

Review

Role of Cellular Lipids in Positive-Sense RNA Virus Replication Complex Assembly and Function

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Abstract: Positive-sense RNA viruses are responsible for frequent and often devastating diseases in humans, animals, and plants. However, the development of effective vaccines and anti-viral therapies targeted towards these pathogens has been hindered by an incomplete understanding of the molecular mechanisms involved in viral replication. One common feature of all positive-sense RNA viruses is the manipulation of host intracellular membranes for the assembly of functional viral RNA replication complexes. This review will discuss the interplay between cellular membranes and positive-sense RNA virus replication, and will focus specifically on the potential structural and functional roles for cellular lipids in this process.

Keywords: RNA viruses; replication; membranes; lipids

1. Introduction

The virosphere is large, complex, and continually expanding. One group of viruses responsible for a wide range of diseases in humans, animals, and plants are classified as positive-sense RNA viruses

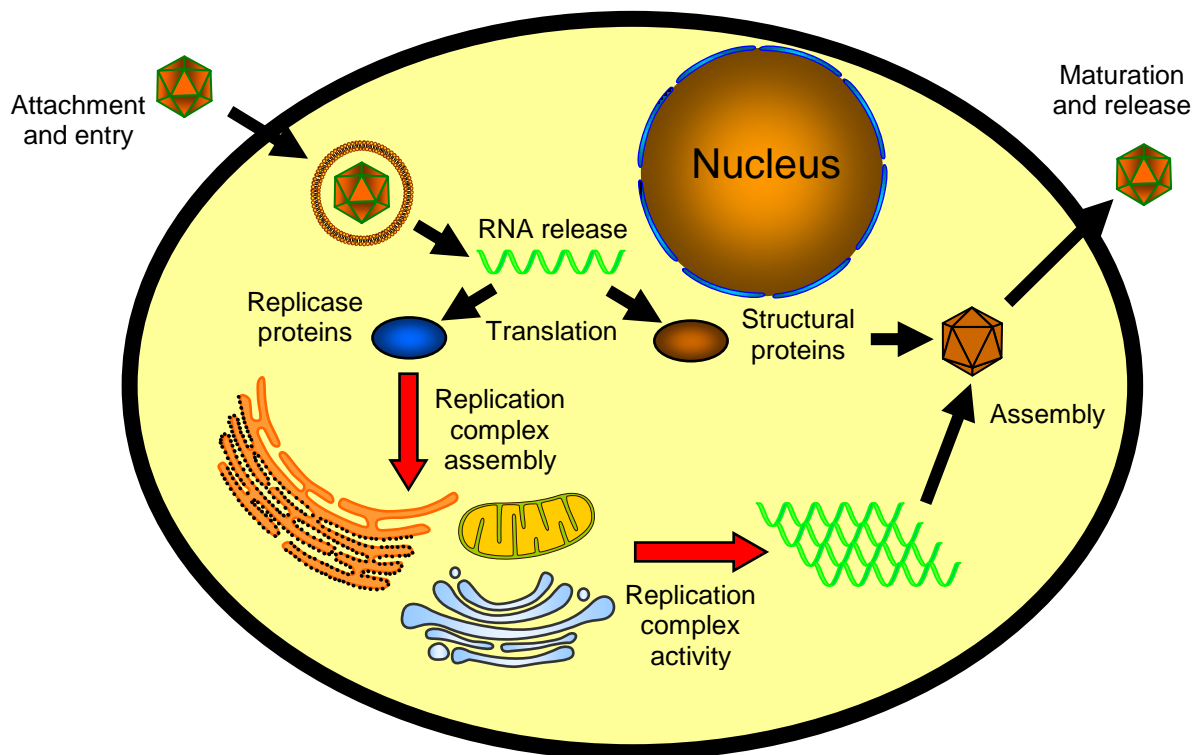
due to their genome structure, which consists of one or more single-stranded RNA molecules that in many respects resemble cellular mRNAs. Clinically important members of this group cause significant morbidity and mortality, include viruses from the *Picornaviridae*, *Flaviviridae*, *Caliciviridae*, *Coronaviridae*, and *Togaviridae* families, and represent a prominent component of the growing list of emerging and potentially devastating health threats [1,2]. Currently approved therapies for infections with these pathogens are limited, and the development of specific viral enzyme-targeted inhibitors is frequently complicated by the inherently high mutation rate of viral RNA polymerases and the rapid development of resistance [3,4]. An alternative approach that has been advocated in the development of novel and potential broadly active antivirals is the targeting of host processes, which range from blockade of cell surface receptors to altering cellular metabolism [5-9]. However, the cell-centric approach to antiviral development requires substantial knowledge and understanding of the host-pathogen interactions that control virus replication.

