killing infected and neighboring cells. Many viruses inhibit apoptosis in order to facilitate their replication and to prevent clearance of the infected cells [153]. Interestingly, some arboviruses utilize cellular caspases during replication or use apoptosis as a measure to release virions from infected cells. WNV infection in *Cx. pipiens* causes apoptosis in the midgut cells, mainly in the anterior region [176]. Infection of *Ae. aegypti* mosquitoes with DENV results in upregulation of apoptosis-related genes. For some genes that function in the apoptosis pathway, silencing results in increased DENV replication [177]. Additionally, DENV infection of both a refractory and susceptible strain of *Ae. aegypti* induces the expression of the apoptotic gene *mx* as soon as 3 hours post blood meal in the refractory, but not in the susceptible strain [178]. These observations suggest that apoptosis acts as an antiviral response in the midgut. Also in the salivary glands, WNV infection causes apoptotic-like cell death, most likely due to the triggering of cellular apoptotic pathways as a result of active virus replication [135,179]. Additionally, infection of *Cx. quinquefasciatus* salivary glands with WNV results in a decrease in a *Culex* apoptosis inhibitor [180]. Although apoptosis is, thus, induced in the salivary glands, virus titers do not rapidly decline over time, suggesting that apoptosis is not effectively clearing the virus infected cells in the salivary glands [179]. Alternatively, virus-induced apoptosis in the salivary glands can perhaps increase the release of transmissible virus into the saliva, thereby actually facilitating virus transmission. Future studies should elucidate the effect of apoptosis on WNV infection of the midgut and release of WNV into the saliva.

The mosquito microbiome

Mosquitoes live in symbiosis with a wide variety of bacteria and fungi, which are abundantly present in the mosquito gut and are referred to as the mosquito gut microbiome [181]. These microorganisms interact with the mosquito gut and have a strong impact on the mosquito's metabolism, but also affect interactions with pathogens [182]. The microbiome of *Ae. aegypti* is important for successful infection of flaviviruses, as has been shown for DENV-2 [181]. The introduction of specific bacterial strains into the mosquito gut microbiome can affect the infection or replication of

flaviviruses in mosquitoes either in a positive or negative way, which in turn may affect their vector competence for certain viruses [181]. Additionally, mosquitoes can be persistently infected with insect-specific viruses that normally do not affect the mosquito life cycle, but can have an effect on the ability of the mosquito to become infected with other viruses [183].

Apart from bacteria in the mosquito midgut, some bacteria are present inside the cells of mosquitoes and are transmitted both horizontally between species and vertically to the mosquito offspring [184]. The best documented intracellular bacterium of mosquitoes is *Wolbachia*. *Wolbachia* is an endosymbiotic bacteria that can affect vector competence for arboviruses (reviewed in [185]). Although there is a large body of literature describing the interactions between flaviviruses and *Wolbachia*, we will here discuss those studies addressing the effect of *Wolbachia* on transmission and replication of WNV only. *Wolbachia* infection increased WNV replication, but resulted in >100 fold less infectious virus production, suggesting that *Wolbachia* interferes with virion assembly within or secretion of virus from mosquito cells [186]. Consequently, *Ae. aegypti* mosquitoes infected with *Wolbachia* have a ~50% reduction in WNV infection and 100% reduction in WNV transmission rates. Infection of *Cx. quinquefasciatus* with *Wolbachia* results in increased resistance to WNV infection via a blood meal. Dissemination rates and transmission rates were significantly lower (2-3 fold) as compared to mosquitoes cleared from *Wolbachia* with tetracycline [187]. However, in another study with *Cx. tarsalis* mosquitoes, infection with *Wolbachia* led to a 1.5 – 2 fold increase in WNV infection rates due to down regulation of Toll-pathway related genes by *Wolbachia* [188]. Moreover, a study on *Wolbachia* in field-caught *Cx. quinquefasciatus* populations showed that densities (*i.e.* the bacterial load) of *Wolbachia* were too low to inhibit WNV infection and did not affect the vector competence of these same mosquito colonies. Thus, for *Cx. quinquefasciatus* the virus inhibitory effect depends on the density of *Wolbachia* in the mosquito [189]. The effect of *Wolbachia* on vector competence of mosquitoes is further dependent on the strain of *Wolbachia* in combination with the species of mosquito used [185]. Taken together infection with *Wolbachia* can have a strong effect on vector competence of mosquitoes for WNV and arguably other arboviruses, although the mechanism remains enigmatic.

Future directions for understanding vector competence

In order to fully understand arbovirus transmission dynamics, it is important to identify the determining factors of vector competence. Especially the influence of the mosquito barriers, immune responses, and microbes on vector competence are still poorly understood. Studying the underlying mechanisms of how these barriers and responses influence vector competence in mosquitoes is therefore important to understand variation in vector competence.

Thus far, most studies on mosquito immune pathways involved in flavivirus infections have been performed with *Aedes* mosquitoes. The limited research on mosquito immune pathways in *Culex* has confirmed the presence of the RNAi, JAK/STAT, Toll, and IMD pathway, as putative antiviral responses towards flavivirus infections. Yet, the relative contribution and effectiveness of these pathways in controlling transmission of WNV and other flaviviruses by *Culex* mosquitoes is still unclear and requires further investigation. Potentially, such studies can indicate novel antiviral responses that are unique for *Culex* species such as the antiviral Vago response that has thus far only been observed in *Culex* mosquitoes [173]. Studies that aim to understand the molecular interactions between the vector and the virus often rely on controlled experiments in cell lines. *Culex* cell lines are available for the species *Cx. quinquefasciatus* (Hsu) and

Cx. tarsalis (Cx.t), but a cell line of *Cx. pipiens* is not available. This limits studies on immune responses and virus replication in *Cx. pipiens* mosquitoes to extrapolations from cell lines of other *Culex* species. Because *Cx. pipiens* is the predominant vector species for WNV in Europe, a *Cx. pipiens* cell line would greatly contribute to research that aims to understand the basis of vector competence for WNV in European mosquitoes. Fortunately, the genome sequence of *Cx. quinquefasciatus*, the first sequenced genome of a *Culex* species, is available and allows for genetic studies with *Culex* mosquitoes. However, as the genome sequence of *Cx. pipiens* is not available, genetic studies should be performed with *Cx. quinquefasciatus* mosquitoes. Therefore, sequencing of the *Cx. pipiens* genome is an important research goal. Alternatively, attempts can be made to perform *de novo* transcriptomics in *Cx. pipiens* and compare the outcomes with the annotation of the *Cx. quinquefasciatus* genome, although such an approach is likely to miss some candidate genes due to genetic variations.

Studies that investigated the effect of temperature on the transmission of WNV by mosquitoes have shown that increasing temperatures result in higher transmission rates [190]. Alternatively, temperature can have an effect on immune regulatory pathways or the microbiome, resulting in altered vector competence [191]. Interestingly, it has been shown that at low temperatures of 18 °C, the RNAi pathway is less effective in *Ae. aegypti* resulting in increased susceptibility to arbovirus infection [192]. Similar studies in *Culex* mosquitoes could highlight why the vector competence changes with alternating temperature or whether it is simply due to the lowered virus replication at lower temperatures. Apart from temperature also the viraemic dose and genetic differences between mosquito populations may affect the induction of innate immune responses. Although it seems natural that a higher viral dose in the initial infection would lead to a stronger induction of immune responses, research supporting this hypothesis is currently lacking. Furthermore, mosquito populations that are more susceptible to WNV infection could have a lower induction of antiviral immune responses upon viral infection. Future studies should try to answer these questions in order to increase our understanding of WNV transmission by mosquitoes. Finally, it has been shown that the composition of the larval environment such as availability of nutrition, larval density, and temperature during development can influence the vector competence of mosquitoes [193]. The effect of nutrition appeared to be negligible, while competition in the larval environment increases the vector competence. Interestingly, warm rearing temperatures decrease the susceptibility of mosquitoes for infection, in contrary to the influence of temperature during the extrinsic incubation period. Whether differences in larval environment also influence innate immune responses, and thereby alter the vector competence in mosquitoes requires further research.

Understanding the molecular mechanisms of virus transmission allows for more directed control approaches *e.g.* by targeting barrier specific genes or application of microorganisms that reduce vector competence. Various vector control strategies have been proposed based on our current understanding of the mosquito's immune system such as 1) the development of transmission blocking vaccines, 2) the generation of refractory mosquitoes through genetic modification of either the mosquito or 3) the mosquito's microbiome and 4) infection of mosquitoes with intracellular bacteria such as *Wolbachia* [185,194,195]. Although no attempts have been made to employ the mosquito's immune system for the control of arboviral disease, promising results have been made in field studies with *Wolbachia*-infected mosquitoes. Studies with *Wolbachia*-infected *Aedes* mosquitoes in Australia and Vietnam have already shown the potential of *Wolbachia* to invade the local mosquito population [196]. While the effect on virus-transmission in the field is yet to be determined, this study indicates the feasibility of releasing *Wolbachia*-infected mosquitoes as an arboviral control strategy. Potentially,

similar release strategies could be performed with genetically modified mosquitoes that employ the mosquito's immune system to further prevent the transmission of arboviruses.

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Chapter III

Mosquito small RNA responses to West Nile and insect-specific virus infections in *Aedes* and *Culex* mosquito cells

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Abstract

Small RNA pathways comprise the main antiviral responses in mosquitoes. The production of viral small-interfering (si)RNAs has been demonstrated in several mosquito species infected with arthropod-borne (arbo) or insect-specific viruses (ISVs). Recently, the production of viral Piwi-interacting (pi)RNAs was observed in *Aedes* spp. mosquitoes and cells infected with flaviviruses, alphaviruses, or bunyaviruses. However, in *Culex* spp. mosquitoes or cells, viral piRNAs have only been shown for bunyaviruses, while no viral piRNAs were produced to alphavirus or flavivirus infections. It remains enigmatic why different small RNA responses are triggered to viruses from different families, and it is unclear how the small RNA response to a virus can differ between *Aedes* and *Culex* spp. mosquitoes. We therefore investigated the small RNA response to West Nile virus (WNV) by parallel deep sequencing of WNV-infected *Cx. tarsalis* Cx.t and *Ae. albopictus* U4.4 cells. As expected, a clear WNV siRNA response was observed in Cx.t and U4.4 cells, while WNV pi-like RNAs, that lack the typical piRNA signature, were only observed in U4.4 cells. Small RNA *de novo* assembly revealed the presence of the ISVs Culex Y virus (CYV), Phasi Charoen-like virus (PCLV), Flock House virus (FHV) and Calbertado virus (CLBOV) in Cx.t cells and CYV, cell fusing agent virus (CFAV) and PCLV in U4.4 cells. We then investigated the small RNA responses to these discovered viruses, and demonstrate that Cx.t cells produce an siRNA response to CYV, PCLV, FHV, and CLBOV. In contrast, piRNAs were only observed for PCLV in Cx.t cells and displayed a classical piRNA signature. In U4.4 cells, an siRNA response was observed to PCLV and CYV, but again a classical piRNA response only occurred to PCLV. Curiously, for CFAV siRNAs only mapped to the 3'UTR, while pi-like RNAs were found in hotspots along the genome. Together, these results indicate that both *Aedes* and *Culex* spp. mosquitoes are capable of establishing a viral siRNA and piRNA response, and demonstrate that the production of viral piRNAs is strongly dependent on the virus family. Furthermore, these findings establish Cx.t cells as a model to study the piRNA pathway of *Culex* spp. mosquitoes.

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Introduction

Mosquitoes serve as primary vectors for most arthropod-borne (arbo)viruses and thereby impose a global health threat to humans and other vertebrates. In mosquitoes, the primary antiviral responses are mediated by small (≤ 30) noncoding RNAs, that can complementary silence viral RNA [104]. Three main classes of small silencing RNAs can be distinguished: micro (mi)RNA, small-interfering (si)RNA, and Piwi-interacting (pi)RNA (Fig. 3.1) (reviewed in [197]). MiRNAs have a length of 22-23 nts and are produced by the nucleases Droscha and Dicer-1 (Dcr1) (Fig. 3.1). They are loaded into an Argonaute-1 (Ago1) RNA-induced silencing complex (RISC) to complementary target mRNAs, which leads to the translational repression or degradation of these mRNAs [197]. Exogenous siRNAs are 21 nts in length and derived from Dcr2 cleavage of double-stranded (ds)RNA of viral or other exogenous origin. SiRNAs are incorporated into an Ago2-RISC complex and complementary target RNA, which is subsequently cleaved by Ago2 and degraded (Fig. 3.1) [101,198]. The input dsRNA can either be viral genomic (vg)RNA in the case of dsRNA viruses and viral dsRNA replication intermediates or secondary RNA structures in the case of ssRNA viruses. For DNA viruses the input RNA can be overlapping mRNA transcripts, secondary RNA structures and/or inverted terminal repeats [199]. The antiviral activity of the siRNA response has been demonstrated for arboviruses from several genera in various cell culture and mosquito models (reviewed in [197]).

The piRNA pathway is known for its transposon suppressing and gene regulating function in the germline and has most extensively been studied in *Drosophila*. In *Drosophila*, primary piRNAs are transcribed as precursors from dedicated loci in the genome, termed piRNA clusters (Fig. 3.1). These precursors are processed by the protein Zucchini, loaded into the PIWI-proteins Piwi and Aubergine (Aub), and processed into mature 25-30 nt long piRNAs [200]. Piwi-bound piRNAs target and silence complementary transposons at the transcriptional level [201], whereas Aub-piRNA complexes initiate the ping-pong piRNA amplification cycle (Fig. 3.1) [202,203]. In the ping-pong amplification cycle, Aub-bound piRNAs base pair with complementary transposon RNAs. The transposon RNA is then cleaved between position 10-11 complementary to the piRNA sequence. Subsequently, the 3' cleavage products are transferred to the PIWI-protein Ago3 and processed into mature secondary sense piRNAs of roughly 25-30 nts. These cleavage events produce the typical ping-pong signature, with antisense piRNAs having a 1U bias, sense piRNAs having a 10A bias, and an overlap between sense and antisense piRNAs of 10 nts [202,203].

While *Drosophila* encodes only three Piwi genes, the important arbovirus vectors *Aedes aegypti* and *Culex quinquefasciatus* encode 7 and 6 Piwi genes, respectively [204,205]. This Piwi gene expansion suggests that the piRNA pathway has additional functions in mosquitoes, beyond transposon control and gene regulation in the germline. The recent discovery that some mosquito species produce viral piRNAs (vpiRNAs) during arbovirus infection raises the exciting possibility that this pathway also contributes to host defence against viruses [206–213]. However, little is known about the mechanisms and functions of the piRNA pathway in mosquitoes [214]. In *Ae. aegypti*, viral piRNA production is dependent on Piwi5 and Ago3, with a minor contribution of Piwi6 [206,207]. Antisense 1U-biased vpiRNAs are mainly bound by Piwi5, while most sense 10A-biased vpiRNAs are bound by Ago3 [207].

In *Aedes* spp. mosquitoes or cells, viral piRNAs have been observed during infections of alphaviruses, flaviviruses and bunyaviruses [206,207,209,211,212]. In contrast, in *Cx. quinquefasciatus* mosquitoes, arboviral piRNAs have thus far only been described for Rift Valley fever virus, a member of the *Phenuiviridae* family (order

Fig. 3.1 Schematic overview of the small silencing RNA responses in mosquitoes.

MiRNAs are produced by Drosha cleavage of transcribed pri-miRNAs in the nucleus to generate pre-miRNAs. Pre-miRNAs are exported to the cytoplasm and processed by Dicer-1 (Dcr1) into mature 22-23 nt miRNAs. MiRNAs are loaded into an Argonaute-1 (Ago1) RNA-induced silencing complex (RISC) and complementary target mRNA to induce the degradation or translational silencing of that mRNA. siRNAs are 21 nts in size and produced by Dcr2 cleavage of dsRNA, loaded into an Ago2-RISC and complementary target RNA to induce RNA degradation. For piRNAs (based on *Drosophila*), piRNA transcripts are transcribed in the nucleus, transported to the cytoplasm and processed by Zucchini with a 1U-bias. Cleavage products are loaded into Piwi, trimmed to 25-30 nts, and complementary target and degrade transposon RNA. Zucchini cleavage products can also be loaded into Aubergine (Aub), cleaved to 25-30 nts, and initiate the ping-pong amplification loop. Aub-piRNA complexes also complementary target transposons, and 3' cleavage products are loaded into Ago3, with a 10A-bias, trimmed to 25-30 nts, and used to complementary target piRNA transcripts. Cleavage products again serve as template for piRNA biogenesis and subsequent loading into Aub. This generates the typical ping-pong signature with primary, antisense piRNAs having a 1U-bias, secondary, sense piRNAs having a 10A-bias, and their overlap being 10 nts.

Bunyavirales) [210], but vpiRNAs have not been observed during alphavirus and flavivirus infection of *Cx. pipiens* [155,214]. It is unclear why different small RNA responses are triggered to viruses from different families, and how the small RNA response to a virus can differ between *Aedes* and *Culex* spp. mosquitoes.

Here, we compared the small RNA responses of *Culex* and *Aedes* mosquito cells during WNV infection in *Cx. tarsalis* Cx.t cells and *Ae. albopictus* U4.4 cells. Moreover, via *de novo* assembly of small RNA reads we identified persistent infections with insect-specific viruses (ISVs) from the *Flaviviridae*, *Phenuiviridae*, *Birnaviridae* and *Nodaviridae* families as well as a virus from the *Narnaviridae*, which also allowed the investigation of small RNA responses across these distinct virus families. We observed an siRNA response to viruses from all these five families in both *Aedes* and *Culex* cells. However, the piRNA response in both cell lines was highly virus-family

dependent. Notably, we observed that the piRNA response to the same virus can differ between *Culex* and *Aedes* mosquito cells, both in regard to the species of small RNAs produced and their relative abundance. These results show that small RNA responses are highly host species and virus family dependent.

Results

Small RNA profiles in WNV-infected *Aedes* and *Culex* mosquito cells

Previous research has implicated that *Cx. pipiens* mosquitoes produce siRNAs but not piRNAs to WNV [155], while *Aedes* spp. cells produce both siRNAs and piRNAs to dengue (DENV) and Zika virus [206,209]. To further investigate the small RNA responses of *Aedes* and *Culex* spp. mosquitoes to flaviviruses, we deep sequenced the small RNAs, of WNV-infected *Cx. tarsalis* Cx.t cells and *Ae. albopictus* U4.4 cells, in parallel. Libraries were prepared from 19-30 nt small RNAs and size-distributions were generated and normalized to the total number of reads per library. A peak of 21 nt small RNAs, likely corresponding to the population of siRNAs, was observed in Cx.t and U4.4 cells (Fig. 3.2). Additionally, a population of 22-23 nt small RNAs was observed in both cell lines, which likely represent miRNAs. Furthermore, a shoulder of 25-30 nt small RNAs, corresponding to a population of putative piRNAs, was observed in U4.4 and to a lesser extent in Cx.t cells. These results confirm that Cx.t and U4.4 cells have an active siRNA response, as shown previously [215,216], and suggest the production of miRNAs and piRNAs in both cell lines.

Fig. 3.2 Small RNA profiles of the *Culex* and *Aedes* mosquito cell lines. Small RNA sequencing libraries of West Nile virus infected Cx.t and U4.4 cells. Shown is the small RNA size distribution of host and non-host reads of 19-32 nts, normalized to the total number of reads in each library.

Small RNA responses to WNV-infection in *Culex* and *Aedes* mosquito cells

To assess the small RNA responses to WNV in *Culex* and *Aedes* spp. mosquito cells, we mapped the small RNA reads from WNV-infected Cx.t and U4.4 cells against the sense (+) and antisense (-) strand of the WNV genome. The titers of WNV in the culture medium at the time of RNA isolation, as determined by end-point dilution assay (EPDA) on Vero cells, were 7.1×10^7 and 3.2×10^8 tissue culture infectious dose 50% (TCID₅₀)/ml for Cx.t and U4.4 cells, respectively. Small RNA size-distributions were generated and normalized to the total number of reads (Fig. 3.3A). In Cx.t cells, a strong 21 nt peak was observed on both the (+) and (-) strand, indicative of a potent

siRNA response to WNV. In U4.4 cells a similar 21 nt siRNA peak was observed on the (+) and (-) strand, albeit less evident as in Cx.t cells. Mapping the 21 nt vsiRNAs along the WNV genome demonstrated that the vsiRNAs target the entire WNV genome in Cx.t and U4.4 cells, although hot- and coldspots could be distinguished (Fig. 3.3B). In U4.4 cells a shoulder of 25-30 nt small RNAs, indicative of vpiRNAs, was observed on the WNV (+) strand but not on the (-) strand (Fig. 3.3A). Interestingly, this shoulder was completely absent in Cx.t cells, suggesting that these cells are unable to produce WNV piRNAs. Mapping of the 25-30 nt putative vpiRNAs from U4.4 cells to the WNV genome indicated that they target specific hotspots in the viral genome (Fig. 3.3C), similarly as demonstrated for other flaviviruses [206,209]. We also mapped the low number of 25-30 nt reads from Cx.t cells, of which the majority mapped to a single region in the genome corresponding to stem-loop-II in the 3' untranslated region (UTR) [106]. To further investigate whether the 25-30 nt small RNAs in Cx.t and U4.4 cells could represent vpiRNAs, sequence logos and overlap signatures were generated of these putative vpiRNA populations (Fig. 3.3D). No 1U- or 10A-bias was observed for the 25-30 nt small RNAs derived from the (+) or (-) strands, respectively. Comparison of the sense- and antisense 25-30 nt small RNAs also did not identify the typical 10 nt overlap expected for vpiRNAs (Fig. 3.3D, lower panels). Absence of a ping-pong signature indicates that these putative vpiRNAs are produced in a ping-pong independent manner and questions whether they are interacting with Piwi proteins. However, as they do resemble the size of piRNAs and the production of Piwi-interacting 25-30 nt small RNAs without a ping-pong signature was previously demonstrated during DENV infection [206], we will refer to these 25-30 nt small RNAs as pi-like RNAs. These results show that U4.4 and Cx.t cells establish a WNV siRNA response, while U4.4 cells, but not Cx.t cells, generate a ping-pong independent, pi-like RNA response to WNV infection.

Fig. 3.3 Small RNA responses to West Nile virus in *Culex* and *Aedes* cells. (A) Small RNA reads of Cx.t and U4.4 cells were mapped to the West Nile virus (WNV) genome. Shown is the small RNA size distribution of sense (+) and antisense (-) mapping reads of 19-32 nts for each cell line normalized to the total number of reads in each library. (B) Distribution of mapped sense and antisense 21 nt small RNAs along the WNV genome in each cell line. (C) Genome distribution of sense and antisense 25-30 nt small RNAs that map to the WNV genome in each cell line. (D) Sequence logos of sense (+) and antisense (-) 25-30 nt small RNAs that map to the WNV genome, and the probability of overlap length in both cell lines.

Discovery of insect-specific viruses by *de novo* assembly of small RNA reads

With the introduction of next-generation sequencing (NGS) technologies and metagenomics into the field of virology, it becomes increasingly clear that many insect cell lines and insects, including mosquitoes, carry persistently infecting ISVs [217–220]. To investigate the presence of ISVs in our Cx.t and U4.4 cells, the libraries of small RNA reads of these two cell lines were subjected to *de novo* assembly. Assembled contigs were filtered for a minimal size of 200 nts and compared to viral sequences in the NCBI database using BLASTx. Contigs with a viral blast hit were re-assembled to produce longer sequences, resulting in 30 and 52 assemblies for Cx.t and U4.4 cells, respectively (Table 3.1).

In Cx.t cells, assemblies were found corresponding to the genomes of WNV, Culex Y virus (CYV), Flock House virus (FHV), Calbertado virus (COLBV), Phasi-Charoen-like virus (PCLV) and a Narnavirus (NV) (Table 3.2). As assemblies covered more than 50% of the viral genomes with a nucleotide identity of more than 90%, it is likely that these viruses replicate in Cx.t cells. The discovery of NV in our Cx.t cells, previously discovered by NGS of field-caught *Cx. pipiens* mosquitoes [221] is surprising, as viruses from the *Narnaviridae* family have only been shown to infect yeast and oomycetes [222]. Mosquitoes can carry a variety of fungi and bacteria, however, there were no signs of fungal or bacterial contamination in our Cx.t cells. Therefore, the assembly of a full-length genome of NV, suggests that NV replicates in Cx.t cells. However, as the Schneider's media used to culture Cx.t cells contains yeast extract, we cannot rule out that NV was co-purified from our Cx.t cells via the culture media.

In U4.4 cells, contigs were retrieved corresponding to the genomes of WNV, CYV, PCLV, cell-fusing agent virus (CFAV) and Semliki Forest virus (SFV) (Table 3.3). Coverages of WNV, CYV and PCLV were >70% with nucleotide identities of >97%, suggesting that these viruses replicate in U4.4 cells. However, the coverage for SFV and CFAV was only ~5% and ~12%, respectively. In addition to exogenous viruses, non-retroviral integrated RNA virus sequences (NIRVS), also referred to as endogenous virus elements (EVEs), can be a source of viral small RNAs [101,223–226]. The low coverage for SFV and CFAV therefore indicates that the virus is replicating at a very low level, or that these short regions correspond to SFV- and CFAV-related NIRVs.

Table 3.1 Discovery of insect-specific viruses by *de novo* assembly of small RNA reads. Small RNA reads from WNV-infected Cx.t and U4.4 cells were used as input for *de novo* assembly. Shown are the number of small RNAs per library, the retrieved contigs from the *de novo* assembly pipeline before- and after filtering for length and presence of a viral BLAST hit, and the final number of assemblies per cell line.

Cell line	Small RNAs	Contigs	Contigs >200 nts	Virus BLASTx hits	Assemblies
Cx.t	19,644,280	866	488	233	30
U4.4	10,971,240	2,061	929	307	52

Table 3.2 Identified viruses by small RNA *de novo* assembly in Cx.t cells. Listed are the viruses for which a contig BLAST hit was retrieved. Shown is the genome size of the subject, the nucleotide coverage of query contigs, the coverage expressed as percentage of query nucleotide coverage length compared to the length of the subject, the number of mismatching nucleotides between the query and the subject, and the percentage nucleotide identity between the query and the subject sequence.

Virus	Virus family	Genome	Segment	Genome size (nts)	Coverage		Mis-match (nts)	Identity (%)
					nts	%		
WNV	Flaviviridae	+ssRNA	N/A	11,050	9,359	85	2	99.97
CYV	Birnaviridae	dsRNA	A	3,429	3,453	100	5	99.86
			B	3,254	3,203	98	6	99.81
FHV	Nodaviridae	+ssRNA	1	3,668	3,668	100	185	94.95
			2	1,400	702	50	73	89.60
CLBOV	Flaviviridae	+ssRNA	N/A	10,735	7,064	66	11	99.84
			L	6,769	3,464	51	84	97.58
PCLV	Phenuiviridae	-ssRNA	M	3,849	2,094	54	42	97.99
			S	1,362	1,387	100	32	97.69
NV	Narnaviridae	+ssRNA	N/A	3,261	2,986	95	31	91.57

Table 3.3 Identified viruses by small RNA *de novo* assembly in U4.4 cells. Listed are the viruses for which a contig BLAST hit was retrieved. Shown is the genome size of the subject, the nucleotide coverage of query contigs, the coverage expressed as percentage of query nucleotide coverage length compared to the length of the subject, the number of mismatching nucleotides between the query and the subject, and the percentage nucleotide identity between the query and the subject sequence.

Virus	Virus family	Genome	Segment	Genome size (nts)	Coverage		Mis-match (nts)	Identity (%)
					nts	%		
WNV	Flaviviridae	+ssRNA	N/A	11,050	10,016	91	1	99.99
CYV	Birnaviridae	dsRNA	A	3,429	3,398	99	10	99.71
			B	3,254	3,197	98	6	99.81
			L	6,769	6,010	89	11	99.82
PCLV	Phenuiviridae	-ssRNA	M	3,849	2,994	78	27	99.10
			S	1,362	1,186	87	27	97.72
CFAV	Flaviviridae	+ssRNA	N/A	10,682	1,280	12	75	94.14
SFV	Togaviridae	+ssRNA	N/A	11,440	637	6	12	98.12

Small RNA profiles of insect-specific viruses in *Culex tarsalis* cells reveal the production of viral siRNAs and piRNAs

As contigs were found for CYV, PCLV, FHV, CLBOV and NV by *de novo* assembly (Table 3.2), we also investigated the small RNA profiles to these viruses in the WNV-infected Cx.t cells. We first mapped the small RNAs of the Cx.t cells-derived small RNA library to the ISV genomes and generated small RNA size distributions for the (+) and (-) strands (Fig. 3.4A). Interestingly, a large number of 21 nt vsiRNAs aligned to the (+) and (-) strands of CYV, FHV, PCLV, CLBOV and NV. Next, we mapped the 21 nt vsiRNAs along the viral genomes for both the (+) and (-) strands (Fig. 3.4B). For CLBOV, NV, and both the A- and B-segments of CYV, the vsiRNAs were distributed all along the viral genome, indicative of replicating viral RNA. For FHV the vsiRNAs were found to map predominantly to specific hotspots on the viral genome, similar to previous results with FHV-infected *Drosophila* cells [227,228]. Reads that mapped to the PCLV L-segment were similarly distributed along the (+) and (-) strand, while for the M-segment the siRNA reads mostly aligned to a hotspot at the 5' end. VsiRNAs that mapped to the S-segment of PCLV were also found in several hotspots, mostly located near the 3' end of the segment. The distribution of small RNAs along the viral genomes in combination with a high coverage after *de novo* assembly indicates that CYV, FHV, PCLV, CLBOV and NV replicate in WNV-infected Cx.t cells.

Curiously, a large shoulder of 25-30 nt vpiRNAs was found to map to both the (+) and (-) strand of the PCLV S-segment, and to a lesser extent to the M-, and L-segments (Fig. 3.4A), implicating that Cx.t cells are capable of producing vpiRNAs despite the absence of WNV piRNAs in these cells (see Fig. 3.3A). Mapping of the 25-30 nt small RNAs along the PCLV L-, M-, and S-segments revealed that these were derived from specific hotspots on the (+) and (-) strands (Fig. 3.4C). Strikingly, analysing the sequence bias and overlap of these 25-30 nt putative-vpiRNAs, revealed strong 10A (+) and 1U (-) biases in combination with a 10 nt overlap between the small RNAs derived from the (+) and (-) strands. This indicates that Cx.t cells produced ping-pong amplified vpiRNAs to this particular ISV. However, no evidence of vpiRNAs was found for CYV, FHV, CLBOV nor NV in Cx.t cells, indicating that the formation of vpiRNAs is highly virus-specific. These results show that Cx.t cells support the replication of ISVs from distinct viral families despite the potent RNAi response in these cells, and are capable of producing vpiRNAs.

➤ **Fig. 3.4 (Next page) Small RNA responses against insect-specific viruses in Cx.t cells.** (A) Size distribution of 19-32 nt small RNAs that map to the genomes of *Culex* Y virus (CYV), Flock House virus (FHV), Phasi Charoen-like virus (PCLV), Calbertado virus (CLBOV) or Narnavirus (NV). Shown is the number of reads mapping to the sense (+) or antisense (-) genome. (B) Genome distributions of 21 nt small RNAs that map to the genomes of CYV, FHV, PCLV, CLBOV or NV. (C) Genome distributions, sequence logos and the probability of overlap length of 25-30 nt small RNAs that map to the genome segments of PCLV.

Small RNA profiles of insect-specific viruses in U4.4 cells demonstrate potent siRNA and virus-dependent piRNA responses

Small RNA *de novo* assembly implicated the presence of PCLV, CYV, CFAV and SFV in U4.4 cells. We therefore investigated the small RNA profiles for these viruses. Small RNAs were mapped to the (+) and (-) strands of the viral genomes, and small RNA size-distributions were generated (Fig. 3.5A). A high number of reads mapped to the genomes of PCLV, CYV, CFAV, and SFV in agreement with the results of the *de novo* assembly (Table 3.3). Peaks of 21 nt vsiRNAs were observed that mapped to both the (+) and (-) strands of CFAV, SFV, the L-, M-, and S-segments of PCLV and the A- and B-segments of CYV (Fig. 3.5A), indicating that U4.4 cells generate an siRNA response to these viruses. Mapping of the 21 nt vsiRNAs along the genomes of PCLV and CYV indicated that vsiRNAs again target hotspots in the L-, M-, and S-segments of PCLV and the A- and B-segments of CYV (Fig. 3.5B). Surprisingly, the 21 nt CFAV siRNAs mapped mostly to the 3'UTR. This suggests either the presence of a CFAV 3'UTR NIRV in U4.4 cells, or the preferential targeting of the CFAV 3'UTR by the siRNA machinery. For SFV, most 21 nt RNAs mapped to two hotspots near the 5' end of the viral genome, but a small number of reads were found along the viral genome. In combination with the low coverage in *de novo* assembly (~5%), this suggests that SFV is replicating at a very low level in U4.4 cells.

A shoulder of 25-30 nt pi-like RNAs was observed for CFAV, SFV and the L-, M-, and S-segments of PCLV, but not against either segment of CYV (Fig. 3.5A). The small RNA profile of CYV is similar to a recent report from CYV-infected U4.4 and Aag2 cells, where also a large number of 21 nt small RNAs and no vpiRNA shoulder was observed, although the authors stated the production of a small number of CYV pi-like RNAs [229]. Mapping the 25-30 nt vpiRNAs along the genome segments of PCLV demonstrated that the pi-like RNAs were derived from hotspots along all the three segments (Fig. 3.5C). Sequence logos of the putative-vpiRNAs that mapped against the (+) or (-) strand of PCLV demonstrated a typical 10A (+) and 1U (-) bias. Furthermore, the putative-vpiRNAs from all three genome segments displayed a 10 nt overlap, indicative of ping-pong amplified vpiRNAs. For CFAV, the population of putative vpiRNAs mapped as separate peaks on the viral genome. Remarkably, these peaks were distributed across the viral genome, suggesting the presence of full-length viral RNA in U4.4 cells, despite the exclusive mapping of siRNAs to the 3'UTR. The putative CFAV piRNAs did not display a piRNA signature nor overlap. The SFV pi-like RNAs mapped to one region at the 5' end, and another region after position ~7,500, which corresponds with the 5' end of the subgenomic mRNA similar to previous vpiRNA profiles of alphaviruses in *Aedes* spp. [207,208,212,230]. However, despite a 10 nt overlap, these SFV pi-like RNAs did not display the expected 10A (+)/1U (-) bias for alphaviruses. Together, these results show that U4.4 cells are persistently infected with PCLV, CYV, CFAV and SFV, and produce differential small RNA populations to viruses from different families.

➤ **Fig. 3.5 (Next page) Small RNA responses against insect-specific viruses in U4.4 cells.** (A) Size distribution of 19-32 nt small RNAs that map to the genomes of Phasi-Charoen-like virus (PCLV), Culex Y virus (CYV), cell fusing agent virus (CFAV) or Semliki Forest virus (SFV). Shown is the number of reads mapping to the sense (+) or antisense (-) genome. (B) Genome distributions of 21 nt small RNAs that map to the genomes of PCLV, CYV, CFAV or SFV. (C) Genome distributions, sequence logos and the probability of overlap length of 25-30 nt small RNAs that map to the genomes of PCLV, CFAV or SFV.

Discussion

Mosquitoes have expanded their repertoire of Piwi proteins and it is currently unknown whether piRNA responses are completely homologous between *Drosophila* and mosquitoes, or even between different mosquito species [204,205]. For example, while *Aedes* spp. mosquitoes and cells are capable of producing vpiRNAs to alphavirus and flavivirus infection [206,207,209], no vpiRNAs have so far been observed to these viruses in *Culex* spp. mosquitoes [155,214]. In the current study, we also observed the production of WNV pi-like RNAs in U4.4 cells, but not in Cx.t cells. In contrast, PCLV-derived vpiRNAs were readily detected in Cx.t cells, indicating that *Cx. tarsalis* mosquitoes are intrinsically capable of producing vpiRNAs. While PCLV-derived vpiRNAs were observed in both Cx.t and U4.4 cells, the ratio between vpiRNAs and vsiRNAs was substantially higher in U4.4 cells. A higher piRNA:siRNA ratio in *Aedes* compared to *Culex* mosquitoes was also observed in a previous study with Rift Valley fever virus, which like PCLV belongs to the family *Phenuiviridae* [210].

Curiously, differences were also observed in the small RNA profiles within the same cell line for viruses from different viral families. In U4.4 cells, WNV- and CFAV-derived pi-like RNAs were produced only against the (+) strand and did not have a ping-pong signature. This is similar to the findings of a previous study with DENV in *Ae. aegypti* Aag2 cells, where pi-like RNAs were equally (+) strand biased and lacked a ping-pong signature [206]. It is known that the production of these DENV pi-like RNAs is dependent on Piwi5 and Ago3, suggesting that these populations of 25-30 nt WNV and CFAV pi-like RNAs are similarly produced by the piRNA machinery [206]. At the same time, however, PCLV-derived vpiRNAs in U4.4 cells did show a clear ping-pong signature, while no CYV-derived vpiRNAs were detected. In Cx.t cells no pi-like RNAs were observed for WNV, CLBOV, CYV, NV or FHV, while vpiRNAs with a ping-pong signature were observed for PCLV. Together, these observations suggest differences in the small RNA responses of *Aedes* spp. versus *Culex* spp. mosquitoes and demonstrate that the production of vpiRNAs in a particular mosquito species is largely divergent for viruses from different families. Possibly, the genomic RNA of flaviviruses and viruses of the family *Phenuiviridae* is differentially available for processing by the small RNA machineries in *Aedes* compared to *Culex* cells. This could be the result of differences in virus replication mechanistics or compartmentalization of replication complexes in *Aedes* versus *Culex* cells. Furthermore, the genome structures of the investigated viruses are quite different (WNV/FHV/CLBOV/NV/CFAV/SFV, (+) ssRNA; PCLV, (-) ssRNA; CYV, dsRNA). Thus, it is possible that differences in small RNA profiles can be explained by differences in the availability of their gRNA as substrate for the piRNA machineries due to the viral coding and replication strategies.

In contrast, abundant siRNAs were detected for all viruses in both U4.4 and Cx.t cells. For WNV, CYV, PCLV and NV, siRNAs mapped all along the viral genome, although hot- and coldspots could be distinguished. However, the majority of FHV siRNAs mapped to the 5' and 3' extremities of the viral genome, most CFAV siRNAs mapped to the 3'UTR and the SFV siRNAs mapped predominantly to two regions near the 5' end of the viral genome. This implicates that, similar to our observations for piRNAs, particular regions of the viral genome are more susceptible for targeting by the siRNA machinery. The importance of RNA-accessibility for the production of small RNAs is further implicated by the hotspot siRNA and piRNA patterns observed for most viruses in mosquito cells [155,157,206,207,209–211,213,215]. Hotspots are potentially more prone to targeting by small RNA machineries due to the formation of preferentially targeted secondary RNA structures. In contrast, coldspots may be targeted less due to

their association with viral- or host proteins, or the formation of tight RNA folding that makes these areas less accessible for nuclease cleavage.

Despite the presence of abundant siRNAs to WNV, CYV, FHV, PCLV, CLBOV and NV, the assembly of nearly full-length genomes of these viruses suggests that they are still able to replicate successfully. It remains enigmatic how viruses can replicate in cells that contain such abundant virus-specific siRNAs. It has recently been hypothesized that the persistent infection of mosquito cells may require an active siRNA response [231,232]. The rationale behind this is that the siRNA response suppresses viral replication to a level that is tolerable to the host cell, thereby sustaining the persistent infection with no or minimal cytopathic effects. As we have not yet quantified the titers of all viruses except WNV, it is indeed possible that virus replication occurs at a very low, persistent level, due to the continuing degradation of viral RNA by the host defence systems. These degradation products could in turn serve as input for the production of small RNAs. Quantifying the virus titers or genome copy numbers will give insight into the correlation between an siRNA response and the establishment of persistent infections, which remains the focus of future research.

As ISVs can interfere with the infection and replication of arboviruses [233–239], the presence of ISVs in mosquitoes and mosquito cell lines can affect the outcome of vector competence and virus replication studies. It is therefore important to investigate the presence of ISVs in both cell culture systems and mosquito colonies used for experiments. Of note, different strains of a particular cell line do not necessarily contain the same persistent infections. For example, Weger-Lucarelli *et al.* identified only CYV in U4.4 cells [218], while our U4.4 cells are additionally infected with the ISVs PCLV and CFAV. Cross-contamination between insect cell lines is a risk, as in most laboratories cell lines are passaged in parallel. To avoid unexpected interference from persistent infections, it is therefore advisable to screen mosquito colonies and mosquito cell cultures on a regulatory interval, for example by NGS or pan-virus family PCR.

In conclusion, we have demonstrated that Cx.t and U4.4 cells are both capable of producing a viral siRNA and piRNA response. This establishes Cx.t cells as the first model cell line to analyse *Culex* piRNAs. Moreover, the divergent production of vpiRNA in different mosquito cell lines to the same virus, suggests functional variation in the piRNA machineries of *Culex* and *Aedes* mosquitoes. Furthermore, differences in small RNA profiles for viruses from distinct viral families implicate an important role of the viral coding strategy in the targeting by small RNA machineries. These results contribute to our understanding of the small RNA machineries in mosquitoes and their implications for infections by arboviruses and ISVs.

Materials and methods

Cell culture and virus infections

Ae. albopictus C6/36 (ATCC CRL-1660) and U4.4 cells [240] were cultured at 28 °C in Leibovitz-L15 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2% tryptose phosphate broth (Gibco) and 1% nonessential amino acids (Gibco). *Cx. tarsalis* Cx.t cells [241] were cultured at 28 °C in Schneider's *Drosophila* medium (Gibco) supplemented with 10% FBS. The infectious-clone used to generate WNV lineage 2, originally originating from south eastern Europe, was described before [157] and the resulting virus is referred to as WNV_{IC}. Briefly, pre-seeded 6-well plates of Vero cells were transfected with ~1 µg of WNV_{IC} plasmid DNA using Lipofectamine 2000 (Invitrogen) to generate the P0 virus stock. Subsequent passages were performed on C6/36 cells and the P2 stock was used for experiments. EPDA on Vero cells were used to determine the TCID₅₀ as previously described [157].

RNA isolation and deep sequencing of small RNAs

Monolayers of U4.4 and Cx.t cells were infected with WNV_{IC} at a multiplicity of infection of 1 in 6-well plates. RNA was isolated at 4 dpi using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Small RNA deep sequencing libraries were prepared as described previously [157]. Briefly, 15 µg total RNA was size-separated by electrophoresis on 15% polyacrylamide, 7 M Urea, 0.5X Tris-boric acid EDTA (TBE) gels. The 19-30 nt RNAs were excised from gel and eluted in 800 µl 0.3 M sodium acetate, precipitated with 80% ethanol and dissolved in 11 µl H₂O. Small-RNA libraries were prepared using the TruSeq small-RNA kit (15016914; Illumina) and sequenced on an Illumina HiSeq 2500 sequencer (Baseclear, Leiden, The Netherlands). Single-end FASTQ reads were generated with the Illumina Casava pipeline version 1.8.3. Initial quality assessments were performed using the in-house filtering protocols of Baseclear and the FASTQC quality control version 0.10.0.

Small RNA analysis

Sequencing libraries were analysed on the Galaxy webserver [242]. Adapters were trimmed using the Clip tool version 10.1. Reads were mapped with Bowtie2 [243] version 2.3.4.2 allowing 1 mismatch with a seed length of 28. Reads were mapped to the viral genomes of WNV_{IC}, Culex Y virus (A-segment JQ659254.1; B-segment JQ659255.1), Flock House virus (RNA-1 NC_004146.1; RNA-2 NC_004144.1), Phasi-Charoen-like virus (L-segment KU936057.1; M-segment KU936056.1; S-segment KU936055.1), Narnavirus (KP642120.1), Semliki Forest virus (KP699763.1), Cell fusing agent virus (NC_001564.2) and Calbertado virus (Aaron C. Brault, personal communication). For analysis of mosquito-derived small RNAs, reads were mapped with Bowtie2 as described above to the genome of *Cx. quinquefasciatus* (CpipJ2) [244] for Cx.t cells or *Ae. albopictus* AaloF1 [245] for U4.4 cells. Genome distributions of siRNAs and piRNAs were produced by filtering reads with a length of 21 or 25-30 nts, respectively, and mapping the genome positions of the 5' end of each read. Sequence signatures were generated using Weblogo3 from mapped sense and reverse-complemented antisense reads that were 3' trimmed to 20 nts. piRNA overlaps were determined using the small RNA signatures tool [246] version 3.1.0 on the mississippi.snv.jussieu.fr Galaxy server using 25-30 nt reads as input and scanning for overlaps of 0-20 nts.

De novo assembly

De novo assembly of small RNA reads was performed with the Metavisitor Oases/Velvet pipeline with k-mers ranging from 15-29 on the mississippi.snv.jussieu.fr Galaxy server on clipped reads, as described in [247]. Retrieved contigs were filtered for a minimum length of 200 nts. Contigs were blasted to all sequence in the NCBI database that match the criteria txid10239[orgn] NOT txid131567[orgn] Not phage[title], which retrieves all non-phage virus sequences, with the BLASTx tool in the NCBI plus toolkit. Contigs with a viral blast hit were assembled using the Contigassembler tool in Vector NTI 11.0 (Invitrogen), and retrieved contigs were blasted against the NCBI non-redundant nucleotide database with BLASTn to identify the viral origin. For comparison of contigs with the viral genome subject sequences, the above mentioned Genbank entries were used.

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Chapter IV

Mosquito co-infection with Zika and chikungunya virus allows simultaneous transmission without affecting the vector competence of *Aedes aegypti*

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Abstract

Zika virus (ZIKV) and chikungunya virus (CHIKV) are highly pathogenic arthropod-borne viruses that are currently a serious health burden in the Americas, and elsewhere in the world. ZIKV and CHIKV co-circulate in the same geographical regions and are mainly transmitted by *Aedes aegypti* mosquitoes. There is a growing number of case reports of ZIKV and CHIKV co-infections in humans, but it is uncertain whether co-infection occurs via single or multiple mosquito bites. Here we investigate the potential of *Ae. aegypti* mosquitoes to transmit both ZIKV and CHIKV in one bite, and we assess the consequences of co-infection on vector competence. First, growth curves indicated that co-infection with CHIKV negatively affects ZIKV production in mammalian, but not in mosquito cells. Next, *Ae. aegypti* mosquitoes were infected with ZIKV, CHIKV, or co-infected via an infectious blood meal or intrathoracic injections. Infection and transmission rates, as well as viral titers of virus-positive mosquitoes, were determined at 14 days after blood meal or 7 days after injection. Saliva and bodies of (co-)infected mosquitoes were scored concurrently for the presence of ZIKV and/or CHIKV using a dual-colour immunofluorescence assay. The results show that orally exposed *Ae. aegypti* mosquitoes are highly competent, with transmission rates of up to 73% for ZIKV, 21% for CHIKV, and 12% of mosquitoes transmitting both viruses in one bite. However, simultaneous oral exposure to both viruses did not change infection and transmission rates compared to exposure to a single virus. Intrathoracic injections indicate that the selected strain of *Ae. aegypti* has a strong salivary gland barrier for CHIKV, but a less profound barrier for ZIKV. This study shows that *Ae. aegypti* can transmit both ZIKV and CHIKV via a single bite. Furthermore, co-infection of ZIKV and CHIKV does not influence the vector competence of *Ae. aegypti*.

Introduction

Zika virus (ZIKV; family *Flaviviridae*, genus *Flavivirus*) is a pathogenic arthropod-borne (arbo)virus that causes neurological disease in humans and congenital syndrome in newborns and infants [58]. In the 60 years after its discovery in 1947, sporadic ZIKV infections were reported in African countries and in parts of Asia [248]. The first large ZIKV outbreak was reported in 2007 on the Yap Islands of Micronesia after which the virus quickly spread to other countries in south-east Asia, such as French Polynesia in 2013, and the Cook Islands and Easter Island in 2014 [249]. In 2015, there was a dramatic increase of reported ZIKV cases in South America, especially Brazil, where over 200,000 cases of infection, six deaths, and over 2,200 incidents of ZIKV associated congenital syndrome were reported [50]. Prior to the ZIKV outbreak in the Americas, flavivirus infections linked to congenital disease were rarely reported. However, a causal relationship between ZIKV infection in pregnant women and subsequent birth malformations, such as microcephaly, have now been confirmed [50,58,59]. The main vector for ZIKV transmission is the *Aedes aegypti* mosquito [32,54,250,251], while *Ae. albopictus* [54,56,252], *Ae. vittatus* [253] *Ae. luteocephalus* [253], and *Ae. hensilli* [254] can transmit ZIKV in laboratory studies.

Other mosquito-borne viruses that circulate concurrently with ZIKV in South America include chikungunya virus (CHIKV; family *Togaviridae*, genus *Alphavirus*), dengue virus (DENV; family *Flaviviridae*, genus *Flavivirus*) and yellow fever virus (family *Flaviviridae*, genus *Flavivirus*). In 2013, CHIKV was introduced into South America via the Caribbean. Since then, over 319,000 cases of infection and 135 deaths have been reported in South America [255]. CHIKV strains that circulate in the Americas are predominantly transmitted by *Ae. aegypti* mosquitoes [31]. Since CHIKV and ZIKV co-circulate in the same geographical regions, individuals can become co-infected with both viruses [256,257]. Co-infections of patients with ZIKV and CHIKV already occurred in South America [258–260], some even reporting triple infection with ZIKV, CHIKV, and DENV [261–263]. Whether a single bite of *Ae. aegypti* can transmit both ZIKV and CHIKV simultaneously, or whether sequential bites of two infected mosquitoes are required for such co-infections in humans, remains unclear.

Here we designed a dual-colour immunofluorescence assay that can concurrently detect ZIKV and CHIKV infection in mammalian and mosquito cells. We analysed the effect of co-infections on virus growth kinetics in mammalian and mosquito cell lines. Furthermore, we studied the effect of co-infection with both ZIKV and CHIKV on the infection and transmission rates of both viruses in *Ae. aegypti* mosquitoes. Finally, mosquito transmission rates after an infectious blood meal and intrathoracic injections were compared to study the effects of the midgut and salivary gland barriers on co- and single-infections.

Results

ZIKV and CHIKV can co-infect and co-replicate in mammalian and mosquito cells

A dual-colour immunofluorescence detection assay was developed to investigate whether ZIKV and CHIKV can infect and replicate in the same mammalian or mosquito cell. Vero (green monkey kidney, mammalian) cells and C6/36 (*Ae. albopictus*, mosquito) cells were seeded as monolayers and inoculated with ZIKV, CHIKV, or co-inoculated and stained for viral antigens at 48 hours post-infection (hpi) (Fig. 4.1). Distinctions between ZIKV and CHIKV infected cells were clear after immunostaining in both Vero and C6/36 cells, indicating that the dual-colour immunofluorescence assay

can be used to score co-infected samples for the presence of ZIKV, CHIKV, or both (Fig. 4.1A & B). ZIKV infected Vero cells displayed localization of the envelope (E (pan-flavivirus α -E (4G2))) protein predominantly near the perinuclear regions / endoplasmic reticulum, corresponding to the replication and assembly sites of flaviviruses [264] (Fig. 4.1A). Most Vero cells in the CHIKV infected sample already lysed due to the strong cytopathic effect (CPE) of CHIKV infection, as indicated by the presence of the E2 envelope protein on the remaining cell projections. Viable CHIKV infected cells showed localization of E2 near the cell boundaries, related to the assembly sites of CHIKV [74] (Fig. 4.1A). The localization of E and E2 was similar in C6/36 cells, with ZIKV-E mostly present near the endoplasmic reticulum surrounding the nucleus, and CHIKV-E2 near the cell membrane (Fig. 4.1B). Co-infection did not alter the localization of ZIKV-E nor CHIKV-E2, indicating that these viruses can co-infect the same cell without obvious interference. These results show that ZIKV and CHIKV are intrinsically capable to co-infect and co-replicate in cells of their mammalian and insect host.

Fig 4.1 Immunofluorescence of ZIKV and CHIKV single- and co-infections in mammalian and mosquito cells. (A) Vero and (B) C6/36 cells were infected with ZIKV^{SUR}, CHIKV³⁷⁹⁹⁷ or both. At 48 hours post-infection (hpi) the monolayers were fixed, permeabilized, stained with antibodies for ZIKV-E (pan-flavivirus α -E (4G2)), CHIKV-E2 and Hoechst33258 (details in materials and methods) and visualized by immunofluorescence. Magnifications are indicated in each picture. Bottom panels indicate zoomed and merged images of co-infected cells.

