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Biochemistry and Molecular Biology of Flaviviruses

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Abstract

Flaviviruses, such as dengue, Japanese encephalitis, tick-borne encephalitis, West Nile, yellow fever, and Zika viruses, are critically important human pathogens that sicken a staggeringly high number of humans every year. Most of these pathogens are transmitted by mosquitos, and not surprisingly, as the earth warms and human populations grow and move, their geographic reach is increasing. Flaviviruses are simple RNA–protein machines that carry out protein synthesis, genome replication, and virion packaging in close association with cellular lipid membranes. In this review, we examine the molecular biology of flaviviruses touching on the structure and function of viral components and how these interact with host factors. The latter are functionally divided into pro-viral and antiviral factors, both of which, not surprisingly, include many RNA binding proteins. In the interface between the virus and the hosts we highlight the role of a noncoding RNA produced by flaviviruses to impair antiviral host immune responses. Throughout the review, we highlight areas of intense investigation, or a need for it, and potential targets and tools to consider in the important battle against pathogenic flaviviruses.

Graphical Abstract

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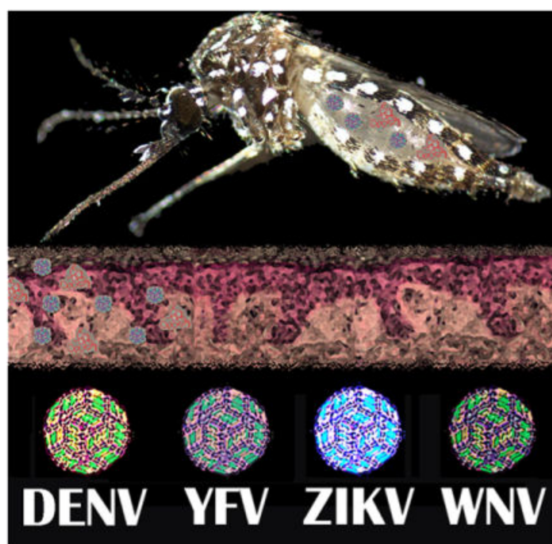

1. INTRODUCTION

The *Flavivirus* genus includes over 50 arthropod-borne viruses (arboviruses).[1] Yellow fever virus (YFV), after which the genus is named, was the first virus demonstrated to cause an arthropod-borne illness.[2] Additional significant human pathogens, including the four dengue viruses (DENV), Japanese encephalitis (JEV), West Nile (WNV), Zika (ZIKV), and tick-borne encephalitis (TBEV) viruses, belong to the genus.[1,3]

Universal countermeasures to combat the spread of flaviviruses are limited to preventing contact between the arthropod vectors and humans using public health approaches. YFV can be effectively combatted with the use of a highly effective vaccine, although this strategy is limited by expense and the willingness of nations to embark on widespread vaccination campaigns. There is one DENV vaccine approved for specific populations in several countries, but concerns about untoward effects have been raised recently.[4,5] Other promising DENV vaccine candidates are in clinical trials,[6,7] and the search for an effective DENV vaccine suggests much more intense study. There are approved JEV vaccines, which are safe and relatively effective.[8] There are no approved vaccines to prevent WNV or ZIKV in humans. No antiviral therapeutics are approved to treat infected individuals nor are any antiviral treatments available that could prevent infection of at-risk populations. There exists significant need to reduce the impact of flaviviruses on the human population; however, this altruistic goal will require a much better understanding of flavivirus biology.

In this review, we focus on the biochemistry and molecular biology of mosquito-transmitted flaviviruses, particularly three (DENV, YFV, and ZIKV) transmitted by *Aedes* mosquitos because of their epidemic potential and toll on human health. Among these we will primarily highlight the four DENV because in terms of public health impact these are the most important mosquito-transmitted viruses. In several sections of the review we focus with an RNA centric lens and highlight the importance of RNA–RNA and RNA–protein interactions in the life cycle of these pathogens. Whenever possible we indicate important areas where

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our understanding of this biology is incomplete and point out exciting new avenues of investigation. We also refer the readers to previous reviews[9–13] and the book entitled *Dengue and Dengue Hemmorhagic Fever* edited by Gubler, Ooi, Vasudevan, and Farrar.[14]

It is our expectation that the study of flavivirus biology will lead to the discovery of fundamental processes operative in human cells. Indeed, there are historical precedents where virology has revealed new molecular biology of the cell (e.g., the discovery of introns in precursors to mRNAs[15,16]). Additionally, we posit that a detailed understanding of these viruses will reveal new targets for prevention and therapy.

1.1. Dengue Viruses

DENV are four closely related but antigenically distinct viral serotypes (DENV1, DENV2, DENV3, and DENV4) that cause very similar disease spectrum in humans.[14] DENV re-emerged in the late 20th and early 21st centuries due to increased geographic distribution of the mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, and the lack of effective vector control methods, vaccines or antiviral treatments.[17,18] Today, DENV circulates in tropical and subtropical regions throughout the world, causing ~100 million cases of dengue fever (DF) and, less commonly, severe dengue.[19]

Dengue is most frequently a subclinical infection, but DF can present as a self-limited illness with a high fever, severe aches, and other flu-like symptoms.[12] Rarely progression to severe dengue can result in plasma leakage that leads to respiratory distress, shock, severe bleeding, and death.[12]

1.2. Yellow Fever Virus

Although many infections are subclinical, yellow fever may progress to a severe, lethal disease. Symptomatic yellow fever begins with an acute illness, termed the “period of infection”, with symptoms including fever, chills, headache, and other flu-like symptoms.[20] This symptomatic period is then followed by the “period of remission” where symptoms lessen.[20] Most symptomatic individuals will clear the virus and recover at this point; however, one-quarter of affected persons progress to the “period of intoxication” marked by symptoms consistent with a hemorrhagic fever with the addition of jaundice, a defining clinical feature.[20] Up to one-half of the patients who reach the “period of intoxication” die from yellow fever, but those who survive fully recover.[20] An excellent vaccine (YFV-17D) exists that prevents yellow fever;[21] however, no treatment is available that may reduce the severity of, or improve the outcome of, yellow fever.

Yellow fever is a historically important human disease that was often experienced as epidemics with severe consequences for local populations. Today, YFV is endemic to regions of Africa and South America,[22] where YFV transmission between primate populations and mosquito vectors in sylvatic cycles serves as a natural reservoir.[23] Fortunately, YFV re-emergence is restrained by local vaccination campaigns, virus surveillance, and internationally coordinated outbreak responses.[24] Recent outbreaks highlight that we are far from controlling yellow fever. An outbreak in Angola and the Democratic Republic of the Congo in 2016 caused 7627 confirmed and reported cases with a case fatality rate of ~14%[25] and stressed the worldwide vaccine supply. More recently, an

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outbreak in Brazil reached worrisomely close to cities with large populations and low vaccination rates such as Sao Paolo.[26]

1.3. Zika Virus

ZIKV was isolated in 1947 in the Ziika Forest in Uganda[27] and remained a footnote among neglected tropical diseases until November 2015 when accumulating cases of microcephaly in Brazil were associated with a ZIKV epidemic several months prior.[28–30] The best evidence suggests that there were multiple introductions of ZIKV into the United States.[31] For general reviews on ZIKV and ZIKV infection (Zika), see Weaver et al. (2016) and Aliota et al. (2017).[32,33] ZIKV genetic material has been amplified from mothers diagnosed with Zika and carrying affected fetuses,[34] from the amniotic fluid bathing an affected fetus,[35] and from neural tissues in fetuses diagnosed with microcephaly.[35,36] Multiple in vivo and in vitro infection models demonstrated that ZIKV infection can disrupt neuro-development.[37–41] Finally, a prospective case-control study found an increased risk for microcephaly associated with congenital ZIKV infection.[42] A recent report suggests that a single amino acid change (S139N) in the ZIKV structural protein, prM, of American strains increases infectivity in human and mouse neural progenitor cells and pathogenicity in mouse models,[43] but the relevance of this variant to the human microcephaly observed in the Americas remains to be determined. ZIKV is the first of the widely distributed arboviruses to be considered as a significant risk factor for human congenital malformations (however, see section 1.4 on JEV).

Although most infections are likely mosquito transmitted, ZIKV can spread directly from person to person through sexual contact or vertically from mother to fetus.[44] This sets ZIKV apart from other pathogenic flaviviruses and creates significant and unexpected public health concerns. Zika is usually asymptomatic, and most symptomatic infections are mild and resemble those observed with dengue: rash, fever, arthralgia, conjunctivitis, myalgia, headache, and retro-orbital pain.[44] There have been reports of hematospermia and symptoms resembling prostatitis following infection,[45] and viral RNA and infectious virus have been detected in the semen of men weeks after clearing of acute symptoms.[46] Most symptomatic ZIKV infections are self-limited and resolve in less than a week; however, there are documented cases of severe Zika in patients with other underlying conditions.[47] Moreover, Zika has been strongly associated with neurological sequelae, most commonly Guillain–Barré Syndrome, but also meningo–encephalitis and myelitis.[44,48]

1.4. Japanese Encephalitis Virus

Among flaviviruses, the antigenically related Japanese Encephalitis Virus (JEV) serogroup includes several neuroinvasive human pathogenic viruses.[49] The namesake, JEV, causes serious illness, and many cases are fatal or result in persistent neurological damage[50] Recently, with the public attention on ZIKV as a teratogenic virus, a renewed interest has developed in observations that JEV can cross the placental barrier with severe consequences for the developing fetus.[51] JEV is maintained in the environment in an avian–mosquito cycle, but zoonosis may be assisted by an intermediate mosquito–pig cycle that brings the virus into closer contact with humans, which are dead-end hosts.[50] JEV is endemic

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throughout India, Southeast Asia, Indonesia, and parts of Australia.[50] Fortunately, there is an effective vaccine.[8,52]

1.5. West Nile Virus

West Nile Virus (WNV), which belongs to the JEV serotype, is broadly distributed throughout the world and maintained in the environment by an avian–mosquito life cycle. Incidental infections may cause disease in humans or agriculturally important animal species including horses.[53] One-quarter of human infections develop nonspecific symptoms of a viral infection.[54] Less than 1% of infections result in West Nile neuroinvasive disease, which may manifest as meningitis, encephalitis, and acute flaccid paralysis.[54] Although full recovery from uncomplicated West Nile fever can be expected, infection is associated with prolonged fatigue. WNV neuro-invasive disease may be lethal or recovery may require specialized care with symptoms persisting beyond a year from infection.[54] Anti-WNV therapeutics or vaccines are in various stages of development.[55]

1.6. Tick-Borne Flaviviruses

Ticks serve as vectors for a variety of flaviviruses. Tick-borne flaviviruses (TBFs) are important agricultural and human pathogens, although humans are dead-end hosts.[56] Tick-borne encephalitis virus (TBEV) is the most frequently encountered human pathogen among TBFs, and viral subtypes vary regionally with a geographic distribution that spans from Europe to Asia.[56] Powassan virus is the only recognized human pathogenic TBF in North America.[57]

TBEV isolates exhibit degrees and types of pathogenicity, for instance, TBEV and Omsk hemorrhagic fever virus (OHFV) share 90% identity at the amino acid level, yet TBEV and OHFV cause encephalitis and hemorrhagic disease, respectively.[58] In vivo evidence relying on viral genetic chimeras located critical determinates of encephalitis caused by TBEV to residues within the open reading frame of the RNA-dependent RNA polymerase (RdRp)/ methyltransferase (NS5 see section 2.5).[58] Comparative studies using chimeric viruses built from the genomes of highly pathogenic and low pathogenic isolates located virulence factors in the variable region of the 3′ UTR (see discussion in section 5).[59,60] Recently, TBEV was reported to preferentially replicate in neuronal dendrites,[61] and cis-acting RNA sequences within the TBEV 5′ UTR are required to direct the localization of TBEV RNAs to dendrites.[62] Studies comparing TBEV isolates should contribute to our understanding of the diverse pathologies associated with flaviviruses.

2. BIOCHEMISTRY AND MOLECULAR BIOLOGY OF FLAVIVIRUSES

2.1. Virion

2.1.1. Viral Surface—Flaviviruses share a common virion structure,[13] and among these the structures of DENV and ZIKV are particularly well characterized. The mature DENV virion is a roughly spherical enveloped 50 nm diameter particle.[63] The outermost layer of the virion is a glycoprotein coat made of repeating units of 180 copies of envelope (E) protein in combination with the viral membrane (M) protein.[63] The surface reveals icosahedral symmetry and consists of 30 “rafts” each consisting of three E dimers.[63–66]

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Elegant early work demonstrated that the E protein of flaviviruses is composed of the stem-membrane domain and three other domains, namely, DI, DII, and DIII, where DI bridges DII and DIII.[67] The fusion loop located at the tip of DII is important for interaction and fusion with the host membrane and is exposed after removal of the pr peptide from the immature form of M (prM). DIII on the other hand is thought to bind to the host receptors.[68–70]

Although the viral envelope is often portrayed as a static structure, the E protein structures exist as a dynamic and heterogeneous population lending to an atomic model of the virion that breathes over time (reviewed by ref 71). Viral breathing[72] may be impacted by environmental[73–75] or viral genetic factors.[66,76,77] Viral breathing may manifest as delayed viral neutralization by specific antibodies[78] and may have a significant role during viral–receptor interactions.[79]

ZIKV shares many structural similarities with other members of its genus, such as the size of the immature and mature particles (namely, 60 and 50 nm in diameter), and the overall architecture of the surface of the virion. Nonetheless, there have been attempts to elucidate structural differences to ZIKV that could explain transmission patterns and tissue tropism not commonly observed with other human flaviviruses.

Two groups have elucidated the structure of mature ZIKV at atomic or near atomic resolution.[80,81] Both groups showed interesting features of the E protein in ZIKV relative to previous structures of DENV, for example, the glycosylation pattern of the envelope protein. Indeed, ZIKV E glycosylation proves more similar to the neurovirulent WNV.[80,81] ZIKV has one glycosylation site at Asn154, whereas DENV has two, at Asn67 and Asn154. These sites have been shown to be important for binding specific host membrane receptors, and differences in the glycosylation pattern of the E protein of various flaviviruses could explain differences in their cellular tropism. ZIKV E protein glycosylation is dispensable for viral propagation in cell culture; however, this post-translational modification is necessary for robust ZIKV viral replication in mouse models and in mosquitos.[82,83] The group of S. Lok noted an insertion of an alanine residue at position 340 in ZIKV DIII that leads to differential positioning of DII and DIII and permits a hydrogen-bond network between five adjacent E proteins not seen in DENV2.[81] The authors conclude that this leads to a more compact structure for ZIKV, which could play a role in ZIKV tropism, transmission, and pathogenesis.

