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MICROREVIEW

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Shaping the flavivirus replication complex: It is curvaceous!

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Abstract

Flavivirus replication is intimately involved with remodelled membrane organelles that are compartmentalised for different functions during their life cycle. Recent advances in lipid analyses and gene depletion have identified a number of host components that enable efficient virus replication in infected cells. Here, we describe the current understanding on the role and contribution of host lipids and membrane bending proteins to flavivirus replication, with a particular focus on the components that bend and shape the membrane bilayer to induce the flavivirus-induced organelles characteristic of infection.

KEYWORDS

flavivirus replication, lipids, membrane curvature, replication complex

1 | INTRODUCTION

The Flavivirus genus is the largest of the *Flaviviridae* family, which are among some of the most significant emergent or re-emergent human pathogens worldwide. Examples include West Nile (WNV), dengue (DENV), Zika (ZIKV), yellow fever (YFV), and Japanese encephalitis (JEV) viruses. These viruses require an arthropod vector where they are transmitted to vertebrates through an infected mosquito during its blood-feeding cycle. Although flaviviruses are responsible for hundreds of millions of infections worldwide, global eradication of flaviviruses remain a challenging task mainly due to the mosquito intermediate vector. Interestingly, disturbances within the vector-vertebrate equilibrium have resulted in a significant interregional spread of these viruses (Chambers, Hahn, Galler, & Rice, 1990). Most flavivirus infections are asymptomatic however, also provoke a range of clinical manifestations from mild flu-like symptoms to severe complications. More specifically, WNV causes meningitis and encephalitis (Sejvar & Marfin, 2006), DENV promotes dengue haemorrhagic fever and dengue shock syndrome (Gubler, 1998), and ZIKV induces microcephaly and Guillain–Barré syndrome (Mlakar et al., 2016). Currently, the only effective vaccines available towards flaviviruses are the tick-borne encephalitis virus and JEV purified, inactivated virus vaccines and the YFV 17D live attenuated virus vaccine (Chambers et al., 1990; Leyssen, De Clercq, & Neyts, 2000; Monath, 1987; Stock, Boschetti, Herzog, Appelhans, & Niedrig, 2012).

The flaviviruses are enveloped viruses containing a single-stranded positive sense RNA ((+)RNA) genome. The 11-kb genome encodes for a single polycistronic open reading frame flanked by an ~100 nucleotide 5′ and 400–700 nucleotide 3′ untranslated region. The (+)RNA translocates to the cytoplasmic surface of the endoplasmic reticulum (ER) where it is then translated by the host ribosome into a polyprotein and processed into 10 proteins (Pijlman et al., 2008). Due to the limited number of viral proteins, host factors such as lipids and proteins are sequestered and exploited to assist in viral replication. Viruses require these host factors for entry, transcription and translation, immune evasion, and finally egress, which are vital stages of the viral life cycle and frequent targets during the design of "novel" antiviral drugs. However, an aspect that is generally overlooked and is a significant hallmark of almost every (+)RNA virus is the formation of virus-induced membrane structures (Mackenzie, 2005; Martín-Acebes, Vázquez-Calvo, & Saiz, 2016; Nagy & Pogany, 2011; Neufeldt, Cortese, Acosta, & Bartenschlager, 2018). In this review, we will focus on the role of these virus-induced membrane structures during flavivirus replication, examine the host factors (lipids and proteins) required for the biogenesis of these structures, and discuss the importance of targeting these structures during antiviral therapy.

2 | VIRUS-INDUCED MEMBRANE PROLIFERATION AND REMODELLING

A common feature of arguably all (+)RNA viruses is the formation of virus-induced membrane "organelles" during replication (Table 1).