Growth kinetics of ZIKV and CHIKV during single- and co-infections of mammalian and mosquito cell lines

Viral co-infections can influence the replication rate in mosquito cell lines and may affect transmission by the mosquito vector [183]. In order to assess whether ZIKV and CHIKV interfere with each other's replication, the growth kinetics and CPE of ZIKV and CHIKV were determined during co- and single-infections in mammalian Vero, *Ae. albopictus* C6/36, and *Ae. aegypti* Aag2 cells (Fig. 4.2 & 4.3). Cells were infected at an MOI of 0.1, washed, and culture fluid samples were collected at 0, 24, 48, 72, and 96 hpi, and titrated by end-point dilution assay (EPDA). In Vero cells, ZIKV reached a peak titer of 8.0×10^7 tissue culture infectious dose 50% (TCID₅₀)/ml within 48 hpi (Fig. 4.3A), whereas CHIKV only reached a titer of 8.7×10^5 TCID₅₀/ml at 24 hpi (Fig. 4.3B). During co-infection in Vero cells, the titer of ZIKV was approximately 3 logs lower at 48 hpi and 72 hpi as compared to single-infection, whereas the titer of CHIKV was not affected by co-infection with ZIKV. In C6/36 cells, ZIKV reached a relatively low peak titer of 9.6×10^5 TCID₅₀/ml at 96 hpi (Fig. 4.3C), whereas CHIKV reached peak titers of 7.7×10^8 TCID₅₀/ml at 48 hpi (Fig. 4.3D). Co-infection resulted in approximately 1 log lower titer of ZIKV at 72 and 96 hpi as compared to single-infection, whereas CHIKV replication was not seemingly affected by co-infection. In Aag2 cells, ZIKV reached a peak titer of 6.7×10^7 TCID₅₀/ml at 96 hpi (Fig. 4.3E), indicating that ZIKV replicates better in *Ae. aegypti* as compared to *Ae. albopictus* cells (compare Fig. 4.3C with 4.3E). In contrast, CHIKV reached peak titers of 1.2×10^6 TCID₅₀/ml at 48 hpi in Aag2 cells, and CHIKV titers rapidly decreased at later time points (Fig. 4.3F). This suggests that CHIKV replicates better in *Ae. albopictus* than *Ae. aegypti* cells (compare Fig. 4.3D with 4.3F). Importantly, co-infections in Aag2 cells did not significantly affect the replication of either ZIKV or CHIKV (Fig. 4.3E & 4.3F). To investigate whether the observed difference in growth kinetics of ZIKV during co- and single-infections in Vero cells was due to altered cell viability, a cell viability assay was performed (Fig. 4.2). Signs of virus induced cytopathic effect were readily observed by bright field microscopy at 24 hpi in the CHIKV and co-infected cells, whereas ZIKV-induced cytopathic effects were only observed after 48 hours (Fig. 4.2A). Additionally, the cell viability of CHIKV infected and co-infected Vero cells was decreased dramatically to 20% at 48 hpi, whereas ZIKV maintained high cell viability up to 48 hpi (Fig. 4.2B). These results suggest that the reduction of ZIKV titers during co-infection with CHIKV is due to the rapid and extensive CPE resulting from CHIKV-induced host shut-off [71], which interferes with ZIKV virion production. Moreover, co-infections did not affect the cell viability in both C6/36 and Aag2 cells until 96 hpi (Cell viability: 80 – 100%).

◀ **Fig 4.2 (Previous page) Cell viability of Vero cells during ZIKV, CHIKV single- or co-infections.** (A-B) Vero cells were infected with ZIKV^{SUR}, CHIKV³⁷⁹⁹⁷, or co-infected at a multiplicity of infection of 0.1. At 0, 24, 48, 72 and 96 hours post-infection (hpi) cells were visualized by (A) bright field microscopy and (B) lysed before measuring the cell viability by CellTiter-Glo assay. Cell viability is presented as percentage normalized to the mock.

Fig 4.3 Growth curves of ZIKV and CHIKV during single- and co-infections. (A-B) Vero, (C-D) C6/36 and (E-F) Aag2 cells were infected with ZIKV^{SUR}, CHIKV³⁷⁹⁹⁷ or co-infected at a multiplicity of infection of 0.1. The virus titers during single- and co-infection were determined by end-point dilution assay (EPDA) at the indicated time points for (A,C,E) ZIKV and (B,D,F) CHIKV. Shown are the mean virus titers $\pm$ standard error of the mean from duplicate samples. The detection limit of the EPDA is indicated by a dashed line.

***Ae. aegypti* is competent to transmit ZIKV and CHIKV**

The viral infectious dose in the blood meal is known to have a strong effect on the mosquito infection rates of mosquito-borne arboviruses [265,266]. Therefore, we determined the dose-dependent infection and transmission rates of ZIKV and CHIKV in *Ae. aegypti*. Female *Ae. aegypti* mosquitoes were offered an infectious blood meal containing 2.0×10^5 , 2.0×10^6 , or 2.0×10^7 TCID₅₀/ml of ZIKV or CHIKV. When comparing single- with co-infections it is important that the infectious blood meals contain equal virus titers and that the mosquitoes engorge similar numbers of infectious particles. To ensure that the infectious blood meals were completely homogenized and to validate our viral dilution series we froze a selection of engorged mosquito bodies directly after the blood meal and determined the virus titer by EPDA. Indeed, mosquitoes infected with increasing doses of infectious virus in the blood meal had higher titers in their bodies. The ingested virus titers of mosquitoes that were infected with the lowest dose were significantly lower than those infected with the two highest doses (ZIKV: $P < 0.01$, CHIKV: $P < 0.01$; Fig. 4.4A), although mosquitoes infected with the two highest doses were not significantly different amongst each other ($P > 0.05$). The infection rates were determined at 14 days post-infection (dpi) by infectivity assay of mosquito bodies and transmission rates by infectivity assay of saliva samples. Inoculation with 2.0×10^5 TCID₅₀/ml in the blood meal resulted in an infection rate of 65.3% for ZIKV (Fig. 4.4B and Table 4.1). Increasing the infectious dose to 2.0×10^6 or 2.0×10^7 TCID₅₀/ml significantly increased the ZIKV infection rate to 92.2% and 100% ($P < 0.01$). For CHIKV, inoculation with 2.0×10^5 , 2.0×10^6 , or 2.0×10^7 TCID₅₀/ml resulted in infection rates of 47.9%, 66.7%, or 81.2%, respectively. Inoculation with the highest CHIKV dose resulted in significantly higher infection rates compared to the lowest dose ($P < 0.001$). These results indicate that the mosquito infectious dose for *Ae. aegypti* is higher for CHIKV than ZIKV. In the same set of experiments, the mosquito saliva was collected by forced salivation assay and scored for the presence of virus to calculate the transmission rates. Transmission rates of 34.7% for ZIKV and 10.4% for CHIKV were reached with an infectious dose of 2.0×10^5 TCID₅₀/ml (Fig. 4.4C and Table 4.1). With a viral titer in the blood meal of 2.0×10^6 or 2.0×10^7 TCID₅₀/ml the transmission rates of ZIKV increased significantly to 68.6% and 68.3% ($P < 0.01$), whereas transmission rates for CHIKV of 5.9% and 21.2% were not significantly different as compared to the lowest dose ($P > 0.05$).

Table 4.1 Infection rates, transmission rates, and median titers of *Ae. aegypti* mosquitoes orally exposed to different doses of ZIKV and CHIKV. Infection and transmission rates determined at 14 days post-infection (dpi) are presented as percentages (number of virus positive mosquito bodies or saliva samples / total number of engorged mosquitoes). Titers were determined for mosquitoes with a fully disseminated infection of ZIKV or CHIKV and are presented as TCID₅₀/ml. The results represent the cumulative data from three independent biological replicates.

Virus	Dose	Infection rate	Transmission rate	Median titer body	Median titer saliva
ZIKV ^{SUR}	2.0×10^5	65(32/49)	35(17/49)	2.0×10^7	1.0×10^3
	2.0×10^6	92(47/51)	69(35/51)	2.0×10^7	1.0×10^3
	2.0×10^7	100(101/101)	68(69/101)	7.1×10^6	1.0×10^3
CHIKV ³⁷⁹⁹⁷	2.0×10^5	48(23/48)	10(5/48)	6.0×10^5	1.0×10^3
	2.0×10^6	67(34/51)	6(3/51)	5.0×10^4	1.0×10^3
	2.0×10^7	81(69/85)	21(18/85)	5.7×10^5	1.0×10^3

Fig 4.4 Infection and transmission rates of *Ae. aegypti* orally infected with different doses of ZIKV or CHIKV. Mosquitoes were inoculated with an infectious blood meal containing a dose of 2.0×10^5 , 2.0×10^6 , or 2.0×10^7 TCID₅₀/ml ZIKV^{SUR} or CHIKV³⁷⁹⁹⁷. (A) Ingested virus titers of *Ae. aegypti* immediately after blood feeding. Each dot represents one mosquito body, and horizontal bars indicate median titers. The detection limit of the end-point dilution assay (EPDA) is indicated by the dashed line. Results were evaluated with a Kruskal-Wallis test. Significant differences between viral doses ($P < 0.05$) are indicated by different letters. (B) Infection and (C) transmission rates of *Ae. aegypti* mosquitoes at 14 days post-infection (dpi) presented as the percentage of engorged mosquitoes. Shown are the mean percentages from three independent replicates. Error bars show the standard error of the mean. Sample size ranged between 48-101 female mosquitoes per treatment. Results were evaluated with Chi-squared test. Significant differences between viral doses ($P < 0.05$) are indicated by different letters. (D-E) Virus titers of CHIKV and ZIKV mosquito (D) bodies and (E) saliva. Each dot represents one mosquito body, and the horizontal bars indicate median titers. The detection limit of the EPDA is indicated by a dashed line. Results were evaluated with a Kruskal-Wallis test. Significant differences between viral doses ($P < 0.05$) are indicated by different letters.

To observe whether increasing infectious doses in the blood meal lead to higher viral titers in the mosquito bodies and saliva, we titrated both the mosquito bodies and saliva samples of mosquitoes with a fully disseminated ZIKV or CHIKV infection (positive body and saliva) at 14 dpi. Median ZIKV titers in mosquito bodies reached 2.0×10^7 , 2.0×10^7 , and 7.1×10^6 TCID₅₀/ml for the respective inoculation doses of 2.0×10^5 , 2.0×10^6 , or 2.0×10^7 TCID₅₀/ml (Fig. 4.4D). Median titers of mosquito bodies inoculated with 2.0×10^7 TCID₅₀/ml of ZIKV were significantly lower compared to median titers of mosquito bodies inoculated with the lower doses ($P < 0.05$), indicating that a higher infectious dose in the blood meal does not necessarily lead to a higher viral load in the mosquito. Median CHIKV titers were approximately 1-3 logs lower than ZIKV titers and reached values of 6.0×10^5 , 5.0×10^4 , and 5.7×10^5 TCID₅₀/ml for the respective inoculation doses of 2.0×10^5 , 2.0×10^6 , or 2.0×10^7 (Fig. 4.4D). No significant differences were found between the median titers of mosquito bodies inoculated with different doses of CHIKV ($P > 0.05$). In addition, median viral titers in virus-positive mosquito saliva samples were determined. All median viral titers of saliva samples were below the TCID₅₀ detection limit, and no significant differences between saliva samples could be observed ($P > 0.05$; Fig. 4.4E). These results show that *Ae. aegypti* is a competent vector for both ZIKV and CHIKV. Moreover, the relatively high mosquito infectious dose for CHIKV indicates that a higher viral dose in the blood meal should be used to study the effects of ZIKV and CHIKV co-infections.

ZIKV and CHIKV are simultaneously transmitted by *Ae. aegypti* upon co-infection

Co-infections of arboviruses can affect their transmission potential by mosquito vectors and even exclude transmission of one particular virus [267,268]. To investigate the effect of co-infection on the infection and transmission of ZIKV and CHIKV, female *Ae. aegypti* mosquitoes were offered an infectious blood meal containing a dose of 2.0×10^7 TCID₅₀/ml of ZIKV, CHIKV, or both. Titrations of engorged mosquito bodies that were immediately frozen after the infectious blood meal, showed that the mosquitoes ingested equal amounts of CHIKV and ZIKV in the single- and co-infections ($P = 0.24$; Fig. 4.5A). At 14 dpi, saliva was collected from the mosquitoes and the infection and transmission rates were determined by infectivity assay. ZIKV infection rates were 100% for the single infection and 97.9% for the co-infection, which was not significantly different ($P = 1.00$; Fig. 4.5B & Table 4.2). Similarly, no significant difference was found between infection rates of orally exposed mosquitoes to CHIKV in single (81.2%) and co-infection with ZIKV (85.4%; $P = 1.00$). In both single- and co-infection, infection rates of mosquitoes orally exposed to CHIKV were significantly lower than those of mosquitoes exposed to ZIKV ($P < 0.05$). In total, 84.4% of mosquitoes that were simultaneously exposed to both viruses were infected with both ZIKV and CHIKV. These results indicate that co-infection of ZIKV and CHIKV does not affect the infection rates in *Ae. aegypti*.

Transmission rates of mosquitoes orally exposed to ZIKV were 68.3% for the single-infection and 72.9% for the co-infection, which was not significantly different ($P = 1.00$; Fig. 4.5C & Table 4.2). For CHIKV, transmission rates were 21.2% for mosquitoes with a single infection and 14.6% for mosquitoes with a co-infection, which was again not significantly different ($P = 1.00$). However, transmission rates of mosquitoes orally exposed to CHIKV were significantly lower than ZIKV exposed mosquitoes ($P < 0.001$). Importantly, 11.5% of mosquitoes that were simultaneously exposed to both viruses, had both ZIKV and CHIKV in their saliva, showing that *Ae. aegypti* can transmit both ZIKV and CHIKV via a single bite. In summary, these results show that simultaneous

exposure to ZIKV and CHIKV can lead to concurrent transmission of both viruses without affecting the infection or transmission rates in *Ae. aegypti*.

Although no effect of co-infection on the infection and transmission rates was observed, there might be an effect on the viral titers in either the mosquito body or saliva that could have an effect on virus transmission. Therefore, viral titers were determined at 14 dpi for both mosquito bodies and saliva samples, of mosquitoes with fully disseminated infections of ZIKV, CHIKV, or both. ZIKV median titers in mosquito bodies reached 7.1×10^6 after single and 2.0×10^7 TCID₅₀/ml after co-infection, whereas CHIKV reached titers of 5.7×10^5 after single- and 6.3×10^5 TCID₅₀/ml after co-infection (Fig. 4.5D & Table 4.2). Median titers of both ZIKV and CHIKV in saliva samples reached $1.0 - 4.6 \times 10^3$ TCID₅₀/ml (Fig. 4.5E & Table 4.2). Compared to single-infection, co-infection did not influence the titers of ZIKV or CHIKV in mosquito bodies or saliva ($P > 0.05$). ZIKV titers were significantly higher than CHIKV titers in both mosquito bodies ($P < 0.01$) and saliva ($P < 0.05$; Fig. 4.5D & E). These results show that co-infection with ZIKV and CHIKV does not affect the transmission potential of *Ae. aegypti* for either virus. Importantly, these experiments demonstrate for the first time that *Ae. aegypti* is intrinsically capable of transmitting ZIKV and CHIKV via a single bite.

Table 4.2 Infection rates, transmission rates and median titers of *Ae. aegypti* mosquitoes orally exposed to ZIKV, CHIKV, or both. Infection and transmission rates of mosquitoes in the co-infection treatment were determined as the percentage of mosquitoes with either ZIKV, CHIKV, or both viruses in their body or saliva, respectively, of mosquitoes within the respective treatment. Titers were determined for mosquitoes with a fully disseminated infection of ZIKV, CHIKV, or both and are presented as TCID₅₀/ml. The results represent the cumulative data from three independent biological replicates.

	Virus	Dose	Infection rate	Transmission rate	Median titer body	Median titer saliva
Single infection	ZIKV ^{SUR}	2.0×10^7	100 (101/101)	68.3 (69/101)	7.1×10^6	1.0×10^3
	CHIKV ³⁷⁹⁹⁷	2.0×10^7	81.2 (69/85)	21.2 (18/85)	5.7×10^5	1.0×10^3
Co-infection	ZIKV ^{SUR}	2.0×10^7	97.9 (94/96)	72.9 (70/96)	2.0×10^7	4.6×10^3
	CHIKV ³⁷⁹⁹⁷	2.0×10^7	85.4 (82/96)	14.6 (14/96)	6.3×10^5	1.0×10^3
	ZIKV ^{SUR} & CHIKV ³⁷⁹⁹⁷		84.4 (81/96)	11.5 (11/96)

Fig 4.5 Infection and transmission rates of *Ae. aegypti* mosquitoes orally inoculated with ZIKV, CHIKV, or both. Mosquitoes were orally infected with an infectious blood meal containing a dose of 2.0×10^7 TCID₅₀/ml ZIKV^{SUR}, CHIKV³⁷⁹⁹⁷, or both. (A) Ingested virus titers of *Ae. aegypti* immediately after blood feeding. Each dot represents one mosquito body, and the horizontal bars indicate median titers. The detection limit of the end-point dilution assay (EPDA) is indicated by a dashed line. Results were evaluated with a Kruskal-Wallis test. Significant differences between treatments ($P < 0.05$) are indicated by different letters. (B) Infection and (C) transmission rates of *Ae. aegypti* mosquitoes at 14 days post-infection presented as the percentage of engorged mosquitoes. The percentage of co-infected mosquitoes and saliva samples is indicated by the dashed line. Shown are the mean percentages from three independent replicates. Error bars show the standard error of the mean. Sample size ranged between 85-101 female mosquitoes per treatment. Results were evaluated with Chi-squared tests. Significant differences between treatments ($P < 0.05$) are indicated by different letters. (D-E) Virus titers of CHIKV and ZIKV mosquito (D) bodies and (E) saliva. Each dot represents one mosquito body, and the horizontal bars indicate median titers. The detection limit of the EPDA is indicated by a dashed line. Results were evaluated with a Kruskal-Wallis test. Significant differences between treatments ($P < 0.05$) are indicated by different letters.

***Ae. aegypti* has a strong salivary gland barrier for CHIKV and a minor barrier for ZIKV**

We observed high infection rates for ZIKV and CHIKV, but the transmission rates for CHIKV were notably lower as compared to ZIKV. This substantial difference in transmissibility of ZIKV and CHIKV by *Ae. aegypti* could be due to the presence of a midgut escape barrier, a salivary gland barrier, or both. To discriminate between these possibilities, female *Ae. aegypti* mosquitoes were intrathoracically injected (to by-pass the midgut barriers) with 2.8×10^3 TCID₅₀ units of ZIKV, CHIKV, or both. After 7 days, mosquito saliva was collected and bodies and saliva were tested for presence of virus by infectivity assay. Injection with ZIKV, CHIKV, and both viruses resulted in all cases in 100% infection rates (Fig. 4.6A & Table 4.3). Transmission rates of mosquitoes injected with ZIKV were similar for the single-infection (77.6%) and after co-infection (68.8%; $P = 1.00$; Fig. 4.6B & Table 4.3). For CHIKV the transmission rates were also similar for the single-infection (22.9%) and the co-infection (27.1%; $P = 1.00$), again suggesting that ZIKV and CHIKV do not interfere. Transmission rates of mosquitoes intrathoracically injected with CHIKV were significantly lower compared to ZIKV ($P < 0.001$). In total, 20.8% of the mosquitoes that were simultaneously exposed to both viruses had both ZIKV and CHIKV in their saliva. Viral titers were again determined for mosquito bodies and saliva samples of mosquitoes with a fully disseminated infection, which were injected with ZIKV, CHIKV, or both viruses simultaneously. ZIKV reached mosquito body titers of 2.0×10^7 TCID₅₀/ml after single and 5.4×10^7 TCID₅₀/ml after co-infections, whereas CHIKV reached titers of 2.0×10^6 after single and 6.3×10^6 TCID₅₀/ml after co-infection (Fig. 4.6C & Table 4.3). Median titers of both ZIKV and CHIKV in saliva samples reached $1.0 - 4.2 \times 10^3$ TCID₅₀/ml (Fig. 4.6D & Table 4.3). Compared to single-infection, co-infection did not influence the titers of ZIKV or CHIKV in mosquito bodies and saliva ($P > 0.05$; Fig. 4.6C & 4.6D). For ZIKV, the transmission rates after intrathoracic injections and blood meal infections are only slightly lower than the infection rates. This suggests that for ZIKV the salivary glands form a minor barrier for accumulation of infectious virus in the saliva. The low transmission rates of CHIKV as compared to the high infection rates after both blood meal infection and intrathoracic injection (Fig. 4.5C & Fig. 4.6B), indicate the presence of a salivary gland barrier that prevents the virus from dissemination into the saliva.

Table 4.3 Infection rates, transmission rates and median titers of *Ae. aegypti* mosquitoes intrathoracically injected with ZIKV, CHIKV, or both. Infection and transmission rates of mosquitoes in the co-infection treatment were determined as the percentage of mosquitoes with either ZIKV, CHIKV, or both in their body or saliva, respectively, out of the total number of injected mosquitoes within the respective treatment. Titers were determined for mosquitoes with a fully disseminated infection of ZIKV, CHIKV, or both and are presented as TCID₅₀/ml. The results represent the cumulative data from three independent biological replicates.

	Virus	Dose	Infection rate	Transmission rate	Median titer body	Median titer saliva
Single infection	ZIKV ^{SUR}	2.8×10^3	100 (49/49)	77.6 (38/49)	2.0×10^7	1.0×10^3
	CHIKV ³⁷⁹⁹⁷	2.8×10^3	100 (48/48)	22.9 (11/48)	2.0×10^6	1.0×10^3
Co-infection	ZIKV ^{SUR}	2.8×10^3	100 (48/48)	68.8 (33/48)	5.4×10^7	4.2×10^3
	CHIKV ³⁷⁹⁹⁷	2.8×10^3	100 (48/48)	27.1 (13/48)	6.3×10^6	1.0×10^3
	ZIKV ^{SUR} & CHIKV ³⁷⁹⁹⁷		100 (48/48)	20.8 (10/48)

Fig 4.6 Infection and transmission rates of *Ae. aegypti* intrathoracically injected with ZIKV, CHIKV, or both. Mosquitoes were infected through intrathoracic injections with a dose of 2.8×10^3 TCID₅₀ units of ZIKV_{SUR}, CHIKV³⁷⁹⁹⁷, or both. (A) Infection and (B) transmission rates of *Ae. aegypti* mosquitoes at 7 days post-infection presented as percentage of the total number of injected mosquitoes. The percentage of co-infected mosquito bodies and saliva samples is indicated by the dashed line. Shown are the mean percentages from three independent replicates. Error bars show the standard error of the mean. Sample size ranged between 48-49 female mosquitoes per treatment. Results were evaluated with Chi-squared tests, and corrected for multiple comparisons with the Bonferroni correction. Significant differences between treatments ($P < 0.05$) are indicated by different letters. (C-D) Virus titers of CHIKV and ZIKV mosquito (C) bodies and (D) saliva. Each dot represents one mosquito body, and the horizontal bars indicate median titers. The detection limit of the end-point dilution assay is indicated by a dashed line. Results were evaluated with a Kruskal-Wallis test, and Dunn's test for multiple comparisons, corrected with the Bonferroni correction. Significant differences between treatments ($P < 0.05$) are indicated by different letters.

Discussion

Since the start of the global spread of ZIKV, this virus co-circulates with CHIKV in many parts of the world. There is an increase in the number of reports describing co-infections of ZIKV and CHIKV (and also DENV) in human patients, but the extent of co-infection in field-collected mosquitoes is not clear. The aim of this study was to assess whether the predominant vector of ZIKV and CHIKV in the Americas, *Ae. aegypti*, is able to transmit both viruses simultaneously, and whether co-infection may change the vector competence for either virus. Here we show that *Ae. aegypti* mosquitoes can indeed simultaneously transmit ZIKV and CHIKV via a single bite. Infection with both ZIKV and CHIKV did not result in lowered infection or transmission rates for either virus, although *Ae. aegypti* was shown to be a more efficient vector for ZIKV as compared to CHIKV. Finally, we show that *Ae. aegypti* mosquitoes have a salivary gland barrier for both CHIKV and ZIKV.

Triple co-infections with ZIKV, CHIKV and DENV have already been reported in patients from Colombia and Nicaragua [261–263] and several cases of ZIKV and CHIKV co-infections in patients have been reported [258–260]. These observations indicate that a single mosquito could take a blood meal containing multiple arboviruses, potentially resulting in the transmission of different viruses simultaneously. Simultaneous transmission of alphaviruses and flaviviruses by *Ae. albopictus* has been reported for DENV and CHIKV [269], and for DENV and Sindbis virus [267]. In another study, CHIKV and DENV co-transmission by either *Ae. aegypti* or *Ae. albopictus* only occurred after sequential blood meals, but not after simultaneous infection in a single blood meal [270]. Furthermore, co-infection of Sindbis virus and DENV greatly decreases both the infection and transmission rates of both viruses [267]. In contrast to these studies, our results clearly show that ZIKV and CHIKV do not interfere with each other in either their infection or transmission by *Ae. aegypti*.

Our results show that ZIKV and CHIKV can replicate simultaneously in a single cell of both the mammalian host and mosquito vector. Furthermore, we show that ZIKV and CHIKV can simultaneously disseminate to the saliva of *Ae. aegypti* mosquitoes, indicating that co-infections do not strongly interfere with virus replication. The effect of co-infection of arboviruses on virus replication is still poorly understood. One explanation for interference between different viruses is superinfection exclusion, where infection of a primary virus excludes secondary infection with the same or a different virus [183]. The primary virus infection could induce the host immune response, claim important cellular factors required for viral replication, or produce defective interfering particles, that suppress replication of a secondary viral infection. Alternatively, primary infection may lead to suppression of the mosquito's antiviral responses, leading to enhanced infection of a secondary virus (reviewed in [183]). Potentially, asynchronous co-infections of CHIKV and ZIKV in *Ae. aegypti* mosquitoes could result in detectable interference and may affect the infection and transmission rates.

However, viral interference was observed in growth curves in Vero cells with co-infection resulting in decreased ZIKV titers. The interference in Vero cells is likely due to a decrease in cell viability as a result of CHIKV infection, leading to high cell death and, thus, less ZIKV production. In contrast to what we observed in mammalian cells, virus replication and cell viability were not affected when C6/36 or Aag2 cells were infected or co-infected by both viruses. The absence of detectable interference between the two viruses on virus replication in mosquito cell lines supports our findings on vector competence of *Ae. aegypti* mosquitoes infected with both viruses. CHIKV replicates in spherules at the plasma membrane [271] whereas flaviviruses replicate in perinuclear regions of the endoplasmic reticulum [272], which may explain the

lack of interference between both viruses. Furthermore, tampering of virus replication by defective interfering particles, which often contributes to viral interference, is less problematic with alphavirus and flavivirus co-infections [183]. Potentially, co-infections of multiple flaviviruses (e.g. DENV and ZIKV) may have a stronger effect on vector competence.

Our results support the role of *Ae. aegypti* as a competent vector for ZIKV and CHIKV. Previous vector competence studies reported infection rates between 70-100% in *Ae. aegypti* [54,250,273] for ZIKV. Infection rates reported here for the ZIKV^{SUR} strain are in line with these findings with 100% infection after administering a high dose blood meal (2.0×10^7 TCID₅₀/ml), and 65-90% with a ten- to hundred-fold lower dose of ZIKV in the blood meal. The transmission rates of 35-70% for the ZIKV^{SUR} strain are higher than some previously reported transmission rates, which ranged between 10-30% for the American strains of ZIKV in Brazilian and Mexican *Ae. aegypti* mosquitoes [54,273]. However, studies with Australian and Poza Rica strains of *Ae. aegypti* reported transmission rates between 70-85% [251,273], which is more in the range of our findings. For the CHIKV³⁷⁹⁹⁷ strain, the infection rates reported here (50-80%) are higher than some previous reports on CHIKV vector competence, which range between 10-30% [274,275]. However, another comprehensive study that investigated the vector competence of ten different *Ae. aegypti* populations for CHIKV reported high infection and dissemination rates between 90-100% [31]. Transmission rates of CHIKV by *Ae. aegypti* mostly range between 40-60% with exceptions to some virus-vector strain combinations that report low transmission rates [31,32,275]. These findings confirm that vector competence is highly variable and dependent on the specific combination of viral strain and mosquito population.

In order for an arbovirus to accumulate in the saliva it has to pass the midgut infection- and escape barriers and the salivary gland infection- and escape barriers [142]. A midgut barrier for CHIKV has previously been reported in *Ae. aegypti* [276] and *Ae. albopictus* [277]. Here, we report infection rates of up to 80% for CHIKV, suggesting that the midgut does not form a strong barrier against CHIKV infection in our *Ae. aegypti* colony. However, CHIKV transmission rates of maximum 20% after an infectious blood meal or intrathoracic injections indicate the presence of a mosquito salivary gland barrier. The presence of a salivary gland barrier is indicated by a low percentage of mosquitoes with a salivary gland infection when a larger percentage of mosquitoes reaches a disseminated infection. We determined transmission rates of CHIKV of maximum 21% after oral exposure while an 81% infection rate was observed, suggesting that the mosquito colony used has a strong salivary gland barrier to the CHIKV³⁷⁹⁹⁷ strain. Additionally, virus-positive saliva samples had a low viral titer for both ZIKV and CHIKV. Although we did not quantify the amount of saliva that the mosquitoes excreted, this suggests that there is indeed a salivary gland barrier for both viruses that prevents the accumulation of high viral titers in the saliva. We determined that the transmission rates after intrathoracic injections were similar to the transmission rates after an infectious blood meal at 7 dpi. Potentially CHIKV and ZIKV may require longer incubation periods between 7 and 14 days to successfully infect the salivary glands and reach the saliva. However, a salivary gland escape barrier has earlier been described for the CHIKV³⁷⁹⁹⁷ strain, where only 60% of the infected mosquitoes had virus-positive saliva at 7 dpi [278].

It is surprising that ZIKV spread so rapidly from its original, natural range to territories in the Pacific (Micronesia, Easter Island, French Polynesia), and subsequently to South- and North-America between 2007 and 2015 [248]. Several hypotheses have been proposed for the rapid dissemination of ZIKV throughout the Americas. First, genetic changes of ZIKV strains could result in adaptations that make the strain that

circulates in the Americas more virulent. Evidence for this comes from a comparative genomic study that indicated 15 amino acid substitutions in epidemic strains compared to pre-epidemic strains [279]. However, an African ZIKV isolate was shown to outcompete the American strain *in vitro* and *in vivo*, suggesting that transmission of the epidemic strains is not enhanced [273]. Secondly, the presence of a large naïve and susceptible human population in combination with high densities of anthropophilic mosquitoes might have accelerated the spread of ZIKV. And thirdly, co-infection of mosquitoes with ZIKV and other arboviruses such as CHIKV and DENV or both may have a positive effect on the vector competence of mosquitoes, resulting in increased transmission rates and faster spread of the viruses. We now show for the first time that mosquito co-infections of ZIKV and CHIKV can indeed occur, without altering the vector competence of *Ae. aegypti* for either virus. Importantly, our results suggest that patients reported with ZIKV and CHIKV co-infections could have been infected with both viruses via the bite of a single *Ae. aegypti* mosquito. However, the proportion of co-infected mosquitoes in a population of *Ae. aegypti* mosquitoes is expected to be extremely small. We therefore consider it unlikely that co-infections of multiple arboviruses contribute to the rapid dissemination of ZIKV across the Americas.

In summary, this study shows that *Ae. aegypti* can transmit ZIKV and CHIKV simultaneously by a single mosquito bite. Additionally, we show that *Ae. aegypti* has a higher vector competence for ZIKV than for CHIKV and that co-infections do not affect the vector competence. By comparison of infections via the blood meal with intrathoracic injections we show that *Ae. aegypti* has a strong salivary gland barrier for CHIKV and a minor salivary gland barrier for ZIKV. Finally, studies with cell lines show that ZIKV virion production is decreased in mammalian, but not mosquito cells, due to induction of cytopathicity by CHIKV. The outcomes of this research provide novel insights into the effects of co-infections on the transmission of arboviruses by mosquitoes.

Materials and Methods

Cell culture

African green monkey kidney Vero E6 (ATCC CRL-1586) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml; Sigma-Aldrich), and streptomycin (100 µg/ml; Sigma-Aldrich) (P/S). Vero cells were cultured as monolayers in T25 cell culture flasks (Greiner Bio-One) at 37 °C with 5% CO₂, and split every 3-4 days. Prior to infections, Vero cells were seeded in DMEM containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (DMEM-HEPES; Gibco) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml), hereafter named DMEM-supplemented. *Ae. albopictus* C6/36 cells (ATCC CRL-1660) were cultured in Leibovitz L-15 medium (Gibco) supplemented with 10% FBS, 2% tryptose phosphate broth (Gibco), and 1% nonessential amino acids (Gibco), hereafter named Leibovitz-complete. *Ae. aegypti* Aag2 cells were cultured in Schneider's *Drosophila* medium (Lonza) supplemented with 10 % FBS, hereafter named Schneider's-complete. Both C6/36 and Aag2 cells were cultured as monolayers in T25 flasks at 28 °C, and split every 3-4 days.

Virus stocks

All proceedings involving infectious virus were executed in the biosafety level 3 laboratory at Wageningen University & Research. An infectious clone derived chikungunya virus 37997 strain (CHIKV³⁷⁹⁹⁷) was used in all studies. To prepare the chikungunya virus 37997 infectious clone (pCHIK_{IC}-37997), the 37997 structural cassette was produced synthetically with AscI/EcoRI overhangs (Baseclear) and cloned into the previously described CHIKV 37997 replicon CHIKrep-FlucEGFP to replace the Fluc-EGFP fusion gene [72]. CHIKV 37997 RNA was *in vitro* transcribed from 5 µg PacI (New England Biolabs (NEB)) linearized pCHIKIC-37997 using SP6 RNA polymerase (NEB) following the manufacturer's protocol. Vero cells were seeded

one day prior to infection in 6-well cell culture plates (Greiner Bio-One) until a confluency of ~80% was reached. The culture medium was replaced for Opti-Mem (Gibco) and 3 µl of *in vitro* transcribed RNA was transfected into Vero cells using 2.5 µl Lipofectamine 2000 (Invitrogen). Four days post transfection the cell culture medium was harvested, centrifuged and stored at -80 °C until further use (P0). In total, 500 µl P0 was used to inoculate a T75 flask (Greiner Bio-One) of C6/36 cells. Four days post infection (dpi) the cell culture medium was harvested (P1), centrifuged and the supernatant was stored in aliquots at -80 °C. Virus titers were determined by EPDA on Vero cells. Zika virus Suriname strain 011V-01621 (ZIKV^{SUR} GenBank accession number, KU937936) [59], was obtained through the European Virus Archive Goes Global catalogue (www.european-virus-archive.com/virus/zika-virus-strain-suriname-2016) as a P3 stock grown on Vero cells. ZIKV P4 was generated by inoculating a pre-seeded T75 flask of Vero cells with 250 µl ZIKV^{SUR} P3. The supernatant was harvested (P4) at 2 dpi, centrifuged to remove cell debris, and the supernatant was stored in aliquots at -80 °C. Virus titers were determined by EPDA on Vero cells.

Virus titrations

Vero cell suspensions were retrieved by detaching Vero cells from a T25 flask with 1 ml of Trypsin-EDTA (Gibco), after which 4 ml of DMEM-supplemented was added. Virus stocks were thawed, vortexed and serial dilutions were made in DMEM-supplemented. Vero cell suspensions were diluted 1:4 with DMEM-supplemented and added to the virus dilutions in a 1:1 ratio. 10 µl of the inoculated dilutions was plated in 6-fold in micro-titer plates (Nunc). EPDAs of samples infected with one virus were scored at 3 dpi based on virus-induced CPE. EPDAs of co-infected samples were fixed with 4% paraformaldehyde and scored by immunofluorescence assay (IFA) at 3 dpi.

Virus growth curves

Cell monolayers were seeded in 6-well plates and infected on the same day for C6/36 and Aag2 cells, or the next day for Vero cells. The cell culture fluid was removed and infections were performed at an MOI of 0.1 ($5.7 \times 10^4 - 2.2 \times 10^5$ TCID₅₀) in standard culture media in a total volume of 1 ml. After 1 h the inoculum was removed and the monolayers were washed twice with 1 ml of Phosphate Buffered Saline (PBS), before addition of 2 ml fresh culture medium. C6/36 and Aag2 cells were maintained at 28 °C and Vero cells were maintained at 37 °C and 5% CO₂. Samples of 100 µl were taken at 0, 24, 48, 72 and 96 hpi and stored at -80 °C until titration by EPDA on Vero cells.

Dual-colour immunofluorescence assays

Cells were fixed with 4% paraformaldehyde/PBS for 1-3 h. Monolayers were washed 3x with PBS, permeabilized by 10 min incubation in 0.1% SDS in PBS, and washed 3x with PBS. Monolayers were stained with α-CHIKV-E2 (Rabbit Polyclonal; 1:5000; [280]) and pan-flavivirus α-E (4G2; Mouse monoclonal; 1:50 [281]) in a 5% FBS solution dissolved in PBS for 1 h at room temperature (RT). Cells were washed 3x with PBS and stained with goat-α-mouse-Alexa Fluor 568 (1:2000; Invitrogen) and goat-α-rabbit-Alexa Fluor 488 (1:2000; Invitrogen) for 1 h at 37 °C. Monolayers were washed 3X with PBS and visualized using an Axio Observer Z1m inverted microscope (Zeiss) in combination with an X-Cite 120 series lamp.

Cell viability assay

Vero cell monolayers were seeded in 96-well plates one day prior to infection and infected at an MOI of 0.1. At the indicated time-point, the medium was removed and replaced with 100 µl of passive lysis buffer (Promega). Cells were lysed by 10 min incubation at RT and lysates were stored at -20 °C until further use. Twenty-five µl of reconstituted CellTiter-Glo Reagent (Promega) was added to 25 µl cell lysate and incubated at RT in the dark for 10 min before measuring the luminescence using a FLUOstar OPTIMA microplate reader (BMG Labtech). Cell viability was calculated by normalizing the average luminescence of the sample to the averaged luminescence of the mock.

Mosquito rearing

In all experiments female *Ae. aegypti* mosquitoes (Rockefeller strain, obtained from Bayer AG) were used. Larvae and adults were maintained at 27±1 °C with 12:12 light:dark cycle

and 70% relative humidity. Adult mosquitoes were provided with 6% ad libitum glucose solution. Human blood (Sanquin) was provided through Parafilm using the Hemotek PS5 feeder (Discovery Workshops). Female mosquitoes were kept together with males for 3 to 6 days in Bugdorm-1 insect rearing cages (30 x 30 x 30 cm, Bugdorm), before females were transferred to buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey) and transported to the Biological Safety Level 3 facility for virus infection assays.

Infectious blood meal

One day before blood feeding, the glucose solution was replaced by water in order to stimulate blood feeding of *Ae. aegypti* females. Virus solutions were made by diluting the virus to the indicated titer (Tables 4.1 & 4.2) in DMEM-supplemented for ZIKV, and Leibovitz-complete for CHIKV. Since CHIKV was grown on C6/36 cells and ZIKV on Vero cells, we compensated for differences in cell culture media by mixing 250 µl of virus solution with 250 µl conditioned media from cultured C6/36 cells for ZIKV and 250 µl conditioned media from cultured Vero cells for CHIKV, after which 500 µl human blood was added. The infectious blood meal was offered through Parafilm using the Hemotek PS5 feeder. Mosquitoes were allowed to feed for 1 h ad libitum in light conditions, at 24 °C and 70% relative humidity. Mosquitoes were anesthetized with 100% CO₂, placed on a CO₂ pad and fully engorged females were selected. Immediately after selection, a selection of mosquitoes was frozen at -80 °C to determine the amount of virus ingested by the mosquitoes. Exposed mosquitoes were maintained at 28 °C. The glucose solution was refreshed every 2-3 days until 14 dpi.

Intrathoracic injections

Virus dilutions of 4×10^7 TCID₅₀/ml were prepared by diluting the ZIKV and CHIKV virus stocks 1:1 with conditioned media taken from cultured C6/36 and Vero cells, respectively, to compensate for differences in growth media. *Ae. aegypti* mosquitoes were anesthetized with 100% CO₂ and placed on a CO₂ pad. Female mosquitoes were selected and injected with 69 nl of the prepared virus stock using a Drummond Nanoject II Auto-Nanoliter Injector (Drummond Scientific). Infected mosquitoes were maintained at 28 °C. The glucose solution was refreshed every 2-3 days until 7 dpi.

Salivation assay

Fourteen days post blood meal or 7 days post injection mosquitoes were anesthetized with 100% CO₂, and placed on a CO₂ pad. Mosquitoes that died within the 7 or 14 days incubation period were discarded. Mosquitoes were immobilized by removing their legs and wings with forceps. The proboscis of each mosquito was inserted into a 200 µl yellow pipet tip (Greiner Bio-One) containing 5 µl of a 1:1 solution of 50% glucose solution and FBS, for a minimum of 45 min. After salivation, the mosquito bodies were added to 1.5 ml Safe-Seal micro tubes (Sarstedt) containing 0.5 mm zirconium beads (Next Advance). Each saliva sample was added to a 1.5 ml micro tube (Sarstedt) containing 55 µl DMEM-supplemented with additional Fungizone (50 µg/ml; Invitrogen), and gentamycin (50 µg/ml; Life technologies), hereafter named DMEM-complete. Mosquito bodies and saliva samples were stored at -80 °C until further processing.

Infectivity assays

Mosquito bodies were taken from the -80 °C freezer and immediately homogenized for 2 min at max speed in a Bullet Blender Storm (Next Advance). Homogenized bodies were centrifuged briefly and resuspended in 100 µl DMEM-complete medium. The homogenate was blended again for 2 min at max speed using the Bullet Blender and centrifuged for 1 min at 14,500 rpm. Mosquito saliva samples were thawed at RT. Of each body homogenate or saliva sample, 30 µl was used to inoculate a Vero cell monolayer in a 96-well plate. After 2-3 h the inoculum was removed and replaced with 100 µl DMEM-complete. For mosquitoes infected with a single virus, the wells were scored for virus induced CPE at 3 dpi. This method was validated by comparing results based on CPE with IFA for the first replicate of mosquitoes, which gave identical results. For mosquitoes that were infected with both viruses, the supernatant was removed and monolayers were fixed with 4% paraformaldehyde in PBS at 3 dpi after which the wells were scored by dual-colour IFA. Bodies and saliva samples of a selection of mosquitoes with a fully disseminated infection of ZIKV, CHIKV, or both, were titrated by EPDA.

Statistics

Kruskal-Wallis tests were used to test for differences between engorged viral titers, and final titers of mosquito bodies and saliva samples. If the outcome of a Kruskal-Wallis test was significant, differences among groups were further tested with Dunn's tests and corrected with the Bonferroni correction for multiple comparisons. Infection and transmission rates were calculated, respectively, by dividing the number of female mosquitoes with infected bodies or with infected saliva by the total number of female mosquitoes in the respective treatment. Mosquitoes with infectious saliva, but uninfected body (<1%), were excluded from the analysis. Differences in infection and transmission rates were tested with Chi-squared tests. Multiple comparisons were corrected with the Bonferroni correction. All statistical analyses were done with the statistical software package R [282]. Power analysis was performed to confirm adequate sample sizes for the vector competence studies using G*Power software.

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Chapter V

Conserved motifs in the hypervariable domain of chikungunya virus nsP3 required for transmission by *Aedes aegypti* mosquitoes

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Abstract

Chikungunya virus (CHIKV) is a re-emerging arthropod-borne (arbo)virus that causes chikungunya fever in humans and is predominantly transmitted by *Aedes aegypti* mosquitoes. The CHIKV replication machinery consists of four non-structural proteins (nsP1-4) that additionally require the presence of a number of host proteins for replication of the viral RNA. nsP3 is essential for CHIKV replication and has a conserved macro, central and C-terminal hypervariable domain (HVD). The HVD is intrinsically disordered and interacts with various host proteins via conserved short peptide motifs: a proline-rich (P-rich) motif that has affinity for SH3-domain containing proteins and duplicate FGDF motifs with affinity for G3BP and its mosquito homologue Rasputin. The importance of these motifs for infection of mammalian cells has previously been implicated. However, their role during CHIKV infection of mosquito cells and transmission by mosquitoes remains unclear. Here, we show that in-frame deletion of the P-rich motif is lethal for CHIKV replication in both mosquito and mammalian cells. However, while mutagenesis of the P-rich motif negatively affects replication both in mammalian and mosquito cells, it did not compromise the infection and transmission of CHIKV by *Ae. aegypti* mosquitoes. Mutagenesis of both FGDF motifs together completely inactivated CHIKV replication in both mammalian and mosquito cells. Importantly, mutation of a single FGDF motif attenuated CHIKV replication in mammalian cells, while replication in mosquito cells was similar to wild type. Surprisingly, CHIKV mutants containing only a single FGDF motif were efficiently transmitted by *Ae. aegypti*. In conclusion, the P-rich motif in CHIKV nsP3 is dispensable for transmission by mosquitoes. A single FGDF motif is sufficient for infection and dissemination in mosquitoes, but duplicate FGDF motifs are required for the efficient infection from the mosquito saliva to a vertebrate host. These results contribute to understanding the dynamics of the alphavirus transmission cycle and may help the development of arboviral intervention strategies.

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Introduction

Alphaviruses (family *Togaviridae*), such as chikungunya virus (CHIKV) and Mayaro virus, are (re-)emerging arthropod-borne (arbo)viruses that impose a global threat on human health [283]. Mayaro virus recently emerged in South America, where a large outbreak was reported in Brazil [283,284]. CHIKV is responsible for several large outbreaks in the Americas [37] and over 150,000 cases of infection were reported in the Americas in 2016 alone [26]. CHIKV disease is characterized by acute fever combined with severe arthralgia, myalgia or rash, and symptoms can last from weeks to several months [37]. Transmission of CHIKV occurs predominantly by *Aedes aegypti* mosquitoes, although some CHIKV strains have incorporated an amino acid substitution in the envelope protein (A226V) that allows transmission by *Ae. albopictus* [25].

Alphaviruses have a single-stranded positive-sense RNA genome that contains two open reading frames, which encode the non-structural proteins (nsP)1-4 and the structural proteins, respectively. nsP4 is the viral RNA-dependent RNA polymerase that together with nsP1-3 forms the viral replication complex. While the roles of nsP1 and nsP2 in viral replication have been extensively documented and reviewed [67,285,286], the exact functions of nsP3 are less clear [71,287]. During infection, nsP3 localizes partially to viral replication complexes [288,289], in accordance with the necessity of nsP3 for virus replication, but nsP3 also localizes towards cytoplasmic foci [122,128,290,291]. Additionally, nsP3 interacts with several host proteins in mammalian and mosquito cells [126,128]. Unravelling the significance and mechanisms of the interactions between nsP3 and these host proteins in mammalian cells has been the focus of several recent studies [123,125,126,292–295], while the role of these interactions during infection of mosquitoes remains to be assessed.

NsP3 consists of three domains: an N-terminal, conserved macro domain, which possesses phosphatase and RNA-binding activity [296], a conserved zinc-binding central domain [297], and a highly phosphorylated [290,298,299], intrinsically disordered [295], hypervariable domain (HVD) at the C-terminus [67]. Despite the lack of conservation in the HVD between the nsP3 of alphaviruses [300], it contains several conserved motifs, of which the Proline-rich (P-rich) PxxPPR or PxxPR, and the duplicate FGDF motifs have been studied most extensively (reviewed in [71,287]). In mammalian cells, the P-rich motifs of Sindbis virus (SINV), Semliki Forest virus (SFV) and CHIKV nsP3 interact with the SH3 domain of the membrane modulating proteins amphiphysin-1/2, and this interaction has been shown to be important for efficient virus replication [292,293]. CHIKV nsP3 has two FGDF motifs that can bind to the nuclear transport factor (NTF) 2-like domain of the stress granule components G3BP1 and G3BP2 in mammalian cells [125,126,301]. This interaction inhibits the formation of cellular stress granules that would otherwise stall host and viral translation, and as a consequence may facilitate virus replication [122–124]. In mammalian cells, mutations in the alphavirus nsP3 HVD that disrupt both of the FGDF motifs result in complete inactivation of CHIKV and also attenuate SFV and SINV [123,125–127]. Furthermore, deletion of a single FGDF motif in CHIKV or SFV nsP3 decreases the affinity for G3BP and attenuates the virus, suggesting that alphaviruses require duplicate FGDF motifs for their successful replication in mammalian cells [125,126].

While the importance of the conserved motifs in the nsP3 HVD for alphavirus replication in mammalian cells has been well-established, little is known about their role during alphavirus transmission by the mosquito vector [302]. Recently, nsP3 and its mosquito interaction partner Rasputin (Rin), the mosquito homologue of G3BP, have been identified as important determinants for alphavirus transmission [119,129].

Chimeric CHIKV equipped with the nsP3 from the *Anopheles*-transmitted alphavirus o'nyong nyong virus (ONNV) showed an increase in infection rate in the CHIKV-refractory vector *An. gambiae* from 0 to 64% [119]. Interestingly, CHIKV chimeras that contained just the C-terminal region of ONNV nsP3, including the HVD, already reached an infection rate of 9-18% [119], suggesting that the nsP3 HVD is an important determinant of mosquito vector specificity. Furthermore, CHIKV nsP3 was shown to co-localize with Rin [129], and Rin co-precipitated with SINV nsP3 [128], indicative of a direct interaction between Rin and nsP3. Interestingly, mutating both FGDF motifs in CHIKV nsP3 together results in complete loss of co-localization of transiently expressed nsP3 and Rin [129], similar to the interaction with G3BP in mammalian cells. However, co-localisation was maintained when only one of the FDGF motifs was mutated [129]. Importantly, knock-down of Rin severely decreased the infection and transmission rates as well as the viral titers of CHIKV in the heads of *Ae. albopictus* after an infectious blood meal [129]. Thus, the conserved motifs in the nsP3 HVD may play a crucial role in infection of the mosquito vector, potentially through their interaction with mosquito host proteins, and ultimately enable the virus to establish a disseminated infection.

Here, we investigated the role of the P-rich and FGDF motifs in the HVD of CHIKV nsP3 during virus replication in mosquito cells and infection of and transmission by the natural CHIKV vector *Ae. aegypti*. Infectious cDNA clones were used to generate CHIKV variants with mutations in either the P-rich or FGDF motif(s). The mutant viruses were assessed for replication kinetics in mammalian (Vero) and *Ae. aegypti* (Aag2) cells. We investigated the infection and transmission of the CHIKV mutants in *Ae. aegypti* mosquitoes through infectious blood meal experiments. These experiments elucidate whether the evolutionary basis for conservation of the P-rich and duplicate FGDF motifs in alphavirus nsP3 lies solely in infection of the mammalian host, or whether these motifs are also required for alphavirus transmission by the mosquito vector.

Results

The CHIKV nsP3 P-rich motif is required for efficient virus replication in mammalian and mosquito cells

For SINV, SFV and CHIKV it has been shown that deletion or mutation of the conserved P-rich motif (Fig. 5.1B) results in decreased replication in mammalian cells [122,292]. To investigate whether the P-rich motif is similarly indispensable for alphavirus replication in mosquito cells, luciferase assays were performed on cells transfected with *in vitro* transcribed RNA of a CHIKV replicon (CHIK_{rep}) with an in-frame deletion of the P-rich motif (Δ PVA) (Fig. 5.1A, Fig. 5.2A). The mutant replicon CHIK_{rep} ^{Δ PVA} was unable to replicate in either mammalian Vero or Aag2 *Ae. aegypti* mosquito cells, whereas wild type CHIK_{rep} was able to replicate in both cell types. We also transfected cells with a CHIKV infectious clone containing the same deletion of the P-rich motif (CHIK_{IC} ^{Δ PVA}), but again we were unable to produce infectious virus in either mammalian and mosquito cells. Together, these results clearly indicate that the P-rich motif is essential for CHIKV replication in both mammalian and mosquito cells. To investigate whether the entire, intact P-rich motif is required for virus production in mammalian and mosquito cells, amino acid substitutions were made in the CHIKV infectious clone (CHIK_{IC}) to produce CHIK_{IC}^{P398A} and CHIK_{IC}^{PPR401AAA} (Fig. 5.1A). These specific mutations are designed to disrupt the residues in the P-rich motif that are conserved among old-world alphaviruses (PxxPPR; Fig. 5.1B-top red boxes). Viral growth curves were determined in duplicate using a multiplicity of infection (MOI) of 0.01 and viral titers were determined by end-point dilution assay (EPDA) on Vero cells.

The results show that both mutants displayed significantly delayed growth kinetics compared to the wild type virus in both Vero (Fig. 5.2B) and Aag2 cells (Fig. 5.2C). Even when virus infections were performed at a higher MOI of 5 this attenuating effect was observed at 24 hpi, although both CHIK_{IC}^{P398A} and CHIK_{IC}^{PPR401AAA} still reached a titer of $\sim 1.0 \times 10^6$ tissue culture infectious dose 50% (TCID₅₀)/ml (Fig. 5.2D). Together, these results indicate that an intact P-rich motif in CHIKV nsP3 is important, but not essential, for virus replication in mammalian and mosquito cells.

Fig. 5.1 The hypervariable domain of alphavirus nsP3 contains conserved P-rich and FGDF motifs. (A) Schematic overview of the viruses used in these experiments. Shown are DNA plasmids used as template for the *in vitro* transcription of chikungunya virus infectious clone (IC) or replicon (rep) RNA. Additionally, the location of deletions and amino acid substitutions used in mutant IC and rep constructs is displayed. (B) Alignment of alphavirus nsP3 amino acid sequences indicates conserved proline rich and FGDF motifs in the hypervariable domain (HVD). Top: zoom-in on the proline rich region of the nsP3 HVD. Red boxes indicate the PxxPPR motif, while blue boxes indicate the PxxPR motif. Bottom: Zoom-in on the FGDF motif-containing region of the nsP3 HVD. Red boxes indicate FGDF motifs. CHIKV chikungunya virus; BFV Barmah forest virus; SFV Semliki Forest virus; WHAV Whataroa virus; EILV Eilat virus; RRV Ross River virus; MAYV Mayaro virus; BeBV Bebaru virus; TALV Tai Forest alphavirus; EEEV Eastern equine encephalitis virus; VEEV Venezuelan equine encephalitis virus; WEEV Western equine encephalitis virus.

Fig. 5.2 The P-rich motif is important but not essential for chikungunya virus replication in mammalian and mosquito cells. (A) Vero and Aag2 cells were transfected with *in vitro* transcribed RNA of CHIK_{rep} or CHIK_{rep}^{ΔPVA} and the relative luciferase expression was quantified at 24 hpt. Bars indicate the mean relative light units (RLU) ± standard error of the mean from three independent experiments. (B/C) Vero (B) and Aag2 (C) cells were infected in duplicate with CHIK_{IC}, CHIK_{IC}^{P398A} or CHIK_{IC}^{PPR401AAA} at a multiplicity of infection (MOI) of 0.01. TCID₅₀/ml was determined by end-point dilution assay (EPDA) on Vero cells at the indicated time-points. (D) Aag2 cells were infected in duplicate with CHIK_{IC}, CHIK_{IC}^{P398A} or CHIK_{IC}^{PPR401AAA} at an MOI of 5 and the TCID₅₀/ml was determined by EPDA on Vero cells at the indicated time-points. Asterisks indicate significant differences compared to the wild type virus by one-way ANOVA with Tukey's post-hoc test on Log₁₀ transformed data at each time-point (α=0.05). The dotted line in panels B-D indicates the EPDA detection limit.