Much emphasis has been put on the thermostability of ZIKV compared to DENV; however, it is not clear how thermostability explains the ability of ZIKV to infect the human genitourinary tract and persist in various bodily secretions such as cervical mucus, sperm, and urine. In fact, viral genetic studies are not fully consistent with the conclusions about thermostability.[84,85] We posit that chemical stability may be an equally if not a more important consideration. With the pH ranging from ~4.4 in the vagina to ~7.9 in the Fallopian tube,[86] one can hypothesize that the unique structural features of ZIKV make it more fit to thrive in various pH conditions of the female reproductive tract.

2.1.2. Interior of the Virion—Contained within the viral envelope is the viral genome complexed with capsid (C) protein, which together form the viral ribonucleoprotein (RNP).

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The composition of the viral interior is presumed to be one viral genome and several hundred copies of C. C is a basic 12 kDa dimeric protein with asymmetric charge distribution, suggesting that one side of the protein, which has a high number of positively charged residues, interacts with viral RNA and the other, which is nonpolar, with the viral membranes and perhaps E protein.[87] The C protein of Kunjin virus (a strain of WNV) forms tetramers on crystallization, creating a positively charged channel with a diameter of 11 Å.[88] Since this channel cannot accommodate double-stranded RNA we posit that C preferentially binds to single-stranded regions of the viral genome. Therefore, we proposed that the virion RNP consists of highly structured regions, primarily but not exclusively the untranslated regions (UTRs), devoid of C protein, and unstructured or poorly structured regions that form a complex with C.[89]

2.1.3. Viral Genome: Primary, Secondary, and Tertiary Structure—The flaviviral genome is a positive-strand RNA that contains a type I cap at its 5′ end and characteristically lacks a poly-A tail at its 3′end.[13] The structure and function of the 5′ cap will be described in more detail in the section on RNA modifications (section 2.1.4). The viral genome encodes a single polyprotein that is co- and post-translationally cleaved by viral and host proteases into 10 mature viral proteins (C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5)[90] (section 2.4). Surrounding the protein-coding region of the viral genome are two highly structured UTR's of ~100nt at the 5′ end of the genome and ~400–700nt at the 3′ end.[90]

The 5′ and 3′ terminal regions of the flavivirus genome consist of multiple RNA sequences and structures that are essential for replication and translation of the viral genome (see review by Gebhard et al.[91]). The 5′ terminal region of the genome can be broken into two domains. The first of these domains is all within the 5′UTR and contains the branched stem-loop A (SLA) structure, a feature conserved throughout the flavivirus genus (Figure 1).[92] The SLA serves as a promoter for viral replication, likely through direct recruitment of the RdRp (NS5).[93–95] Downstream of the SLA is a uridine-rich region that acts as a spacer between the two 5′ terminal domains and enhances viral replication.[94] The second domain of the 5′ terminal region extends from the 5′UTR into the C ORF. This domain contains the 5′ upstream of AUG region (5′UAR) that folds into a second stem-loop structure (SLB), the downstream of AUG region (DAR), the C coding region hairpin (cHP), the 5′ cyclization sequence (5′CS), and the downstream of 5′CS pseudoknot (DCS-PK).[96–100] All of these elements have been shown to play roles in viral RNA replication, some through direct interactions with corresponding elements in the 3′UTR (5′UAR, DAR and 5′CS) (Figure 1).[91] The cHP has also been shown to play an important role in start codon recognition.[99] Recently, Liu et al. (2016) identified a conserved RNA switch, the 5′-UAR-flanking stem (UFS), which extends the SLB structure and enhances recruitment of NS5 and RNA synthesis.[101]

The 3′ terminal region of the flaviviral genome can be broken into three distinct domains, all of which reside within the 3′UTR.[97,102] Domain 1, previously known as the variable region, is the least conserved in the 3′UTR and is most notable for the presence of two stem-loop structures (SLI and SLII) that form pseudoknots (PK1 and PK2) (Figure 1).[91,103,104] Because of their resistance to the 5′ to 3′ exonuclease, Xrn1, these two structures are

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known as exonuclease-resistant (xr)RNA1 and xrRNA2 (Figure 1). These two structures are important for the formation of two forms of the subgenomic flaviviral RNA (sfRNA). These sfRNAs (sfRNA1 and sfRNA2) play critically important roles in the suppression of host innate immunity and adaptation to different hosts (see section 5 and published reviews[91,103,104]). The xrRNA structures from Murray Valley encephalitis virus and ZIKV have been solved at atomic resolution, and provide an elegant explanation for the exonuclease resistance of these RNAs.[105,106]

Domain 2 of the 3′ terminal region contains either one (e.g., ZIKV and YFV) or two (e.g., DENV and JEV) conserved dumbbell structures, DB1 and DB2 (Figure 1), which harbor sequences that are essential for both replication and translation.[107–109] These dumbbell structures are predicted to form pseudoknot structures (PK3 and PK4) that are essential for their role in translation and may also function as additional Xrn1-stalling sites in a species-specific manner (see section 5).[104,107,110–113]

Domain 3 of the flavivirus genome is highly conserved in all members of the flavivirus genus and contains the 3′DAR, 3′CS, and 3′UAR sequences that interact with the corresponding regions located at the 5′ end of the genome and are required for circularization and replication of the viral genome (Figure 1). Finally, this domain contains the 3′ stem-loop structure (3′SL), which is flanked on its 5′ end by the short hairpin (sHP)[97,90,114] and is required for RNA synthesis.[115,116]

Circularization of the flavivirus genome is mandatory for replication and is mediated by direct interaction of the RNA sequences and structures present in the 5′ and 3′ terminal regions[102,117,118] (Figure 1). The presence of inverted complementary sequences in the C gene (5′CS) and the 3′UTR (3′CS) was first proposed by Hahn et al. and has since been shown to be indispensable for mediating circularization of the genome.[97,102] In addition to the 5′-3′CS interaction, direct interactions between the 5′UAR and the 3′UAR and between the 5′DAR and the 3′DAR are also required.[102] These interactions have been characterized by extensive mutagenesis and have been directly visualized using atomic force microscopy.[96] Gamarnik and colleagues provided compelling data that the equilibrium between linear and circular forms of the genome are essential for efficient flaviviral replication.[91,119]

Identification and characterization of the aforementioned sequences in the 5′ and 3′terminal regions of the flavivirus genome have been enabled by the implementation of phylogenetic and computational prediction analyses followed by mutagenesis in reverse genetics systems to assess the effect on viral growth kinetics. Secondary structures predicted in silico have been verified by chemical and enzymatic structure probing methodologies (reviewed in refs 91 and 103). To date, these studies have primarily been directed at the 5′ and 3′ terminal regions that together account for less than 10% of the full viral genome. One notable exception is a study by Proutski et al., where the authors utilized a computational approach to identify local RNA secondary structure across the viral genome and identified a multitude of potential secondary structures throughout the previously unexplored open reading frame.[120] The presence of structures in this region is suggestive of additional functional elements that may play a role in the viral life cycle outside of their protein coding function.

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Recently, the application of selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) chemistry and next-generation sequencing to predict local secondary structures has enabled full genomic structure mapping of viruses (e.g, human immunodeficiency virus (HIV) and hepatitis C virus (HCV)).[121–124] Although these studies relied on purified, refolded RNA to characterize structures in these viruses, the rapid advancement in this technology now allows for full genome secondary structure prediction in vivo.[125,126] Full genomic analysis of structures in the flavivirus genome native state within the virion and in an active infection may allow us to unravel the critical elements responsible for toggling between translation, replication, and packaging. A deeper understanding of the molecular switch between these critical processes may provide insight into potential constraints placed on the viral sequences and provide new opportunities to block these fundamental steps in the viral life cycle.

2.1.4. Viral Genome: RNA Modifications—RNA modifications are becoming increasingly recognized as important regulators of cellular gene expression.[127] In flaviviruses, RNA methylation by viral enzymes has documented roles in infection.[128,129] Recent studies also demonstrate that flavivirus genomes are methylated by host methyltransferases (MTase) with important consequences for infection.

2.1.4.1. Methylation of the Flavivirus Genome 5′ End Is Critical for Virus Infection: RNA polymerase II transcripts are modified during synthesis with a cap structure (m^7GpppN) added to the initial base of the RNA through a 5′-5′ linkage (reviewed in ref 130). Multiple enzymes participate in these reactions: (i) RNA triphosphatase, (ii) guanylyltransferase, and (iii) N^7-guanine MTase. The 5′ γ-phosphate is removed by the RNA triphosphatase to make it a substrate for capping by a guanylyltransferase. Subsequent guanosine cap methylation at N^7 produces a "type 0" cap structure.[131,132] The mRNA is additionally acted upon by a ribose MTase which adds a 2′-O-methyl group to the penultimate nucleotide, yielding a type 1 cap.[133] The cap structure is a critical effector of mRNA metabolism that is important for several processes, whereas 2′-O-methylation serves to differentiate mRNA from foreign molecules.[134]

Flaviviruses possess all of the required enzymes needed to cap newly synthesized genomes in the cytoplasm. The NS5 protein harbors RNA-polymerase activity as well as putative guanylyltransferase and MTase activities.[135–138] NS3 is a second critical virus enzyme that, in addition to protease and helicase activities, contributes RNA triphosphatase activity required for capping.[139,140] The pathway of cap formation is similar between cellular and viral RNAs. The 5′ γ-phosphate is dephosphorylated by NS3, and NS5 appends a Gp cap. NS5 subsequently executes two methylations to initially yield an $\underline{m}^7$GpppA type 0 cap followed by a mature m^7GpppA$\underline{m}$ type 1 cap.[136] The flavivirus MTase is unique compared to cellular MTase because it preferentially methylates the cap of only viral RNA.[141] Elegant biochemical assays have determined that m^7GpppA-RNA is preferred as a 2′-O-methylation substrate by NS5 compared to GpppA-RNA. This preference dictates the sequence of methylation reactions performed by NS5.[142]

Mutational analysis of NS5 has revealed amino acid residues that are important for N^7-guanine methylation, 2′-O-methylation, or general methyltransferase activity. The 2′-O-

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methylation activity can be disrupted without affecting viral viability. In contrast, N^7-guanine methylation is absolutely required for virus infection.[137,143] There are several possible explanations for why m^7G-cap methylation is required by flaviviruses. First, it is likely that the cap stimulates viral translation initiation, although, as will be discussed below when describing translation of flaviviruses (section 2.3.1), initiation is likely to be mediated by noncanonical mechanisms. Second, the cap structure protects RNA from 5′ to 3′ exonucleases like Xrn1[144] and likely reduces the extent of viral RNA decay. Protection from exonuclease activity, however, is not absolute as a significant fraction of viral genomes is degraded to form sfRNA (see sections 2.1.3 and 5). Finally, N^7-guanine methylation could prevent detection of flaviviral genomes by hypothetical cellular factors that recognize unmethylated caps as foreign RNA.

Why is 2′-O-methylation important for flavivirus infections? Diamond, Shi, and colleagues found that a WNV 2′-O-methylation-deficient mutant virus (E218A) is attenuated in primary cells and immunocompetent mice while type I interferon (IFN) receptor-deficient mice and cells are completely susceptible to infection by the mutant virus.[145] These authors further demonstrated that murine IFIT1 and IFIT2 proteins more effectively restrict mutant WNV without 2′-O-methylation in comparison to WT virus. Several reports have characterized human IFIT1 as a factor that recognizes the type 0 cap structure and inhibits translation, likely through blocking access of initiation factors.[146,147] The resulting slowdown of viral protein synthesis has been linked to a heightened innate immune response that ultimately prevents productive infection.[148,149] Taken together, these reports indicate that cap 2′-O-methylation allows cells to detect and restrict nonself RNAs using IFIT proteins.

2.1.4.2. Internal Adenosine Methylation in Flavivirus Infection: A widespread modification to cellular mRNA is methylation of the N^6 position of adenosine, referred to as (m^6A).[150–152] This modification is found at internal mRNA nucleotides as well as the penultimate nucleotide of RNA pol II RNAs that begin with A, resulting in m^6Am due to 2′-hydroxyl methylation.[131] Methylase and demethylase enzymes capable of adding or removing m^6A have been identified and are known as "writers" and "erasers", respectively. Moreover, several "reader" effector proteins that bind m^6A in RNA have been characterized.[152,153] A recent technical innovation which has enabled identification of m^6A sites involves use of m^6A-specific antibodies to immunoprecipitate modified RNA molecules. This facilitates identification of m^6A locations across the transcriptome.[154–156]

To date there have been two studies addressing m^6A in flaviviruses,[157,158] including YFV, DENV, WNV, and ZIKV. Locations of m^6A in the genomes of WNV, YFV, DENV, and two ZIKV strains were mapped by Gokhale et al. This analysis revealed multiple regions within flaviviral genomes that are methylated, including significant m^6A within the coding region of NS5.[157] Lichinchi and colleagues also localized m^6A in ZIKV RNA and probed functional roles for erasers, writers, and readers in infection.[158] RNAi-mediated knockdown of METTL3 and METTL14 methyltransferases elevated ZIKV infection of 293T cells, while ALKBH5 demethylase knockdown inhibited infection. In addition, overexpression of YTDHF1/2 reduced levels of ZIKV RNA secreted from infected cells, suggesting that these proteins act as restriction factors, possibly by inducing decay of viral RNA. Lichinchi et al.

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also observed that ZIKV infection changes patterns of m^{6}A in host mRNA, implying that ZIKV may alter cellular gene expression through regulating m^{6}A.[158] These initial studies have just scratched the surface, and many open questions remain concerning roles for m^{6}A in flavivirus infection.

In addition to providing interesting new insight into virus biology, studies detailing the molecular mechanisms of viral RNA methylation may facilitate approaches to treat and/or prevent infections in humans. Therapeutics designed to target flavivirus MTase enzymes may be potent drugs used to treat symptomatic infections. In addition, recombinant flaviviruses without 2′-O-MTase enzymatic activity are attenuated and protect against infection with virulent viruses, making them potentially attractive candidate vaccine strains.[159,160]

2.2. Viral Attachment and Entry into the Cell

In this section, we briefly cover steps in the viral life cycle from attachment to release of the viral genome to the cytoplasm of the infected cell. We refer the reader to other reviews of these steps.[161,162]

2.2.1. Attachment—The viral envelope recognizes one or more cell surface factors to initiate viral entry. The complement of insect or mammalian host factors that engage the viral envelope during entry as well as the conformational changes induced in the viral E protein during entry are of great interest as potential antiviral targets for vaccine and drug development. Heparin sulfates were proposed as a viral attachment factor for DENV,[163] and DC-SIGN was the first identified receptor for DENV entry in mammalian cells,[164] while heat shock family proteins were identified as part of the receptor complex in mosquito cells.[165,166] Subsequent research has shown that the four DENV serotypes can enter the cell using many different receptors in mammalian and mosquito systems (reviewed in ref 167). Similar conclusions have been reached for the attachment of other mosquito-borne flaviviruses to mammalian and insect cells.[168–175]

The Amara lab selected a 293T cell line that was poorly permissive for DENV and designed a cDNA screen that identified genes which enhanced DENV infection when overexpressed.[79] The screen identified a suspected viral receptor, L-SIGN, and identified novel flaviviral receptors in the TIM and TAM family of phosphatidylserine receptors.[79] Overexpression of TIM1 or TIM4 enhances DENV2 absorption, infection, and virus production by more than 100-fold relative to the parental cell line.[79] Overexpression of TIM1 carrying mutations in residues important for binding phosphatidylserine, however, does not enhance infection.[79] Since in mature DENV2 virions E protein obscures phosphatidylserine on the envelope and is therefore predicted to block binding to TIM1,[63] one could postulate that conformational changes in E protein induced by endosome acidification could make phosphatidylserine available for TIM and/or TAM binding.