TABLE 1 Summary of +ssRNA virus membranes and the roles of host proteins and lipids in viral replication and membrane remodelling

Virus	Membrane structure	Size of membrane	Cellular origin	Host factor	Role in replication	Reference
West Nile virus	CM/PC: Randomly folded membranes. VP: Cluster of single membrane vesicles within the lumen of the ER	50–150 nm per vesicle	CM/PC derived from ER and ER-Golgi intermediate VP: trans-Golgi	FASN	FASN activity aids RC membrane formation	(Martín-Acebes et al., 2011
				Cholesterol	RC membrane formation	Mackenzie et al., 2007
				Ceramide	Viral replication	Aktepe et al., 2015
				Lyso-PChol and Phospholipase A2	Viral replication and VP formation	Liebscher et al., 2018
				Reticulon 3.1A	CM/PC proliferation and VP formation	Aktepe et al., 2017
Dengue virus	VP: Cluster of single and double membrane vesicles within the lumen of the ER	80–150 nm per vesicle	ER	FASN and fatty acids	FASN activity aids RC membrane formation	Heaton et al., 2010
				Cholesterol	Viral entry and replication	Poh et al., 2012
				RTN3.1A	VP architecture	Aktepe et al., 2017
Enterovirus (poliovirus and coxsackievirus)	Cluster of vesicle or rosette-like structure	70–400 nm	ER, trans-Golgi, and lysosome	ARF1, GBF1	Viral replication and organelle formation	Hsu et al., 2010
				PI4KIIIIβ	PtdIns4P enrichment in RC membranes	Hsu et al., 2010
				PtdIns4P	Replication protein binding to membranes & RC membrane formation	Hsu et al., 2010
Alphaviruses (Semliki Forest, Sinbis, and Chikungunya virus)	Spherule-lined cytopathic vacuoles	600–4,000 nm; spherules 50 nm	Endosome and lysosome	PI4K	Membrane formation by stimulating phosphatidyl choline synthesis	Perez, Guinea, & Carrasco, 1991
				Amphiphysin	SH3-medited interaction with nsP3 promotes membrane curvature via binding to surface lipids	Neuvonen et al., 2011
Coronaviruses (mouse hepatitis virus)	Double membrane vesicles structure	More than 200 nm per vesicle	Probably rough ER or ER–Golgi intermediate	LC3-I	RC formation by hijacking autophagy	Reggiori et al., 2010
				EDEM1 and OS9	Edemosome formation	Reggiori et al., 2010
Hepatitis C virus	Membranous web: Cluster of single and double membrane vesicles embedded in a membranous matrix	80–150 nm per vesicle	Probably the ER	RTN3.1A	Inhibits HCV replication by interacting with NS4A to inhibit dimerisation	Wu, Ke, Hsu, Yeh, & Horng, 2014
				Phosphatidylinositol 4-kinase III alpha	Stimulates phosphatidylinositol 4-phosphate production and replication complex formation	(Berger et al., 2009, Berger, Kelly, Jordan, Tartell, & Randall, 2011)
Tomato bushy stunt virus	Inward vesicular structures like multivesicular bodies (peroxisomal multivesicular bodies)	80–150 nm in diameter	Peroxisome and ER	Vps23p, Brol	p33 recruits these components to the peroxisome which in turn assembles and protects the RC	Barajas, Jiang, & Nagy, 2009
				Vps4p AAA+ ATPase	Aids in the viral RC formation by interacting with the viral RNA	Barajas, de Castro Martín, Pogany, Risco, & Nagy, 2014
				Erg25, SMO1, and 2	Sterol synthesis and RC formation	Sharma, Sasvari, & Nagy, 2010

(Continues)

TABLE 1 (Continued)

Virus	Membrane structure	Size of membrane	Cellular origin	Host factor	Role in replication	Reference
Brome mosaic virus	Spherular ER membranes	50–70 nm of spherules	Presumably the ER; however, specific membrane sites remain poorly understood	RTN3	RTN is incorporated into the interior spherules and maintains an open channel	Diaz et al., 2010
				ESCRT-III complex, Snf7	RNA replication attenuation alongside alterations in spherule formation.	Diaz, Zhang, Ollwerther, Wang, & Ahlquist, 2015
				OLE1, Δ9 fatty acid desaturase	Essential for viral replication. Lower levels of phospholipids may affect membrane fluidity	Lee, Ishikawa, & Ahlquist, 2001

Note. CM/PC: convoluted membranes/paracrystalline arrays; FASN: fatty acid synthase; VP: vesicle packet; ER: endoplasmic reticulum; RC: replication complex; PChol: phosphatidylcholine; RTN: reticulon.