The P-rich motif is not required for the transmission of chikungunya virus by *Ae. aegypti* mosquitoes

The P-rich motif is conserved in most if not all mosquito transmitted alphaviruses and also in the insect-specific alphaviruses Taï Forest Alphavirus (TALV) and Eilat virus (EILV) (Fig 5.1B). As TALV and EILV are proposedly unable to replicate in a vertebrate host [303,304], conservation of a P-rich motif for these viruses suggests its importance for alphavirus infection in the invertebrate vector. To investigate whether the P-rich motif of CHIKV nsP3 is involved in virus transmission by mosquitoes, female *Ae. aegypti* were offered an infectious blood meal containing either CHIK_{IC}, CHIK_{IC}^{P398A} or CHIK_{IC}^{PPR401AAA} (Fig. 5.3A). After 14 days, the infection and transmission rates were determined by infectivity assay on Vero cells (Fig. 5.3B). For all three viruses, similar infection (88-96%; $P > 0.37$) and transmission (6-7.7%; $P = 1.00$) rates were obtained, indicating that the P-rich motif is not required for virus transmission by mosquitoes.

To investigate whether putative differences could be observed earlier after infection of the mosquito vector, we also determined the infection and transmission rates after 7 days (Fig. 5.3C). Again, infection (72-76%; $P > 0.61$) and transmission (18-23%; $P > 0.47$) rates were obtained that were not significantly different when compared to the wild type virus. In order to determine whether the mutations in CHIK_{IC}^{P398A} and CHIK_{IC}^{PPR401AAA} were preserved during infection of the mosquito, the nsP3 sequences were amplified by RT-PCR for a selection of mosquitoes with CHIKV-positive saliva. Presence of the expected mutations in nsP3 was confirmed, indicating that the mutant viruses did not revert to wild type during replication in the mosquito vector (Fig. S5.1). To assess whether the titers in the mosquito bodies would perhaps be decreased due to the mutations in the P-rich motif, CHIKV titers were determined in the bodies of mosquitoes with virus-positive saliva (Fig. 5.3D). However, no difference in viral titer between CHIK_{IC}, CHIK_{IC}^{P398A} and CHIK_{IC}^{PPR401AAA} ($P > 0.99$) was observed. Together, these results indicate that the CHIKV nsP3 P-rich motif is not required for the transmission by *Ae. aegypti* mosquitoes.

< Fig. 5.3 (Previous page) The P-rich motif is not required for transmission of chikungunya virus by *Aedes aegypti* mosquitoes. (A) Schematic experimental set-up. Female *Ae. aegypti* mosquitoes were infected through an infectious blood meal containing 1.0×10^7 TCID₅₀/ml of CHIK_{IC}, CHIK_{IC}^{P398A} or CHIK_{IC}^{PPR401AAA}. At 14 days post infection (dpi) (B) or 7 dpi (C) the infection and transmission rates were determined by infectivity assay on Vero cells. Bars represent cumulative numbers from three (B) or two (C) independent experiments. n = total number of mosquitoes used per treatment. Statistics were performed by Fisher's exact test ($\alpha=0.05$). (D) TCID₅₀/ml of CHIKV in the bodies of *Ae. aegypti* mosquitoes with CHIKV-positive saliva at 7 dpi were determined by end-point dilution assay on Vero cells. Statistics were performed by one-way ANOVA with Tukey's post-hoc test on Log₁₀ transformed data ($\alpha=0.05$). ns, not significant.

Chikungunya virus nsP3 requires duplicate FGDF motifs for replication in mammalian cells and a single FGDF motif in mosquito cells

Research has indicated that mutating both FGDF motifs together significantly attenuates SFV and renders CHIKV unable to replicate in mammalian cells [125,126]. Here, we investigated whether mutations in one (nsP3-FG_N, nsP3-FG_C) or both (nsP3-FG_{NC}) FGDF motifs would interfere with the replication of CHIKV in mosquito cells. These amino acid mutations were previously shown to disrupt the interaction of nsP3 with G3BP [125,305], and the co-localization of nsP3 with Rin [129]. We tested the effect of mutating the FGDF motifs on CHIKV replication using an infectious clone that contains an in-frame insertion of the mCherry reporter gene in nsP3 (CHIK_{IC}nsP3mC; Fig. 5.1A). *In vitro* transcribed, capped RNA from CHIK_{IC}nsP3mC, CHIK_{IC}nsP3mC-FG_N, CHIK_{IC}nsP3mC-FG_C or CHIK_{IC}nsP3mC-FG_{NC} was used to transfect Vero and C6/36 mosquito cells. At 36 hours post-transfection (hpt), the cells were fixed and analysed for mCherry fluorescence as a marker for virus replication (Fig. 5.4A). Red fluorescence was observed in cells transfected with RNA from the infectious clones with mutations in a single FGDF motif (CHIK_{IC}nsP3mC-FG_N and CHIK_{IC}nsP3mC-FG_C) and wild type CHIKV. However, mammalian and mosquito cells transfected with RNA from CHIK_{IC}nsP3mC-FG_{NC} did not show any red fluorescence, indicating that a minimum of one functional FGDF motif is essential for CHIKV replication in both mammalian and mosquito cells.

To assess whether the RNA of CHIK_{IC}nsP3mC-FG_{NC} was of sufficient quality, we co-transfected replication-competent RNA from CHIK_{IC}, which does not express mCherry. In this experiment, CHIK_{IC} RNA is able to complement the CHIK_{IC}nsP3mC-FG_{NC} RNA, leading to mCherry expression. Indeed, upon co-transfection red fluorescence could be observed in some cells (Fig. 5.4B), indicating that the quality of CHIK_{IC}nsP3mC-FG_{NC} RNA was fine but that the RNA was replication-incompetent due to the mutation of both FGDF motifs. To confirm this result using a more sensitive assay, Vero and C6/36 cells were transfected with CHIKV replicons expressing firefly luciferase CHIK_{rep} or CHIK_{rep}-FG_{NC} (Fig. 5.1A) and firefly luciferase activity was measured at 24 hpt (Fig. 5.4C). In both Vero and C6/36 cells, only the wild type replicon CHIK_{rep} produced above-background luciferase levels, indicating that indeed mutation of both FGDF motifs together results in complete inactivation of CHIKV in mammalian and mosquito cells. To assess the effect of single FGDF mutations on virus production in mammalian and mosquito cells, we performed viral growth curves on Vero and Aag2 cells at an MOI of 0.01 using CHIK_{IC}, CHIK_{IC}-FG_N and CHIK_{IC}-FG_C (Fig. 5.4D/E). These viruses do not contain the mCherry reporter gene (Fig. 5.1A). It is important to note that we determined the viral titers for the inoculum and the samples for the mammalian growth curves by EPDA on Vero cells. For the growth curves on mosquito cells, the titrations were performed by EPDA on Aag2 cells. As expected, CHIK_{IC}-FG_N and CHIK_{IC}-FG_C were severely attenuated in Vero cells (Fig. 5.4D). However, the result was very different

in Aag2 cells; CHIK_{IC}-FG_N and CHIK_{IC}-FG_C displayed remarkably similar growth rates as compared to CHIK_{IC} (Fig. 5.4E). In summary, duplicate FGDF motifs are very important for CHIKV replication in mammalian cells, while a single FGDF motif is sufficient, but required, for efficient CHIKV replication in mosquito cells.

Fig. 5.4 At least one FGDF motif is required for chikungunya virus replication in mammalian and mosquito cells.

(A) Vero and C6/36 cells were transfected with *in vitro* transcribed RNA of CHIK_{IC}nsP3mC, CHIK_{IC}nsP3mC-FG_N, CHIK_{IC}nsP3mC-FG_C, or CHIK_{IC}nsP3mC-FG_{NC}. Cells were fixed at 36 hours post transfection (hpt), stained with Hoechst, and fluorescence was observed by fluorescence microscopy. (B) Vero cells were transfected with *in vitro* transcribed RNA of CHIK_{IC}nsP3mC or CHIK_{IC}nsP3-FG_{NC} either individually or co-transfected with *in vitro* transcribed RNA of CHIK_{IC}. Cells were fixed at 36 hpt, stained with Hoechst and fluorescence was observed by fluorescence microscopy. (C) Vero and C6/36 cells were transfected with *in vitro* transcribed RNA of CHIK_{rep} or CHIK_{rep}-FG_{NC} and the relative luciferase expression was quantified at 24 hpt. Bars indicate the mean relative light units (RLU) $\pm$ standard error of the mean, normalized to the wild type replicon from at least three independent experiments. (D) Growth curves of CHIKV_{IC}, CHIKV_{IC}-FG_N and CHIKV_{IC}-FG_C on Vero cells infected in duplicate with a multiplicity of infection (MOI) of 0.01 based on end-point dilution assay (EPDA) on Vero cells. At the indicated time-points the TCID₅₀/ml was determined by EPDA on Vero cells. (E) Growth curves of CHIKV_{IC}, CHIKV_{IC}-FG_N and CHIKV_{IC}-FG_C on Aag2 cells infected in duplicate with an MOI of 0.01 based on infectivity on Aag2 cells. At the indicated time-points the TCID₅₀/ml was determined by EPDA on Aag2 cells. Statistics were performed by one-way ANOVA with Tukey's post-hoc test on Log₁₀ transformed data at each time-point ($\alpha=0.05$). Asterisks indicate significance compared to the wild type virus. The dotted line in panels D-E indicates the EPDA detection limit.

Requirement of FGDF motifs for chikungunya virus transmission by *Aedes aegypti*

While duplicate FGDF motifs do not appear to be required for virus replication in mosquito cells (Fig. 5.4E), they might be important to sustain transmission of CHIKV by mosquitoes. Therefore, we infected *Ae. aegypti* mosquitoes via an infectious blood meal with 2.8×10^5 TCID₅₀/ml CHIK_{IC}, CHIK_{IC}-FG_N or CHIK_{IC}-FG_C and determined the infection and transmission rates at 7 dpi by infectivity assay on Vero and Aag2 cells (Fig. 5.5A). When scored on Vero cells the mosquito infection rate of CHIK_{IC}-FG_C (54.8%; $P < 0.001$) was significantly lower as compared to CHIK_{IC} (73.7%), while the infection rate of CHIK_{IC}-FG_N (72.6%; $P = 0.89$) was similar to wild type (Fig. 5.5B). Next, the saliva of infected mosquitoes was tested for the presence of virus. This showed that the transmission rates of both CHIK_{IC}-FG_N (7.4%; $P < 0.001$) and CHIK_{IC}-FG_C (9.6%; $P < 0.001$) were significantly lower as compared to CHIK_{IC} (25.7%), indicating that duplicate FGDF motifs are important for CHIKV transmission by *Ae. aegypti* to the vertebrate host.

However, given that CHIK_{IC}-FG_N and CHIK_{IC}-FG_C were severely attenuated on Vero cells (Fig. 5.4D), we also determined the infection and transmission rates by infectivity assay on Aag2 cells, using exactly the same mosquito bodies and saliva samples (Fig. 5.5C). The infection rate did not change much, and CHIK_{IC}-FG_C (57%; $P < 0.01$) still reached a lower infection rate than CHIK_{IC} (74.7%) or CHIK_{IC}-FG_N (78.5%; $P = 0.49$). Interestingly, however, when scored on Aag2 cells the transmission rates were very similar for CHIK_{IC} (19.3%), CHIK_{IC}-FG_N (16.3%; $P = 0.54$) and CHIK_{IC}-FG_C (13.7%; $P = 0.21$). To directly compare the infectivity of the mosquito bodies and saliva samples between Vero and Aag2 cells, the ratio between the number of positive samples on Vero and Aag2 cells was determined for each replicate and normalized to the ratio for mosquitoes inoculated with wild type CHIK_{IC} (Fig. 5.5D). Indeed, the ratio of Vero/Aag2 positive samples was lower for CHIK_{IC}-FG_N and CHIK_{IC}-FG_C as compared to wild type CHIK_{IC} (FG_N 0.18; FG_C 0.33).

To determine whether the mutations in CHIK_{IC}-FG_N and CHIK_{IC}-FG_C were preserved during replication in the mosquito, the nsP3 sequences of a selection of mosquitoes with CHIKV-positive saliva were amplified by reverse-transcription (RT)-PCR. Presence of the expected mutations in nsP3 was confirmed, indicating that the mutant viruses did not revert to wild type (Fig. S5.1). Together, these results imply that, while CHIK_{IC}-FG_N and CHIK_{IC}-FG_C are perfectly able to infect the mosquito body and disseminate to ultimately reach the mosquito saliva, their virions are less able to replicate in vertebrate cells. These results are in agreement with the observed differences between the growth curves on Vero and Aag2 cells (compare Fig. 5.4D with Fig. 5.4E). To assess the effect of mutating one FGDF motif on the viral load in the mosquito body, the viral titers in mosquito bodies of which the saliva was virus positive were determined by EPDA on Aag2 cells (Fig. 5.5E). Viral loads of mosquitoes infected with CHIK_{IC}, CHIK_{IC}-FG_N or CHIK_{IC}-FG_C were not significantly different (2.7 - 5.0×10^6 TCID₅₀/ml; $P > 0.65$). Thus, a single FGDF motif in nsP3 is sufficient for the infection and dissemination of CHIKV in *Ae. aegypti* mosquitoes, but duplicate FGDF motifs are required for efficient infection of the vertebrate host by mosquito bite.

Fig. 5.5 A single FGDF motif is sufficient for the transmission of chikungunya virus by *Aedes aegypti* mosquitoes. (A) Schematic experimental set-up. Female *Ae. aegypti* mosquitoes were infected through an infectious blood meal containing 2.8×10^5 TCID₅₀/ml of CHIK_{IC}, CHIK_{IC}-FG_N, CHIK_{IC}-FG_C. At 7 days post infection (dpi) the infection and transmission rates were determined by infectivity assay on (B) Vero and (C) Aag2 cells. Bars represent cumulative numbers from three independent experiments. n = total number of mosquitoes used per treatment. Statistics were performed by Fisher's exact test ($\alpha=0.05$). (D) Ratio between the number of positive bodies or saliva samples in Vero compared to Aag2 cells. Statistics were performed by Kruskal-Wallis test with Dunn's post-hoc test ($\alpha=0.05$). (E) TCID₅₀/ml in the bodies of *Ae. aegypti* mosquitoes with CHIKV-positive saliva at 7 dpi were determined by end-point dilution assay on Aag2 cells. Statistics were performed by one-way ANOVA with Tukey's post-hoc test on Log₁₀ transformed data ($\alpha=0.05$). ns, not significant.

The interaction with mammalian G3BP and mosquito Rasputin depends on the presence of FGDF motifs in the HVD of chikungunya virus nsP3

We have previously shown that mutations in a single FGDF motif do not abrogate the co-localization of CHIKV nsP3 and the mosquito homolog of G3BP, Rin, in insect cells [129]. However, in mammalian cells, it has been suggested that mutation of just the N-terminal FGDF motif of nsP3 already dismantles the interaction with G3BP [125,126]. We therefore investigated the effect of mutating either of the two FGDF motifs on the co-localization of CHIKV nsP3 with G3BP in mammalian cells and Rin in mosquito cells. Because there is no anti-Rin antibody available, Aag2 cells were first transfected with a plasmid expressing an EGFP-tagged Rin (pPUB-Rin-EGFP, Fig. 5.6A). Vero (Fig. 5.6B) and Aag2 (Fig. 5.6C) cells were infected with CHIK_{IC}nsP3mC, CHIK_{IC}nsP3mC-FG_N or CHIK_{IC}nsP3mC-FG_C. At 24 hpi, cells were fixed and permeabilized and Vero cells were immuno-stained for G3BP-1. In both mammalian and mosquito cells mutation of either the N-terminal or the C-terminal FGDF motif did not abrogate the co-localization of nsP3 with G3BP and Rin.

As co-localization observed by fluorescence microscopy does not prove that there is a physical protein-protein interaction we investigated the importance of the FGDF motifs for interaction of nsP3 with G3BP in mammalian cells and Rin in mosquito cells by co-immunoprecipitation (co-IP). First, Vero cells were transfected with CMV-driven plasmids expressing the nsP3EGFP fusion proteins nsP3, nsP3-FG_N, nsP3-FG_C, nsP3-FG_{NC} or just EGFP as a control (Fig. 5.6A). At 24 hpt cells were lysed and lysates were subjected to co-IP with α -GFP beads followed by western blot detection of nsP3EGFP and G3BP in both the cell lysates and co-IP samples (Fig. 5.6D). The expression of the nsP3 fusion proteins was similar for nsP3-FG_N, nsP3-FG_C and nsP3-FG_{NC} while expression of wild type nsP3 was lower. Yet after co-IP, G3BP was only enriched for wild type nsP3, despite the presence of less nsP3 in the co-IP fraction. G3BP was not enriched for nsP3-FG_N, nsP3-FG_C or nsP3-FG_{NC}, suggesting that nsP3 requires duplicate FGDF motifs to establish high-affinity for G3BP in mammalian cells.

Next, Aag2 cells were transfected with PUB-driven plasmids expressing the nsP3EGFP fusion proteins nsP3, nsP3-FG_C, nsP3-FG_N or nsP3-FG_{NC} and co-transfected with pPUB-Rin-mC, encoding a Rin-mCherry fusion protein (Fig. 5.6A). Cells were lysed, subjected to co-IP with α -GFP beads followed by detection of nsP3EGFP and Rin-mC in the cell lysates and precipitated samples (Fig. 5.6E). Expression of the nsP3EGFP fusion proteins was similar for nsP3, nsP3-FG_N, nsP3-FG_C, nsP3-FG_{NC}. Expression of Rin-mC was similar for nsP3, nsP3-FG_N, nsP3-FG_C transfected samples, while Rin-mC expression was highest in cells transfected with nsP3-FG_{NC}. Similar amounts of nsP3, nsP3-FG_N and nsP3-FG_C but more nsP3-FG_{NC} was precipitated. Interestingly, however, Rin-mC was only enriched for nsP3, nsP3-FG_N and nsP3-FG_C, but not for nsP3-FG_{NC}. This demonstrates that nsP3 requires only one FGDF motif for interaction with Rin. These results suggest that differences in the affinities of FGDF motifs for G3BP and Rin may underlie the difference for the required number of FGDF motifs between mammalian and mosquito cells.

Fig. 5.6 At least one FGDF motif is required for the interaction of nsP3 with G3BP and mosquito Rin. (A) Schematic overview of the used plasmids expressing Rasputin (Rin) fused to EGFP Rin-EGFP or mCherry Rin-mC and CHIKV nsP3 EGFP fusion proteins of wild type and FGDF single- and double-mutant nsP3. CMV cytomegalovirus promoter; PUB *Aedes aegypti* poly-ubiquitin promoter; NTF2 nuclear transport factor 2-like domain; RRM RNA recognition motif; RGG arginine glycine rich region; HVD hypervariable domain. (B) Vero cells were infected with CHIK_{IC}nsP3mC, CHIK_{IC}nsP3mC-FG_N or CHIK_{IC}nsP3mC-FG_C. At 24 hours post infection (hpi) cells were fixed, permeabilized, stained with α-G3BP and visualized by fluorescence microscopy. (C) Aag2 cells were transfected with pPUB-Rin-EGFP and at 24 hours post transfection (hpt) cells were infected with CHIK_{IC}nsP3mC, CHIK_{IC}nsP3mC-FG_N or CHIK_{IC}nsP3mC-FG_C. At 24 hpi cells were fixed and visualized by fluorescence microscopy. (D) Vero cells were transfected with CMV-driven plasmids expressing EGFP, nsP3EGFP, nsP3EGFP-FG_C, nsP3EGFP-FG_N or nsP3EGFP-FG_{NC}. At 24 hpt cells were lysed and lysates were subjected to co-immunoprecipitation (co-IP) with α-GFP beads. Lysates and co-precipitates were subjected to western blot with α-G3BP and α-GFP antibodies. (E) Aag2 cells were transfected with PUB-driven plasmids expressing EGFP, nsP3EGFP, nsP3EGFP-FG_N, nsP3EGFP-FG_C or nsP3EGFP-FG_{NC} and co-transfected with pPUB-Rin-mC. At 24 hpt cells were lysed and lysates were subjected to co-IP with α-GFP beads. Lysates and co-precipitates were subjected to western blot with α-GFP and α-mCherry antibodies.

Discussion

The delicacy of alphavirus-vector interactions has been well-illustrated by the discovery that a single A->V mutation in the CHIKV E1 envelope protein could change the viral vector specificity from *Ae. aegypti* to *Ae. albopictus* [25]. Additionally, for VEEV a single amino acid substitution in the E2 protein could determine its potential to infect *Ochlerotatus taeniorhynchus* mosquitoes [306]. Here, we investigated the importance of the conserved P-rich and FGDF motifs in the HVD of CHIKV nsP3 for infection of mosquito cells and transmission by *Ae. aegypti* mosquitoes.

Deletion of the P-rich motif in the nsP3 HVD renders CHIKV unable to replicate in both mammalian and mosquito cells, while amino acid substitutions of the core residues in the P-rich motif resulted in a significant decrease of replication in both cell types. This is similar to previous findings with SINV where deletions in the SINV nsP3 HVD that disrupt the P-rich motif were reported to decrease growth kinetics and plaque size in C7/10 mosquito cells [127], an indication for reduced replication potency or cytopathicity, or both. However, mutations of the P-rich motif did not affect CHIKV infection or transmission by *Ae. aegypti* mosquitoes. The nsP3 P-rich motif has high affinity for SH3-domain containing proteins and is known to interact with amphiphysin-I/II [292,295] in mammalian cells, and proposedly also with the *Drosophila* homolog of amphiphysin [292], although no evidence has so far been reported for this interaction in insect cells. We indeed demonstrate that mutation of the P-rich motif results in decreased virus production in mosquito cells, which suggests that nsP3 interacts with mosquito SH3-domain containing proteins, although these interactions do not appear to be essential for transmission by the mosquito vector. It was previously shown that deletions of the P-rich motif of SFV and SINV result in decreased affinity for amphiphysins and lowered replication efficiencies in mammalian cells [292]. Therefore, the decrease in replication of CHIK_{IC}^{P398A} and CHIK_{IC}^{PPR401AAA} in mammalian cells may be due to reduced affinity for amphiphysins or other cellular proteins that contain an SH3 domain. Of note, both the P398A and PPR401AAA mutations do not mutate all prolines in the CHIKV PxxPPR motif, thus the remaining prolines may still partially interact with host proteins. This may underlie the strong attenuation of CHIK_{IC}^{P398A} and CHIK_{IC}^{PPR401AAA} after low MOI infection, but not after high MOI infection where the remaining proline(s) may be sufficient to establish successful replication. Complete inactivation of CHIKV after deletion of the entire P-rich motif implies that such interactions are crucial to establish successful virus infection. It has been demonstrated that the arginine residues downstream of the P-rich motif of CHIKV, which are not conserved in SFV and SINV nsP3, partially determine the affinity for amphiphysin-II [295]. This might explain why deletion of the whole P-rich motif, including the C-terminal arginines downstream of PxxPPR completely abrogates CHIKV replication, while mutating just the core residues in the P-rich motif results in a less dramatic effect.

Mutation of both FGDF motifs together fully abrogated CHIKV replication in both mammalian and mosquito cells. This indicates that the FGDF motifs not only play an important role during infection of mammalian cells, as previously reported [123–126,293,294], but that they also determine the successful infection of mosquito cells. Furthermore, we show for CHIKV that a single FGDF motif is sufficient to infect mammalian cells, although the replication and infection efficiencies are dramatically decreased compared to wild type CHIKV. In contrast, in mosquito cells, CHIK_{IC}-FG_N and CHIK_{IC}-FG_C replicated similarly to the wild type CHIK_{IC} and were efficiently transmitted by *Ae. aegypti* mosquitoes after an infectious blood meal. This indicates that CHIKV requires only a single FGDF motif for infection of the mosquito vector. Thus, duplicate

FGDF motifs appear to be required for the efficient infection from the mosquito's saliva to a subsequent vertebrate host, which supports the conservation of duplicate FGDF motifs for most mosquito-borne alphaviruses.

The mosquito protein Rin, a homolog of mammalian G3BP, was previously identified as an important regulator of CHIKV transmission, as knock-down of Rin severely decreased the transmission of CHIKV by *Ae. albopictus* mosquitoes [129]. In our study, we confirmed that, during CHIKV infection, a single FGDF motif is sufficient for the co-localization of nsP3 with Rin, similar to previous experiments with transiently expressed nsP3 [129]. Mutation of both FGDF motifs in nsP3 together abolished its ability to co-localize and interact with Rin, suggesting that the inability of CHIKV_{IC}-FG_{NC} to replicate in mosquito cells is due to absence of the nsP3-Rin interaction. Furthermore, we demonstrate that only one FGDF motif is required for the interaction between nsP3 and Rin, in contrast to mammalian cells where the interaction between nsP3 and G3BP was only observed for wild type nsP3 and not for nsP3 with one mutated FGDF motif. For SFV nsP3 it has been shown that mutation of just the phenylalanine of FG_N largely abrogates the interaction with G3BP while mutating the phenylalanine of FG_C only decreases the affinity of nsP3 for G3BP [125]. We mutated not only the first phenylalanine of FG_C but also the glycine to alanine, both of which have been shown to be important for G3BP binding [305]. This may explain why we could not observe an interaction between G3BP and either nsP3-FG_N or nsP3-FG_C.

G3BP can be present in monomeric, dimeric and polymeric forms [307] and the two FGDF motifs of alphavirus nsP3 each bind one NTF2-like domain of two separate G3BP monomers [125,305]. As a result, mutation of one FGDF motif results in decreased affinity for G3BP and attenuates alphavirus replication [123,125,126]. It is uncertain whether mosquito Rin forms a dimer, although the crystal structure of the *Drosophila* Rin NTF2-like domain was demonstrated in dimeric form [308]. Thus, a single FGDF motif might be sufficient for the interaction with monomeric Rin to support CHIKV replication in mosquito cells and in *Ae. aegypti* mosquitoes. Alternatively, the FGDF motifs of nsP3 might have a higher affinity for Rin as compared to G3BP, resulting in sufficient recruitment of Rin to replication complexes in mosquito cells with only a single FGDF motif. As Vero and Aag2 cells were cultured at 37 °C and 28 °C respectively, lowered affinity of the FGDF motifs for G3BP as compared to Rin may partially be explained by the higher incubation temperature. Potentially, the higher temperature at which mammalian Vero cells are cultured decreases the stability of the nsP3-G3BP interaction, thus resulting in lower affinity of nsP3 for G3BP as compared to Rin.

The exact reason for recruitment of Rin/G3BP by nsP3 is still speculative, although several hypotheses such as recruitment of viral gRNA followed by shielding of the viral replication complex from degrading enzymes [126] and prevention of antiviral stress granule formation [122,123] have been brought forward. Possibly, the interaction with G3BP/Rin is particularly crucial early after infection and a certain level of G3BP/Rin has to be bound by nsP3 to successfully form viral replication complexes. Interestingly, while arthritogenic alphaviruses recruit G3BP via their FGDF motifs, neurotropic alphaviruses lack FGDF motifs and instead recruit members of the Fragile X syndrome (FXR) family via a different motif in the nsP3 HVD [126,293]. Proteins of the FXR family are abundantly expressed in neuronal cells [309], which may therefore be more susceptible to infection by alphaviruses that use FXR family proteins for their replication. Whether a direct correlation exists between the use of FXR or G3BP for replication and the symptoms caused by alphavirus infection should be the focus of future research. Mosquitoes possess a homolog of one FXR family protein, called Fragile X syndrome-related protein 1 (FMR1). Based on our results, we hypothesize that the

nsP3 of neurotropic alphaviruses may interact with mosquito FMR1, similarly to the CHIKV nsP3-Rin interaction. Future research should investigate the possibility of an interaction between nsP3 and mosquito FMR1 and its importance for the transmission of neurotropic alphaviruses by mosquitoes.

Concluding, our results increase the understanding of the functionality of conserved motifs in the alphavirus nsP3 HVD in the context of the arboviral dual-host life cycle. Understanding the underlying mechanisms of G3BP/Rin recruitment by alphavirus nsP3 remains the focus of future research and may well contribute to the development of antiviral drugs and compounds that target this crucial interaction.

Materials and Methods

Cell culture

African green monkey kidney Vero E6 cells (ATCC CRI-1586) were cultured at 37 °C with 5% CO₂ in Dulbecco's Modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml; Sigma-Aldrich) and streptomycin (100 µg/ml; Sigma-Aldrich). *Ae. albopictus* C6/36 (ATCC CRL-1660) cells were cultured at 28 °C in Leibovitz L-15 medium (Gibco) supplemented with 10% FBS, 2% tryptose phosphate broth (Gibco) and 1% nonessential amino acids (Gibco). *Ae. aegypti* Aag2 cells were cultured at 28 °C in Schneider's Drosophila medium (Lonza) supplemented with 10% FBS. During experiments gentamicin (50 µg/ml; Life technologies) was added to the culture medium of C6/36 and Aag2 cells.

Recombinant viruses

Previously reported infectious clone derived chikungunya virus 37997 strain (CHIKV³⁷⁹⁹⁷) [149] was used in all experiments. Gateway-compatible pIB vectors with CHIKV nsP3 sequences containing the mutations P398A, PPR401AAA, FG479AA, FG497AA and FG479AA/FG497AA were described previously [129]. To insert an internal fluorescent reporter gene into nsP3, mCherry was PCR amplified from CHIK_{rep}-nsP3mC [122] with primers 1,2 (primer sequences are listed in Table 5.1) containing BspEI overhangs and cloned as BspEI fragment into the pIB-nsP3 vectors. This introduces the mCherry sequence between amino acids 367-368 of the CHIKV nsP3 gene. The Firefly luciferase gene was amplified from the previously described pCHIK_{rep}-FlucEGFP [122] with primers 7,8 and used to replace the FlucEGFP sequence in the same pCHIK_{rep}-FlucEGFP to generate pCHIK_{rep}. Mutant nsP3 containing mutations P398A and PPR401AAA in the P-rich motif were cloned from the corresponding pIB vectors as AgeI/NheI fragment into pCHIK_{rep}-Fluc to create pCHIK_{rep}-nsP3mC^{P398A} and pCHIK_{rep}-nsP3mC^{PPR401AAA}. nsP3 containing mutations in the FGDF motifs were PCR amplified using primers 3,4, nsP4 was PCR amplified using primers 5,6 and the two PCR products were fused by overlapping-PCR using primers 3,6. Subsequently, the nsP3-nsP4 fusion PCR products were cloned as AgeI/AscI fragment into pCHIK_{rep}-Fluc to create pCHIK_{rep}-nsP3mC-FG_N (FG479AA), pCHIK_{rep}-nsP3mC-FG_C (FG497AA) and pCHIK_{rep}-nsP3mC-FG_{NC} (FG479AA & FG497AA). The Fluc sequence was replaced with the CHIKV³⁷⁹⁹⁷ structural cassette from pCHIK_{IC} through AscI/EcoRI cloning to generate pCHIK_{IC}-nsP3mC infectious clones of all mutants. The internal mCherry was removed by BspEI digestion and vector self-ligation. An overview of the recombinant CHIKV encoding plasmids used in this study is shown in Fig. 5.1A. Recombinant viruses were generated from the pCHIK_{IC} infectious clones as described previously [149]. Briefly, pCHIK_{IC} plasmids were linearized by PacI digestion to serve as template for an SP6 RNA polymerase (NEB) transcription reaction and the resulting *in vitro* transcribed RNA was used to transfect pre-seeded Vero cell monolayers with Lipofectamine 2000 (Invitrogen) to generate the P0 stock. Subsequent passages were performed on C6/36 cells.

Expression plasmids

Gateway-compatible and OpIE-2 promoter driven pIB insect-cell expression plasmids expressing EGFP, nsP3EGFP, nsP3EGFP-FG_N, nsP3EGFP-FG_C and nsP3EGFP-FG_{NC} were reported previously [129]. The *Ae. aegypti* poly-ubiquitin (PUB) promoter was amplified from pPUB-Fluc [310] using primers 9,10 (Table 1) containing BspHI and SacI restriction

sites and cloned as BspHI/SacI fragment into pIB to generate the Gateway-compatible pPUB vector. Gateway-cloning (Invitrogen) was used to generate pPUB-EGFP, pPUB-nsP3EGFP, pPUB-nsP3EGFP-FG_N, pPUB-nsP3EGFP-FG_C and pPUB-nsP3EGFP-FG_{NC}. Plasmid pIB-Rin-mC, expressing the mosquito Rin fused C-terminally to an mCherry red fluorescent reporter gene, was reported previously [129]. EGFP was PCR amplified from pEGFP-N1 with primers 13,14 (Table 5.1) containing KpnI and SacII overhangs and cloned as KpnI/SacII fragment into pIB-Rin-mC to generate pIB-Rin-EGFP. The PUB-promoter was amplified from pPUB-EGFP with primers 11,12 (Table 5.1) containing EcoRI overhangs and cloned as EcoRI/EcoRI fragment into pIB-Rin-mC and pIB-Rin-EGFP to generate pPUB-Rin-mC and pPUB-Rin-EGFP respectively.

Table 5.1 Primers used in this study

#	Primer name	Sequence (5'→3')
1	mCherry-F	ATTCCGGAAATGGTGAGCAAGGGCGAG
2	mCherry-R	GTTCCGGAATCTTGACAGCTCGTCCATG
3	nsP3-F	TAAACAGCGTAGCTATACCG
4	nsP3_end-R	CCCACCTGCCCTATCTAGTC
5	nsP3_end-F	GACACGGACGACGAATTATG
6	nsP4-R	TTCCATGGCGCGCCGCTAG
7	Fluc-F	GGCCACCTTTGCAAGCTCT
8	Fluc-R	GAATTCCTCCACGGCGATCTTCCG
9	pPUB-F	AATCATGAATCTTTACATGTAGCTTGTGCA
10	pPUB-R	AAGAGCTCGGTTGAAATCTCTGTTGAGCA
11	pPUB-EcoRI-F	GGACCGGTATGGTGAGCAAGGGCGAGG
12	pPUB-EcoRI-R	GGGAGCTCCCTTGACAGCTCGTCCATGCC
13	GFP-KpnI-F	GGGGGGTACCATGGTGAGCAAGGGCGAGGAG
14	GFP-SacII-R	GGGGCCGCGGCTACTTGACAGCTCGTCCATGCCGAG

Virus growth curves

Vero or Aag2 cell monolayers were seeded one day prior to infection in a 6-well plate. The amount of inoculum for the specific MOI was calculated based on EPDA on either Vero or Aag2 cells. Cell monolayers were infected with 1 ml of inoculum in either DMEM- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) supplemented with 10% FBS, streptomycin and penicillin (DMEM-supplemented) or Schneider's Drosophila medium supplemented with 10% FBS and gentamycin (50 µg/ml) (Schneider's-supplemented). After 2 h the inoculum was aspirated, monolayers were washed with phosphate-buffered saline (PBS) and 2 ml of fresh culture medium was added. Vero cells were incubated at 37 °C with 5% CO₂ and Aag2 cells were incubated at 28 °C. At the indicated time points post infection 30 µl supernatant samples were taken and after one freeze-thaw cycle (-80°C) the infectious virus titre was determined by EPDA on either Vero or Aag2 cells.

End-point dilution assays

Vero cells were detached by incubation with Trypsin-EDTA (Gibco) and diluted 1:4 in DMEM-supplemented, while Aag2 cells were detached by force and diluted 1:8 with Schneider's supplemented. Samples were thawed, vortexed and 10-fold serial dilutions were made in either DMEM-supplemented or Schneider's-supplemented. Cell suspensions were added to the dilutions 1:1 and 10 µl of inoculated dilution was plated 6-fold in micro-titer plates (Nunc). When samples from mosquitoes were titrated, Fungizone (50 µg/ml; Invitrogen) and gentamycin (50 µg/ml; Life Technologies), were added to the medium and cell fractions prior to titrations (DMEM-complete, Schneider's-complete). Titrations on Vero cells were scored by CHIKV-induced CPE, while titrations on Aag2 cells were scored by α-CHIKV-E2 based immunofluorescence assay.

Immunofluorescence assay

Vero or Aag2 cells were fixed by >10 min incubation in 4% paraformaldehyde in PBS. Cells were permeabilized by >10 min incubation in 0.1% sodium dodecyl sulphate in PBS, washed three times with PBS and blocked by incubation in 5% FBS in PBS for 30 min. Primary antibody binding was performed with α -CHIKV-E2 (rabbit polyclonal; 1:5000 [280]) or α -G3BP1 (1:500; G6046 Sigma Aldrich) diluted in 5% FBS in PBS for 1 h at room temperature (RT). Cells were washed three times with PBS and incubated with the secondary antibody goat- α -rabbit-Alexa Fluor 488 (1:1000; Invitrogen) in 5% FBS in PBS for 1 h at 37°C. Cells were washed three times with PBS and visualized using an Axio Observer Z1m inverted microscope (Zeiss) in combination with an X-Cite 120 series lamp.

Infectious blood meal experiments

Ae. aegypti mosquitoes (Rockefeller strain, obtained from Bayer AG,) were reared as described previously [149] and transported to the Biological Safety level 3 facility in buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey) for virus infection assays. Mosquitoes were starved for one day and then offered an infectious blood meal from a 1:1 mixture of virus and human blood (Sanquin) in a total volume of 1 ml via a Hemotek feeder covered with a Parafilm membrane. Mosquitoes were allowed to feed for 1 h under light conditions at 24 °C and 70% relative humidity (RH). Mosquitoes were anesthetized with CO₂ and fully engorged females were selected and maintained in buckets at 28 °C, 12:12 light:dark cycle, 70% relative humidity and provided with a cotton pad soaked in 6% glucose solution. After 7 or 14 days, mosquitoes were anesthetized with 100% CO₂, and immobilized by removing their legs and wings. The proboscis of each stripped mosquito was inserted into a 200 μ l pipet tip containing 5 μ l of a 1:1 mixture of 50% glucose and FBS. Mosquitoes were allowed to salivate for >45 min, after which the bodies were individually transferred to 1.5 ml Safe-Seal micro tubes (Sarstedt) that contained a small scoop of 0.5 mm zirconium beads (Next Advance). Saliva samples were added to 1.5 ml micro tubes (Sarstedt) that contained 60 μ l DMEM-complete. All samples were stored at -80 °C until further processing. For most experiments, transmission was determined at 7 dpi, as wild type CHIKV reaches relatively high transmission rates at this time-point. This therefore presents a good resolution for the detection of a putative attenuation of a mutant virus.

Infectivity assay

Frozen mosquito bodies were homogenized for 2 min at maximum speed in a Bullet Blender Storm (Next Advance). Homogenized bodies were centrifuged for 1 min at 14,500 rpm, after which 100 μ l of DMEM-complete was added and the homogenization was repeated. Finally, the mosquito debris was pelleted by 1 min centrifugation at 14,500 rpm. Thirty microliters of mosquito body supernatant or saliva samples were used to inoculate either Vero or Aag2 cell monolayers in 96-well plates. After 2-3 h incubation the inoculum was removed and replaced with fresh DMEM-complete or Schneider's-complete. Infectivity was scored at 3 dpi through virus-induced CPE in Vero cells or by CHIKV-E2-based immunofluorescence in Aag2 cells, as described above.

Verification of nsP3 mutants

Mosquitoes with CHIKV-positive saliva were selected from mosquitoes inoculated with CHIK_{IC}, CHIK_{IC}^{P398A}, CHIK_{IC}^{PPR401AAA}, CHIK_{IC}-FG_N or CHIK_{IC}-FG_C. A selection of mosquitoes was made to represent each replicate experiment. Thirty μ l of mosquito sample supernatant was used to inoculate a well of a 96-well plate pre-seeded with C6/36 cells. At 3 dpi RNA was isolated with TRIzol reagent (Invitrogen) and 300 ng total RNA was subjected to one-step RT-PCR with primers 3,4 using SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen) following the manufacturer's protocol. RT-PCR products were observed on agarose gel to confirm the infection of mosquitoes with CHIKV. To investigate the presence and preservation of the expected mutations, RT-PCR products were subjected to digestion with SacII (NEB), NotI (NEB) or ApeKI (NEB) according to the manufacturer's protocol. Digestions were observed on agarose gel.

Transfections

Transfections of mosquito cells with DNA plasmids were performed with Fugene-HD (Promega) in serum-free media following the manufacturer's protocol. Transfections of Vero cells with DNA plasmids and transfections of C6/36, Aag2 and Vero cells with *in vitro* transcribed RNA were performed using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco). At 4 hours post transfection (hpt) with Lipofectamine 2000 the transfection mix was replaced with fresh culture media.

Luciferase measurements

The cell culture volume was aspirated and cells were lysed by 20 min incubation in 1X passive lysis buffer (Promega) at RT. Ninety μ l of cell lysate was pipetted into an opaque 96-well plate and 90 μ l of Steady-Glo reagent (Promega) was added. After 10 min the luciferase activity was measured using a FLUOstar Optima microplate reader (BMG Labtech).

Co-immunoprecipitation and western blotting

Pre-seeded Vero or Aag2 cells were transfected with plasmids expressing nsP3 or Rin or both. One dpt cells were detached, washed once with PBS and lysed by 30 min incubation in lysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40) supplemented with Complete protease inhibitors (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were cleared by 10 min 20,000 g centrifugation at 4 °C and diluted 5:2 with dilution buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA). Twenty-five μ l GFP-Trap_A beads (Chromotek) were added to 500 μ l dilution buffer and pelleted by 2 min 2,500 g centrifugation at 4 °C. Washing was repeated twice. Cell lysates were added to the equilibrated beads and incubated for 1-2 h at 4 °C with overhead rotation. Beads were washed thrice by resuspension in dilution buffer followed by 2 min 2,500 g centrifugation at 4 °C. Bound protein complexes were eluted by resuspension in 2X SDS-loading buffer containing 10% β -mercaptoethanol followed by 10 min incubation at 95 °C. Beads were removed by 5 min 3,000 g centrifugation and eluates were subjected to western blotting. Briefly, proteins were size-separated by SDS-PAGE and semi-dry blotted onto Immobilon-P membranes (Merck Millipore). Membranes were blocked overnight at 4 °C by incubation in 1% milk powder/PBS-0.05%-Tween-20 (PBST). Blocked membranes were probed for 1 h at RT with primary antibodies α -G3BP (1:1000; G6046 Sigma Aldrich), α -GFP (1:2000; A6455 Molecular Probes), α -mCherry (1:1000; ab183628 Abcam) diluted in 1% milk powder/PBST. Membranes were washed thrice for 5 min with PBST and probed for 1 h at RT with alkaline phosphatase (AP) conjugated secondary antibody goat- α -rabbit-AP (1:2500; D0487 Dako) diluted in PBST. Membranes were washed thrice for 5 min in PBS-T and developed with nitroblue tetrazolium (NBT)/BCIP (5-bromo-4-chloro-3-indolylphosphate) (Roche) until the desired signal was achieved.

Statistics

Statistical analysis were performed in SPSS Statistics 23. Virus titers in mosquito bodies and supernatants from growth curve experiments were checked for normality by Kolmogorov-Smirnov test. Data that did not follow a normal distribution was Log₁₀ transformed and confirmed for normality. Means were compared by one-way ANOVA with Tukey post-hoc test ($\alpha=0.05$). Data that did not follow a normal distribution, despite Log₁₀ transformation was analysed by Kruskal-Wallis test with Dunn's post-hoc test ($\alpha=0.05$). Two-tailed Fisher's exact tests were used to compare infection and transmission rates and performed with GraphPad QuickCalcs ($\alpha = 0.05$). Ns non-significant; * $P < 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

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Supplemental information

Fig. S5.1 Verification of CHIK_{IC} nsP3 mutants *in vivo* in *Ae. aegypti* mosquitoes. RNA was isolated from the bodies of mosquitoes with CHIKV-positive saliva. The nsP3 gene was amplified by RT-PCR (primers indicated by arrows) and digested with SacII, NotI or ApeKI to screen for preservation of the expected mutations.

Chapter VI

Noncoding subgenomic flavivirus RNA is processed by the mosquito RNAi machinery and determines West Nile virus transmission by *Culex pipiens* mosquitoes

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Abstract

Flaviviruses, such as Zika virus, yellow fever virus, dengue virus and West Nile virus (WNV) are a serious concern for human health. Flaviviruses produce an abundant noncoding subgenomic flavivirus RNA (sfRNA) in infected cells. SfRNA results from stalling of the host 5'→3' exoribonuclease XRN1/Pacman on conserved RNA structures in the 3'UTR of the viral genomic RNA. SfRNA production is conserved in insect-specific, mosquito-borne, tick-borne and flaviviruses with no-known vector, suggesting a pivotal role for sfRNA in the flavivirus life cycle. Here we investigated the function of sfRNA during WNV infection of *Culex pipiens* mosquitoes and evaluated its role in determining vector competence. An sfRNA1-deficient WNV was generated that displayed similar growth kinetics as wild type WNV in both RNA interference-competent and -compromised mosquito cell lines. Small RNA deep sequencing of WNV-infected mosquitoes indicated an active small-interfering RNA based antiviral response for both the wild type and sfRNA1-deficient virus. Additionally, we provide the first evidence that sfRNA is an RNAi substrate *in vivo*. Two reproducible small RNA hotspots within the 3'UTR/sfRNA of the wild type virus mapped to RNA stem loops SL-III and 3'SL, which stick out of the 3D sfRNA structure model. Importantly, we demonstrate that sfRNA-deficient WNV displays significantly decreased infection and transmission rates *in vivo* when administrated via the blood meal. Finally, we show that transmission and infection rates are not affected by sfRNA after intrathoracic injection, thereby identifying sfRNA as a key driver to overcome the mosquito midgut infection barrier. This is the first report to describe a key biological function of sfRNA for flavivirus infection of the arthropod vector, providing an explanation for the strict conservation of sfRNA production.

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Introduction

Viruses from the genus *Flavivirus* (family *Flaviviridae*), such as the highly pathogenic dengue virus (DENV) [311], the emerging Zika virus [312] and West Nile virus (WNV) [45,313] are important threats to human health and are collectively responsible for millions of annual reported cases of infection [16]. Flaviviruses are positive-sense single-stranded RNA (ssRNA) viruses that can infect many different hosts including insects, ticks, birds and mammals [81]. They can be further divided based on their vector species into mosquito-borne flaviviruses (MBF), tick-borne flaviviruses (TBF), insect-specific flaviviruses (ISF) and flaviviruses with no-known vector (NKVF). The epidemic potential of flaviviruses was clearly demonstrated when WNV was first detected in New York City in 1999, reaching the West Coast of the United States only 4 years later, causing the largest outbreak of WNV induced neuroinvasive disease to date [41]. In southern Europe, lineage I WNV outbreaks were reported as early as the 1960s [19], but more recently a novel pathogenic lineage II WNV isolate was identified [45,314,315]. In 2010, in Greece, an exceptionally severe outbreak of this lineage II WNV strain resulted in 197 patients with neuroinvasive disease and 33 deaths [45]. Understanding the determinants of transmission of these flaviviruses is important to evaluate their epidemic potential and is the first step in designing novel ways to break the flavivirus transmission cycle.

The overall capacity of a mosquito to become orally infected after an infectious blood meal and to transmit the virus to the next vertebrate host is expressed as the vector competence [100]. For a successful infection of the mosquito vector, arboviruses have to overcome the midgut infection and escape barriers, as well as the salivary gland barriers [142]. After ingestion of a viremic blood meal, the midgut-epithelial cells have to become infected. These cells have a strong RNA interference (RNAi) response that restricts virus infection and acts as a bottleneck on virus population diversity [104,165]. Next, the virus has to replicate in the midgut cells, produce progeny virus, and escape from the midgut cells through the basal lamina into the haemolymph. Subsequently, the virus disseminates to other organs and ultimately infects the salivary glands. After sufficiently high virus titers in the saliva have been reached, viruses can be transmitted to a new vertebrate host via a bite by the infected mosquito.

Vector competence is influenced by viral, environmental (e.g. temperature and the microbiome) and vector-related factors, including vector genetics and antiviral responses [100]. In arthropods, antiviral responses are predominantly mediated by RNAi [101,104] and also by Toll, IMD, JAK/STAT signalling pathways and apoptosis [102,153]. Three distinct insect small RNA pathways can be discriminated: the 21 nucleotide (nt) small interfering RNA (siRNA) response, the ~21-22 nt microRNA (miRNA) pathway, and the 25-30 nt PIWI-interacting RNA (piRNA) response [212,316,317]. In *Culex* mosquitoes – the predominant group of WNV vectors – virus specific siRNAs are highly abundant whereas viral piRNAs (vpiRNAs) appear to be absent [155,165]. For flaviviruses, three putative viral suppressors of RNAi (VSRs) have been identified *in vitro*: the NS4B and NS3 protein of DENV-2 and the noncoding subgenomic flavivirus RNA (sfRNA) for WNV [103,117,318].

During viral replication in both vertebrate and invertebrate host cells, flaviviruses produce a highly abundant noncoding sfRNA of approximately 0.5 kb in size. SfRNA is produced via a unique mechanism involving degradation of the viral genomic (vg)RNA by the host 5'→3' exoribonuclease XRN1/Pacman [106]. XRN1 stalls at stem-loop (SL) and dumbbell (DB) RNA structures within the flaviviral 3' untranslated region (UTR), resulting in the accumulation of sfRNA [319–322]. The stalling of XRN1 occurs due to steric hindrance caused by pseudoknots (PK) and other tertiary RNA

structures [107]. During WNV infection, three species of sfRNA, named sfRNA1, 2 and 3, are produced by stalling of XRN1 on SL-II, SL-IV and DB-1, respectively [319,322]. The formation of sfRNA is important for replication in insect and several vertebrate cells, since sfRNA mutants that produce only sfRNA3 have attenuated replication rates, even in Dicer-2 deficient C6/36 cells [106,112,321,322]. In mammalian cells, sfRNA is essential for flavivirus induced cytopathicity and pathogenicity [106]. Importantly, sfRNA acts as an antagonist of both interferon and retinoic acid-inducible gene-I-like receptor dependent innate immune responses [109,112]. Accordingly, WNV mutants deficient in sfRNA production are attenuated in mice [106,322].

Despite the clear functions of sfRNA in flavivirus pathogenesis in vertebrates, the role of sfRNA in the arthropod vector is poorly understood. SfRNA production is conserved for all MBFs and TBFs and although recent RNA structure analysis of ISF 3'UTRs failed to identify XRN1 resistant structures [323,324], it was previously described that ISFs and NKVFs have conserved SL structures with putative pseudoknot structures in their 3'UTR [105]. Indeed, sfRNA production has been experimentally shown for several NKVFs and the ISF cell fusing agent virus [113]. The conservation of sfRNA production by MBFs, TBFs and ISFs emphasizes the putative importance of sfRNA production in arthropods [105]. So far it has been demonstrated that sfRNA acts as a suppressor of miRNA- and siRNA-mediated RNAi *in vitro* in both mammalian and arthropod cells [115,117]. The highly abundant sfRNA most likely acts as a decoy substrate for Dicer to prevent it from cleaving other double-stranded (ds)RNA molecules. Recently, sfRNA has been demonstrated to mildly suppress the RNAi pathway *in vivo*, but the biological significance of this suppression requires more detailed studies [116]. Based on the high abundance of sfRNA in infected arthropod cells, and the conservation of sfRNA in most, if not all, flavivirus genomes known to date [105], including those restricted to replication in arthropods, we hypothesize that sfRNA is a crucial factor for flavivirus transmission by the arthropod vector.

In this study the importance of sfRNA for dissemination and transmission of WNV by *Culex pipiens* mosquitoes was investigated. By using a lineage II WNV infectious clone deficient in sfRNA formation, we investigated replication kinetics in mammalian cells and both RNAi-competent and -deficient mosquito cell lines. The putative role of sfRNA in modulating the RNAi response *in vivo* was determined by a deep sequencing approach of viral small RNAs. Notably, the importance of sfRNA in WNV infection and transmission by *Culex* mosquitoes was studied *in vivo* and the role of the midgut epithelium was assessed by comparison of infections via the blood meal and via intrathoracic injections. The outcomes of this research highlight the importance of sfRNA for flavivirus transmission by mosquitoes and provide a biological explanation as to why sfRNA production is strictly conserved in the *Flavivirus* genus.

Results

An sfRNA1-deficient clone of WNV is not attenuated *in vitro*

WNV mutants that are deficient in the formation of full-length sfRNA (sfRNA1) cause progressively less cytopathic effect (CPE) in cell culture when only shorter and/or less abundant sfRNA species can be produced [106,322]. To study the effects of sfRNA on transmission independent of effects on replication and cytopathicity, we used a mutant that is only deficient in sfRNA1. This mutant has previously been shown to have little effect on the replication rate and cytopathicity of WNV, as opposed to sfRNA1+2 deficient mutants which are attenuated in both insect and vertebrate cell lines [106,112,322]. An infectious clone of WNV (WNV_{ic}) was compared to an sfRNA1-deficient infectious clone of WNV (WNVΔSF1), that was generated by mutating the

top loop of SL-II (Fig. 6.1A). The wild type virus (WNV_{GR}) was used for comparison in the growth curve and cell viability experiments. As expected, Northern blot analysis on RNA isolated from Vero cells using a 3'UTR-specific probe indicated that WNV_{IC} predominantly produced sfRNA1. Conversely, WNVΔSF1 only produced sfRNA2 as a result of mutated SL-II, resulting in XRN1 stalling on SL-IV (Fig. 6.1B). An unexpected additional band was observed for WNV_{IC} between sfRNA1 and sfRNA2, which was not produced in cells infected with WNVΔSF1 (Fig. 6.1B). Comparison with the flaviviruses Usutu virus (USUV) and WNV New York 1999 (WNV_{NY99}) demonstrated that the size of full-length sfRNA from WNV_{IC} was similar, confirming that the top band is sfRNA1 (data not shown). One-step growth curves in Vero cells demonstrated that WNVΔSF1 had growth kinetics similar to those of WNV_{IC} and the wild type WNV_{GR} virus isolate (Fig. 6.1C). In addition, WNVΔSF1 caused a comparable level of CPE comparable to that of WNV_{GR} and WNV_{IC}, as observed by a steady decrease in cell viability between 1 and 3 dpi (Fig. 6.1D). These results validate WNVΔSF1 as a model to study the effects of sfRNA without interference from altered cytopathicity and/or replication.