ZIKV has presented a challenging case study for understanding viral entry in the many cell types infected by this virus and represents an area of intense investigation. ZIKV may infect a subset of monocytes in most individuals;[176,177] however, it also infects cells in placental tissues,[34,38,178] eventually leading to severe neural infection in the developing fetus.

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[36,40,41,179] Using mammalian in vitro models, AXL protein was proposed to be an important ZIKV receptor;[178,180,181] however, in other systems ZIKV readily infects AXL$^{-/-}$ neural progenitor cells in vitro[182] and causes pathogenesis in a mouse model lacking AXL,[183] demonstrating that AXL cannot be the sole ZIKV receptor. The heterogeneity of receptor usage by ZIKV is consistent with the observations regarding DENV receptors.

2.2.2. Antibody-Dependent Enhancement—Severe dengue disease has been associated with the presence of non-neutralizing anti-DENV antibodies produced after infection with a heterologous DENV serotype. This phenomenon, termed antibody-dependent enhancement (ADE),[184] provides an alternative DENV entry mechanism using antibody–Fc receptor interactions (reviewed by ref 185). This phenomenon may not be restricted to DENV serotypes, and enhancement may be mediated by antibodies to heterologous flaviviruses (see refs 186–190 and references therein).

On the basis of these observations, an interesting hypothesis emerged that may explain how ZIKV crosses the placental barrier leading to severe fetal infection. In Brazil, pregnant women with pre-existing immunity to DENV were likely infected with ZIKV. The heterologous anti-DENV antibodies could enhance ZIKV infection in a subset of the mother's Fc receptor expressing cells, which then embed into the placenta and expose the fetus to ZIKV. It was relatively straightforward to provide in vitro evidence to support the hypothesis,[191–198] and a mouse model is supportive;[199] however, in vivo evidence in primates and humans does not corroborate these findings, leaving the issue unresolved.[200–202] Recent work from the Ooi and Harris groups may provide an explanation for the difficulty in confirming ADE in human cases since it appears that the phenomenon may be only operative in a very narrow range of antibody titers.[189,190]

2.2.3. Entry—Flavivirus adsorption to the cell surface occurs via interaction with attachment factors. Subsequently, receptor-mediated endocytosis is the primary route by which flaviviruses are internalized.[203–206] WNV and DENV2 virions have been observed by electron microscopy to be attached to electron-dense invaginations on the cell surface consistent with clathrin-coated pits in both mammalian[203] and mosquito cell lines.[205] Clathrin-dependent entry by WNV and DENV2 was blocked by overexpression of dominant negative EPS15, a factor required for receptor-mediated endocytosis,[207] in mammalian cells[203] and mosquito cells.[205] Knockdown of endogenous EPS15 also inhibited infection.[208] Finally, live-cell virus imaging using fluorescently labeled DENV2 and yellow fluorescent protein-labeled clathrin showed that the virions associated with and entered by clathrin-coated endocytosis.[204]

Flaviviruses within endosomes depend on the cellular vacuolar ATPase (vATPase) in order to deposit their genomes into the cytoplasm and productively infect a cell. The eukaryotic vATPases are multisubunit protein complexes that pump protons into the endosome, resulting in reduced endosomal pH.[209] Using in vitro models of flavivirus infection, it was observed that bafilomycin, a vATPase chemical inhibitor, blocked infection of Japanese encephalitis virus (JEV) and WNV in mammalian and insect cell lines.[203,210] The vATPase cannot assemble unless all subunits are present,[209] and the observation that RNAi-mediated depletion of several individual subunits of the vATPase reduced WNV, DENV, and YFV

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infections support the requirement for the vATPase complex during flavivirus infection.[208,211–213] These compelling in vitro data were corroborated by in vivo experiments showing that DENV2 propagation was inhibited when vATPase activity was inhibited in mosquitos[214] and in mammalian in vivo models.[215,216] The shared requirement for the vATPase for diverse flaviviruses has sparked great interest in this enzyme as a target for broadly acting antiflavivirals. Unfortunately, chloroquine, an inhibitor of endosome acidification that is approved for clinical use, was ineffective as an anti-DENV treatment in clinical trials.[217]

Endosome acidification causes the pH-dependent fusion of the viral membrane with the endosomal membrane. The model for fusion of the viral and cellular membranes is based upon elegant studies that illuminated the structure of the DENV2 E protein in the mature virion[63,65,218] and the postfusion E protein[219] and has been reviewed in ref 220. In response to acidification of the endosome, structural rearrangements in the E protein bring the viral and endosomal membranes into sufficiently close apposition such that spontaneous fusion with the cellular endosomal membrane occurs with the subsequent release of the viral RNP into the cytoplasm.[220]

2.2.4. Uncoating of the Viral RNP into the Cytoplasm—Although the disassembly of the viral RNP is far from understood, we proposed a model where elongating ribosomes drive the release C from the genomic RNA.[89] Mechanisms of uncoating requiring degradation of C are not consistent with the fact that initial viral DENV translation does not require C degradation.[221] In this context, it is important to note that most flaviviruses have a very low specific infectivity (infectious unit to genome unit ratio), and thus, we presume that many genomes that enter a cell will not result in productive infection. A substantial fraction of incoming genomes have been shown to be degraded, and this depends on ubiquitination, which is required for uncoating.[221] The very early interactions between the viral genome and the host RNA binding proteins, which are critical to determine the course of viral infection, are incompletely understood.

2.3. Translation of the Viral Genome

As described above, the flaviviral ORF encodes for a polyprotein that is processed by viral and host proteases into three structural (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Figure 2). There are, however, cases described in which different proteins are produced by ribosome frameshifting. In WNV, an alternative reading frame in NS4B produces a protein named N-NS4B/WARF4,[222,223] and in Japanese encephalitis virus (JEV), a frameshift results in a C-terminally extended form of NS1 termed NS1′.[224] Finally, in insect-specific flaviviruses, a protein named *fifo* is synthesized from an alternative reading frame spanning parts of NS2A and NS2B.[225]

2.3.1. Flavivirus Translation Initiation—As mentioned above, the viral genome is capped on the 5′ end with a type 1 cap (m^7GpppAm),[226] and this protects viral mRNAs from degradation by Xrn1.[227] In addition, 2′O-methylation of the viral RNA masks the genome from identification by IFIT proteins as foreign RNA.[145] Although the cap is required for efficient viral translation initiation,[228] eIF4E, which supports cap-dependent

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translation of most cellular mRNAs[229] and strongly discriminates m^{7}Gppp and Gppp, is not required for efficient DENV2 translation.[230] It was proposed by Edgil et al. that the primary mode of DENV translation initiation utilizes eIF4E but that this can switch to an alternative translation mode when this protein is limiting.[230] This model was based on the assumption that cap recognition is always mediated by eIF4E, but this assumption has been disproven by important studies that demonstrate that eIF3D, a subunit of the eIF3 protein complex, can recognize the cap and mediate cap-dependent translation initiation in some mRNAs with structured 5′UTRs.[231] We propose that DENV2 employs noncanonical mechanisms to mediate cap-dependent translation initiation, possibly via direct recruitment of the eIF3 complex.

Initiation of translation is likely enhanced by structures in the 3′UTR[232] that recruit a poorly understood repertoire of host proteins.[233] For instance, the viral 3′UTR recruits poly(A) binding protein (PABP) in a poly(A)-independent manner, presumably to support circularization of the viral mRNA for efficient translation.[234]

2.3.2. Flavivirus Translation Elongation and Termination—Once the 40S ribosomal subunit and associated factors are recruited to the viral RNA, it scans the 5′UTR until reaching the AUG start codon. Selection of the AUG is aided by a secondary structure element, the aforementioned cHP, located 14 nucleotides downstream of the start codon and stalls the 40S ribosomal subunit to ensure correct start codon selection.[99]

After the large (60S) ribosomal subunit joins, the 80S ribosome is poised for elongation, a process that has been viewed as somewhat unidimensional, but that is likely to be similar to translation initiation–versatile, regulated, and different for distinct classes of mRNAs.[235] Indeed, studies with flaviviruses may shed light into noncanonical modes of translation elongation.[213,236,237] For example, the acidic phosphoproteins RPLP1 and RPLP2 of the ribosomal stalk, which are thought to recruit elongation factors to the ribosome, are exquisitely required for translation of DENV2 RNA but are not required for global cell protein synthesis.[236] Indeed, our work has identified a region in the prM-E coding sequences where elongation is exquisitely sensitive to RPLP1 and RPLP2 depletion.[236] As discussed above, flaviviral ORFs also have "slippery sequences" that cause the ribosome to change its reading frame, creating noncanonical proteins. Detailed studies of these noncanonical elongation mechanisms could reveal similar ones operative among specific classes of cellular mRNAs, which may share properties with the flavivirus genome.

Once the ribosome encounters the stop codon, translation termination occurs with the help of release factors eRF1 and eRF3, which catalyze the release of the polypeptide chain, leading to ribosome disassembly and recycling.[235,238] Very little is known about flavivirus translation termination, and future studies should determine whether the conventional model for cellular translation termination is able to explain this step of flavivirus translation.

2.3.3. Translation at the Endoplasmic Reticulum—While it is possible that translation initiation starts in the cytosol, the elongating ribosome is likely to stall when the transmembrane domain in the C-terminus of the immature capsid protein (Figure 2) emerges from the ribosome exit tunnel and is bound by the signal recognition particle (SRP), which

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delivers the entire complex to the translocon in the endoplasmic reticulum (ER) membrane. An alternative view is that the flaviviral genome is recruited to the ER and initiates translation using ER-associated ribosomes; this has been proposed for some cellular mRNAs (for two perspectives on this issue see refs 239–241). Several components of the SRP-translocon pathway were found to be important for flavivirus propagation by RNAi and CRISPR loss of function genome-scale screens (SRP54, SRP9, SRP14, SSR1, SSR2, SSR3, SEC61, SEC63);[211,213,242,243] however, this model still needs empirical testing because translocation to the ER can also occur in an SRP pathway-independent manner.[244]

Regardless of whether or not flavivirus translation initiates on ER-associated ribosomes, flavivirus polyprotein synthesis takes place in association with ER membranes. Fractionation of Kunjin infected cells followed by actinomycin treatment revealed that incorporation of radiolabeled leucine occurred mainly in the rough ER, consistent with translation in that organelle.[245] Immunofluorescence labeling of Kunjin E protein identified protein accumulation on intracellular membranous networks that were consistent with ER.[246] Radiolabeled E protein was isolated most efficiently in the detergent-soluble fraction, demonstrating that viral proteins cannot be easily separated from membrane fractions.[246] In addition, DENV[247] and YFV[248] prM and E were shown to be translated as products protected from protease digestion, consistent with translation into the lumen of the ER. These observations were recently extended in ribosome-profiling studies[249] performed by our group in collaboration with the Nicchitta and Vasudevan laboratories.[250] In these experiments, DENV-infected cells were separated into ER and cytosolic fractions to characterize viral and cellular translation in these distinct compartments. This revealed DENV RNA was highly enriched in ER fractions and, late in infection, became the dominantly translated RNA on the ER.[250]

2.4. Polyprotein Processing

The viral genome has a single open reading frame that is translated into a long polyprotein; however, efficient processing by host and viral proteases ensures that the mature viral proteins are produced (Figure 2). Processing is sufficiently rapid that no one has reported observing the full-length polyprotein as a single product during infection. A concise characterization of YFV polyprotein synthesis and maturation was presented by Rice and colleagues in YFV-17D-infected human SW-13 cell lines.[251] Immunoprecipitation using YFV protein-specific rabbit antisera targeting either C, prM, or E proteins after at least 40 min of continuous radiolabeling detected specific bands that corresponded to C, prM, or E proteins but no significant precursors.[251] Using cell-free models of mRNA translation, the flavivirus TBEV E protein was efficiently cleaved in reactions containing an isolated ER fraction.[252] These data suggest that processing of structural proteins requires ER factors and that when these factors are present processing is very efficient or unprocessed fusion proteins are readily degraded.

On the other hand, unprocessed intermediates of NS proteins can be observed in certain conditions. For instance, in a pulse-chase experiment, a form of NS1 with higher molecular mass accumulated first and subsequently disappeared, coincident with the accumulation of mature NS1.[251] This suggested that NS1-NS2A is originally produced as a metastable

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precursor that is subsequently processed into NS1 and NS2A. Similar analyses suggested efficient processing between NS2A and NS2B and between NS2B and NS3. Finally, a large precursor protein corresponding to NS3-NS4A-NS4B-NS5 as well as a number of processed intermediates and the mature viral proteins are observed,[251] suggesting multiple paths to their production.

Cellular and viral proteases coordinate flavivirus polyprotein processing (Figure 2). The viral protease catalytically active holoenzyme is the trypsin-like NS3 protease in complex with its cofactor NS2B.[253,254] Sites sensitive to YFV protease have in common two basic residues (Arg being the most common) flanked by amino acids with short side chains (often Gly).[90] The dual cleavage between C and prM requires a coordinated two-step cleavage in which the viral protease, NS3, and cellular signalase cleave the C protein from the cytosolic and ER lumen side, respectively; releasing mature C into the cytosol and leaving a small part of the immature C protein in the ER membrane.[255–257] Processing between prM-E and E-NS1 is mediated by the endogenous signalase and does not require the viral protease.[247,248,252,258] A distinct cleavage between NS1–NS2A requires an unknown cellular protease.[258] The remaining cleavage sites, NS2A–NS2B and NS2B–NS3, are mediated by NS3 in cis,[259] while processing between NS3–NS4A and NS4B–NS5 is mediated by NS2B and NS3 in trans.[260–263] The mature NS4A and NS4B proteins are generated by cleavage at two sites by NS2B/NS3 and the cellular signalase,[260] leaving a 2 kDa protein named 2K peptide, which is inserted in the ER membrane (Figure 2).