Pestivirus and hepacivirus replication occurs within a perinuclear matrix defined as the membranous web (Egger et al., 2002); bromoviruses induce numerous intraluminal ER membrane invaginations (Lee & Ahlquist, 2003); and alphaviruses replicate in spherule-lined cytoplasmic vacuoles derived from the lysosome and endosomes (Froshauer, Kartenbeck, & Helenius, 1988). Flaviviruses are no exception to this process as they induce the formation of vesicle packets (VPs) and convoluted membranes/paracrystalline arrays (CM/PC) (Mackenzie, Jones, & Young, 1996). WNV-induced ER rearrangements are first observed before the end of the latent period (9 to 10 h.p.i) and is apparent with an increase in cytoplasmic vacuoles (Ishak, Tovey, & Howard, 1988; Ng, 1987; Ng & Hong, 1989; Westaway, Mackenzie, Kenney, Jones, & Khromykh, 1997). As the infection progresses, membrane structures protruding the rough ER membranes proliferate with whorls of fibres in various vacuoles (Ng, 1987; later confirmed as replicating RNA by ultrastructural studies) and are followed by the formation of microtubule paracrystals (Ng & Hong, 1989). These structures have been confirmed as three continuous membranous structures: VP, CM/PC (Mackenzie et al., 1996; Westaway et al., 1997).

The VPs are groups of 70- to 100-nm vesicles proliferating and enclosed by the rough ER. They contain electron dense material (viral dsRNA) and viral proteins (NS1, NS2A, NS3, NS4A, and NS5; Cortese et al., 2017; Junjhon et al., 2014; Mackenzie et al., 1996; Mackenzie, Khromykh, Jones, & Westaway, 1998; Miorin et al., 2013; Welsch et al., 2009; Westaway et al., 1997; Westaway, Khromykh, & Mackenzie, 1999), which are proposed to form the replication complex (RC) and therefore the region mediating RNA replication. The WNV VPs consist of numerous vesicles that are either connected to the rough ER or interconnected to one another by pores that open to the cytoplasm via a membranous neck (Gillespie, Hoenen, Morgan, & Mackenzie, 2010). The neck acts as a passage for the movement of cytoplasmic content into the VP and the transport of newly transcribed viral RNA out to the cytoplasm (Gillespie et al., 2010). Furthermore, the newly synthesised RNA translocates to the CM/PC where it is proposed to undergo translation and proteolytic processing by the viral protease NS2B-3 and host signalase (Westaway et al., 1997). CM/PC structures are continuous with the rough ER with the WNV CM/PC containing markers from the ER–Golgi intermediate compartment (Mackenzie, Jones, & Westaway, 1999). Interestingly, WNV-infected mammalian and insect cells contain both VP and CM structures (Ng, 1987); however, these structures were only observed in DENV-infected mammalian cells. DENV-infected insect cells contain VPs but do not contain CM structures (Junjhon et al., 2014).

The requirement for virus-induced membranes by (+)RNA viruses is not entirely understood but believed to play similar roles between different viruses. The VPs will conceal the dsRNA and the RCs, preventing detection by pathogen recognition receptors (Overby, Popov, Niedrig, & Weber, 2010; Uchida et al., 2014), as well as antiviral proteins such as PKR (Samuel et al., 2006) and MxA (Hoenen et al., 2014; Hoenen, Liu, Kochs, Khromykh, & Mackenzie, 2007). Furthermore, the process of compartmentalisation increases the local concentration of replicative components, narrows RNA replication and translation to specific sites, acts as a scaffolding for RC anchoring to

membranes, and tethers the viral RNA during unwinding (Mackenzie, 2005; Miller & Krijnse-Locker, 2008). Combined, these functions act as a central hub for viral replication that promotes exponential replication; however, the identification and role of host lipid and protein factors required to form these structures are not entirely understood.

