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# Structure-guided paradigm shifts in flavivirus assembly and maturation mechanisms

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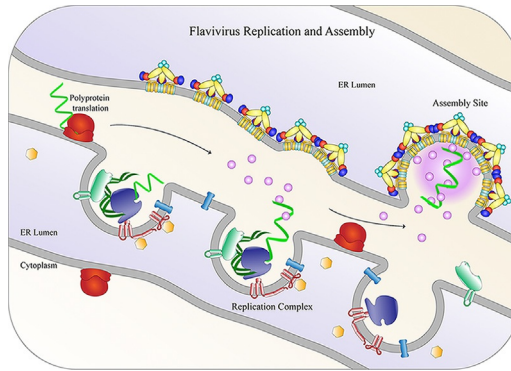
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## Abstract

The flavivirus genus encompasses more than 75 unique viruses, including dengue virus which accounts for almost 390 million global infections annually. Flavivirus infection can result in a myriad of symptoms ranging from mild rash and flu-like symptoms, to severe encephalitis and even hemorrhagic fever. Efforts to combat the impact of these viruses have been hindered due to limited antiviral drug and vaccine development. However, the advancement of knowledge in the structural biology of flaviviruses over the last 25 years has produced unique perspectives for the identification of potential

therapeutic targets. With particular emphasis on the assembly and maturation stages of the flavivirus life cycle, it is the goal of this review to comparatively analyze the structural similarities between flaviviruses to provide avenues for new research and innovation.



## Abbreviations

<b>ADE</b>	antibody-dependent enhancement
<b>C</b>	capsid protein
<b>C + <math>\alpha</math>5</b>	capsid protein plus helix $\alpha$ 5
<b>cryo-EM</b>	cryo-electron microscopy
<b>DAX</b>	death domain associated protein
<b>DENV</b>	dengue virus
<b>DI</b>	E domain I
<b>DII</b>	E domain II
<b>DIII</b>	E domain III
<b>E</b>	envelope protein
<b>ER</b>	endoplasmic reticulum
<b>ESCRT</b>	endosomal sorting complexes required for transport
<b>HCV</b>	hepatitis C virus
<b>HIV</b>	human immunodeficiency virus
<b>HMAb</b>	human monoclonal antibody
<b>hnRNP k</b>	heterogeneous nuclear ribonucleoprotein K
<b>ILVs</b>	intraluminal vesicles
<b>JEV</b>	Japanese encephalitis virus
<b>KUNV</b>	Kunjin virus
<b>LRP1</b>	low-density lipoprotein receptor-related protein 1
<b>M</b>	membrane protein
<b>MVBs</b>	multivesicular bodies
<b>NC</b>	nucleocapsid core
<b>NCL</b>	nucleolin
<b>NS</b>	non-structural proteins
<b>prM</b>	pre-membrane protein
<b>SLEV</b>	Saint Louis encephalitis virus

<b>SVP</b>	subviral particle
<b>TBEV</b>	tick-borne encephalitis virus
<b>TGN</b>	<i>trans</i> -Golgi network
<b>TMD</b>	transmembrane domain
<b>USUV</b>	Usutu virus
<b>VLP</b>	virus-like particle
<b>WHO</b>	World Health Organization
<b>WNV</b>	West Nile virus
<b>WT</b>	wild type
<b>YFV</b>	yellow fever virus
<b>ZIKV</b>	Zika virus



## 1. Introduction

Flaviviruses, such as dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV), pose a significant threat to human health with a reported 40% of the global population (3.1 billion) at risk of infection each year (Brady et al., 2012). DENV alone accounts for 390 million infections annually with almost one quarter of all cases showing symptoms, including dengue hemorrhagic fever or dengue shock syndrome (Bhatt et al., 2013). There are four distinct serotypes of DENV (DENV1–4), and previous studies have hypothesized that severe symptoms such as hemorrhagic fever or shock syndrome are the result of sequential infection with multiple serotypes (Kuhn et al., 2002). Studies have found that sub-neutralizing antibodies that were cross-reactive between DENV serotypes actually increased the number of cells infected and facilitated higher virus production through a phenomenon known as antibody-dependent enhancement (ADE) (Kou et al., 2011; Quinn et al., 2013). Mechanistically, ADE increases viral uptake into FcR-bearing macrophages (Flipse et al., 2016) and also increases the expression of early host dependency factors, resulting in increased viral infection and production (Chan et al., 2019). Due to the conservation of both protein sequence and structural epitopes across other flaviviruses, it has also been shown that ADE can facilitate the increased infectivity of ZIKV in individuals previously vaccinated for YFV (Malafa et al., 2020). While the intricacies of ADE remain obscure, they also present a great opportunity for new discoveries in flavivirus biology. For more information on ADE in flaviviruses, readers are encouraged to see an excellent review by Kulkarni (2019).

ZIKV was first isolated from humans in 1947 (MacNamara, 1954) and remained in relative obscurity until it caused major epidemics on Yap Island in 2007 (Duffy et al., 2009), in Oceania in 2013–14 (Cao-Lormeau et al., 2014), Brazil in 2015 (Zanluca et al., 2015) and finally throughout the Americas in 2016 when the World Health Organization (WHO) declared ZIKV “a public health emergency of international concern” (WHO, 2016). Today, ZIKV persists as a prominent threat to public health with thousands of cases reported annually in South America alone (Black et al., 2019). Similarly, WNV has become endemic in the Western hemisphere with an average of more than 2000 cases per year in the United States (Hasan et al., 2018). Both JEV and YFV have been successfully controlled through vaccination, but diseases resulting from these viruses are still prominent worldwide (Lindenbach et al., 2013). The WHO has estimated that roughly 200,000 global cases of YFV occur each year, with about 30,000 resulting in death (WHO, 2019a). In addition, the WHO has estimated there to be approximately 70,000 annual cases of JEV worldwide, with roughly 20,000 deaths (WHO, 2019b).

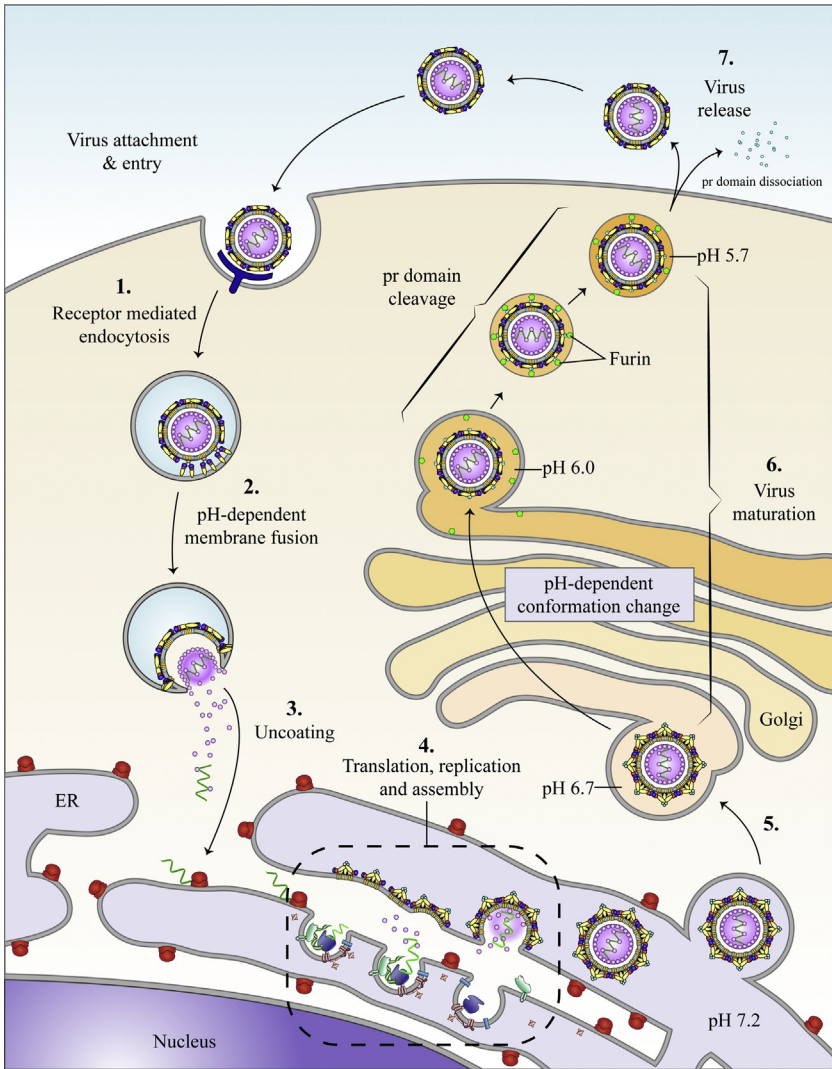
Of the 53 unique species reported to date in the flavivirus genus (ViPR, 2020) (from the Latin word *flavus*, meaning yellow, due to the jaundice induced by YFV), most are transmitted via mosquitos or ticks. With global temperatures on the rise, the geographical reach of flaviviruses is increasing due to the broadening habitats of their insect vectors (Barrows et al., 2018). Efforts to combat the spread of most flaviviruses have been limited to simple contact prevention measures, with mosquito netting and protective clothing initiatives being the most widely utilized tactics. Unfortunately, with the increased restriction of pesticide use in developing countries, as well as an increase in mosquito resistance to available pesticides, efforts to control the spread of flavivirus vectors has been diminishing over the last 30 years, and with it an increase in flavivirus infections has been observed (Fotakis et al., 2020; Wang et al., 2013). While YFV and JEV can both be effectively combatted through vaccination, there are no vaccines for WNV or ZIKV, and the single available DENV vaccine (from Sanofi Pasteur) has recently demonstrated untoward side effects through ADE, limiting its use and global acceptance (Barrows et al., 2018). Other DENV vaccines from the National Institute for Allergy and Infectious Diseases and Takeda Pharmaceutical Company are currently in clinical trials and are showing promising results (Biswal et al., 2019; Whitehead et al., 2017).

Though there have been many studies aimed at the development and identification of small molecule inhibitors capable of preventing and treating

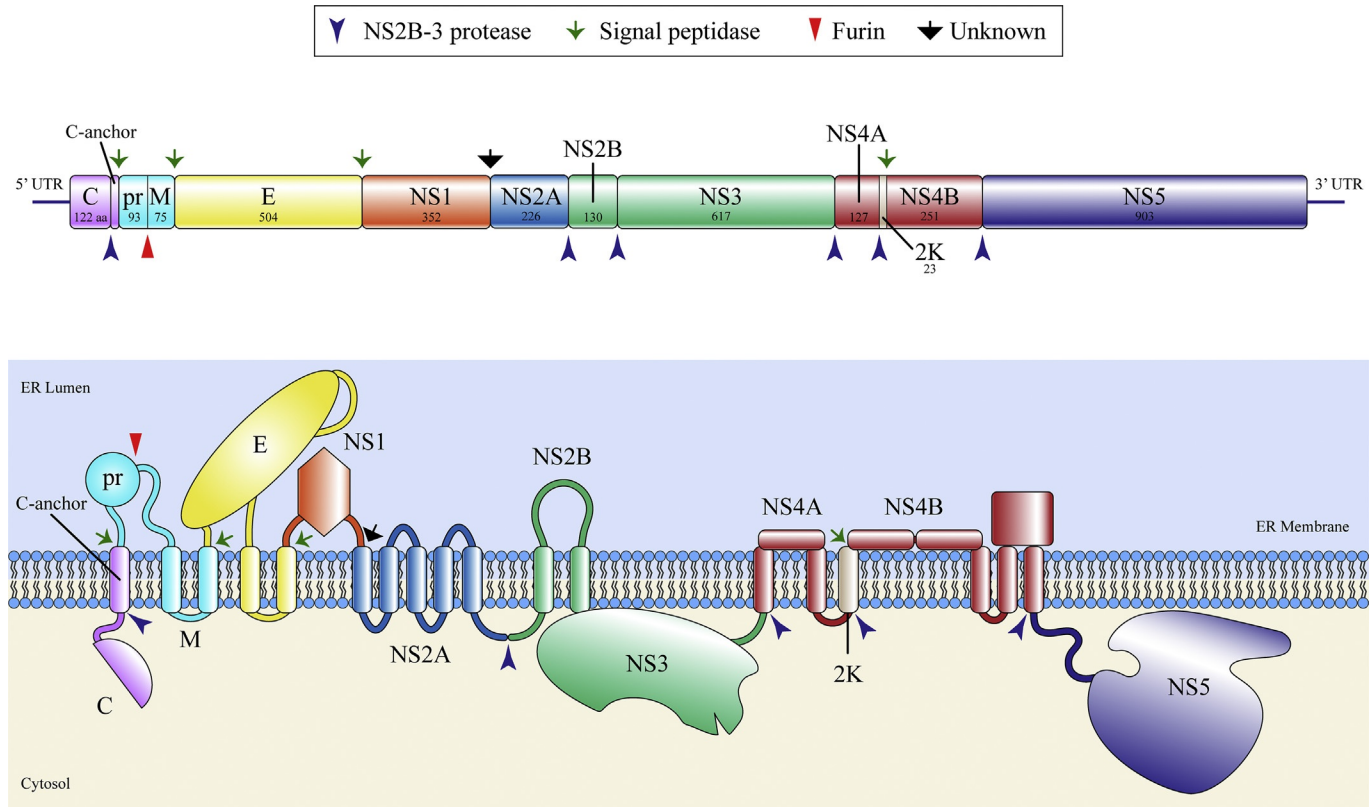
flavivirus infections (reviewed extensively in [Pierson and Diamond, 2020](#)), there are currently no antiviral therapeutics clinically approved for the treatment of flavivirus infected individuals, nor for use in preventative measures in at risk populations. There exists a significant need to produce therapeutics and vaccines capable of reducing the spread of flaviviruses. However, such a goal requires greater understanding of flavivirus biology and, more specifically, flavivirus structure. Therefore, it is the focus of this review to analyze the current available knowledge of the assembly and maturation phases of the flavivirus life cycle to provide avenues for new research and innovation.