Diamond and colleagues performed a screen for WNV host factors, which identified several subunits of the signalase (or signal peptidase complex (SPC)), of which the authors followed up on the role of signal peptidase complex subunit 1 (SPCS1).[243] In SPCS1 KO cells WNV E protein and a high molecular weight protein reactive to anti-E antibodies is observed, suggesting that polyprotein processing is affected.[243] Using overexpression constructs, the role of the signalase at the signal sequence for prM is observed to be uniquely sensitive to SPCS1 knockout, and lack of SPCS1 causes all tested viral subunits to be reduced.[243] Also, signalase activity between NS4A and NS4B requires SPCS1, but NS1–NS2A processing is not clearly affected.[243] Surprisingly, the cleavage between E and NS1 is much more dependent on SPCS1 when prM is present in the construct. Therefore, placement of NS1 in a more internal position on the construct renders it more dependent on SPCS1 for cleavage. The signalase activity may be context dependent or the composition of the signalase may vary.

Part of the maturation of viral proteins is the addition of post-translational modifications (e.g., ubiquitination). While we do not cover this area here, we refer the readers to a recent review[264] and to section 3.4.

In summary, the processing of the viral polyprotein and the accumulation of mature viral proteins involves many processes that are beautifully coordinated during infection and represent intriguing targets for development of novel therapeutics.

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2.5. Viral Replication

Newly translated viral proteins co-opt the invading viral genome(s) and change its primary function from translation to replication (Figure 3).[89] We posit that the high local concentration of viral NS proteins in the vicinity of the translating viral RNA and perhaps the effect of these proteins on cellular membranes drives the first critical switch for the genome from translation to replication. It is possible that this switch occurs more than once during early infection (see legend for Figure 3). The positive strand viral genome is copied into a negative strand viral antigenome, which in turn serves as a template for new genomes but to date has not been shown to have any protein coding capacity. It must be noted that an infectious unit (one plaque forming unit) could require multiple genomes to enter a cell, and thus, the specialization of genomes could be more complex than portrayed in this parsimonious model.

Viral replication requires an assemblage of viral RNA structures, viral proteins, and cellular factors. Major advances in understanding the viral and cellular factors that contribute to viral replication were derived by studies using a variety of flavivirus replicons. Khromykh and Westaway designed the first flavivirus replicon, a self-replicating, noninfectious viral-like RNA, based on the Kunjin flavivirus genome. The Kunjin-based replicon contained the 5′UTR and the 3′UTR.[265] In addition, the coding sequence for the C-terminal transmembrane domain from the E protein was retained as it was known to function as the signal sequence for the NS proteins,[258] and the replicon contained the coding sequences for all NS proteins.[265] The authors determined that the viral RNA coding for the first 20aa of C protein is necessary to sustain replication.[265] Next, the authors demonstrated that the replicon could be modified by addition of exogenous coding sequences for selectable or screening markers.[265] In a subsequent publication, the coding region for the viral NS5 protein was deleted, and RNA replication was observed only when NS5 was expressed in trans,[266] demonstrating that a synthetic viral-like RNA could be manipulated to aid study of viral replication.

Flavivirus replication depends on conserved RNA elements within the genome. As discussed in section 2.1.3, sequences at both the 5′ and the 3′ terminal regions and their long-term interactions are essential for RNA synthesis (Figure 1).[96–98,267,268]

2.5.1. Replication Complex—Viral RNA synthesis occurs in association with membranous rearrangements termed replication complexes (RC). The models we present here have been inspired by pioneering work of the Ahlquist and Bartenshlager groups on the structure of replication complexes for model (+) stranded RNA viruses.[269–272] Dramatic intracellular membrane rearrangements were observed in flavivirus-infected tissues and cells,[273] and these rearranged membranes were associated with accumulation of the viral replication intermediate, dsRNA, and viral nonstructural proteins necessary for replication,[274,275] suggesting that the viral membrane rearrangements have an important role in viral replication. Viral RNA synthesis and viral protein biogenesis, while both occurring in association with ER membranes, may involve membrane compartments with distinct characteristics.[275] Recently, the ultrastructure of DENV and ZIKV replication complexes in human and mosquito cell lines was visualized using electron tomography.[272,276,277] DENV-

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induced formation of invaginations into the ER lumen creating 80 nM–120 nm spherules that were often connected to the cytosol by a single, small pore.[272,276] Modification of the lipid composition of the ER membrane is required to alter membrane curvature and form vesicle packets, and this is likely mediated by viral NS proteins (see discussion in section 2.5.2).[278] The RC contains a small pore that is open to the cytosol such that the interior of the RC is contiguous with the cytoplasm (Figure 3).[272,276] Electron-dense structures consistent with ribosomes were observed on nearby membranes, suggesting an ER origin,[272] although the replication complex may arise by redistributing components of the ERGIC, Golgi, and trans-Golgi network compartments.[279–283] Ribosomes were not observed within the invaginations, suggesting that viral translation does not occur within these structures. In addition, virions were observed within the ER lumen but never within nor budding from the replication structures, suggesting the invaginations were not precursors to virions. The consensus is that these structures are sites of significant viral genome replication. Interestingly, ZIKV RC resembled those of DENV but were found to be uniquely surrounded by microtubules and intermediate filaments.[277] In Figure 3 we summarize these data and highlight how three critical functions of viral genomes, translation, replication, and viral assembly, occur in distinct compartments of the ER.

2.5.2. Viral Components of the Replication Complex—The composition of the RC is an area of important investigation and has been reviewed recently.[284] Here we focus on the viral components of the RC and point out that the cellular components are much less well understood.

2.5.2.1. NS1: Viral replication requires NS1 through a poorly defined mechanism. Immunofluorescent labeling determined that DENV NS1 is similarly distributed compared with viral dsRNA and electron microscopy studies localized immunogold labeled DENV NS1 and dsRNA to rearranged intracellular membranes.[274] In addition, Kunjin NS1 similarly colocalized with dsRNA and immunoprecipitation (IP) of viral dsRNA also coimmunoprecipitated (co-IP) NS1.[275] NS1 is known to be in the lumen of the ER (Figure 2) and must connect with components in the RCs via transmembrane interactions (Figure 3). The Rice laboratory defined a role for NS1 during viral replication. A temperature-sensitive YFV NS1 mutant was isolated, which results in continuous accumulation of viral negative strand RNA at the permissive temperature but decreased negative strand RNA accumulation after shifting to the nonpermissive temperature.[285] YFV NS1, expressed in trans, during an infection by a packaged, NS1-deficient YFV genome complements RNA accumulation;[286] however, structural proteins were present in these experiments, and given the duration of the experiments an impact of NS1 at viral attachment and entry could not be ruled out. Diamond and colleagues somewhat clarified this issue using an analogous WNV model of NS1 trans-complementation by showing that viral attachment and entry are not significantly changed, although newly synthesized viral E protein is slightly reduced.[287] NS1 localizes to sites of RNA synthesis, can be purified with other replication components, and is required for viral replication; however, unraveling the mechanism by which NS1 supports viral RNA synthesis requires further investigation.

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2.5.2.2. NS2A: NS2A is a poorly understood component of the replication complex. NS2A is a hydrophobic membrane protein lacking any known enzymatic activity.[288] Similar to NS1, electron micrographs of Kunjin-infected cells reveal that immunogold-labeled dsRNA and Kunjin NS2A colocalize during infection to the same rearranged intracellular membranes.[289] In addition, multiple nonstructural proteins and the viral 3′UTR were shown to bind GST-tagged NS2A,[289] suggesting a role during viral replication. Finally, Shi and colleagues demonstrated that DENV NS2A is critical for RNA synthesis but not for translation of a DENV2 replicon.[288,290] Given these data and specifically the many interactions between replication components and NS2A, it follows that the latter may function as a scaffold protein that organizes the replication complex.

2.5.2.3. NS4A and NS4B: NS4A and NS4B are polytopic membrane proteins[278,291] generated by coordinated post-translational cleavages from host and viral proteases.[251,292] Both viral proteins are components of the RC,[272,289] and NS4A plays an important role during replication. Deletion of Kunjin NS4A[293] or mutations introduced into the DENV replicon at the first transmembrane α helix of NS4A[294] abolish viral replication. The mechanism(s) by which NS4A function are poorly understood.

Overexpression of WNV NS4A induced intracellular membrane rearrangements consistent with membrane rearrangements observed during viral infection.[295] Subsequently, the Bartenschlager lab showed that expression of GFP-tagged DENV NS4A is sufficient to induce similar membrane rearrangements.[278] A genetic study identified that NS4A connects luminal and cytosolic components of the replication complex, and further studies found that overexpressed NS4A oligomerizes, and NS4A interacts with NS4B.[294,296] These data suggest that NS4A is an important scaffold supporting the replication complex. Furthermore, DENV1 NS4A was evaluated using a substitution assay in which short amino acid sequences from JEV NS4A were swapped into the corresponding positions of a DENV1 clone.[297] Efficient replication of a chimeric DENV1/JEV clone occurred only in the presence of mutations in the DENV1 NS4B ORF, suggesting that these two viral proteins interact.[297] The NS4A–NS4B interaction was better characterized by Shi and colleagues, who showed that NS4A and NS4B co-IP each other in the absence of other viral components.[298] They went on to show that a 36aa region of NS4A and a 62aa region of NS4B, each containing a transmembrane domain, are necessary for the interaction and using NMR identified the amino acids that mediate the interaction.[298] Lindenbach and Rice discovered that DENV NS1 complements NS1-deficient YFV but only when YFV NS4A is mutated, suggesting that NS4A connects luminal NS1 to the cytosolic NS3 and NS5 replication machinery.[299] On the basis of the experiments described above, it is believed that NS4A may be a driver of the ER invaginations where RCs are assembled and may help organize the luminal, transmembrane, and cytoplasmic components of the RCs.

NS4B is a glycosylated[300] membrane-associated[291] viral protein that is essential for viral replication.[293] The intracellular distribution pattern of GFP-tagged WNV NS4B suggested that NS4B can by itself cause membrane rearrangements.[301] Furthermore, NS4B has been reported to interact with NS3,[302–304] suggesting potential interactions with the RC. Recent high-throughput chemical screening campaigns identified NS4B as an important target for antiviral drug discovery. Using a DENV replicon-based chemical screen, Shi and colleagues

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reported that an NS4B inhibitor (NITD-618) blocks DENV replication but does not inhibit other flaviviruses.[305,306]

The work summarized above suggests that NS4B and NS4A support viral replication by multiple mechanisms including maintaining the replication complex structure, organizing the replicase, and modifying the activity of enzymatic components of the replicase (Figure 3).

2.5.2.4. NS3 and NS5: NS3 is modular protein with at least three enzymatic activities: a protease activity, which was described above (section 2.4), participates in the maturation of viral proteins, an ATPase-driven helicase domain within NS3 is assumed to unwind dsRNA during viral RNA synthesis, and a 5′ RNA triphosphatase activity, the first step required for RNA capping by NS5 (section 2.1.4). Select mutations engineered into the conserved amino acid motifs within the DENV2 NS3 helicase domain block ATPase activity and helicase activity and virus production.[307]

During viral infection NS3 and NS5, which is the protein with RdRp activity, form a complex that is important for efficient replication. IP of NS3 consistently co-IPs NS5 and vice versa.[251] In addition, the 3′ terminal stem loop of the JEV genome forms a complex with NS3 and NS5.[308] Finally, the ATPase activity of DENV1 NS3 is enhanced by addition of NS5, although NS5 does not have ATPase activity.[309]

As described above, viral RNA synthesis occurs in RCs. The RdRp and the mRNA capping methyltransferase activities are provided by the viral NS5 protein. Purified recombinant NS5 has RdRp activity when provided a positive strand RNA as template.[310] The methyltransferase activity was first demonstrated using recombinant NS5[136] and subsequent genetic studies have validated this observation (reviewed in ref 128). A WNV-based replicon that lacks NS5 was complemented by expression of WT NS5 in trans but not by an NS5 carrying mutation of either the conserved GDD motif in the polymerase active site or the conserved *S*-adenosyl methionine binding domain.[311]

2.5.3. Model for Genome Amplification—The substrate for negative strand synthesis is likely in the panhandle conformation where the 5′ and 3′ ends of the genome are in close proximity.[102] In this conformation, NS5 bound to the SLA structure in the 5′ end is in the vicinity of the 3′ end of the genomic RNA, which is the initiation site for RNA synthesis (reviewed in ref 89). The proper positioning of the polymerase is likely influenced by 3′SL structure at the 3′ end of the genome, which has been shown to also interact with NS5.[312] Elegant structural and reverse genetic studies indicate the possibility of NS5 dimerization[313] and position the C-terminal 18 residues close to the thumb subdomain, which could be important for initiation of RNA synthesis.[314]

We propose that once negative strand synthesis commences formation of the genome panhandle structure is disfavored, preventing reinitiation of negative strand RNA synthesis. This leads to the suggestion that a single copy of the negative strand will be synthesized from the incoming genome in one RC (Figure 3; see discussion in Garcia-Blanco et al. (2016)[89]). This is consistent with data that indicate that replication complexes increase in

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number but do not increase in size during infection.[272,315] On the other hand, each antigenome is likely to serve as the template for the synthesis of multiple nascent genomes within one RC (Figure 3). Each nascent genome displaces the preceding one, and each promoter region is likely to be associated with NS5 while in the RC; however, once a genome diffuses beyond the pore of the RC and moves through the cytoplasm there is competition between different fates as depicted in Figure 3. Changing the relative local concentration of NS5 and genomes and a relatively stable concentration of translation initiation factors can lead to oscillations between replication and translation for a specific genome RNA. Eventually as the concentration of C rises the process of assembly will become more competitive (#3 in Figure 3). A bipartitie nuclear localization signal (NLS) has been shown to translocate C to the nucleolus,[316,317] and it is possible that the removal of capsid from the cytoplasm early in replication may favor translation and replication over assembly at these early times (see discussion in ref 318).

