Compartmentalized replication organelle of flavivirus at the ER and the factors involved

Yali Ci1,2 · Lei Shi1,2

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Abstract
Flaviviruses are positive-sense single-stranded RNA viruses that pose a considerable threat to human health. Flaviviruses replicate in compartmentalized replication organelles derived from the host endoplasmic reticulum (ER). The characteristic architecture of flavivirus replication organelles includes invaginated vesicle packets and convoluted membrane structures. Multiple factors, including both viral proteins and host factors, contribute to the biogenesis of the flavivirus replication organelle. Several viral nonstructural (NS) proteins with membrane activity induce ER rearrangement to build replication compartments, and other NS proteins constitute the replication complexes (RC) in the compartments. Host protein and lipid factors facilitate the formation of replication organelles. The lipid membrane, proteins and viral RNA together form the functional compartmentalized replication organelle, in which the flaviviruses efficiently synthesize viral RNA. Here, we reviewed recent advances in understanding the structure and biogenesis of flavivirus replication organelles, and we further discuss the function of virus NS proteins and related host factors as well as their roles in building the replication organelle.

Keywords Flavivirus · Replication organelle · ER rearrangement · Compartmentalization · Membrane remodeling · Nonstructural proteins · Host factors

Introduction
RNA viruses pose a great threat to human beings. Many well-known viruses, such as influenza virus, HIV, dengue virus (DENV), rabies virus, poliovirus and the current global epidemic SARS-CoV-2, are RNA viruses. RNA viruses have a very high mutation rate during their replication, which ensures their fitness for survival and spread in hosts. Flaviviruses are positive-sense single-stranded RNA viruses that have infected humans and caused severe symptoms for several hundred (probably over thousand) years, and comprise 70 members, including yellow fever virus (YFV), Zika virus (ZIKV), DENV and West Nile virus (WNV). Currently, an estimated 400 million DENV infection cases occur annually around the world, and 40% of the world’s population in over 100 counties is at risk of infection [1–3]. During the recent outbreak of ZIKV in America, more than 500,000 cases were reported at the peak of the pandemic in 2016 [4]. Undoubtedly, flaviviruses epidemic have become a great challenge to public health.

Flavivirus is one of the four genera of the Flaviviridae family, and the blood-borne hepatitis C virus (HCV) is another related member belong to the genus Hepacivirus of Flaviviridae family. To avoid confusion, “flavivirus” hereafter in the present review refers to just the members of the Flavivirus genus and does not include HCV. Flaviviruses are also known as arboviruses, because most flaviviruses can be transmitted by arthropod vectors (mosquitoes or ticks). Mosquito-borne flaviviruses can be vertically transmitted between mosquito generations. Meanwhile, mosquito- and tick-borne flaviviruses are horizontally transmitted between mosquitoes/ticks and humans by bites, which makes cross-species transmission of flavivirus possible.
Flaviviruses are enveloped viruses with diameters of approximately 50 nm. The flavivirus genome is a positivesense single-stranded RNA ~11 kb long in length that can be directly translated into a viral polyprotein precursor. The polyprotein precursor is processed by viral protease (NS3 and NS2B) and host proteases (e.g., signal peptidase) and cleaved into three structural proteins and seven nonstructural proteins. Structural proteins (capsid, E and prM/M) assemble viral particles. The capsid proteins associate with viral genomic RNA to form nucleocapsids. E and M proteins are virus envelope proteins involved in flavivirus entry and fusion. Nonstructural proteins participate in virus replication in the cell.

Flaviviruses enter the cell through endocytosis. Although various membrane factors are implied to be involved in binding to flaviviral E proteins for virus attachment, specific cellular receptors for flaviviruses entry are still unidentified (for review, see [3, 5]). The E protein is also the fusogen that mediates the fusion between the viral envelope and endosomal membrane to release the virus nucleocapsid. Subsequent replication and virion assembly of flaviviruses occur at the ER. Then, viral particles are transported into the Golgi apparatus for maturation and finally released outside of the cell through the cellular secretory pathway (Fig. 1).