## 1.1 Flavivirus life cycle

All flaviviruses are enveloped viruses roughly 50 nm in diameter that contain a positive sense, single stranded RNA genome ([Lindenbach et al., 2013](#)). Like most enveloped viruses, flaviviruses utilize a myriad of cell attachment mechanisms to gain access into a host cell and initiate infection, including glycan and phosphatidylserine receptor-mediated endocytosis as well as antibody enhanced endocytosis ([Perera et al., 2008](#); [Sirohi and Kuhn, 2017](#); [Smit et al., 2011](#)) ([Fig. 1](#)). Once the virus attaches to the host cell it gains entry via clathrin-mediated endocytosis ([Perera et al., 2008](#); [Smit et al., 2011](#)), and the membrane of the virus fuses with the membrane of the endosome during pH-dependent conformational changes of the viral envelope (E) proteins ([Mukhopadhyay et al., 2005](#)). This in turn releases the viral genome from the nucleocapsid core (NC) of the virus into the cell cytoplasm where it is transported to the endoplasmic reticulum (ER) to begin the process of viral protein translation and genome replication ([Garcia-Blanco et al., 2016](#)). The flavivirus +ssRNA genome encodes ten viral proteins ([Fig. 2](#)). These include the three structural proteins—envelope protein (E), pre-Membrane/Membrane protein (prM/M) and capsid protein (C)—and the seven non-structural (NS) proteins—NS1, NS2A/B, NS3, NS4A/B and NS5 ([Lindenbach et al., 2013](#)). While the structural proteins are used in the production of infectious particles and transport of the viral genome, the non-structural proteins aid in the replication of the virus genome and evasion of the host's immune defenses ([Barrows et al., 2018](#)). The virus assembles at the endoplasmic reticulum (ER) as an immature particle ([Zhang et al., 2003b](#)) and is then trafficked through the trans-Golgi network (TGN) where it undergoes pH-dependent maturation ([Junjhon et al., 2014](#)). During the maturation phase, the pr domain of



**Fig. 1** Flavivirus life cycle: Flaviviruses initiate infection through a myriad of interactions with host cell receptors. (1) Once attached, the virus is internalized into the cell via clathrin-mediated endocytosis. (2) The E glycoproteins then undergo conformational changes to allow fusion between the viral envelope and the endosomal membrane, which ultimately leads to the release of the RNA into the cytoplasm. (3) The viral RNA genome is released and transported to the ER where it is translated. (4) Viral RNA replication occurs in virus-induced membrane invaginations known as replication complexes. These structures shield viral replication products and are spatially congruent with sites of virion assembly along the ER membrane. (5) Immature virions are assembled and bud into the ER lumen and are subsequently trafficked through the TGN. (6) The low pH of the TGN promotes virion maturation through glycoprotein conformational changes and subsequent cleavage of the pr domain from the prM protein. (7) After furin cleavage, the secretory vesicle containing the virion fuses with the plasma membrane, where the pr domain is released and the particle emerges into the extracellular environment as a fully mature and infectious virus.



**Fig. 2** Flavivirus genome and polyprotein domain structure: The ~11 kb flavivirus open reading frame genome (top) is translated into a single polyprotein along the ER membrane (bottom). This polyprotein is processed into ten functional proteins by both host and viral proteases (protease cleavage sites are labeled with arrows), generating three structural proteins (C, pr/M/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). As the name suggests, the structural proteins function to encapsulate and protect the viral RNA, forming an enveloped virion. In contrast, the non-structural proteins facilitate RNA genome replication and assist in cellular immuno-evasion. The amino acid length of each protein is indicated by the small number within each gene “box” (top).



prM is cleaved by the host protease furin, and the particle is ultimately released from the cell as a fully mature infectious virus particle (Elshuber et al., 2003).



## 2. Flavivirus polyprotein translation and processing

The flavivirus genome contains a single open reading frame that is translated into a singular, large polyprotein. During translation, the polyprotein is woven through the ER, where both the structural and non-structural proteins are anchored by various signal sequences and transmembrane domains (Lindenbach et al., 2013) (Fig. 2). While current consensus places the site of flavivirus genome translation at the ER membrane, it is entirely possible that translation could begin in the cytosol. However, the elongation ribosome would likely stall when the signal recognition particle (SRP) binds to the signal sequence of C protein after it emerges from the ribosome exit tunnel. Indeed, studies using RNAi and CRISPR loss of function screening have found components of the SRP-translocon pathway to be important for virion propagation (Krishnan et al., 2008; Marceau et al., 2016; Zhang et al., 2016). Regardless of whether translation begins on the ER or in the cytoplasm, it has been shown that polyprotein synthesis is certainly associated with the ER membrane in a CAP-dependent manner (Barrows et al., 2018; Boulton and Westaway, 1976; Markoff, 1989; Ng et al., 1983; Ruiz-Linares et al., 1989). Interestingly, no studies to date have observed full-length polyproteins as a single product during flavivirus infection, suggesting that processing of the polypeptide is quite swift. For example, immunoprecipitation studies by Chambers et al. (1990a) targeting YFV C, prM and E proteins found that processing of the polyprotein can be observed in as little as 40 min after transfection.

The mature viral proteins used in the replication and assembly of infectious virus particles are produced through efficient luminal and cytosolic processing by both viral and host proteases (Fig. 2). The flavivirus protein NS3 undergoes autocatalytic processing to separate itself from NS4A and subsequently cleaves NS2B from NS2A to form the viral protease complex NS2B-3 (Erbel et al., 2006; Kouretova et al., 2017). This complex also cleaves the C and C-anchor, NS4A-B and NS4B-NS5 junctions on the cytosolic portion of the polyprotein. Additionally, it has been shown that the host protease signal peptidase cleaves the C-anchor and prM, prM-E, E-NS1, and NS4A-B junctions on the ER luminal portion of the

polyprotein (Amberg et al., 1994; Amberg and Rice, 1999). The protease responsible for cleavage of the luminal junction between NS1 and NS2A has not yet been identified (Falgout et al., 1989).

As mentioned, cleavage between the C and prM proteins requires a tandem effort by the NS2B-3 protease and host signal peptidase (Fig. 2). This produces a soluble C protein on the cytosolic side of the ER capable of interacting with the viral genome during assembly but leaves a small portion of the immature C protein in the ER membrane. This small portion of C, otherwise known as C-anchor (Byk and Gamarnik, 2016), C-helix  $\alpha 5$  (Tan et al., 2020), or C-signal peptide (Lobigs et al., 2010), has previously been thought to play no role in the formation of virus particles and functioned only for polyprotein translocation and organization during translation. However, a recent study in ZIKV has shown that the cleavage of C-anchor by NS2B-3 might not be as efficient as once thought, and the short peptide is somehow removed from the membrane and retained in a secondary sub-population of mature cytosolic C protein, ultimately aiding in the assembly and budding of immature virions (Tan et al., 2020—explored in greater detail in Section 4 of this review). (Please note: the specific nomenclature of “C-anchor” is used herein to generally describe the signal peptide of C protein (Fig. 2). However, when discussing the models or results of other groups, the nomenclature of C-anchor will be changed to the nomenclature used by those cited groups.)

Due to the hydrophobic signal peptide on the C-terminus of C protein (C-anchor), the prM protein is translocated into the luminal side of the ER membrane during translation. The prM protein contains C-terminal transmembrane domains (TMDs) made up of two transmembrane helices forming a hairpin loop. Together, these helices function as an ER membrane anchor for the mature protein after host signal peptidase cleavage (Lindenbach et al., 2013). The prM TMDs contain both a stop-transfer sequence, and a signal sequence, which translocates the downstream E protein into the ER lumen during translation. Similarly, E protein contains two transmembrane helices that make up its C-terminal TMDs, with both a stop-transfer sequence and a signal sequence also present. Previous studies have shown that only a few minutes after polyprotein translation and protease cleavage, the prM and E proteins form a stable heterodimer anchored to the ER membrane (Allison et al., 1995; Konishi and Mason, 1993; Lorenz et al., 2002). Interestingly, the presence of TMD anchors made of two transmembrane helices on the C-terminus of all flavivirus E proteins is quite unique among class II viral fusion proteins, all of which contain a TMD

anchor made of only one transmembrane helix (Langosch et al., 2007; White et al., 2008). Once processed, prM and E are free to begin forming virion envelopes along the ER membrane and C is free to complex with viral genomic RNA to form the nucleocapsid core (NC). Together, these structures subsequently combine to form immature virions.

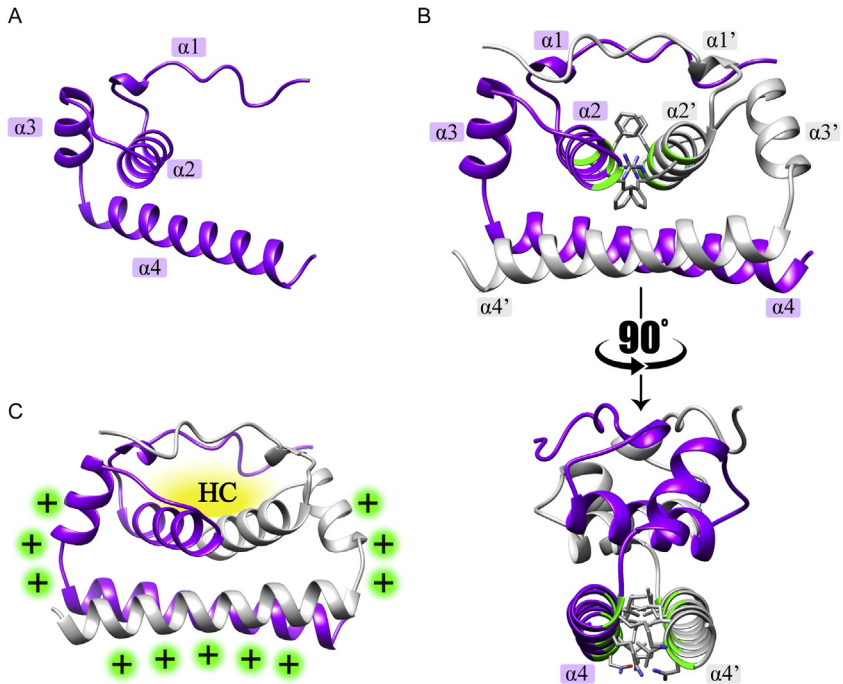
While the processing and maturation of flavivirus structural proteins is detailed above, discussion about the same processes for the flavivirus non-structural proteins is outside the scope of this review. For a more in depth look at non-structural protein processing and maturation, readers are referred to a recent review from Barrows et al. (2018).



### 3. Flavivirus structural proteins: Structure and function

#### 3.1 Capsid (C) protein

The flavivirus C protein is composed exclusively of  $\alpha$ -helices and is roughly  $\sim 122$  residues in length ( $\sim 100$  residues when excluding C-anchor) (Ma et al., 2004). To date, the crystal structures of C protein have been identified for JEV (Poonsiri et al., 2019), ZIKV (Shang et al., 2018), and WNV (Dokland et al., 2004), with the structure of DENV C protein determined using nuclear magnetic resonance spectroscopy (Jones et al., 2003). For each of these protein structures, only residues  $\sim 20$ – $100$  have been clearly resolved due to the flexibility of the N-terminal region of C protein (residues 1–19) and cleavage of the C-anchor by the NS2B-3 protease during polyprotein processing (residues  $\sim 101$  onward). Interestingly, all solved C protein structures share very high structural and charge distribution homology with one another regardless of their highly divergent amino acid sequences (Faustino et al., 2019). The monomeric form of C is exclusively  $\alpha$ -helical with four distinct helices (helix  $\alpha 1$ – $\alpha 4$ ) forming a right-handed bundle (Fig. 3A). When comparing the known structures of C proteins, helix  $\alpha 1$  has been shown to be the most flexible and has been postulated to use this flexibility to drive membrane association during assembly (Faustino et al., 2019). C protein monomers were shown to readily dimerize in solution (Pong et al., 2011), which is assumed to be the basic building blocks of nucleocapsid cores (NCs) during the complexing of C with genomic RNA for virus assembly (Kiermayr et al., 2004). As shown in Fig. 3B, the C dimer is formed through interacting hydrophobic interfaces between the “core” helices  $\alpha 2$ – $\alpha 2'$  and the “bottom” helices  $\alpha 4$ – $\alpha 4'$ , where  $\alpha 2'$  and  $\alpha 4'$  are the helices from the second monomer.