Fig. 6.1 Generation and characterization of an sfRNA1-deficient WNV. (A) 2D RNA structure model of the 3'UTR/sfRNA of WNV_{GR}. Pseudoknot interaction is indicated by black lines. The nucleotides mutated in WNVΔSF1 are shaded. (B) Northern blot of RNA harvested from Vero cells infected with WNV_{IC}, WNVΔSF1 or mock treated. Numbers at right are kilobases. (C) Vero cells were infected with WNV_{GR}, WNV_{IC} or WNVΔSF1 at a multiplicity of infection (MOI) of 1. Supernatants were taken at 0, 24, 48, and 72 hours post-infection (hpi) and titrated by end-point dilution assay. Shown are the mean titers of two independent experiments $\pm$ standard error of the mean. Statistics were done using a two-way-ANOVA. (D) Vero cells were infected with WNV_{GR}, WNV_{IC} or WNVΔSF1 at an MOI of 5 and at the indicated time-points the cell viability was measured by CellTiter-Glo assay.

An sfRNA1-deficient WNV replicates efficiently in RNAi-competent mosquito cells

To investigate whether sfRNA1 is required for replication of WNV in mosquito cells, one-step virus growth curves were performed on *Cx. tarsalis* Cx.t, *Aedes albopictus* U4.4 and Dicer2-deficient *Ae. albopictus* C6/36 cells [230]. WNV_{GR}, WNV_{IC} and WNVΔSF1 replicated similarly in all three mosquito cell lines, with maximum titers reaching $\sim 4 \times 10^8$ tissue culture infectious dose 50% (TCID₅₀)/ml at 3 days post-infection (dpi) in U4.4 cells and $\sim 1 \times 10^8$ TCID₅₀/ml at 4 dpi in both C6/36 and Cx.t cells (Fig. 6.2A), however WNVΔSF1 reached slightly lower titers at 3 and 4 dpi in U4.4 and 4 dpi in C6/36 cells. These results indicate that sfRNA1 is not required for efficient WNV replication in mosquito cells, consistent with the observations in mammalian cells (Fig. 6.1C). Furthermore, the similar virus titers in RNAi-competent Cx.t and U4.4 cells as compared to the RNAi-deficient C6/36 cells indicate that there is no obvious effect of an active RNAi response on either WNV_{IC} nor WNVΔSF1 virus production. Northern blot analysis indicated that abundant levels of sfRNA1, but low levels of sfRNA2 are produced in WNV_{IC} infected U4.4, C6/36 and Cx.t cells (Fig. 6.2B). However, cells infected with WNVΔSF1 produced high amounts of sfRNA2 and much lower levels of sfRNA1, particularly in the Cx.t cells. Quantification of sfRNA1 and sfRNA2 signal intensities demonstrated an sfRNA1/sfRNA2 ratio of ~ 3 for WNV_{IC} versus 0–0.4 for WNVΔSF1 infected cells (Fig. 6.2C). These results indicate that WNVΔSF1 is largely defective in sfRNA1 production and can successfully replicate in RNAi-competent mosquito cells.

Fig. 6.2 One-step growth curves and sfRNA production in mosquito cells. (A) *Ae. albopictus* U4.4 and C6/36 and *Cx. tarsalis* Cx.t cells were infected with WNV_{GR}, WNV_{IC} or WNVΔSF1 at a multiplicity of infection of 1. Supernatants were taken at 0, 1, 2, 3 and 4 days post-infection (dpi) and titrated by end-point dilution assay. Shown is the mean titer $\pm$ standard error of the mean (SEM) from two independent experiments. Statistics were done using a two-way-ANOVA, * = $P < 0.05$. (B) Northern blot of RNA isolated 4 dpi from U4.4, C6/36 or Cx.t cells using a 3'UTR specific probe. Numbers at right are kilobases. (C) Mean signal ratio of sfRNA1/sfRNA2. Shown are the mean results $\pm$ SEM from two independent experiments. Statistics were performed using a two-tailed unpaired t-test.

Viral siRNAs derived from the 3'UTR are produced in North-American and north-western European *Cx. pipiens* mosquitoes infected with WNV_{GR}

While sRNA suppresses reporter-based RNAi in mosquito cells [115,117], and *in vivo* in mosquitoes and interacts with the RNAi machinery in mammalian cell lines [116], it is still unclear whether during an *in vivo* infection in mosquitoes the 3'UTR/sRNA is processed by the RNAi machinery. To investigate if WNV_{GR} 3'UTR-derived small RNAs are produced in *Cx. pipiens* mosquitoes, we analysed the vsRNA profiles generated in a set of independent experiments, with WNV_{GR} in north-western European (NWE) and North-American (NA) *Cx. pipiens* strains (Fig. 6.3) [155]. Size distributions of vsRNAs showed that 21 nt viral small interfering (vsi)RNAs were highly abundant and no obvious >25 nt vsRNAs were produced (Fig. 6.3A), indicating that no WNV-specific piRNAs are produced in *Cx. pipiens* mosquitoes. A small shoulder of 25-32 nt sRNAs was present, however these sRNAs did not have the 10A-biased piRNA signature [155]. Hot- and coldspot analysis of the 21 nt vsRNAs along the WNV genome indicated that vsiRNAs were produced across the whole length of the genome (Fig. 6.3B). NA mosquitoes produced smaller amounts of vsiRNAs and mapped differentially across the genome, indicating that different mosquito populations can influence the sRNA profile. Detailed hot-coldspot analysis on the viral 3'UTR showed that in both NWE and NA mosquitoes two hotspots were present in SL-III and the 3'SL, respectively, while NA mosquitoes also displayed a hotspot in SL-IV (Fig. 6.3C). These results show that the 3'UTR of the WNV_{GR} gRNA is targeted by the RNAi machinery and processed into 21 nt vsRNA, indicating that sRNA may be processed by the RNAi machinery *in vivo*.

◀ **Fig. 6.3 (Previous page) Deep sequencing of siRNAs from WNV_{GR} infected North-American and north-western European mosquitoes that target the 3'UTR.** (A) Size distributions of 15-32 nt small RNAs mapping to the viral genome of WNV_{GR}, normalized to the total number of clipped reads in the library. (B) 21 nt small RNA reads were aligned to the viral genome and normalized to the total number of clipped reads in the library. Positive strand reads are displayed in black and negative strand reads in grey. (C) Detailed view of the 21 nt small RNA distribution on the 3'UTR. Black arrows indicate the vsiRNA hotspots that targets the 3'UTR in both mosquito colonies.

Cx. pipiens mosquitoes mount an active RNAi response against wild type and sfRNA-deficient WNV

Our experiments clearly show that an sfRNA-deficient mutant replicates efficiently in RNAi-competent mosquito cells, although the 3'UTR appears to be processed by the RNAi machinery *in vivo*. To investigate if sfRNA is a substrate for Dicer and/or influences the mosquito RNAi response *in vivo*, small RNA populations of 19-24 nts were sequenced from pools ($n > 12$) of WNV_{IC} or WNVΔSF1 blood meal infected mosquitoes at 14 dpi (Fig. 6.4). RNAs larger than 25 nts were excluded from deep sequencing since it was previously shown that *Culex* mosquitoes do not produce 25-30 nt vpiRNAs (Fig. 6.3A) [155,165]. To rule out potential effects of differentiation between the two mosquito pools, we first verified the virus titer and vgRNA levels at 14 dpi. Virus titers in the two pools of mosquitoes were marginally different, with a slightly but not significantly higher titer for WNV_{IC} (1.1×10^6 vs. 6.3×10^5 TCID₅₀/ml, $P = 0.272$, Fig. 6.4A), while qPCR on the vgRNA demonstrated that the number of viral genome copies were similar (Fig. 6.4B). Northern blot analysis on total RNA isolated from pools of WNV-positive mosquitoes confirmed that also in mosquitoes WNV_{IC} predominantly produced sfRNA1 while WNVΔSF1 only produced sfRNA2 (Fig. 6.4C). The vsRNA size distribution was very similar in WNV_{IC} and WNVΔSF1 infected mosquitoes (Fig. 6.4D & E). A distinctive peak of 21 nt vsRNAs was observed for both WNV_{IC} and WNVΔSF1 (Fig. 6.4D & E). This 21 nt vsRNA population was present on both the (+) and (-) strands, indicating that they can be regarded as vsiRNAs. WNV_{IC} produced slightly higher total levels of vsiRNAs than WNVΔSF1 (Fig. 6.4D), although when normalized to the number of viral reads (Fig. 6.4E) there was no difference in the percentage of 21 nt reads. This indicates that the difference in percentage of total reads can be explained by the slightly evaluated titer of WNV_{IC} although the number of viral genome copies were similar (Fig. 6.4A & B). Next, we investigated whether sfRNA affected the genome distribution of vsRNAs by mapping the 21 nt reads along the vgRNA for the (+) (Fig. 6.4F) and (-) strand (Fig. 6.4G). The vsiRNA genome distributions were highly similar between WNV_{IC} and WNVΔSF1 on both the (+) and (-) strands, as illustrated by the mirrored hot-coldspot plots (Fig. 6.4F & G), providing no direct evidence that sfRNA1 interferes with the antiviral RNAi response *in vivo*.

SfRNA-derived vsiRNAs should be (+) strand biased due to the nature of sfRNA biogenesis [106]. Indeed, two distinct hotspots were identified that occur on the (+) strand in WNV_{IC} infected mosquitoes, but not on the (+) strand of WNVΔSF1 infected mosquitoes (Fig. 6.4H) nor on the (-) strands of both viruses were identified (Fig. 6.4I). Hotspots 10642 and 10976 were present only in WNV_{IC} infected mosquitoes and not in mosquitoes infected with WNVΔSF1, indicating that the vsiRNAs from these hotspots are derived from sfRNA1. Interestingly, these sfRNA-specific hotspots co-occurred with predicted secondary structures of SL-III (10,642) and the 3'SL (10,976) (Fig. 6.4H & 6.5A). Finally, KUN-miR-1, previously discovered *in vitro* [325], was also found at position 11,030, indicating that KUN-miR-1 could have a potential function *in vivo* in mosquitoes. These results show for the first time that sfRNA is processed by the mosquito RNAi machinery and that an sfRNA1-defective WNV mutant has a differential vsiRNA profile in the viral 3'UTR.

Fig. 6.4 Small RNA sequences of WNV_{IC} or WNVΔSF1 blood fed mosquitoes. (A) WNV titers of mosquito bodies used for small RNA sequencing. Statistics were performed using an unpaired t-test. The error bars indicate standard error of the mean. (B) WNV genome copies in RNA samples used for small RNA sequencing were determined by qRT-PCR. (C) Northern blot from the same RNA sample that was used for deep sequencing using a 3'UTR-specific probe. Numbers at the right are kilobases (D/E) Size distributions of small RNA reads mapping to the viral genome of WNV_{IC} or WNVΔSF1 blood fed mosquitoes, normalized to (D) the total number of reads or (E) the total number of viral reads in the library. (F/G) The 5' ends of the reads were aligned to the viral genome and normalized against the number of viral reads. (F) Positive-strand and (G) negative-strand reads were mapped for WNV_{IC} and WNVΔSF1. (H/I) Detailed view of the 21 nt RNA distribution on the 3'UTR for the (H) positive strand and (I) the negative strand. Black arrows indicate siRNA hotspots that are sfRNA-specific. The open arrow indicates the presence of Kun-miR-1.

3D modelling of WNV sRNA reveals SL-III and the 3'SL as accessible dsRNA substrates

Our small RNA deep sequencing data clearly shows that sRNA is processed by the RNAi machinery into vsiRNAs, but does not explain why vsiRNA hotspots occur in certain RNA structures. To better understand why some regions of the 3'UTR/sRNA are preferentially targeted by RNAi, a 3D-model of the WNV_{GR} 3'UTR/sRNA was created using RNAComposer [326] (Fig. 6.5). Each secondary structure was colour coded and the two most significant vsiRNA hotspots were coloured in green (Fig. 6.5A). The pseudoknot interactions in the XRN1/Pacman stalling structures SL-II and SL-IV result in very tight tertiary folding (Fig. 6.5B), potentially making these structures inaccessible to processing by host nucleases. In contrast, SL-III sticks out of the 3'UTR coil and could thereby present an accessible dsRNA substrate for Dicer and/or other nucleases. Similarly, tight tertiary folding occurs for the dumbbell RNA structures DB1 and DB2 (Fig. 6.5C), which also display tight folding via pseudoknot interactions with the main coil of the 3'UTR/sRNA. The 3'SL, however, sticks out of the 3'UTR coil and could thereby also present an accessible dsRNA substrate. These results provide insight into the tertiary fold of the 3'UTR/sRNA and explain why vsiRNAs are mostly derived from SL-III and the 3'SL.

Fig. 6.5 Three-dimensional RNA structure model of sRNA reveals that SL-III and the 3'SL are more accessible to the mosquito RNAi machinery. (A) Schematic presentation of the WNV_{GR} sRNA secondary RNA structure with projections of the vsiRNA hotspots. Pseudoknot interactions are indicated by black lines and pseudoknot-interacting bases are coloured in red. SRNA-specific vsiRNAs are indicated in green and each SL and DB was given a separate colour. (B/C) 3D RNA structure model of (B) the first 240 and (C) the last 288 nts of WNV sRNA. Colours match the colour scheme of the secondary structures in panel A.

sRNA1 determines WNV transmission by mosquitoes

The conservation of sRNA production in all flaviviruses suggests an important biological function in both mammalian and insect hosts. Importantly, the sequence of the flaviviral 3'UTR can be phylogenetically divided based on the virus vector species [323], indicating a role for sRNA in infection and/or transmission of the arthropod vector. To investigate if sRNA is important for dissemination and transmission of WNV, *Cx. pipiens* mosquitoes were allowed to feed for 1 h on a blood meal containing either WNV_{IC} or WNVΔSF1. After 14 days the titer of WNV in the mosquito bodies was determined by EPDA and the presence of WNV in the saliva was determined by inoculating Vero cells (Fig. 6.6A). Importantly, the infection rate, as determined by the percentage of engorged mosquitoes with a measurable virus titer, was significantly lower for WNVΔSF1 than for WNV_{IC} (13% vs 32%, $P < 0.001$, Fig. 6.6B, Table 6.1).

In addition, the transmission rate, as determined by the percentage of saliva-positive mosquitoes out of the total number of engorged mosquitoes, was significantly lower for mosquitoes infected with WNVΔSF1 than for WNV_{IC} (10% vs 26%, $P < 0.001$, Fig. 6.6B, Table 6.1), indicating that sRNA1 production was important for transmission when mosquitoes were orally infected. Dissemination rates, as determined by the percentage of saliva-positive mosquitoes out of the number of virus-positive mosquitoes was not affected by sRNA1 (82% vs. 79%, $P = 1.000$, Fig. 6.6B, Table 6.1). This indicates that sRNA1 mainly affects infection of the midgut and consequently increases the transmission rate. The viral titers of mosquito bodies that were positive for the presence of virus by EPDA were not significantly different between WNV_{IC} and WNVΔSF1 ($P = 0.360$, Fig. 6.6C). However, the viral titers in the saliva were significantly higher for WNV_{IC} infected as compared to WNVΔSF1 infected mosquitoes (2.2×10^5 vs. 8.0×10^3 TCID₅₀/ml, $P < 0.01$, Fig. 6.6C). These results suggest that sRNA1 determines WNV transmission by increasing the overall infection rates and by boosting viral titers in the mosquito saliva.

Table 6.1 Four replicate blood feeding experiments demonstrate that sRNA determines the infection and transmission rates of WNV in *Cx. pipiens* mosquitoes.

Infection, transmission and dissemination rates of orally infected *Cx. pipiens* mosquitoes with either WNV_{IC} or WNVΔSF1. In addition, the sample size (n) as the number of engorged females after a blood meal, absolute numbers of positive bodies and saliva samples are shown for each replicate. Rep. replicate.

Virus	Rep.	n	Positive bodies	Positive saliva	Infection (%)	Transmission (%)	Dissemination (%)
WNV _{IC}	1	33	11	11	33	33	100
	2	39	20	14	51	36	70
	3	24	5	4	21	17	80
	4	44	9	8	20	18	89
	Total	140	45	37	32	26	82
WNV ΔSF1	1	37	8	6	22	16	75
	2	40	3	3	8	8	100
	3	23	2	2	9	9	100
	4	49	6	4	12	8	67
	Total	149	19	15	13	10	79

Fig. 6.6 Transmission and infection rates of WNV_{IC} and WNVΔSF1 in *Cx. pipiens* mosquitoes after an infectious blood meal. (A) Schematic overview of the experimental procedure. (B) Infection (Bodies), transmission (Saliva) and dissemination (Saliva/Bodies) rates of *Cx. pipiens* mosquitoes infected through an infectious blood meal containing WNV_{IC} (n=140) or WNVΔSF1 (n=149). Infection, transmission and dissemination rates are presented as percentages of the total number of mosquitoes. Data represents cumulative numbers from four independent experiments. Statistics were performed using Fisher exact tests. ns, not significant. (C) Titers of WNV-positive mosquito bodies and saliva samples of WNV_{IC} or WNVΔSF1 blood meal infected mosquitoes. Shown are the mean titers ± standard error of the mean. Statistics were performed using a two-tailed unpaired Mann-Whitney U test.

SfRNA1 is a key driver to overcome the mosquito midgut infection barrier

The infection rate of mosquitoes and the subsequent dissemination to the salivary glands are important parameters for arbovirus transmission [100]. Clearly, WNVΔSF1 displayed significantly reduced transmission rates, which could be the result of 1) reduced virus dissemination through an effect of sfRNA1 on a post-midgut barrier or 2) the consequence of reduced initial infection rates. Although our data suggests that the main effect of sfRNA occurs in the midgut, we discriminated between these two possibilities by intrathoracic injections of *Cx. pipiens* mosquitoes with 7×10^3 TCID₅₀ units of either WNV_{IC} or WNVΔSF1. In this way, the midgut barriers are bypassed to allow the study of post-midgut virus dissemination and transmission. At 14 dpi an infection rate of 100% was achieved for mosquitoes injected with either WNV_{IC} or WNVΔSF1 (Fig. 6.7A, Table 6.2), suggesting that both viruses are equally capable to establish a systemic, fully disseminated infection when the midgut infection barrier is bypassed. Next, the saliva of all mosquitoes was tested for the presence of WNV. There was no significant difference in transmission rates (94% vs. 90%, $P = 0.696$, Fig. 6.7A, Table 6.2) or dissemination rates (94% vs. 90%, $P = 0.696$, Fig. 6.7A) between WNV_{IC} and WNVΔSF1, despite a significant difference in average body titers (WNV_{IC}:

2.4×10^7 , WNV Δ SF1: 4.4×10^6 , $P < 0.001$, Fig. 6.7B). Titration of the mosquito saliva showed no significant differences (WNV_{IC}: 4.4×10^5 , WNV Δ SF1: 1.0×10^6 , $P = 0.873$). Additionally, at 1, 6 and 14 dpi, no significant difference between the transmission rates of WNV_{IC} and WNV Δ SF1 was observed (1 dpi 14% vs. 11%, $P = 1.000$; 6 dpi 100% vs. 100%, $P = 1.000$; 14 dpi 100% vs. 86%, $P = 0.492$; Fig. 6.7C). To rule out the possibility that the lack of difference between WNV_{IC} and WNV Δ SF1 was due to a too high viral dose, we repeated the experiment with a 10-fold lower injection dose of 7.7×10^2 TCID₅₀ per mosquito and scored the infection and transmission rates at an earlier time point of 7 dpi (Fig. 6.7D). Again, no difference between WNV_{IC} and WNV Δ SF1 was observed in either the infection or transmission rates, indicating that there is no clear post-midgut effect of sRNA1 on transmission. These results demonstrate that sRNA1 does not play a determining role in post-midgut dissemination to the salivary glands. Instead, sRNA1 is vital to efficiently overcome the midgut infection and/or escape barriers resulting in enhanced virus dissemination and subsequent transmission.

Fig. 6.7 Intrathoracic injections of *Cx. pipiens* mosquitoes with WNV_{IC} and WNV Δ SF1. (A) Infection (Bodies), transmission (Saliva) and dissemination (Saliva/Bodies) rates of WNV_{IC} (n=34) and WNV Δ SF1 (n=50) in *Cx. pipiens* mosquitoes after intrathoracic injections with 7.7×10^3 TCID₅₀ per mosquito. Data represents cumulative numbers from three independent experiments. Statistics were performed using Fisher exact tests. (B) Titers of WNV-positive mosquito bodies and saliva samples of mosquitoes injected with WNV_{IC} or WNV Δ SF1. Shown are the mean titers $\pm$ standard error of the mean. Statistics were performed using a two-tailed unpaired Mann-Whitney U test. (C) Transmission rates in *Cx. pipiens* mosquitoes of WNV_{IC} or WNV Δ SF1 after intrathoracic injection at 3, 6 and 14 days post-infection (dpi). Statistics were performed using Fisher exact tests. (D) Infection and transmission rates at 7 dpi in *Cx. pipiens* mosquitoes after intrathoracic injection of a low dose (7.7×10^2 TCID₅₀ vs. 7.7×10^3 TCID₅₀) of WNV_{IC} or WNV Δ SF1. Statistics were performed using Fisher exact tests. ns, not significant.

Table 6.2 Three replicate intrathoracic injection experiments indicate no effect of sRNA on post-midgut dissemination of WNV in *Cx. pipiens* mosquitoes. Infection, transmission and dissemination rates of *Cx. pipiens* mosquitoes that were intrathoracically injected with either WNV_{IC} or WNVΔSF1. In addition, the sample size (n), absolute numbers of positive bodies and saliva samples are shown for each replicate. Rep. replicate.

Virus	Rep.	n	Positive bodies	Positive salivas	Infection (%)	Transmission (%)	Dissemination (%)
WNV _{IC}	1	15	15	15	100	100	100
	2	13	13	11	100	85	85
	3	6	6	6	100	100	100
	Total	34	34	32	100	94	94
WNVΔSF1	1	19	19	17	100	89	89
	2	21	21	18	100	86	86
	3	10	10	10	100	100	100
	Total	50	50	45	100	90	90

An sRNA1+2-deficient WNV has attenuated replication in *Culex* cells and decreased transmission by *Culex* mosquitoes

We showed that sRNA1 is required for successful passing of the mosquito midgut barrier, but WNVΔSF1 still produces the shorter sRNA2. To investigate whether combined abrogation of both sRNA1 and sRNA2 production would further attenuate WNV, we generated an infectious clone with mutations in both SL-II and SL-IV (WNVΔSF1+2; Fig. 6.8A). The mutation introduced in SL-IV resides in the conserved three-way-junction that was previously shown to be important for XRN1 resistance [107]. Northern blot analysis of RNA isolated from C6/36 mosquito cells infected with WNV_{IC} or WNVΔSF1+2 demonstrated that the double mutant indeed fails to produce sRNA1 and sRNA2 (Fig. 6.8B). The effect of sRNA1+2 deletion on replication was tested by performing growth curves on Cx.t and Vero cells with WNV_{IC} and WNVΔSF1+2. No difference in replication was observed in Vero cells, although WNVΔSF1+2 presented slightly lower titers (Fig. 6.8C). However, in Cx.t cells, WNVΔSF1+2 was severely attenuated in virus growth, indicating that sRNA is important for successful replication in *Culex* (Fig. 6.8D). Since we showed that deletion of sRNA1 production results in decreased transmission rates in *Cx. pipiens* after an infectious blood meal, we hypothesized that combined abrogation of sRNA1 and sRNA2 production further attenuates WNV in mosquitoes. To test this hypothesis, *Cx. pipiens* mosquitoes were given an infectious blood meal with 5.0×10^6 TCID₅₀/ml of either WNV_{IC} or WNVΔSF1+2. The titer of this blood meal was slightly lower than in the previous experiments due to a lower plateau titer of WNVΔSF1+2 in C6/36 cells. WNV_{IC} infected mosquitoes reached an infection rate of 16% and a ~14% transmission rate, consistent with the data in Fig. 6.6. However, the WNVΔSF1+2 blood fed mosquitoes reached significantly lower infection (2%, $P = 0.004$) and transmission (2%, $P = 0.04$) rates, indicating that sRNA1 and sRNA2 are vital for infection of mosquitoes. Sequencing of the 3'UTR of the WNVΔSF1+2 infected mosquitoes showed that the mutations in SL-II and SL-IV were still present, indicating that no reversions occurred (data not shown). In conclusion, these results provide the first evidence that sRNA plays an important role in mosquito transmission of WNV, which could underpin the strict conservation of sRNA production by flaviviruses.

Fig. 6.8 Transmission and infection rates of *Cx. pipiens* infected with WNV_{IC} and WNVΔSF1+2. (A) 2D RNA structure model of SL-II and SL-IV of WNV_{GR}. Pseudoknot interaction is indicated by black lines. Nucleotides mutated in WNVΔSF1+2 are shaded. Arrows indicate the starting point of sfRNA1 and sfRNA2. (B) Northern blot of RNA harvested from C6/36 cells infected with WNV_{IC} or WNVΔSF1+2. (C) Vero or (D) Cx.t cells were infected with WNV_{IC} or WNVΔSF1+2 at a multiplicity of infection of 1. Supernatants were taken at 0, 1, 2, 3, and 4 days post-infection (dpi) and titrated by end-point dilution assay. Shown are the mean titers of two replicate samples $\pm$ standard error of the mean. (E) Infection (Bodies), transmission (Saliva) and dissemination (Saliva/Bodies) rates of *Cx. pipiens* mosquitoes infected through an infectious blood meal containing WNV_{IC} (n=73) or WNVΔSF1+2 (n=83). Infection, transmission and dissemination rates are presented as percentages of the total number of engorged female mosquitoes. Data represents cumulative numbers from three independent experiments. Statistics were performed using Fisher exact tests.

Table 6.3 Three replicate blood feeding experiments demonstrate that deficiency in *sRNA1* and *sRNA2* results in decreased WNV infection and transmission rates in *Cx. pipiens*. Infection, transmission and dissemination rates of orally infected *Cx. pipiens* mosquitoes with either WNV_{IC} or WNVΔSF1+2. In addition, the sample size (n) as the number of engorged females after a blood meal, absolute numbers of positive bodies and saliva samples are shown for each replicate. Rep. replicate.

Virus	Rep.	n	Positive bodies	Positive saliva	Infection (%)	Transmission (%)	Dissemination (%)
WNV _{IC}	1	23	8	7	35	30	88
	2	28	3	2	11	7	67
	3	22	1	1	5	5	100
	Total	73	12	10	16	14	83
WNV ΔSF1+2	1	18	0	0	0	0	0
	2	36	2	2	6	6	100
	3	29	0	0	0	0	0
	Total	83	2	2	2	2	100

Discussion

Flavivirus epidemics occur as a result of a complex three-way interplay between virus, host and vector and are influenced by a range of environmental factors. Where and when flavivirus outbreaks will occur cannot be readily predicted, but it is clear that the ability of a mosquito population to transmit a certain flavivirus is essential for efficient viral spread from one vertebrate host to another. This vector competence is primarily dictated by the specific virus-vector combination, yet the viral determinants of vector competence are not well defined. For WNV₀₂ it has been described that a single amino acid change in the envelope glycoprotein E resulted in increased mosquito transmission rates, as compared to the original NY99 strain [327]. This example illustrates that flaviviral products (*i.e.* viral proteins, viral RNA) may determine vector competence by overcoming infection barriers to establish a transmissible infection in the mosquito [118].

Here, we have identified a novel determinant of WNV vector competence, a noncoding viral RNA whose abundant production in infected cells is widely conserved in the large group of flaviviruses. This sRNA, as well as the flavivirus 3'UTR it is derived from, contains various conserved RNA structures that may influence virus transmission by the arthropod vector [105,328]. SfRNA production is highly conserved amongst all members of the genus *Flavivirus*, including the NKVFs, ISFs, TBFs and MBFs [105,106,113]. Despite the many functions of sRNA described in mammalian cells, the biological significance for sRNA production in the arthropod vector has remained unknown [105]. We now demonstrate that sRNA determines WNV transmission by *Cx. pipiens* mosquitoes. An sRNA1-deficient WNV mutant, WNVΔSF1, was attenuated in the mosquito and had significantly decreased infection and transmission rates. A mutant deficient in sRNA1 and sRNA2, WNVΔSF1+2, was also attenuated for replication in *Culex* cells and demonstrated decreased transmission and infection rates in *Cx. pipiens* mosquitoes after an infectious blood meal. From these studies we conclude that sRNA is produced abundantly in the mosquito to facilitate efficient transmission to the next host.

It has been firmly established that infection of and escape from midgut epithelial cells present crucial barriers for arbovirus transmission (recently reviewed in [142]). Our results show that sfRNA affects the transmission rate at the level of the mosquito midgut, since WNV transmission was negatively affected by sfRNA deficiency when the virus was administered through an infectious blood meal, but not after intrathoracic injection. After administration through intrathoracic injections, the infection and transmission rates between WNV_{IC} and WNVΔSF1 were not significantly different at all tested virus doses and time points, although we did observe a slight difference in overall virus titer in the mosquito bodies. This may be an indication of a potential additional post-midgut effect of sfRNA on virus dissemination, which is supported by the lower titers in the saliva for WNVΔSF1 after infection via an infectious blood meal. However, the transmission rates of wild type and sfRNA-defective WNV in the injection experiment were always close to 100%, and no significant difference in virus titer in the saliva was observed.

Several intracellular mechanisms could be involved in establishing the mosquito midgut barrier against arbovirus infection, such as RNAi, Toll/IMD/JAK/STAT signalling pathways or apoptosis. The importance of antiviral RNAi in midgut cells for virus transmission and population diversity has convincingly been shown in *Culex* mosquitoes [104,165]. We aimed to determine the strength of the mosquito RNAi response by analysing the vsiRNA reads relative to the viral genome in a direct comparison between WNV_{IC} and WNVΔSF1. The abundance of vsiRNA is dependent on i) the efficiency of the putative viral RNAi suppressor function of sfRNA and ii) the amount of available viral dsRNA substrate in the mosquito body, which is a function of viral RNA replication and thus dependent on the success of RNAi suppression. In our study we found no direct correlation between the expression of sfRNA1 and the abundance of (v)siRNAs produced *in vivo*. We cannot formally exclude the possibility that sfRNA1 facilitates WNV replication by RNAi suppression at an early stage, so that later more viral dsRNA can be processed into vsiRNA, but the similar levels of WNV_{IC} and WNVΔSF1 viral genomic RNA in infected mosquitoes do not support this.

The effect of sfRNA on midgut-infection most likely occurs during initial infection, which takes place in a small number of cells [136]. This 'single cell' interaction requires very high-resolution sequence data to detect a potential RNAi suppression by sfRNA, which can explain why we did not find this correlation in our experiments. Sequencing of individual midguts of WNV_{IC} and WNVΔSF1 blood fed mosquitoes could provide more detail on the mechanism of interplay between the RNAi response and sfRNA production in the mosquito midgut. A recent study showed that sfRNA mildly suppressed the dsRNA-induced silencing in *Cx. quinquefasciatus* mosquitoes infected with Kunjin virus [116]. The silencing suppression was demonstrated to be significant, although the difference between the wild type and the sfRNA deficient virus was not large. Since complete knockdown of RNAi is associated with increased mosquito mortality upon arbovirus infection, it is quite possible that sfRNA only mildly modulates the RNAi response to facilitate virus transmission without negatively affecting mosquito fitness [116]. Thus, because vsiRNA abundance cannot provide conclusive evidence for RNAi suppression by sfRNA, we reasoned that analysing the distribution of the vsiRNA on the viral genome would be more informative to reveal potential differences between WNV_{IC} and WNVΔSF1. However, sfRNA also did not affect the genome distribution of vsiRNAs on either the (+) or (-) strand of the WNV genome. Despite the clear importance of sfRNA in WNV transmission by the mosquito, WNVΔSF1 replicated equally efficient in RNAi-competent Cx.t and U4.4 cells, suggesting that the observed effect of sfRNA on transmission *in vivo* may be independent of the RNAi response.

SfRNA was previously demonstrated to be a substrate for Dicer *in vitro* and able to suppress dsRNA- and short hairpin RNA-induced RNAi silencing in *Ae. albopictus* U4.4 and *Drosophila melanogaster* S2 cells [115]. Detailed analysis of the vsiRNAs that mapped to the 3'UTR of WNV_{IC} and WNVΔSF1 now show for the first time that sfRNA is processed into vsiRNAs *in vivo* in mosquitoes, as demonstrated by two sfRNA-specific hotspots in the 3'UTR. The hotspot in SL-III of the 3'UTR was consistently found in independent experiments in two different mosquito colonies. However, the same hotspot was not found in USUV infected *Cx. pipiens* mosquitoes [155] (data not shown), even though SL-III is present in the 3'UTR of USUV. Analysis of the WNV_{GR} 3'UTR sequence by Vmir did not predict either of the two hotspots as potential miRNAs, while it did predict the presence of the previously discovered KUN-miR-1 that is derived from the 3'SL [325,329,330] (data not shown). Additionally, alignment of the SL-III region from the 3'UTR of several flaviviruses from the Japanese encephalitis virus (JEV) serogroup (including WNV, USUV, JEV and Kunjin virus) showed that the sequence of this hotspot is poorly conserved (data not shown). This indicates that the SL-III derived viral small RNA is unlikely to have an important biological function as a miRNA/siRNA, but leaves open the possibility that processing of SL-III by Dicer may ultimately have an effect on virus dissemination in the mosquito midgut, *e.g.* via an RNA decoy mechanism. The hotspot in the 3'SL only occurred in WNV_{IC} infected mosquitoes and not in WNVΔSF1 infected mosquitoes, indicating that this small RNA is derived from sfRNA1 even though sfRNA2 also contains the 3'SL and is produced by WNVΔSF1. Perhaps the absence of sfRNA1 decreases the amount of substrate to be processed into small RNAs. Alternatively, the different folding of the shorter sfRNA2 as compared to sfRNA1 results in different processing by the RNAi machinery.

When the 3D RNA structure of sfRNA is modelled, both SL-III and the 3' SL are clearly sticking out of the compact fold of the predicted sfRNA tertiary structure. These exposed stem loops could potentially make them easily accessible substrates for Dicer and/or other host nucleases. Further studies are required to fully understand the molecular interplay of sfRNA, and the RNA structures within this molecule, with RNAi and the relative importance of this interaction for flavivirus transmission *in vivo*.

Our finding that sfRNA is very important in mosquito infection is in line with recent research that studied the mutation rate of flavivirus 3'UTR RNA sequences in the insect host. It was demonstrated for DENV2 that the XRN1/Pacman resistant RNA structures SL-I and SL-II (equivalent to WNV SL-II and SL-IV, respectively) evolve differently depending on the origin of the host cell. In mosquito cells, SL-II incorporated mutations very rapidly compared to the upstream SL-I, which was conserved in mosquito cells but mutated more frequently during infection of mammalian cells [324]. This suggests that the XRN1/Pacman-resistant SL-I is under strong selective pressure to be maintained in the insect host, most likely to safeguard production of sfRNA1. Consistent with this notion, it was recently described that sfRNA plays a role in flavivirus transmission during a DENV epidemic. A DENV clade from Puerto Rico was outcompeted by a new clade that produced higher sfRNA:vgRNA ratios, indicating that sfRNA production correlates with increased epidemiological fitness [109]. Our results support and extend this hypothesis by demonstrating for the first time that sfRNA is required for successful flavivirus transmission by mosquitoes.

In conclusion, we have shown that sfRNA is a key driver to overcome the mosquito midgut infection barrier to establish a transmissible infection. Moreover, we provide novel evidence that sfRNA is processed by the RNAi machinery *in vivo* in mosquitoes. Our results provide the first biological explanation for the highly conserved nature of sfRNA amongst all flaviviruses, foremost those that replicate in mosquitoes,

which may offer novel targets to interfere with the complex flavivirus transmission cycle in nature.

Materials and Methods

Cell culture

Aedes albopictus U4.4 and C6/36 (ATCC CRL-1660) mosquito cells were cultured in Leibovitz L-15 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2% tryptose phosphate broth (Gibco) and 1% nonessential amino acids (Gibco). *Culex tarsalis* Cx.t cells [241] (CDC, Fort Collins) were cultured in Schneider's medium (Gibco) supplemented with 10% FBS. All mosquito cells were maintained as a monolayer in T25 cell culture flasks at 28 °C. African green monkey kidney Vero E6 (ATCC CRL-1586) cells were cultured in Dulbecco's modified Eagle medium containing HEPES (DMEM-HEPES; Gibco) supplemented with 10% FBS, penicillin (100 U/ml; Sigma-Aldrich) and streptomycin (100 µg/ml; Sigma-Aldrich). Cells were maintained as a monolayer in T25 cell culture flasks at 37 °C and 5% CO₂.

Generation of infectious clones and virus stocks

The wild type WNV lineage II isolate used in this study was isolated in south-eastern Europe (Genbank KC496015.1) and is further referred to as WNV_{GR}. The infectious clone of WNV_{GR}, based on the same virus isolate, was supplied by Tamás Bakonyi from the Department of Microbiology and Infectious Diseases, Szent István University, Hungary [331]. The infectious clone is further referred to as WNV_{IC}. A sub clone of the virus was made by PCR amplification of the NS4-3'UTR region with Phusion polymerase (New England Biolabs) using primers FW 5'-TGGCTGAAGTCCCAGGAACGA-3' and RV 5'-TGGAAGTCCGAGCTCATCGCT-3'. The resulting amplicon was cloned into pJET1.2 (Fermentas) and site-directed mutagenesis was used to produce pJET1.2/subGR₁₀ΔSF1 with mutations in the pseudoknot site of SL-II by Phusion PCR using primers FW 5'-GAAGTCACTAGACGGTGCTGCCTGCG-3' and RV 5'-CTAGTGAGCTTCCGGTGGCAGCATTATC-3'. Site-directed mutagenesis on pJET1.2/subGR₁₀ΔSF1+2 with primers FW 5'-CTCTAGTGTGGCACTCTGCGGAGAGTGCAG-3' and RV 5'-CGCAGAGTGCCACACTAGAGTGTGGTCTGAC-3' was used to produce pJET1.2/subGR₁₀ΔSF1+2 with mutations in SL-II and SL-IV. The NS4-3'UTR region of pJET1.2/subGR₁₀ΔSF1 or pJET1.2/subGR₁₀ΔSF1+2 was cloned using SphI-HF (New England Biolabs) and Pfl23II (Fermentas) into WNV_{IC} to produce WNVΔSF1 and WNVΔSF1+2. Virus stocks of WNV_{IC}, WNVΔSF1 and WNVΔSF1+2 were produced by transfecting Vero cell monolayers with 800 ng infectious clone DNA using 2 µl of Lipofectamine 2000 (Invitrogen) in 24-well plates. Three days post transfection (dpt), the supernatant was harvested and used to inoculate a T75 flask of C6/36 cells seeded one day in advance. Six dpi the supernatant of C6/36 cells was harvested and stored at -80°C. All viruses used in this study are from a second passage (P2).

Cell-viability assay

Cell monolayers were washed with 1X phosphate-buffered saline (PBS) and lysed using Passive Lysis buffer (PLB; Promega) for 10 min at RT. Subsequently CellTiter-Glo 2.0 reagent was mixed with the PLB in a 1:1 ratio and incubated in the dark for 10 min at RT before measuring the luminescence using a FLUOstar OPTIMA microplate reader (BMG Labtech).

Virus titrations

TCID₅₀ was determined by EPDA on Vero cells as described previously [154]. Briefly, Vero cell monolayers were detached using Trypsin (Invitrogen) and diluted in DMEM supplemented with 10% FBS, penicillin and streptomycin. 10-fold dilutions were made of the virus samples in culture media and Vero cell suspension (1.0 × 10⁵ cells) was added in a 1:1 ratio. For each dilution, 10 µl was plated in 6-fold into a 60-well Micro-Well plate (Nunc) and scored 3 dpi for presence of WNV either by CPE or immunostaining using anti-E monoclonal antibodies.

Virus growth curves

Monolayers of indicated cell types were infected in a 6-well plate at an MOI of 1 by adding 1 ml of diluted virus to the cells. Cells were incubated in the presence of virus for 2 h and washed three times with 1X PBS. 2 ml of fresh cell culture medium was added and cells were

incubated at either 37 °C with 5% CO₂ (Vero) or 28 °C (mosquito cells). At the indicated time post infection 30 µl of samples of cell culture medium were frozen at -80 °C until further processing. Virus samples were titrated on Vero cells by EPDA.

RNA isolation and Northern blot

Total RNA was isolated from cell monolayers using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. For RNA isolations performed on mosquito bodies, bodies were homogenized in a Bullet Blender using zirconium beads (Next Advance) for 2 min at max speed. 100 µl DMEM was added, samples were centrifuged 2 min at 12,000 g and the pellet was homogenized in 500 µl TRIzol Reagent. An additional 75% ethanol wash step was included to ensure a clean RNA pellet. Isolated RNA was quantified with a Nanodrop UV-photo spectrometer and stored at -80°C. 5 µg total RNA was separated by denaturing gel electrophoresis in 6% polyacrylamide / 7M Urea / 0.5X TBE gel for 1.45 h at 150 V constant voltage. RNA was semi-dry-blotted on Hybond-N membrane (Amersham biosciences) for 60 min at 0.2 A constant amperage, UV cross-linked by 1 min UV-light exposure and hybridized overnight with DIG-labelled probes in modified church/10% formamide buffer at 50 °C. DIG-labelled probes were made by PCR using Phusion polymerase (New England Biolabs) with DIG-labelled nucleotides (Roche) (FW 5'-GCAGTCTGCGACAGTGCC 3'; RV 5'-GTTGTGCAGAGCAGAAGATC-3'). Blots were stained with AP-labelled α-DIG antibodies for 30 min (#11093274910; Roche) and developed with NBT-BCIP solution (Roche) until a sufficient signal was achieved. Blots were scanned with a Bio-Rad Gel Doc scanner. Quantification of band signal intensities was performed in ImageJ by transforming the image into 8-bit followed by analysing the band intensity using the measure function. After deducting the background signal from the sfRNA1 and sfRNA2 band intensities, the sfRNA1:sfRNA2 ratio was determined.

RT-PCR and qRT-PCR

First strand synthesis was performed with 500-1,000 ng total RNA per reaction using Superscript III (Invitrogen) and random hexamers (Roche) following the manufacturer's protocol. RT-PCR reactions were performed on 0.2 µl cDNA using GoTaq green (Promega) according to the manufacturers protocol. qRT-PCR was performed with SYBR Select Master Mix (Applied biosystems) on a Rotagene 3000 (Corbett Research). Briefly, the cDNA was diluted 5X in ddH₂O and 1 µl was used as a template with primers targeting either the vgRNA (FW 5'-TGCACTCTAGTCTAGTGTGGG-3'; RV 5'-TCTTGAATGTAGCCATGAGTGCC-3') or the Cx. *pipiens* 40S rRNA (FW 5'-ATGGTTTTCGGATCAAAGGT-3'; RV 5'-GACGTGCTTGCCGAGAAC-3') at an annealing temperature of 57 °C.

Deep sequencing of small RNAs

Total RNA was isolated as described above and 15 µg of RNA per sample per lane was separated by gel electrophoresis on 15% polyacrylamide / 7M Urea / 0.5X TBE gel. For the sequencing of small RNAs from WNV_{ic} and WNVΔSF1 infected northern European mosquitoes the 19-24 nt RNAs were cut from gel using radioactively-labelled RNA oligos, loaded in the adjacent lanes of the gel as rulers. Subsequently, the gel slices were crushed using gel-breaker tubes and small RNAs were eluted in 800 µl 0.3 M NaOAc, precipitated with 80% EtOH and dissolved in 11 µl H₂O. Small RNA deep-sequencing libraries were prepared using the TruSeq small RNA kit (#15016914; Illumina) according to the manufacturer's protocol, as described in [155]. Briefly, after adapter ligation, reverse transcription and PCR amplification, the cDNA libraries were gel purified from 6% polyacrylamide / 1X TBE gel, eluted in 500 µl 0.3 M NaOAc, precipitated with 80% EtOH and dissolved in 11 µl 10 mM Tris-HCl pH 8.5. The small-RNA library was sequenced on an Illumina HiSeq 2500 (Baseclear) and single-end FASTQ reads were generated using the Illumina Casava pipeline version 1.8.3. Initial quality assessment were performed by Baseclear using in-house filtering protocols and the FASTQC quality control tool version 0.10.0. Further sequence analysis was performed on the Galaxy server [242]. Adapter sequences were removed from each read using the Clip tool version 1.0.1 and mapped to either the WNV_{ic} or WNVΔSF1 genome with Bowtie version 1.1.2. Size profiles of the viral small RNAs were retrieved from all the mapped reads allowing 1 mismatch. For genome profiles, the 5' ends of the 21 nt virus reads were mapped along the viral genome.

Mosquito rearing

A *Culex pipiens* colony originating from Brummen, the Netherlands was established in 2010 and maintained at 23 °C, 60% relative humidity (RH), and a 16:8h light:dark cycle. Mosquitoes were maintained in Bugdorm cages provided with 6% glucose solution *ad libitum*. Hemotek PS5 feeders (Discovery Workshops) filled with bovine or chicken whole blood (KemperKip) were used to administer a blood meal for egg production. Egg rafts were allowed to hatch in tap water supplemented with Liquifry No. 1 (Interpet Ltd). Larvae were fed with a 1:1:1 mixture of ground koi food, ground rabbit food and bovine liver powder (Sigma-Aldrich).

In vivo infections

Infectious blood meal: 2 days before blood feeding, the mosquitoes were starved by providing them with a tissue soaked in water. 1 ml whole-chicken blood (Kemperkip) was mixed with 1 ml virus solution in Leibovitz-L15 supplemented with 10% FBS to a final concentration of 4.0×10^7 TCID₅₀/ml. 2-7 days old mosquitoes were allowed to feed *ad libitum* through a Parafilm membrane using a Hemotek feeder in a controlled dark room at 24 °C, 70% RH for 1 h. After blood feeding, mosquitoes were anesthetized with 100% CO₂ and engorged females were selected on a CO₂ pad. Intrathoracic injections: 2-5 day old mosquitoes were anesthetized with 100% CO₂ and placed on a CO₂ pad and $\sim 7.7 \times 10^3$ or $\sim 7.7 \times 10^2$ TCID₅₀ virus was injected in a total volume of 69 nl using a Drummond Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company). Blood fed or injected mosquitoes were incubated at 28 °C with a 16:8 light dark (L:D) cycle and fed with 6% sugar water during the course of the experiment. At 14 dpi (or as indicated) mosquitoes were anesthetized with 100% CO₂ and the legs and wings were removed from the mosquito bodies. Mosquitoes were salivated by putting the proboscis in 5 µl of 3% sugar water, 50% FBS for 45 min. After salivating, the mosquito bodies were added to an 1.5 ml Eppendorf tube containing 0.5 mm zirconium beads (Next Advance) and frozen at -80 °C. 55 µl of HEPES-buffered DMEM/10%FBS/PS/Gentamycin was added to the saliva samples. Mosquito homogenates and saliva samples were stored at -80 °C until further processing.

WNV infectivity assay

WNV infectivity assays were performed as described previously [154]. Briefly, mosquito bodies were homogenized in a Bullet Blender (Next Advance) (two rounds of 2 min at max speed). 100 µl of HEPES-buffered DMEM/10%FBS/PS/gentamycin was added to the mosquito homogenates and 30 µl mosquito homogenate was used to inoculate Vero cell monolayers in 96-well plates. For saliva samples, 30 µl saliva in DMEM/10%FBS/PS/gentamycin was used to inoculate a Vero cell monolayer in a 96-well plate. Three dpi wells were scored for presence of virus by CPE and immunostaining using anti-E monoclonal antibodies [332,333].

RNA structure modelling in silico

Secondary structures were folded using the Mfold webserver with standard settings and flat exterior loop type [334]. The secondary RNA structure was visualised using the VARNA RNA editing package [335]. Tertiary structure folding was performed using the RNAComposer webserver with standard settings [326]. The tertiary RNA structure was visualized using PyMol viewer [336].

Statistics

All statistical analysis were performed in Graphpad/Prism 5. Significant differences between virus infection and transmission rates were determined by Fisher's exact test ($\alpha=0.05$). T-tests were performed using a 95% confidence interval. D'Agostino and Pearson omnibus normality test was used to check for Gaussian distributions. In the case that the data did not follow a Gaussian distribution, a Mann-Whitney U-test was used ($\alpha=0.05$) to replace the t-test. Statistics on the virus growth curves were done using a two-way-ANOVA.

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Chapter VII

Functional RNA during Zika virus infection

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Abstract

Zika virus (ZIKV; family *Flaviviridae*; genus *Flavivirus*) is a pathogenic mosquito-borne RNA virus that currently threatens human health in the Americas, large parts of Asia and occasionally elsewhere in the world. ZIKV infection is often asymptomatic but can cause severe symptoms including congenital microcephaly and Guillain-Barré syndrome. The positive single-stranded RNA genome of the mosquito-borne ZIKV requires effective replication in two evolutionary distinct hosts – mosquitoes and primates. In addition to some of the viral proteins, the ZIKV genomic RNA and functional RNAs produced thereof aid in the establishment of productive infection and the evasion of host cell antiviral responses. ZIKV has evolved to contain a nucleotide composition and RNA modifications, such as methylation and the formation of G-quadruplexes that allow effective replication in both hosts. Furthermore, a number of host factors interact with the viral genome to modulate RNA replication. Importantly, the ZIKV genome produces a noncoding subgenomic flavivirus RNA due to stalling of host 5'→3' exoribonucleases on viral RNA structures in the 3' untranslated region. This subgenomic flavivirus RNA exerts important proviral functions such as antagonizing the innate interferon response and RNA-interference. Here, we discuss the ZIKV genomic RNA and functional RNAs thereof to assess their significance during ZIKV infection. Understanding the details of the ZIKV infection cycle will aid in the development of effective antiviral strategies and safe vaccines.

Introduction

The paradigm of the predominant role of RNA as a messenger in the cellular process of translating genetic information (DNA) into functional molecules (proteins) has completely changed since the discovery of the mechanism of RNA interference (RNAi) more than a decade ago [337]. Since then, it has become increasingly clear that RNAs, large and small, are key intracellular molecules that execute vital, direct roles in *e.g.* organismal development, cell survival, cell to cell communication and innate immune responses against invading pathogens [338]. Regulation of these processes takes place at multiple levels involving an expanding range of newly discovered functional RNAs, *e.g.* small interfering (si)RNA [339], micro (mi)RNA [340], PIWI-interacting (pi)RNAs [214], but also longer noncoding RNA [341] and circular RNAs [342]. Noncoding RNA molecules have many, mainly undiscovered functions, and are involved in a diverse range of intimately regulated pathways, including those related to viral diseases [343].

Fig. 7.1 Schematic overview of features contained in the ZIKV genomic RNA. (A) The 5' cap of the Zika virus (ZIKV) RNA is methylated by the methyl-transferase domain of the viral protein NS5. Shown is the chemical structure of the 5' cap on the ZIKV genomic (g)RNA. (B) The ZIKV genome can cyclise through base pairing between the 5' and 3'UTRs. Cyclisation is essential for virus replication as it creates a template for the viral polymerase NS5 for virus replication. Shown is the secondary structure of the 5'UTR of the ZIKV Natal strain and the formation of the RNA 'panhandle' after cyclisation through base pairing with complementary sequences in the 3'UTR. (C) The ZIKV gRNA can be internally methylated by host methylases. Indicated are the chemical structures of the two types of internal methylation that can be observed in the ZIKV gRNA. (D) The ZIKV gRNA has a bias in the use of codons and dinucleotides in a way that CpG and UpA dinucleotides are suppressed. (E) The ZIKV genome contains Guanine-quadruplexes that can be formed in regions that contain consecutive blocks of at least two adjacent guanine residues. (F) The ZIKV 3'UTR contains resistant RNA structures that can stall the degradation by 5'→3' exoribonucleases such as XRN1, resulting in the formation of two species of sfRNA.

The genomes of RNA viruses are efficiently organized to contain the minimal set of genes required for replication and suppression of host innate immune responses. Despite their small genome size, RNA viruses have evolved unique ways to manipulate their host and the cells they replicate in involving viral proteins as well as viral RNA molecules. Advances in chemical probing, RNA crystallography and bioinformatics have accelerated the understanding of the intricate roles that viral RNAs play in the infection process [344,345].

Zika virus (ZIKV), which is a mosquito-borne RNA virus of the family *Flaviviridae* (genus *Flavivirus*), has emerged as a medically important pathogen since the start of a large-scale outbreak in Brazil in 2015. ZIKV infections now threaten human health in the Americas, large parts of Asia and Africa, with severe symptoms including congenital microcephaly and Guillain-Barré syndrome. The history of ZIKV, its biological properties and further disease symptoms have been extensively reviewed by others [346,347]. However, the diverse roles that host and virus-derived RNAs play during the ZIKV infection cycle are continuously being uncovered. The ZIKV genome is a single-stranded (ss) positive sense (+) RNA of ~10.7 kilo bases in length that encodes seven non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B, 5) and three structural proteins (Capsid, precursor-Membrane, Envelope) (Fig. 7.1). The ZIKV genomic RNA (gRNA) is flanked by highly structured 5' and 3' untranslated regions (UTRs) of respectively ~107 and ~428 nucleotides in size. The 5'UTR contains a methylated 5' cap structure, while the 3'UTR lacks a poly-(A) tail and terminates with CU_{OH} at the end of a conserved RNA hairpin.

During ZIKV infection, the methyltransferase (MTase) of the viral NS5 caps the viral gRNA. The importance of 5' cap methylation for initiation of genome translation and protection from degradation is relatively well understood [348,349]. Additionally, further methylation of the gRNA by host MTases was recently described and appears to affect viral replication [350,351]. Bioinformatic analysis has also shown that the ZIKV gRNA contains a bias for certain codons and dinucleotides that may contribute to viral fitness. Furthermore, ZIKV produces two species of 3'UTR-derived, noncoding subgenomic flavivirus RNA (sfRNA) during infection [108,111,352] that may be important for virus transmission and pathogenesis, as has been described for other flaviviruses [106,112,157].