3 | HOST FACTORS REQUIRED FOR FLAVIVIRUS REPLICATION AND MEMBRANE MORPHOGENESIS

In addition to modulating the innate immune response during infection, viral proteins manipulate multiple pathways to regulate cellular homeostasis. Genome-wide RNA interference and CRISPR screens in WNV- and DENV-infected cells demonstrated that each virus possesses overlapping as well as unique requirements of host proteins to aid in viral replication. These studies identified host proteins that required for the regulation of the cytoskeleton networks, cell trafficking, RNA processing, host translation, protein modification and degradation, stress response, signal transduction, apoptosis, and lipid metabolism (Krishnan et al., 2008; Marceau et al., 2016; Savidis et al., 2016; Zhang et al., 2016). Krishnan et al. (2008) identified 305 host proteins that regulate WNV infection of which 283 are host susceptibility factors and 22 are host resistance factors. Interestingly, enrichment of carbohydrate and lipid metabolism regulatory genes were specific to WNV, indicative of the importance of lipid metabolism during WNV replication in mammalian cells (Krishnan et al., 2008). However, Perera et al. (2012) demonstrated by high-resolution mass spectrometry in DENV-infected mosquito cells that 15% of cellular metabolites and 85% of isolated RC membrane metabolites were significantly different compared with uninfected cells (Perera et al., 2012).

4 | LIPIDS

Lipids are a diverse group of naturally occurring organic compounds that are synthesised from fatty acids and their derivatives. Lipids are one of the most abundant type of cellular molecules that display innumerable amounts of biochemical and physiological cellular functions. They are the main constituent of cellular membranes (plasma membrane, ER, Golgi, endosome, and lysosomes); however, the lipid composition constructing these structures vary among tissue types (Klose, Surma, & Simons, 2013; Muro, Atilla-Gokcumen, & Eggert, 2014). Historically, it was believed that the primary role of lipids was limited to membrane morphogenesis and energy production; however, advances in lipidomics has led to the discovery of lipids in various cellular functions. These include structural changes and stability (induce and stabilise membrane curvature), protein modification (glycosylation), signalling platforms (such as lipid rafts), and inflammation (Kusumi et al., 2012; Muro et al., 2014). The integration of specific lipid classes into membrane leaflets allows them to adjust their fluidity, plasticity, and topology, which further aids in maintaining membrane curvature and regulates signalling.

Viral replication is a complex process that requires and regulates many host factors including lipid metabolism and redistribution (Stapleford & Miller, 2010). Viruses interact with host lipids to enhance replication; however, certain lipids that are advantageous for one virus could be detrimental for another. WNV (Medigeshi, Hirsch, Streblow, Nikolich-Zugich, & Nelson, 2008) requires cholesterol rich-microdomains to facilitate entry, whereas JEV and DENV entry is significantly blocked by cholesterol enrichment (Lee, Lin, Liao, & Lin, 2008), suggesting a preferential requirement of lipids between viruses within the same family. Furthermore, (+)RNA viruses depend on lipids to induce viral "organelles" that are integral for efficient viral replication. Proliferations of virus-induced membrane structures and the stabilisation of the RC is maintained by interacting with lipids of targeted host organelles by regulating their lipid composition (Martín-Acebes, Blázquez, De Oya, Escribano-Romero, & Saiz, 2011). Additionally, it has been observed that ceramide plays an instrumental role in benefitting WNV replication but an apparent inhibitory role for DENV (Aktepe, Pham, & Mackenzie, 2015). These reports not only highlight the need to further understand the role of different lipid classes to flavivirus replication but also the contribution they have on each individual virus.

4.1 | Fatty acid synthase

Fatty acid synthase (FASN) is a cytoplasmic, multifunctional protein that catalyses fatty acid synthesis. In the presence of NADPH, FASN primarily synthesises palmitate—a long-chain fatty acid (C 16:0)—from acetyl-CoA and malonyl-CoA (Wakil, 1989). Martín-Acebes et al. (2011) showed that WNV replication is dependent on FASN activity by using two FASN inhibitors: cerulenin and C75. Upon infection with WNV, FASN is recruited and localises with the WNV RC. Inhibition of FASN activity attenuates WNV replication, which suggests a preferential requirement of fatty acids in regulating WNV-induced membrane proliferation (Martín-Acebes et al., 2011). DENV also requires FASN activity for membrane biogenesis. During DENV infection, FASN is recruited to sites of viral compartments by interacting with the viral NS3 protein, which in turn stimulates the activity of FASN to increase fatty acid biosynthesis (Heaton et al., 2010). Collectively, WNV and DENV preferentially require fatty acid biosynthesis alongside the mevalonate pathways to form the CM/PC and VP. Expression of FASN catalyses the conversion of acetyl-CoA and the generation of palmitoyl-CoA, the first two steps in regulating the fatty acid synthesis pathway (Smith, 1994, Tong, 2005, Heaton et al., 2010). The newly generated fatty acids are modified or incorporated into the ER, thus leading to the expansion of the viral membranes and regulating membrane fluidity and curvature (Heaton et al., 2010). Furthermore, fatty acid biosynthesis together with cholesterol synthesis can aid in viral membrane biosynthesis.