**Fig. 3** Flavivirus capsid protein: (A) A single capsid monomer (ZIKV C protein—PDB ID: 6C44) with each helix ( $\alpha 1$ – $\alpha 4$ ) labeled within the structure. (B) Hydrophobic interactions (residues highlighted in green) between helices  $\alpha 2$  and  $\alpha 2'$  (top) and helices  $\alpha 4$  and  $\alpha 4'$  (bottom—rotated ninety-degrees horizontally) stabilizing the ZIKV capsid dimer. The helices of one capsid protomer within the dimer ( $\alpha 1$ – $\alpha 4$ ) are colored in purple, while the helices of the other capsid protomer ( $\alpha 1'$ – $\alpha 4'$ ) are colored in light gray. (C) Side view of the ZIKV capsid dimer. The positive exterior of the dimer is shown by the green (+) symbols, which has been hypothesized to interact with the viral RNA during assembly. The central hydrophobic cleft (HC), which has been shown to interact with lipids, is indicated in yellow. C-anchor is not present in this structure of C protein. For an understanding of the C protein structure with C-anchor included, readers are referred to [Tan et al. \(2020\)](#).

The flexible N-terminal region (first 20 residues) is intrinsically disordered, highly positively charged, and extends away from the monomer core in most C protein structures, whereas the internal connecting region between helices  $\alpha 1$  and  $\alpha 2$ , as well as all of helix  $\alpha 2$ , are mostly hydrophobic ([Ma et al., 2004](#); [Samsa et al., 2009, 2012](#)). This creates a “hydrophobic cleft” ([Fig. 3C](#)) in the central core of the C dimer that may allow the protein to interact with lipids. [Samsa et al. \(2009\)](#) found that

DENV C proteins were capable of binding to ER derived organelles known as lipid droplets, and suggested that such a process could be an essential means of C protein sequestration during early infection to prepare the protein for genome encapsulation at a later stage. The group was able to identify two residues within C protein, namely L50 and L54, that mediated lipid association. They also observed that mutation of these residues resulted in a reduced association of C protein with lipid droplets as well as a reduction of infectious virus particles when compared to wild type (WT). Such a finding suggests that interactions between lipids and C proteins through the internal hydrophobic cleft might also aid in the formation of virus particles during assembly, and not only as a means of sequestration. However, no empirical data demonstrating the necessity of specific C-lipid interactions during flavivirus assembly has been produced.

Surrounding the hydrophobic cleft of the capsid dimer is a relatively large hydrophilic region made up of the  $\alpha 3$  and  $\alpha 4$  helices (Fig. 3C). These faces contain numerous basic lysine and arginine residues resulting in a nearly uniform positive exterior. This positive surface has been postulated to interact with negatively charged nucleic acids and is thought to bind the viral genomic RNA during viral packaging and assembly. Indeed, several previous studies have shown that C protein dimers are capable of indiscriminately binding a range of nucleic acids through interactions with their phosphate backbones, including single or double stranded DNA or RNA (Byk and Gamarnik, 2016; Li et al., 2018; Samuel et al., 2016). However, no studies have confirmed the binding of genomic RNA by this surface during virus assembly. In addition to the hydrophilic faces made by helices  $\alpha 3$  and  $\alpha 4$ , the intrinsically disordered N-terminal domain is also positively charged and was unresolved in any of the published crystal structures because of its flexibility. The presence of a disordered, positively charged region on the N-terminus of C protein is reasonable since numerous other nucleic acid binding proteins also contain disordered, positive peptide regions. Two examples are the lysine-rich regions of eukaryotic histones and the arginine-rich capsid regions of non-enveloped icosahedral viruses such as the human astrovirus (Erler et al., 2014; Krishna, 2005; Requiao et al., 2019). An alanine scanning mutagenesis study from Samsa et al. (2012) demonstrated that efficient DENV virus production relied specifically on the conservation of the basic regions in the N-terminus of C, and not the conservation of specific amino acids. Moreover, while flaviviruses share low sequence homology in their C proteins, the two basic motifs in the N-terminal region are highly conserved (Faustino et al., 2019). Altogether, these data suggest that the positive nature of

the C protein N-terminal region might serve as an additional conserved nucleic acid binding component during genome packaging and particle assembly.

The main role of C protein is to interact with and encapsidate the viral genome prior to assembly and packaging. However, C protein has been shown to participate in numerous other functions outside of viral genome interactions that effectively aid (and sometimes hinder) viral propagation. The most noteworthy feature of C protein cellular localization, aside from its association with lipid droplets, is its ability to localize in the nucleus of flavivirus-infected cells (Bhuvanakantham et al., 2009; Mori et al., 2005; Tsuda, 2006; Wang et al., 2002; Westaway et al., 1997). This observation indicated that C protein could migrate away from sites of replication during flavivirus infection where it could participate in a myriad of secondary functions. A study by Bhuvanakantham et al. (2009) found that the nuclear transport protein importin- $\alpha$  was able to bind to the WNV C protein and mediate its translocation from the cytoplasm to the nucleus. Another study using DENV found that once C protein made its way into the nucleus, it functioned like a histone mimic and was capable of binding to core histones to form heterodimers, effectively disrupting the formation of nucleosomes (Colpitts et al., 2011). Binding of C proteins to core histones and the disruption of nucleosome formation could potentially impede nuclear function. This in turn could result in altered gene expression, decreased DNA transcription, an increase in DNA damage and even a shift in RNA translation favoring viral mRNA over cellular mRNA.

Moreover, the DENV C was shown to bind nucleolin (NCL), the multifunctional nuclear protein involved in ribosome biogenesis, protein transport, chromatin remodeling, translational control, and even RNA processing (Balinsky et al., 2013). Such an interaction was hypothesized to be crucial for efficient viral propagation after the same study found that NCL knockdown resulted in a reduction of infectious DENV. The DENV C was also shown to bind the heterogeneous nuclear ribonucleoprotein K (hnRNP k) in the nucleus and shuttle it into the cytoplasm (Chang et al., 2001). The downstream effect of this cytoplasmic shuttling of hnRNP K is currently unknown, but the silencing of hnRNP K has been shown to decrease DENV propagation when compared to wild type, indicating a potentially crucial role for the C protein-hnRNP K interaction / translocation during flavivirus infection (Brunetti et al., 2015). Furthermore, the DENV and JEV C proteins were shown to bind and shuttle the nucleolar phosphoprotein B23 to the cytoplasm, as well as retard its movement into

and out of the nucleus (Moore et al., 2013; Tsuda, 2006). Subsequently, it was reported that the overproduction of B23 proteins reduced virus propagation, suggesting that C protein's involvement in B23 sequestration might create a more hospitable environment for flavivirus multiplication (Tsuda, 2006). The WNV C protein was also reported to bind the nuclear helicase DDX56, which was ultimately found to have a positive influence on virion assembly and maturation (Xu et al., 2011).

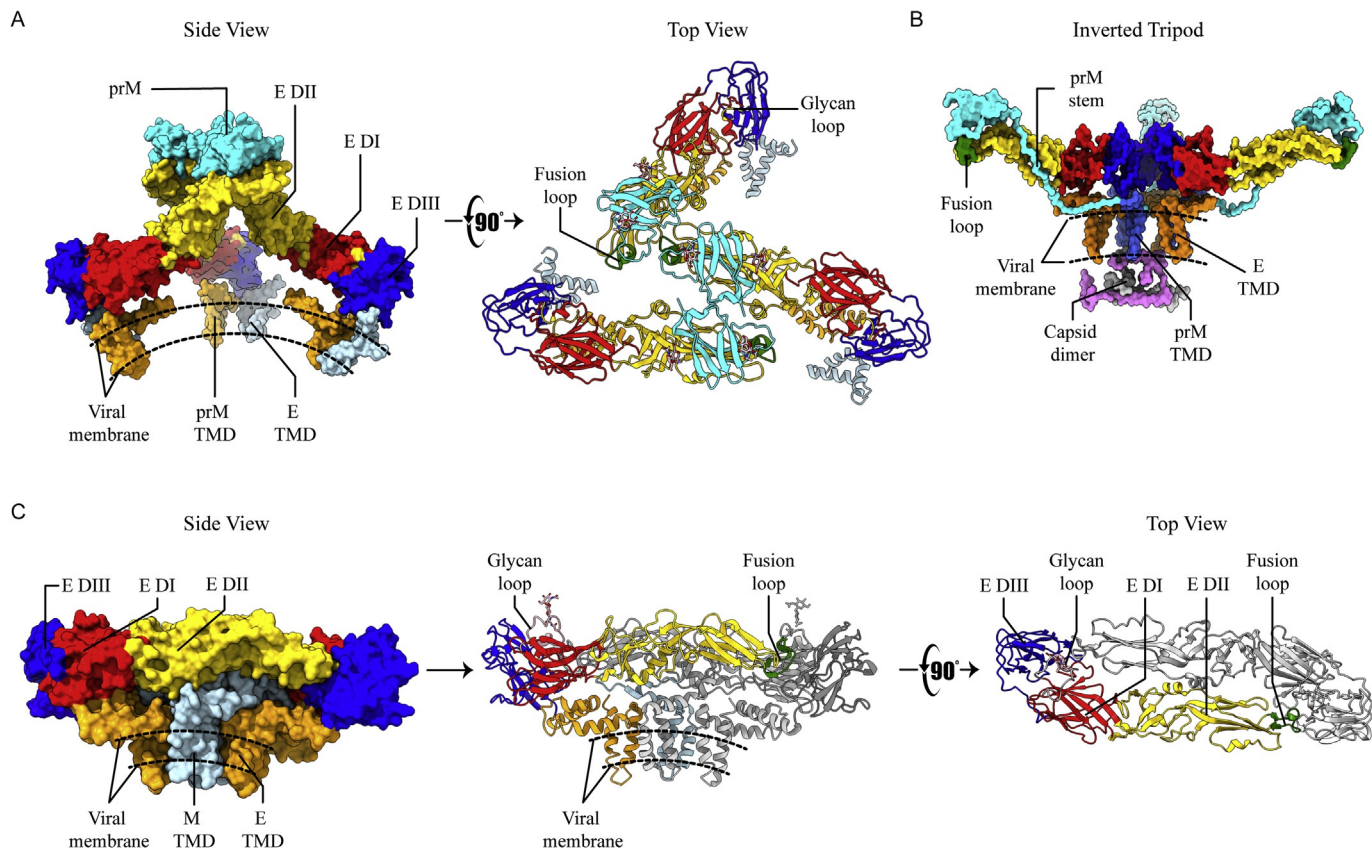
As demonstrated, there are numerous studies showcasing the benefits of nuclear localization of flavivirus C proteins for virus propagation. However, other studies have identified several C protein nuclear interactions that suggest a deleterious effect on virus proliferation. WNV C was shown to interact with and sequester the HDM2 protein to the nucleolus, inducing p53-dependent apoptosis (Yang et al., 2007). WNV C expressed in H1299 cells also generated cytotoxic effects due to cell cycle arrest at the G2 phase (Oh et al., 2006). Similarly, WNV C protein was found to be capable of activating caspase-9 and caspase-3, as well as binding to Hsp-70, which resulted in mitochondria-mediated apoptosis and disruption of protein folding machinery (Oh and Song, 2006; Yang et al., 2002). In DENV, the observation of binding between C protein and core histones also identified the binding of C to the death domain associated protein (DAXX), which encouraged cell death via apoptosis (Netsawang et al., 2010). Considering the virus dependency on cellular machinery and longevity for sufficient viral propagation yields, such negative effects on host cell health seem to be inhibitory from an evolutionary perspective. Nonetheless, some studies have shown that certain C protein induced apoptotic pathways can be negated by other pathways also activated by C protein (Li et al., 2012; Oh et al., 2006; Urbanowski and Hobman, 2013). This would suggest that the flavivirus C protein possibly contains functional redundancy to prevent premature cellular termination prior to the yield of appropriate viral progeny. However, these hypothetical divergences are yet to be investigated.

Altogether, the flavivirus C protein is a small, simple protein that is capable of undertaking numerous cellular responsibilities during the virus life cycle. The continued investigation of this interesting little protein will surely reveal new pathways of activation and potential avenues for therapeutic intervention. Moreover, further investigation of this protein's structure and its various interacting partners will undoubtedly produce a better understanding of flavivirus assembly.

### 3.2 Pre-membrane/membrane protein (prM/M)

The flavivirus prM protein is a seven  $\beta$ -stranded membrane bound glycoprotein that is connected to the double alpha-helical TMDs by a long flexible linker known as the prM “stem.” This protein consists of roughly 168 amino acids, depending on the specific virus being analyzed. The first  $\sim 91$  residues on the N-terminal end of prM constitute the “pr” domain of the protein which is cleaved during viral maturation by the host protease furin (Lin et al., 2018; Pierson and Diamond, 2012). The pr domain has been shown to contain three disulfide bonds (C34–C68, C45–C80 and C53–C66) that stabilize the domain, as well as a glycan at N69 (Li et al., 2008). Structurally, the pr domain functions as a protective “cover” that is positioned over the fusion loop of E protein within the prM–E heterodimer (Fig. 4A and B) where it ultimately prevents the premature fusion of immature virions to endosomal membranes prior to their release into the extracellular environment as fully mature particles (Guirakhoo et al., 1991; Heinz et al., 1994). Studies in tick-borne encephalitis virus (TBEV—another flavivirus) have confirmed this role by showing that the cleavage of the pr domain is essential for the future infectivity of virions (Elshuber et al., 2003). Residues  $\sim 92$ –125 constitute the “ecto-domain” of M protein that remains in the virion structure after furin cleavage of the pr domain. The remaining 43 residues ( $\sim 126$ –168) constitute two membrane spanning alpha helices and have been implicated in ER retention. The first transmembrane helix (residues  $\sim 126$ –147) serves as a stop-transfer sequence, while the second transmembrane helix (residues  $\sim 149$ –168) functions as a signal sequence for host signalase cleavage during polyprotein processing. Several residues within the prM protein have been shown to be key in the heterodimerization of prM with E protein after translation. These studies demonstrated that a tyrosine residue at position 78 in WNV (Tan et al., 2008), a histidine residue at position 99 in JEV (Lin and Wu, 2005), and a histidine residue at position 130 in DENV2 (Pryor et al., 2004) were all paramount in the formation and stability of prM–E heterodimers as well as efficient viral assembly. Another study reported that mutations at residues 120, 123 and 127 in DENV2 prM resulted in reduced viral titers via plaque assay and reduced virus assembly efficiency via ELISA analysis (Hsieh et al., 2011). Together, these results suggest that the prM/M protein is crucial to the generation of prM–E heterodimers and ultimately the formation and maturation of flavivirus virions.