2.6. Flavivirus Assembly and Egress

The late stages of the flaviviral life cycle include the assembly of viral components into virion particles, maturation of these into infectious particles, and release from the cell by secretion. Flavivirus assembly occurs at the ER where viral genomes assemble with C, prM, and E proteins and bud into the ER lumen, taking ER membranes with the developing virion. Using electron tomography virions were observed budding into the lumen of the ER in both mammalian and mosquito cell lines.[272,276] Coexpression of prM and E can initiate viral budding in the absence of other viral proteins or genomes to form virus-like particles (VLPs).[319]

The coordinated processing of the two cleavages at the junction of the flavivirus structural proteins C–prM by host and viral proteases is important in regulation of the assembly of the YFV.[257] Similarly, studies on Murray Valley encephalitis virus evinced that the coordination of cytosolic and luminal cleavages at the C–prM junction is key to efficient incorporation of nucleocapsid (the viral RNP) during assembly.[494]

Nonstructural proteins have important but poorly understood roles in packaging of the viral RNP. Using a systematic genetic approach, Bartenschlager and colleagues identified mutations in DENV NS1 that reduce viral production without strongly inhibiting viral RNA replication.[320] Of 46 amino acids selected for alanine-scanning mutagenesis, approximately one-half inhibit replication of the genome,[320] which is consistent with the established role of NS1 during replication (see section 2.5.2). Of the remaining half, five of the alanine substitutions cause limited impact on replication but strong reduction in viral production, and the authors noted that these mutations increase levels of intracellular virus relative to extracellular virus.[320] Although recombinant VLPs formed in the absence of NS1,[319] Bartenschlager and colleagues provided evidence that NS1 modulates export of fully assembled and infectious virus.[320]

The nonstructural protein NS2A plays an important role in viral assembly. A mutant YFV NS2A does not affect expression of viral proteins or RNA synthesis but strongly inhibits production of infectious virus.[321] Analysis of the secreted viruses showed that this NS2A mutant leads to the production of VLPs that lack capsid and viral RNA.[321] The role of

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NS2A in viral assembly was also observed in DENV[290] and Kunjin virus,[322] suggesting that this is a conserved role for NS2A.

NS3 is required for Kunjin virus RNA packaging into viral particles.[323] Similar dependence on NS3 was observed in viral assembly using a YFV replicon-based trans-packaging system.[324] Mutation of a conserved tryptophan at position 349 in the helicase domain of NS3 to alanine (W349A) has no effect on the expression of viral proteins or amplification of RNA in transfected cells; however, it blocks the production of infectious virus particles.[324] Interestingly, the role of NS3 in virus assembly does not strictly require helicase or protease activity, although mutant forms of NS3 are less efficient in rescuing production of infectious virus particles. This reduction in efficiency is consistent with optimal assembly requiring cleavage at the C–prM junction by the NS2B-3 protease.[325,326]

Studies of Kunjin virus have revealed that the polymerase activity of NS5 is a prerequisite for assembly. Cells transfected with DNA-based Kunjin replicons produce RNA genomes via nuclear transcription, and these genomes can be translated whether or not NS5 RdRp is active.[327] Packaging into virions, however, depends on NS5 RdRp activity as no infectious virions were produced in cells transfected with a (GDD) Kunjin genomic RNA, which has a deletion of the NS5 gene RNA-polymerase motif GDD.[327] This defect could be complemented in trans and is not observed when replicon had WT NS5.[327] These intriguing data suggest coupling between RNA synthesis and packaging, thus suggesting heretofore unknown connections between these processes (Figure 3).

Flaviviruses are initially assembled as immature particles that mature during exit from the cell. The structure of the immature DENV and YFV particles was resolved using cryo-EM and showed that, in both viruses, spikes formed by the prM protein protrude from the surface and increase the diameter of the virion to about 60 nm, which is larger than mature virions (50 nm).[328] In the immature particles E protein is arranged as trimers around the prM spike.[328] Mature Murray Valley encephalitis virus induces syncytia formation in C6/36 cells at low pH; however, immature virus does not,[329] which suggests that the immature prM spike prevents premature viral fusion with endosomal membranes during egress. The maturation of flaviviruses begins upon exposure to the acidic environment of the Golgi and trans-Golgi network. This pH triggers conformational rearrangement of the prM and E that makes prM accessible to cleavage by the cellular protease furin, producing the M protein and the pr peptide, which remains bound to the viral envelope at acidic pH and continues to prevent premature viral fusion.[330] A pH shift from acidic to neutral upon exit of the virus to the extracellular space causes release of the pr peptide from viral surface, resulting in infectious mature virus (reviewed in ref 331).

3. PRO-VIRAL HOST FACTORS

Successful viral infection requires commandeering of many cellular factors to work for the virus (e.g., ribosomal components) and the neutralization of many other cellular factors that normally protect from infection (e.g., interferon stimulated gene products). In this review, we refer to the former as pro-viral factors and the latter as antiviral factors. The limited size of the flaviviral genomes restricts the number of available proteins they can express, so these

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viruses are dependent on a large number of pro-viral host factors for all stages of the viral life cycle. Indeed, this dependency is shared by all viruses, even relative giants like Pandoraviruses, which have genomes larger than 2 megabases.[332] The study of host factors, which is the study of how viruses and cells interact, has led to critically important insights on the biology of both viruses and cells (e.g., the discovery of introns in pre-mRNAs[15,16]). The hope is that the understanding of virus–cell interactions will continue to shed light on new biology (e.g., noncanonical mechanisms of translation initiation) and will also open up novel strategies to decrease viral pathogenesis by interfering with essential virus–cell interactions (e.g., blocking CCR5 to treat HIV infection).[333] In this review, we broadly cover the current knowledge of host factors, pro- and antiviral, that impact flavivirus replication with an emphasis on RNA binding proteins. We also refer the reader to other reviews.[11,334,335]

Functional genomic approaches such as genome-scale RNA interference-mediated knockdowns and CRISPR-mediated gene knockouts have contributed to a remarkable expansion in the identification of host factors.[336,337] Additionally, methods that map physical interactions between viral and host components (both RNA and proteins) have also greatly expanded our understanding of host factors.[338,339]

3.1. Pro-Viral Host Factors: From Attachment to Expression of the Viral Proteins

The dependency on host factors for attachment, entry, and uncoating was described in detail in section 2.2, where we discussed these early steps in the viral life cycle. The only point we make here is that flaviviruses, like all viruses, are the ultimate Darwinian tinkerers and will use any host factor (protein, RNA, and even lipids) to their advantage. An extreme example is how DENV uses antibodies and Fc receptors to efficiently enter cells and mediate severe infection during ADE (see references cited in section 2.2.2).

The primary aim of any virus is to replicate itself, and without exception this requires translation of viral proteins. Indeed, we classify viruses in part by the number and properties of the steps required to convert the genome into mRNA(s).[340] Inherent to this requirement is the critical role of the cellular translational machinery, and at the center of this machinery sits the ribosome. Unfortunately, the flavivirus field has largely ignored the ribosome as a unique host factor. As described above, we identified the ribosomal stalk proteins RPLP1 and RPLP2 as critically important for flavivirus translation,[213,236] and these studies are leading to the identification of cellular mRNAs that share this requirement and eventually to understand the role of these poorly understood proteins. As our view of the ribosome shifts from a monotonic machine to a more modular one, it is likely we will identify new ribosomal components that are exquisitely required by flaviviruses. Indeed, the same is true about requirements for the initiation, elongation, and termination factors that work with ribosomes to mediate translation (see discussion in section 2.3).

3.2. ER-Associated Pro-Viral Factors

The co- and post-translational processing of the complex flavivirus polyprotein requires coordination of ribosomes, the Sec61 translocon, ER proteases, ER-associated chaperones, and enzymes that mediate post-translational modifications. Translation is directed to the ER

by recognition of a signal sequence by the signal recognition particle and transferred to the translocon through the SRP receptor.[341,342] The ER translocon is an ER-localized complex composed of three subunits: SEC61alpha, SEC61beta, and SEC61gamma. The ribosome, translocon, TRAP, and OST complexes guide the nascent polypeptides, ensuring that the polypeptide is synthesized with the correct topology on ER membranes (structure reviewed in ref 343). Biochemical and genetic evidence support roles for the ER translational machinery during flavivirus infection. RNAi-based knockdown of SRP54 or any of the three translocon subunits has been shown to inhibit WNV, DENV2, and YFV infection.[211–213] In addition, DENV infection was inhibited by loss of the signal sequence receptor,[242] while deletion of SEC61beta blocks WNV, DENV2, JEV, and ZIKV,[243] and chemical inhibition of the translocon inhibits DENV infection (see ref 344).

The co- and post-translational processing of the flavivirus polyprotein requires cellular membranes,[252] consistent with a role for the ER signalase. Indeed, a role for the signalase during YFV polyprotein processing was predicted when the sequence of YFV-17D was determined,[90] and a pan-flavivirus role for signalase was proposed based on sequence conservation at processing sites.[345] Early efforts to understand polyprotein processing considered the sequence, composition, and topology of the polyprotein and noted which processing sites were reasonable targets for signalase.[248,257] As discussed above, functional genomics validated the requirement for individual subunits of the signalase for polyprotein processing.[243]

The complex topology of the viral proteins requires cellular chaperones and chaperone-like activities, which help proteins to achieve a final folded form. The HSP70 and HSP90 families of cellular chaperones are necessary for DENV entry.[346,347] In addition, the HSP70 family of chaperones and cochaperones is necessary for DENV NS5 stability[348] and for YFV polyprotein processing.[349] A screen using a cDNA-based overexpression strategy identified DNAJC14 as an important cochaperone that impacts polyprotein processing.[350,351]

The IRE1alpha ER stress sensor is repressed by binding the HSP70 family member GRP78/BIP.[352] Accumulation of unfolded proteins sequesters GRP78/BIP leading to activation of the unfolded protein response (UPR), including activation of IRE1alpha, leading to increased expression of chaperones.[352] Knockout of ER stress sensor IRE1α reduced viral infection in mouse embryonic fibroblasts,[353] suggesting that the UPR plays an important role during viral infection (reviewed in ref 354). It is likely that the role of chaperones extends throughout viral infection and may be a particularly important arm of the UPR supporting viral infection.

ER membrane complex (EMC) subunits scored as potent pro-viral factors in an RNAi-screen for YFV-17D host factors but were not characterized further.[213] *EMC2* and *EMC3* were identified in a gene-editing-based screen for WNV-induced cell death.[355] *EMC2* knockout cell lines were developed, and they are fully permissive to WNV infection; however, expression of WNV proteins C, E, and NS3 is somewhat reduced after a 36 h infection. The role of the EMC for the virus was not further characterized, and the role of the EMC for the cell was unexplored in these cells.

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Functional genomic screening uncovered that knockdown or knockout of ER–membrane complex (EMC) subunits reduces DENV2, JEV, WNV, and ZIKV infection.[181,242,243] Carette and colleagues identified EMC1–7 subunits as hits in a functional genomics screen for DENV pro-viral factors using gene-editing technologies.[242] Brass and colleagues showed that DENV2, YFV-17D, and ZIKV infection is inhibited by knockdown or knockout of several EMC subunits.[181] They showed that DENV and ZIKV attachment is not blocked by EMC knockdown; however, the pattern of internalized DENV E protein in DENV-infected cells is altered shortly after infection in EMC knockdown cells,[181] which suggests a defect up to and including viral RNA synthesis. For ZIKV, attachment is not altered by EMC knockdown; however, entry appears to be blocked, leaving virus on the surface of the cell.[181] Nonetheless, the mechanism(s) for flavivirus dependence on EMC is(are) not fully known and requires further study. The cellular function(s) of the EMC is also incompletely understood, but its requirement for proper expression of membrane resident and secreted proteins[356] and its location on the ER suggest an ER-associated chaperone-like activity. Recently, the EMC has been shown to have transmembrane domain insertase activity.[357] It is also possible that an undefined role of the EMC in lipid synthesis[358] may alter membrane protein maturation and expression. These proposed cellular functions are consistent with observed blocks of flavivirus entry and viral protein biogenesis. Studies on the flaviviral requirement for EMC should lead to a better understanding of this obscure protein complex. Furthermore, since the EMC is critically required by many flaviviruses, we envision that anti-EMC compounds could become pan-flavivirus antivirals.

3.3. Cellular Lipids and Autophagy

As previously discussed, the viral membrane phosphatidylserine composition may be a determinant of viral entry,[79] cellular membrane fractions are required for polyprotein processing in cell free assays,[252] and the virus replicates on ER-derived intracellular membranes.[272] Thus, it is not surprising that cellular lipids are major host factors impacting the virus life cycle.

Autophagy is a process by which cytoplasmic material, intracellular membranes, or organelles is engulfed by double-membrane vesicles originating from the ER and subsequent shuttling of this engulfed material to the lysosome for recycling.[359] Autophagic vesicle assembly requires synthesis of phosphatidylinositol-3-phosphate by the type III phosphatidy-linositol-3-kinase (PI3K) and recruitment of LC3-II, Beclin, and other AuTophaGy-related (ATG) proteins to ER membranes.[359] DENV infection induces accumulation of the autophagy marker LC3-II, the autophagic PI3K inhibitor 3-methyladenine reduces DENV virus production, the autophagy activator rapamycin enhances DENV virus production, and DENV virus production is reduced in ATG5−/− mouse embryonic fibroblasts.[360] Furthermore, treatment of an innate immunedeficient mouse line with the autophagy activating drugs rapamycin or nicardipine results in enhanced specific infectivity with a mouse-adapted DENV virus and significantly decreases mouse survival[361]

Studies with a viral-like DENV replicon demonstrated that inhibition of autophagy by 3-methyladenine treatment or Beclin knockdown impacts a step in the viral life cycle

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subsequent to translation of incoming viral RNA and independent of virion assembly and secretion.[362] Obviously, these experiments do not exclude that steps not interrogated by the replicon can also be impacted. Interestingly, the autophagy dependency is suppressed by addition of exogenous lipid-BSA, suggesting that DENV requires autophagy because autophagy generates free fatty acids, a potential source of energy for viral replication.[362] Kirkegaard and colleagues reported an interesting role for autophagy during viral production.[361] The authors noted the suppression of 3-methyladenine treatment by lipid–BSA mixture; however, when autophagy was inhibited with spautin-1, a lipid-BSA mixture no longer rescued replication,[361] suggesting a more complex involvement of autophagy in DENV infection. Indeed, spautin-1-treated and DENV-infected cells show normal viral replication, viral assembly, viral budding, and virion processing by furin-like proteases, but the maturation of the virus immediately prior to release is blocked resulting in virions with decreased infectivity.[361]

Not surprisingly, cellular proteins that modulate membrane lipid composition are important for flavivirus infection. The DENV envelope contains an estimated 8000 lipid molecules of the sphingolipid, sterol, and phospholipid families.[363] In addition, phosphatidylserine and cholesterol enhance DENV infection,[79,364] suggesting that the envelope lipid composition is optimal for infectivity.[365] A targeted functional genomics approach identified proteins involved in fatty acid synthesis as pro-DENV factors, and chemical inhibitors of fatty acid synthesis reduced DENV production.[366] In addition, the cellular protein fatty acid synthase was redistributed to NS3-positive membranes, and radiolabeling of newly synthesized lipids was increased during DENV infection.[366] A whole-cell lipidomic profiling showed that lysophospholipids and ceramides are specifically increased in infected C6/36 *Aedes albopictus* mosquito cells.[367] A postnuclear, membrane-enriched, viral RNA-containing cell fraction that was presumed to be enriched for DENV RCs was also analyzed, and in this fraction sphingolipids are enriched at least 4-fold and phosphatidylethanolamine 10-fold.[367] The inhibition of enzymes that participate in cholesterol[368–370] and sphingolipid[371] biogenesis reduce flavivirus infection in human cells. Furthermore, lovastatin delays DENV infection and increases survival in an animal model.[372] Nonetheless, a randomized, double-blind, placebo-controlled clinical trial failed to demonstrate efficacy of lovastatin (cholesterol biosynthesis inhibitor) against DENV infection in 300 Vietnamese adults positive for DENV.[373] It is still unclear which lipid classes are absolutely required for infection or if there are lipid classes, and associated host proteins that may be commonly required by multiple pathogenic flaviviruses.