4.2 | Cholesterol

Mammalian cellular membranes are composed of lipid bilayers containing phospholipids and cholesterol. Modification of membrane fatty acids, phospholipids, and cholesterol content disrupts membrane fluidity and affects a variety of cellular functions (Smith, 1994). Cholesterol biosynthesis is regulated within the ER by the sterol regulatory element-binding protein, a membrane-bound transcription factor. Sterol

regulatory element-binding proteins play a key role in activating genes that upregulate cholesterol synthesis and uptake, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and FASN (Simons & Ikonen, 2000). Additionally, cellular cholesterol is further regulated by the intake of extracellular cholesterol (Goldstein & Brown, 1984) and the efflux of intracellular cholesterol (Schmitt & Tampé, 2002).

WNV and DENV have both shown dependence to cholesterol to mediate viral replication (Mackenzie, Khromykh, & Parton, 2007; Poh et al., 2012; Ye, 2007). Mackenzie et al. (2007) demonstrated that WNV up-regulates cholesterol biosynthesis during infection, and this increase caused a redistribution of cholesterol to the viral RCs. The manipulation of cholesterol levels by reducing HMGCR and altering cellular geranylgeranylated protein concentration drastically reduced viral replication, presumably by reducing HMGCR levels contributing to membrane biogenesis (Mackenzie et al., 2007). In contrast, depletion of intracellular cholesterol with U18666A (a multiaction inhibitor that acts by inhibiting HMGCR activity and the release of cholesterol from late endosome and lysosome [Cenedella, 2009]) inhibits DENV entry and replication by trapping virions in late endosomes/lysosomes; however, the formation of VPs and RCs was not altered (Poh et al., 2012). These results suggest that cholesterol depletion affects WNV and DENV at different stages of their replication cycles and that a combination of multiple lipid classes is involved in the shaping of the VPs. Further, reducing individual lipid components is not sufficient to completely prevent VP formation, and a combined reduction of fatty acids, sterols, and sphingolipids are required.

4.3 | Sphingolipids and ceramide

Sphingolipids are generally composed of a long-chain sphingoid base, amide-linked fatty acids, and a polar (carbohydrate) head group at the 1-position, except for ceramide and sphingomyelin, which contains a hydroxy at the 1-position and a phosphorylcholine head group, respectively (Hannun & Bell, 1989). Sphingolipids consist of four main members: sphingomyelin, ceramide, sphingosine, and sphingosine-1-phosphate. Due to the central position occupied by ceramide in the sphingolipid pathway, it is considered as the central metabolic hub for sphingolipid biosynthesis and catabolism (Hannun & Obeid, 2008). Removal of phosphorylcholine from sphingomyelin by the hydrolytic activity of acid sphingomyelinase results in the production of ceramide through the sphingomyelinase pathway (Utermöhlen, Herz, Schramm, & Krönke, 2008). Ceramide is also synthesised in the ER through the de novo pathway by the catalysis of serine and palmitoyl-CoA by serine-palmitoyl-coenzyme A transferase, which acts as the first committed step in ceramide biosynthesis. Finally, recycling of complex sphingolipids through the salvage pathway can convert sphingosine into ceramide by the enzymatic activity of ceramide synthase (Merrill & Wang, 1992).

Over the past few decades, sphingolipids have been shown to regulate cellular homeostasis almost at every level. Ceramide has been shown to regulate cell senescence (Venable, Lee, Smyth, Bielawska, & Obeid, 1995), cell stress responses such as differentiation, cell-cycle arrest, and apoptosis and induces membrane curvatures (Obeid, Linardic, Karolak, & Hannun, 1993). Sphingomyelin does not have the tendency to form membrane curvature due to the phosphorylcholine head group. However, the formation of ceramide by cleaving the phosphorylcholine head group causes a structural change resulting in a cone-shaped lipid structure, which has the tendency to induce spontaneous negative curvature. The presence of ceramide on one leaflet of a lipid bilayer enhances membrane bending (negative curvature) and the tendency to form hexagonal phases II structures (Goñi & Alonso, 2002; Krönke, 1999; Utermöhlen et al., 2008). The unique structure of ceramide can also influence cellular signalling by effecting membrane microdomain function (such as lipid rafts) and vesicular transport.