**Fig. 4** See figure legend on next page.

Aside from the dimerizing and protective interactions with E protein during virus assembly, very little is known about the functions of flavivirus prM/M proteins, if any exist. One study did find that the ecto-domain of M proteins from DENV1-4, WNV, and even JEV were able to interact with the human dynein light chain component Tctex-1, and that the silencing of Tctex-1 significantly reduced viral progeny (Brault et al., 2011). The authors then suggested that Tctex-1 might influence virus assembly or maturation through its interactions with M. This conclusion is somewhat controversial since the flavivirus prM/M protein is considered to be an exclusively ER luminal protein while Tctex-1 is a cytosolic component of the cell transport system, significantly decreasing the probability of a natural interaction between these two proteins. Nonetheless, Brault et al. (2011) suggest that these two proteins could come together during viral induced ER stress and ER membrane permeabilization. Such a hypothesis has yet to be tested and supported, however. Another group reported that DENV2 prM is capable of interacting with “hypothetical” mosquito proteins using a yeast 2-hybrid screening library (Mairiang et al., 2013). Although, no human orthologs of the identified “hypothetical” protein are known to exist and no interacting partners for M protein were identified at all. These results could simply be an artifact of experimentation, or they

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**Fig. 4** Mature and immature flavivirus glycoproteins: (A) Side view surface representation of the immature ZIKV (PDB ID: 5U4W) prM-E heterodimer trimeric spike (left). The prM protein is colored cyan, the E protein DI is colored red, DII is colored yellow, and DIII is colored blue. The E protein TMDs are colored orange while the prM/M TMDs are colored light blue. The viral membrane is indicated by the curved dotted line. On the right side of panel (A) is a top view ribbon representation of the trimeric spike. This structure is color coded the same as the surface structure on the left but includes a depiction of the fusion loop (green) and glycan loop (pink). (B) Side view surface representation of the Fab stabilized immature ZIKV (PDB ID: 6LNT) prM-E heterodimer inverted tripod described in Section 4 (Tan et al., 2020). In this structure, density was observed below the TMDs of prM and E protein (light blue and orange, respectively) that fit C protein (purple). The prM stem was also visualized (cyan). (C) Side view surface representation of the mature dimeric M-E heterodimers (PDB ID: 6C08) (far left). The E protein domains are color coded as follows: DI in red, DII in yellow, and DIII in blue. The E TMDs are colored orange while the M TMDs are colored light blue. The viral membrane is indicated by the curved dotted line. In the center of panel (C), a ribbon representation of the dimeric M-E heterodimer displays the glycan loop (pink) and the fusion loop (green). One heterodimer is color coded the same as the surface representation on the left, while the other heterodimer is colored gray. A 90-degree rotated top view of the dimeric heterodimer is present on the far-right side of panel (C), displaying the same color scheme as the ribbon structure in the center.

could suggest that prM/M does not interact with any host proteins and solely functions to stabilize and secure the maturation of E protein during virus assembly and egress. Either way, further investigation into the non-canonical functions of prM and M is warranted.

### 3.3 The envelope protein (E)

From the first published X-ray crystal structure of TBEV E protein in 1995 (Rey et al., 1995), our knowledge of E protein and flavivirus structure has expanded exponentially over the last 25 years. As such, the structures of E protein within the immature and/or mature virions have been identified for DENV1 (Fibriansah et al., 2014; Kostyuchenko et al., 2013), DENV2 (Zhang et al., 2004, 2013b), DENV3 (Wirawan et al., 2019), DENV4 (Kostyuchenko et al., 2014), JEV (Wang et al., 2017), YFV (Zhang et al., 2003a), WNV (Zhang et al., 2007, 2013a), ZIKV (Prasad et al., 2017; Sirohi et al., 2016), and TBEV (Zhang et al., 2003b). Additionally, the crystal structure of the Usutu virus (USUV—another flavivirus) pre-fusion E protein has also been recently published (Chen et al., 2018). The flavivirus E protein contains roughly  $\sim 500$  residues, weighs approximately  $\sim 53$ – $60$  kDa depending on the number of glycosylation sites present, and has been described as an elongated protein with a length of approximately  $\sim 170$  Å (Tang et al., 2015; Wu et al., 2003). Each E monomer is organized into three structurally conserved yet distinct domains (Domains I, II and III), as well as a fourth stem-anchor region. The stem is made up of three flexible alpha helices and the anchor consists of two antiparallel transmembrane helices, herein denoted as the E protein TMDs (Fig. 4A–C). Several studies have produced soluble E proteins that display the three ecto-domains but lack the TMDs and part of the stem region (Cherrier et al., 2009; Modis et al., 2004; Volk et al., 2011; White et al., 2010). The primary function of the flavivirus E protein is to facilitate virus attachment and cellular entry through viral-endosomal membrane fusion. Flavivirus entry is initiated by the interaction of the E protein with receptor molecule/s followed by endocytosis and low-pH induced conformational changes of the dimeric E protein on the mature virus to form fusogenic trimers. The exposed fusion loop on the fusogenic trimers interacts with the endosomal membrane resulting in subsequent fusion of the virus and endosomal membrane and the release of viral genome into the host cytosol (Hienz and Stiasny, 2017). However, a detailed description of these stages of the flavivirus life cycle

is outside the scope of this review. Nonetheless, the individual structures and functions of each E protein domain as they pertain to virus assembly and maturation are outlined in the sections below. For a more in depth understanding of the functional roles of E protein in flavivirus cell entry, readers are encouraged to explore an excellent review by [Laureti et al. \(2018\)](#).

### 3.3.1 *E domain I*

The N-terminus of E protein constitutes domain I (DI) and forms an eight-stranded  $\beta$ -barrel structure that is located centrally within the protein ([Roby et al., 2015](#); [Tang et al., 2015](#)) (Fig. 4). The central location of this domain allows it to function like a hinge, aiding in the numerous conformational changes that E protein must undergo during the maturation and fusion steps of the flavivirus life cycle. DI is made up of approximately 120 residues split into three major segments (residues 1–51, 137–189, and 285–302) ([Wu et al., 2003](#)) and has been shown to contain type-specific non-neutralizing epitopes ([Chiou et al., 2012](#); [Crill and Chang, 2005](#)). DI is flanked on both sides by the other two ecto-domains of E protein, with the elongated domain II (DII) on one side and the immunoglobulin-like domain III (DIII) on the other. This positioning allows DI to act as both a bridge-like hinge and a core stabilizer for the other ecto-domains of E protein ([Yu et al., 2013](#)). This domain also contains an asparagine residue capable of accepting glycosylation during virus maturation. However, the location of this glycosylation site is not conserved across all flaviviruses. For example, the four DENV serotypes all contain an N-linked glycosylation site at N153, while TBEV contains one at position N154 ([Goto et al., 2005](#); [Yoshii et al., 2013](#)). Other studies have even shown DI glycosylation to differ between various strains of the same virus, with most WNV strains (such as NY99) displaying glycosylation at N154, but other strains such as Kunjin virus (KUNV) containing none ([Alsaleh et al., 2016](#); [Maharaj et al., 2019](#)). Similarly, some African lineage ZIKV contain no glycosylation sites while all Asian lineage ZIKV strains are glycosylated at N154 ([Carbaugh et al., 2019](#); [Ladner et al., 2016](#)). Studies have demonstrated that the glycosylation of DI is related to proper virion production, pH sensitivity, and even neuroinvasiveness ([Carbaugh and Lazear, 2020](#)). Additionally, a study by [Goo et al. \(2017\)](#) found that a single residue mutation in the hinge region between DI and DII is capable of attenuating both WNV and ZIKV, suggesting that the ability for DI to be conformationally flexible is imperative to flavivirus pathogenesis.

### 3.3.2 E domain II

DII is elongated and contains two  $\beta$ -stranded areas connected by flexible loops and two short helices near DI. DII is the site of the M-E & M-E heterodimer oligomerization interface in the mature virion, with a buried interface surface area of  $1490 \text{ \AA}^2$  (Füzik et al., 2018; Rey et al., 1995; Roby et al., 2015; Tang et al., 2015) (Fig. 4C). Along with DI, the DII of all four DENV serotypes has been shown to contain another asparagine residue capable of accepting glycosylation during virus maturation at N67 (Chambers et al., 1990b; Johnson et al., 1994; Lee et al., 2010). The DII N67 glycosylation site is unique to DENV and has been proposed to interact directly with the host cell receptor DC-SIGN during virus attachment and entry (Pokidysheva et al., 2006). At its tip, DII contains a highly conserved fusion loop, including residues W101, L107, and F108, that is responsible for the fusion of the viral and endosomal membranes during virus entry (Allison et al., 2001; Füzik et al., 2018; Hasan et al., 2018). This hydrophobic fusion loop (residues  $\sim 100$ –109) is hidden from the aqueous environment by the prM protein in the immature virion (Heinz et al., 1994). After maturation, the fusion loop of one E monomer is then shielded by the hinge region between DI and domain III (DIII) of the adjacent E monomer within a dimer, as well as the glycosylated moiety at N153/154 if present (Hasan et al., 2018; Mukhopadhyay et al., 2005; Sirohi and Kuhn, 2017). When the virus enters the target host cell endosome, the DI-DII hinge region undergoes pH-dependent conformational changes that expose the fusion loop, allowing it to fuse the viral and endosomal membrane together to initiate infection (Zhang et al., 2004). DII has also been shown to contain many flavivirus cross-reactive peptides that stimulate neutralizing antibodies, and which have been used as targets for potential vaccine candidates (Chiou et al., 2012).

### 3.3.3 E domain III

The E protein DIII is connected to DII by a flexible linker that places the domain on the opposite side of DI from DII and is located near the C-terminus of the E protein (Mukherjee et al., 2006; Nguyen et al., 2019; Volk et al., 2011). DIII contains roughly 100 residues that together construct six  $\beta$ -strands and ultimately yield a  $\beta$ -barrel shape which folds into an immunoglobulin-like highly conserved structure (Fig. 4). This domain protrudes away from the mature virus structure to form an apophysis, which has been proposed to contain epitopes for the binding of both host receptors and neutralizing antibodies (Mandl et al., 2000). Indeed, several

studies have identified that DIII does contain linear antigenic epitopes that can bind to specific neutralizing antibodies (Huerta et al., 2008; Roehrig et al., 2013; Sirohi and Kuhn, 2017). These epitopes have also been found to facilitate cell receptor binding with heparan sulfate, ribosomal protein SA, low-density lipoprotein receptor-related protein 1 (LRP1), and even some carbohydrate receptors to initiate virus entry (Huerta et al., 2014; Roehrig et al., 2013). It has been previously shown that the neutralizing epitopes present in DIII are particularly conserved amongst several viruses. For example, the DIII epitopes are comprised of residues 306, 331, 333, 337, 360, and 373–399 in JEV (Wu et al., 2003), residues 306, 307, 308, 330, 332, 366 and 391 in WNV (Kanai et al., 2006), and residues 307, 333–351 and 383–389 in DENV (Lin et al., 2015). Additional studies have confirmed that DIII of ZIKV and TBEV E proteins are also capable of stimulating neutralizing antibodies (Gallichotte et al., 2019; Pulkkinen et al., 2018). Regardless of the high level of sequence and structural conservation across all flaviviruses, DIII has successfully been used as an antigen for serological specific diagnostic testing and has garnered attention as a possible candidate for vaccine development (Chávez et al., 2010). For example, truncated DIII antigens from JEV, WNV and TBEV were able to specifically capture antibodies directed toward each respective virus despite the well-known cross reactivity between these viruses (Beck et al., 2015).

### 3.3.4 E stem-anchor region

The stem-anchor region includes the stem (residues ~396–451) that is made of three membrane associated helices and a transmembrane anchor (residues ~452–504) made of two antiparallel helices that serve as a membrane tether (Zhang et al., 2003a,b). The two transmembrane helices of the anchor (residues ~457–479 and ~482–504) form the E protein TMDs and function similar to the TMDs of the prM protein. The E protein stem has been implicated to play a crucial role during the membrane fusion of flavivirus entry. For fusion to occur, it requires the relocation of DIII and the elongation of DII to expose the fusion loop at the distal end of DII and prepare it for membrane interaction within a single M-E heterodimer (Chao et al., 2014). Together with two other M-E heterodimers, this is accomplished through the reordering of the stem region via a “zippering” motion, changing its buried conformation in the outer leaflet of the viral membrane to one that can stabilize the fusion trimer (Stiasny et al., 2013; Zhang et al., 2013b). This effectively inverts the E monomers, causing the transmembrane

anchors to cluster around the newly exposed fusion loops of the trimeric fusion stalk, driving membrane fusion and genome release. Disruption of domain III relocation through incubation with exogenous free domain III or the inhibition of stem “zippering” through incubation with stem-derived peptides has been shown to inhibit infection of DENV1 and DENV2 (Liao and Kielian, 2005; Schmidt et al., 2010). Another study found that the replacement of DENV2 E protein stem-anchor region with that of JEV E protein increased the secretion of DENV E proteins similar to that of stem-anchor truncated E proteins (Chang et al., 2003). This suggested that the ER membrane retention ability of virus specific stem-anchor regions (i.e., DENV E protein ecto-domain+DENV E protein stem-anchor) might be essential for virus assembly and without it (i.e., DENV E protein ecto-domain+JEV E protein stem-anchor), the proteins are readily secreted.