The small genomes of viruses relative to other organisms requires that they appropriate cellular machinery to complete their replication cycle, which for positive-sense RNA viruses is depicted schematically in Figure 1. For example, no virus encodes the complete set of nucleic acid and protein constituents necessary for autonomous translation of viral RNAs, which represents an important initial step in virus replication, and therefore positive-sense RNA viruses utilize diverse and often elaborate mechanisms to subvert the cellular translation apparatus to their benefit [10,11]. Another step in the life cycle of positive-sense RNA viruses that highlights the importance of cell components and virus-host interactions is the requirement of host-derived intracellular membranes for RNA replication [12-14]. This requirement is completely independent of their structural role during the encapsidation and assembly of enveloped viruses. The conclusion that cellular membranes are essential host factors in viral RNA replication is based primarily on four sets of observations. First, positive-sense RNA virus replication is associated with dramatic intracellular membrane rearrangements, which are readily demonstrated by electron microscopy [15-39]. Second, viral proteins with known or hypothesized enzymatic activity linked to genome amplification, which are referred to collectively as replicase proteins, co-partition with intracellular membrane fractions. Furthermore, these membrane fractions retain viral replicase enzymatic activity that can be measured *in vitro* [18,26,28,40-45]. Third, detergents can disrupt, whereas phospholipids can enhance, *in vitro* viral replicase activity [28,42-45]. And fourth, pharmacologic or genetic disruption of lipid metabolism has been shown to modulate positive-sense RNA virus replication [46-63].

Although the observations outlined above provide substantial validation for the important role that cellular membranes play in positive-sense RNA virus replication, they provide only circumstantial evidence for the specific membrane components involved in the process and their precise molecular functions with respect to viral genome replication. Intracellular membranes contain diverse protein and lipid constituents and play a variety of roles during normal cellular physiology and metabolism, which include facilitating the spatial separation of cellular processes and the consequent differential concentrations of crucial cellular components, providing structural integrity to maintain organelle shape, and contributing functional co-factors for multiple processes such as signal transduction and biosynthesis. In this review, we will discuss three potential roles that lipid constituents of host cell membranes play in positive-sense RNA virus genome replication. These include: (i) providing a

scaffold for targeting and assembly of RNA replication complexes; (ii) inducing alterations in membranes structure to potentially shield viral RNA replication intermediates from cellular innate antiviral pathways; and (iii) serving as functional co-factors for optimal enzymatic activity of viral replicase proteins. We will use examples of plant, insect, and mammalian viruses to highlight specific aspects of these potential roles, and will focus primarily on lipids rather than membranes in general. Several excellent reviews have recently been published [12-14] for readers interested in further exploring the connections between positive-sense RNA viruses and cellular membranes. In addition, readers with a particular interest in hepatitis C virus, one of the most clinically relevant positive-sense RNA viruses whose connection with cellular lipid metabolism is becoming increasingly apparent, are encouraged to explore the companion article by McLauchlan *et al.* forming part of this special issue of *Viruses*.