Here, we review the composition and methylation patterns of the ZIKV gRNA, interactions between ZIKV RNA and host proteins, and the production of sfRNA and how these functional RNAs support viral replication and aid in the evasion of antiviral responses. We do not cover the field of siRNA, miRNA and piRNAs, as these antiviral small RNAs have been very well reviewed elsewhere [101,104,353] and to our knowledge no significant advances have been made in studies specifically investigating ZIKV. Further, we discuss novel insights into the RNA structure, folding and evolution of the ZIKV UTRs, with a specific focus on the 3'UTR and the colinear sfRNA. Finally, we indicate areas for future research on RNA during ZIKV infection that may lead to better understanding of the biology of this unique pathogen and opportunities for the development of antiviral treatments and novel vaccines.

RNA methylation during ZIKV infection

Many chemical modifications of RNA exist, which can contribute to RNA structure, function, stability and regulation. An important chemical modification is RNA methylation, which protects the RNA from rapid degradation and controls the efficiency of messenger RNA (mRNA) translation. In addition to the well-described methylation of the 5' end of the ZIKV gRNA (capping) [348,349], it was recently described that, similar to other flaviviruses, the ZIKV gRNA can also be methylated at internal adenosines [350,351]. Here we discuss both RNA methylation strategies, with specific emphasis on RNA methylation during ZIKV infection.

The 5' end of the ZIKV gRNA contains a cap structure that consists of a guanosine which is bound to the first nucleotide of the RNA via a 5'-5' triphosphate bond. Methylation of the 5' cap can take place at the nitrogen on position 7 (N-7) of guanosine (cap-0) and at the ribose 2'-oxygen (2'-O) of the first nucleotide (cap-1), and is performed sequentially by the N-terminal MTase domain of NS5 [349,354–356]. The effects of viral cap methylation have been studied for multiple viruses from different families, and cap methylation was found to be essential for viral RNA stability, translation, and evasion from host antiviral pathogen recognition receptors. By using a methylated 5' cap, viral RNAs mimic cellular RNAs, and in this way the viral RNAs are not recognized as non-self by the host. This allows the viral RNAs to be translated by the hosts translational machinery [349,357].

Generally, N-7 cap methylation deficient flaviviruses cannot replicate. Studies on West Nile virus (WNV) and dengue virus (DENV) indicated that N-7 methylation is essential for viral replication, pointing out the importance of the flaviviral MTase (NS5) as a target for the development of antiviral drugs [355,358–361]. Flaviviruses deficient in 2'-O methylation are attenuated, however replication still occurs, albeit less efficiently. During WNV infection in vertebrate cells, 2'-O cap methylation helps to evade the antiviral interferon (IFN)-induced proteins with tetra-tricopeptide repeats (IFIT) response [362]. IFITs are IFN stimulated genes, and the encoded proteins inhibit viral infections by binding cellular and viral RNAs and proteins (reviewed by [363]). A WNV mutant, deficient in 2'-O methylation activity, was attenuated due to its inability to escape suppression by IFIT proteins [362,364]. Furthermore, it has also been reported that DENV and Japanese encephalitis virus (JEV) mutants deficient in 2'-O methylation are attenuated, likely due to increased sensitivity to IFN and IFIT1 [365,366]. Deficiency in 2'-O methylation as a basis for vaccine development and abolishing N-7 methylation by targeting the NS5 MTase are two important strategies that have been proven effective against multiple flaviviruses (DENV, JEV, WNV) [355,360,361,365,366] and could potentially contribute to the development of ZIKV vaccines or antiviral treatments.

The high structural similarity between the MTases of different flavivirus species, including that of ZIKV, provides an appealing target for the development of antiviral drugs that can simultaneously combat multiple flaviviruses [348,367–369]. The conserved domains and binding pockets within the ZIKV MTase can contribute to further understand the mechanisms and functions of ZIKV RNA methylation, and are utilized as a basis for computational drug design [348,367,370,371].

Although the flaviviral MTase methylates viral RNA [349], it is still unclear whether the MTase is able to methylate host mRNAs and, as a consequence, can also alter host RNA functions. NS5 consists of an N-terminal MTase domain and a C-terminal RNA-dependent RNA polymerase (RdRp) domain. In eukaryotes, capping and synthesis of RNA are coupled, because the capping machinery directly binds to the RNA polymerase complex [372,373]. It is also expected that flaviviral capping and

methylation are coupled to RNA replication [349]. Full-length NS5 crystal structures of JEV, DENV and ZIKV have been generated and show that the interface between viral MTase and RdRp is important in regulating virus replication, however more studies are needed to fully understand the functional coupling between the MTase and RdRp domains [368,369,374]. Provided that viral RNA methylation and replication are indeed coupled, and given the fact that the viral RdRp strictly discriminates between cellular and viral RNAs using the viral sequence, structure and cyclisation of the UTRs [84,375–379], it is likely that host RNA methylation by the viral MTase does not readily occur. However, DENV NS5 can methylate host ribosomal RNA *in vitro* [380] and 2'-O methylation of synthetic RNAs by ZIKV MTase does not seem to be dependent on a cis-acting RdRp [348]. Clearly, more elaborate studies are required to investigate the potential role of the viral MTase in host RNA methylation *in vivo*.

Besides 5' cap methylation, it was shown for DENV and ZIKV that viral RNA can also be methylated internally by the viral NS5 MTase, on position 2'-O of adenosine [348,380]. However, the biological significance of internal 2'-O methylation is still unclear. In addition to internal 2'-O methylation, a second internal RNA methylation has been reported. It was shown for multiple flaviviruses (DENV, yellow fever virus (YFV), WNV and ZIKV) that their viral RNAs can be methylated on position N-6 of adenosine (m⁶A) by cellular MTases [350]. m⁶A methylation is a common internal modification in eukaryotic mRNAs [381] and in nuclear replicating RNA viruses [382–384]. Recently, it was discovered that also members of the *Flaviviridae* such as hepatitis C virus, DENV and ZIKV, which all replicate in the cytoplasm [87], have conserved m⁶A-modified regions within their RNA genome [350]. For hepatitis C virus, m⁶A negatively affects particle production during viral infection. However, whether the observed effect of m⁶A on particle production is more widely distributed among the *Flaviviridae*, and whether other conserved m⁶A sites in *Flaviviridae* genomes have a significant impact on the viral life cycle, still remains to be explored.

For the flaviviral genomes analysed, m⁶A methylation seems to be more prevalent towards the 3' end of the viral RNA genome. In addition, methylation did not only occur in the coding region, but also in the 3'UTR of the DENV and YFV genomes. For the two investigated ZIKV genomes, no m⁶A was present in the 3'UTR, but m⁶A methylation does become more prevalent towards the 3' end of the coding region. Preferential disposition of m⁶A towards the 3' end was also reported for mammalian mRNA transcripts, and it has been suggested that this may contribute to regulation of the 3'UTR and the transcriptome [385,386], which might also be of importance during ZIKV infection.

Interestingly, comparison between the genomes of ZIKV isolates from Puerto Rico and Dakar, belonging to the Asian and African lineage, respectively [17], showed dissimilar m⁶A patterns in the RNA regions encoding E, NS1, NS3 and NS5 [350]. ZIKV was first considered to cause only mild disease symptoms that were quickly resolved, however, during the recent outbreak in the Americas, ZIKV-induced disease was observed to be much more severe, with symptoms including microcephaly and Guillain-Barré syndrome [346]. Further understanding of m⁶A-modifications and their putative effects on RNA function may help to clarify the differences in human pathogenicity between the two different ZIKV lineages.

ZIKV RNA as well as host RNA transcripts can become chemically altered through m⁶A modification. The addition of m⁶A occurs by host MTases and can be removed by demethylases, resulting in effects on viral replication and host functions. A recent study showed that increased levels of m⁶A in ZIKV RNA due to silencing of host demethylases negatively influenced viral replication of the African ZIKV isolate MR-766, whereas m⁶A depletion due to knock-down of host MTases resulted in increased

viral replication [351]. These findings indicate that alterations in ZIKV RNA methylation by host enzymes during ZIKV infection can have significant impact on ZIKV production. Alterations in m⁶A on ZIKV RNA by host MTases and demethylases can therefore be identified as a post-transcriptional mechanism to regulate ZIKV infection. In addition to this first layer of control, m⁶A modifications were also found to be employed by the host YTH domain protein family (YTHDF) to regulate ZIKV replication. YTHDF proteins can directly bind m⁶A in ZIKV RNA, with YTHDF2 binding the most ZIKV RNAs [351]. In mammalian cells, it is known that YTHDF2 can induce degradation of m⁶A-containing RNA via deadenylation as the initial step of decay [387]. As knock-down of YTHDF2 resulted in increased ZIKV replication, the protein might also be involved in destabilisation and degradation of ZIKV viral RNA, in this way exerting a regulatory function during ZIKV infection [351].

Finally, another regulatory mechanism might involve methylation of host mRNA transcripts. During ZIKV infection, selective alterations in m⁶A positions were found to be introduced in host transcripts [351]. These modifications could alter gene translation, splicing and stability of transcripts. One hypothesis is that these preferential depositions of m⁶A modifications on host mRNA promote expression of host proteins involved in virus detection and antiviral responses. Alternatively, m⁶A modifications of host transcripts during viral infection could also facilitate viral replication. For instance, host transcripts that are involved in an antiviral response could be suppressed by selective m⁶A modifications induced by ZIKV infection. Thus, the effect of m⁶A modifications on host transcripts during ZIKV infection might work both ways, potentially triggering both pro-viral and anti-viral events, and the exact role of virus and host is yet to be determined [351].

ZIKV codon and nucleotide usage patterns

Coding regions in the genomes of most organisms are biased in their use of synonymous codons, which may represent the optimal use of transfer tRNA frequencies, translational accuracy and translation elongation rates [388]. Also the use and organisation of nucleotides is biased, with most notably a strong under-representation of T_pA (U_pA in RNA) in most organisms and C_pG dinucleotides in vertebrates and plants [389,390]. Many viruses have evolved similar biases in their genomic sequences [391–393], possibly to increase their translation and replication rates and help evade non-self detection by the host cell. The constraints imposed by evolutionary quite distant vertebrate and invertebrate host cells have similarly shaped the genomes of ZIKV and other flaviviruses [394,395]. This section reviews the codon usage and nucleotide composition of the ZIKV genome to better understand the molecular evolution of the virus and discusses the possibilities to exploit some of these characteristics in the search for antiviral treatments and vaccine development.

Codon usage bias

ZIKV isolates can be assigned to three different clusters, the East-African, West-African and Asian cluster, the latter which has recently been introduced into the Americas [396]. Analysis of the relative synonymous codon usage between ZIKV isolates also groups these geographically distinct isolates into three genetically diverse clusters [397–401]. By measuring the distance in codon usage from the hypothetical equal use of synonymous codons, these studies also showed that the ZIKV genome has a relatively low codon usage bias and a stable composition between isolates [397–401]. This relatively low codon bias is a common feature for arthropod-borne RNA

viruses and it has been suggested to be beneficial for effective replication in vertebrate and invertebrate host cells, which have diverse codon preferences [402].

Codon usage patterns within the ZIKV open reading frame were, however, significantly different from the codon usage observed in the different host (human and mosquito) genomes. In comparison to human genomes, codons ending with an adenosine were over-represented in the ZIKV genome. Similar to human coding sequences, codons with a C_pG dinucleotide were severely under-represented in the ZIKV genome, which is not the case in coding sequences from the mosquito host. Altogether the general codon usage pattern of ZIKV is slightly more similar to the pattern observed in human cells than to the pattern of the two main ZIKV mosquito vectors *Aedes aegypti* and *Aedes albopictus* [397,399–401]. Furthermore, the base composition of the ZIKV genome displays a preference of purines (G + A) compared to pyrimidines (C + U), with a respective ratio of 1.31 [398]. The relatively low codon usage bias, which is associated with the above described preferences in nucleotide composition suggests mutational pressure and not natural selection as the main driver to shape ZIKV codon usage bias [398]. Recent studies identified RNA-editing by double-stranded RNA-specific adenosine deaminase ADAR as a mechanism that may have contributed to the mutational pressure on the ZIKV genome [403,404].

Dinucleotide bias

As described above, C_pG dinucleotides are markedly suppressed in the ZIKV genome compared to their used frequencies in mosquito coding sequences. In fact, both U_pA and C_pG dinucleotides are strongly suppressed throughout the ZIKV genome compared to the expected unbiased values (Fig. 7.2A). U_pA dinucleotides are suppressed in the coding regions of most organisms including vertebrates and arthropods. U_pA dinucleotides in cytoplasmic mRNA and viral RNA can be recognised by RNA-degrading enzymes and it has been hypothesized that the degree of U_pA dinucleotides in cellular RNA is used to regulate RNA turn-over [405,406].

The cytosine in a C_pG dinucleotide in plant and vertebrate DNA can be methylated, making it more likely to deaminate into a thymine. This selectively reduces C_pG dinucleotide frequencies in their genomes and increases C_pA and T_pG dinucleotides on the complementary strand [407,408]. This pattern and specifically the under-representation of C_pG dinucleotides is mimicked by many viruses that infect plants and vertebrates [391,409]. In contrast, many invertebrates lack significant DNA methylation activity and consequently do not suppress C_pG dinucleotide frequencies. Consistent with the hypothesis for virus mimicry, invertebrate viruses including insect-specific flaviviruses show little to no suppression of C_pG dinucleotides [395,409], whereas ZIKV and other vertebrate-infecting flaviviruses do contain markedly reduced C_pG dinucleotide frequencies (Fig. 7.2A). Analysis of the (di)nucleotide composition of flaviviral genomes can therefore help to classify flaviviruses [410] and even reliably assign the host range for (newly discovered) flavivirus based on their genome sequences [411]. Importantly, synonymously increasing C_pG or U_pA dinucleotide frequencies in genomes of other single stranded RNA viruses strongly reduced the replicative fitness of these viruses [412–414], though it is currently unknown why RNA viruses such as ZIKV are biased in the use of C_pGs.

Taken together, the relatively low codon usage bias and nucleotide composition with a strong under-representation of C_pG (Fig. 7.2B) and U_pA (Fig. 7.2C) dinucleotides in the ZIKV gRNA is rather typical for a mosquito-borne flavivirus and provides possibilities for attenuation through synonymous recoding. Synonymously altering the frequency of occurrence of codons or dinucleotides in viral genomes has been used to successfully attenuate viruses from diverse families, including the *Flaviviridae*

(reviewed by [415]). Random recoding of NS5 in tick-borne encephalitis virus resulted in mildly reduced replication rates in cell culture and attenuated pathogenesis in a mouse model [416]. DENV was attenuated by codon pair deoptimization [417], however this consequently introduced unfavourable dinucleotides, which is now believed to have caused the observed attenuation [418,419]. Interestingly, DENV was not attenuated in C6/36 mosquito cells, suggesting that these cells have a higher tolerance to the introduced changes [417]. This provides an interesting avenue for rationally designed attenuated vaccines, which in the case of arboviruses such as ZIKV may simply be grown in cells from their invertebrate hosts.

Fig. 7.2 Dinucleotide bias in ZIKV and other flaviviruses. (A) The ZIKV genome contains a bias for certain dinucleotide pairs. Shown is the mean ratio between the observed di-nucleotide pair and the expected di-nucleotide frequencies with the standard deviation from 41 ZIKV genome sequences. (B) Comparison of C_pG and U_pA dinucleotide frequencies indicates a host/vector-specific pattern of C_pG and U_pA between different groups of flaviviruses. Shown are the ratios between the observed and the expected C_pG and U_pA dinucleotide frequencies against the G/C content of the viral genome. ZIKV is indicated by an arrow.

G-quadruplexes in the ZIKV genome

DNA and RNA sequences rich in guanine residues can contain adjacent residues of guanines referred to as G-blocks. When a G-block consists of four or more guanines, they can associate through Hoogsteen base-pairing to form a planar structure referred to as a G-tetrad. Two or more G-tetrads can stack to form a so-called G-quadruplex (G4) [420]. In human genomes, G4s function as controllers of telomere lengths, regulate DNA replication and regulate translation of host mRNAs (Reviewed in [420]). Furthermore, G4s pose resistance to cellular nucleases, although G4 specific nucleases such as yeast KEM1/SEP1 (= human XRN1) and human GQN1 have been identified [421,422]. Recent research has identified 64-78 putative G4 RNA structures in the (+) strand of the ZIKV genome based on bioinformatic analysis [423]. Interestingly, no G4s were identified on the (-) strand of the ZIKV genome. Seven of the putative ZIKV G4-structures are conserved within the genomes of >58 flaviviruses, suggesting an important function in the flavivirus life cycle. These conserved G4s are located in the prM, E, NS1A (2x), NS3, NS5 (2x) regions of the viral genome. Additionally, a ZIKV-specific G4 site was observed in the 3'UTR. Formation of G4s in the NS3 and NS5 regions was confirmed *in vitro* by biochemical analysis, while presence of G4s in other regions of the ZIKV genome are only based on *in silico* analysis and should be confirmed through laboratory studies [423].

The functional significance of G4s in the ZIKV genome has not been assessed, but G4s have been implicated in the life cycle of other viruses (reviewed in [420]). G4s serve as regulators of human immunodeficiency virus (HIV) replication by repressing the activation of transcription and promoting strand transfer and template switching by the viral reverse transcriptase [424,425]. Furthermore, the nuclear capsid protein of HIV and potentially the E1 protein of human papillomavirus, bind to and potentially unwind G4 sites, indicating that G4s can act as binding sites for viral proteins [420,426]. Finally, G4 containing aptamers have been implicated as antiviral compounds for single stranded RNA viruses as shown for Influenza virus, where G4-aptamers bind to and inhibit NS1 and for Hepatitis A virus where G4 hexanucleotides act as an inhibitor of the 3C protease [427,428]. The presence of G4s on the (+) strand and not the (-) strand of ZIKV suggests that G4s may contribute to the translation efficiency of the (+) strand, similar to what has been described for HIV, or involve the production of (-) strand from (+) strand templates. Alternatively, G4 sites in the ZIKV genome may act as binding sites for viral and cellular host factors that contribute to virus replication. The E protein of ZIKV has been shown to interact with the viral gRNA [429]. The E-RNA interaction sites do contain putative G4 sites [423], although no experimental evidence has been performed to study the link between G4s and E interaction.

Functions of RNA structures in the ZIKV UTRs

The flavivirus genome is flanked by a 5' and 3'UTR. For ZIKV the 5'UTR has a size of ~107 nucleotides while the 3'UTR is longer with a size of 428 nucleotides. The flavivirus 5'UTR comprises a Y-shaped RNA stem loop (SL) structure called SL-A and a shorter SL that terminates in the start codon AUG called SL-B [430]. SL-A is required for the initiation of virus replication from the 3' end and directly interacts with the viral polymerase NS5 to facilitate virus replication [376]. Additionally, the presence of an intact SL-A is required for both N-7 and 2'-O methylation of the 5' cap [349]. SL-B is rich in U-A residues and is important for a long-range RNA interaction with the 3' U-A rich region that contributes to virus replication [431]. Downstream of the 5'UTR, in the Capsid coding region, a cyclisation sequence (CS) is present that is complementary to the CS in the 3'UTR [431]. Base pair interaction between the 5' and the 3' CS results in the formation of an RNA panhandle [84]. The formation of this panhandle is important for virus replication but does not alter the translation of viral RNA [432]. Base pairing of the 5' and 3' CS brings the 5' and 3' U-A rich regions in close proximity that thereby also base pair, forming a more stable panhandle. The formation of this panhandle reforms the structure of the 3'SL in such a way that it becomes accessible as a template for the viral RdRp and thereby facilitates virus replication [376]. Recently, it was shown that an RNA sequence upstream of the 5' U-A rich region is required for proper RdRp recruitment and acts as a switch between genome cyclisation and RdRp recruitment [377].

The ZIKV 3'UTR is highly structured and contains three SLs (SL-I, SL-II, 3'SL), one dumbbell (DB-1) and a pseudo-dumbbell (ψ -DB) RNA structure [279]. Additionally, the ZIKV 3'UTR contains four short SLs of which the function remains unknown. The 3'UTR of ZIKV contains four predicted pseudoknot interactions, which are tertiary interactions between nucleotides within a RNA loop with a sequence outside of the loop. These four pseudoknots are located in the ZIKV SL-I, SL-II, ψ -DB and DB-1 RNA structures (Fig. 7.3A) [108]. The 3'SL contains a conserved pentanucleotide motif (5' CACAG 3') and a terminal 5' CU 3', that functions as the recognition site for the viral RdRp [379].

Several studies have identified proteins that bind to RNA structures in the 3'UTR, which have been extensively reviewed previously [105]. Here we will discuss host proteins that interact with the flavivirus 3'UTR that have been implicated in the replication cycle of ZIKV. A recent study identified the interaction between ZIKV gRNA and the protein Musashi-1, which is an important regulator of translation in stem cells [433]. The RNA binding domain of Musashi-1 binds to three binding sites located in the ZIKV 3'UTR and increases ZIKV replication, possibly by deregulating the expression of neuronal proteins. Perhaps, binding of Musashi-1 to the ZIKV 3'UTR contributes to the fetal neurotropism during ZIKV infection [433].

Many of the identified 3'UTR interacting proteins are located in stress granules (SGs). SGs are cytoplasmic aggregates that form under environmental stress and can function as triage centres to evaluate the fate of host and also viral RNAs [434]. It was recently shown that both DENV and ZIKV suppress the formation of SGs in infected cells, most likely by preventing the sequestration of viral RNA into SGs [435] or via SG inhibition by different ZIKV viral proteins [436]. Furthermore ZIKV and DENV infection prevent the phosphorylation of eIF2 α to block translational shut-off of host and viral translation [435]. The SG components Caprin 1, G3BP1/2 and USP10 bind to the SL-I, SL-II region of the 3'UTR of DENV [110,437]. Additionally, poly(A)-binding protein binds to the region upstream of the 3'SL and increases the translation efficiency for DENV [438]. Furthermore, TIA-1 and TIAR have been identified as interaction partners

Fig. 7.3 Conservation of the primary sequence, secondary structure and prediction of the tertiary structure of the ZIKV 3'UTR / sfRNA. (A) Secondary RNA structure of the ZIKV Natal strain 3'UTR / sfRNA. Black lines connected to green coloured bases indicate pseudoknot interactions, while black lines connected to white bases indicate base pair interactions between the 5' sequence and the three-way junction. Nucleotides with a red border indicate residues of which the primary sequence is not conserved amongst all ZIKV strains, while nucleotides also filled in red indicate residues of which both the primary and secondary structure is not conserved. (B) Northern blot detection of ZIKV sfRNA. Vero (mammalian) or C6/36 (mosquito) cells were infected with ZIKV. Total RNA was isolated and separated on a polyacrylamide gel. The sfRNA species were detected with a 3'UTR specific probe. (C) *In silico* prediction of the ZIKV 3'UTR / sfRNA 3D structure. I: complete sfRNA. II: SL-I and SL-II. III: Ψ -DB, DB-1 and 3' SL. Colours of the structure elements match the colours in panel A. Pseudoknot interactions are indicated in green. (D) Variation in the primary sequence and secondary structure of the ZIKV 3'UTR / sfRNA based on the comparison of 39 Asian, five East-African and five West-African ZIKV genome sequences. Given is the % of sequences in which the primary sequence or secondary structure varies from the ZIKV Natal strain.

with the minus strand 3'SL for WNV and the interaction with TIAR was suggested to be important for virus replication [439]. Depletion of G3BP1, TIAR or Caprin-1 was shown to lower ZIKV replication [436], indicating that these SG components are required for an efficient ZIKV replication cycle.

Processing bodies (PBs) are cytoplasmic foci containing a subset of proteins and RNAs that are involved in the post transcriptional processing, regulation and degradation of mRNAs. PBs are often observed adjacent to SGs and some processes are coupled, as indicated by the exchange of proteins between PBs and SGs [440]. Using a combined transcriptomics and proteomics approach, it was observed that the expression and translation of some PB components, including the dead-box helicase DDX6, is upregulated during ZIKV infection in human stem cells [441]. However, ZIKV infection does not alter the morphology and abundance of PBs in infected cells [436]. DDX6 binds to the RNA DBs of DENV, and this interaction is important for successful virus replication [437]. Potentially the interaction between the ZIKV 3'UTR and DDX6 is also important for ZIKV replication. Other PB components that have a known interaction with the flaviviral 3'UTR are XRN1, which will be discussed in the next section, and DDX3. DDX3 interacts with both the 5' and 3'UTR of JEV which is crucial for efficient virus translation [442]. Inhibition of DDX3 reduced the replicative fitness of both WNV and DENV, making DDX3 an interesting target for antiviral compounds [443]. Finally, La, MOV34, NF- κ B2, PTB and eEF1a have been shown to bind to the 3'SL of flaviviruses [83]. The interaction with eEF1a has been well studied and is involved in facilitating minus strand RNA synthesis for WNV [444]. As there is much similarity between the UTRs of flaviviruses, it would be interesting to study the importance of these host factors in the context of ZIKV infection.

The SL and DB RNA structures in the flaviviral 3'UTR are duplicated in the genomes of most mosquito-borne flaviviruses, *e.g.* they have two SL and two DB RNA structures [324,328]. The two main explanations for the presence of these duplicated RNA structures is A) their putative interaction with dimeric proteins or B) their requirement to sustain replication in a dual-host system with vertebrate hosts and invertebrate vectors, where the first structure is required for replication in the mammalian host whereas the duplicated structure is important in the invertebrate host [328]. Interestingly, DENV acquired different mutations in the 3'UTR when the virus was passaged in either mammalian or mosquito cells [324]. In mammalian cells, mutations arose mostly in the SL-I region of the 3'UTR, while in mosquito cells mutations occurred predominantly in SL-II. In mammalian cells the dominant mutation was A59G, which was predicted to stabilize the first pseudoknot interaction, while in mosquito cells a higher variability in mutations and occasionally even deletions in SL-II were observed.

Mutations in SL-I did not affect replication in either mammalian or mosquito cells, while mutations and deletions of SL-II increased the replication in mosquito cells and decreased replication in mammalian cells [324]. It was proposed that the mutations in SL-II are integrated to form RNA structures required for virus replication in mosquito cells suggesting that SL-II has important roles in the virus life cycle in mosquitoes [324]. Alternatively, mosquito cells may select for presence of SL-I while the absence of selection for an intact SL-II results in the accumulation of mutations in that region of the 3'UTR.

Subgenomic flavivirus RNA

During flavivirus infection the flaviviral gRNA is prone to 5'→3' degradation by the cellular exoribonuclease XRN1 in mammalian cells or Pacman in insect cells [106,445,446]. XRN1 preferably degrades single-stranded RNAs with a 5' mono-phosphate, but is strongly inhibited by the presence of a 5' cap or 5' triphosphate [447]. However, degradation by XRN1 is a frequently occurring process as during viral replication uncapped, 5' mono-phosphate RNAs may exist before addition of the 5' cap. Alternatively, 5' mono-phosphate gRNA can arise after demethylation by de-capping enzymes or through internal cleavage by endonucleases such as Dicer2 or PIWI proteins. In addition to the above described 5' modifications, tertiary RNA structures that are present in the 5' end of the flaviviral 3'UTR exhibit resistance to degradation by XRN1. Pseudoknots formed by some of the SLs and DBs in the 3'UTR of flaviviruses fold tightly into an XRN1 resistant RNA structure (xrRNA) [107,108]. These xrRNAs stall XRN1, leading to the formation of sfRNA [106,321]. During the infection cycle, the ongoing degradation of the viral gRNA results in the accumulation of considerable amounts of sfRNA [106]. The mechanism of sfRNA generation and the functions of sfRNA in the flavivirus lifecycle have extensively been reviewed elsewhere [105,320,448]. Here we will discuss the recent findings that concern ZIKV sfRNA and extrapolate knowledge on sfRNA from other flaviviruses to discuss the functional significance of sfRNA production in the ZIKV life cycle.

Stalling on an xrRNA does not completely inhibit all downstream RNA degradation by XRN1. For DENV, SL-I, SL-II, and DB-1 have been identified as xrRNAs and subsequent stalling of XRN1 therefore results in the accumulation of three distinct sfRNAs (sfRNA1, 2, and 3) of different lengths [319], although some studies have shown the presence of a fourth species of sfRNA through stalling on DB-2 [352]. For WNV three RNA structures, SL-II, SL-IV and DB-1, have been identified as xrRNAs leading to the formation of three sfRNA species during infection [106,322]. The YFV 3'UTR contains a single XRN1 stalling site on the SL-E structure. Interestingly, YFV produces two species of sfRNA with a common 5' end, indicating that the YFV sfRNA2 is 3' truncated and lacks the 3'SL [321], although the mechanism of this 3' truncation and the fate of the cleaved 3'SL are unclear.

Recently, the formation of two sfRNA species was observed by us (Fig. 7.3B) and others during ZIKV infection by XRN1 stalling on SL-I and SL-II respectively [108]. The ZIKV 3'UTR also contains the ψ -DB and DB-I RNA structures (Fig. 7.3A), that contain predicted pseudoknot interactions, however these RNA structures were not identified as xrRNAs [108]. Two 3'UTR-derived RNAs with a size smaller than that of sfRNA2 have been detected during ZIKV infection, however, it is unclear whether these RNAs are the result of XRN1 stalling on the ZIKV ψ -DB or DB-I [352]. Interestingly, it was shown that the formation of ZIKV sfRNA1 might not require the stalling of XRN1 *per se*, as XRN1 knockdown decreased the abundance of sfRNA2 but did not affect the abundance of sfRNA1 [108]. The complete pseudoknot interaction of SL-I was required for the production of sfRNA1 [108], which suggests that other cellular nucleases could be involved in the formation of ZIKV sfRNA1.

Resolving the xrRNA1 structure from ZIKV [108] and the xrRNA2 structure from Murray Valley encephalitis virus [107] by X-ray crystallography has provided detailed knowledge on the requirements for folding of the SLs associated with sfRNA1 and 2 formation. XrRNA1 folds as a SL with a side bulge at the middle of the loop, forming a three-way-junction with a ring-like structure (Fig. 7.3C).

The structure contains a pseudoknot interaction between the top of the SL and a downstream RNA sequence. The base pairs just preceding the xrRNA1 stem interact with the two free bases in the three-way-junction to pull the 5' end through the ring-like RNA, resulting in the formation of an 'RNA knot' that prevents XRN1 from pulling the RNA into its active site. Additionally, two A-U base pair interactions between the bottom-stem and the top of xrRNA1 contribute to the tight 3D folding of xrRNA1 [108]. The pseudoknot, central cytosine and three-way-junction interactions are required for the efficient stalling of XRN1, while the A-U base pairing does not affect the formation of sfRNA1 [107,108]. The folding of xrRNA2 mostly follows xrRNA1, with a similar ring-like three-way-junction in the middle of the SL and a pseudoknot interaction from the top of the loop with a downstream sequence [107]. The nucleotides 5' of the SL base pair with nucleotides within the three-way junction, similarly as in xrRNA1, to block access for the XRN1 active-site to the nucleotides within xrRNA2. The sequence elements required for the knot-like RNA structures of xrRNA1 and xrRNA2 are highly conserved in the genomes of flaviviruses, underlining the importance of XRN1 stalling and the formation of sfRNAs in the virus transmission cycle [106,323,324].

XRN1 has been shown to have a higher affinity for DNA sequences containing G4s [421] and hydrolyses templates containing G4s faster than monomeric templates [449], suggesting that G4s might contribute to increased degradation by XRN1. Thus, the presence of G4s in the (+) RNA strand of the ZIKV genome might contribute to increased degradation of the (+) strand specifically. However, others have suggested that the observed preference of XRN1 for G4 substrates is due to XRN1 degrading RNA up to the G4 complexes where it then stalls, resulting in more observed G4-RNA binding to XRN1 [446]. The ZIKV 3'UTR also contains a predicted G4 site in the 3'SL but its role, if any, in the production of sfRNA and viral replication is not known.

Functional significance of sfRNA production during ZIKV infection

The formation of sfRNA is vital for flavivirus replication and engineered mutants lacking the formation of one or multiple sfRNAs become increasingly attenuated in mammalian and insect cells [106,112,321,322]. SfrNA performs various functions in the flavivirus cycle and several pathways have been described to be affected by sfRNA (extensively reviewed by [105,448]). The stalling of XRN1 or Pacman on xrRNA structures in the 3'UTR results in reduced cellular XRN1 ribonuclease activity. This subsequently decreases the degradation of cellular and viral uncapped-mRNA, and correlates with increased host and viral mRNA stability [450]. This effect has been suggested to promote virus replication by the accumulation of viral mRNAs and increased availability of host factors [450]. Dysregulated mRNA stability during flavivirus infection may function to aid in virus replication through the disruption of PBs, dysregulation of host immune responses and might contribute to virus induced cytopathicity [450]. Dysregulation of host mRNA stability due to stalling of XRN1 is associated with diverse members of the *Flaviviridae* family, including DENV and WNV from the genus *Flavivirus*, the *Hepacivirus* hepatitis C virus and the *Pestivirus* bovine viral diarrhoea virus [450,451]. It is thus highly likely that this also occurs during ZIKV infection, as ZIKV also effectively stalls XRN1 during infection [108].

SfRNA has been identified as an antagonist of the innate type-I IFN response, which is an important antiviral pathway that restricts early-phase virus infections in mammalian cells [452]. WNV sfRNA antagonizes the IFN regulatory factor (IRF)-3 and IRF-7 dependent IFN response in both mammalian cells and in mice [112]. The mechanism of IFN antagonism of WNV sfRNA is still unclear, although it was shown to be PKR, RNase L and RIG-I independent [112,453]. In contrast, DENV sfRNA antagonizes IFN induction through binding to the RIG-I activating enzyme TRIM-25,

thereby suppressing the RIG-I dependent IFN response [109]. Additionally, binding of DENV sfRNA to the stress granule components Caprin1, G3BP1 and G3BP2 was linked to suppression of the IFN response [110]. Finally, JEV sfRNA was shown to suppress IFN-signalling by blocking the nuclear translocation of IRF-3 [454]. Recently, ZIKV sfRNA was indicated as a mild suppressor of RIG-I and MDA-5 dependent IFN induction after transient expression of the ZIKV 3'UTR and transfection of either a RIG-I or MDA-5 agonist [111]. As antagonism of the IFN response is often paired with increased pathogenicity [455–457], as observed during WNV infection [112], ZIKV sfRNA production might contribute to (neuro)-virulence or persistence during ZIKV infection. It would be interesting to assess whether ZIKV sfRNA production is indeed positively correlated with increased virulence and pathogenicity *in vivo*. Deletions as small as 10 nucleotides in the ZIKV 3'UTR DB-1 structure decreased viral RNA synthesis and increased the sensitivity to type I IFN inhibition [458]. It remains to be investigated whether the observed attenuation is related to a direct function of the 3'UTR or if it is a consequence of altered sfRNA abundance or specific sfRNA activity in innate immune modulation.

In mosquito cells, the most prominent innate immune response to virus infection is the RNAi response [104]. This pathway is initiated when dsRNA molecules are recognized and cleaved by the endonuclease Dicer2 into 21 nucleotide siRNAs and loaded into the Ago2 protein to target complementary mRNAs [101]. Although the ZIKV genome is a single (+) RNA strand, viral replication inevitably results in the formation of dsRNA intermediates and both the gRNA and sfRNA contain various dsRNA structures that can be recognized and processed by Dicer2. Indeed, WNV sfRNA was identified as a Dicer2 substrate in both mammalian and mosquito cells [115]. Deep sequencing of small RNAs from *Culex* mosquitoes infected with WNV or a WNV-mutant that does not produce sfRNA1 indicated that sfRNA is indeed processed by the RNAi machinery *in vivo* [157]. SfRNA is highly abundant during infection (sfRNA:viral gRNA ~10:1) [106]. This led to the hypothesis that sfRNA functions as a decoy Dicer substrate, thus preventing the degradation of viral gRNA and consequently facilitating viral gRNA replication [115]. An interaction between sfRNA and both Dicer2 and Ago2 was established by RNA co-immunoprecipitation in mammalian cells [116]. In *Culex* mosquitoes, WNV sfRNA was indeed shown to suppress dsRNA induced RNA silencing by suppression of RNAi based silencing of a target gene [116]. However, the RNAi suppressor function of sfRNA could not be confirmed by deep sequencing of small RNAs from WNV infected *Culex* mosquitoes, as the amount of siRNAs produced in WNV infected mosquitoes was highly abundant [116,157]. Furthermore, a WNV sfRNA1-mutant was not attenuated in RNAi-competent mosquito cells, which questions how effective sfRNA is as RNAi suppressor *in vivo* [157]. As the RNAi suppressor function of sfRNA is not required for replication *per se*, the importance of the interaction between sfRNA and the RNAi machinery requires further investigation. Perhaps the RNAi suppressor function of sfRNA is vital for replication in certain parts of the mosquito *e.g.* the midgut and/or salivary glands. Based on the structural similarity between ZIKV and WNV sfRNAs, ZIKV sfRNA might also suppress RNAi in a similar fashion as has been described for WNV. During WNV infection in live mosquitoes, the two RNA structures most targeted by Dicer were the SL-III and 3'SL structures, potentially since they are more freely available than the SL-I, SL-IV and DB structures [157]. Although ZIKV and DENV sfRNAs lack the SL-III structure, they do have the highly conserved 3'SL structure, indicating that indeed ZIKV sfRNA could theoretically be processed by the RNAi machinery, leading to suppression of the RNAi response. It is expected that small RNA sequencing data for ZIKV infection in mosquitoes will become available very soon and then this question can be resolved.

Transmission of arboviruses, such as WNV and ZIKV, by mosquitoes requires the virus to overcome certain mosquito barriers of which the midgut and the salivary gland barriers most significantly affect virus transmission [142]. As sfRNA antagonizes the insect RNAi response and the mammalian IFN response, the formation of sfRNA might be important to overcome the mosquito barriers and thereby contribute to efficient transmission of flaviviruses by mosquitoes. Importantly, sfRNA1 is required for successful transmission of WNV by mosquitoes [157]. A mutant WNV that lacks the formation of sfRNA1 could not efficiently infect *Culex* mosquitoes after an infectious blood meal while infection after injection, which bypasses the midgut barrier, was not affected by the production of sfRNA1, suggesting that WNV sfRNA1 is important for the infection of the mosquito midgut. Since ZIKV also produces abundant sfRNA1 and sfRNA2 in mosquito cells [108,352], ZIKV sfRNA may prove important for the efficient transmission of the virus by mosquitoes.

As the 3'UTR and sfRNA of ZIKV and other mosquito-borne flaviviruses contain duplicated SL and DB regions, it has been hypothesised that one of the duplicates is required for effective infection in either the vertebrate host while the other may contribute more during infection of the mosquito host [323,324]. In support of this hypothesis, it was recently shown that the ratio between the various sfRNA species of DENV alternates after several replication cycles in either mammalian or mosquito cells [352]. DENV grown on human cells mainly produces sfRNA1, while DENV grown on mosquito cells produces sfRNA 1, 2, 3 and 4. Interestingly, when the human-adapted DENV was passaged five times on mosquito cells the formation of sfRNA1, 3 and 4 was observed, while five passages of the mosquito-adapted DENV on human cells resulted in formation of mainly sfRNA1. This suggests that full-length sfRNA is mainly important in mammalian cells where it might act as an antagonist of IFN signalling, while sfRNA2, 3 and 4 would be more important in mosquitoes. However, ZIKV infection in mammalian and mosquito cells resulted in the production of equally abundant sfRNA1 and 2, suggesting that ZIKV sfRNA1 and 2 are functional in both the mammalian and mosquito host [352].

Comparison of the primary and secondary structure of the 3'UTR from 49 ZIKV isolates

Distinct DENV-2 strains, with variations in the nucleotide sequence of the 3'UTR, were shown to produce different amounts of sfRNA [109]. The amount of sfRNA that was detected by RT-PCR during infection positively correlated with the epidemiological fitness of the DENV strain, potentially due to increased IFN antagonism in the mammalian host or increased transmission efficiency by the mosquito vector [109,112,118]. To investigate the conservation of primary sequence and the secondary structures in the ZIKV 3'UTR, 95 sequences were retrieved from Genbank of which 49 had a (near)-complete 3'UTR. Nucleotide sequence alignment of the 49 complete ZIKV 3'UTRs of both African (10) and Asian (39) ZIKV isolates indicated some nucleotide sequence variation across the 3'UTR (Fig. 7.3D). Nevertheless, when mapping these nucleotide variations onto the secondary structure of the contemporary ZIKV Natal isolate 3'UTR, these variations were mostly located in predicted non-structured regions (Fig. 7.3D, black lines). Additionally, a few complementary variations were observed that due to their complementarity do not affect the secondary structure. Nucleotide variations that affect the secondary RNA structure were only observed in the DB-1 structure and at the proximal end of the 3'SL (Fig. 7.3A, red dots; Fig. 7.3D, red lines). The nucleotide variations in DB-1 are predicted to maintain the DB's overall secondary structure, but decrease the number of base pair interactions in the DB stems. Nucleotide variation at the end of the ZIKV 3'SL is surprising, as these nucleotides are highly

conserved in the genomes of flaviviruses and are indispensable for virus replication [379]. Therefore, these nucleotide variations are most likely the result of sequencing errors at the proximal end of the viral genome. If this is true, we can conclude that the 3'UTR structure is very conserved and that mutations occur in non-basepairing nucleotides. Three long-range interactions between the ZIKV 5' and 3'UTR have been predicted *in silico* in addition to the classical flavivirus CS between the DBs and the 3'SL (Fig. 7.1A) [279]. The sequence variations in the 3'UTR between different ZIKV isolates are not expected to affect either of these long-range RNA interactions, suggesting that all described ZIKV isolates have equal genome cyclisation possibilities. Furthermore, as the sequence variation observed between ZIKV isolates is predicted to maintain the secondary and tertiary structures of the 3'UTR, it is unlikely that sfRNA production will greatly vary between ZIKV isolates.

Concluding remarks and future perspectives

Here we have reviewed the functional RNAs with an important (predicted) role in the ZIKV replication cycle. Insights into the activity of these RNAs are increasing our understanding of ZIKV replication, pathogenesis and transmission and may aid the development of novel antiviral drugs and safe vaccines [443,458]. ZIKV has only recently gained full attention of the scientific community, therefore more functional RNAs and RNA-based virus-host interactions in the ZIKV transmission cycle will likely be discovered. As the dual-host transmission cycle of ZIKV requires adaptation to the evolutionary very distant mammalian host and the mosquito vector, investigating the functional RNAs in both host types may help to explain how ZIKV has evolved. As mosquitoes possess the ability for internal m⁶A methylation [459], the presence and role of m⁶A methylation of host and viral RNA during ZIKV infection in mosquito cells requires further studies. Methylation, including m⁶A, can change secondary RNA structures and therefore alter protein-RNA interactions [460]. Similarly, G4-quadruplexes might attract G4-binding proteins and block the interaction with other RNA binding proteins [420]. Therefore, future studies should investigate whether RNA modulations of host and viral RNA during ZIKV infection allows for binding of host and viral proteins specific to m⁶A methylated sites, or whether it abolishes certain RNA-protein interactions. Further, the ease of genome engineering ZIKV infectious clones [458] provides an exciting opportunity to generate potent live-attenuated vaccines with higher safety profiles. The rational design of viruses with altered C_pG dinucleotide bias in (certain parts of) the ZIKV genome may yield viruses with an attenuated phenotype in humans while their growth properties in mosquito cells are not compromised. Finally, while the role of sfRNA in suppression of innate immunity, host-switching, pathogenicity in vertebrates and transmission by mosquitoes has been investigated for a limited number of other flaviviruses, it will be very important to address these questions for ZIKV. Answering these questions will bring us closer to fully understand the complex world of RNA molecules affecting the ZIKV infection cycle.

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Chapter VIII

Subgenomic flavivirus RNA binds the antiviral mosquito DEAD-box helicase ME31B and determines Zika virus transmission by *Aedes aegypti*

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Abstract

The majority of medically important flaviviruses, such as Zika (ZIKV), West Nile (WNV), yellow fever and dengue virus are transmitted between humans, predominantly by *Aedes* and *Culex* spp. mosquitoes. During infection of mammalian and mosquito cells, flaviviruses produce a noncoding subgenomic flavivirus RNA (sfRNA). The production of sfRNA is conserved for all flaviviruses, including insect-specific flaviviruses, which implies an important function of sfRNA in the insect vector. We previously showed for WNV that sfRNA increases the efficiency of transmission by *Cx. pipiens* mosquitoes. As the production of sfRNA is highly conserved, its role in transmission may be generic for mosquito-borne flaviviruses. However, it is currently unknown if sfRNA also determines the transmission of flaviviruses by *Aedes* spp. mosquitoes and knowledge on the mechanism that underlies the sfRNA-mediated transmission of flaviviruses is lacking. Here, we demonstrate that sfRNA is essential for the transmission of ZIKV by *Ae. aegypti* mosquitoes. By determining the mosquito infection and transmission rates both after an infectious blood meal and after intrathoracic injections, we implicate ZIKV sfRNA as a driver to overcome the midgut barrier. Importantly, to better understand the molecular mechanism of sfRNA-mediated virus transmission, we employed an RNA-affinity purification method to detect *Ae. aegypti* proteins that specifically bind to ZIKV or WNV sfRNA. We uncover that sfRNA interacts with several proteins of processing bodies and stress granules via its affinity for the mosquito ME31B protein and demonstrate that ME31B displays antiviral activity during ZIKV and WNV infection in *Ae. aegypti* cells. These results establish sfRNA as a generic determinant of flavivirus transmission by both *Culex* and *Aedes* spp. mosquitoes and contribute to our understanding of the molecular interactions of this fascinating noncoding RNA.

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Introduction

Annually, human-infecting arthropod-borne (arbo)viruses are responsible for over 400 million cases of human infection, and with a continuing increase in global temperatures, trade and travel, this number is expected to further increase [2–4,8–10]. Mosquito-transmitted flaviviruses (family *Flaviviridae*), such as Zika (ZIKV), West Nile (WNV), dengue (DENV) and yellow fever virus (YFV) are arboviruses of serious concern for human health [7,190]. ZIKV became an urgent global threat for human health after its introduction in South America, resulting in over 200,000 cases of ZIKV infection between 2015 and 2017 [461]. The ZIKV outbreak in the Americas correlated with an increased incidence of microcephaly and other neurological disorders, and was declared a public health emergency of international concern (PHEIC) by the World Health Organisation in 2016 [60]. Currently, ZIKV is considered a causative agent of microcephaly in the unborn child of ZIKV-infected pregnant women and Guillain-Barré syndrome in adults [52]. Furthermore, ZIKV can be sexually transmitted [63] and ZIKV infection may negatively affect the fertility of males [52]. WNV was first introduced into the western-hemisphere via New York in 1999, where it caused a major outbreak in humans in the United States (US) [462]. To date, over 48,000 cases of West Nile disease have been reported in the US alone [463–465] and WNV is endemic in southern Europe, Africa, Australia, North-America and South-America [19–21]. ZIKV is mainly transmitted by *Aedes* spp. mosquitoes, of which *Ae. aegypti* is the primary vector species, and mostly infects humans and non-human primates [466]. In contrast, WNV is transmitted by *Culex* spp. mosquitoes (predominantly *Culex pipiens*) and mostly infects birds. Occasionally, blood feeding of a WNV-infected mosquito leads to infection of humans or other non-avian vertebrates, that may develop severe symptoms despite low levels of viremia. These non-avian vertebrates are considered as dead-end hosts for WNV [48,190].

Flaviviruses have a single-stranded positive-sense RNA genome that contains one open reading frame (ORF) that is translated into a single polyprotein. This polyprotein is post-translationally processed into the viral structural and non-structural proteins. The ORF is flanked by highly-structured 5' and 3' untranslated regions (UTRs), that are essential for virus replication [82,83]. During flavivirus infection, the viral genomic RNA is readily degraded by host 5'→3' exoribonucleases such as XRN1 [106]. However, when these XRNs encounter the highly-structured 3'UTR they stall on XRN-resistant RNA structures, which results in a residual RNA molecule referred to as subgenomic flavivirus RNA (sfRNA), that is very abundant in infected cells [106,108,321,467]. X-ray crystallography revealed that XRNs stall on tightly folded RNA structures, which form a “*molecular knot*” that is resistant to degradation by XRN [107,108]. The 3'UTR of ZIKV contains several secondary and tertiary RNA structures: duplicated stem-loop (SL) structures SL-I and SL-II, two dumbbell (DB)-like structures Ψ -DB and DB-1 and a 3'SL [468]. ZIKV produces at least two species of sfRNA in infected cells through stalling of XRN on SL-I and SL-II, respectively [108,352,468]. Although the 3'UTRs of WNV and ZIKV are highly similar, some differences are apparent. The WNV 3'UTR contains a UA-rich SL-I at its 5' end and the duplicated SL structures SL-II and SL-IV are separated by the additional SL-III. This SL region is followed by duplicated DB-structures DB-1 and DB-2 and a 3'SL [83]. During infection with WNV, three species of sfRNA can be discriminated as result from stalling on SL-II, SL-IV and DB-1, respectively [106,157,322].

Significant functions of sfRNA have been implicated in mammalian cells, such as induction of cytopathicity [106,469], evasion of the type I/II interferon response [109,111,112], decreased mRNA turnover, [450] and enhanced virus replication

[106,322]. Lately, the importance of sfRNA formation for the infection and subsequent transmission by the mosquito vector has been pinpointed. We previously showed that sfRNA1-deficient WNV-mutants display decreased infection and transmission efficiencies in *Culex* mosquitoes after an infectious blood meal. However, after intrathoracic injections, equal infection and transmission efficiencies were observed, indicative of a role for sfRNA during infection of the mosquito midgut [157]. Moreover, it was recently demonstrated in *Ae. aegypti*, that DENV-2 strains that produce lower amounts of sfRNA have reduced transmission efficiencies, suggesting that sfRNA may also have important functions during infection of *Ae. aegypti* mosquitoes [470]. Currently, direct evidence for sfRNA-mediated infection of and transmission by *Ae. aegypti* mosquitoes is lacking, and the molecular mechanism(s) by which sfRNA mediates the infection of mosquitoes remains enigmatic. Identification of mosquito proteins that interact with sfRNA may elucidate the pathways involved in sfRNA-mediated flavivirus infection of mosquitoes.

Here, we investigated whether ZIKV sfRNA is a determinant to overcome the midgut barrier through comparison of mosquito infection via blood feeding and intrathoracic injections. By RNA-affinity purification we identified mosquito proteins that interact with the sfRNA/3'UTR of both ZIKV and WNV, and map the RNA structures in sfRNA/3'UTR required for this interaction. Through RNA interference (RNAi)-based silencing of these sfRNA-interacting mosquito proteins we determined the significance of the interactions with ZIKV and WNV sfRNA for virus replication in *Ae. aegypti* cells. These results present the first description of sfRNA-interacting proteins of mosquitoes and provide important insights into sfRNA-mediated transmission of flaviviruses by mosquitoes. Furthermore, our results establish sfRNA as a generic determinant of flavivirus transmission by both *Aedes* and *Culex* spp. mosquitoes.

Results

Zika virus sfRNA1 is dispensable for replication in *Ae. aegypti* mosquito cells

To investigate the role of ZIKV sfRNA on the transmission by *Ae. aegypti* mosquitoes, an sfRNA1-deficient ZIKV-mutant was generated using a ZIKV infectious clone [471]. The pseudoknot interaction in SL-I, which is required for the formation of sfRNA1 [108], was mutated (Fig. 8.1A), to generate ZIKVΔSF1. We also attempted to generate ZIKVΔSF1+2 through mutation of the pseudoknot in SL-II (Fig. 8.1A), however, for this mutant we were unable to generate infectious virus from the transfection of *in vitro* transcribed, capped RNA (data not shown). To demonstrate that ZIKVΔSF1 is indeed sfRNA1-deficient, we infected *Ae. albopictus* C6/36 and U4.4, as well as *Ae. aegypti* Aag2, and *Ae. pseudoscutellaris* AP-61 cells with ZIKV or ZIKVΔSF1 at a multiplicity of infection (MOI) of 1. At 72 hours post-infection (hpi), RNA was isolated and subjected to northern-blotting with a 3'UTR-specific probe. Infection with ZIKV resulted in the production of sfRNA1 and sfRNA2 in all four cell types, however, in cells infected with ZIKVΔSF1 only sfRNA2 was produced, indicating that ZIKVΔSF1 is indeed sfRNA1-deficient (Fig. 8.1B). Moreover, the production of sfRNA was much lower in C6/36 cells for both ZIKV and ZIKVΔSF1, in accordance with the poor ZIKV growth rates in these cells, as reported previously [149].

Fig. 8.1 A ZIKV-mutant defective in sfRNA1 production is not attenuated in Aag2 cells. (A) Schematic overview of the ZIKV sfRNA secondary structure. SL stem loop; DB dumbbell, adapted from [468]. Numbers indicate the nucleotide position in the 3' untranslated region (UTR), counting from the 3' end. Black lines indicate pseudoknot interactions. Insets: the SL-I and SL-II RNA structures. Mutated nucleotides in ZIKVΔSF1 and ZIKVΔSF1+2 are shaded. (B) Northern blot using a 3'UTR-specific probe on total RNA from ZIKV or ZIKVΔSF1 infected C6/36, Aag2, AP-61 and U4.4 cells. (C) Aag2 cells were infected with ZIKV or ZIKVΔSF1 at a multiplicity of infection of 5 and the titer in the supernatant was determined at 24 and 48 hours post infection (hpi) by end-point dilution assay (EPDA) on Vero cells. Shown are the mean viral titers ± standard error of the mean from duplicate replicates. Dotted line indicates the EPDA detection limit.