3.4. Ubiquitin System

Ubiquitin is a widespread post-translational modification (PTM) that regulates protein function and degradation.[374] Modification of proteins with the small, 76 amino acid ubiquitin molecule requires a set of three enzymes known as E1, E2, and E3. The first of these, E1, activates ubiquitin in an ATP-dependent manner and then transfers the molecule to an E2 conjugating enzyme. E2 ubiquitin interacts with an E3 ubiquitin ligase, which dictates the protein substrate specificity, and ubiquitin is transferred to the substrate via a covalent isopeptide bond involving the ε-amino group of a substrate lysine residue or, less frequently, other amino acids. Proteins can be polyubiquitinated since ubiquitin itself can serve as a

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substrate; polyubiquitination is frequently recognized as a signal for protein degradation mediated by the proteasome complex.[374] As ubiquitination has been broadly implicated in multiple cellular processes, including regulation of innate immunity,[375] it is not surprising that viruses have evolved mechanisms to interface with the ubiquitin system.

There are a number of reports that detail interactions between the ubiquitin system and flaviviruses. In *Aedes aeypti*, depletion of multiple proteasomal subunits reduced production of infectious DENV without affecting viral RNA levels, suggesting a role for the proteasome at a late stage of the life cycle.[376] Similar results were obtained in human THP-1 cells infected by an antibody-dependent route,[377] and the proteasome inhibitors, bortezomib and MG132, were shown to reduce infection by DENV, ZIKV, and WNV.[378,379] Notably, other groups have reported that the ubiquitin system is important for early events in flavivirus infections. Wang et al. showed that several proteasome inhibitors caused internalized JEV particles to be mislocalized to lysosomes, resulting in loss of infection.[380] Similarly, Byk et al. reported that inhibition of E1 enzyme activity blocked DENV uncoating, possibly due to retention of internalized virions in endosomes.[221] This observation supports an earlier study that observed reduced DENV infection due to inhibition of the E1-activating enzyme, UBE1.[381] Another study reported that the E3 ubiquitin ligase, Nedd4, stimulated JEV replication without affecting virus entry,[382] suggesting that the ubiquitin system is important at multiple phases of the flavivirus life cycle. Viral proteins have also been reported to be modified by ubiquitin with contrasting outcomes for infection. Liu et al. discovered that DENV NS3 is modified with polyubiquitin.[383] This PTM promoted association of NS3 with its cofactor, NS2B, resulting in elevated enzymatic activity against the antiviral PRR protein, STING. In another study, the E protein of DENV was reported to be modified by a specific ubiquitin molecule (Ub3881) in mosquitos, resulting in accelerated E turnover and reduced production of infectious virus.[384] Finally, there are well-documented reports of DENV and ZIKV inducing degradation of STAT2 protein in a proteasome-dependent manner to interfere with signal transduction by type I IFN.[385,386] Taken together, it is clear that the ubiquitin system is critical for flavivirus infection and components of this pathway represent potential therapeutic targets for blocking virus transmission.[387] This consideration and that for any drug targeting host factors must carefully weigh potential toxicity to the patient.

3.5. RNA Binding Proteins as Pro-Viral Host Factors

Given the central role of RNA in the life cycle of flaviviruses it is not surprising that host RNA binding proteins (RBPs) play important roles as pro-viral host factors. In order to understand the molecular mechanisms of flaviviral pathogenesis, both in vitro and in vivo approaches have been employed to identify flaviviral RNA interacting proteins. These include host RBPs interacting with DENV,[233,234,283,388–401] ZIKV,[158,402,403] JEV,[404–410] WNV,[411–414] and YFV.[283,388,411] Here we review some salient recent findings and discuss their potential roles in viral pathogenesis; additionally. we refer the reader to recent reviews.[103,334,415]

Host RBPs act on various stages of the DENV life cycle with either pro-viral or antiviral properties. Several proteins were reported to impact on the translation and/or the replication of DENVs. PABP associates with the nonpolyadenylated 3′ end of DENV2 RNA and

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promotes DENV2 translation.[234] In addition, NFKB2 was reported to enhance viral translation via the interaction with the DENV2 3′UTR.[394] NFKB2 is pro-viral because the knockdown of NFKB2 reduced DENV2 RNA level in infected cells. Silencing of the polypyrimidine tract binding protein PTBP1 using siRNA did not impair the translation of a DENV2 luciferase reporter mRNA; instead, pro-viral PTBP1 was involved in viral RNA synthesis and replication.[388] This pro-viral role for DENV2 is in contrast with a demonstrated anti-JEV role for PTBP1.[404] Recently, a growing list of DENV RNA binding proteins has been reported. The majority of these RBPs were involved in promoting viral replication. This was mainly demonstrated by measuring virus titer or viral RNA levels in infected cells of which RBP levels were depleted using genetic tools. For example, silencing of helicase DDX6 led to a decrease of DENV2 viral RNA accumulation and reduced viral particle production.[233] Although DDX6 might contribute to viral replication given that it is localized close to the DENV replication site,[233] a recent report suggests that DDX6 represses activation of interferon-stimulated genes (ISGs).[416] This finding may very well explain why DENV2 replication is less efficient in DDX6 knocked down cells since reduced DDX6 primes cells to establish an antiviral state and thus could restrict DENV2 replication. The same result was observed with DENV2 and WNV infection when LSM1, another component of P bodies that also interacts with the DENV 3′UTR, was silenced.[391,417] Additionally, the DEDD RNA exonuclease ERI3 binds to the 3′UTRs of DENV and YFV and is required for viral RNA synthesis.[283] In summary, although the functional significance for many of these RBPs has been established, understanding the detailed molecular mechanisms by which these RBPs contribute to viral propagation requires further investigation.

Little is known about RBPs interacting with ZIKV RNA. Lichinchi et al. demonstrated that binding of ZIKV genomes by the m^6A readers, YTHDF1, YTHDF2, and YTHDF3, was driven by high levels of m^6A through the full-length ZIKV RNA (see section 2.1.4). Knockdown of YTHDF1 and 2 increased ZIKV infection. Inversely, overexpression inhibited the infection, suggesting that these proteins may destabilize ZIKV RNA acting as antiviral factors.[158] Unlike YTHDF proteins, the translational regulator Musashi-1 (MSI1) has a pro-viral function evidenced by reduction of infection induced by MSI1 knockdown in several neural cells;[402] however, the exact mechanism used by MSI1 to improve postentry events in ZIKV infection is unclear. The interaction between MSI1 and the ZIKV 3′UTR is in part mediated by the presence of three MSI1 putative binding sites (nucleotides 10478, 10668, and 10773). A triple-mutant ZIKV 3′UTR lacking the three putative binding sites has decreased binding to MSI1.[402] G3BP1 and Caprin-1 are pro-viral factors for ZIKV infection,[403] which is opposite to what was observed for DENV2 (see ref 389 and discussion of antiviral factors). Hou et al. showed that pull down of G3BP1 from ZIKV-infected cell lysates leads to enrichment of viral genomes by RT-qPCR.[403] Nonetheless, since G3BP1 binds to ZIKV capsid,[403] the possibility that the interaction of G3BP1 and viral genome is mediated by capsid cannot be ruled out. In summary, the study of RBPs interacting with the ZIKV genome and/or sfRNA is at an early stage and is likely to reveal important details of the host–virus interface that may be relevant to unique aspects of ZIKV pathogenesis.

Several RBPs have been reported as necessary for efficient JEV infection. La protein, a multifunctional RBP, binds to both the JEV 5′UTR and the 3′SL in the 3′UTR. Reduction

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of viral progeny in human cells depleted of La protein indicated that La protein is required for JEV infection; however, the molecular mechanism by which La protein promotes JEV replication is unclear.[409,410] The heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2) participates in post-transcriptional regulation in the nucleus and cytoplasm. This protein interacts with JEV C protein and NS5 and also binds to the 5′ end of the negative strand of JEV. During the course of JEV infection, knockdown of hnRNPA2 inhibited production of negative and positive strand JEV RNAs, suggesting a positive role in replication of JEV RNA.[406] Finally, murine Mov34, a member of a protein family involved in RNA transcription and translation, interacts with the JEV 3′UTR, although it remains unclear whether this protein participates, negatively or positively, in JEV propagation.[408]

In the case of WNV, eEF1A and the stress granule protein TIAR were reported as pro-viral factors. eEF1A interacts with the WNV 3′ terminal SL and facilitates the synthesis of negative strand RNA. Moreover, eEF1A interacts with the same region in DENV and YFV RNA.[411] TIAR, a member of the RNA recognition motif (RRM) family of RBPs, binds to the WNV 3′ (−) SL RNA and facilitates the amplification of viral genome from the minus-strand template.[412,413]

4. ANTIVIRAL FACTORS

4.1. Host Factors Involved in Intrinsic and Innate Immunity

Intrinsic immunity confers resistance to viruses using restriction factors that are usually constitutively expressed and that target a narrow spectrum of different viruses.[418,419] A broader definition could include the absence of a pro-viral factor such as a deletion in the CCR5 gene in some human populations leading to HIV resistance.[333] Innate immunity usually refers to immune mechanisms that more broadly target multiple pathogens (viruses) and, in many cases, are induced upon infection. We will discuss host factors that act in both of these types of immunity and will emphasize the role of RBPs. We also discuss how viruses counteract these factors and, again in an RNA-centric way, focus on flaviviral countermeasures mediated by the sfRNA. We refer the reader to many recent and extensive reviews of innate immunity and flaviviruses.[420–426]

When viruses invade cells, they bring with them and produce pathogen-associated molecular patterns (PAMPs) that are not normally present in a cell and which are recognized as foreign. Flaviviral PAMPs include the viral genome and the viral replicative intermediate, dsRNA, which may be detected by pattern recognition receptors (PRRs) including TLR3, RIG-I, and MDA5. Detection of intracellular infection activates interferon expression, which enables the host to control infection. Overexpression of TLR3 in HEK-293 cells enhances the IL-8 and IFN-α/β production and restricted DENV replication and cytopathic effect.[427] RIG-I and MDA5 were including ISG54 and ISG56. Knockout of either RIG-I or MDA5 in mouse embryonic fibroblasts was not sufficient to entirely blunt ISG54 and ISG56 expression after DENV infection; however, knockdown of MDA5 in a RIG-I null background abolished DENV-induced ISG54 and ISG56 expression.[428] In addition, IPS-1/MAVS/Cardif knockout mouse embryonic fibroblasts showed complete block of ISG54 expression after DENV challenge,[428] and DENV2 challenge in IPS-1/MAVS/Cardif knockout mice results in increased viremia and reduced IFNα and IFNβ expression,[429]

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demonstrating that IPS-1/MAVS/Cardif are involved in innate immune signaling for the primary DENV infection. Downstream DENV-induced innate immune signaling requires the transcriptional activators IRF3 and IRF7 as well. DENV2 infection was cleared efficiently in IRF3 or IRF7 single-knockout mice; however, DENV2 challenge of IRF3 and IRF7 double-knockout mice resulted in higher viremia as well as delayed expression of a variety of ISGs.[430] Nonetheless, mice that lack IRF3, IRF5, and IRF7 survive DENV infection[431] unlike those that lack the type I IFN receptor, which succumb to DENV (see below). The survival of these triple-KO mice is likely mediated by IRF1-dependent activation of both type I and type II interferon pathways.[431]

A fascinating recent development uncovered the fact that DENV infection can lead to mitochondrial damage and release of mitochondrial DNA into the cytoplasm, which is sensed as a PAMP by the DNA sensor cGAS.[432] As will be discussed below, flaviviruses deploy countermeasures to many immune mechanisms, and in this case DENV inactivates both cGAS and its downstream effector STING countering their activation of interferon-signaling pathways.[432,433]

Detection of viral infection by the cellular innate immune response is essential to protect the host and leads to cytokine expression including interferons. Flavivirus infection is controlled by type I interferon (IFN) signaling, with type II IFN signaling contributing too. More recently, the importance of type III (λ) IFN in immunity to flaviviruses has become appreciated.[434–438] Mice are not natural hosts to DENV or YFV infection, and viral challenge in healthy, adult mice results in an acute and rapidly cleared viremia with little or no outward signs of infection. In an effort to identify mouse models of flaviviral infection for the purpose of understanding the host–virus interaction, several of the major IFN-signaling pathways responsible for host clearance of flavivirus infection were determined. Harris and colleagues first identified that DENV2 challenge, using a mouse-adapted DENV isolate, could cause significant pathology in mice that mirrored severe dengue in humans using the type I and type II IFN receptor double-knockout (AG129) mice.[439] Although infection by either wild-type DENV2 or mouse adapted DENV2 both resulted in similar viremia, the mouse-adapted DENV2 caused increased vascular permeability and reduced survival,[439] suggesting that the AG129 mouse and adapted DENV viral infection model may be an effective tool to study dengue-induced vascular permeability associated with severe dengue in humans. Ryman and colleagues demonstrated the relative contribution of type I and type II IFN signaling while developing a mouse model of YFV infection.[440] Type II IFN signaling deficient (IFN gamma receptor knockout) mice did not display significant signs of YFV infection using either WT or attenuated YFV strains.[440] Pathogenic YFV challenge in type I IFN signaling deficient (IFN type I receptor knockout) mice caused weight loss requiring euthanasia for all reported mice, although attenuated YFV-17D did not cause significant disease.[440] Therefore, type I IFN signaling is important for YFV control in these mouse models. The contribution of type II IFN signaling was observed only in AG129 mice where attenuated YFV-17D induced significant weight loss requiring euthanasia,[440] suggesting that clearance of YFV required both type I and type II IFN signaling. STAT-family proteins mediate downstream IFN signaling. Although DENV challenge in STAT1 or STAT2 individual knockout mice does not cause lethal phenotype, DENV challenge in

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STAT1, STAT2 double-knockout mice results in a lethal phenotype similar to that observed in AG129 mice.[441]

The cellular innate immune response to flaviviruses requires detecting viral infection by the pattern recognition receptors, TLR3, RIG-I, and MDA5, and cytokine signaling through IFN-signaling pathways to clear infection. The relevance of findings derived from studies in mouse models to mechanisms of resistance and pathogenesis in humans remains to be determined. Future studies using human cells and human genetics should be used to test this relevance.