Flaviviruses have evolved to exploit ceramide to establish infection by modulating these molecules to mediate entry and egress into cells. JEV and WNV both require ceramide enriched membrane-platforms to enter and egress from cells, respectively. The increase in membrane-bound ceramide by sphingomyelinase treatment significantly increased JEV infection, whereas reduction in ceramide levels had converse effects (Tani et al., 2010). Further, Martín-Acebes et al. (2014) demonstrated that WNV virions possess a unique membrane envelope composition enriched in sphingolipids and the reduction of ceramide by inhibiting sphingomyelinase reduced virion release (Martín-Acebes et al., 2014). Apart from entry and egress, Perera et al. (2012) suggested that the negative curvature-enhancing lipids such as ceramide may be active in the formation of the membranes induced during DENV infection (Perera et al., 2012). Mass spectrometry analysis on DENV-infected cells revealed that membrane remodelling was directly linked with the induction of unique lipids (such as sphingolipids) during replication where 85% of these lipids were different in viral membranes compared with uninfected cells (Perera et al., 2012). Furthermore, it was demonstrated that the down regulation of ceramide has contrary effects on WNV and DENV replication. Reduction of intracellular ceramide levels attenuated WNV replication but enhanced DENV replication (Aktepe et al., 2015). These results suggest that a regulated flux of sphingomyelin-to-ceramide conservation is essential for flavivirus infection, where a regulated balance of sphingolipids is required during specific stages of viral replication. Caution should be taken when designing therapeutic agents against flaviviruses as clearance of one virus may be detrimental for another.

4.4 | Phospholipids

Two lipidomic analyses on WNV-infected mammalian cells and DENV-infected mosquito cells have revealed a distinct modulation of the phospholipid landscape upon infection (Liebscher et al., 2018; Perera et al., 2012). Both these studies observed a significant decrease in phosphatidylcholine (PChol) abundance with an increase in lyso-PChol. This change is indicative of increased activity of the host enzyme Phospholipase A2, a role that has been recently shown for at least WNV (Liebscher et al., 2018). In contrast to ceramide, lyso-PChol exhibits the capacity to induce positive membrane curvature due to its relatively large hydrophilic head group in relation to its hydrocarbon tail (Fuller & Rand, 2001). Therefore, the combination of ceramide and lyso-PChol to induce both negative and positive curvature would align with the requirements of the invagination from the ER to form the VP. A more recent study has also revealed a highly dynamic regulation of lipid homeostasis in DENV-infected mosquitoes

(Chotiwan et al., 2018), which again showed major changes in phospholipids, glycerophospholipids, glycerolipids, and sterol lipids. This study additionally showed that the entire repertoire of lipid biochemistry contributes to DENV replication including lipid synthesis, lipolysis, lipid conversion, β-oxidation, and/or redistribution to provide the optimal lipid environment and platforms facilitating efficient virus replication.

5 | HOST MEMBRANE-SHAPING PROTEINS DURING VIRAL REPLICATION

In addition to lipids, host proteins are crucial in almost every step of (+) RNA virus replication, including membrane biogenesis. Alongside host lipids, proteins are required to bend and stabilise virus-induced membranes. Although host proteins are vital for flavivirus replication, studies involving host proteins to modulate cellular membranes during infection remains under investigation. CRISPR and RNA interference screens have identified key host proteins in the replication of many flaviviruses; however, very few studies have subsequently investigated the role of identified proteins in the biogenesis and stability of the viral RC. The reticulon (RTN) protein is the most extensively studied host membrane-shaping protein during (+)RNA viruses, including WNV and DENV (Aktepe, Liebscher, Prier, Simmons, & Mackenzie, 2017).