Flavivirus replication in the cell induces remarkable ER rearrangement, described as dilated ER cisternae with multiple invaginated vesicles (Ve), convoluted membranes (CM) and paracrystalline arrays (PC), which is a characteristic structural hallmark of flavivirus infection [6–9]. Compartmentalized and aggregated membranous networks derived from host cellular ER form flavivirus replication platforms. Together with flavivirus RC and viral RNA, they constitute flavivirus replication organelles in the cell. The present review discusses the architecture and biogenesis of flavivirus replication organelles, the factors involved and the underlying mechanism.

**Membranous replication organelle of flavivirus**

Flaviviruses are not the only viruses that take advantage of the host intracellular membrane system to replicate. Actually, the replication of almost all positive-strand RNA viruses is associated with cellular membranous organelles (for review, see [10]).

Utilization of the host intracellular membrane system to establish viral replication organelles is essential and has many merits. First, a membranous structure is needed and propitious to the assembly of viral RC. Usually, virus-encoded NS proteins are responsible for replication. To efficiently synthesize virus genome, virus NS proteins assemble
into RC to function. Many NS proteins are integral membrane proteins located on cellular organelles. Thus, viral RC assembly on the membranous structure is intrinsically essential. Second, membranous structures, such as single-membrane vesicles (SMVs) or double-membrane vesicles (DMVs), are often formed in the replication of a variety of viruses. These vesicles form a relatively enclosed space and environment. On one hand, such an enclosed structure concentrates the enzyme and substrates for higher reaction efficiency. On the other hand, the compartments also protect the viral genome from recognition, modification and degradation by host cellular immune system and then to evade or delay the host immune response [11–13]. Third, enveloped virus replication sites on the membrane could be spatially close to virion assembly sites, which is convenient for the subsequent virion assembly step. In addition to proximity, some viral replication factors also regulate virion assembly; thus, virus replication and assembly are tightly associated with each other.

As mentioned above, flaviviruses reorganize host cell ER to build viral replication organelles. The ER is a major location for protein synthesis and lipid metabolism in the cell, providing numerous conveniences for viral replication (for review, see [14]). Soon after the translation and processing of flavivirus-encoded proteins, they could directly assemble in situ and initiate viral replication without further translocation to other organelles. The virion assembly of flaviviruses is also completed at the ER, although the assembly site is considered to be close to but different from the replication compartments. Thus, viral protein translation, viral replication and assembly are spatially linked together.

**Architecture of flavivirus replication organelle**

Since the 1960s, the flaviviruses replication has been linked to the ER. Electron microscopy (EM) and biochemical studies provided solid evidence. DENV, Japanese encephalitis virus (JEV) and ZIKV were found to replicate around the ER in different cultured mammalian cells (neurons, lymphoblasts and kidney cells) and infected animal tissues [15–20]. DENV also replicated on the ER of mosquito (Aedes albopictus) cells, indicating a conserved replication site between the mammal host and arthropod vector [21]. Membrane fraction analysis from sucrose gradient density ultracentrifugation also demonstrated that DENV viral proteins were associated with membranes and localized on the ER [19]. Structurally, dilated ER cisternae enclosed with vesicles and virions are often described in early EM studies.

With the development of EM, the fine tuning and three-dimensional structure of flavivirus replication compartments has been successfully resolved (Fig. 2). Using electron tomography, Ralf Bartenschlager group showed that DENV replication compartments are SMVs invaginated toward the dilated ER lumen with a diameter of 80–90 nm, and multiple invaginated vesicles enclosed in the ER lumen form vesicle packets (VPs) [7, 22]. However, these vesicles are not completely sealed structures. A narrow neck (~10 nm in diameter) connects the invaginated vesicle with the cytosol to allow for the exchange of materials such as proteins, nucleic acids (newly synthesized viral RNA) and other molecules. CM are also found in the center of aggregated membrane networks. Similar ultrastructure of TBEV, WNV, and ZIKV replication compartments was also recently observed by EM [23–26].

Although HCV belongs to the *Flaviviridae* family and is a different genus, the HCV replication organelle is quite different from flaviviruses [27]. The larger DMV (~200 nm diameter on average) in the cytoplasm of HCV-infected hepatoma cells is considered the replication organelle. Considering the different structures and formation mechanisms, we focus on flavivirus replication organelles in the present review.