IFN signaling leads to the expression of interferon stimulated genes (ISGs) whose products are the effectors of the antiviral state (reviewed by Schoggins and Rice[442]). MacDonald, Rice, and colleagues pursued identification of cellular genes that had antiflaviviral activity.[350] The screening and validation resulted in the identification of one antiviral human gene: DNAJC14,[350] which when overexpressed reduces replication of YFV-17D, YFV-Asibi, Langant, and WNV and also the related member of the *Flaviviridae* family-HCV.[350] In a subsequent publication, DNAJC14 overexpression was shown to inhibit polyprotein processing between NS3 and NS4A consistent with the role of DNAJC14 as a cochaperone of HSP70 chaperone proteins.[349] In parallel studies, Rice and colleagues examined the activity of ISGs on a broad set of viruses and discovered many that have antiflaviviral activity.[443,444]

4.2. RNA Binding Proteins As Antiviral Factors

Anti-DENV host RBPs can exert their activity via direct or indirect pathways. MCPIP1, a RNA binding nuclease, degrades DENV2 RNA through binding to the 3′UTR and restricts viral replication. The same MCPIP1-mediated antiviral effect was also observed for JEV.[445] Alternatively, host RBPs can indirectly antagonize DENV through host immune responses. G3BP1, G3BP2, and CAPRIN1, which are stress granules associated proteins, are important for ISG mRNA translation, and they are required for host cells to establish an antiviral state.[389] In addition, TRIM25, a ubiquitin ligase, regulates RIG-I pathways against viral infection.[395] YBX1 negatively regulates DENV2 translation and is an antiviral host factor against DENV2.[397] The action of YBX1 represents a type of intrinsic immunity by an RBP reminiscent of the effect of UPF1 on Semliki Forest virus[446] and probably represents a widespread antiviral strategy.

Besides PTB and MCPIP1 mentioned above, IFIT1 and far upstream element binding protein 1 (FBP1) have been reported to bind to JEV RNA and inhibit translation. As discussed in section 2.1.4, IFIT1 recognizes 5′ caps that have not been methylated in the 2′ position of the penultimate nucleotide and inhibits translation of a JEV defective in 2′-O MTase activity.[407] FBP1 interacts predominantly with the JEV 3′UTR and weakly with the JEV 5′UTR. Overexpression and knockdown experiments suggested that FBP1 negatively regulates JEV infection. Mechanistically, the increased activity of a JEV replicon reporter in FBP1 knocked down cells suggested that FBP1 inhibits viral translation.[405] Due to preferential binding to the JEV 3′UTR, it would be interesting to study the specific interaction between FBP1 and JEV sfRNA and to test if this interaction could sequester FBP1 as a mechanism to promote viral translation (see section 3.5).

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4.2.1. Antiviral Modulators in Mosquitos—Mosquitos mount an innate immune response structured around three major pathways: Toll, IMD, and Jak/STAT.[447] In vivo RNAi depletion experiments support antiflavivirus properties for each of the pathways.[448–453] Activation of each pathway triggers a specific cascade that results in the translocation from the cytoplasm to the nucleus of a distinct transcription factor: the NFκB transcription factors Rel1a and Rel2 for Toll and IMD, respectively, and the dimerized STAT for Jak/STAT. Transcription regulation for each pathway significantly overlap with genes regulated upon DENV infection,[452,454–456] further supporting the antiviral function of each pathway. These pathways show significant similarities to NFκB and Jak/STAT-regulated pathways critical in mammalian innate immunity.

Regulated immune effectors include antimicrobial peptides (AMPs), factors involved in phagocytosis and the complement system, although the impact of each class remains to be addressed for different viruses. Interestingly, many subsets of genes are identically regulated in amplitude and direction by different pathways, suggesting a potential synergistic response.[457] A biochemical cascade that bridges the RNAi, IMD, and Jak/STAT pathways has been discovered in *Culex* mosquitos. Dicer-2 sensing of the viral dsRNA activates the TNF-associated factor (TRAF) that cleaves off the inhibitor fragment of Rel2, resulting in its binding to the promoter of Vago and transcription activation.[458] Vago then elicits the Jak/STAT pathway that antagonizes another flavivirus, WNV.[450]

RNA interference (RNAi) functions in diverse organisms including mammals[459,460] and mosquitos.[461] RNAi is a conserved and well-studied pathway for post-transcriptional gene regulation[462] and has potent antiviral activity in invertebrates.[463] The antiviral effect of RNAi in mosquitos against DENV infection has been demonstrated using infection of *Aedes aegypti* mosquitos with DENV2.[464] Knockdown of endogenous Dicer2 enhanced DENV2 viral production by 10-fold and reduced the time necessary for the virus to be produced from 10 to 7 days.[464] Additionally, mosquitos genetically engineered to express an inverted viral mRNA, which would produce antiviral siRNAs, showed a drastic reduction in virus production.[465] Nevertheless, infected mosquitos harbor low levels of virus-derived siRNAs,[466,467] which may limit the impact of RNAi on antiviral immunity.

The function of RNAi as an antiviral mechanism in mammals is controversial. Cullen and colleagues created genetic Dicer knockout (No-Dice) 293T cell lines, which were unable to generate mature miRNAs, and these cells were infected with many viruses to the same degree as the parental Dicer-positive cell line, suggesting that a large number of viruses, among these several flaviviruses, are not strongly inhibited by nor robustly dependent upon miRNAs for viral propagation.[468] A caveat to be considered is that this result could represent a proclivity of 293T cells and the particular miRNAs expressed in these cells. Indeed, some studies have shown that specific miRNAs can mediate antiviral effects (reviewed in ref 469); for example, one such study shows that human miR532-5p represses WNV infection by downregulating proviral human host factors.[470] Nodamura virus infection of mammalian cells requires a protein known to inhibit RNAi, suggesting that RNAi may play antiviral roles against this virus.[471] Recent work with human enterovirus-71 suggests that very effective anti-RNAi mechanisms prevented the antiviral effect of RNAi from being detected; the same could be true for flaviviruses.[472] It should be noted that some studies

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suggest that specific miRNAs can be proviral for flaviviruses in mammalian cells,[473] which had been also noted for the related hepatitis C virus.[469] It is possible, therefore, that RNAi plays antiviral and proviral roles for flaviviruses in mammalian cells. In summary, the jury is still out on whether or not RNAi plays a major antiviral role, akin to the interferon system, in mammalian cells.

5. VIRAL COUNTERMEASURES TO ANTIVIRAL HOST FACTORS

In the natural mammalian hosts flaviviruses acquired strategies to blunt the antiviral innate immune response, which contributes to efficient viral replication. As discussed above, all viral proteins and the viral genome have a role in viral replication and assembly of viral progeny. In addition, it is likely that the virus invests similarly in restraining host immune pathways, such that many, if not all, viral proteins and the viral genome play an active role as inhibitors of the innate immune response.

Flaviviruses are no exception, and several immune pathways are repressed by flavivirus infection. Only some examples will be provided here, and for a more in-depth examination of this subject we refer the reader to several reviews.[420,425,474] DENV2 viral infection reduces IFN-β or IFN-γ induced STAT1 phosphorylation.[475] DENV2 NS2A, NS4A, or NS4B, when overexpressed, are capable of blocking IFN-β-induced innate immune activation and render normally nonpermissive cells permissive to Newcastle disease virus.[475] Overexpression of DENV2 NS2A, NS4A, or NS4B act synergistically, resulting in maximal inhibition of IFN-β activity when all three viral proteins are expressed simultaneously.[475] Exogenous expression of NS4B was able to block STAT1 phosphorylation induced by either IFN-β or IFN-γ; however, it was unable to block TNF-α activation of an alternative NFκB pathway.[475]

Type I and type II IFN signaling, including concomitant IFN-induced STAT1 and STAT2 phosphorylation and translocation to the nucleus, are inhibited by Kunjin virus or WNV infection and expression of Kunjin or WNV replicons.[476] Expression of Kunjin virus NS2A, NS2B3, NS4A, and NS4B are each able to block IFN-α activity.[476] WNV NS1 expression inhibits TLR3-dependent NFκB activity and downstream cytokine production and relieved the antiviral action of TLR3 on vesicular stomatitis virus.[477] dsRNA-induced cytokine (TNF-α, IL6, and IFN-β) production is reduced by exogenous treatment with recombinant WNV E protein, and the effect is observed in TLR3 or TRIF knockout cell lines,[478] suggesting that E protein impacts steps after activation of TLR3. Interestingly, the E protein activity is linked to E protein glycosylation, such that the greatest anti-innate immune activity is observed by E protein produced by insect cells.[478]

The mechanisms that mediate the observed blocks of innate immune pathways are not completely understood, but in a few cases viral proteins are known to target specific antiviral proteins. For instance, DENV and ZIKV infection reduces total STAT2 protein, and this is likely mediated by NS5 binding STAT2 promoting proteasome-dependent STAT2 degradation.[385,386] In a mouse model of DENV infection, DENV-induced innate immune signaling requires the transcriptional activators IRF3 and IRF7[430] and DENV protease blocks IRF3 activation.[479] Although the viral sensor RIG-I and the adaptor protein MAVS

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were unchanged, the mediator of IRF3 activation (MITA/STING) was degraded in the presence of DENV protease.[479] In another study DENV NS4A was shown to interact with MAVS, preventing its interaction with RIG-I and downstream IRF3 activation and IFN production.[480] Viral protein antagonism of the host antiviral response provides part of the picture.

In mosquitos, only one study directly tested the impact of flaviviral nonstructural proteins on RNAi, and the authors concluded that none of these proteins inhibit RNAi.[481] There is evidence for flaviviral inhibition of the mosquito immune response. Infection with JEV in *Aedes albopictus* C6/36 cells inhibits STAT phosphorylation.[482] DENV infection of mosquitos and mosquito Aag2 cells inhibited some immune signaling molecules and production of antimicrobial peptides.[483,484] As in mammals, evasion of the innate immune system likely requires the combined activity of individual viral factors each dedicated to block specific branches of the innate immune system.

5.1. Subgenomic Flaviviral RNA: A Versatile Countermeasure

A portion of the newly synthesized positive strand viral genomes are degraded, usually by the 5′ to 3′ exoribonuclease, Xrn1, until nuclease resistant structures in the 3′UTR stall Xrn1, leaving a stable noncoding sfRNA.[227] See Figure 1 and discussion of xrRNA structures in section 2.1.3. Although the exact length of the sfRNA differs between flaviviruses, all viruses within the genus are presumed to make an sfRNA.[227] This has not been actually demonstrated for all flaviviruses, especially for those with no known vector or those that infect insects only.

Interestingly, the xrRNA2 structure is absent from DENV4 and YFV, which raises the possibility that these viruses may be employing alternative strategies to mitigate fitness costs associated with replication in alternate hosts.[485,486] The Gamarnik laboratory demonstrated that the evolutionary pressure on xrRNA2 sequences from DENV2 is different in mammalian and mosquito cells.[487] In mosquitos mutations that disrupt Xrn1 resistance appear, resulting in shorter sfRNA species, sfRNA2, sfRNA3, and sfRNA4 (see Figure 1), while in human cells sfRNA1 is the predominant product.[113] Mosquito-adapted DENV2 that produced shorter sfRNAs had a fitness disadvantage and triggered an increased immune response (IFNβ and ISG15 gene expression) in human cells, but the same virus did not have an altered replication rate when grown in mosquito cells. Thus, duplication of xrRNA structures, as suggested by higher phylogenetic similarity between xrRNA1 from different viruses than between xrRNA1 and 2 from the same virus,[487] may enable a quick switch in production of different sfRNA species with host-specific functions. It should be noted that ZIKV adapted to either human or mosquito cells produced the same sfRNA species.[113]

The sfRNA has a role in innate immune evasion. Infection of WT mouse embryonic fibroblasts by a mutant WNV that cannot make sfRNAs is less efficient than comparable infection by WT WNV; however, viral replication is independent of sfRNA synthesis in innate immune compromised (IRF3/IRF7 double knockout) mouse embryonic fibroblasts.[488] Using a lethal mouse model of WNV infection, the mutant WNV was significantly less pathogenic than the WT WNV in normal mice.[488] The pathogenic impact of the mutant virus could be recovered in type I IFN signaling deficient (IFNAR knockout or IRF3/IRF7

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double knockout) mice.[488] Finally, transfected in vitro transcribed sfRNA could dampen the antiviral impact of IFN treatment on an IFN-sensitive viral infection.[488] These data indicate that the sfRNA inhibits innate immunity in different ways (Figure 4).

Subsequent studies established specific mechanisms by which the sfRNA can inhibit innate immunity. The WNV or DENV sfRNA blocked RNA interference in mammalian and mosquito cell lines, suggesting that the sfRNA could be a RNA silencing suppressor.[481,489] Our group showed that DENV2 sfRNA binds the cellular RBPs G3BP1, G3BP2, and CAPRIN1 and inhibits their function as enhancers of the translation of interferon-stimulated mRNAs, leading to a blunted antiviral state.[389] Transfection of in vitro transcribed DENV2 3′UTR RNA blocked protein accumulation of IFN-β-induced IFITM2 but did not block expression of the IFITM2 mRNA, while mutations in the DENV 3′UTR that interrupted sfRNA synthesis increased the IFN sensitivity of a DENV replicon.[389] Interestingly, DENV3, YFV, and WNV sfRNAs do not bind G3BP1, G3BP2, and CAPRIN1[389] but still have anti-immune activity.[488] This finding led us to propose that the sfRNA is a versatile noncoding RNA that can rapidly evolve different sequences and structures, which can bind and sequester different host RBPs.[103] Indeed, the first domain of the sfRNA, which appears to be most important for its anti-immune function,[389] is the so-called flavivirus variable region (VR). The VR and the two xrRNA1 and xrRNA2 structures within it are not required for the mechanics of viral replication and thus are not as constrained as sequences such as the 3′SL. Given the disproportionate importance of RBPs in innate immunity the ability of sfRNAs to bind and sequester different RBPs as it evolves is likely an effective mechanism to sample alternative ways to disable the immune system.[490] Thus, we propose that the sfRNA provides a potent strategy that can use many different tactics.