To assess whether full-length sfRNA formation is essential for the replication of ZIKV in *Ae. aegypti*, we compared the growth kinetics of ZIKV and ZIKVΔSF1 in Aag2 cells. Aag2 cells were infected with ZIKV or ZIKVΔSF1 at an MOI of 5 and viral titers were determined at 24 and 48 hpi (Fig. 8.1C). At both timepoints, ZIKV and ZIKVΔSF1 reached similar titers, indicating that full-length sfRNA formation is not essential for the replication of ZIKV in Aag2 cells. This finding justifies the use of ZIKVΔSF1 to study the effect of full-length sfRNA formation on ZIKV transmission by *Ae. aegypti* mosquitoes, without obvious interference from attenuated virus replication.

Zika virus sfRNA is essential for the efficient infection of and transmission by *Ae. aegypti*

We have previously shown that sfRNA is required for the efficient transmission of WNV by *Culex* mosquitoes and identified sfRNA as a key determinant of midgut-dependent infection [157]. As several structural elements (e.g. XRN-resistant SLs, DBs and the 3'SL) of sfRNA are highly conserved within viruses of the genus *Flavivirus* [323,324], we hypothesized that the function of sfRNA as a determinant of mosquito infection and transmission is shared between flaviviruses. To test this hypothesis, we analysed whether sfRNA is also required for the transmission of ZIKV by *Ae. aegypti* mosquitoes (Fig. 8.2). *Ae. aegypti* females were allowed to feed on an infectious blood meal containing 3.0×10^6 tissue culture infectious-dose 50% (TCID₅₀)/ml of ZIKV or ZIKVΔSF1 in three independent biological replicates (Fig. 8.2A). The viral titers of the individual blood meals were determined by end-point dilution assay (EPDA) to confirm that indeed equal titers were administered of both ZIKV and ZIKVΔSF1 (Fig. 8.2B). At 14 days post inoculation, the infection and transmission rates of exposed mosquitoes were determined by infectivity assay on Vero cells (Fig. 8.2C). In the current study, the infection rate was defined as the percentage of virus-positive mosquito bodies from the total number of engorged mosquitoes and the transmission rate was defined as the percentage of virus-positive saliva samples out of the total number of engorged mosquitoes.

Fig. 8.2 Full-length sfRNA production is essential for ZIKV infection of and transmission by *Ae. aegypti*. (A) Schematic overview of the experimental set-up. (B) Titers of the infectious blood meals used in the three replicate experiments were determined by end-point dilution assay (EPDA) on Vero cells. Shown is the mean titer $\pm$ standard error of the mean (SEM). Statistics were performed by unpaired t-test. (C) Female *Ae. aegypti* were allowed to feed on an infectious blood meal containing 3.0×10^6 TCID₅₀/ml of ZIKV or ZIKVΔSF1. Engorged females were incubated for 14 days at 28 °C and infection and transmission rates were determined by infectivity assay on Vero cells. Dissemination rates were determined by comparing the number of virus-positive saliva samples to the number of virus-positive bodies. Shown are the mean infection, transmission and dissemination rates $\pm$ SEM from three independent experiments. Statistics were performed by Fisher's exact test on cumulative data. (D) Viral titers in the bodies of mosquitoes with ZIKV-positive (Inf.) and ZIKV-negative (Neg.) saliva and titers of ZIKV-positive saliva samples were determined by EPDA on Vero cells. Shown are the average titers $\pm$ SEM. Statistics were performed by unpaired t-test or Mann-Whitney U test. ns, not-significant. Dotted line indicates the EPDA detection limit.

ZIKV reached an infection rate of 66%, while a significantly lower percentage of infected mosquitoes was observed for ZIKVΔSF1 (44%; $P = 0.0003$), demonstrating that full-length sfRNA is essential for efficient ZIKV infection in *Ae. aegypti*. A transmission rate of 31% was reached for ZIKV, while ZIKVΔSF1 reached a significantly lower transmission rate of only 5% ($P < 0.0001$). We then calculated the dissemination rate as percentage of mosquitoes with ZIKV-positive saliva out of the population of ZIKV infected mosquitoes. A dissemination rate of 47% was reached for ZIKV infected mosquitoes, opposed to only 11% for the ZIKVΔSF1 infected mosquitoes ($P < 0.0001$), indicating that full-length sfRNA is very important for ZIKV dissemination to the saliva.

To assess if the production of full-length sfRNA would affect the viral load in both the mosquito body and saliva, we determined the viral titers in saliva-positive mosquito bodies, saliva-negative mosquito bodies and in the virus-positive saliva samples (Fig. 8.2D). The titers of ZIKV and ZIKVΔSF1 were similar in the bodies of both saliva-positive ($3.6\text{--}4.6 \times 10^6$ TCID₅₀/ml; $P = 1.00$) and saliva-negative mosquitoes ($1.8\text{--}2.2 \times 10^6$ TCID₅₀/ml; $P = 0.63$). This indicates that sfRNA is mostly required to establish an initial infection in the mosquito, and does not generate an obvious replicative advantage after infection has been established. The titers of ZIKV and ZIKVΔSF1 in the saliva samples were not significantly different ($P = 0.11$). However, the mean ZIKV titer was still 6-fold lower in the saliva of ZIKVΔSF1 exposed mosquitoes (4.1×10^3 vs. 6.3×10^2), suggesting that sfRNA may also play a role in the further dissemination of ZIKV into the saliva.

ZIKV sfRNA is required to overcome the midgut and salivary gland barriers of *Ae. aegypti* mosquitoes

The major hurdle for the transmission of mosquito-borne flaviviruses, and arguably all arboviruses, is the midgut [142,190]. The significance of the midgut as a barrier for a certain virus can be demonstrated by intrathoracic virus injections, which bypasses the midgut and establishes a systemic infection via the mosquito haemolymph. To investigate the role of full-length ZIKV sfRNA in overcoming the midgut barrier, female *Ae. aegypti* mosquitoes were injected with ~ 400 TCID₅₀ of ZIKV or ZIKVΔSF1. After 7 days, the infection and transmission rates of exposed mosquitoes were determined by infectivity assay on Vero cells (Fig. 8.3A). As expected, all injected mosquitoes (100%) were infected with either ZIKV or ZIKVΔSF1. Interestingly, similar transmission rates were reached for ZIKV and ZIKVΔSF1 (55% vs. 68%; $P = 0.32$). The viral titers in the virus-positive bodies and saliva samples were determined by EPDA on Vero cells and demonstrated that ZIKV and ZIKVΔSF1 reached similar titers in the bodies of *Ae. aegypti* (Fig. 8.3B; $1.1\text{--}1.2 \times 10^7$ TCID₅₀/ml; $P = 0.49$). This demonstrates that sfRNA is not essential for replication of ZIKV in the mosquito body after passing the midgut barrier in *Ae. aegypti*. However, the titers of the mosquito saliva samples were significantly lower for ZIKVΔSF1 as compared to ZIKV (3.6×10^3 vs. 6.3×10^4 TCID₅₀/ml; $P = 0.01$), suggesting that full-length sfRNA is required for ZIKV to overcome the salivary gland barrier in *Ae. aegypti* mosquitoes.

Fig. 8.3 ZIKV sfRNA is required to overcome the midgut- and salivary gland barriers.

(A) Female *Ae. aegypti* mosquitoes were intrathoracically injected with ~400 TCID₅₀ of ZIKV or ZIKV Δ SF1. At 7 days post injection, infection and transmission rates were determined by infectivity assay on Vero cells. Dissemination rates were determined by comparing the number of virus-positive saliva samples to the number of virus-positive bodies. Shown are the mean infection, transmission and dissemination rates. Statistics were performed by Fisher's exact test. (B) Viral titers in the bodies of virus-positive bodies and saliva samples were determined by end-point dilution assay (EPDA) on Vero cells. Shown are the average titers $\pm$ standard error of the mean. Statistics were performed by Mann-Whitney U test. ns, not-significant. Dotted line indicates the EPDA detection limit.

ZIKV and WNV sfRNA do not modulate the expression of Toll or JAK/STAT regulated genes early after infection of *Ae. aegypti* cells

While we have now established that sfRNA is required for the infection and transmission of both ZIKV by *Ae. aegypti* (see Fig. 8.2 & 8.3) and WNV by *Cx. pipiens* mosquitoes [157], the underlying mechanism remains unclear. Recently it was demonstrated for DENV that sfRNA slightly modulates the expression of genes involved in the antiviral Toll pathway [171], which correlated with enhanced transmission by *Ae. aegypti* mosquitoes [470]. We therefore hypothesized that sfRNA may suppress mosquito innate immune pathways, to increase flavivirus infection and transmission rates. To test this hypothesis, Aag2 cells were either infected with WNV, WNV Δ SF1+2, ZIKV or ZIKV Δ SF1 at an MOI of 1, treated with heat-inactivated bacteria (positive control), or left untreated (Mock). At 24 hours post treatment, RNA was extracted from the cells and subjected to qRT-PCR with primers specific to the mRNA of the Toll regulated genes *CecG* and *DefC* [472,473] or the JAK/STAT regulated gene *TEP22* [470]. The values were normalized to the *rpS7* reference gene [474]. Furthermore, the viral genomic (vg)RNA copies of WNV and ZIKV in infected cells were quantified by qRT-PCR and also normalized to the *rpS7* reference gene (Fig. 8.4A-H). As expected, addition of heat-inactivated bacteria significantly upregulated the expression of *CecG*, *DefC* and *Tep22*, indicating that Aag2 cells are capable of Toll and JAK/STAT-signalling (Fig. 8.4B-D & F-H). However, even though WNV and WNV Δ SF1+2 reached high levels of vgRNA replication in infected cells (Fig. 8.4A), no induction of *CecG*, *DefC*, nor *TEP22* expression was observed (Fig. 8.4B-D). Similarly, the replication of ZIKV or ZIKV Δ SF1 (Fig. 8.4E) did not induce the expression of *CecG*, *DefC*, nor *TEP22* (Fig. 8.4F-H). These results indicate that full-length sfRNA is not essential to prevent or suppress the induction of a Toll or JAK/STAT signalling response, and questions the role of these innate immune responses during the early stage of WNV and ZIKV infection of mosquitoes.

Fig. 8.4 SfrRNA does not modulate the expression of Toll and JAK/STAT regulated genes early after infection of *Ae. aegypti* cells. Aag2 cells were infected with (A-D) WNV, WNVΔSF1+2, (E-H) ZIKV, ZIKVΔSF1 at a multiplicity of infection of 1, treated with heat-inactivated bacteria (+) or left untreated (mock). At 24 hours post treatment, total RNA was extracted and subjected to qRT-PCR to quantify the expression of the Toll-regulated genes (B,F) CecG and (C,G) DefC, the JAK/STAT regulated gene (D,H) TEP22 and the copies of (A,E) viral genomic (g)RNA. Gene expression was normalized to the rpS7 reference gene and the untreated mock control. Shown is the mean normalized relative expression of two replicates from three independent biological experiments ± standard error of the mean. Statistics were performed by One-way ANOVA with Tukey's post-test. Cq Cycle of quantification. ns, not significant.

Streptavidin-binding aptamers as a tool for sfrRNA-affinity purification

RNA-affinity purification has been used successfully to identify mammalian RNA-binding proteins that interact with the sfrRNA/3'UTR of several flaviviruses [105,437,475,476]. We therefore hypothesized that in mosquitoes, host proteins may similarly interact with sfrRNA, to mediate flavivirus replication and transmission. We performed sfrRNA-affinity purification using the S1m aptamer, which has high-affinity for streptavidin (SA) [477], to identify RNA-binding proteins for both ZIKV and WNV sfrRNA (Fig. 8.5A). We produced *in vitro* transcribed RNA corresponding to the sequences of 4XS1m-ZIKV-sfrRNA, 2XS1m-ZIKV-sfrRNA and ZIKV-sfrRNA. The RNAs were incubated with SA-coated beads to allow RNA-to-bead coupling, after which the beads were washed and collected by centrifugation. Input, unbound and bound RNA was extracted and analysed by gel-electrophoresis (Fig. 8.5B; left panel). The highest percentage of RNA-to-bead coupling was achieved for 4XS1m-ZIKV-sfrRNA while nearly no bead-bound RNA was observed for ZIKV-sfrRNA lacking aptamers. We then produced *in vitro* transcribed RNA of 4XS1m-WNV-sfrRNA and 4XS1m-Control, which corresponds to a region in WNV NS2A of equal length as WNV sfrRNA, similar as the control RNA in the study of Ward *et al.* [437] (Fig. 8.5A). Both 4XS1m-WNVsfrRNA and 4XS1m-Control

showed high-affinity for the SA-beads (Fig. 8.5B; right panel). For all aptamer-fused RNAs, multiple bands were observed after gel-electrophoresis, as expected for the highly structured 4XS1m aptamer [477].

As both sfRNA and the S1m aptamers are highly structured RNAs, the fusion of 4XS1m to ZIKV or WNV sfRNA could interfere with folding of either RNA fragment. To identify sfRNA binding proteins it is essential that the RNA structures resemble those during a natural infection. To verify the folding of ZIKV and WNV sfRNA in fusion with the 4XS1m aptamers, an *in vitro* XRN-stalling assay was performed on 4XS1m-Zika-sfRNA, 4XS1m-WNV-sfRNA and the 4XS1m-Control. *In vitro* addition of XRN resulted in the accumulation of RNA corresponding to ZIKV-sfRNA1 for 4XS1m-ZIKV-sfRNA and WNV-sfRNA1 for 4XS1m-WNV-sfRNA (Fig. 8.5C). As expected, no sfRNA could be detected for the 4XS1m-Control. This result indicates that the complex RNA structures required for XRN stalling of ZIKV and WNV sfRNA are correctly folded in the presence of the 4XS1m sequence, thereby validating the 4XS1m aptamer system to study ZIKV and WNV sfRNA-binding proteins.

Fig. 8.5 A streptavidin-binding aptamer approach for the detection of sfRNA-binding proteins. (A) Schematic overview of the constructs used to produce 4XS1m-aptamer-fused ZIKV sfRNA, WNV sfRNA and the WNV NS2A Control. Numbers represent the nucleotide position in the 3'untranslated region, counting from the 3'end. (B) Streptavidin beads were incubated with *in vitro* transcribed RNA of 4XS1m-ZIKV-sfRNA, 2XS1m-ZIKV-sfRNA, ZIKV-sfRNA, 4XS1m-WNV-sfRNA or 4XS1m-Control. RNA was extracted from input (I), unbound (U) and bound (B) RNA and analysed on 0.5X TBE/poly-acrylamide gel. (C) *In vitro* transcribed RNA of 4XS1m-ZIKV-sfRNA, 2XS1m-ZIKV-sfRNA, ZIKV-sfRNA, 4XS1m-WNV-sfRNA or 4XS1m-Control was treated with 0, 0.1 or 1 unit (U) of *in vitro* exoribonuclease (XRN) and analysed on 0.5X TBE/poly-acrylamide gel.

Identification of ZIKV and WNV sfRNA binding proteins in *Ae. aegypti* cells

To investigate which mosquito proteins interact with ZIKV and WNV sfRNA, we employed an sfRNA affinity pull-down in combination with a mass spectrometry approach in three independent biological replicates (Fig. 8.6A). Briefly, SA-beads were coated with 4XS1m-ZIKV-sfRNA, 4XS1m-WNV-sfRNA or 4XS1m-Control. RNA-coated beads were incubated with lysates of non-infected Aag2 cells to allow RNA-protein interactions to establish. Samples were washed five times, RNA-bound proteins were eluted with RNase A and eluates were analysed by mass spectrometry to identify and quantify the RNA-bound proteins. The mean relative abundance from the three independent biological replicate experiments was computed as fold difference in abundance in the sfRNA samples compared to the control sample, and statistically compared by students t-tests. Volcano plots were generated for proteins detected for ZIKV and WNV sfRNA where proteins with a ≥ 10 -fold relative enrichment in the sfRNA samples compared to the control sample. Proteins with a P -value of ≤ 0.05 were considered as significant (Fig. 8.6B & C). The complete list of proteins detected by mass spectrometry as putative interacting partners of the two sfRNAs is included in Table S8.1 (ZIKV) and Table S8.2 (WNV). Both the ZIKV (Fig. 8.6B) and WNV (Fig. 8.6C) sfRNA samples were significantly enriched for three proteins: ME31B (ortholog of human DDX6), Ataxin-2 (ATX2) and AAEL018126. Additionally, 3 and 14 proteins were ≥ 10 -fold enriched with lower significance in the ZIKV and WNV samples, respectively (Fig. 8.6B & C; insets). Notably, Lingerer (Lig) and LSM12, the latter which was previously identified as a DENV 3'UTR binding protein in mammalian cells [437], were ≥ 10 -fold enriched in both datasets.

To further investigate the biological processes that may be modulated by sfRNA, those proteins that were ≥ 3 -fold enriched for both WNV and ZIKV sfRNA were further analysed. Twenty-five proteins matched these criteria, and for 24 proteins an ortholog in *Drosophila* could be determined. The putative protein-protein interaction network of these sfRNA binding proteins was determined via the STRING database with high confidence level (≥ 0.7) using these *Drosophila* orthologs, as protein-protein interactions are poorly described for *Ae. aegypti*. The sfRNA binding proteins were significantly enriched in the computed protein-protein interactions ($P = 2 \times 10^{-15}$), indicating that these proteins are likely biologically connected and involved in similar processes in *Ae. aegypti*. Interestingly, two main interaction networks were implicated (Fig. 8.6D). Analysis of the functions of these 24 *Drosophila* orthologs using the Panther Gene Ontology server, implicated their involvement in posttranscriptional regulation of gene expression ($P = 6.4 \times 10^{-6}$) and cytoplasmic translation ($P = 3.9 \times 10^{-2}$). Most likely, the identified complex of ME31B, PABP, LSM12, TYF, LARP and REF2 is mostly involved in posttranscriptional regulation of gene expression, while the complex of primarily ribosomal proteins is involved in cytoplasmic translation (Fig. 8.6D). In summary, these results suggest that ZIKV and WNV sfRNA interact with these two mosquito protein networks to mediate the transmission of flaviviruses by their mosquito vector.

SfRNA has high affinity for the DEAD-box helicase ME31B

To confirm the observed interaction of ME31B, ATX2 and AAEL018126 with sfRNA, we attempted to clone the cDNAs of *Ae. aegypti* ME31B, ATX2 and AAEL018126 into a mosquito poly-ubiquitin (PUB) promoter-driven plasmid. Additionally, we included LSM12 as a potential sfRNA-binding candidate, as it was >10 fold enriched for both WNV and ZIKV sfRNA and its mammalian ortholog was previously characterised as a binding protein of the DENV-2 3'UTR [437]. Furthermore, we included the 60s ribosomal protein (60sRp) as a positive control, as it bound equally to ZIKV sfRNA, WNV sfRNA and the control RNA in our mass spectrometry approach (Table S8.1 & S8.2). ME31B, ATX2, LSM12 and 60sRp were successfully cloned into PUB-driven plasmids and fused N-terminally to EGFP-2A-3XFLAG (3F) or EGFP (Fig. 8.7A). The foot-and-mouth disease virus 2A peptide sequence is a ribosomal skipping element [478], resulting in the production of separate EGFP-2A, and the 3XFLAG-fusion protein. Unfortunately, we were unable to PCR-amplify the AAEL018126 gene, which may be due to low gene expression, or discrepancies between the reported genome sequence of *Ae. aegypti* AagL5.1 and the sequence in our Aag2 cells. Next, Aag2 cells were transfected with pPUB-3F-ME31B, pPUB-3F-ATX2, pPUB-3F-LSM12 or pPUB-3F-60sRp and lysates were subjected to RNA-affinity purification using SA-beads coated with 4XS1m-WNV-sfRNA, 4XS1m-ZIKV-sfRNA or 4XS1m-Control, similar as described above. Input and RNase A-eluted proteins were subjected to western blot analysis with α -FLAG antibodies (Fig. 8.7B).

Similar to the results from mass spectrometry, 3F-ME31B was highly enriched for WNV and ZIKV sfRNA, but not for the control RNA (Fig. 8.7B), confirming that sfRNA has strong and specific affinity for ME31B. Surprisingly, and in contradiction to the mass spectrometry results, 3F-ATX2 was not enriched for WNV nor ZIKV sfRNA (Fig. 8.7B). For both 3F-LSM12 and 3F-60sRp no obvious enrichment was observed for either sfRNAs and the control RNA (Fig. 8.7B). As ATX2 is predicted to interact with ME31B (see Fig. 8.6D), we hypothesized that ATX2 could indirectly interact with sfRNA via ME31B. To test this hypothesis, we performed a similar RNA-affinity purification using lysates of Aag2 cells co-transfected with both pPUB-EGFP-ME31B and pPUB-3F-ATX2 (Fig. 8.7C). With these co-transfected cell lysates, both 3F-ATX2 and EGFP-ME31B were enriched with both WNV and ZIKV sfRNA, confirming that ATX2 interacts with sfRNA indirectly via ME31B.

► **Fig. 8.7 (Next page) *Ae. aegypti* ME31B and ATX2 interact with ZIKV and WNV sfRNA.** (A) Schematic overview of the used plasmid constructs. (B) RNA-affinity purification to confirm WNV and ZIKV sfRNA binding proteins. Lysates of Aag2 cells transfected with pPUB-3F-ME31B, pPUB-3F-ATX2, pPUB-3F-LSM12 or pPUB-3F-60sRp were prepared. Lysates were subjected to RNA-affinity purification using streptavidin beads coated with *in vitro* transcribed RNA of 4XS1m-WNV-sfRNA, 4XS1m-ZIKV-sfRNA or 4XS1m-Control. RNA-bound proteins were eluted with RNase A and detected by western blot with α -Flag antibodies. (C) ATX2 interacts with ZIKV and WNV sfRNA indirectly via ME31B. Lysates of Aag2 cells transfected with pPUB-3F-ATX2, pPUB-EGFP-ME31B or both pPUB-3F-ATX2 and pPUB-EGFP-ME31B were prepared. Lysates were again subjected to RNA-affinity purification as in panel B and RNA-bound proteins were detected by western blot with α -Flag and α -GFP antibodies.

ME31B is an antiviral gene that inhibits ZIKV and WNV genomic RNA replication in *Ae. aegypti* cells

The confirmed interaction of sfRNA with ME31B, ATX2 and possibly AAEL018126, implicates a putative role of these mosquito proteins during flavivirus infection. We therefore investigated whether gene silencing of ME31B, ATX2 or AAEL018126 would affect the replication of ZIKV or WNV Aag2 cells. Therefore, cells were transfected with dsRNA against ME31B, ATX2, AAEL018126 or luciferase (Luc) as negative control, and re-transfected after 2 days to reach high silencing efficiencies [206]. Total RNA was isolated from a proportion of the transfected cells and gene silencing efficiencies of 75-90% were determined by qRT-PCR (Fig. 8.8A). The remaining cells were plated into 96-well plates and infected with ZIKV, ZIKVΔSF1, WNV or WNVΔSF1+2 at an MOI of 0.1. At 48 hpi, viral titers in the supernatant were determined by EPDA, total RNA was extracted from cell monolayers and subjected to qRT-PCR to quantify the vgRNA copies. Interestingly, silencing of ME31B resulted in significantly higher vgRNA levels for ZIKV, ZIKVΔSF1, (Fig. 8.8B) WNV and WNVΔSF1+2 (Fig. 8.8C), while silencing of ATX2 and AAEL018126 did not affect ZIKV nor WNV replication (Fig. 8.8B & C). To further investigate the effect of ME31B silencing on virus replication, the viral titers were determined in the ME31B and control silenced cells. For both ZIKV and ZIKVΔSF1, silencing of ME31B slightly, but not significantly, increased the viral titers. Similarly, for WNV and WNVΔSF1+2, silencing of ME31B correlated with higher viral titers, but did not result in a statistically significant difference. Together, these results indicate that ME31B inhibits ZIKV and WNV genomic RNA replication in Aag2 cells, irrespective of full-length sfRNA formation.

Fig. 8.8 Depletion of ME31B increases WNV and ZIKV replication in *Ae. aegypti* cells. (A) Confirmation of silencing efficiencies by qRT-PCR. Aag2 cells were transfected with dsRNA against Luciferase (Luc), ME31B, Ataxin-2 (ATX2) or AAEL018126 (AAEL) and re-transfected after 48 hours. RNA was harvested and subjected to qRT-PCR with gene-specific primers, normalized to the RpS7 reference gene and the relative gene expression was computed compared to the dsLuc transfected control. Unpaired t-tests were used to compare silencing efficiencies. (B-C) Effect of gene silencing on viral RNA replication of ZIKV and WNV. At 4 hours post second transfection, cells were infected with (B) ZIKV, ZIKVΔSF1, (C) WNV, or WNVΔSF1+2 at a multiplicity of infection (MOI) of 0.1. Viral genome copies were determined at 48 hours post-infection (hpi) by qRT-PCR normalized to the rpS7 reference gene, and the relative viral genomic (g)RNA copies were computed compared to the dsLuc transfected control. (D-E) Effect of ME31B silencing on the replication of ZIKV and WNV. At 4 hours post second transfection, cells were infected with (D) ZIKV, ZIKVΔSF1, (E) WNV, or WNVΔSF1+2 at an MOI of 0.1. Viral titers were determined at 48 hpi by end-point dilution assay on Vero cells. One-way ANOVA with Tukey's post-hoc test was used to compare ¹⁰Log transformed viral titers and relative viral gRNA copies.

ME31B binds specifically to the DB-1 structure of ZIKV and the orthologous DB-2 structure of WNV sfRNA

As sfRNA may bind to ME31B to antagonise its antiviral effects, we expected that sfRNA-deficient flaviviruses would benefit more from ME31B silencing than wild type flaviviruses. However, we observed similar effects on wild type and sfRNA-deficient ZIKV and WNV. As ZIKV Δ SF1 and WNV Δ SF1+2 still produce small amounts of sfRNA2 and sfRNA3, respectively, we cannot exclude that the amount of sfRNA still produced by these mutants is sufficient for ME31B binding. To investigate the mosquito ME31B binding site on WNV and ZIKV sfRNA, we performed an RNA-affinity purification using 3' truncated sfRNAs (Fig. 8.9). As expected, ME31B was again enriched for full-length or Δ 3'SL sfRNA of ZIKV and WNV, but not the control RNA. However, when DB-2 of WNV and the orthologous DB-1 of ZIKV was deleted this enrichment was lost, implicating that ME31B binds to DB-2 and the orthologous DB-1 of the WNV and ZIKV sfRNA, respectively. Together, these results indicate an interaction of ME31B and ATX2 with WNV and ZIKV sfRNA, that depends on the specific affinity the DB-structures in sfRNA for ME31B. As ZIKV DB-1 and WNV DB-2 are still present in sfRNA2 of ZIKV and sfRNA3 of WNV, respectively, this may explain why ME31B-silencing resulted in similar increases in the replication of wild type and sfRNA-deficient ZIKV and WNV.

Fig. 8.9 ME31B binds to DB-1 of ZIKV sfRNA and the orthologous DB-2 of WNV sfRNA. Lysates of Aag2 cells transfected with pUB-3F-ME31B were subjected to RNA-affinity purification with 4XS1m-aptamer fused truncated-sfRNAs. A schematic overview of the used truncations is included in the figure. RNA-bound proteins were eluted with RNase A and detected by western blot with α -Flag antibodies.

Discussion

The molecular determinants of arbovirus transmission by mosquitoes are largely unknown, and few interactions between arboviruses and their vectors have been described in detail. All flaviviruses produce a noncoding sfRNA through stalling of host XRN3 on RNA structures in the viral 3'UTR in both the mammalian and mosquito hosts. We previously showed that the production of sfRNA is essential for the transmission of WNV by *Cx. pipiens* mosquitoes, but the underlying molecular mechanism remains enigmatic [157]. As the production of sfRNA is conserved for all flaviviruses [105,106,114], sfRNA may act as a generic determinant of flavivirus transmission by their insect vector(s).

In the present study, we demonstrate that the production of full-length sfRNA is also essential for the transmission of ZIKV by *Ae. aegypti* mosquitoes. We showed that ZIKV sfRNA is essential to overcome the midgut infection barrier, as infection rates were decreased for ZIKVΔSF1 compared to ZIKV after an infectious blood meal, but not after intrathoracic injections. Furthermore, the titers of ZIKV in the saliva of ZIKVΔSF1 injected mosquitoes were decreased compared to mosquitoes injected with ZIKV, indicating that full-length sfRNA formation is also necessary for the efficient dissemination of ZIKV to the saliva. These results are similar to our results with WNV in *Cx. pipiens*, that identified WNV sfRNA as a key driver to overcome the midgut barrier, and also implicated a role for WNV sfRNA in dissemination to the saliva [157]. This suggests that sfRNA-mediated infection of and transmission by mosquitoes is a general phenomenon for flaviviruses in both *Aedes* and *Culex* spp. mosquitoes.

While several molecular mechanisms of sfRNA-mediated infection of and transmission by mosquitoes have been proposed [116,157,468,470,479], none of these has been convincingly confirmed yet. SfRNA is not efficiently packaged into virions and is dependent on sufficient vgRNA replication for its formation [106]. Therefore, functions of sfRNA can only be executed at a later stage of replication in infected cells. SfRNA could act as a viral suppressor of mosquito innate immune responses, such as the RNA interference (RNAi) response [104]. *In vitro* and *in vivo* in *Culex* mosquitoes, sfRNA suppresses Dicer slicing activity, possibly by acting as a decoy Dicer substrate [115,116]. However, in our previous *in vivo* study with WNV-infected *Culex* mosquitoes no significant difference in the abundance of viral small interfering RNAs was observed between wild type and sfRNA1-deficient WNV [157]. Furthermore, sfRNA1-deficient WNV replicated similarly to wild type WNV in RNAi-competent mosquito cells, indicating that sfRNA1 formation is not essential to evade the RNAi response [157]. Other innate immune responses of mosquitoes include the immune deficiency (Imd), JAK/STAT, and Toll signalling-pathways [102], of which the latter two have previously been implicated as antiviral during flavivirus infection of mosquitoes [169–172,174]. Thus, sfRNA may modulate these innate immune responses to enhance mosquito infection and transmission efficiencies. For DENV-2, in *Ae. aegypti* mosquitoes, it has been shown that increased levels of sfRNA production correlate with suppression of genes regulated by the Toll-pathway, but not those regulated by the Imd and JAK/STAT pathways [470]. However, the differences in mRNA levels were minor (<2 fold), thus, it remained questionable whether sfRNA causes sufficient differences in the protein levels of these immune regulatory pathways. In the present study, we did not observe an induction of Toll- or JAK/STAT regulated gene expression at 24 hpi with wildtype and sfRNA-deficient ZIKV and WNV in *Ae. aegypti* cells. Thus, sfRNA-mediated infection of mosquitoes is likely not the result of suppressed Toll- or JAK/STAT induction. However, previous studies with DENV indicated a strong induction of Toll- and JAK/STAT regulated genes at 48 hpi in Aag2 cells [480]. Therefore, we cannot exclude whether ZIKV and

WNV infection may induce innate immune pathways at a later time-point post-infection or whether evasion of the Toll- and JAK/STAT pathways by sfRNA is DENV specific.

Alternatively, sfRNA could act as a “molecular sponge” by interacting with mosquito RNA-binding proteins to antagonize or redirect their functionality, as previously hypothesized [105]. Here we have identified several *Ae. aegypti* proteins that bind to ZIKV and WNV sfRNA. The most significant sfRNA-interacting proteins, ME31B and ATX2, are associated with ribonucleoprotein (RNP) granules, such as stress granules (SGs) and processing bodies (PBs) [481]. SGs are formed when translation is impaired due to stress and typically contain stalled mRNAs and proteins involved in translation and mRNA turnover [434]. SGs function by re-organizing proteins and mRNAs to either prevent mRNA degradation, facilitate the formation of messenger-RNP complexes and allow the translation of stress-responsive mRNAs whose translation is cap-independent [434]. Contrarily to SGs, PBs are constitutively present. They function as storage hubs for mRNAs that are translationally repressed, mostly representing mRNAs encoding regulators of mRNA metabolism, cell cycle, translation, and histone modifications [482]. PBs contain many proteins involved in mRNA turnover, such as the decapping proteins DCP1/2, exoribonucleases such as XRN1/Pacman and other proteins involved in mRNA decay such as ME31B, EDC3 and LSMs [483]. Not surprisingly, many viruses interfere with PB assembly [483], including members of the *Flaviviridae* family [484–486]. Silencing of ME31B leads to the disassembly of PBs, but ME31B is dispensable for SG formation [487,488]. Thus, the increased genomic RNA replication of ZIKV and WNV upon ME31B silencing found in this study suggests that PBs may have antiviral activity during flavivirus replication in *Ae. aegypti* cells.

ME31B is a member of the DEAD-box family of RNA helicases, and is involved in mRNA decapping, translation inhibition, mRNA storage and miRNA-mediated mRNA decay [489,490]. We observed increased replication of ZIKV and WNV upon ME31B silencing, thereby elucidating an antiviral role of this protein. We demonstrate that ME31B binds directly to sfRNA, which is produced by XRN-mediated 5'→3' degradation of decapped vgRNA. Interestingly, sfRNA has been reported to co-localize with XRN1, which normally localizes to PBs, in cytoplasmic foci [106]. Curiously, a complex of PB-related proteins, such as ATX2, Lig, poly-(A) binding protein (PABP), and LSM12 were also enriched for sfRNA by mass spectrometry. Furthermore, the significantly enriched protein AAEL018126 has closest homology to the *Drosophila* twenty-four gene (tyf; ~29% homology), which has known interactions with ATX2 and LSM12 [491]. As ATX2 was observed to bind sfRNA via interaction with ME31B, a complex of PB proteins, which also includes AAEL018126, is likely co-purified with ME31B. Interestingly, ATX2 is also found in SGs and is essential for SG-formation [492]. Together, our results support a model in which sfRNA modulates PBs and/or SGs through sequestration of ME31B during infection of *Ae. aegypti*. Future studies should focus on elucidating a direct link between PB/SG-modulation and sfRNA-mediated infection and transmission of flaviviruses by mosquitoes.

Previous studies have applied RNA-affinity purification to identify sfRNA/3'UTR binding proteins in mammalian cells [109,437,475,493]. Mammalian orthologs of ME31B (DDX6), LSM12, PABP (PABP1) and the ribosomal proteins, have been observed to interact with the 3'UTR of flaviviruses, suggesting that the binding or sequestration of these proteins serve common functions in mammalian and mosquito cells. However, interactions of sfRNA with mammalian orthologs of ATX2 (ATXN2), AAEL018126 (no reported orthologs) and Lig (UBAP2) have not been described. Thus, sfRNA may exhibit both similar and differential functions in mammalian versus mosquito hosts. Alternatively, differences in the methodology of RNA-affinity purification, such as the use of tobramycin aptamers or biotin-labelled RNAs, may affect experimental results.

We applied a label-free mass-spectrometry approach, which is less sensitive compared to labelled methods and therefore results in more false-negatives [494]. While this makes it likely that the significantly enriched proteins that we identified are indeed biologically relevant, it is possible that more *Ae. aegypti* sRNA-binding proteins could have been identified using a more sensitive detection method.

In conclusion, we have established sRNA as a generic determinant of flavivirus transmission by not only *Cx. pipiens*, but also *Ae. aegypti* mosquitoes. We demonstrated that sRNA is essential to overcome both the midgut-, and salivary-gland barriers in infected mosquitoes. Via RNA-affinity purification several *Ae. aegypti* sRNA-binding proteins were identified, that connect sRNA to the modulation of PBs, SGs, mRNA turnover, and protein translation. These results contribute to our understanding of the underlying molecular mechanism(s) of this intriguing noncoding RNA, and how it fine-tunes the infection of vector mosquitoes to ensure efficient transmission to vertebrate hosts.

Materials and Methods

Cell lines

African green monkey kidney Vero E6 cells (ATCC CRL-1586) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml; Sigma-Aldrich) and streptomycin (100 µg/ml; Sigma-Aldrich) and incubated at 37 °C with 5% CO₂. For experiments, Vero cells were incubated in DMEM-HEPES (Gibco) supplemented with penicillin and streptomycin (DMEM-supplemented). For experiments with mosquito samples, additional Fungizone (50 µg/ml) and gentamycin (50 µg/ml; Life Technologies) were added (DMEM-complete). *Ae. aegypti* Aag2 and *Ae. pseudoscutellaris* AP-61 cells were cultured in Schneider's *Drosophila* medium (Lonza) supplemented with 10% FBS and incubated at 28 °C. *Ae. albopictus* C6/36 (ATCC CRL-1660) and *Ae. albopictus* U4.4 cells were cultured in Leibovitz L-15 medium (Gibco) supplemented with 10% FBS, 2% tryptose phosphate broth (Gibco) and 1% nonessential amino acids (Gibco) and incubated at 28 °C. During experiments gentamicin (50 µg/ml) was added to the culture medium of Aag2 and C6/36 cells.

Viruses

Infectious clone derived WNV GR₁₀ strain (WNV_{ic}; GenBank KC496015.1) and sRNA-deficient mutants WNVΔSF1 and WNVΔSF1+2 were reported previously [157]. All WNV stocks used were of a second passage on C6/36 cells. A pCC1-BAC-based and SP6 promoter driven infectious clone of the BeH819015 ZIKV isolate from Brazil [471] was retrieved from prof. Dr Andres Merits and hereafter named pZIKV_{ic}. The NS5-3'UTR region was PCR amplified using primers 1,2 (All primers used are listed in Table 8.1) and cloned into pJET1.2 (Thermo Scientific) to create pJET1.2-ZIKVNS5-3'UTR. Site-directed mutagenesis using pJET1.2-ZIKVNS5-3'UTR as template and primers 3,4 was used to mutate the pseudoknot in SL-I (GGGG10423CCCC) to generate pJET1.2-ZIKVNS5-3'UTRΔSF1. Subsequently, site-directed mutagenesis using pJET1.2-ZIKVNS5-3'UTRΔSF1 as template and primers 5,6 was used to mutate the pseudoknot in SL-II (CGG10503ACC) to generate pJET1.2-ZIKVNS5-3'UTRΔSF1+2. The NS5-3'UTR region of pJET1.2-ZIKVNS5-3'UTRΔSF1 and pJET1.2-ZIKVNS5-3'UTRΔSF1+2 was cloned as BstBI/FseI (NEB) fragment into pZIKV_{ic} to generate pZIKVΔSF1 and pZIKVΔSF1+2. pZIKV_{ic}, pZIKVΔSF1 or ZIKVΔSF1+2 were linearized by AgeI digestion and used as template for SP6-based *in vitro* RNA transcription (NEB), with m⁷G_{ppp} RNA cap structure analog (NEB) following the manufacturer's protocol. In attempt to generate infectious virus, pZIKV_{ic}, pZIKVΔSF1 or pZIKVΔSF1+2-derived, *in vitro* transcribed, capped RNA was transfected into a pre-seeded monolayer of Vero cells in 6-well plates using Lipofectamine 2000 (Invitrogen). Six days post transfection (dpt) the supernatant was harvested and used to inoculate a T25 flask of Vero cells, which was harvested as P1 stock 3 days post inoculation. P2 and P3 stocks were generated on AP-61 cells and stored at -80 °C until further use. Virus titers of stocks were determined after one freeze-thaw cycle by EPDA on Vero cells.

Construction of RNA-aptamer and protein expression plasmids

Aptamer constructs were based on the design by Leppek *et al.*, 2014 [477]. The ZIKV sfRNA sequence was PCR amplified from pZIKV_{IC} using primers 7,8 containing a T7 promoter overhang and cloned into pJET1.2 to generate pJET-T7-sfRNA_{ZIKV}. pJET-T7-sfRNA_{ZIKV} was digested with PstI (NEB) and XbaI (NEB), which removes the original T7 promoter and a residual XbaI site, to generate pT7-sfRNA_{ZIKV}. Complementary oligos 9,10 containing the S1m-aptamer sequence were designed to generate BamHI and BglII overhangs. Oligos were annealed and ligated with T4 DNA ligase (Promega) to produce 1x-S1m, 2x-S1m and 4x-S1m DNA fragments that were ligated into pT7-sfRNA_{ZIKV} to generate pT7-S1m-sfRNA_{ZIKV}, pT7-2XS1m-sfRNA_{ZIKV} and pT7-4XS1m-sfRNA_{ZIKV}. Next, the WNV sfRNA and NS2A sequences were amplified with primers 11,12 and 13,14, respectively, from pWNV_{IC}. PCR products were cloned as NdeI/XbaI (NEB) fragments into pT7-4XS1m-sfRNA_{ZIKV} to replace the ZIKV sfRNA sequence and generate pT7-4XS1m-sfRNA_{WNV} and pT7-4XS1m-NS2A. EGFP was PCR-amplified from pEGFP-N1 with primers 15,16 that introduce attB1/2, AscI, EcoRI and NdeI sites and Gateway cloned (Invitrogen) into pDONR207 to generate pDONR-EGFP. Complementary oligos 17,18 containing the foot-and-mouth disease (FMDV) 2A ribosomal skipping element followed by a three-tandem repeat FLAG-tag (2A-3XFLAG) were annealed and ligated as AscI/EcoRI (NEB) fragment into pDONR-EGFP-3F. The genes coding for ME31B (AAEL008500), ATX2 (AAEL007805), LSM12 (AAEL007820) and 60sRp (AAEL010821) were PCR-amplified from Aag2 cells total RNA-derived cDNA using Q5 High-Fidelity DNA polymerase (NEB) and primers 19,20 (ME31B), 21,22 (ATX2), 23,24 (LSM12) or 25,26 (60sRp). PCR products were cloned as EcoRI/NdeI (ATX2, 60sRp, LSM12) or MfeI/NdeI (ME31B) fragment into pDONR-EGFP-3F and Gateway cloned into the previously described mosquito expression vector pPUB [495] to generate pPUB-3F-ME31B, pPUB-3F-ATX2, pPUB-3F-LSM12 and pPUB-3F-60sRp. The ME31B PCR product was additionally cloned as Mfe/NdeI fragment into pDONR-EGFP and subsequently Gateway cloned into pPUB to generate pPUB-EGFP-ME31B.

Infectious blood meals and mosquito processing

Ae. aegypti mosquitoes (Rockefeller strain, obtained from Bayer AG) were reared as described previously [149] and transported to the biological safety level 3 facility in buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey) before virus infection experiments. Before blood feeding, mosquitoes were starved for one day by offering them a cotton pad wetted in water. Infectious blood meals composed of a 1:1 mixture of virus and human blood (Sanquin) were prepared and added to a Hemotek feeder system covered by a Parafilm membrane. Mosquitoes were allowed to feed ad libitum for 1 h under light conditions at 24 °C and 70% relative humidity. Mosquitoes were anesthetized with 100% CO₂ and fully engorged females were selected and maintained in buckets at 28 °C in 12:12 light:dark cycle, 70% relative humidity and provided with a cotton pad wetted in 6% glucose solution that was refreshed every 2-3 days. After 14 days mosquitoes were anesthetized with 100% CO₂ and immobilized by removal of their legs and wings. The proboscis of each stripped mosquito was inserted into a 200 µl pipet tip containing 5 µl of a 25% glucose and 50% FBS solution. Mosquitoes were allowed to salivate for >45 min, after which the bodies were individually transferred to 1.5 ml Safe-Seal micro tubes (Sarstedt) that contained a small scoop of 0.5 mm zirconium beads (Next Advance). The saliva samples were added to 1.5 ml micro tubes (Sarstedt) that contained 60 µl DMEM-complete. All samples were stored at -80 °C until further processing.

Intrathoracic injections

Ae. aegypti mosquitoes were anesthetized with 100% CO₂ and kept on a CO₂ pad. Female mosquitoes were selected and injected with 69 nl (~400 TCID₅₀) ZIKV or ZIKVΔSF1 using a Drummond Nanoject II injector (Drummond Scientific) with glass capillaries (3.5" Drummond # 3-000-203-G/X; Drummond Scientific). Injected mosquitoes were placed in buckets and incubated at 28 °C in 12:12 light:dark cycle, 70% relative humidity, and provided with a cotton pad wetted in 6% glucose solution that was refreshed every 2-3 days. After 7 days, mosquitoes were processed as described above.

Infectivity assay

Frozen mosquito bodies were homogenised in a Bullet Blender Storm (Next Advance) for 2 min at maximum speed. Homogenised bodies were centrifuged for 1 min at 14,500 rpm, 100 µl of DMEM-complete was added and the homogenization was repeated. The mosquito debris was collected by 1 min centrifugation at 14,500 rpm and 30 µl of mosquito body supernatant or saliva sample was used to inoculate a pre-seeded Vero cell monolayer in a 96-well plates. After 2-3 h the inoculum was removed and replaced with fresh DMEM-complete and infectivity was scored at 3-4 dpi based on ZIKV-induced CPE.

Virus titrations

The TCID₅₀/ml was determined by EPDA on Vero cells. Briefly, Vero cells were detached with Trypsin (Gibco), and diluted in DMEM-supplemented or DMEM-complete for mosquito body and saliva samples. Tenfold dilutions of virus-containing supernatants were prepared in DMEM-supplemented/complete and added 1:1 to Vero cell suspension. Each dilution was plated in 6-fold into a 60-well Micro-Well plate (Nunc) and scored at 3-4 dpi by ZIKV or WNV-induced cytopathic effect (CPE).

Transfections and dsRNA-mediated gene silencing

Transfections of mosquito cells with DNA plasmids were performed with Fugene HD (Promega) in serum-free media following the manufacturer's protocol. Transfections of Vero cells with *in vitro* transcribed RNA were performed using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco). At 4 hours post transfection (hpt) the transfection mix was aspirated and replaced with fresh culture media. For silencing experiments, dsRNA was transfected into pre-seeded Aag2 cell monolayers in 24-well plates using Fugene HD. After 48 hours, the cell monolayers were re-transfected with dsRNA to ensure proper silencing efficiency. At 6 hours post re-transfection, cells were split into 96-well plates and a fraction was stored for RNA isolation and quantification of gene silencing by qRT-PCR. The 96-well plates of freshly-seeded silenced cells were inoculated with WNV, WNVΔSF1+2, ZIKV or ZIKVΔSF1 at an MOI of 0.1. Viral titers in the supernatant were determined by EPDA on Vero cells at 48 hpi.

***In vitro* RNA synthesis**

In vitro RNA was produced with either T7 or Sp6 RNA Polymerase (NEB) according to the manufacturer's protocols. To generate linear templates for production of 51m-aptamer containing RNAs, the corresponding plasmids were used as template for a PCR reaction with forward primer 27 and the corresponding reverse primer 28-30. Truncated sRNA species were generated by using forward primer 27 in combination with reverse primers 31-36. PCR products were gel-purified and additionally purified by phenol-chloroform-extracted before proceeding with T7 *in vitro* RNA synthesis. For generation of dsRNA for gene-silencing, partial gene sequences of ME31B, ATX2 and AAEL018126 were amplified with primers 37-44, that introduce flanking T7 promoters. PCR products were purified by phenol-chloroform extraction and 1 µg DNA was used as template for *in vitro* RNA synthesis using T7 RNA polymerase.

XRN-stalling assays

Prior to XRN stalling assay, the RNA was renatured by incubation at 56 °C for 5 min, 2 min incubation at RT and stored on ice. *In vitro* XRN stalling assays were performed with Terminator 5' phosphate-dependent exonuclease (Epicentre) according to the manufacturer's protocol and the reaction was terminated by addition of 100 mM EDTA.

qRT-PCR

Total RNA was extracted with TRIzol reagent and RNA yield was quantified by nanodrop. Equal amounts of total RNA were treated with RQ1-RNase-free DNase (Promega) to remove residual DNA contamination and subjected to first-strand synthesis using Superscript II reverse transcriptase (Invitrogen) and random hexamers (Roche) according to the manufacturer's protocols. The resulting cDNA was diluted 1:5 and subjected to relative quantification by qRT-PCR using SYBR Select Master mix (Invitrogen) and primers 45-62 on a CFX96 Real-Time PCR system (BioRad). Cycling conditions were 95 °C (2 min), [95 °C (15 s), 55 °C (30 s), 72 °C (30s)] x40, 72 °C (30 s), followed by a 55-95 °C melt curve (0.5 °C/5 s) to verify amplicon homogeneity. Data analysis was performed using Bio-Rad CFX

Maestro software with relative quantification to a standard curve, consisting of 10-fold serial dilutions of Aag2 cells total-RNA derived cDNA and normalized to the rpS7 reference gene.

RNA electrophoreses & northern-blotting

Total RNA was size-separated by denaturing gel-electrophoresis in 6% polyacrylamide 7 M urea/TBE gel. Gels were stained with ethidium bromide and visualized with a Bio-Rad Gel Doc scanner. Northern-blotting and probe synthesis was performed as described previously [157]. Briefly, RNA was semidry blotted onto a Hybond-N membrane (Amersham Biosciences), UV cross-linked and hybridized overnight with PCR-generated digoxigenin (DIG)-labelled probes in modified Church/10% formamide buffer at 50 °C. Blots were probed with alkaline-phosphatase (AP)-conjugated α -DIG antibodies (11093274910; Roche) and developed with nitroblue tetrazolium (NBT)-BCIP (5-bromo-4-chloro-3-indolylphosphate) solution (Roche) until sufficient signal was achieved. The DIG-labelled WNV 3'UTR specific-probe was described previously [157]. The ZIKV 3'UTR specific DIG-labelled probe was generated by PCR with DIG-labelled nucleotides (Roche), primers 61,62 and pZIKV_{IC} as template.

RNA-affinity purification

RNA-affinity purification was based on the 4X S1m optimized streptavidin-binding RNA-aptamer system which has high affinity for streptavidin [477]. For samples prepared for liquid chromatography-mass spectrometry (LC-MS/MS) all subsequent steps were performed in LoBind tubes (Eppendorf). For RNA-affinity purification with overexpressed proteins the protocol was downscaled. Fifty-percent Streptavidin Sepharose High Performance bead-slurry (SA-beads; GE Healthcare) was equilibrated by washing thrice with SA-RNP lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 2 mM DTT, 2 mM vanadylribonucleosid complex RNase inhibitor (VRC; NEB) supplemented with Halt Protease and Phosphatase inhibitor cocktail (ThermoFisher) for samples analysed by LC-MS/MS or Complete protease inhibitor cocktail (Roche)) by 1 min 1,500 rpm centrifugation at 4 °C followed by resuspension of the SA-beads. The final pellet of equilibrated SA-beads was resuspended 1:1 in SA-RNP lysis buffer. Per 100 μ l equilibrated SA-beads 20 μ l of DNase-treated *in vitro* transcribed 4XS1m aptamer-containing RNA was dissolved to a total volume of 50 μ l in SA-RNP lysis buffer. RNA was renatured by incubation at 56 °C for 5 min, 37 °C for 10 min, and room-temperature (RT) for 2 min, and stored on ice until further use. Five percent of the total volume was stored in TRIzol (Invitrogen) to analyse the quality of the input-RNA. To the remaining renatured RNA 100 μ l equilibrated 50% SA-bead slurry was added and supplemented with 80 U RNasin (Promega). RNA and bead mixtures were incubated at 4 °C for 2.5 h with overhead rotation, with manual mixing every 15 mins to allow for RNA to bead coupling. RNA-bound beads were collected by 2 min 1,000 rpm centrifugation at 4 °C. Five percent of the supernatant was stored in 500 μ l TRIzol to analyze the quality of the unbound RNA. To prepare Aag2 cell lysates for RNA-affinity purification the culture medium of Aag2 cell monolayers was aspirated and the cells were washed once with PBS. Cells were detached by scraping, pelleted by 5 min 1,600 rpm centrifugation and washed twice with PBS. Aag2 cell pellets were lysed in SA-RNP lysis buffer supplemented with 1% NP-40 by 20 min incubation on ice. Nuclei were lysed by forcing the lysates through a 0.8 $\times$ 0.4 mm needle and the cell debris was removed by 15 min 14,000 rpm centrifugation at 4 °C. Protein concentrations were determined by Bradford Protein Assay (Bio-Rad) following the manufacturer's protocol and absorption was measured using a FLUOstar Optima microplate reader (BMG Labtech). Cell lysates (2.7 – 3.0 mg protein) were pre-cleared by incubation with 25 μ l Avidin agarose beads (Thermo Pierce) at 4 °C for 30 min under overhead rotation. Beads were removed by 1 min 14,000 rpm centrifugation at 4 °C and the supernatant was added to 50 μ l 50% SA-beads and incubated for 2.5 h at 4 °C under overhead rotation. Beads were removed by 1 min 14,000 rpm centrifugation at 4 °C and the supernatant was added to a new tube and supplemented with 40 U RNasin. For protein-to-RNA coupling, 100 μ l pre-cleared lysate was added to $\sim$ 50 μ l of RNA-bound SA-beads and incubated at 4 °C for 3.5 h under overhead rotation. Beads were collected by 1 min 1,000 rpm centrifugation at 4 °C and resuspended in SA-wash buffer (20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM MgCl₂, 2 mM DTT) supplemented with VRC and protease inhibitors. A total of five wash steps were performed followed by a final wash with SA-wash buffer supplemented with only protease inhibitors. The final pellet was eluted by addition of 30 μ l SA-elution buffer (20 mM

Tris-HCl, 30 mM NaCl, 5 mM MgCl₂, 2 mM DTT) supplemented with protease inhibitors and 0.6 µg RNase A (Invitrogen) followed by 10 min incubation at 4 °C. Beads were removed by 1 min centrifugation at 6,000 rpm at 4 °C, ~30 µl eluate was collected and the residual beads were removed by 1 min centrifugation at 6,000 rpm at 4 °C. The final eluate was supplemented with 10 µl 4X SDS loading buffer (278 mM Tris-HCl, 18% glycerol, 4.5% SDS, 0.02% bromophenol blue, 10% β-mercapto-ethanol). Samples were stored at -20 °C until further use.

Mass-spectrometry

RNA-affinity purified proteins were size-separated shortly (1 cm) on a Mini-PROTEAN TGX Precast gel (Bio-Rad) by SDS-PAGE. Gels were stained for 1 h with Coomassie Brilliant Blue at RT and de-stained overnight with demineralized-water. Gels were washed thrice with demineralized-water and cysteine reduction was performed by incubation in 25 ml 50 mM NH₄HCO₃ (pH 8.0)/10 mM DTT for 2 h at 60 °C. Alkylation was performed by incubation in 25 ml 0.1 M Tris/HCl (pH 8)/20 mM C₂H₄INO for 30 min at RT under dark conditions. Each lane was cut into 1 mm² pieces and proteins were digested by overnight incubation with 250 ng Trypsin in a total volume of 75-150 µl 50 mM NH₄HCO₃ (pH 8.0) at RT. The pH was decreased to 2-4 by addition of C₂HF₃O₂ and peptides were concentrated with in-house C18 µColumns. High-quality protein identification was performed by nano LC-MS/MS using reversed phase nano LC (Proxeon) in combination with an Orbitrap XL. Peptides were identified and quantified with MaxQuant and filtering was performed in Perseus to retrieve proteins with ≥2 peptide hits, of which ≥1 unique peptide(s).