**Crucial factors involved in flavivirus replication organelle formation**

To replicate in the cell efficiently, flaviviruses remodel the ER structure. The remodeled ER structure is quite different from the normal structure. Generally, the ER network comprises flattened sacs (cisternae) connected to the nuclear envelope as well as loose interconnected membrane tubes and sheets. Vesicles containing cargos, such as newly synthesized proteins and lipids, bud from the ER and are transported to other organelles. Many cellular factors in the cytoplasm participate in this ER membrane budding process, including membrane curvature induction (protrusion toward the cytoplasm), maintenance and vesicle shedding. Flavivirus infection induces aggregated and tangled ER networks in the cell. Importantly, a flavivirus-induced vesicular structure invaginated toward the ER lumen is a diametrically opposite process of vesicle budding. Clearly, some factors participate in this ER reorganization process during flavivirus replication, including viral proteins, host proteins and lipids. Considering the rareness of the invaginated vesicular structure at the ER under physiological conditions, the assumption that flavivirus-encoded proteins play dominant roles establishing viral replication organelles is reasonable. Nevertheless, host factors such as proteins and lipids may facilitate the process.

**Flavivirus NS proteins**

Flavivirus genomic RNA encodes ten viral proteins, seven of which are NS proteins (NS1, 2A, 2B, 3, 4A, 4B and 5) that carry out virus replication (Fig. 3). Among the seven
NS proteins, NS1 is the only one localized in the ER lumen. NS2A, 2B, 4A and 4B are integral membrane proteins that reside on the ER membrane. NS3 and NS5 are two soluble enzymes with protease, NTPase, helicase and polymerase activity in the cytoplasm. NS3, NS5 and viral RNA need to be recruited to the replication compartments and assembled into functional RC, since they do not possess an ER anchor. Thus, virus replication can be accomplished.

In summary, nonstructural proteins have three functions. First, nonstructural proteins (NS3 and NS5) have enzymatic functions in viral double-stranded RNA (dsRNA) unwinding, RNA synthesis, modification and viral polyprotein processing. Second, nonstructural proteins remodel the host cell...
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ER structure to build viral replication organelles, which is the prerequisite structural basis for replication. Evidence has demonstrated that flavivirus NS proteins are essential and sufficient to establish replication organelles [6, 28, 29]. Third, some nonstructural proteins also play roles as scaffold proteins in virus replication and assembly. ER membrane-anchored NS proteins recruit soluble enzymes (NS3 and NS5 in the cytoplasm) to the ER to form RC, or bring newly synthesized viral RNA and structural proteins together to assemble the virion.

Below, we discuss the role of each flavivirus NS protein in the establishment of replication organelles in detail.

**NS1**

Flavivirus NS1 is the first NS protein translated after the structural proteins. After translation, signal peptidase and unidentified host proteases cleave between E/NS1 and NS1/2A, producing the individual NS1 protein. NS1 is the only NS protein localized in the ER lumen, where proteins are glycosylated. The molecular weight of NS1 ranges from 46 to 55 kD depending on glycosylation [30, 31]. Meanwhile, as the ER is the gateway of cellular secretion route, NS1 hitchhike the secretory pathway and is secreted into the extracellular milieu. Thus, it is not surprising that DENV NS1 was first found as soluble complement fixing (SCF) antigen 50 years ago [32, 33].

In addition to substantial studies focusing on secreted NS1, biochemical and structural studies also provide valuable functional information of NS1. Evidence has indicated the association of secreted JEV NS1 with extracellular membranous particles [34]. GPI linkage of DENV NS1 with the membrane was also reported [35]. Structural studies demonstrated that secreted NS1 forms dimers or hexamers associated with lipid membrane or lipoprotein particles [36, 37]. Based on these data, researchers have tried to establish the linkage between NS1 function and the immune response or vascular dysfunction.