### 3.3.5 *prM-E heterodimer TMDs*

Unlike C protein, the prM and E proteins are located within the ER lumen and are membrane anchored due to their C-terminal double TMDs (Fig. 4). These proteins remain tethered to the ER after polyprotein translation and processing to await encapsulation of the flavivirus NC. Aside from acting as simple anchors, the TMDs of YFV prM and E proteins were previously shown to contain ER retention sequences that served to maintain the proteins within the ER and prevent degradation (Op De Beeck et al., 2004). Similarly, the length and hydrophobicity of the DENV E TMDs were found to act as ER membrane retention signals, preventing premature degradation of the proteins prior to adequate viral protein production for assembly (Hsieh et al., 2010). Cryo-electron microscopy (cryo-EM) studies on both the immature and mature flavivirus structures have revealed that the antiparallel hairpin conformation of the prM and E heterodimer TMDs penetrate the outer viral membrane leaflet and reach into the inner leaflet, but do not extend out into the intra-particle space (Kostyuchenko et al., 2013; Zhang et al., 2003a,b, 2013a). The effects of this specific TMD orientation on possible assembly mechanisms is explored in greater detail within Section 4 of this review. Additionally, the exchange of E TMDs between JEV and TBEV resulted in the increased secretion of subviral-like particles (SVPs—particles with no C proteins or genomes) at the expense of infectious virions, suggesting a possible role for the TMDs during flavivirus assembly and NC incorporation (Blazevic et al., 2016).



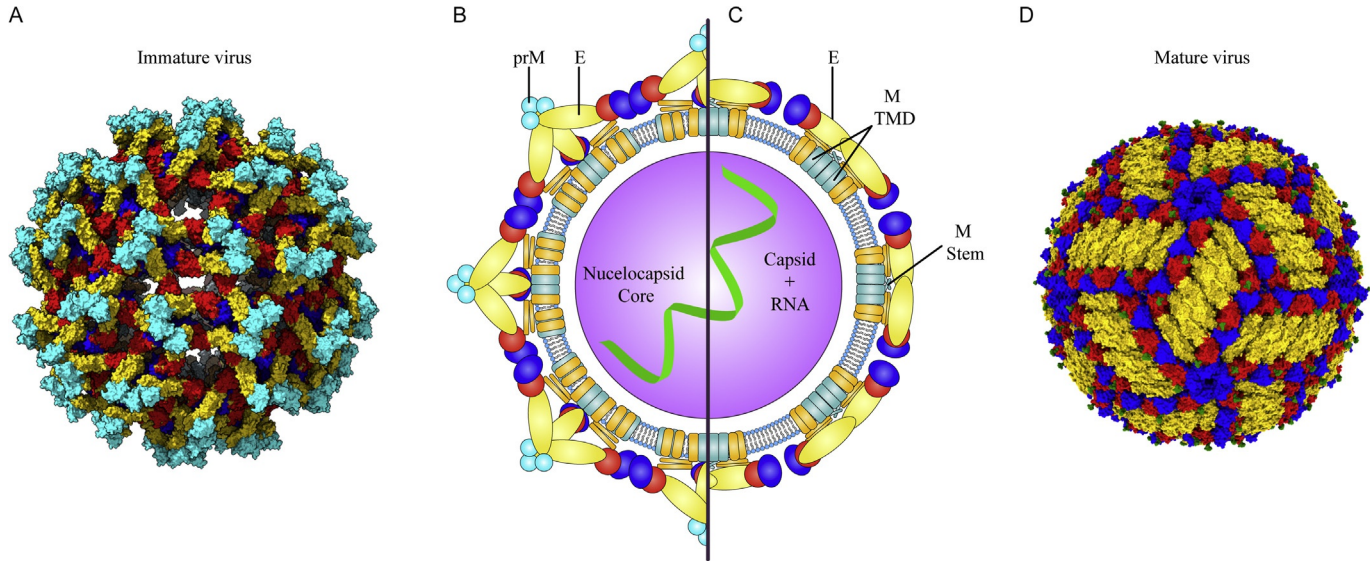


## 4. Flavivirus assembly and budding

To date, we know remarkably little about the biochemical and structural mechanisms that drive flavivirus assembly. The broad concepts such as the location and components of assembly are known, but the minute details of specific interacting partners and their effects on the construction of flavivirus particles remain obscure. Flavivirus assembly has been shown to occur at the ER membrane where the viral RNA genome assembles with C proteins to form nucleocapsid cores (NCs) (although no naked NCs have ever been seen), which then bud into the ER lumen where they are encapsulated by the ER membrane and multiple copies of prM and E proteins, forming an immature virus particle. Several structures of these ~60 nm, non-infectious “immature” virions have been solved for numerous flaviviruses (Kostyuchenko et al., 2013; Li et al., 2008; Prasad et al., 2017; Zhang et al., 2003b) (Fig. 5). The immature flavivirus contains 180 copies of prM/E-heterodimers arranged into 60 trimeric “spikes” that protrude outward from the viral membrane. Three copies of the prM peptide sit at the top of each trimeric spike where they protect the fusion loops located at the distal end of each E protein monomer from premature low pH-mediated membrane fusion. Prior to maturation, this “spikey” immature virus particle is glycosylated in the ER and is then transported into the Golgi where maturation occurs.

Exactly how the genomic RNA locates and interacts with C proteins (or vice versa) to generate these immature virions was previously unknown, since no packaging sequence within the genome had been identified and C protein had been shown to indiscriminately bind any free nucleic acid (Pong et al., 2011; Teoh et al., 2014). These findings were seemingly contradictory to viral genomic screening data showing that flaviviruses specifically packaged viral RNA (Khromykh et al., 2001). It has been suggested that this RNA packaging specificity is accomplished through the coupling of genomic replication and encapsulation. Indeed, studies using electron-tomography have shown that the sites of genomic replication (which are virus modified ER invaginations known as replication complexes) are found in close proximity to sites of virion budding (Junjhon et al., 2014; Welsch et al., 2009). Some of the replication complexes were even shown to contain pores connecting the sites of replication to the sites of viral budding. However, proximity alone cannot sufficiently explain how flavivirus virions are capable of specifically packaging newly synthesized viral RNAs.

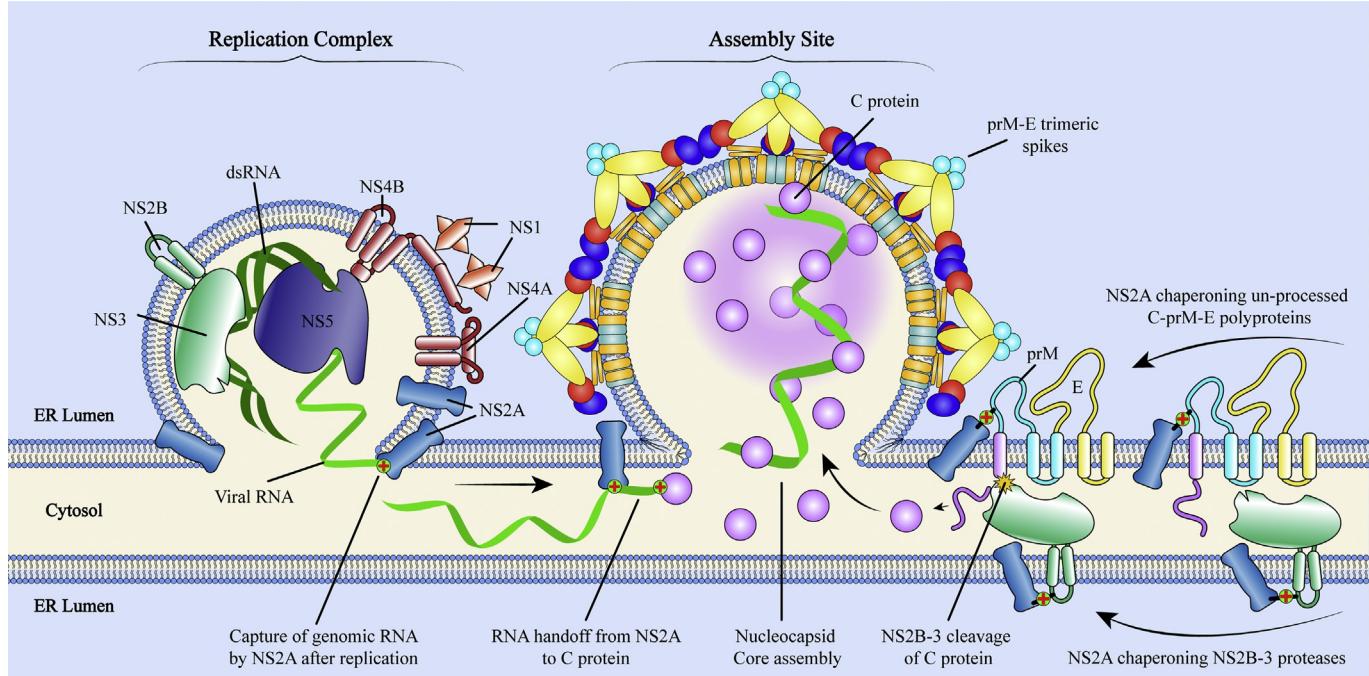




**Fig. 5** Mature and immature flavivirus structure: (A) Surface representation of the immature ZIKV (PDB ID: 5U4W) particle with prM and each E protein domain color coded as follows: prM (cyan), E protein domain I (red), E protein domain II (yellow), E protein domain III (blue). (B) Cross-section diagram of the immature flavivirus particle showing prM-E heterodimer trimeric spikes. The diagram is color coded to match panel A. Additionally, the prM TMDs (cylinders along the lipid bilayer) are colored in light blue, while the E protein TMDs are colored orange. (C) Cross-section diagram of the mature flavivirus particle showing dimeric M-E heterodimers. Color scheme is the same as panel (B). (D) Surface representation of the mature ZIKV (PDB ID: 6C08) particle with each E protein domain color coded the same as in panel (A), but also includes the fusion loop shown in green.

A recent study by Xie et al. (2019) has addressed this issue by observing that the last 285 nucleotides of the 3'UTR genomic RNA in DENV was capable of binding to the NS2A protein via residues R94, K95 and K99. The authors subsequently put forward an assembly model (Fig. 6) through which the newly synthesized RNA is immediately bound by NS2A upon its exit from the replication complexes and shuttled to assembly sites. In parallel, other NS2A proteins recruit newly translated and unprocessed C-prM-E polyproteins, as well as previously complexed NS2B-3 proteases, to the site of assembly through interactions with prM and NS3, respectively. Such a nucleation of NS2A proteins to recruit various interacting partners could be made possible through NS2As previously demonstrated ability to oligomerize along the ER membrane (Patkar and Kuhn, 2008; Voßmann et al., 2015). Once all of the proteins are brought together by NS2A, the recruited NS2B-3 protease sequentially cleaves C from the C-prM-E polyprotein in tandem with host signalase, allowing it to bind to the readily available genomic RNA conveniently located nearby via parallel NS2A recruitment. The newly processed prM and E proteins can then arrange as heterodimers and begin forming the viral envelope while other C proteins previously sequestered to lipid droplets (Samsa et al., 2009) begin binding to the genomic RNA forming NCs. The NS3 helicase was also hypothesized to help in the condensing of the genomic RNA for NC formation. The binding of NS2A to viral RNA, as well as the binding of NS2A to prM and NS3 in DENV was also found to exist in ZIKV (Zhang et al., 2019), further supporting the model of NS2A assisted flavivirus assembly put forward by Xie et al. (2019). Nonetheless, many different aspects of this assembly model remain obscure, such as how lipid droplet sequestered C proteins are recruited to sites of assembly, how RNA and C proteins complex to form NCs, how the NCs are structured, and also how the NC's initiate the budding process. Additionally, the active recruitment process of NS2B-3 protease and C-prM-E polyprotein by NS2A to sites of assembly has not been confirmed through biochemical or electron microscopy studies. More research is needed to fully support this model of flavivirus assembly.