5.1 | Reticulon

The RTN family of proteins are membrane-associated proteins that are prominently found on the ER membrane, where they induce ER membrane shaping and stabilise highly curved ER membrane tubules (Voeltz, Prinz, Shibata, Rist, & Rapoport, 2006). Previous studies have established that RTNs are required for brome mosaic virus (BMV) spherule and enterovirus 71 (E71) RC formation and replication. The BMV 1a protein interacts with RTN and incorporates RTN into the spherule interior where they are necessary for spherule formation and maintain an open channel to the cytoplasm (Diaz, Wang, & Ahlquist, 2010). Furthermore, the E71 2C protein, which associates with host membrane vesicles to induce viral RCs, interacts with host RTN3 protein to aid in infectivity, viral protein, and dsRNA synthesis (Tang et al., 2007).

Recent studies have demonstrated the importance of RTN 3.1A during flavivirus replication. During flavivirus (WNV, DENV, and ZIKV) infection, RTN3.1A is required to induce (WNV and ZIKV) or preserve (DENV) RC architecture by directly (WNV) or indirectly (DENV and ZIKV) interacting with the viral NS4A protein (Aktepe et al., 2017). The abolishment of this interaction by silencing RTN3.1A destabilises and promotes the degradation of NS4A, which further affects the aptitude of these viruses to remodel intracellular membranes. This translates into a significant attenuation in viral translation and the generation of viral infectious particles (Aktepe et al., 2017). Together,

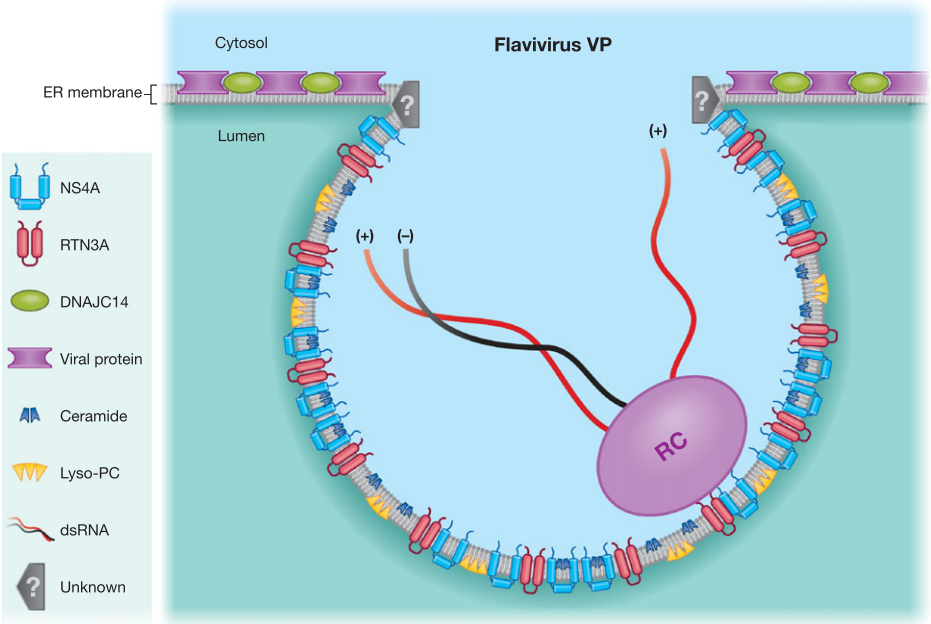

FIGURE 1 Hypothetical model for the flavivirus replication complex (RC); vesicle packet (VP). Flavivirus infection constructs membrane proliferations termed VPs which house the viral RC. The construction and stability of the RC promotes an exponential increase in viral replication. Translation of viral proteins on the surface of the ER acts as a platform for protein–protein and protein–lipid interactions. Viral proteins interact with DNAJC14, which acts as a chaperone to modulate VP formation (Yi et al., 2011, Yi et al., 2012), potentially on cholesterol-rich microdomains (Mackenzie et al., 2007). We speculate that the viral protein NS4A (based on its predicted topology, structure, and membrane remodelling capacity [Roosendaal et al., 2006, Miller et al., 2007]) induces membrane curvature while interacting with the host RTN3.1A protein (Aktepe et al., 2017). We suggest that the biogenesis and recruitment of the cone-shaped lipids ceramide (Aktepe et al., 2015) and lyso-PChol (Liebscher et al., 2018) stabilises and provides the required curvature for the formation of the VP. Viral and host proteins supporting the VP neck structure (an integral component of the VP that allows for the passage of nucleotides, proteins, and viral RNA) is currently unknown; however, components of the ESCRT complex may be recruited to aid in the stabilisation of the neck (Barajas et al., 2009, Barajas et al., 2014, Diaz et al., 2015)

these studies demonstrate a novel mechanism by which BMV, E71, and flaviviruses remodel cellular membranes by recruiting host proteins to the RCs.