However, all the above mentioned studies cannot answer the question “What is the role of NS1 in flavivirus replication?” Neither the induction of the host immune response by secreted NS1 nor the NS1-lipid association seems to have a direct link to flavivirus replication. Flavivirus NS1 has been proved to be essential for virus replication in viral genomic RNA synthesis for two decades [38, 39]. DENV NS1 association with the intracellular membrane, particularly with VPs but not with mature virus particles, and NS1 colocalization with viral dsRNA by cryo-EM provide additional evidence supporting its role in the virus replication complex [39]. Recently, ZIKV NS1 was shown to remodel ER structure in mammalian cells, resembling the flavivirus replication compartments [29]. These data vigorously argue that NS1 is directly involved in the establishment of flavivirus replication organelles and unravel the essential role of NS1 in virus replication mechanistically [29, 40].

The essential role of NS1 in viral replication depends on its association with the lipid membrane. Structural studies demonstrated that flavivirus NS1 has three regions facing the lipid membrane: β-roll, greasy finger and wing flexible loop [37, 41, 42]. Several hydrophobic residues in these regions of NS1 could insert into the lumenal leaflet of the ER membrane to introduce membrane curvature toward the ER lumen, resulting in the formation of invaginated vesicles (Fig. 4) [29]. Actually, membrane curvature induction by asymmetric hydrophobic insertion into the lipid bilayer is
Flavivirus replication occurs on the cytoplasmic side of the ER, where viral RNA and enzymes (NS3 and NS5) exist. It has long been mystery how NS1 in the ER lumen, sequestered by the ER membrane, plays an essential role in viral RNA synthesis. Some studies have shown that NS1 interacts with other flavivirus NS proteins to participate in virus replication, suggesting that NS1 acts as a cofactor rather than an important player in viral replication. However, this cannot explain why point mutations in the NS1 membrane association region block virus replication. The model by which NS1 induces ER invagination to establish replication organelles converges with several lines of previous studies, including NS1 association with lipids, NS1 ER lumen location around replication compartments and NS1 essential role in viral RNA synthesis.

Flavivirus NS1 is a multifunctional protein involved in various processes (for review, see [48–51]). We do not discuss these processes in detail here due to the relatively weak connection with flavivirus replication organelles.

NS2A

NS2A (~ 23 kD) is a membrane protein localized at the ER with five membrane span regions [52]. A recent report suggested a single-transmembrane topology for ZIKV NS2A, which differs from that of other flaviviruses [53]. The NS2A N-terminus localizes in the ER lumen, and the C-terminus localizes in the cytosol. Immunofluorescence and immuno-EM demonstrated that NS2A localized around the virus-induced membrane (VP, CM and PC) [54].

Functionally, mutation scanning analysis indicated that DENV and YFV NS2A is involved in both viral RNA synthesis and virion assembly [55–57]. Several reports have identified multiple residues of WNV and ZIKV NS2A affecting viral replication [58–60]. These residues lie throughout the NS2A sequence without obvious clustering patterns, and the underlying mechanisms are not well known. On the other hand, the role of flavivirus NS2A in virion assembly seems more evident [61–63]. In two recent studies, the models in which DENV and ZIKV NS2A may act as an important scaffold protein in virion assembly are proposed: NS2A facilitates virion formation by recruiting c-prM-E structural proteins and viral RNA to the assembly site [64, 65].

A few studies have suggested the membrane activity of DENV NS2A. Dens25, a peptide in the N-terminus of DENV2 NS2A located in the ER lumen, possesses

Fig. 4 Flavivirus NS1 induces invaginated vesicles at the ER. Several hydrophobic residues in β-roll, greasy finger and wing flexible loop of NS1 can insert into lumenal leaflet of the ER membrane to induce the curvature. Thus, ER lumen located NS1-induced invaginated vesicles at the ER by the mechanism of asymmetrically insertion into the lipid membrane.
membrane insertion properties in vitro, suggesting its possible involvement in membrane modulation [66, 67]. The data also showed that DENV NS2A exhibited pore forming activity in the bacterial expression system [68]. However, the correlation of these observations with flavivirus replication is ambiguous. Thus, whether NS2A plays a role in replication organelle establishment remains unclear and should be determined.