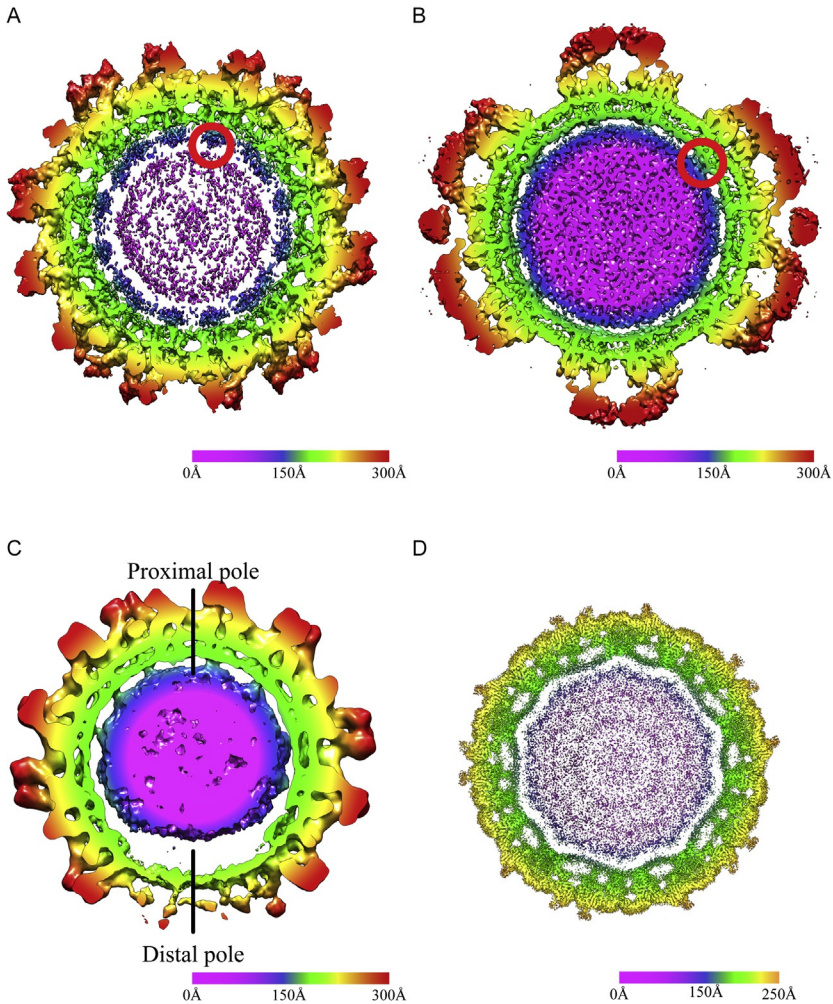
The lack of tangible interactions between the C proteins of the NC and the prM/E heterodimers of the envelope has been another confounding aspect of flavivirus assembly. Unlike the structurally similar viruses of the Alphavirus genus (family *Togaviridae*), whose envelope proteins make physical contact with the C proteins of their NCs (Basore et al., 2019), no such interactions have been identified in the immature or mature



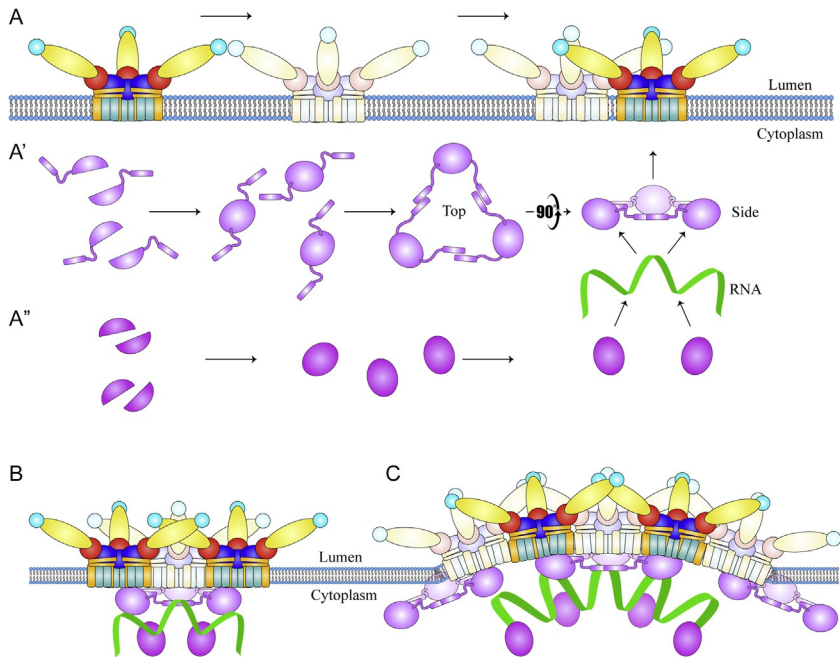
**Fig. 6** NS2A assisted assembly model—This model was originally proposed by Xie et al. (2019): The viral RNA (light green ribbon) is immediately captured by NS2A (blue dumbbells) upon its release from replication complexes (left), where it is transported to assembly sites (right). In parallel, other NS2A proteins recruit un-processed C-prM-E polyproteins and NS2B-3 proteases to the assembly site. Here, NS2B-3 (green) cleaves the C-prM-E polyprotein (purple, cyan and yellow) in tandem with host signalase (not shown). This facilitates in the dimerization of soluble C proteins, which are then able to receive the genomic RNA from NS2A, forming a NC. Meanwhile, the processed prM and E proteins are able to form heterodimers and subsequently form trimeric spikes on the luminal side of the membrane (color code similar to Figs. 3 and 5). The NC then buds into the nascent viral envelope at the ER lumen.

structures of flaviviruses even when the surface resolution of these structures has approached near atomic resolution. The closest any previous study has come to identifying molecular connections between the NC and envelope was in 2017, when it was shown for the first time that the immature ZIKV structure contained residual density below the TMDs of prM/E that fit C protein (Prasad et al., 2017) (Fig. 7A). This residual density effectively bridged the gap between the NC and the viral envelope previously observed in other flavivirus structures and suggested that the NC could potentially interact with the envelope glycoproteins during the assembly process via their TMDs. Additionally, a recent cryo-EM study by Tan et al. (2020) using an antigen-binding fragment (Fab) from the human monoclonal antibody (HMAb) DV62.5 to bind and stabilize the immature ZIKV particle was able to increase the resolution of the C-RNA NC density and show that C protein does actually come into close contact with the TMDs of the envelope glycoproteins (Fig. 7B). It should be noted that the 60 dimeric C protein densities observed in the immature Fab-stabilized ZIKV structure from Tan et al. (2020) are absent in the previously published mature ZIKV structures (Kostyuchenko et al., 2016; Sevvana et al., 2018; Sirohi et al., 2016) (Fig. 7). This study also found that the C-anchor (referred to as helix  $\alpha 5$  in the manuscript), which was previously thought to play no role in the flavivirus life cycle apart from the translocation of prM into the ER lumen, remains intact within a sub-population of mature C protein to facilitate C oligomerization and subsequently drive flavivirus assembly. These two C protein populations: (1) fully cleaved C and (2) C lacking NS2B-3 cleavage and retaining the C-helix  $\alpha 5$  (C +  $\alpha 5$ ), work in conjunction with one another to form the NC and interact with the prM/E TMDs. Using these C populations as unique building blocks, the authors proposed a model for flavivirus assembly (Fig. 8). Firstly, both C and C +  $\alpha 5$  form homodimers within their respective populations in the cell cytoplasm after polyprotein translation and processing. In parallel, the processed prM and E proteins form heterodimers which then interact with two other prM/E heterodimers to form an “inverted tripod” in the ER lumen (Fig. 4B). These tripods then coalesce on the ER membrane to form a “forest” of tripods that will eventually turn into the viral envelope. On the cytosolic side, the multiple copies of C +  $\alpha 5$  proteins laterally interact via helix  $\alpha 5$  to form a triangular “trimer of dimers” known as an “assembly unit.” Each assembly unit is then capable of interacting with the tips of the prM/E TMDs within an inverted tripod, effectively forming a lattice along the ER membrane that induces membrane curvature and ultimately leads to viral budding.





**Fig. 7** Structural comparisons of flavivirus particles: Unique structural elements have been identified within immature flavivirus structures throughout the last 20 years (panels A–C). Meanwhile, the structural elements observed in mature flavivirus structures have remained somewhat constant, but the resolution has increased dramatically (Panel D). (A–D) Central section looking down the fivefold axis of the cryo-EM map. The central sections are contoured and colored radially from magenta (NC core), blue (capsid), green (TM) to yellow and red (ecto-domain). (A) Cross-section structure of the immature ZIKV particle at 9 Å, published by [Prasad et al. \(2017\)](#) (EMDB: 8505). Residual density (blue blob in red circle) underneath the prM-E spikes was shown to fit capsid protein. This was the first time any connections between the flavivirus NC and the envelope glycoproteins had been observed. (B) Cross-section structure of the antibody stabilized immature ZIKV particle at 8 Å, published by [Tan et al. \(2020\)](#) (EMDB: 0932). Density fitting C protein (red circle) was resolved to  $\sim 9.5$  Å and showed that C protein retained its helix  $\alpha 5$  (C-anchor). (C) Asymmetric reconstruction and cross-section structure of the immature KUNV particle at  $\sim 20$  Å, published by [Therkelson et al. \(2018\)](#) (EMDB: 8983). Contact between the NC and the viral envelope was observed at the “proximal pole” (top), indicating an interaction during virus assembly/budding. A lack of density at the “distal pole” (bottom) indicated that symmetrical incorporation of the glycoproteins is sterically hindered at the bud neck (also see [Fig. 8](#)). (D) Cross-section structure of the mature ZIKV particle at 3.1 Å, published by [Sevvana et al. \(2018\)](#) (EMDB: 7543). No contacts were observed between the NC and envelope.



**Fig. 8** Capsid mediated assembly model—This model was originally proposed by [Tan et al. \(2020\)](#): All “A” panels occur in parallel. (A) Cleaved prM and E proteins oligomerize to form prM-E heterodimers, which in turn coalesce to form “inverted tripods.” These inverted tripods then associate to form trimeric spikes. A’ Un-processed C proteins retaining their helix  $\alpha 5$  ( $C + \alpha 5$ ) dimerize in the cytoplasm and subsequently form trigonal trimers through interactions between each helix  $\alpha 5$ . The positively charged exterior of each dimer facilitates membrane interaction, which ultimately leads to binding with the prM-E TMDs. A’’ Fully processed C proteins (not containing helix  $\alpha 5$ ) also dimerize and work in tandem with the  $C + \alpha 5$  proteins to assist the condensation of genomic RNA. (B) The  $C + \alpha 5$  trimers facilitate proper spacing between prM-E inverted tripods, which promotes trimeric spike formation. (C) Proper prM-E inverted tripod spacing and trimeric spike formation through  $C + \alpha 5$ -prM-E TMD interactions initiates membrane bending and drives viral budding, forming immature flavivirus particles. Color scheme is similar to [Figs. 4 and 5](#).

Meanwhile, the fully cleaved C protein population interacts with the genomic RNA to form the NC. The two populations work together, one ushering along the formation of the envelope, and the other condensing the genome for encapsulation. The authors suggested that the function of  $C + \alpha 5$  protein during the assembly processes is to provide the correct spacing between the prM/E tripods, which it does through the generation of “assembly units” via inter-helix  $\alpha 5$  interactions. Using mass spectrometry analysis, [Tan et al. \(2020\)](#) found that only  $\sim 45\%$  of the C proteins within

immature virions retained their helix  $\alpha 5$ . This, along with the weaker densities of helix  $\alpha 5$  observed within the Fab stabilized cryo-EM structure, suggested that some of the triangular assembly units might contain C proteins without helix  $\alpha 5$ . While such a suggestion does not fully align with the author's model (i.e., strict delineation of C and C +  $\alpha 5$  function during virus assembly), a mixed population of C protein assembly units would not necessarily refute their model either. For instance, most of the C proteins within each assembly unit could still contain a helix  $\alpha 5$  and would thus facilitate assembly unit formation. Nonetheless, further studies are needed to determine the ratio of C to C +  $\alpha 5$  within flavivirus particles and the consequences of their mixed interactions (if any) during flavivirus assembly.

In the immature structure published by [Tan et al. \(2020\)](#), a single C protein dimer seems to contact three prM TMDs from within an inverted tripod of prM/E heterodimers. These interactions were hypothesized to occur via residues 141–146 of the prM TMDs, and residues 21–36 and 58–63 of C protein. A simple spatial comparison between the prM/M TMDs in the immature and mature particles shows that they shift from a trimeric to a dimeric structure ([Kostyuchenko et al., 2013](#)). Therefore, [Tan et al. \(2020\)](#) suggest that their observed C–prM interactions would break during the maturation process and the shift from a trimeric interacting partner to a dimeric one. This could effectively negate any C–prM interactions between the NC and envelope in the mature structure or at least sufficiently weaken such interactions, leading to weaker density during icosahedral symmetry averaging and particle reconstruction. Thus, no connections would be seen in the mature virus structures. However, the loss of core–envelope connectivity after assembly could actually be advantageous for the virus, since the loss of C–prM interactions during maturation might “free up” the M and E TMDs for the conformational changes that they must undergo during future membrane fusion steps. While these results undoubtedly aid in the continued quest to understand flavivirus assembly, further testing of the hypothesized interacting residues of prM and C protein via mutagenesis screening is necessary.