The influence of the sfRNA on evolution of viral disease within the population has recently been described. DENV2 strain PR-2B replaced DENV2 strain PR1 as the most common DENV2 strain circulating in Puerto Rico after an epidemic in 1994.[395] In vitro analysis of these two viruses revealed that the ratio of DENV2 sfRNA to genomic RNA ratio was increased for the PR-2B strain.[395] The PR-2B sfRNA was able to attenuate the antiviral activity of IFN treatment on DENV replication to a greater degree than the PR1 sfRNA, and sfRNA binding to cellular factors disrupted innate immune activation which may have contributed DENV2 PR-2B aggressive spread in the population.[395]

In mosquitos, sfRNA is produced through the same interaction with Xrn1, although sfRNA quantity and size may differ between human and mosquito cell.[106,110,113,485,491] It was recently demonstrated that sfRNA could increase mosquito transmission.[492,493] WNV mutants that were depleted of the longer species of sfRNAs produced a lower infection rate of mosquito saliva, a proxy for transmission.[492] DENV2 isolates that produced lower sfRNA quantity in salivary glands had also a lower saliva infection rate.[493] Using infectious clones, it was further demonstrated that the DENV2 3′UTR sequence determined the sfRNA quantity in salivary glands. Mosquito transmission is determined by the immune response (reviewed in ref 447). The multiple domains in the sfRNA may act as a decoy for Dicer2 and limit RNAi efficiency. Mosquitos infected with a sfRNA-deficient WNV mutant had a higher dsRNA-mediated RNAi efficiency than when infected with the wild type;[489] however, lower RNAi efficiency of the wild type was only 3-fold less and highly variable. In

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mosquitos, sfRNA was shown to be processed into siRNA; however, the presence of sfRNA did not alter populations of siRNAs mapping to the rest of the viral genome, arguing against an impact of sfRNA on RNAi.[492] Other studies tested the impact of sfRNA on RNAi in mosquitos, but many questions remain on its importance (reviewed in Yeh and Pompon, in press). Alternatively, DENV2 infectious clones that produced higher sfRNA quantity in salivary glands due to two 3′UTR substitutions inhibited some components of the Toll pathway exclusively in this tissue.[493]

The viral sfRNA may interfere with mammalian post-transcriptional gene regulation and innate immune activation. The viral sfRNA may block the mosquito RNAi machine as well. Although the role of the sfRNA remains an active area of interest for the flavivirus field, the evidence thus far suggests that viral genome-derived sfRNA is a valuable viral tool that contributes to reducing antiviral defenses.

6. CONCLUDING REMARKS

In this review, we attempted to comprehensively cover the biochemistry and molecular biology of flaviviruses, and in some sections we emphasized the RNA biology of these viruses. We hope this review provides a good window for those who want to explore the literature about these fascinating and important human pathogens.

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Biographies

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Kuo-Chieh Liao is a 2012 Ph.D. graduate of the Mogridge group at the University of Toronto, Canada. His thesis focused on characterizing the interaction between host mammalian cells and bacterial toxins. He obtained his bachelor degree at National Chiao Tung University in Taiwan. He is currently a senior research fellow in the Pompon–Garcia-Blanco group at Duke NUS Medical School in Singapore. He studies the roles of host RNA binding proteins in dengue pathogenesis.

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Ruben Soto-Acosta received his B.A. degree in Chemistry, Bacteriology, and Parasitology from the Autonomous University of Chihuahua, Mexico. He holds a M.S. degree in Pharmacology and a Ph.D. degree in Infectomic and Molecular Pathogenesis under the direction of Dr. R. M. del Angel from the Center of Research and Advanced Studies from the National Polytechnic Institute (CINVES-TAV) in Mexico City, Mexico. In 2015, he joined the Bradrick–Garcia-Blanco laboratory at the University of Texas Medical Branch as a postdoctoral fellow, where he is analyzing RNA binding proteins interacting with ZIKV sfRNA and its implications in ZIKV replication.

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Rafael Kroon Campos studied Biology from Pontificia Universidade Catolica de Minas Gerais, Brazil, where he received his B.S. degree working on coinfection of vaccinia viruses. He obtained his M.S. of Microbiology degree from the Universidade Federal de Minas Gerais, Brazil, working on mimiviruses and their virophages, work which was awarded best thesis of the Microbiology Department. He is currently a Ph.D. degree student at Duke University with Drs. Shelton S. Bradrick and Mariano A. Garcia-Blanco as mentors and works on human factors required for flavivirus infection. His research interests relate to molecular biology, virology, and RNA translation.

Shih-Chia Yeh graduated from the National Taiwan University with her Ph.D. degree in Entomology. After receiving her Ph.D. degree, she worked as a postdoctoral fellow in the Institute of Zoology at the National Taiwan University to do white spot syndrome virus (WSSV) research and then moved to National Chung Hsing University to do insecticide resistance study. Currently, she is a senior research fellow in the Pompon–Garcia-Blanco laboratory at Duke-NUS Medical School, Singapore, where she studies Dengue virus transmission by mosquitos.

Geraldine Schott-Lerner is a graduate student at the University of Texas Medical Branch pursuing her Ph.D. degree in Biochemistry and Molecular Biology with Drs. Shelton S. Bradrick and Mariano A. Garcia-Blanco as mentors. She obtained her B.S. degree in Microbiology at the University of Houston-Downtown. Her current research focus is the study of the regulation of alternative splicing of the Interleukin 7 receptor transcripts and its implications in multiple sclerosis.

Julien Pompon obtained his Ph.D. degree at the University of New Brunswick in Canada and received a fellowship from the Fondation pour la Recherche Médicale (FRM) to do a postdoctoral fellowship at the Institute of Molecular and Cellular Biology (IMBC) in Strasbourg, France. He studied mosquito immunity in the context of pathogen–mosquito interactions. He went on to be a senior research fellow with Dr. Mariano A. Garcia-Blanco at Duke-NUS Medical School, where he is now Assistant Professor of Emerging Infectious

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Diseases and co-PI of the Pompon–Garcia-Blanco laboratory. His work focuses on molecular entomology to study mosquito transmission of viruses.

October M. Sessions received his Ph.D. degree in Molecular Genetics and Microbiology in 2009 from Duke University with Dr. Mariano A. Garcia-Blanco as mentor. He studied the host factors required for dengue virus propagation. After his graduate studies, he continued his study of dengue virus as a postdoctoral fellow in the laboratory of Dr. Ooi Eng Eong at Duke-NUS Medical School, where he characterized the host transcriptomic response to dengue viral infection. In 2014, he became Assistant Professor at Duke-NUS. The current focus of his laboratory is deciphering the mechanisms that define the pathogenic potential of flaviviruses.

Shelton S. Bradrick earned his Ph.D. degree in Pathology and Microbiology at the University of Nebraska Medical Center, where he studied the pathogenesis of coxsackievirus B3 heart infection under the direction of Dr. Jose R. Romero. He subsequently joined the laboratory of Dr. Matthias Gromeier for postdoctoral training at Duke University Medical Center, where he studied hepatitis C virus (HCV). He continued his studies on HCV and interferon responses with Dr. Mariano A. Garcia-Blanco at Duke University with the support of a K01 Award from the National Institute of Diabetes and Digestive and Kidney Diseases. He currently focuses his research on molecular host–pathogen interactions involving mosquito-borne flaviviruses at the University of Texas Medical Branch in collaboration with Dr. Garcia-Blanco.

Mariano A. Garcia-Blanco obtained his M.D. and Ph.D. degrees in Molecular Biophysics and Biochemistry from Yale University, New Haven, CT, with Dr. Peter Lengyel as mentor. He did a postdoctoral fellowship with Dr. Phillip A. Sharp at the Massachusetts Institute of Technology focusing on RNA binding proteins that act on premessenger RNA splicing. He spent the first 24 years of his independent research career at Duke University, where he was Professor of Molecular Genetics and Microbiology, and Medicine, Charles D. Watts Professor, and Director of the Center for RNA Biology. In 2014, he became Professor and Chair of Biochemistry and Mildred Hajek Vacek and John Roman Vacek Distinguished Chair at the University of Texas Medical Branch. There he collaborates with Dr. Bradrick to study the interface between host and virus and the post-transcriptional regulation of immune pathways. Since 2006 he has been Professor of Emerging Infectious Diseases at Duke-NUS Medical School, Singapore, where he coleads a laboratory with Dr. Pompon to study host–flavivirus interactions with an emphasis on mosquitoes. He was a Sackler Foundation Scholar and was elected to the Association of American Physicians, fellow of the AAAS, and fellow of the American Academy of Microbiology.

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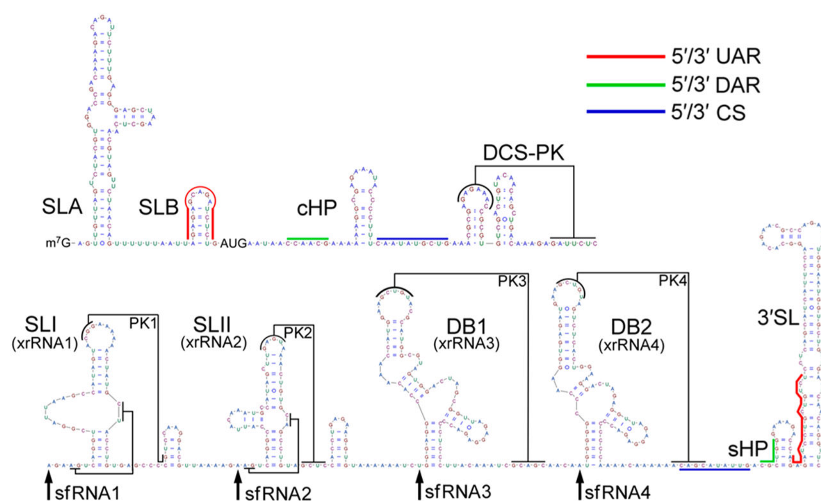

Figure 1.

Secondary and tertiary structures within the DENV2 (New Guinea C) genome. (Top) Structures within the 5′UTR and proximal capsid-coding region are shown. 5′cap structure (m7G) and AUG start codon are indicated in black. Sequences that participate in long-range tertiary interactions with sequences in the 3′UTR are highlighted with colored lines. (Bottom) Known 3′UTR structures are shown. Arrows indicate 5′ ends of identified subgenomic flavivirus (sf)RNAs.

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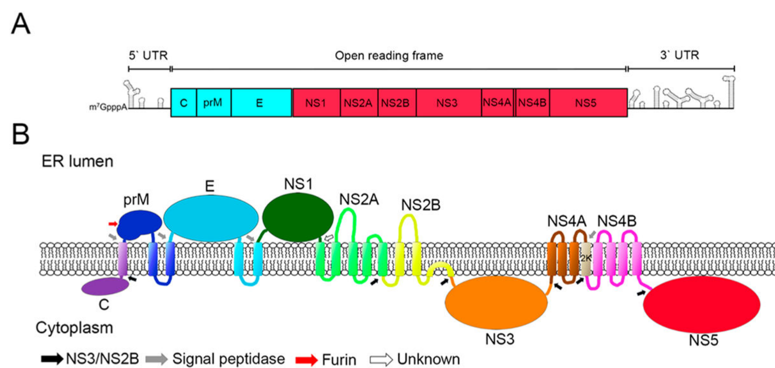

Figure 2.

Flaviviral genome and polyprotein. (A) Flaviviral genome. Flaviviruses have a single-stranded (+) RNA genome of approximately 11 kb. Genome is capped but not polyadenylated. It encodes three structural (blue) and seven nonstructural (red) proteins which are translated from a single ORF. In between NS4A and NS4B, the genome also encodes a small peptide of 2 kDa (2K peptide). 5′ and 3′ UTRs are known to have complex structure, with several hairpins, which are important for translation, RNA synthesis, and sfRNA formation. (B) Flaviviral polyprotein topology and predicted transmembrane domains. Flavivirus polyprotein is integrated into the ER membrane. Viral proteins prM, E, and NS1 are mainly on the luminal side and C, NS3, and NS5 on the cytoplasmic side. Proteins NS2A, NS2B, NS4A, and NS4B have several transmembrane domains spanning across the ER, and thus, large parts of these proteins are on each side and on the ER membrane. 2K peptide is entirely inserted in the ER membrane. Polyprotein is cleaved co- and post-translationally at multiple sites. Cleavages on the cytoplasmic side are done by the viral protease NS3 and its cofactor NS2B, and cleavages on the ER lumen side are done by the signal peptidase complex. Polyprotein also has an additional furin protease cleavage in prM that gives rise to the mature M protein in the Golgi and one additional site between NS1 and NS2A that is cleaved by an unknown enzyme.

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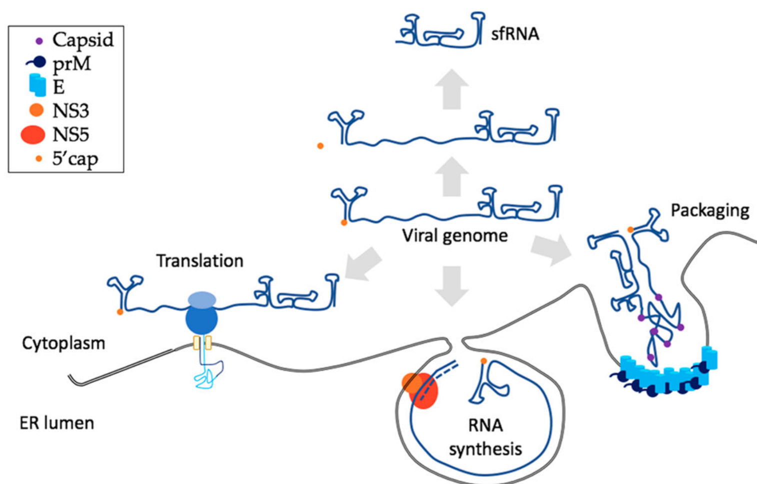

Figure 3.

Multiple roles of the flavivirus genome. Viral genomes are translated, used as templates for negative strand synthesis, packaged into virions, and partially degraded to form subgenomic flaviviral RNAs (sfRNA). First three of these roles take place in association with the ER.

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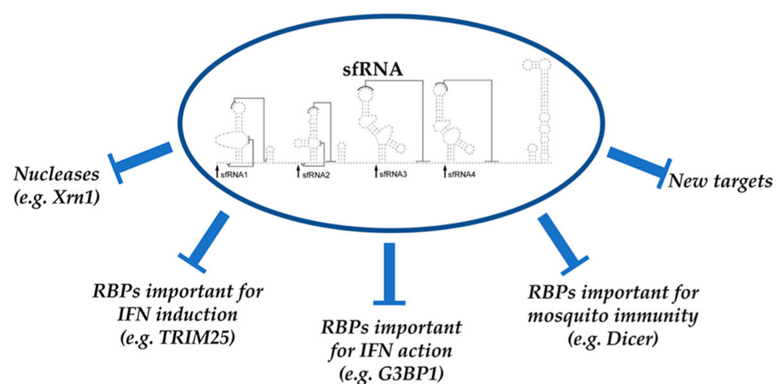

Figure 4.
sfRNA can impact many components of the immune system in mosquitos and humans. sfRNA sequesters Xrn1, leading to changes in the cellular transcriptome, binds to TRIM25, dampening IFN production, soaks up RBPs that are required for efficient ISG mRNA translation, and inhibits RNAi (see text for references).