**NS2B**

Flavivirus NS2B (~14 kD) is a multitransmembrane protein localized at the ER with four proposed membrane-spanning regions (the C-terminal two helices may be half-buried in the membrane) [69]. Immunofluorescence and cryo-EM showed that Kunjin virus NS2B colocalized with viral dsRNA around virus-induced membranes [70, 71]. Flavivirus NS2B has a well-known role as an essential cofactor of NS3 protease by increasing its protease activity more than 3000-fold [72–76]. The NS2B-NS3 protease complex structure revealed that the cytosolic region of NS2B wrapped around the NS3 protease domain, stabilizing NS3 protease folding [77–80]. Furthermore, as NS3 has no membrane tethered region, NS2B recruits NS3 to the replication site at the ER [81]. Thus, NS2B plays dual roles in NS3 function: a cofactor of the NS3 protease domain and a recruiter of NS3 to the ER.

A study also showed that NS2B alters membrane structure in vitro. Bacterially expressed JEV and DENV NS2B form oligomers and permeabilize the cell membrane [82, 83]. Interestingly, several mutations in JEV NS2B transmembrane region also attenuated viral RNA synthesis and virion assembly to varying extents independent of NS3 protease activity, one of which was proven to weaken the NS2A-2B interaction [84]. It seems that NS2B has extra functions other than the NS3 cofactor and recruiter in virus replication. However, the role of NS2B, especially in flavivirus-induced membrane structure, is vague and needs to be explored.

**NS4A**

NS4A (~16 kD) is a small membrane protein with three proposed membrane-spanning regions [85]. Flavivirus NS4A was reported to localize around virus-induced membrane structure in virus-infected cells [54, 86]. The NS4A N-terminus is located in the cytosol after cleavage from NS3, and the C-terminus lies in the ER lumen. The last C-terminal transmembrane region, also known as the 2 K peptide, is the leading signal sequence to introduce the N-terminus of NS4B into the ER lumen.

Evidence has shown that NS4A can induce membrane rearrangement, which may be related to virus replication organelle formation. The 2 K peptide, which is between NS4A and NS4B, probably plays a role in NS4A-induced membrane alteration, although the opposite contribution was reported in DENV and WNV [87, 88]. Moreover, the N-terminal cytoplasmic domain of DENV NS4A contains an amphipathic helix (AH) that may interact with the membrane [89]. AH is a common motif associated with membrane structure comprising hydrophobic and polar residues that form a polar/nonpolar interface. The side chains of hydrophobic residues in the AH insert into the lipid bilayer, and the polar residues interact with the polar region of the membrane. Thus, AH can sense, induce or maintain membrane curvature. DENV NS4A N-terminal AH bound to a highly curved lipid membrane specifically in vitro [90, 91]. It is possible that NS4A interacts with the cytoplasmic leaflet of the ER membrane to sense or modulate the ER structure through its N-terminal AH motif. Molecular dynamics (MD) simulations also suggest that DENV NS4A could induce membrane undulation [92]. In addition, the oligomerization of DENV NS4A mediated by its first TM region is important for virus replication, and mutation in this region reduced NS4A oligomerization and attenuated virus replication [93]. In addition, it is still under discussion whether NS4A interacts with NS1 to facilitate the construction of flavivirus replication organelle [40, 94].

**NS4B**

NS4B (~27 kD) is an integral membrane protein with three transmembrane regions [95]. NMR experiment also suggests a 5-transmembrane model of NS4B using reconstitute micelles in vitro [96]. The NS4B N-terminus lies in the ER lumen, and the C-terminus is located in the cytoplasm. Its colocalization with NS3 and viral dsRNA on the ER implies its association with the viral membrane-bound replication complex [95]. NS4B participates in DENV replication, as lethal mutations (P104R, K143A) in NS4B have been identified [97]. This could be due to the dimerization of DENV NS4B through the cytosolic loop (129–165 aa) and the C-terminal region (166–248 aa), which is important for replication complex formation [98]. A study also demonstrated that DENV NS4B interacted with NS3 and promoted NS3 helicase activity by dissociating it from single-stranded RNA [99].