The idea that delayed or incomplete cleavage of C protein is capable of regulating virion assembly presented by [Xie et al. \(2019\)](#) and [Tan et al. \(2020\)](#), respectively, has been supported by other studies. For example, the cleavage by the luminal host signal peptidase at the C–anchor/prM junction is thought to only occur after NS2B-3 cleavage at the C/C–anchor junction on the cytoplasmic side of the ER ([Lee et al., 2000](#); [Lobigs et al., 2010](#); [Lobigs and Lee, 2004](#)). One possible outcome of the sequential

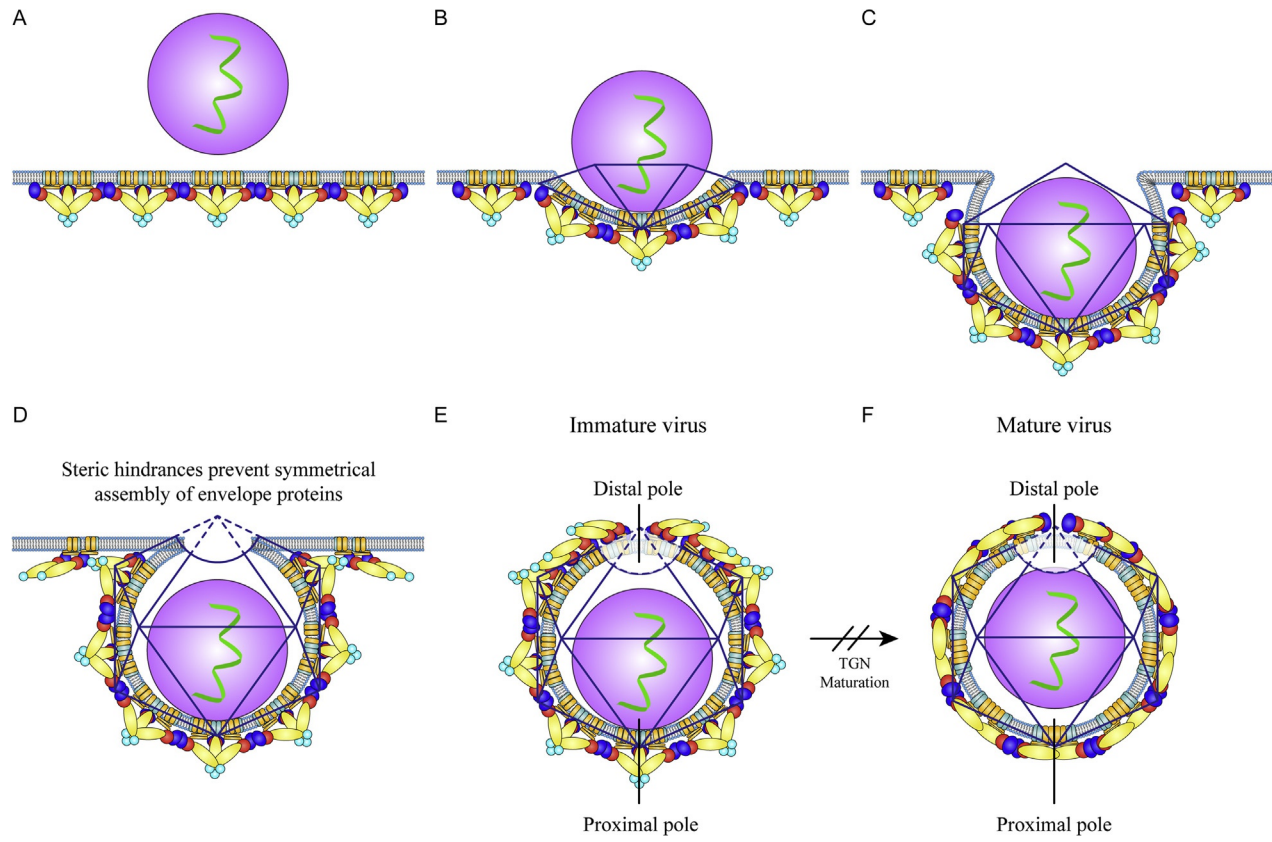
cleavage is that the first cleavage allows C-RNA interaction through NS2B-3, and the delayed second cleavage allows nucleocapsid incorporation into budding membranes containing viral glycoproteins. Consequently, a study in YFV evinced that the serial processing of the two cleavage junctions is indeed an important regulator of virion assembly (Amberg and Rice, 1999). Another study in Murray Valley encephalitis virus showed that the coordination of C-prM cleavage, firstly at the cytosolic side then at the luminal side, was essential for the efficient incorporation of the NC into virions (Lobigs and Lee, 2004). These studies all demonstrate the necessity of NS2B-3 cleavage *prior* to signalase cleavage for efficient viral assembly. However, the two models proposed above seem to require either the delay in C/C-anchor cleavage via NS2B-3 before NS2B-3 is able to associate with NS2A (Xie et al., 2019), or the cleavage of signalase at the luminal C-anchor/prM junction *prior* to NS2B-3's cytosolic cleavage of the C/C-anchor junction (Tan et al., 2020). The model proposed by Xie et al. (2019) simply requires that NS2B-3 delay its cleavage of the C/C-anchor junction and does not seem to contradict the previously published literature, but the model by Tan et al. (2020) does. This is not to say that either model is correct or incorrect, but instead it is to simply showcase the need for more rigorous experimentation to adequately understand the assembly processes of flaviviruses.

Another study offers a similar perspective on the assembly mechanisms of flaviviruses as Tan et al. (2020) but showcases the need for the development of better tools to aid in our probing of the flavivirus life cycle. Therkelson et al. (2018) utilized asymmetric single particle (SPA) reconstructions of immature KUNV and ZIKV to investigate the possibility of there being unique structural elements within flaviviruses that were “lost” during canonical symmetry imposition and reconstruction. While no direct C protein contacts with the glycoprotein TMDs were observable due to their low resolution ( $\sim 20$  Å), a globular density corresponding to the NC was shown to be pushed up against one side of the viral membrane (referred to as the “proximal pole” — Fig. 7C). Small protrusions aligning with the glycoprotein TMDs were also observed at this proximal pole. However, the resolution was too low to make any claims about what the density could represent. Interestingly, the eccentric location of the NC density in the asymmetric particle is contradictory to the concentric location of the NC in previously published immature virion structures using imposed icosahedral symmetry (Prasad et al., 2017; Tan et al., 2020). Such a discrepancy between the asymmetric and symmetric structures



suggests that any unique positioning of the NC during flavivirus assembly could be “averaged out” during particle reconstruction and symmetry imposition, artificially removing any asymmetric interactions between the NC and glycoprotein TMDs. This concept was supported when the same study (Therkelson et al., 2018) found that the asymmetric structure of mature KUNV contains an NC that was aligned concentrically with its glycoprotein shell, similar to other icosahedral mature flavivirus structures (Sevvana et al., 2018). Because the location of the NC was different only in the asymmetric immature particle, the authors posited that flavivirus assembly and budding occur via an asymmetric interaction between the proximal pole of the NC and the glycoprotein shell (Fig. 9), which forces the ER membrane around the NC. Steric hinderance at the bud neck (referred to as the “distal pole”) prevents any glycoprotein TMDs from interacting with the NC, thus manifesting as an eccentrically located core in the asymmetric immature particle (Figs. 9D and E). The residual “scar” of steric hinderances at the bud neck can be seen in the lack of defined density at the distal pole of the immature virion (Fig. 7C), indicating a loss of icosahedral symmetry and lack of glycoprotein spikes. Altogether, the study by Therkelson et al. (2018) has shifted the paradigm of what it means to “solve” a virus structure, and numerous virus structures that have already been “solved” could be hiding countless unique elements that might reveal insights into their biology. Therefore, a combination of asymmetric and localized 3D reconstruction protocols along with tomographic reconstructions will be essential to understand various viral processes, including flavivirus assembly.

The studies outlined above demonstrate that the assembly of immature flavivirus virions is a highly intricate process that remains unclear. Consequently, the process of flavivirus budding is similarly opaque. Most enveloped viruses bud at the plasma membrane but some, such as hepatitis C virus (HCV), vaccinia virus, and of course flaviviruses bud into internal compartments such as ER-derived membrane vesicles (Bartenschlager et al., 2004), membrane sheets (Chlanda et al., 2009), or even directly into the ER itself (Mukhopadhyay et al., 2005), respectively. Theoretically, virus budding can be divided into two unique stages: (1) Membrane deformation: the “envelopment” of the membrane around an assembling virion; and (2) Membrane fission: the severing of the bud neck. As seen in the flavivirus immature structure (Figs. 4A and 5), the prM and E structural proteins bind to the viral membrane via their TMDs and form a spherical edifice. It has been generally presumed that the energy provided by protein-protein



**Fig. 9** See figure legend on next page.

and/or protein-membrane interactions is sufficient to drive the membrane envelopment of flavivirus NCs. However, similar to how membrane deforming proteins cooperate with clathrin scaffolds during flavivirus endocytosis (Hackett and Cherry, 2018), other cellular factors may be recruited to help with internal membrane bending and fission during viral budding.

The most widely investigated internal membrane budding pathway is the endosomal sorting complexes required for transport (ESCRT) pathway, which has been found to include five unique complexes using proteins encoded by over 30 different genes (Votteler and Sundquist, 2013). These unique complexes form various interacting networks with other cellular components and coordinate the sorting of specific cargo by aiding in the budding of intraluminal vesicles (ILVs) into multivesicular bodies (MVBs) through the constriction and severing of bud necks (Li et al., 2015). Beyond MVB and ILV formation, ESCRT complexes have been found to aid in cell abscission, autophagy, exosome secretion and even human immunodeficiency virus (HIV) budding at the plasma membrane (Carlton et al., 2008; Filimonenko et al., 2007; Lee et al., 2007; Rusten et al., 2007). Additionally, the ESCRT pathway has also been shown to play an important role in the re-assembly of the nuclear envelope during cell division (Olmos et al., 2015; Vietri et al., 2015), showcasing its ubiquitous presence in many locations around the cell. These supplementary functions of the ESCRT pathway, aside from its canonical role during ILV formation, demonstrate that ESCRT factors could be involved during membrane invagination or budding events along various internal compartments. Indeed, two independent groups observed that yeast ESCRT factors are required for the

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**Fig. 9** Asymmetric assembly model—This model was originally proposed by Therkelson et al., 2018: (A) Glycoproteins assemble along the ER membrane as prM-E heterodimer trimeric spikes. (B) The unstructured NC interacts with the trimeric spikes through their TMDs and initiates the budding process of an icosahedron. (C) As the NC pushes deeper into the ER membrane, more glycoproteins are wrapped into the virion envelope. (D) During the final stages of the budding process, the virion is unable to incorporate the final copies of the envelope glycoproteins due to the steric hinderance of the narrowing bud neck. (E) Highly distorted density at the distal pole of the immature virion due to the lack of glycoproteins suggests that icosahedral symmetry is incompletely fulfilled during budding. The NC also remains in close proximity with the envelope at the proximal pole after budding. (F) Viral maturation through the TGN produces dramatic conformational changes in the glycoproteins, allowing furin to cleave the pr peptide from the particle. As a result, the NC is shifted to the center of the particle, displaying no contact points with the envelope. Color scheme is similar to Figs. 3 and 5.

formation of peroxisome replication compartments of both tomato bushy stunt virus and brome mosaic virus (Barajas et al., 2009; Diaz et al., 2015). Contrasting results were observed in an HCV study, where ESCRT factors were not used for the construction of replication complexes but were instead found to play a critical role in the formation of virus particles (Corless et al., 2010). Subsequently, a mass-spectrometry analysis of JEV and DENV interacting partners reported that several ESCRT factors were recruited to sites of replication complexes along the ER membrane (Tabata et al., 2016). Upon further analysis using siRNA knockdown, the ESCRT proteins TSG101, CHMP2/3, and CHMP4 were all found to play essential roles in flavivirus propagation but did not affect genomic RNA replication. This suggested that the ESCRT proteins were involved in the processes of virus budding and not the formation of flavivirus replication complexes. As such, further biochemical analysis of ESCRT-mediated flavivirus particle formation could lead to a greater understanding of the flavivirus budding process and could even present useful targets for antiviral drug development.



## 5. Flavivirus maturation and egress

In contrast with the immature flavivirus particle, as well as other enveloped viruses such as influenza (Krammer et al., 2018), HIV (Seabright et al., 2019), measles (Ferren et al., 2019), and coronaviruses (Schoeman and Fielding, 2019), the mature flavivirus particle contains a smooth, spike-less surface that closely resembles a golf ball (Fibriansah et al., 2014; Kostyuchenko et al., 2013, 2014; Mukhopadhyay et al., 2003; Wang et al., 2017; Zhang et al., 2013b) (Fig. 5). Initial structure prediction models hypothesized that flaviviruses contained  $T=3$  icosahedral symmetry (Caspar and Klug, 1962). However, this was found to be inaccurate after the first flavivirus structure (DENV) was published in 2002 using cryo-EM (Kuhn et al., 2002). This report demonstrated that while flaviviruses do indeed contain icosahedral symmetry, they lack the predicted  $T=3$  quasi-equivalent environment. Flavivirus mature particles are roughly  $\sim 50$  nm in diameter and contain 180 copies of E protein organized into 90 antiparallel homodimers containing twofold symmetry that lie flat against the surface of the lipid bilayer (Kuhn et al., 2002). The E protein ecto-domains predominate the surface of the virion while the smaller M proteins reside underneath these ecto-domains alongside the stem and TMDs of each E protein. Groups of three E protein homodimers are arranged parallel to one another, forming 30 protein “rafts” around the particle. These rafts are what give flaviviruses their characteristic

“herringbone” pattern. Furthermore, these rafts almost completely cover the viral membrane, seemingly rendering the lipids below them inaccessible from the viral exterior.