Western blot analysis

Protein samples were size-separated by SDS-PAGE and semi-dry blotted onto Immobilon-P membranes (Merck Millipore). Membranes were blocked overnight at 4 °C with 1% milk powder/PBS-0.05%-Tween-20 (PBST). Membranes were probed for 1 h at RT with primary antibodies α-GFP (1:2000; A6455 Molecular Probes) or α-FLAG (1:2000; F3165 Sigma Aldrich) diluted in 1% milk powder/PBST. Membranes were washed thrice for 5 min with PBST and probed for 1 h at RT with AP-conjugated secondary antibody goat-α-rabbit-AP (1:2500; D0487 Dako) or goat-α-mouse-AP (1:2500; A5153 Sigma-Aldrich) diluted in PBST. Membranes were washed thrice for 5 min with PBST and developed with nitro blue tetrazolium NBT/BCIP until the desired signal was reached.

Statistics & software

Statistical analysis were performed in IBM SPSS Statistics 23. Titers of mosquito body and saliva samples were assessed for normality with Kolmogorov-Smirnov test. Data that did not follow a normal distribution was Log₁₀ transformed and re-assessed for normality. Normally distributed data was compared by unpaired two-tailed t-test (α=0.05) and data that did not follow a normal distribution was analysed by Mann-Whitney U test (α=0.05). Silencing efficiencies and relative gene expression as determined by qRT-PCR were compared by unpaired two-tailed t-test (α=0.05). Gene expression of JAK/STAT and Toll-pathway regulated genes were compared by one-way ANOVA with Tukey's post-hoc test (α=0.05). Viral titers and genome copies after silencing of sfRNA-binding candidates were assessed for normality with Kolmogorov-Smirnov test. Data that did not follow a normal distribution was Log₁₀ transformed and re-assessed for normality. Normally distributed data was compared by one-way ANOVA with Tukey's post-hoc test (α=0.05) and data that did not follow a normal distribution was analysed by pairwise Kruskal-Wallis one-way ANOVA test (α=0.05). Infection, transmission and dissemination rates were compared by two-tailed Fisher's exact test (α=0.05) using GraphPad QuickCalcs. Graphs were produced in GraphPad Prism 5. Protein interaction networks were produced on the STRING database V10.5 [496] using the closest orthologs in *Drosophila* listed on Vectorbase [497] with a minimum interaction score of 0.7.

Table 8.1: Primers used in this study

#	Primer name	Sequence (5'→3')
1	SubZika-F	GGCTAGATTCTAGAGTTCGAAGCCCTTG
2	SubZika-R	GGCCAAGTTTTCCGCGAGGTATC
3	Zika-PK1-F	GCCACAGCTTCCCCAAGCTGTGCAGCCTGTGA
4	Zika-PK1-R	GCACAGCTTTGGGGAAGCTGTGGCTGACTAGCA
5	Zika-PK2-F	CGCCATGGCAACCAAGAAGCCATGCTGCCTGT
6	Zika-PK2-R	ATGGCTTCTTGTTGCCATGGCGTTCTCGGC
7	T7-ZIKV sfRNA-F	GGCTGCAGTAATACGACTCACTATAGGGAGAGGTACCGGATC- CCATATGATGTTGTCAGGCCTGCTAGTCAGC
8	T7-ZIKV sfRNA-R	GGGAGCTCTCTCGGAATTCTAATCTCTAGAGACCCATGGATTTCCTCA- CACC
9	S1m-F	/5PHOS/GATCCGTAGAAAATGCGGCCGCCGACCAGAATCATG- CAAGTGCCTAAGATAGTCGCGGGTCGGCGGCCGCATCTGCTGGGA
10	S1m-R	/5PHOS/GATCTCCAGCAGATGCGGCCGCCGACCCGCGACTATCT- TACGCACTTGATGATTCTGGTCGGCGGCCGCATTTTCTACG
11	WNV-sfRNA-F	GGCATATGATGGAAGTCAGGCCAGATTAATGCTGC
12	WNV-sfRNA-R	GGGAATTCTAATCTCTAGAGATCTGTGTTCTAGCACCACC
13	WNV-NS2A-F	GGCATATGTACAACGCCGACATGATTGATCCTTTTCA
14	WNV-NS2A-R	GGGAATTCTAATCTCTAGACTGTACACATCAAGGTTTAGG- CATTTTAGTCCA
15	attB1-EGFP-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATTAACCATGGT- GAGCAAGGGCGAGGAG
16	attB2-EGFP-R	GGGGACCACTTTGTACAAGAAAGCTGGGTACATATGGAATTCGGCGC- GCCACTTGACAGCTCGTCCATGCCGA
17	2A-3XFLAG-F	CGCGCCAACCTTTGACCTGCTCAAGTTGGCCGGCGACGTCGAGTC- CAACCCAGGGCCCGACTACAAAGACCATGACGGTGATTATAAAGAT- CATGACATCGATTACAAGGATGACGATGACAAGG
18	2A-3XFLAG-R	AATTCCTTGTATCGTCATCCTTGTAAATCGATGTCATGATCTTTATAAT- CACCGTCATGGTCTTTGTAGTCGGGCCCTGGGTTGGACTCGACGTCG- CCGGCCAACCTTGAGCAGGTCAAAGTTGG
19	ME31B-F	CAATTGATGATGACTGAAACGCTGAATTCTAATAATCATCTCAG
20	ME31B-R	CATATGTTATTGCTAATGTTCTGTTCTTCTCTGTGTGC
21	ATX2-F	GGTGGGAATTCATGCATGCGGTACATCACA
22	ATX2-R	GGTGGCATATGCTACTGAGCGGATGGATGCT
23	LSM12-F	GAATTCATGGCTGGTCTAGTTCAGGATTGTTTTCTATTGG
24	LSM12-R	CATATGCTATTTGGCAGAACTGTTGCGGATGG
25	60sRp-F	GGTGGGAATTCATGGGTAGGGAGGACAAAGCTA
26	60sRp-R	GGTGGCATATGTTAATCGAACAGACCGAAGCCCC
27	T7-F	TAATACGACTCACTATAGGGAGAGGTACCGG
28	NS2A-RNA-R	CTGTACACATCAAGGTTTAGGCATTTTAGTCCA
29	WNV-sfRNA-RNA-R	AGATCCTGTGTTCTAGCACCACCAG
30	ZIKV-sfRNA-RNA-R	AGACCCATGGATTTCCTCCACACC
31	ZIKV SL-II-R	TTTTTTGACTCAGTGTCTCTGAGGGG
32	ZIKV ψ DB-R	GAAGGGTGGGGAAGGTGCGC

33	ZIKV DB2-R	AATATGCTGTTTTGCGTTTTCCGGGG
34	WNV SL-IV-R	TTAACCCAGTCCACCTGGGG
35	WNV DB1-R	TTTTTGACGCGGGGTCTCCTTAACC
36	WNV DB2-R	GTCAATATGCTGTTTCTTTTGGTGTTTTTGGCAC
37	dsME31B-F	TAATACGACTCACTATAGGGAGACCAGTGGCGAGGGCTAAAAATGGT
38	dsME31B-R	TAATACGACTCACTATAGGGAGACCACAGTTCTTCACGCTCAGTGGG
39	dsATX2-F	TAATACGACTCACTATAGGGAGACCACCAAGGTGCCCCGAAAAAG
40	dsATX2-R	TAATACGACTCACTATAGGGAGACCACAAGTGCTGGTGATGGATGGG
41	dsAAEL018126-F	TAATACGACTCACTATAGGGAGACCATTCTCGCAAACTGGCAACG
42	dsAAEL018126-R	TAATACGACTCACTATAGGGAGACCCTCTCCCATGTCGATGACGG
43	dsLuc-F	GTAATACGACTCACTATAGGGACTTACGCTGAGTACTTC
44	dsLuc-R	GTAATACGACTCACTATAGGGGAAATCCCTGGTAATCCG
45	qPCR-rpS7-F	ATGGTTTTCGGATCAAAGGT
46	qPCR-rpS7-R	CGATAGCCTTCTTGCTGTTG
47	qPCR-CecG-F	GAAGCAGGTGGCCTCAAGAA
48	qPCR-CecG-R	TTAGCCCCAGCTACAACAGG
49	qPCR-DefC-F	CTGCTCGTCACTCCTCCATC
50	qPCR-DefC-R	GCACTGAGGCAAAGAGCAAC
51	qPCR-TEP22-F	AACGTGGCCTTTGCCAATTC
52	qPCR-TEP22-R	CACTACCACCGTCGTTGACA
53	qPCR-ZIKV-F	GAGACGAGATGCGGTACAGG
54	qPCR-ZIKV-R	CGACCGTCAGTTGAACTCCA
55	qPCR-WNV-F	TGCACTCCTAGTCCTAGTGTTTGGG
56	qPCR-WNV-R	TCTTGAATGTAGCCATGAGTGCC
57	qPCR-ME31B-F	CCCGAAAATGGCAGAGACCT
58	qPCR-ME31B-R	TCCTCTGGTCGAGGCACATA
59	qPCR-ATX2-F	ATGCAAACGATGGGCAAACC
60	qPCR-ATX2-R	GTTGGTTGGGTGAGGTGTGA
61	qPCR-AAEL018126-F	GGCAACGATACCGATTGTGC
62	qPCR-AAEL018126-R	GCTACTACTGGGCACGGTTT
63	ZIKV-probe-F	AGTTCGCTACTTGGGTGAAGAAGGG
64	ZIKV-probe-R	GGAAACTCATGGAGTCTCTGGTCTTTCC

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Table S8.1. 30 most enriched putative ZIKV sfRNA binding proteins, as detected by mass spectrometry.

Name (some based on orthology with <i>Drosophila</i>)	Gene IDs	Log difference	-Log(P-value)	Log LFQ (protein abundance)						Proteins	Peptides	Unique peptides
				ZIKV-sfRNA 1	ZIKV-sfRNA 2	ZIKV-sfRNA 3	NS2A 1	NS2A 2	NS2A 3			
ME31B	AAEL008500	2.534	2.809	7.64	7.47	7.01	5.10	4.30	5.13	2	28	28
Ataxin-2	AAEL007805	2.227	2.264	6.83	6.36	7.30	5.21	4.30	4.30	6	11	11
Lingerer	AAEL009607	1.515	0.924	6.37	6.77	4.30	4.30	4.30	4.30	16	4	4
AAEL018126	AAEL018126	1.318	3.291	5.53	5.45	5.87	4.30	4.30	4.30	14	6	6
Lsm12	AAEL007820	1.075	0.934	4.30	5.95	5.87	4.30	4.30	4.30	1	2	2
AAEL024098	AAEL024098	1.039	0.745	6.23	6.16	6.90	5.53	4.30	6.35	1	9	9
Nup358	AAEL020592	0.880	1.006	5.39	5.78	5.55	5.48	4.30	4.30	4	7	7
RpS19	AAEL010756	0.811	0.319	6.54	6.62	4.30	6.43	4.30	4.30	3	9	9
RpL10a	AAEL013221	0.763	0.332	6.51	7.29	6.84	6.77	4.30	7.28	2	8	8
eIF3i	AAEL013144	0.718	0.935	5.38	5.38	4.30	4.30	4.30	4.30	1	4	4
RpL23	AAEL015006	0.707	0.427	4.30	6.42	4.30	4.30	4.30	4.30	1	5	5
Prohibitin	AAEL013952	0.677	0.432	6.20	6.59	5.82	5.86	4.30	6.42	4	8	8
RpS10	AAEL002047	0.673	0.427	4.30	6.32	4.30	4.30	4.30	4.30	1	4	4
Heat shock cognate 70	AAEL019403	0.653	0.334	6.38	6.83	6.24	6.89	4.30	6.31	3	18	14
Ref2	AAEL026243	0.631	0.427	4.30	6.19	4.30	4.30	4.30	4.30	1	3	3
Tubulin	AAEL002848	0.617	0.346	6.41	6.64	5.81	6.15	4.30	6.57	1	14	5
RpLP1	AAEL005027	0.589	0.427	4.30	6.07	4.30	4.30	4.30	4.30	5	4	4
eIF3-S5	AAEL009101	0.588	0.427	4.30	6.06	4.30	4.30	4.30	4.30	1	3	3
La related protein	AAEL009070	0.586	0.418	5.79	5.66	4.30	5.39	4.30	4.30	3	5	5
eIF3k	SAV_STRAV	0.585	0.244	7.55	8.84	7.53	8.20	5.70	8.26	1	4	4
RpI35	AAEL006115	0.578	0.427	4.30	6.03	4.30	4.30	4.30	4.30	1	2	2
Piwi5	AAEL000823	0.569	0.280	6.05	6.00	4.30	6.04	4.30	4.30	1	4	4
PABP	AAEL013233	0.561	0.512	5.63	5.78	5.91	5.45	4.30	5.89	1	7	7
RpL10	AAEL010318	0.535	1.229	7.13	7.10	6.55	6.47	6.24	6.47	2	27	27
Hsc70-3	AAEL025362	0.529	0.223	6.75	7.03	5.64	6.73	4.30	6.81	2	6	6
Vap33	AAEL026215	0.517	0.413	5.62	6.16	5.56	5.96	4.30	5.53	10	12	10
disulfide-isomerase A6	AAEL024800	0.512	0.427	4.30	5.84	4.30	4.30	4.30	4.30	2	2	2
cathepsin I	AAEL010065	0.508	0.304	5.65	5.88	4.30	5.71	4.30	4.30	3	7	7
Otefin	AAEL002833	0.497	0.263	6.02	5.70	4.30	5.92	4.30	4.30	1	4	4
60sRp	AAEL001580	0.493	0.427	4.30	5.78	4.30	4.30	4.30	4.30	1	4	4
	AAEL010821	0.129	0.222	7.43	7.48	7.77	7.37	7.12	7.81	1	13	13

Table S8.2. 30 most enriched putative WNV sRNA binding proteins, as detected by mass spectrometry.

Name (some based on orthology with <i>Drosophila</i>)	Gene IDs	Log difference	-Log(p-value)	Log LFQ (protein abundance)					Proteins	Peptides	Unique peptides
				WNV-sRNA 1	WNV-sRNA 2	WNV-sRNA 3	NS2A 1	NS2A 2	NS2A 3		
Me31b	AAEL008500	2.407	2.852	7.34	6.97	7.44	5.10	4.30	5.13	2	28
Ataxin-2	AAEL007805	1.602	1.806	5.70	6.53	6.39	5.21	4.30	4.30	6	11
RpS19	AAEL010756	1.590	1.049	6.66	6.48	6.66	6.43	4.30	4.30	3	9
Protein lingerer	AAEL009607	1.390	0.927	4.30	6.54	6.23	4.30	4.30	4.30	16	4
RpL23	AAEL015006	1.378	0.887	5.98	4.30	6.75	4.30	4.30	4.30	1	5
RpS10	AAEL002047	1.253	0.922	6.36	4.30	6.00	4.30	4.30	4.30	1	4
	SAV_STRAV	1.239	0.587	8.91	7.81	9.17	8.20	5.70	8.26	1	4
RpL38	AAEL005451	1.184	0.935	6.10	4.30	6.05	4.30	4.30	4.30	1	3
Tubulin	AAEL008103	1.127	0.743	6.94	6.04	7.01	6.23	4.30	6.08	1	11
	AAEL002848	1.092	0.686	6.94	6.37	6.98	6.15	4.30	6.57	1	14
RpLP1	AAEL005027	1.082	0.924	6.06	4.30	5.78	4.30	4.30	4.30	5	4
RpL10a	AAEL013221	1.045	0.490	7.28	6.92	7.28	6.77	4.30	7.28	2	8
Lsm12	AAEL007820	1.037	0.916	5.68	4.30	6.03	4.30	4.30	4.30	1	2
AAEL018126	AAEL018126	1.026	3.406	5.22	5.51	5.24	4.30	4.30	4.30	14	6
	AAEL005515	1.026	0.858	6.12	5.83	5.64	5.91	4.30	4.30	4	7
ATP synthase alpha	AAEL012175	1.025	0.612	6.87	6.07	7.12	6.28	4.30	6.41	1	13
	AAEL009691	1.011	0.432	7.67	6.50	6.98	6.28	4.30	7.53	4	43
Prohibitin	AAEL013952	1.002	0.683	6.45	6.20	6.92	5.86	4.30	6.42	4	8
Ref2	AAEL026243	0.986	0.933	5.84	4.30	5.72	4.30	4.30	4.30	1	3
	AAEL019669	0.953	1.040	5.55	5.85	5.63	4.30	4.30	5.56	1	5
	AAEL013069	0.928	0.639	6.41	6.07	6.87	6.05	4.30	6.22	1	10
	AAEL025999	0.926	0.647	6.15	4.30	6.00	4.30	4.30	5.07	2	2
	AAEL011447	0.923	0.734	6.43	6.15	6.46	5.85	4.30	6.12	1	8
arginyl-tRNA synthetase	AAEL000199	0.920	0.930	5.60	4.30	5.76	4.30	4.30	4.30	1	7
	AAEL008738	0.904	0.769	6.42	6.16	6.21	5.74	4.30	6.03	1	13
	AAEL002832	0.885	0.405	6.47	4.30	6.50	4.30	4.30	6.02	1	2
Heat shock cognate 70	AAEL019403	0.877	0.484	6.82	6.59	6.72	6.89	4.30	6.31	3	18
	AAEL003396	0.863	0.643	6.39	5.85	6.62	6.01	4.30	5.96	1	6
	AAEL015065	0.859	0.465	7.31	6.35	5.97	6.40	4.30	6.36	4	59
	AAEL021422	0.852	0.821	5.19	4.30	5.96	4.30	4.30	4.30	2	3
60sRp	AAEL010821	0.063	0.100	7.503	7.672	7.310	7.374	7.116	7.806	1	13

Chapter IX

General discussion

Abstract

In a world of increasing global trade and travel, in combination with higher environmental temperatures due to climate change and urbanization, the incidence of arboviral disease is expected to further increase. Arboviruses are dependent on the presence of competent insect vectors for their transmission to vertebrate hosts, including humans. Understanding the essential interactions between arboviruses and their vectors, could provide valuable insights for the development of novel arbovirus intervention strategies. So far, vector competence studies have implicated barriers and immune responses in the mosquito, that can impact the transmission of arboviruses. However, mosquitoes largely remain a black box in this perspective, as details of the virus-vector interactions that underlie vector competence are still unclear. This thesis aims to better understand the arboviral and mosquito factors, as well as arbovirus-vector interactions, that determine the outcome of exposure of mosquitoes to arboviruses. In this concluding discussion section, the findings described in this thesis are placed within the broader perspective of arbovirus transmission by mosquitoes. The implication of the findings for concepts of vectorial capacity, vector competence and the significance of mosquito immune responses are discussed. Also, an updated mechanistical model is provided for the sfRNA-mediated infection of mosquitoes by flaviviruses. Furthermore, this chapter addresses the knowledge gaps and perspectives for future research regarding virus-vector interactions. Finally, the societal relevance of the results described in this thesis is discussed and suggestions for arbovirus intervention strategies are presented.

Vectorial capacity, possible roles for viral determinants?

This thesis focussed on viral determinants that positively affect the vector competence, *i.e.* the biological ability to transmit a virus, of mosquitoes for arboviruses. As discussed in Chapter II, vector competence is an important factor for the arbovirus transmission cycle, as incompetent vectors are unable to transmit the virus to a vertebrate host. Yet, competent vectors may not always contribute to arbovirus transmission in the field, *e.g.* if there is only a low number of competent mosquitoes, or when there is no contact between competent mosquitoes and vertebrate hosts. Vectorial capacity, however, takes into account all physiological, ecological and environmental factors regarding the vector, host, and virus, that play a role in arbovirus transmission. Therefore, vectorial capacity is a much more accurate predictor for the contribution of a certain mosquito species to arbovirus transmission [100,498]. The vectorial capacity can be modelled (Equation 9.1) with m =number of female mosquitoes per host, a =daily blood feeding rate, b =vector competence, p =probability of daily survival of the vector, n =extrinsic incubation period (EIP) of the virus, *i.e.* the average time between exposure to an infectious blood meal and virus transmission via the mosquito saliva. This formula describes the daily number of potential infectious bites that arise from all mosquitoes that bite on one particular viraemic individual [499].

$$\text{Vectorial capacity} = \frac{ma^2bp^n}{-\ln(p)} \quad (1)$$

Equation 9.1 Modelling vectorial capacity. Based on [498,500].

Arboviruses can modulate the vector competence (b) of mosquitoes through inhibition of mosquito immunity, increasing virus replication efficiencies in mosquito cells and strategies to overcome the main mosquito barriers (Chapter II) (reviewed in [102,142]). However, arboviruses may also impact other parameters that influence the vectorial capacity. Through behavioural manipulation of the infected mosquito vector, arboviruses can affect, for example, the feeding rate (a) to increase their odds of transmission [501]. Unfortunately, the description of arbovirus-induced behavioural changes is limited. WNV infected *Culex pipiens* mosquitoes showed less frequent host-seeking behaviour [502], and decreased mobility of *Aedes aegypti* has been reported during ZIKV and DENV infection [503,504]. Furthermore, altered blood-feeding behaviour, including more frequent probing, has been reported in dengue virus (DENV), La Crosse virus and Sindbis virus (SINV) infected *Ae. aegypti* mosquitoes [505–509]. Decreased host-seeking behaviour and reduced mobility could result in a smaller number of feeding events, thus, lowering the vectorial capacity. On the other hand, decreased host-seeking and mobility of infected mosquitoes could increase their probability of survival, by avoiding dangerous physical encounters, and thereby instead enhance the vectorial capacity. Future research should investigate in more detail the contribution of arbovirus-induced behavioural changes to the vectorial capacity of mosquitoes and the virus-vector interactions that underlie these changes.

Arbovirus infection can potentially also affect the vectorial capacity by influencing the survival of infected mosquitoes. While mosquitoes are generally quite tolerant to arbovirus infection, a modest reduction in mosquito survival has been demonstrated in studies where mosquitoes were infected with flaviviruses, alphaviruses or bunyaviruses [508,510–516]. However, other studies observed no effects on survival in mosquitoes infected with eastern equine encephalitis virus (EEEV), DENV and West Nile virus (WNV) [515,517,518]. Also, various studies with malaria-infected mosquitoes

did not show significant effects on mosquito survival, questioning the contribution of parasite-manipulation to mosquito survival rates (reviewed in [501]). The different outcomes of studies with arbovirus-infected mosquitoes indicate that survival is highly dependent on the virus-vector combination and on experimental conditions. Possibly, arbovirus-vector interactions, such as the interaction of chikungunya virus (CHIKV) non-structural protein 3 (nsP3) with Ras GTPase-activating protein-binding protein (G3BP)/Rasputin (Rin) and amphiphysins (Chapter V), and Zika virus (ZIKV)/WNV subgenomic flavivirus RNA (sfRNA) with ME31B (Chapter VIII), can modulate the survival of their mosquito vector to increase the vectorial capacity. To determine if ZIKV and WNV sfRNA or the interactions of CHIKV nsP3 with host-proteins may have an effect on the survival of infected mosquitoes, the survival rates during the studies included in this thesis (Chapter V, VI, VIII) were analysed. Mosquitoes infected with ZIKV (*Ae. aegypti*) and WNV (*Cx. pipiens*) mutants that produce shorter species of sfRNA did not have significantly different survival rates compared to mosquitoes infected with wild type viruses (Fig. 9.1; dark grey (ZIKV) and light grey (WNV) bars). Similarly, for CHIKV (*Ae. aegypti*), mosquitoes infected with virus mutants that are impaired in their interaction with amphiphysins (398 & 401) or G3BP/Rin (FG_N & FG_C) also displayed survival rates similar to those of mosquitoes infected with wild type CHIKV (Fig. 9.1; white bars). However, as these experiments only lasted for 7 or 14 days, there may still be effects by either sfRNA or nsP3 on long-term survival rates.

Fig. 9.1 Survival of wild type and mutant ZIKV, WNV or CHIKV infected *Cx. pipiens* and *Ae. aegypti* mosquitoes during the studies included in this thesis. Survival rates were determined by comparing the number of engorged female mosquitoes, with the number of salivated mosquitoes to retrieve the numbers of mosquitoes that succumbed during the experiment. Survival rates were calculated for blood-fed mosquitoes that were incubated for 14 days (WNV & ZIKV) or 7 days (CHIKV) only. Data is from experiments with ZIKV wild type and ΔsfRNA1 (ΔSF1)-mutant (*Ae. aegypti*; triplicate; Chapter VIII), WNV wild type, ΔSF1-, and ΔsfRNA1+2 (ΔSF1+2)-mutants (*Cx. pipiens*; triplicate; Chapter VI), CHIKV wild type and mutants of the P-rich motif (398 & 401) (*Ae. aegypti*; duplicate; Chapter V), and CHIKV wild type and mutants of the FGDF-motifs (FG_N & FG_C) (*Ae. aegypti*; triplicate; Chapter V).

Finally, arboviruses may impact the vectorial capacity through modulation of the EIP (n). The EIP differs per virus-vector combination and is temperature-dependent. For example, at 28 °C CHIKV has a relatively short EIP of 6-8 days in *Ae. aegypti* [519], and in some individual mosquitoes, virus can already be detected in the saliva at 2 days post infection (dpi) [31,32]. ZIKV has a substantially longer EIP at 28 °C in *Ae. aegypti*, namely 10-15 days [520], although sometimes ZIKV-positive saliva can be detected as early as 7 dpi [56,273,466]. WNV has a similar EIP of ~10-14 days in *Culex* mosquitoes at 28 °C [190,521]. Viral factors that shorten the EIP may further determine the vectorial capacity, due to enhanced virus replication rates, increased dissemination or increased progeny virus production. In this thesis, the effects of flavivirus sRNA and CHIKV nsP3 on vector competence were determined at a static time-point of 7 or 14 dpi (Chapter V, VI & VIII). Time-course experiments could resolve whether or not sRNA or nsP3 affect the EIP, which could have implications for their influence on the vectorial capacity. In conclusion, future studies that aim to identify determinants of arbovirus transmission, should investigate effects on both the vector competence and on the vectorial capacity of the mosquito vector.

Vector competence: significance of the midgut and salivary gland barriers

The mosquito midgut- and salivary glands impose infection- and escape barriers to arbovirus infection and transmission (Chapter II) [142]. In *Cx. pipiens*, a clear midgut infection barrier for WNV was observed (Chapter VI), while the effect of the salivary gland barrier was less clear. The presence of a midgut-infection barrier for WNV in *Cx. pipiens* has been demonstrated by others [154–156,521], indicating that the ability of WNV to overcome the mosquito midgut is an important determinant for the vector competence of *Cx. pipiens*. Other studies have also shown that the salivary glands of *Cx. pipiens* do not form a significant barrier for WNV [154–156,521]. In *Ae. aegypti* a dose-dependent midgut infection barrier was observed for both CHIKV and ZIKV (Chapter IV). Furthermore, the presence of a salivary gland barrier was clearly observed for CHIKV (Chapter IV & V), while it had less impact on the transmission of ZIKV (Chapter IV & VIII). This illustrates that the significance of mosquito barriers is highly dependent on the combination of virus and vector, in agreement with other studies (reviewed in [142,522]). It remains unclear why CHIKV experiences a stronger salivary gland barrier than ZIKV in *Ae. aegypti*. Potentially, ZIKV has evolved strategies to better infect or establish successful replication in the salivary glands. Of note, the experiments conducted in this thesis did not discriminate between the infection and escape barriers of both the midgut and salivary glands. These could have been discriminated by additionally measuring the viral titers in midgut-deprived carcasses and isolated salivary glands respectively. It is therefore also possible that CHIKV experiences a midgut escape barrier, as reported by others [277,523], instead of a salivary gland infection barrier.

The midgut and salivary glands serve as barriers for arbovirus infection, even in competent vectors, as also in a successful arbovirus transmission cycle, only a small number of midgut cells become infected, initially [141,524,525]. Possibly, also only a low number of virions escapes from the midgut into the circulatory system [142]. Escape of a low number of virions from the midgut through the basal lamina into the circulatory system is sufficient to induce a systemic infection. This is clearly demonstrated by intrathoracic injection experiments, where a low viral titer (typically $<10^3$ TCID₅₀) is introduced into the mosquito haemolymph, which is usually sufficient to establish a systemic infection (Chapters IV, VI, VIII). Therefore, the initial stage of

infection of and replication in the mosquito midgut followed by dissemination into the haemolymph is critical.

Fig. 9.2 Visualization of three different explanations for a mosquito midgut and salivary gland infection barrier. After a mosquito takes an infectious blood meal with an arbovirus, only a few midgut cells become infected, suggesting that the wild type virus fitness lies just above the infection-threshold. From the input virus particles in the engorged blood meal, only a fraction is able to enter the midgut cells, a percentage of viral genomes can establish a replication complex, and again a selection of initial replication complexes may reach the state of translation, progeny virus production and release from the midgut epithelial cells. Viruses may be defective at the level of entry, replication, translation or release, thereby reaching below the midgut-infection threshold, resulting in an inability to infect a given mosquito. Asterisks indicate viral strains that are able to establish an infection cycle in a mosquito.

Arbovirus infection of and release from the midgut is highly dose-dependent (Chapter IV) [265,526–528], indicating that the midgut-barrier imposes an infection-threshold that requires a certain viral dose to be overcome. Generally, researchers conclude the presence of a midgut infection barrier when low percentages of infections are achieved after oral inoculation. However, this does not differentiate between failure to infect, failure to replicate in or failure to be released from the midgut cells (Fig. 9.2). Decreased infection rates at a lower dose of virus inoculum, may therefore be explained by fewer events of virus entry into a midgut epithelial cell, or by a reduced number of successful virus infection events, *i.e.* infections that result in effective virus replication, virion production, and virus release. In the latter case, there is potentially a threshold for a minimum number of infected RNA copies required to establish a successful replication cycle, due to the fact that per copy of viral genomic RNA there is a minimum replication efficiency and a minimum efficiency of translation. In other words, infection with multiple virions in a single midgut cell may result in the establishment of multiple viral replication complexes, that together overcome the threshold of minimal replication efficiency required for virus production. Similarly, a threshold of progeny virus production and release from the midgut cells could require a certain efficiency to ultimately establish a successful infection of the mosquito (Fig. 9.2).

In line with this threshold hypothesis, a study that aimed to understand differences in vector competence of *Ae. aegypti* for different strains of DENV did not observe differences in midgut binding affinities [529]. The fact that DENV strains with high and low infection rates have similar midgut-binding efficiencies, suggests that differences in infection rates can be caused post-entry, at the stage of virus replication, RNA translation, or progeny virus production. Decreases in virus replication may be related to intracellular immune signalling, as discussed in Chapter II, or the ability to recruit host factors that are essential to initiate a successful replication cycle, as exemplified by our studies on nsP3 and sfRNA (Chapters V & VIII). For alphaviruses, interaction of nsP3 with the host factors G3BP and Rin is essential to establish an infection in the vertebrate and invertebrate host, respectively [123,125–127] (Chapter V). Similarly, the production of sfRNA for flaviviruses is essential for midgut infection in the invertebrate host, likely through inhibition of innate immune responses and/or the recruitment of host factors for virus replication (Chapter VI & VIII). As various innate immune pathways have been implicated in the defence of mosquitoes to arbovirus infection (Chapter II) (reviewed by [102,197]), the ability of arboviruses to evade these host defence mechanisms is expected to play a key role. This can have important implications for the range of vector species that are capable of transmitting a certain arbovirus. For example, there is a large difference in the vector competence of *Culex* and *Aedes* mosquitoes for a number of arboviruses. Both CHIKV and ZIKV fail to infect *Culex* mosquitoes [252,273,530,531], while WNV can be transmitted by both *Culex* and *Aedes* spp. mosquitoes [190,532]. The mechanisms that exclude infection of *Culex* mosquitoes with CHIKV and ZIKV are still unclear, but likely involve the interaction with midgut receptors, host factors required for virus replication, or the evasion of immune responses that differ between *Culex* and *Aedes* mosquitoes. The next section discusses the role of small RNA-mediated innate immune responses in determining the transmission of arboviruses by mosquitoes.

Significance of mosquito small RNA responses for arbovirus transmission

The siRNA response

Small RNA responses are important in the antiviral defence of insects [101,197], and the small-interfering (si)RNA response has been implicated as the most potent antiviral response in mosquitoes [104]. Unfortunately, our understanding of siRNA-mediated immunity in insects mainly relies on findings in *Drosophila*, while less is known about the specific mechanism of the siRNA and other small RNA responses in mosquitoes. Studies have demonstrated that administration of virus-complementary double-stranded (ds)RNA into mosquitoes triggers resistance to infection with flaviviruses and alphaviruses [533,534]. Also, the production of siRNAs has been illustrated during mosquito infection with flaviviruses, alphaviruses and bunyaviruses (Chapter VI) [155,209,211,535]. As silencing of Dicer-2 (Dcr2) increases replication and infection of flaviviruses and alphaviruses in mosquitoes and mosquito cells [164,536], the antiviral effect of the RNA-interference (RNAi) pathway is Dcr2-mediated. However, while Argonaute-2 (Ago2) knockdowns demonstrated that siRNA-mediated silencing restricts the replication of alphaviruses [534,536,537] and bunyaviruses [210,211], it remains questionable to which extent flaviviruses are restricted by an Ago2-mediated siRNA response [164,209,214]. WNV and ZIKV replicated similarly in RNAi-competent and Dcr2-deficient C6/36 cells (Chapters IV, VI & VIII), suggesting that flaviviruses are not particularly sensitive to the siRNA response. Also, fully-disseminated, WNV-infected mosquitoes produced abundant WNV-siRNAs (Chapter VI), suggesting that siRNAs do not necessarily have potent antiviral activity to arbovirus infection. This apparent insensitivity of flaviviruses to RNAi may be due to the presence of flavivirus-encoded suppressors of RNAi or shielding of the viral replication complex from targeting by the siRNA machinery. Moreover, not all siRNAs may in fact be equally capable of silencing, as illustrated in a study with Semliki Forest virus (SFV) where abundant hotspot siRNAs were less efficient in RNA silencing than coldspot siRNAs [216]. As demonstrated and discussed in Chapters VI, VII and VIII, the role of siRNA as an RNAi-suppressor of flaviviruses *in vivo* is questionable. Furthermore, although flavivirus NS4B and Capsid have been proposed as proteinaceous RNAi-suppressors *in vitro* outside the context of flavivirus replication [103,166], their biological relevance for *in vivo* flavivirus infection has thus far not been confirmed by others [115,538]. In summary, there is no obvious known RNAi-suppressor of flaviviruses that explains the apparent insensitivity to an active siRNA response. Alternatively, the ability of flaviviruses to replicate in cells and mosquitoes that produce viral (v)siRNAs in abundance, could be due to insensitivity or inaccessibility of flaviviruses to RNAi targeting of viral replication complexes. This appears paradoxical, as these vsiRNAs are produced from cleaved viral RNA, and the presence of both sense and antisense siRNAs suggests that they are derived from dsRNA-replication intermediates. However, it is unclear which proportion of viral RNA molecules is processed by the RNAi-machinery and if these viral RNAs represent replication competent RNAs or rather free sense and antisense RNA molecules. Importantly, the differences in the siRNA response to viruses from different families illustrate that the impact of the siRNA response strongly depends on the virus-vector combination and demonstrate that the siRNA response is not always equally potent to have antiviral activity *in vivo*.

The piRNA response

Besides siRNAs, mosquito vectors can produce viral Piwi-interacting (pi) RNAs during arbovirus infection, suggesting that the piRNA-response may act as an additional layer of mosquito host defence [206–213]. While several studies did not implicate strong antiviral effects of vpiRNAs on arbovirus infection [207,209,211,212], recent research has identified Piwi4 as an antiviral protein during arbovirus infection [209,539,540]. Thus, the piRNA response may in fact have important implications for arbovirus infection of and the subsequent transmission by their mosquito vectors.

Similar to the siRNA response, the activity of the piRNA response is highly dependent on the combination of virus and mosquito species (Chapter III). For flaviviruses, which have (+) strand RNA genomes, production of viral (v)piRNAs has been observed in *Aedes* spp. mosquito cells, which are (+) strand biased, Piwi5- and Ago3-dependent, but lack a ping-pong signature (Chapter III) [206,209]. Interestingly, for alphaviruses (that are also (+) strand RNA viruses), vpiRNAs observed in *Aedes* spp. cells are similarly (+) strand biased and Piwi5/Ago3-dependent, mainly map to the subgenomic mRNA, but do in fact show a classical ping-pong signature [207,212]. In flavivirus infected cells little to no free (-) strand gRNA is detected, as the (-) strand is covered by the viral polymerase and is always base paired to the newly synthesized (+) RNA and the partially displaced (+) strand in the replicative form of the gRNA [272]. Similarly, for alphaviruses, the (+) strand could be more accessible due to their replication characteristics [67,285,541]. Hypothetically, the presence of predominantly free (+) strand gRNA results in a bias of the piRNA machinery into (+) pi-like RNAs. Importantly, no flavivirus or alphavirus pi-like RNAs are observed in *Culex* cells and in *Culex* mosquitoes (Chapter III) [155,214]. This remarkable observation indicates that the piRNA response can differ drastically between *Aedes* and *Culex* mosquitoes and suggests that the piRNA machineries differ between mosquitoes. Indeed, *Aedes* mosquitoes encode 7 Piwi proteins, as compared to 6 Piwi proteins for *Culex* mosquitoes, of which the majority has no known function [204,205,214]. Alternatively, alphavirus and flavivirus RNA may be differentially available to targeting in *Aedes* and *Culex* cells, for example due to post-transcriptional modifications such as methylation of the viral RNA (Chapter VII).

For negative-strand RNA viruses the production of vpiRNAs with a classical ping-pong signature has been demonstrated in mosquito cells (Chapter III) [210,211,213]. Negative strand RNA viruses generally produce an excess of (-) genomes [542], although the majority should notably be shielded by the bound nucleoproteins [543]. Antisense piRNAs produced from the (-) strand may result in ping-pong amplification via (+) mRNA transcripts, thus leading to the ping-pong piRNA signature as observed for bunyaviruses in insect cells (Chapter III) [210,211,213]. In RNAi-competent *Drosophila* and mosquito cells, no vpiRNAs have been observed for most dsRNA viruses, despite the presence of abundant 21 nt siRNAs (Chapter III) [215,229,544]. An exception is Bluetongue virus (BTV), for which pi-like RNAs have been observed in *Culicoides sonorensis* midges and *Ae. aegypti* cells [213]. The replication and transcription of such dsRNA viruses takes place within viral particles or core particles [545], which may protect their RNA from targeting by the piRNA machinery, although it is unclear why this would only allow siRNA but not piRNA production. Future research should investigate the mechanisms that underlie these differences in small RNA responses between different mosquito species cell lines and viruses from distinct families, to better understand the implications of the different small RNA responses to arbovirus transmission and vector specificity.

Subgenomic flavivirus RNA as a determinant of virus transmission

All viruses from the genus *Flavivirus*, which includes the tick-borne, mosquito-borne, insect-specific, and no-known vector flaviviruses, produce a noncoding sfRNA as a result of stalling of 5'→3' exoribonucleases on the viral 3' untranslated region (UTR) [105,106,114]. The conservation of sfRNA formation for viruses that are restricted to insects, implies an important function for sfRNA during infection of the insect host. However, while sfRNA had been implicated in evasion of mammalian immune responses and was demonstrated to interact with mammalian host factors [106,109,111,112,437], knowledge on the importance of sfRNA formation during infection of the mosquito vector was lacking at the start of this PhD thesis.

The results in Chapters VI & VIII demonstrate that the formation of sfRNA is essential for flavivirus infection of and transmission by both *Culex* and *Aedes* spp. mosquitoes. Essentially, full-length sfRNA formation is required to overcome the midgut- and salivary gland barriers for both WNV in *Cx. pipiens* and ZIKV in *Ae. aegypti*. This establishes sfRNA as a generic determinant for flavivirus transmission by mosquitoes. Moreover, DENV- and ZIKV-mutants that contain mutations or deletions in the 3'UTR and thereby indirectly produce lower amounts of sfRNA (via unknown mechanisms), are also attenuated in mosquitoes [470,546–548], supportive of sfRNA as a determinant of flavivirus transmission by mosquitoes. As discussed above, the mosquito midgut and salivary glands present an infection threshold, and overcoming these thresholds likely requires an optimal replication and immune evasion strategy. SfRNA is not incorporated into virions and is only produced after sufficient (~12–24 h) virus replication has occurred [106]. There is, however, a possibility that sfRNA-containing vesicles were co-purified during generation of the WNV and ZIKV virus stocks, but as no evidence of sfRNA incorporation into extracellular vesicles has been reported, this scenario is not likely. Therefore, the dependency on sfRNA-production for flavivirus infection of mosquitoes appears to exist both post-entry and post-initial replication. Visualisation of virus infected cells *in vivo*, in blood-fed mosquitoes, via the use of a fluorescent reporter-labelled virus [471,549] or immunostaining, could increase the understanding of sfRNA-mediated infection and transmission of flaviviruses. For example, infections with a GFP-labelled wild type and sfRNA-deficient flavivirus may reveal either a lower number of initially infected cells for the sfRNA-deficient virus, a slower dissemination through the midgut, or attenuated release from the midgut into the haemolymph. Such findings could guide research that aims to further elucidate the mechanism of sfRNA-mediated flavivirus transmission, by revealing the tissue and/or timepoint where the effect of sfRNA is most significant.

The sfRNA-mediated advantageous effect on flavivirus infection was most strongly observed *in vivo*, while full-length sfRNA formation was not essential for virus replication in *Culex* or *Aedes* cell lines (Chapter VI & VIII). The ΔsfRNA1-mutant viruses still produce sfRNA2 (ZIKV) or sfRNA2 and sfRNA 3 (WNV), and these shorter versions may still be sufficient to rescue replication in mosquito cells, but not *in vivo* during infection of mosquitoes. In contrast, ΔsfRNA1+2-mutants produce insufficient quantities of sfRNA to facilitate efficient replication in both cell culture and mosquitoes. Together, these findings implicate that full-length sfRNA-production is necessary for optimal virus replication, but the advantage of sfRNA production is too subtle to detect in an experiment with mutant viruses that are only deficient in sfRNA1-formation in a relatively non-selective cell culture system. Possibly, sfRNA-mediated infection and transmission depends on (A) the recruitment or sequestration of mosquito host factors (Chapter VIII), (B) altered host mRNA-transcription via inhibition of XRN1 [450] or (C)

the suppression of innate immune responses such as RNAi [115,116], Toll and JAK/STAT signalling [470,479]. The next paragraphs present the current knowledge and a working model of sfRNA-mediated infection of mosquitoes.

Significance of host factor recruitment or sequestration by sfRNA

Because host factors are essential for the replication of arguably all viruses [550,551], the necessity of sfRNA for mosquito infection may rely on its interaction with mosquito host factors. Chapter VIII uncovered that ZIKV and WNV sfRNA and/or their 3'UTR specifically binds with the *Ae. aegypti* DEAD-box helicase protein ME31B (Chapter VIII), which is the mosquito ortholog of human DDX6. DEAD-box helicases have two conserved domains, that contain motifs involved in ATP-binding and hydrolysis and RNA binding activity. An RNA helicase is designated a DEAD-box helicase when motif II, which possesses nucleoside triphosphate and magnesium-binding activity and coordinates nucleotide hydrolysis, contains a DExD/H-signature [489]. *Ae. aegypti* ME31B shares >85% identity with *Drosophila melanogaster* ME31B and the closest human ortholog is DDX6, of which the first domain (part of the helicase core) shares 89% identity. The *Cx. quinquefasciatus* ortholog of *Ae. aegypti* ME31B shares 90% amino acid sequence identity, and shares >75% identity with *D. melanogaster* ME31B, suggesting that functions of ME31B are largely conserved between *Aedes* and *Culex* mosquitoes.

ME31B is an essential factor for processing body (PB) formation and positively regulates mRNA-decapping and -turnover [487,489,490]. ME31B is also known to be involved in miRNA-mediated silencing of mRNAs [490,491,552]. Besides ME31B, ZIKV and WNV sfRNA interact, directly or indirectly, with other PB proteins and proteins involved in mRNA translation (Chapter VIII). PBs have been implicated in viral RNA decay and the RNAi response, as proteins involved in these responses aggregate in PBs [440,553]. However, recent findings suggest that PBs are not involved in mRNA degradation *per se*, but rather function as a storage hub of non-translating mRNA and mRNA under translational repression [482,554,555]. Proteins that bind to these non-translating mRNAs often involve decapping and translational suppressor proteins that, as a consequence, co-aggregate with their target mRNAs in PBs [482]. Disruption of PBs by flavivirus infection may alter the availability of host proteins, due to the release of the mRNAs that encode for these proteins from PBs and, consequently, facilitate virus replication. Indeed, for a number of viruses a correlation between PB-disassembly and enhanced virus replication has been reported [483,484,486].

Interestingly, various PB components, including the mammalian ME31B ortholog DDX6, are recruited to WNV and hepatitis C virus (HCV) replication sites in mammalian cells [484,485]. Furthermore, DDX6 is required for efficient replication of the flaviviruses DENV and ZIKV [437,556]. Also, orthologs of ME31B have been implicated in the transport of viral RNA to the endoplasmic reticulum (ER), where flavivirus replication organelles reside [97], and positively regulate the translation of several viruses [557]. SfRNA and/or the entire 3'UTR binds to PB components, such as ME31B, poly-(A)-binding protein (PABP) and LSM12 (Chapter VIII). Binding of PB components to the 3'UTR may recruit them to virus replication complexes to facilitate early virus replication and/or translation [83]. While recruitment can correlate with enhanced virus replication, the underlying mechanisms remain enigmatic, especially since various PB components are involved in mRNA turnover.

However, the results also show that ME31B displays antiviral activity during ZIKV and WNV infection in *Ae. aegypti* cells (Chapter VIII). While this may initially appear contradictory with previous observations where DDX6 enhanced flavivirus replication [437,556], it is not necessarily a discrepancy. The effect of ME31B on flavivirus infection

of mosquito cells may differ from the effect of its ortholog DDX6 in mammalian cells, especially since only domain I is conserved between *Ae. aegypti* and humans. Indeed, ME31B was also reported as an antiviral protein during infection of Rift Valley Fever virus or La Crosse virus in *Drosophila* [558], in support of an antiviral activity of ME31B in insect cells. As ME31B and its orthologs regulate mRNA degradation and are also involved in miRNA-mediated silencing of mRNAs [490,491,552], sequestration of ME31B by sfRNA at the late stage of virus replication may block miRNA-mediated suppression of viral mRNA translation, viral RNA slicing or other antiviral responses that are regulated via ME31B, and thereby facilitate virus translation. Furthermore, as ME31B is essential for the formation of PBs [487], the enhanced replication of ZIKV and WNV observed after ME31B silencing may be an indirect effect of PB-disruption, due to the release of proteins or mRNAs that encode proteins involved in translation or by inhibiting PB-mediated antiviral responses.

Besides PB-associated proteins, several proteins involved in translation, such as ribosomal proteins were also enriched in the ZIKV and WNV sfRNA RNA-affinity purification (Chapter VIII). As flaviviruses lack a poly-(A) tail, they rely on the recruitment of viral proteins to their structured 5' and 3'UTRs to assemble translation initiation complexes [83]. For example, PABP binds to the DB-1 - DB-2 region in the DENV 3'UTR [438] and eukaryotic elongation factor (eEF)-1 α binds to the 3'SL of WNV and DENV [559,560]. As our RNA-affinity purification does not discriminate between sfRNA and the 3'UTR, the identified sfRNA binding proteins may also bind to the 3'UTR to facilitate translation of the vgRNA.

Mechanistic model of sfRNA-mediated enhancement of mosquito infection

It is challenging to combine the sfRNA-mediated infection of mosquitoes, the affinity of the 3'UTR/sfRNA for ME31B and the antiviral nature of ME31B in a single model, since several questions remain unanswered. First of all, increased virus replication in Aag2 cells after silencing of ME31B does not necessarily translate to an *in vivo* infection of *Ae. aegypti*. This could be experimentally investigated by dsRNA-mediated silencing of ME31B in *Ae. aegypti*. By comparing infection- and transmission efficiencies in ME31B silenced and non-silenced mosquitoes, the effect of ME31B on the competence of *Ae. aegypti* for wild type and sfRNA-deficient flaviviruses could be assessed. Secondly, the relative contribution of respectively the 3'UTR and sfRNA to ME31B sequestration is unknown. Although the structure of the 3'UTR and sfRNA are colinear and presumably identical, base pairing with the viral 5'UTR, differential binding of viral or host factors by these structures or their expected intracellular localization (3'UTR – replication complexes, sfRNA – cytoplasmic foci [106]) may affect their affinity for ME31B. Pull-down experiments of overexpressed, tagged ME31B in flavivirus-infected *Ae. aegypti* cells could be used to detect ME31B-associated vgRNA and sfRNA respectively through qRT-PCR on precipitated fractions. Thirdly, it is uncertain if depletion of ME31B in the silencing experiments (Chapter VIII) increases virus replication either directly, due to decreased ME31B protein levels, or indirectly, through the inability of cells to form PBs. In the latter case, sfRNA may disturb the formation of PBs via its interaction with ME31B to facilitate virus replication by inhibiting a putative PB-mediated antiviral effect. It would therefore be valuable to investigate the formation of PBs in *Ae. aegypti* cells during infection of wild type and sfRNA-deficient flaviviruses.

Fig. 9.3 Mechanistic model of sfRNA-mediated enhancement of mosquito infection. Flavivirus virions enter the midgut epithelial cells and form invaginated replication complexes in the endoplasmic reticulum (ER). (1) In the early stage of replication, released (+) strand vRNA molecules bind ME31B (ortholog of human DDX6) to recruit processing-body (PB) components to viral replication complexes. (2) As ME31B is essential for PB formation, recruitment of ME31B results in the dissociation of PBs, and the release of translationally repressed host and/or viral mRNAs. (3) Recruitment of PB components involved in decapping and mRNA degradation, including decapping enzymes (DCP) and the exoribonuclease Pacman (ortholog of human XRN1), induces decapping of the free (+) viral genomic RNA followed by degradation by Pacman. Pacman stalls on resistant stem-loop RNA structures in the 3'UTR, leading to the formation of sfRNA. (4) SfRNA competes with the 3'UTR for binding of PB components and due to its high abundance sequesters PB components such as ME31B, LSM12, ATX2, PABP and Pacman into cytoplasmic foci. (6) The decreased turnover of free (+) strand, increases the number of (+) vRNAs for translation, resulting in a switch from virus replication to translation.

In a hypothetical mechanistic model (Fig. 9.3), flaviviruses recruit PB components, such as ME31B, PABP and LSM12, to sites of virus replication to facilitate early virus replication via the interaction of the 3'UTR with ME31B (Fig. 9.3 - 1). This may simultaneously disrupt PBs, resulting in release of suppressed host mRNAs, which may thereby be available for translation (Fig. 9.3 - 2). As a downside, this strategy would expose the viral RNA to decapping-enzymes and subsequent 5'→3' degradation by XRNs. To overcome this, flaviviruses stall these XRNs on conserved RNA structures in the 3'UTR, which produces an abundant sfRNA that itself also has affinity for ME31B (Fig. 9.3 - 3). SfRNA sequesters ME31B and the associated decapping and RNA-decay factors, to prevent further degradation of the viral RNA, that thereby instead aggregate in cytoplasmic foci [106] (Fig. 9.3 - 4). In line with this hypothesis, inhibition of XRN1

and increased mRNA stability is observed in flavivirus infected cells and is sfRNA-dependent [450]. Thus, sfRNA potentially acts as a negative feedback loop to the RNA-decay machinery, where the degradation of vgRNA directly blocks this same degradation pathway.

In this model sfRNA could also act as a switch from virus replication to translation, as was previously postulated [105]. During flavivirus replication, the translationally active (+) strand may be prone to degradation, as it lacks a poly-(A) tail, and is not protected through base pairing or covering by the viral polymerase (as mentioned above). In the early stage of infection, recruitment of PB components, including proteins involved in mRNA decay, may result in degradation of (+) strands and the formation of sfRNA (Fig. 9.3 – 1 & 3). However, increasingly abundant sfRNA competes with the 3'UTR for the recruitment of PB components (Fig. 9.3 – 4 & 5). As this competition decreases the exposure of replication complexes to proteins involved in mRNA turnover, more (+) strand becomes available for translation, while mRNA-decay is suppressed in correlation with the abundance of sfRNA, as has been demonstrated during flavivirus infection (Fig. 9.3 – 6) [450]. In conclusion, sfRNA may modulate PBs and PB-factors to benefit virus replication and translation, which can be essential to successfully establish a replication cycle in a highly selective environment such as the mosquito midgut. Alternatively, or concurrently, sfRNA may modulate mosquito innate immune pathways to assist in overcoming the midgut and salivary gland barriers. The next section discusses the current knowledge on the correlation between sfRNA production and the suppression of innate immune responses.