For the membrane rearrangement, DENV NS4B possesses several membrane active regions, comprising both the proposed transmembrane region and non-transmembrane regions [100]. Overexpression of WNV NS4B could induce ER-derived foci formation in the cytoplasm by confocal microscopy, suggesting its possible role in virus-induced membrane structure [101]. The role of NS4B in flavivirus-induced ER remodeling needs further study.
**NS3 and NS5**

NS3 and NS5 are viral enzymatic proteins carrying out protein processing, dsRNA unwinding, RNA synthesis and modification. NS3 (~68 kD) has an N-terminal protease domain and a C-terminal helicase domain to cleave the viral polyprotein and separate the dsRNA intermediate during viral RNA amplification, respectively. NS5 (~105 kD) has an N-terminal methyltransferase and a C-terminal RNA-dependent RNA polymerase (RDRP) domain with 5′RNA capping, cap methylation and RNA synthesis activities. These two proteins are soluble proteins without membrane anchors. Thus, for efficient virus replication, NS3 and NS5 should be retained around replication compartments on the ER by other factors. Interestingly, ZIKV NS3 localized on mitochondria when expressed alone, whereas NS2B could recruit NS3 to the ER, where flavivirus replicates [81]. Flavivirus NS5 has both cytoplasmic and nuclear localization, whose nuclear localization may relate to virus replication and the host immune response [102–105]. The factor recruiting NS5 to the replication site at the ER needs to be identified. To date, no evidence indicates that NS3 and NS5 are directly involved in the formation of flavivirus-induced membrane structures [28]. Surprisingly, in addition to its enzymatic function, NS3 seems to play a role in virion assembly. Mutation of several residues in DENV and YFV NS3 does not affect virus replication but abolishes infectious particle assembly, although the underlying mechanism remains to be explored [106, 107].

**Flaviviruses compartmentalized replication organelle formation**

The formation of flavivirus compartmentalized replication organelle is a complicated process involving membrane remodeling (replication compartment formation on the ER), protein complex assembly (virus RC assembly) in the replication compartment, RC-viral RNA recognition, RNA synthesis and release (Fig. 5). Protein–membrane, protein–protein and protein–RNA interactions together contribute to the formation of flavivirus replication organelles. Thus, the ER membrane, viral nonstructural proteins and viral RNA form a compact and highly ordered structure to accomplish virus replication.

Compartmentalized ER structure is the platform of flavivirus replication. Multiple NS proteins in flaviviruses have been shown to interact with lipid membranes. NS1, as a membrane associated protein, may play a key role in replication compartment formation through hydrophobic insertion into luminal leaflet of the ER membrane. The other four smaller multitransmembrane NS proteins residing on the ER membrane, NS2A, 2B, 4A and 4B, all possess membrane

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**Fig. 5** Flavivirus replication organelles at the ER. Membrane active NS proteins induce invaginated replication compartments at the ER, and RC (NS3, NS5 and viral RNA etc.) assemble in such compartments to carry out viral RNA synthesis. Host factors may also contribute to the establishment of flavivirus replication organelles.
binding or permeabilizing activity in the model membrane system or bacterial expression system [83]. However, the data from the model system in vitro need to be carefully evaluated to distinguish whether the membrane activities of these NS proteins are actually related to replication compartment formation. Some may be involved in other processes, such as virion assembly or virus-induced cytopathic effects. A more complicated scenario is that a single NS protein may not be sufficient to establish replication compartments alone. Evidence has shown that NS1, NS2B, NS4A and NS4B form homodimers or oligomers to function. Furthermore, there are broad interactions among these NS proteins, including NS1 and NS4, NS2A and NS2B, NS4A and NS4B [40, 108–112]. It is likely that cooperation of multiple NS proteins induces and fine tunes the exquisite architecture of replication organelles.