6 | DNAJC14: A CRUCIAL PROTEIN INVOLVED IN YFV RC FORMATION

Although DNAJC14 does not possess membrane bending capabilities, it is a vital protein required by YFV to aid in RC assembly and further docking of the RC to a specific subcellular ER membrane location. A study by Yi and colleagues revealed a central role for the host protein DNAJC14 in formation of the YFV RC (Yi et al., 2011; Yi, Yuan, Rice, & MacDonald, 2012). DNAJC14 (also known as DRIP78, Jiv, or HDJ3) is a chaperone molecule within the Hsp40 family of protein chaperones. It was observed that DNAJC14 co-localises with YFV dsRNA within the RC, and that endogenous levels of DNAJC14 are vital for replication, but overexpression of DNAJC14 was inhibitory to a broad range of flaviviruses (Yi et al., 2011). A 1:1 stoichiometry of viral protein to DNAJC14 allows proper folding of the viral protein and thus RC formation; however, overexpressing of DNAJC14 disrupts the normal stoichiometry and interrupts RC formation. Subsequent studies determined that recruitment of DNAJC14 to the YFV RC was associated with detergent-resistant membrane domains within the ER, demonstrating an importance in RC formation in membrane biogenesis (Yi et al., 2012). This characterisation of DNAJC14 highlights the importance of host factors in regulating flavivirus replication and the protein–protein and protein–lipid interactions that drive and shape RC formation and stabilisation.

7 | CONCLUSION

Flaviviruses have evolved to utilise host lipids and proteins to generate virus-induced membrane compartments to assist in replication. WNV and DENV co-opt FASN, phospholipids, cholesterol, and RTN3.1A to aid in membrane biogenesis, however differentially require ceramide during this process. FASN is recruited to sites of viral replication to stimulate an increase in fatty acid biosynthesis, which are modified and/or incorporated into the ER. Free fatty acids and cholesterol within the ER may lead to the expansion of the viral membranes and regulate membrane fluidity and curvature. Furthermore, we speculate that sphingolipids may have differential roles during flavivirus membrane morphogenesis. Cone-shaped and inverted cone-shaped lipids possess a tendency to change the physical properties of membranes by inducing stress onto the inserted side of the bilayer to form either positive or negative membrane curvature (Dowhan & Bogdanov, 2002). The insertion of cone shaped ceramide lipids on one leaflet of the lipid bilayer enforces spontaneous negative membrane curvature (Figure 1; Goñi & Alonso, 2002; Krönke, 1999; Utermöhlen et al., 2008). The attenuation observed during WNV replication in the absence of ceramide may be linked to CM/PC and VP abnormalities. Although Perera et al. (2012) suggests that ceramide may be active in the formation of the membranes induced during DENV infection, we have established that depleting endogenous ceramide levels enhanced DENV replication. Unlike WNV, DENV may require diverse classes of lipids to proliferate viral membranes. Additionally, we have demonstrated that both WNV and DENV require RTN3.1A to form viral membranes. Alongside the negative curvature induced by lipids, RTN3.1A inserts into the lipid bilayer to induce positive curvatures to counteract the negative curvature, thus aid in the expansion of the CM/PC and VP (Figure 1). Together, the coalescence of these factors are most likely driven by the association of host and viral proteins within specific membrane platforms (i.e., DNAJC14 within detergent-resistant membranes) to initiate RC formation and stability.

Overall, the differential requirement of lipids and host proteins may in fact explain the subtle differences we observe in membranous structures during flavivirus replication. Disruption of these structures are detrimental for viral replication and thus could be a target for antiviral therapy; however, care must be taken when establishing new antivirals as treatment options. Treating one flavivirus infection may increase infection severity of a secondary infection, thus causing detrimental results.

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