The lipid bilayer is roughly polygonal in shape and surrounds the NC with an approximately  $\sim 20 \text{ \AA}$  gap between the NC and the inner leaflet, similar to what was observed in the immature virion (Kostyuchenko et al., 2013; Li et al., 2008; Zhang et al., 2003b). The presence of discernable features within the viral membrane suggest that there might be some lateral order among the phospholipids, but this concept remains undefined. The flavivirus membrane is shown to be “bent” angularly at the distal ends of both the M and E TMDs, reducing the thickness of the membrane from  $\sim 40$  to  $\sim 30 \text{ \AA}$  due to the short length of these helices (Zhang et al., 2013a). Both the M and E protein TMDs are shown to be oriented vertically within the mature virion, each forming a coiled-coil hairpin loop but showing no interactions between the two proteins. The three peripheral membrane helices of the E protein stem region and the singular peripheral membrane helix of M protein lie parallel to the outer leaflet of the viral membrane underneath the E ecto-domains. Previous studies in DENV have found that helices one and three of the E protein stem region are amphipathic, allowing them to interact with the viral membrane, while simultaneously neutralizing any electrostatic repulsion between the phospholipid head groups in the membrane and the ecto-domains of the E protein rafts (Zhang et al., 2013b). The single helix forming the M protein stem was also shown to be partially buried in the viral membrane, suggesting that it too is amphipathic and might aid in the neutralization of electrostatic repulsions (Zhang et al., 2004).

The mature M and E proteins were shown to contain hydrophobic interactions that stabilized their dimeric interface (Zhang et al., 2004). Indeed, a thorough study in DENV by Zhang et al. (2013b) identified numerous residues in both the M and E proteins that interacted with one another to form three “binding pockets” that functioned to stabilize the envelope of mature flavivirus virions. Their study concluded that residue V2 of M interacted with residues L216, L218 and M260 of E protein, forming pocket 1. Pocket 2 was generated via interactions between residues H7, M10 and L12 of M protein with residues H209 and W212 of E. Lastly, W19 of M protein was found to be encircled by E protein residues W206 and H261, forming pocket 3. The authors suggested that these three interaction points between M and E provided enough strength within the dimeric heterodimers that the whole particle was ultimately stabilized as a

result. The authors also went on to explain that the identified interacting residues were highly conserved among all flaviviruses, with a few minor exceptions, demonstrating the necessity of these interactions in the stabilization of mature virions. Another study supported this claim when mutations at residues H214 and H263 of WNV E protein (the equivalent of DENV E protein residues H209 [pocket 2] and H261 [pocket 3], respectively) reduced viral cell entry, suggesting that these mutants produced stability issues within the mature virions (Nelson et al., 2009). Additionally, a study in DENV demonstrated that another residue in E protein, namely H98, also played a significant role in the stabilization and maturation of virions (Zheng et al., 2010). After substituting H98 with an alanine, Zheng et al. (2010) observed a significant reduction in virus infectivity and prM processing. Together, these studies show that several histidine residues within E protein could very well serve as “pH sensors” for the virus, effectively *locking* E protein into place while the virion is in a neutral or basic environment and each histidine is deprotonated. When the environment turns acidic, such as in the late endosome after virus entry, each histidine would then become protonated and positively charged, subsequently repelling the other basic residues within each binding pocket and *unlocking* the E protein to freely undergo the conformational changes needed for membrane fusion.

Comparisons between each of the solved structures of mature flaviviruses has revealed a strikingly conserved organization with minor discrepancies only evident at the atomic scale. Irrespective of the relatively low sequence homology between ZIKV and DENV E proteins (54%), the root mean square deviation (r.m.s.d.) of corresponding C $\alpha$  atoms of the M and E proteins in the mature structures of these two viruses was found to only be 1.8 Å (Sevvana et al., 2018; Sirohi et al., 2016). Such a high level of structural conservation between these two viruses could indicate that a specific conformation of E protein is necessary for proper protein function, regardless of sequence similarity. The same study also observed that the largest r.m.s.d between the mature ZIKV and DENV structures was at sites of glycosylation (up to 6 Å) (Sevvana et al., 2018; Sirohi et al., 2016). This discrepancy makes sense given the differences in the location, size, and number of glycosylation sites between ZIKV and DENV. For example, ZIKV only contains one glycosylation site at N154, while DENV contains two sites at N67 and N153 (Carbaugh et al., 2019; Goto et al., 2005). Moreover, the glycosylation region is five amino acids longer in ZIKV than in DENV and maintains poor sequence homology across all flaviviruses.

The differences in glycan location and number has also been postulated to be a defining factor in the pathogenic or tropic nature of specific flaviviruses (Sirohi et al., 2016). For example, the glycosylation at N154 in WNV was found to be a requirement for neuroinvasiveness (Alsaleh et al., 2016; Maharaj et al., 2019). More research into the structural, and by extension pathological, differences in flavivirus glycosylation is required to understand their significance more fully.

As can be seen above, numerous studies have identified the structural differences between the immature and mature flavivirus particles. However, several conformational changes must occur to transform the particle from a “spikey” conformation into its final, smooth form. After the immature flavivirus buds into the ER lumen, the prM-E heterodimers are arranged into 60 trimeric spikes with the E protein DII of each trimer protruding away from the virion. At the tip of each trimeric spike, prM sits atop the fusion loops at the distal end of each E protein DII, protecting them from premature membrane fusion. While still in the ER lumen, the immature virus is decorated with carbohydrate moieties at various locations. Studies have confirmed that the prM protein is glycosylated at N69 (Li et al., 2008), with circumstantial evidence showing additional N-linked glycosylation at N7, N31, and N52 (Courageot et al., 2000). The purpose of prM glycosylation has not been extensively studied, but one possibility could be that decorated prM proteins are required for the proper folding and chaperoning of E protein through the TGN during the maturation process. Additionally, the E protein of DENV has been shown to be glycosylated at N67 and N153 (Goto et al., 2005). The N67 site is unique to DENV, with most other flaviviruses showing glycosylation only at N154 (the N153 complement) and some displaying no glycosylation at all (Alsaleh et al., 2016; Maharaj et al., 2019; Yoshii et al., 2013). Functionally, E protein glycosylation was reported to facilitate cell receptor binding and even protection of the fusion loop as an “epitope shield.” For a more in depth look at the consequences of flavivirus glycosylation, readers are encouraged to examine a thorough review by Yap et al. (2017).

After formation and glycosylation in the ER lumen at pH  $\sim$ 7.2, the immature virion is trafficked to the Golgi where it then makes its way through the TGN. Here, the environmental pH is incrementally lowered from pH  $\sim$ 6.7 in the *cis*-Golgi to pH 6.0 in *trans*-Golgi. The low pH in the TGN initiates the rotation of the elongated E protein DII within each prM-E heterodimer, effectively collapsing each trimeric spike into an anti-parallel “dimeric” prM-E heterodimer (Li et al., 2008; Yu et al., 2008).



At this stage, the E protein DII is still capped by the pr peptide of prM, but it is now pointed parallel to the membrane surface instead of perpendicular as observed in the immature virion. Zhang et al. (2013b) hypothesized that the rotation of E protein from a position perpendicular to the viral membrane to a parallel one is mediated by prM and driven by a decrease in pH. Their model suggests that the flexible linker between the pr peptide and M stem-TMD region acts like a “drawstring.” The energy required to tighten the drawstring is supplied via the diminishing pH in the TGN and results in the rotation of E DII and the collapse of the 60 trimeric spikes into 90 dimers. After the prM drawstring lowers E, the pr peptide at the tip of each E protein DII then binds to the adjacent E protein DI, locking the prM-E dimeric heterodimers in place and exposing the furin cleavage site on the pr peptide. This pH-dependent “locking” mechanism between the pr peptide and adjacent E protein DI is somewhat supported by previous studies showing that the pH dependent conformational changes of E proteins prior to furin cleavage of the pr peptide are indeed reversible (Yu et al., 2008).

The presence of a reversible mechanism suggests that the dimeric “lock” requires a proton “key” and that such an interaction would most likely need to occur between a negatively charged residue and a histidine. The declining pH of TGN trafficking would maintain protonation of the H residue, allowing it to interact with the hypothetical negative residue and maintain a connection between the pr peptide of one heterodimer and the E protein DI of an adjacent heterodimer. Upon furin cleavage and subsequent release from the cell, the environmental pH would return to neutral and the H residue would become deprotonated, releasing pr from the virion. However, no mutagenesis studies have identified the residue(s) responsible for such a mechanism. Nonetheless, the structure of the low pH immature DENV particle published by Yu et al. (2008) can provide a structural foundation from which possible interacting residues can be identified. While no side chain data is available within the PBD file (PDB: 3C6R), negative residue E60 of prM is in close proximity with the E protein DI residue H149 in the adjacent prM-E heterodimer. Residue E60 of prM is highly conserved across most flaviviruses, with the exception of JEV and WNV, which contain an aspartate (Yoshii et al., 2012). Similarly, residue H149 of E protein is also highly conserved, except for JEV and WNV which contain a threonine (Chakraborty, 2016). Of course, protein sequence alignment does not necessarily translate to structural similarity, but the previously discussed structural homology of flaviviruses



(Sirohi et al., 2016) is a strong indicator that these residues align in similar positions within virions and could present interacting partners capable of aiding in the low pH dimerization of prM-E heterodimers. Thus, the hypothetical locking mechanism proposed by Zhang et al. (2013b) would benefit greatly from mutagenesis studies testing the necessity of the aforementioned residues.

Once the prM-E heterodimers are lowered into their dimeric form, the host protease furin cleaves the exposed pr peptide from M, leaving the rest of M (residues 1–75) attached to the viral membrane (Lin et al., 2018; Pierson and Diamond, 2012). Residues 1–20 of the M protein (the M “stem” region) are then somehow internalized underneath the ectodomains of the E protein (the same location of the rest of M protein—residues 21–75, including the M TMDs) where they remain throughout the rest of virus life cycle. It was hypothesized that this change in position of the M-stem, from one flanking the E monomer to one underneath the E ecto-domain, was facilitated by the “hole” between the two dimerized E monomers (Zhang et al., 2003b, 2013b). This hole would have been previously blocked by H27 and H244, one from each E monomer, but could subsequently be opened by the protonation and subsequent charge repulsion of both histidine residues under low pH conditions. After viral release from the cell and a return to physiological pH, the hole would again close, preserving the M-stem underneath the E ecto-domains (Zhang et al., 2013b).

The last step of flavivirus maturation involves the fusion of the TGN transport vesicle containing the virus with the plasma membrane, effectively releasing the virion into the extracellular environment. At this point, the pr domain completely dissociates from the virus particle due to the deprotonation of E protein residue H244, which is then no longer capable of binding to N63 of pr, leaving a fully mature flavivirus behind (Li et al., 2008). Indeed, the substitution of E protein H244 with an alanine residue reduced the release of DENV virus like particles by blocking prM-E interactions, demonstrating the necessity of pr in preventing premature membrane fusion during virus maturation and egress (Zheng et al., 2014). It is worth noting that the fully mature state and fully immature state structures of many flaviviruses have already been solved, but the only intermediate structure between these two states that has been published to date is that of DENV (Yu et al., 2008). More studies probing into the intermediate structural states of flaviviruses would undoubtedly expand our understanding of their very intricate maturation process. Nonetheless, it has

interestingly been shown that the proteolytic processing of pr is not 100% efficient, and that a vastly heterogeneous population of virions ranging from completely mature to completely immature is released after flavivirus infection, particularly with DENV (Li et al., 2008; Stadler et al., 1997; Yu et al., 2008). Indeed, it has been previously shown that the prM content of released DENV can be as high as 50% (Dejnirattisai et al., 2015). Analysis of the morphological distribution of ZIKV progeny from infected Vero cells overexpressing furin also showed that most particles displayed a “smooth” appearance, suggesting a relatively robust proteolytic action from the overexpression of furin (Pierson and Diamond, 2012). Such a distribution of viral morphologies might be necessary for some unknown biological function, but no advantages have been identified to date.



## 6. Conclusions

Within the last 10 years, significant advancements have been made in understanding the structure of flaviviruses and the functions of their various components. Increased resolution through cryo-EM and comparisons between symmetric and asymmetric reconstructions have identified unique structural elements within flavivirus particles. Crystallography and NMR studies of individual flavivirus structural proteins have produced greater insight into the functions and interactions between these proteins during virus assembly and maturation. Comparisons between mature and immature virus structures have displayed the significant conformational changes that must occur during the maturation phase, displaying possible therapeutic targets. The use of antibodies for structural stabilization and a vast number of high-resolution immature and mature virion structures in complex with antibodies produced valuable epitope information (not discussed in this review—see Rey et al., 2018). The abundance of structural data has even fueled countless biochemical and mutagenesis studies that in turn probe structurally identified interactions for their importance in virus assembly and maturation. Altogether, the progress being made in the field of flavivirus structural biology is remarkable, and the implications for antiviral therapeutic development are promising.

Nevertheless, there exists substantial room for improvement. The intricate mechanisms of flavivirus assembly and maturation require deeper exploration and more rigorous experimentation to fully understand. For example, the structure of the viral RNA and the functional elements that drive assembly, as well as the host proteins responsible for aiding in

replication complex and assembly site ER membrane invagination remain poorly understood. The role of lipids and lipid generation during virus assembly and budding are also currently unknown. Additionally, the biochemical mechanisms capable of producing the conformational changes observed between immature and mature virions have yet to be identified (even though some models have been proposed—see [Zhang et al., 2013b](#)). The elucidation of the systems mentioned above, as well as many more not described here, would significantly improve our understanding of flaviviruses and their pathogenesis. Such an advancement in knowledge would undoubtedly aid in the development of antiviral therapeutics and vaccine innovation.

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