Once the replication compartments form, RC may assemble in these invaginated vesicles at the ER. Two enzymes, NS3 and NS5, need to be recruited to replication compartments by ER membrane-bound NS proteins. As mentioned above, NS2B recruits NS3, whereas recruiter of NS5 has not been identified. NS3 is a potential factor to recruit NS5 to the flavivirus replication site at the ER as they interact with each other. In addition, the cooperation between NS3 (RNA helicase) and NS5 (RNA polymerase) is pivotal and essential for viral RNA synthesis, and they regulate their enzymatic activity reciprocally [113–115]. The stoichiometric proportion of NS3–NS5 in RC would matter for better efficiency. It may not be equal, considering equivalent amounts of NS3 and NS5 are translated and processed from the same viral polyprotein precursor, while a certain amount of NS5 is localized in the nucleus rather than on the ER. Additionally, long-range viral dsRNA (~11 kb) unwinding probably requires the cooperation of multiple helicases in view of the limited progressivity of flavivirus NS3 helicase on the nucleic acid strand [113, 116]. Therefore, more NS3 than NS5 may be present in the replication compartments. It is intriguing to clarify the proper ratio and how to maintain this ratio between helicase and polymerase to efficiently synthesize viral RNA. Other NS proteins may also be involved in the RNA synthesis process, facilitating NS3 and NS5 function. For example, DENV and WNV NS4A/B interacted with NS3 and regulated NS3 helicase activity [117–119]. Collectively, precise assembly of RC consisting of certain NS proteins with appropriate proportions in the replication compartment is critical for viral RNA synthesis.

Viral RNA also needs to be recruited to the replication compartment, and newly synthesized positive-strand RNA is released from the compartment. The 5′ and 3′ UTRs of flavivirus RNA form a series of stem-loop structures, which might be recognized by viral proteins for RNA unwinding, synthesis and packaging into viral particles. Moreover, 3′ UTR of DENV genomic RNA is found to play a crucial role in replication compartment biogenesis [120]. Of all NS proteins, NS3 and NS5 are major viral RNA-interacting NS proteins. Apart from NS3 and NS5, NS2A was found to bind to the viral RNA 3′ UTR and coordinate virus packaging. Whether NS2A or other NS proteins interact with viral RNA to facilitate RNA replication is not yet well understood.

**Host cellular factors**

Host cellular factors, including proteins and lipids, are important players regulating flavivirus replication organelle assembly. They positively facilitate or negatively attenuate virus replication.

**Protein factors**

Lots of host protein factors interact with flavivirus-encoded proteins and viral RNA, and many participate in the host immune response to virus infection (not discussed here). As flaviviruses replicate on the ER, ER proteins are widely reported to participate in and regulate flavivirus replication, including the ER membrane complex (EMC), Hrd1, OST, reticulon, atlastin, etc. [121–126]. Among these proteins, several host protein factors are involved in flavivirus replication by modifying ER structure.

**Reticulon**

Reticulon (RTN) is a family of membrane proteins (RTN1–4 in mammals) localized on the ER or plasma membrane (RTN4). ER-associated RTNs are considered as ER-shaping proteins that induce and stabilize curvature of ER tubules by inserting the hairpin-like transmembrane region into the cytoplasmic leaflet of the ER membrane [127]. A study found that RTN 3.1A is recruited to viral replication sites upon WNV, DENV and ZIKV infection [128]. Knockdown of RTN3.1A attenuated flavivirus replication and interfered with virus induced-membrane remodeling. RTN 3.1A specifically interacts with WNV NS4A but not with DENV or ZIKV NS4A, suggesting different regulatory mechanisms.

**Atlastin**

Atlastin (ATL) is another protein family that plays a crucial role in ER morphogenesis, particularly in the fusion process of ER tubules. Atlastins are dynamin-related GTPases-mediating homotypic fusion of the ER membrane [129, 130]. Studies suggest atlastin also facilitates flavivirus replication [131, 132]. Silencing of atlastin attenuates ZIKV replication.
and decreases membrane packets. ATL3 interacts with ZIKV NS2A and NS2B-NS3 [131]. In another study, ATL family proteins played distinct roles in the flavivirus life cycle. ATL2 knockdown blocked flavivirus replication due to defects in replication organelles. Whereas ATL3 specifically impaired virion maturation and secretion through the ADP-ribosylation factor 4 (Arf4)-related trafficking pathway [132].

**BPI fold containing family B member 3 (BPIFB3)**

BPIFB3 is a protein localized to the ER whose function is poorly understood [133]. It belongs to the lipid-binding antimicrobial proteins of the LBP/BPI superfamily based on sequence homology. Silencing of BPIFB3 inhibits flavivirus replication and blocks the formation of replication organelles. EM and biochemical evidence demonstrates that depletion of BPIFB3 promotes reticulophagy (autophagy selectively clears and degrades ER components) [134]. It is not clear whether BPIFB3 directly interacts with viral proteins to regulate flavivirus replication.