To suppress or not suppress: sfRNA as an innate immune suppressor in mosquitoes

SfRNA is important for the infection and subsequent transmission of flaviviruses by *Culex* and *Aedes* mosquitoes, but the mechanism whereby sfRNA enhances virus transmission remains enigmatic (Chapters VI, VII, VIII). The demonstration of sfRNA as an *in vitro* Dcr2-decoy molecule [115], inhibitor of RNAi-based silencing and evidence of a direct interaction of sfRNA with Dcr2 and Ago2 in infected mosquitoes [116], hints towards sfRNA as an RNAi suppressor *in vivo*. However, sfRNA appears not to be essential for replication in RNAi-competent cell lines and does not suppress the accumulation of WNV siRNAs in *Cx. pipiens* at 14 dpi (Chapters VI & VIII). Nonetheless, as RNAi is an important innate immune response in insects [101,104], and sfRNA is processed by the mosquito RNAi-machinery *in vivo* [157], it cannot be ruled out that sfRNA antagonizes the RNAi response in one way or another to mediate flavivirus infection of mosquitoes. No affinity of ZIKV nor WNV sfRNA for the proteins of the RNAi pathway was detected in our RNA-affinity purification experiments, however RNAi suppression does not necessarily rely on sequestration or binding of RNAi-effector proteins [101,561]. Midgut infection and the establishment of virus replication occurs early after exposure to an infectious blood meal, approximately within 1-3 days [140,145]. Release of flaviviruses from the midgut generally occurs in a window of 2-7 days post exposure [140,145]. Therefore, a crucial inhibition of the RNAi response by sfRNA expectedly occurs within 1-7 days after a blood meal, which may explain why there was no correlation between sfRNA production and siRNA suppression at 14 dpi (Chapter VI). It would be informative to analyse the population of vsiRNAs early after infection of mosquitoes with a wild type and sfRNA-deficient flavivirus, specifically in isolated mosquito midguts. In combination with quantification of viral RNA copy numbers by qRT-PCR this should reveal whether sfRNA formation correlates with lower levels of vsiRNAs relative to the abundance of vgRNA. To add to the complexity, recent findings suggest that the RNAi response may not play a significant role in arbovirus

restriction in mosquito midguts. In *Drosophila* loading of siRNAs into Ago2-RISC involves the proteins Loquacious (loqs) and r2d2, however, in mosquitoes, the presence of another factor, named loqs2 is required for efficient siRNA-mediated silencing [562]. Loqs2 is expressed in the mosquito body, but not in the midgut and consequently the siRNA pathway does not restrict DENV or ZIKV infection in mosquitoes, but does suppress virus dissemination [562]. Therefore, suppression of the RNAi response in the mosquito midgut by sfRNA may be of lesser significance, although an effect of sfRNA on virus dissemination through inhibition of systemic RNAi may still affect virus transmission.

In *Drosophila*, siRNAs are amplified in haemocytes, which produce secondary siRNAs that are responsible for the systemic spread of the siRNA-signal [563]. Secondary siRNA production depends on the conversion of viral RNA into viral DNA, which is subsequently used as template for secondary siRNA production. Similar reverse transcription pathways that lead to the formation of viral DNA are also present in mosquitoes [231,232,564]. Systemic RNAi can inhibit the replication and subsequent dissemination of a virus infection [563,565]. Importantly, secondary siRNAs have a 5' triphosphate [566], which disables their cloning in conventional small RNA deep sequencing due to incompatibility in the adapter ligation step. Consequently, conventional deep sequencing data lack information on the population of secondary siRNAs [563], indicating that secondary siRNAs may have been excluded in our deep sequencing approach during WNV infection of *Cx. pipiens* (Chapter VI). Modulation of the RNAi pathway through inhibition of secondary siRNA amplification could be involved in systemic infection after midgut release, resulting in increased infection and transmission efficiencies. As haemocytes are quite refractory to virus infection, secondary siRNA amplification in haemocytes depends on the acquisition of viral RNA, possibly through phagocytosis [563,567]. Potentially, uptake of sfRNA by haemocytes could inhibit the formation of secondary siRNAs, resulting in increased midgut escape, dissemination and subsequent virus transmission. Future research should investigate the correlation between sfRNA production and the abundance of secondary siRNAs in flavivirus infected mosquitoes.

Other mosquito immune responses, such as the Toll, JAK/STAT and Imd pathways, have been implicated in the defence of mosquitoes to arboviruses (Reviewed in [102]). SfRNA has been demonstrated as a suppressor of mammalian immune signalling pathways [109–112], thus sfRNA may similarly antagonize homologous immune pathways in mosquitoes. Although DENV-2 sfRNA was observed to suppress the JAK/STAT pathway in mosquitoes [470], no effect of sfRNA production on expression of JAK/STAT nor Toll-pathway regulated genes was observed (Chapter VIII). In combination with the minor effect of sfRNA on the expression of JAK/STAT, Toll and Imd regulated genes by Pompon *et al.* [470], this makes the putative antagonistic effect of sfRNA on these pathways quite unlikely. Inhibition of innate immune signalling pathways by a noncoding RNA molecule such as sfRNA, most likely occurs via the interaction with cytoplasmic immune receptors or proteins involved in immune signalling. However, no interaction between ZIKV or WNV sfRNA and any proteins directly involved in the JAK/STAT, Toll and Imd pathways was detected (Chapter VIII). Therefore, future research should instead focus on clarifying the correlation between sfRNA and RNAi-suppression, and unravel the significance of the sfRNA-interacting proteins that have been discovered in Chapter VIII for flavivirus infection of and transmission by mosquitoes.

Unbiased methods to identify mosquito and viral factors required for arbovirus transmission

Viruses are dependent on a large number of host factors for a successful replication cycle [550,551]. Such factors can be identified from either the virus-perspective, by mutagenesis of the viral genome, or from the mosquito-perspective, by host-gene mutagenesis, deletion, or knockdown. In this thesis, the roles of flavivirus sRNA and CHIKV nsP3 for virus transmission by mosquitoes were investigated from a virus-perspective (Chapter IV, VI & VIII). These two molecules were investigated specifically, as previous findings were supportive for their role as a determinant of transmission by mosquitoes. Interestingly, novel techniques allow the implementation of strategies that, instead, screen the complete viral genome for essential regions [568,569]. Basically, these methods randomly introduce mutations/insertions in the viral genome to generate a virus-mutant library that can be used to identify regions that are essential or non-essential for replication in the tested system. By infection of both mosquito cells and mosquitoes with such a virus-mutant library, novel determinants of mosquito infection and transmission may be identified. In other words, regions that are essential for mosquito infection, but not replication in mosquito cell lines could be uncovered, which would suggest a specific role of such RNA/protein for infection of the mosquito vector. Regions in the viral genome that are essential for mosquito infection can form the basis for studies on arbovirus-vector interactions and may be required to establish an interaction with mosquito host factors

Alternatively, studies that employ a mosquito-perspective screen, could be used to identify proteins that are essential for arbovirus transmission. Genome-wide loss-of-function screens in human cells, which assess the effect of knockdown of every single gene on virus replication (e.g. by siRNA, dsRNA, or CRISPR/Cas9-based silencing), have implicated a number of proviral and antiviral host factors for arbovirus replication, and brought forward interesting candidates for antiviral drug development [570–573]. Similarly, genome-wide loss-of-function screens in *Drosophila* cells with WNV and SINV implicated host factors involved in virus infection in insect cells [574,575]. Although transcriptome analysis of arbovirus infected mosquitoes and mosquito cells have been performed [152,170,171,180,576], genome-wide loss-of-function screens have not been described for arboviruses in mosquitoes or mosquito cells. Such studies could implicate important pathways and host factors that are essential for arbovirus infection of and transmission by mosquitoes. As novel genetic tools, such as CRISPR-Cas9, are now available to generate knockouts in mosquitoes [577,578], significant hits from an *in vitro* screen could be investigated *in vivo* for their significance to arbovirus transmission by mosquitoes. A combination of the described methods could greatly expand our understanding of arbovirus-vector interactions, which may lead to the development of novel methods to combat arbovirus transmission in the field.

Societal significance of the findings in this thesis

The increasing global burden of arboviral disease raises the need for improved arbovirus intervention strategies. Such strategies focus on either preventing disease of exposed human individuals or decreasing the chance of exposure of an individual to an infected mosquito [579,580]. The first category comprises vaccines and antiviral drugs and will not be discussed in detail (for detailed reviews on ZIKV, WNV or CHIKV vaccines please read [38,48,64]).

Vaccination is a highly effective preventative strategy and effective human arbovirus vaccines are available for JEV, YFV and TBEV. However, for many arboviruses,

vaccine development has not yet completed the full set of clinical trials and vaccines are therefore not commercially available. A promising DENV vaccine is based on viral attenuation via deletion of 30 nts in the 3'UTR (DENV Δ 30), corresponding to the DB-2 RNA structure (essential for interaction with ME31B; Chapter VIII) [581]. Interestingly, the DENV-4 Δ 30 vaccine strain is attenuated in infection and transmission by *Ae. aegypti* [548] and the 30 nts deletion reportedly results in decreased formation of sfRNA [547]. The results in this thesis (Chapter VIII) suggest that the DENV Δ 30 mutants may fail to interact with ME31B/DDX6, which can underlie their reduced replication and pathogenicity in mammalian cells, and their restricted transmission by mosquitoes [437,548,581]. A similar strategy has already been used for the generation of a ZIKV vaccine, where deletions in DB-1 (homologous to DENV DB-2) led to an attenuated virus that is immunogenic and protective in mice. Importantly, this ZIKV vaccine strain was also incapable of infecting mosquitoes, again emphasizing the importance of an interaction between sfRNA/3'UTR and ME31B for the infection of mosquitoes [458,546]. Based on the results in Chapters VI & VIII, a similar strategy could be implemented to generate live-attenuated vaccine strains for WNV, and potentially other flaviviruses. Attenuated strains with mutations of RNA structures essential for the production of sfRNA combined with deletion of DB-2 to abolish the interaction with ME31B/DDX6 will be highly attenuated in mammalian cells [106,322,437], and will have the additional advantage of being poorly transmitted by mosquitoes.

Similarly, the necessity of CHIKV, and potentially other alphaviruses, to establish an interaction between nsP3 and G3BP/Rin (Chapter V) [123,125–127] as well as amphiphysins or other SH3-domain containing host proteins (Chapter V) [122,292] could be exploited to generate live-attenuated vaccines. CHIKV strains with one mutated G3BP-interacting motif are attenuated in mammalian cells, but not in mosquito cells (Chapter V). Mutating one G3BP-interacting motif, in combination with mutation of the amphiphysin-interacting motif could allow the amplification of virus-mutants in mosquito cells, that replicate poorly in mammalian cells. This has the potential to generate a safe live-attenuated CHIKV vaccine strain that generates a potent immune response, as the virus is immunogenically identical to wild type CHIKV. It remains a fact that vaccination is quite expensive and therefore more difficult to implement in developing countries. Several antiviral treatments have been described that can be used to treat patients that suffer from arboviral disease. However, as antivirals are often used as a reactive rather than a prophylactic treatment, they should always be accompanied by preventative measures. As arboviruses rely on their transmission on arthropod vectors, intervention strategies that focus on vector control can be highly effective. Vector control strategies operate by either reducing the exposure of humans to vector mosquitoes, diminishing the vector population size, or lowering the vector competence. Strategies such as the sterile insect technology (SIT) and gene drive approaches can be implemented to diminish the vector population size [582–584]. SIT comprises the release of overwhelming numbers of sterilized male mosquitoes that compete with wild males for mating with wild females. As most female mosquitoes mate only once in their lifetime, and mating with a sterile male does not produce any offspring, this strategy can successfully decrease the mosquito population in the treated area [582]. A downside to SIT is the difficulty of getting public acceptance, as releasing thousands of mosquitoes in arbovirus-threatened areas can be counter-intuitive. Another major drawback of SIT is its efficacy, which is limited to one generation, thus requiring the prolonged release of sterile male mosquitoes. This drawback may be overcome by the use of gene-drive approaches. Gene-drive approaches are based on selfish genes that spread within a population without necessarily being beneficial for the organism [582–584]. Basically, these

technologies use nuclease- or recombination-based integration events to ensure that the offspring inherits a copy of the synthetic gene on both chromosomes (in case of a diploid organism). As the synthetic gene includes the elements required for its own integration, such as nucleases and guide sequences, the synthetic gene can drive itself through a population of insects. In combination with a gene that prevents development of the mosquito offspring, this technology can potentially be used to decrease the population density of mosquitoes with high efficacy over a prolonged period. However, there are still many questions concerning the efficacy, risks, environmental impact and ethics of releasing genetically-modified mosquitoes that need to be addressed before large-scale implementation of this technique becomes feasible and societally acceptable [585].

An alternative strategy is the release of mosquitoes with lowered vector competence and/or vectorial capacity. This strategy relies on knowledge of arbovirus-vector interactions and is limited to the known determinants of mosquito vector competence for arboviruses. A well-studied example is the use of the intracellular bacteria *Wolbachia*, as discussed in Chapter II. Several studies have demonstrated that the infection of mosquitoes with certain strains of *Wolbachia* can make them resistant to arbovirus infection (reviewed in [185]). The release of *Wolbachia*-infected mosquitoes has been successfully implemented in semi-field studies [196,586,587]. Results with *Wolbachia*-infected mosquitoes are sometimes contradicting [188] and their efficacy as vector control strategy is debateable [185,585]. However, the findings demonstrate the potential of strategies aimed at reducing the vector competence of mosquitoes for arboviruses. Mosquitoes and mosquito cell lines, can also be infected with insect-specific viruses (ISVs) (Chapter III). As infection of mosquitoes with ISVs can decrease the vector competence for arboviruses [233,239,588], ISVs may be used as a parallel strategy to *Wolbachia* to generate arbovirus-refractory mosquito strains. The results in this thesis uncover that both the interaction between alphavirus nsP3 and mosquito Rin [129] (Chapter V), as well as the production of sfRNA for flaviviruses are important for virus transmission by mosquitoes (Chapters VI and VIII). Such determinants may be useful targets for the production of refractory mosquito strains. For example, mosquitoes could be genetically modified to express miRNAs or siRNAs either transiently or induced upon arbovirus infection, that target these viral determinants, or their interaction partners (e.g. ME31B/Rin) to lower the vector competence for arboviruses. Mosquitoes expressing synthetic miRNAs that target the vRNA of DENV and CHIKV have already been demonstrated to successfully reduce the infection and transmission in *Ae. aegypti* mosquitoes [589]. The expression of DENV complementary dsRNA has demonstrated to effectively decrease viral infection and transmission in *Ae. aegypti* [163,590]. In combination with a gene-drive approach, an introduced antiviral trait could spread through a given mosquito population, resulting in a decreased vector competence of field mosquitoes to arboviruses. The implementation of arbovirus-intervention strategies that target key arbovirus-vector interactions presents a valuable tool for future arboviral control strategies, and evaluating its potential should be the focus of future research.

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Summary

Samenvatting

Summary

Mosquitoes spread a number of pathogenic viruses, such as Zika virus (ZIKV), West Nile virus (WNV), and chikungunya virus (CHIKV). These viruses belong to a larger group of arthropod-borne (arbo) viruses. Typical for arboviruses is that they have to infect and replicate in both their vertebrate host, in which they cause disease, and their arthropod vector, such as mosquitoes and ticks, to establish an efficient transmission cycle. Arboviruses are responsible for over 400 million estimated cases of human disease each year. Importantly, only a few mosquito species are capable of transmitting medically important arboviruses, and competent mosquitoes belong primarily to the *Aedes* and *Culex* genera. ZIKV and WNV both belong to the genus *Flavivirus* (family *Flaviviridae*). ZIKV is transmitted by *Aedes* mosquitoes, predominantly *Ae. aegypti*, while WNV is transmitted by *Culex* mosquitoes, mainly *Cx. pipiens*. ZIKV infection usually leads to mild symptoms, although in severe cases ZIKV can cause Guillain-Barré syndrome in adults and microcephaly in the unborn child of infected pregnant women. WNV infection often proceeds asymptomatic, but in severe cases patients can develop neuroinvasive disease, which can be fatal. CHIKV is an *Alphavirus* (family *Togaviridae*) and is transmitted mostly by *Ae. aegypti* and *Ae. albopictus* mosquitoes. Patients with CHIKV infection typically present high fever in combination with joint- and muscle pain, which can be very severe and may last for several months.

The incidence of arboviral diseases is increasing, concurrent with expanding global trade and travel and elevated environmental temperatures. Unfortunately, we still poorly understand the molecular mechanisms and virus-mosquito interactions that underlie the transmission of arboviruses by mosquitoes, which is important to combat the incidence of arboviral disease.

For the successful transmission by a mosquito, arboviruses have to infect and replicate in the cells of the mosquito midgut after an infectious blood meal. Progeny virus then has to disseminate into the mosquito's circulatory system, and infect secondary tissues to establish a systemic infection. Subsequently, the virus needs to infect and replicate in the mosquito salivary glands and end up in the saliva to be injected into the next vertebrate host during a new blood meal. It is well known that especially the midgut and salivary glands form a barrier for arbovirus transmission, however we do not understand the interactions of arboviruses with these barriers at a molecular level. Furthermore, mosquitoes react to arbovirus infection with various innate immune responses, and the molecular interactions between arboviruses and these immune responses also lack a detailed understanding. Therefore, this thesis investigates the molecular determinants of transmission by mosquitoes for human-infecting arboviruses. The role of virus vector-interactions at the midgut- and salivary gland barriers is assessed for ZIKV, CHIKV and WNV. Furthermore, viral molecules are identified that determine the transmission of these arboviruses by interacting with specific mosquito factors.

The main antiviral response of mosquitoes is mediated by small RNAs (≤ 30 nts in size). Interestingly, different small RNA responses are activated in response to viruses from different viral families. Furthermore, the small RNA response to a virus can differ between *Aedes* and *Culex* species mosquitoes. Therefore, the small RNA responses of *Culex* versus *Aedes* cells are compared by investigating the small RNAs produced to WNV infection in *Aedes* U4.4 and *Culex* Cx.t mosquito cells. Via *de novo* assembly of small RNAs, the presence of insect-specific viruses is uncovered in both *Aedes* and *Culex* cell lines, which may have implications for arbovirus research using such cell lines as insect-specific viruses and arboviruses may interfere during co-infection. It is shown that both *Aedes* and *Culex* cells are able to produce two types

of small RNA responses to virus infection: small-interfering RNAs and Piwi-interacting RNAs. Importantly, the production of Piwi-interacting RNAs is strongly dependent on the virus family and the production of Piwi-interacting RNAs to one particular virus can differ between *Aedes* and *Culex* cells.

Similarly to insect-specific viruses, two or more arboviruses can also co-infect mosquitoes, but the putative virus-virus interactions have not been studied in much detail. With the introduction of ZIKV into the Americas in 2015, where CHIKV was co-circulating, patients with co-infections of ZIKV and CHIKV were reported. However, it was unclear whether it was possible for a single mosquito to transmit multiple arboviruses simultaneously. Therefore, the infection of *Ae. aegypti* mosquitoes with ZIKV and CHIKV are compared during separate and co-infections. *Ae. aegypti* mosquitoes are shown to be highly competent for both ZIKV and CHIKV. Importantly, it is demonstrated that one *Ae. aegypti* mosquito can transmit both ZIKV and CHIKV via a single bite, without significant interference between the two viruses.

As viruses are obligatory parasites of their host cells, they recruit and interact with host proteins to establish a successful replication cycle. CHIKV non-structural protein 3 (nsP3) interacts with Ras GTPase-activating protein-binding protein (G3BP) and amphiphysin in mammalian cells via conserved amino acid motifs. Artificial interference with the interaction of nsP3 with G3BP and amphiphysin decreases CHIKV replication in mammalian cells, but the importance for replication in mosquito cells and infection of mosquitoes was unknown. By mutating the core amino acid residues in nsP3 that are required for the interactions with G3BP and amphiphysin, the importance of these interactions is investigated. The results show that the amino acid motif required for interaction with amphiphysin is required for efficient replication of CHIKV in both mammalian and mosquito cells, but is dispensable for virus transmission by *Ae. aegypti* mosquitoes. Curiously, it is demonstrated that duplicated amino acid motifs are required for the interaction of nsP3 with mammalian G3BP, while only a single motif is sufficient for the interaction with Rasputin, the mosquito G3BP ortholog. Consequently, CHIKV with only one Rasputin/G3BP-interacting motif is still efficiently transmitted by *Ae. aegypti* mosquitoes, but attenuated in mammalian cells.

In counter defence to insect immune responses, insect viruses often contain suppressors of innate immunity, that interact with host molecules to promote viral replication and/or antagonize antiviral effects. During the replication in vertebrate and invertebrate cells, flaviviruses produce a noncoding subgenomic flavivirus RNA (sfRNA) due to the stalling of exoribonucleases on resistant RNA structures in the viral 3' untranslated region. This sfRNA is highly abundant in infected cells and has been implicated as a suppressor of innate immune responses, inducer of apoptosis and enhancer of virus replication in mammalian cells. Importantly, the formation of sfRNA is conserved for all viruses from the genus *Flavivirus*, including insect-specific flaviviruses, which suggests an important role of sfRNA during infection of the invertebrate vector.

Vector competence studies with wild type and sfRNA-deficient WNV and ZIKV demonstrates that sfRNA is essential for the infection and transmission of WNV by *Cx. pipiens* and ZIKV by *Ae. aegypti* mosquitoes. This establishes sfRNA as a generic determinant of flavivirus transmission by mosquitoes. In contrast, sfRNA-deficient ZIKV and WNV is not attenuated in mosquito cell lines, suggesting a function of sfRNA in the mosquito irrespective of effects on virus replication. Comparison of infectious blood meals with infections via intrathoracic injections that bypass the midgut barrier, identifies sfRNA as a key determinant for ZIKV and WNV to overcome the mosquito midgut barrier, as well as the salivary gland barrier. Small RNA deep sequencing of wild type and sfRNA-deficient WNV demonstrates that sfRNA is processed by the RNA-interference machinery *in vivo* in mosquitoes, but sfRNA does not significantly

antagonize the RNA-interference response. Importantly, an *in vitro* sfRNA-affinity purification method identifies the specific interaction of ZIKV and WNV sfRNA with three mosquito proteins, including the DEAD-box helicase ME31B. Silencing of ME31B results in increased ZIKV and WNV viral genomic RNA replication in mosquito cells, implicating an antiviral function of mosquito ME31B. Moreover, we show that ME31B is bound specifically to a dumbbell RNA structure in ZIKV and WNV sfRNA, thus sfRNA recruits or sequesters ME31B. As ME31B is essential for the assembly of processing bodies, that may be antiviral, and is involved in mRNA turnover and suppression of translation, sequestration of ME31B by sfRNA may underlie the sfRNA-mediated transmission of flaviviruses by mosquitoes.

In conclusion, this thesis significantly contributes to our understanding of arbovirus transmission by identifying two essential arboviral transmission-determinants and expands the current knowledge of arbovirus-vector interactions.

Samenvatting

Muggen verspreiden een aantal ziekteverwekkende virussen, zoals het Zika virus (ZIKV), het West Nile virus (WNV) en het chikungunya virus (CHIKV). Deze virussen behoren tot een grotere groep van virussen die geleedpotige infecteren, de zogeheten arbovirussen. Typerend voor arbovirussen is dat ze zowel hun gewervelde gastheer, waarin ze ziekte veroorzaken, als hun geleedpotige vector, zoals muggen en teken, moeten infecteren. Daarnaast zijn ze ook genooddaakt om te repliceren in zowel de gewervelde gastheer als de geleedpotige vector om een efficiënte transmissiecyclus te volbrengen.

Arbovirussen zijn jaarlijks verantwoordelijk voor, naar schatting, meer dan 400 miljoen menselijke ziektegevallen. Echter zijn slechts een aantal muggensoorten in staat om arbovirussen over te brengen die ziekte in mensen teweegbrengen. Van deze 'competente' muggensoorten behoren de meeste soorten tot het geslacht *Aedes* of *Culex*. ZIKV en WNV behoren beide tot de zogeheten *flavivirussen* (familie *Flaviviridae*). ZIKV wordt overgedragen door *Aedes* muggen, voornamelijk *Ae. aegypti*, terwijl WNV wordt overgedragen door *Culex* muggen, voornamelijk *Cx. pipiens*. Infectie met ZIKV leidt meestal tot milde symptomen, hoewel ZIKV in ernstige gevallen het syndroom van Guillain-Barré in volwassenen en microcefalie in het ongeboren kind van geïnfecteerde zwangere vrouwen kan veroorzaken. Infectie met WNV verloopt vaak zonder symptomen, maar in ernstige gevallen kunnen patiënten een infectie in het zenuwstelsel ontwikkelen, wat fataal kan zijn. CHIKV behoort tot de zogeheten *alphavirussen* (familie *Togaviridae*) en wordt voornamelijk overgedragen door *Ae. aegypti* en *Ae. albopictus* muggen. Patiënten met een CHIKV infectie ontwikkelen doorgaans hoge koorts in combinatie met gewrichts- en spierpijn, wat zeer ernstig kan zijn en enkele maanden kan aanhouden. Het aantal door arbovirussen veroorzaakte ziektegevallen neemt momenteel toe, parallel met de toenemende internationale handel, de toename van internationale reizen en hogere omgevingstemperaturen. Helaas is de kennis over de interacties tussen arbovirussen en muggen, en de onderliggende moleculaire mechanismen zeer beperkt. Dat terwijl deze interacties ten grondslag liggen aan de overdracht van arbovirussen door muggen. Omdat dergelijke kennis erg belangrijk is om ziektegevallen door arbovirussen te bestrijden, is het uitermate belangrijk dat we beter begrijpen welke interacties tussen virussen en hun muggen vector van belang zijn en hoe deze interacties op moleculair niveau werken.

Om succesvol door een mug te worden overgedragen moeten arbovirussen na opname tijdens een bloedmaaltijd de cellen van het muggen-darmkanaal infecteren, en zichzelf in deze cellen vermenigvuldigen. De nieuwe virusdeeltjes moeten er vervolgens in slagen om de bloedsomloop van de mug te bereiken, om van daaruit secundaire weefsels te infecteren en een systemische infectie te veroorzaken. Vervolgens moet het virus de cellen van de muggen-speekselklieren bereiken, deze infecteren en zichzelf wederom vermenigvuldigen in deze cellen. Nieuw gemaakte virusdeeltjes moeten vervolgens terecht komen in het speeksel van de mug, om tijdens een nieuwe muggenbeet op een vertebrate gastheer in diens bloedbaan te worden geïnjecteerd. Het is inmiddels bekend dat met name de cellen van het muggen darmkanaal en de cellen van de muggen speekselklier een barrière vormen voor arbovirussen. Echter, de interacties van arbovirussen met deze barrières worden nog niet op moleculair niveau begrepen.

Naast deze barrières hebben muggen ook een aangeboren immuunsysteem, wat geactiveerd kan worden tijdens infectie met een arbovirus, om zodoende de infectie te onderdrukken of een succesvolle infectie te voorkomen. Ook de moleculaire interacties tussen arbovirussen en de onderdelen van het aangeboren immuunsysteem

van de mug worden niet in detail begrepen. In dit proefschrift worden daarom de moleculaire determinanten onderzocht die essentieel zijn voor de overdracht van mensen infecterende arbovirussen door muggen. Met name de rol van arbovirus-mug interacties die een rol spelen bij het overkomen van de darm- en speekselklier barrières worden onderzocht tijdens infecties met ZIKV, CHIKV en WNV. Bovendien worden virale moleculen geïdentificeerd die de overdracht van deze arbovirussen bepalen door interacties aan te gaan met specifieke factoren van de mug.

Het belangrijkste antivirale mechanisme van muggen wordt aangestuurd via kleine RNA moleculen (≤ 30 nucleotiden in grootte). Interessant is dat in insecten verschillende kleine RNA responsen worden geactiveerd als reactie op infectie met virussen van verschillende virale families. Bovendien kan de kleine RNA respons tijdens infectie met één specifiek virus verschillen tussen *Aedes* en *Culex* muggen. Om deze verschillen beter te begrijpen worden in hoofdstuk 3 de kleine RNA responsen van *Culex* en *Aedes* muggencellen vergeleken. Dit werd gedaan door de kleine RNA moleculen te analyseren die geproduceerd worden tijdens WNV infectie in *Aedes* U4.4 en *Culex* Cx.t cellen. Door deze kleine RNA moleculen te assembleren wordt de aanwezigheid van insect-specifieke virussen aangetoond in zowel de *Aedes* als de *Culex* cellen. Dit heeft belangrijke implicaties voor onderzoek naar arbovirussen waarvoor dergelijke cellijnen worden gebruikt, aangezien insect-specifieke virussen kunnen interfereren met arbovirussen tijdens een co-infectie. Daarnaast wordt aangetoond dat zowel *Aedes* als *Culex* cellen in staat zijn om twee soorten kleine RNA responsen tegen virusinfectie te produceren: de zogeheten kleine-interfererende (si)RNAs en Piwi-interacterende (pi)RNAs. Een belangrijke observatie is dat de productie van piRNAs sterk afhankelijk is van de infecterende virusfamilie en dat de productie van piRNAs tijdens infectie van één specifiek virus kan verschillen tussen *Aedes* en *Culex* cellen. Deze resultaten vergroten ons begrip van het door kleine RNAs aangestuurde immuunsysteem van muggen, en de rol die dit systeem speelt in de overdracht van arbovirussen door muggen.

Net als co-infectie tussen arbovirussen en insect-specifieke virussen, kunnen co-infecties van muggen met twee of meer arbovirussen ook voorkomen, echter de mogelijke virus-virus interacties zijn nog niet in detail bestudeerd. Toen ZIKV in 2015 in Noord-Amerika werd geïntroduceerd, waar CHIKV gelijktijdig circuleerde, werden patiënten gerapporteerd met co-infecties van zowel ZIKV als CHIKV. Het was echter onduidelijk of deze patiënten mogelijk door één enkele mug gelijktijdig waren geïnfecteerd met meerdere arbovirussen. Om deze mogelijkheid te onderzoeken, worden in hoofdstuk 4 *Ae. aegypti* muggen geïnfecteerd met ZIKV, CHIKV of zowel ZIKV als CHIKV. De resultaten laten zien dat *Ae. aegypti* muggen zeer competent zijn om zowel ZIKV als CHIKV over te dragen. Belangrijk is dat wordt aangetoond dat één *Ae. aegypti* mug tegelijkertijd zowel ZIKV als CHIKV kan overdragen via één enkele beet, zonder dat daarbij significante interferentie tussen de twee virussen optreedt.

Omdat virussen impliciet afhankelijk zijn van een gastheer cel, rekruteren ze en interacteren ze met gastheerfactoren om een succesvolle replicatiecyclus tot stand te brengen. Het niet-structurele proteïne 3 (nsP3) van CHIKV interacteert met het Ras GTPase-activerende eiwit bindende proteïne (G3BP) en het eiwit amphiphysin in vertebrate cellen via in nsP3 geconserveerde aminozuurmotieven. Het kunstmatig muteren van deze geconserveerde aminozuurmotieven in nsP3 verbreekt de interacties met G3BP en amphiphysin en vermindert daardoor de replicatie van CHIKV in vertebrate cellen. Echter, het belang van deze aminozuurmotieven voor CHIKV replicatie in muggencellen en overdracht door muggen was aan het begin van dit proefschrift nog onbekend. Door wederom deze geconserveerde aminozuurmotieven in nsP3 te muteren wordt in hoofdstuk 5 het belang van de interactie met G3BP en amphiphysin voor virus

replicatie in muggen cellen en de overdracht door muggen onderzocht. De resultaten laten zien dat het aminozuurmotief dat vereist is voor de interactie met amphiphysin vereist is voor efficiënte replicatie van CHIKV in zowel vertebate als muggencellen, maar dit motief is niet nodig voor overdracht door *Ae. aegypti* muggen. Opvallend is dat twee gedupliceerde aminozuurmotieven nodig zijn voor de interactie van nsP3 met G3BP in vertebate cellen, terwijl slechts één motief nodig is voor de interactie met Rasputin, de ortholoog van G3BP in muggen. Als gevolg wordt een CHIKV mutant met slechts één intact Rasputin-interacterend motief nog steeds efficiënt overdragen door *Ae. aegypti* muggen, maar is dit mutant virus verzwakt in vertebate cellen.

In de strijd tussen virussen en het immuunsysteem van insecten, produceren insectenvirussen vaak factoren die het aangeboren immuunsysteem onderdrukken, meestal door een interactie aan te gaan met gastheermoleculen die onderdeel uitmaken van dit immuunsysteem, om virale replicatie te bevorderen. Tijdens de replicatie in cellen van gewervelde en ongewervelde organismen produceren flavivirussen een niet-coderend subgenomisch flavivirus RNA (sfRNA). Dit sfRNA wordt geproduceerd door gastheer exoribonucleasen te blokkeren via resistente RNA structuren in de virale 3' niet getransleerde regio. SfRNA is in overvloed aanwezig in geïnfecteerde cellen en is aangewezen als onderdrukker van het aangeboren immuunsysteem, activator van apoptose en verhoogd de efficiëntie van virusreplicatie in vertebate cellen. Belangrijk is dat de productie van sfRNA geconserveerd is voor alle flavivirussen, inclusief insect-specifieke flavivirussen, wat een belangrijke rol van sfRNA tijdens infectie van de insecten gastheer suggereert.

In hoofdstukken 6 en 8 wordt door middel van overdrachtstudies met wild-type en sfRNA-deficiënt WNV en ZIKV aangetoond dat sfRNA essentieel is voor de infectie en overdracht van WNV door *Cx. pipiens* en ZIKV door *Ae. aegypti* muggen. Dit laat zien dat sfRNA een determinant is van flavivirus overdracht door muggen. Daarentegen zijn sfRNA-deficiënt ZIKV en WNV niet verzwakt in muggen cellen, wat suggereert dat sfRNA een specifieke rol speelt in de mug, onafhankelijk van effecten op virusreplicatie. Door infecties via een bloedmaaltijd te vergelijken met intrathoracale injecties, welke de muggendarm barrière omzeilen, wordt sfRNA geïdentificeerd als determinant voor ZIKV en WNV om deze darmbarrière te passeren. Daarnaast laten de resultaten zien dat sfRNA ook belangrijk is voor ZIKV en WNV om de speekselklier barrière door te komen. Door de kleine RNAs te onderzoeken tijdens infectie van muggen met wild-type en sfRNA-deficiënt WNV wordt aangetoond dat sfRNA in muggen wordt afgebroken door het kleine RNA gereguleerde immuunsysteem, echter dit heeft geen zichtbaar effect op de efficiëntie van ditzelfde immuunsysteem. Met behulp van een *in vitro* sfRNA-affiniteitszuiveringsmethode zijn eiwitten in kaart gebracht die een specifieke interactie aangaan met het sfRNA van ZIKV en WNV. Dit toont aan dat sfRNA met tenminste drie muggen eiwitten interacteert, waaronder de DEAD-box helicase ME31B. Het verminderen van de ME31B aanwezigheid in muggencellen resulteerde in verhoogde RNA replicatie voor ZIKV en WNV, wat een antivirale functie van ME31B impliceert in muggen. Bovendien wordt aangetoond dat ME31B bindt aan een specifieke RNA structuur in ZIKV en WNV-sfRNA. Aangezien ME31B essentieel is voor de opbouw van cellulaire "processing bodies", welke antiviraal kunnen zijn, en betrokken is bij mRNA-afbraak en het onderdrukken van eiwit translatie, kan het binden van ME31B door sfRNA ten voordelen zijn voor flavivirus infectie in muggen cellen. Bovendien zou de interactie van sfRNA met ME31B ten grondslag kunnen liggen aan de door sfRNA geassisteerde overdracht van flavivirussen door muggen.

Concluderend draagt dit proefschrift aanzienlijk bij aan ons begrip van arbovirus overdracht via muggen door twee essentiële arbovirale transmissiedeterminanten te identificeren en de huidige kennis van arbovirus-muggen interacties te verbeteren.

Appendices

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List of abbreviations

60sRp	60s ribosomal protein
ADAR	adenosine deaminase acting on RNA
<i>Ae.</i>	<i>Aedes</i>
Ago	Argonaute
<i>An.</i>	<i>Anopheles</i>
AP	alkaline phosphatase
Arbovirus	arthropod-borne virus
ATX	Ataxin
Aub	Aubergine
BeBV	Bebaru virus
BFV	Barmah Forest virus
cDNA	complementary DNA
CecG	Cecropin G
CFAV	Cell fusing agent virus
CHIKV	chikungunya virus
CLBOV	Calbertado virus
CMV	cytomegalovirus
co-IP	co-immunoprecipitation
CPE	cytopathic effect
Cq	cycle of quantification
CRISPR	clustered regularly interspaced short palindromic repeats
CS	cyclisation sequence
<i>Cx.</i>	<i>Culex</i>
CYV	Culex Y virus
DB	dumbbell
DCP	mRNA-decapping protein
Dcr	Dicer
DDX6/3	DEAD-box helicase 6/3
DefC	Defensin C
DENV	dengue virus
dpi	days post infection
ds	double-stranded
EDC	enhancer of decapping
EEEV	eastern equine encephalitis virus
eEF1a	eukaryotic elongation factor 1a
EGFP	enhanced green fluorescent protein
EILV	Eilat virus
EPDA	end-point dilution assay
ER	endoplasmic reticulum
EVE	endogenous viral elements
FHV	Flock House virus
FMR1	Fragile X syndrome-related protein 1
FXR	Fragile X syndrome
G3BP	Ras GTPase-activating protein-binding protein
G4	G-quadruplex
GQN1	G-quadruplex nuclease 1
gRNA	genomic RNA
HIV	Human-immunodeficiency virus
hpi	hours post-infection

hpt	hours post-transfection
HVD	hypervariable domain
IC	infectious clone
IFIT	interferon-induced protein with tetra-tricopeptide repeats
IFN	interferon
IMD	immune deficiency
IRF	interferon regulatory factor
ISF	insect-specific flavivirus
ISV	insect-specific virus
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JEV	Japanese encephalitis virus
kb	kilo base
LARP	La-related protein 1
Lig	Lingerer
LSM	like-Sm proteins
Luc	luciferase
MAYV	Mayaro virus
MBF	mosquito-borne flaviviruses
mC	modified Cherry
MDA-5	melanoma differentiation-associated protein 5
ME31B	maternal expression at 31B
miRNA	micro RNA
MOI	multiplicity of infection
MOV34	Moloney leukaemia virus-34
mRNA	messenger RNA
MTase	methyltransferase
NA	North-American
NF-kB2	nuclear factor kappa-B2
NGS	next-generation sequencing
NIRVS	non-retroviral integrated RNA virus sequences
NKVF	no-known-vector flaviviruses
NS	non-structural
nsP	non-structural protein
nt	nucleotide
NTF2	nuclear transport factor 2
NV	Narnavirus
NWE	north-western European
ONNV	O'nyong nyong virus
ORF	open reading frame
PABP	poly-(A) binding protein
PBS	phosphate-buffered saline
PCLV	Phasi Charoen-like virus
PHEIC	public health emergency of international concern
piRNA	Piwi-interacting RNA
PIWI	P-element-induced Wimpy testis
PK	pseudoknot
PKR	protein kinase R
PLB	passive lysis buffer
P-rich	proline-rich
PTB	polypyrimidine tract-binding protein
PUB	poly-ubiquitin

(q)RT-PCR	(quantitative) reverse-transcription polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
REF2	RNA and export factor binding protein 2
rep	replicon
RGG	arginine glycine rich region
RH	relative humidity
RIG-I	retinoic acid-inducible gene I
Rin	Rasputin
RISC	RNA-induced silencing complex
RLU	relative light units
RNAi	RNA-interference
rpS7	ribosomal protein S7
RRM	RNA recognition motif
RRV	Ross River virus
RT	room temperature
S1m	modified streptavidin aptamer
SA	streptavidin
SEM	standard error of the mean
sfRNA	subgenomic flavivirus RNA
SFV	Semliki Forest virus
SG	stress granule
sgRNA	subgenomic RNA
SINV	Sindbis virus
siRNA	small-interfering RNA
SL	stem-loop
Spp.	species
ss	single-stranded
STRING	search tool for the retrieval of interacting genes/proteins
TALV	Taï Forest alphavirus
TBE	tris/borate/ethylenediaminetetraacetic acid
TBF	tick-borne flaviviruses
TCID50	tissue culture infectious dose 50%
Tep22	Testicular protein 22
TIA-1	T-cell-restricted intracellular antigen 1
TIAR	T-cell intracellular antigen-related
TRIM	tripartite motif-containing protein
TRIM-25	tripartite motif-containing protein 25
TYF	twenty-four
UBAP2	ubiquitin-associated protein 2
US	United States
USP10	ubiquitin specific peptidase 10
USUV	Usutu virus
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
vgRNA	viral genomic RNA
vpiRNA	viral piwi-interacting RNA
VSR	viral suppressor of RNA-interference
vsRNA	viral small RNA
WEEV	western equine encephalitis virus
WHAV	Whataroa virus
WNV	West Nile virus

XRN	exoribonuclease
xrRNA	XRN-resistant RNA
YFV	yellow fever virus
YTHDF	YTH domain protein family
ZIKV	Zika virus

Table with an overview of viruses named in this thesis including names, abbreviations, taxonomy and genome organisation.

Virus name	Abbr.	Virus order	Virus family	Virus genus	Genome organisation
Barmah Forest virus	BFV	N/A	<i>Togaviridae</i>	<i>Alphavirus</i>	(+) ssRNA, non-segmented
Bluetongue virus	BTV	N/A	<i>Reoviridae</i>	Orbivirus	dsRNA, ten segments
Bovine viral diarrhoea virus	BVDV	N/A	<i>Flaviviridae</i>	<i>Pestivirus</i>	(+) ssRNA, non-segmented
Bunyamwera virus	BUNV	<i>Bunyavirales</i>	<i>Peribunyaviridae</i>	<i>Orthobunyavirus</i>	(-) ssRNA, three segments
Calbertado virus	CLBOV	N/A	<i>Flaviviridae</i>	<i>Flavivirus</i>	(+) ssRNA, non-segmented
Cell fusing agent virus	CFAV	N/A	<i>Flaviviridae</i>	<i>Flavivirus</i>	(+) ssRNA, non-segmented
Chikungunya virus	CHIKV	N/A	<i>Togaviridae</i>	<i>Alphavirus</i>	(+) ssRNA, non-segmented
Culex Y virus	CYV	N/A	<i>Bimaviridae</i>	<i>Entomobirnavirus</i>	dsRNA, two segments
Dengue virus	DENV	N/A	<i>Flaviviridae</i>	<i>Flavivirus</i>	(+) ssRNA, non-segmented
Eastern equine encephalitis virus	EEEV	N/A	<i>Togaviridae</i>	<i>Alphavirus</i>	(+) ssRNA, non-segmented
Eilat virus	EILV	N/A	<i>Togaviridae</i>	<i>Alphavirus</i>	(+) ssRNA, non-segmented
Flock House virus	FHV	N/A	<i>Nodaviridae</i>	<i>Alphanodavirus</i>	(+) ssRNA, two segments
Foot-and-mouth disease virus	FMDV	<i>Picornavirales</i>	<i>Picornaviridae</i>	<i>Aphthovirus</i>	(+) ssRNA, non-segmented
Hepatitis A virus	HAV	<i>Picornavirales</i>	<i>Picornaviridae</i>	<i>Hepatovirus</i>	(+) ssRNA, one segment
Hepatitis C virus	HCV	N/A	<i>Flaviviridae</i>	<i>Hepacivirus</i>	(+) ssRNA, non-segmented
Human immunodeficiency virus	HIV	<i>Ortervirales</i>	<i>Retroviridae</i>	<i>Lentivirus</i>	(+) ssRNA, non-segmented
Influenza virus	IV	<i>Articulavirales</i>	<i>Orthomyxoviridae</i>		(-) ssRNA, eight segments
Japanese encephalitis virus	JEV	N/A	<i>Flaviviridae</i>	<i>Flavivirus</i>	(+) ssRNA, non-segmented
Kunjin virus	KUNV	N/A	<i>Flaviviridae</i>	<i>Flavivirus</i>	(+) ssRNA, non-segmented
La Crosse virus	LACV	<i>Bunyavirales</i>	<i>Peribunyaviridae</i>	<i>Orthobunyavirus</i>	(-) ssRNA, three segments
Mayaro virus	MAYV	N/A	<i>Togaviridae</i>	<i>Alphavirus</i>	(+) ssRNA, non-segmented
Murray Valley encephalitis virus	MVEV	N/A	<i>Flaviviridae</i>	<i>Flavivirus</i>	(+) ssRNA, non-segmented

Narnavirus	NV	N/A	Narnaviridae	Narnavirus	(+) ssRNA, non-segmented
O'nyong nyong virus	ONNV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
Phasi-Charoen-like virus	PCLV	Bunyavirales	Phenuiviridae	Phasivirus	(-) ssRNA, three segments
Rift Valley fever virus	RVFV	Bunyavirales	Phenuiviridae	Phlebovirus	(-) ssRNA, three segments
Ross River virus	RRV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
Schmallenberg virus	SBV	Bunyavirales	Peribunyaviridae	Orthobunyavirus	(-) ssRNA, three segments
Semliki Forest virus	SFV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
Sindbis virus	SINV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
Tai Forest alphavirus	TALV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
Tick-borne encephalitis virus	TBEV	N/A	Flaviviridae	Flavivirus	(+) ssRNA, non-segmented
Usutu virus	USUV	N/A	Flaviviridae	Flavivirus	(+) ssRNA, non-segmented
Venezuelan equine encephalitis virus	VEEV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
West Nile virus	WNV	N/A	Flaviviridae	Flavivirus	(+) ssRNA, non-segmented
Western Equine encephalitis virus	WEEV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
Whataroa virus	WHAV	N/A	Togaviridae	Alphavirus	(+) ssRNA, non-segmented
Yellow fever virus	YFV	N/A	Flaviviridae	Flavivirus	(+) ssRNA, non-segmented
Zika virus	ZIKV	N/A	Flaviviridae	Flavivirus	(+) ssRNA, non-segmented

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About the author

Giel Petrus Maria Göertz was born on June 20th 1991 in Boxmeer, The Netherlands. He went to primary school De Canadas and afterwards attended the athenaeum at the high school Het Elzendaalcollege, both in the picturesque town of Boxmeer. In 2009 Giel went to the Wageningen University to follow the Bachelor program of Molecular Life Sciences, where he discovered his interest for the smallest forms of 'life': bacteria and viruses. He continued with the Master Molecular Life Sciences with a specialisation in Biomedical Research. Giel performed his MSc thesis in the Laboratory of Virology, where he investigated how the non-structural protein

2 of chikungunya virus inhibits the innate interferon response; work that was later published in the Journal of Virology. Subsequently, he went to Brisbane, Australia for his internship in the laboratory of Prof. Dr Alexander Khromykh. Here Giel studied how a noncoding subgenomic flavivirus RNA could interfere with the interferon response of vertebrate cells. In 2014 Giel graduated as Master of Science and immediately started his PhD studies in the Laboratory of Virology under the supervision of Dr Gorben Pijlman and Prof. Dr Monique van Oers. In his PhD he continued to unravel the mysteries surrounding the noncoding subgenomic flavivirus RNA, and discovered that it plays a key role in the transmission of flaviviruses by mosquitoes. Giel also investigated co-infection of mosquitoes with chikungunya and Zika virus, and unravelled the importance of mosquito-virus interactions for alpha- and flaviviruses. During his PhD Giel was an active member of the PE&RC PhD council where he participated in the career committee, social events committee and the research committee. He was also a PhD representative during the staff meetings at the laboratory of virology. In 2016 Giel co-initiated and co-organized the first Dutch arbovirus network meeting (DARN), which unites Dutch scientists working on arboviruses. The DARN has since evolved into an annually recurring event. Giel was also actively involved in education and guided student PGO-groups in the course Molecular Virology for 4 years and supervised the practical course of Immunotechnology for 4 years. Moreover, he was the daily supervisor of 10 MSc and 1 BSc thesis student. In 2016 Giel won the award for best main-dish at the annual virology international dinner.

List of publications

Published

1. **G.P. Göertz**, G.P. Pijlman. (2015). "Dengue non-coding RNA: TRIMmed for transmission". *Cell Host and Microbe* 18 (2): 133-134
2. **G.P. Göertz**, J.J. Fros, P. Miesen, C.B.F. Vogels, M.L. van der Bent, C. Geertsema, C.J.M. Koenraadt, R.P. van Rij, M.M. van Oers, G.P. Pijlman. (2016). "Noncoding subgenomic flavivirus RNA is processed by the mosquito RNA interference machinery and determines West Nile virus transmission by *Culex pipiens* mosquitoes." *Journal of Virology* 90 (22): 10145-10159
3. C.B.F. Vogels, J.J. Fros, **G.P. Göertz**, G.P. Pijlman, C.J.M. Koenraadt. (2016). "Vector competence of northern European *Culex pipiens* biotypes and hybrids for West Nile virus is differentially affected by temperature". *Parasites and Vectors* 9 (1): 393
4. **G.P. Göertz***, C.B.F. Vogels*, C. Geertsema, C.J.M. Koenraadt, G.P. Pijlman. (2017). "Mosquito co-infection with Zika and chikungunya virus allows simultaneous transmission without affecting vector competence of *Aedes aegypti*". *PLoS Neglected Tropical Diseases* 11 (6): e0005654
5. C.B.F. Vogels*, **G.P. Göertz***, G.P. Pijlman, C.J.M. Koenraadt. (2017). "Vector competence of European mosquitoes for West Nile virus". *Emerging Microbes and Infection* 6 (11): e96
6. C.B.F. Vogels, **G.P. Göertz**, G.P. Pijlman, C.J.M. Koenraadt. (2017). "Vector competence of northern and southern European *Culex pipiens pipiens* mosquitoes for West Nile virus across a gradient of temperatures". *Medical and Veterinary Entomology* 31 (4): 358-364
7. **G.P. Göertz**, S.R. Abbo, J.J. Fros, G.P. Pijlman. (2018). "Functional RNA during Zika virus infection". *Virus Research* 254 (1): 41-53
8. **G.P. Göertz**, K.L. McNally, S.J. Robertson, S.M. Best, G.P. Pijlman, J.J. Fros. (2018). "The MTase-like domain of chikungunya virus nsP2 inhibits the interferon response by promoting the nuclear export of STAT1". *Journal of Virology* 92 (17): e01008-18
9. **G.P. Göertz**, M. Lingemann, C. Geertsema, M.H.C. Abma-Henkens, C.B.F. Vogels, C.J.M. Koenraadt, M.M. van Oers, G.P. Pijlman. (2018). "Conserved motifs in the hypervariable domain of chikungunya virus nsP3 required for transmission by *Aedes aegypti* mosquitoes". *PLoS Neglected Tropical Diseases* 12 (11): e0006958

Submitted

10. **G.P. Göertz**, P. Miesen, G.J. Overheul, R.P. van Rij, M.M. van Oers, G.P. Pijlman. (2019). "Mosquito small RNA responses to West Nile and insect-specific virus infections in *Aedes* and *Culex* mosquito cells. *Submitted*.
11. **G.P. Göertz**, J. van Bree, A. Hiralal, B.M. Fernhout, C. Steffens, S. Boeren, T.M. Visser, C.B.F. Vogels, C.J.M. Koenraadt, M.M. van Oers, G.P. Pijlman. (2019). "Subgenomic flavivirus RNA binds the antiviral mosquito DEAD/H-box helicase ME31B and determines Zika virus transmission by *Aedes aegypti*. *Submitted*.

* These authors contributed equally.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Vector competence of European mosquitoes for West Nile virus (2014-2017)

Writing of project proposal (4.5 ECTS)

- The role of non-coding flaviviral RNA in virus replication and dissemination in mosquitoes (2015)

Post-graduate courses (3.8 ECTS)

- Laboratory training in small RNA library preparation; RIMLS, Nijmegen (2015)
- Next generation sequencing data analysis; LUMC, UMCG (2015)
- EBSA BSL3 Course: a look behind the walls; AGES, Vienna (2015)
- Comparisons and contrasts between animal and plant vector-borne viruses; ASV, Madison (2017)

Laboratory training and working visits (0.9 ECTS)

- Training in preparation of MS/MS samples; Biochemie, WUR (2017)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Journal of Virology: arbovirus vector competence (2014)
- PLoS Pathogens: *Wolbachia* and dengue replication in mosquitoes (2016)

Deficiency, refresh, brush-up courses (3.9 ECTS)

- Bedrijfsbushulpverlening / emergency services (2014-2018)
- Basic statistics (2015) (2014-2017)

Competence strengthening / skills courses (4 ECTS)

- PhD Carousel: being a vital PhD, speedreading, communication in science, essentials in time-management; WGS (2015)
- Project & time-management; PE&RC (2016)
- Teaching and supervising thesis students; WGS (2016)
- Career perspectives; WGS (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (3 ECTS)

- First years PhD weekend (2014)
- PE&RC Day (2014-2017)
- Mid-term PhD weekend (2017)

Discussion groups / local seminars / other scientific meetings (4.8 ECTS)

- Dutch symposium on emerging Bunyaviruses (2014)
- Rob Goldbach virology lecture (2014, 2016, 2018)
- WEES Seminars (2014-2018)
- Dutch annual virology symposium (2015-2018)

- Dutch annual ecology meeting (2016)
- Dutch arboviral network meeting (2016-2018)
- PE&RC career meetings (2017)
- KNAW masterclass one-health approach to infectious diseases (2017)

International symposia, workshops and conferences (12.3 ECTS)

- 1st International Meeting on Arboviruses and their Vectors (2015)
- Positive-Strand RNA Viruses Meeting (2016)
- Zika Virus Summit (2016)
- International Conference of Entomology (2016)
- 36th American Society for Virology meeting (2017)
- 2nd International Meeting on Arboviruses and their Vectors (2017)

Lecturing / Supervision of practicals / tutorials (10.8 ECTS)

- PGO Molecular virology (2014-2017)
- Immunotechnology practical course (2015-2018)

Supervision of MSc students (5 ECTS)

- Stef van der Krieken: influence of West Nile virus GR10 sfRNA on stress granule formation
- Marit Lingemann: chikungunya virus and stress granule inhibition
- Mark Corten: silencing of RNAi related genes in *Culex* cells
- Winnie van den Boogaard: importance of conserved domains in chikungunya nsP3 for virus replication
- Julian Bakker: alphavirus nsP3 interactions with human G3BP and mosquito Rasputin
- Bas Fernhout: determination of sfRNA binding partners in mosquito cells
- Anwar Hiralal: determination of sfRNA binding partners in mosquito cells
- Gwen Nowee: interactions between alphavirus nsP3s and host proteins
- Joyce van Bree: identification of host proteins interacting with ZIKV and WNV sfRNA
- Carmen Steffens: interaction of subgenomic flavivirus RNA with mosquito immune pathways

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