**Lipids**

Flavivirus infection significantly changes host cell lipid metabolism by adjusting lipid composition and distribution, arguing the importance of lipids [135–138]. Lipids are involved in almost all aspects of the flavivirus life cycle including entry, fusion, replication, assembly and virus crosstalk with the host cell immune response [139–145]. However, only limited studies provided clues to show that specific lipids may be involved in flavivirus replication organelles formation. As the flavivirus replication site, the ER is also an important hub for lipid synthesis and transport. Flaviviruses not only remarkably reorganize ER structure, but they also change the lipid metabolism and composition of the ER. Lipidomic analysis demonstrated that flavivirus infection resulted in pronounced lipid composition changes in the membrane fraction where RC exists in mosquito cells [135]. In another lipidomic study, ZIKV infection induced striking changes of sphingolipids in mammalian cells. Sphingolipid ceramide redistributed to ZIKV replication sites and sensitized cells to ZIKV infection [146]. But the relationship between sphingolipids and ZIKV replication organelles is not clear.

Lipids can contribute to flavivirus replication organelles in various ways. First, lipids intrinsically affect replication organelle structure themselves. Lipids have their own shapes determined by the headgroup and acyl tail. Spontaneous membrane curvature can be induced by the asymmetric distribution of different lipids on the bilayer, depending on lipid type, structures and composition [147]. Cone-shaped ceramide and lysophosphatidylcholine (LPC) naturally possess the ability to induce negative and positive curvature respectively, and both are upregulated in DENV-infected mosquito cells [135]. Lipid composition changes in the ER upon flavivirus infection may assist in the formation of highly curved flavivirus replication organelles [148]. Second, specific lipids on the viral replication membrane may facilitate replication by recruiting related protein factors. Lipids could determine the specificity and increase the affinity of membrane associated protein factors. For example, phosphoinositides, which are a small fraction of cellular lipids, play important roles in signal transduction, intracellular trafficking, organelle structure and homeostasis maintenance. Positive-strand RNA viruses such as enterovirus and HCV regulate PI4P production and distribution, forming PI4P-rich membrane structures essential for virus replication [149]. For flavivirus, evidence showed that the peptide (dens25) of DENV NS2A strongly interacts with the negatively charged lipid membrane, which might be related to membrane rearrangement.

In summary, although many lipids have been reported to affect flaviviruses replication, and some of them redistribute and concentrated to flaviviruses replication sites [135, 146, 150], no direct evidences were provided to support that these lipids contribute to biogenesis of replication organelles. The role of lipids in the establishment of flavivirus replication organelles should be investigated.

**Conclusion and outlook**

Flavivirus-induced compartmentalized replication organelles derived from the ER are a characteristic cellular hallmark, and are the prerequisite and essential structural basis of flavivirus replication. Both viral and host cellular factors are involved, in which viral NS proteins may play dominant roles. The establishment and function of flavivirus replication organelles required multidimensional protein–membrane, protein–protein and protein–RNA interactions as well as orchestration of these protein–membrane, protein–protein and protein–RNA complexes.

Previous studies have extensively explored the function of each flavivirus NS protein, RNA element and host factor in viral replication. However, the functions of individual factors are inevitably affected and modified by other units in the complexes. Thus, such studies could be less comprehensive and inaccurate in consideration of multicomplexes working together rather than monofactor to fulfill the virus replication. To better understand the mechanism of flavivirus replication, integral studies using complexes consisting of multiple factors are necessary. Undoubtedly, the systems
and techniques for studying the complexes are more complicated and challenging. Reconstitution of the proteo-liposome system in vitro with more NS proteins in combining with advanced EM and super-resolution microscopy will help to understand the process and underlying mechanism of flavivirus-induced ER membrane remodeling. The live cell imaging and single molecular techniques will help to monitor the real-time dynamics of replication organelles as well as viral RNA synthesis in situ.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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