Figure 1. Schematic of positive-sense RNA virus replication cycle. General steps include: (i) attachment and entry; (ii) release of genome into cytoplasm; (iii) translation of genomic viral RNA into replicase or structural proteins; (iv) assembly of replication complex on host intracellular membrane; (v) amplification of viral genome via dsRNA intermediate; (vi) genome encapsidation; and (vii) maturation and release.



1.1. Lipids and replication complex targeting and assembly

The interior of a cell is a highly organized environment, where membrane-bound organelles and specific organelle membranes represent one mechanism whereby cells enforce spatial constraints on particular metabolic processes. For example, the enzymes involved in electron transport and respiration

are concentrated on the inner mitochondria membrane. Similarly, viruses may use intracellular membranes as simple molecular scaffolds on which to assemble their replication complexes, thereby increasing replication efficiency by concentrating essential viral and potential cellular components within a smaller microenvironment in the cell. Furthermore, membranes may provide a convenient platform to coordinate various steps in the replication cycle (see Figure 1), such as the demonstrated coupling of viral RNA replication, translation, and genome packaging [64-66]. However, although positive-sense RNA virus replication complexes have been found associated with cellular membranes derived from a number of intracellular organelles, including the endoplasmic reticulum, Golgi apparatus, endosomes, lysosomes, peroxisomes, chloroplasts, and the mitochondria (reviewed in ref [12-14]), individual viruses show a particular membrane selectivity (Table 1). This selectivity suggests a specific “receptor•ligand”-type interaction between viral replicase proteins and intracellular membrane components. Several membrane-specific targeting signals within individual virus-encoded replicase proteins have been characterized in detail [26,67-70], but only a limited number of cellular membrane components have been shown to be important in viral RNA replication complex targeting and assembly, most of which are membrane proteins [71-73]. However, preferential interactions between positive-sense RNA virus replicase proteins and membrane phospholipids have been described [44,74], suggesting that lipids may also contribute to replication complex targeting and assembly. We recently demonstrated that the membrane-targeting viral replicase protein of Flock House virus, a model alphanodavirus that assembles its replication complexes on outer mitochondrial membranes [25], preferentially interacts with anionic phospholipids, and in particular cardiolipin [74]. Although most phospholipids are widely distributed throughout intracellular membranes, cardiolipin is found predominantly, if not exclusively, in mitochondrial membranes [75,76], suggesting a potential role for this particular lipid in Flock House virus RNA replication complex targeting and assembly. The ubiquitous nature of cellular phospholipids and the substantial technical challenges associated with studying lipid-membrane protein interactions at the molecular and structural levels has thus far hampered our ability to more fully examine the potential role that cellular lipids play in positive-sense RNA virus replication complex assembly.

1.2. Lipids and membrane structure alterations

Positive-sense RNA viruses induce a number of distinctive membrane structures, which include the “membranous webs” of hepatitis C virus [24], the clustered vesicles of picornaviruses [27,29], the double-membrane vesicles of coronaviruses [15,20,21], flaviviruses [22,23], and arteriviruses [15,16], and the spherule-like cytopathic vacuoles of togaviruses that resemble cellular multivesicular bodies [30-32]. One virus whose membrane-induced structures have been examined in detail is Flock House virus, which induces spherule-like invaginations within the outer mitochondrial membrane (Figure 2). These structures, termed virus-induced “mini-organelles”, have been examined using tomographic electron microscopy to provide unprecedented detail and develop three-dimensional models of viral RNA replication complexes [38]. Similar tomographic models have also recently been described for dengue virus [23] and SARS-coronavirus [20], two positive-sense RNA viruses that extensively modify endoplasmic reticulum membranes.

Table 1. Examples of diverse intracellular membranes used by positive-sense RNA viruses to assemble functional RNA replication complexes

| Family | Virus | Membrane(s) | References |
|-----------------------|---|---|------------|
| <i>Arteriviridae</i> | Equine arteritis virus | Endoplasmic reticulum | [15,16] |
| <i>Bromoviridae</i> | Alfalfa mosaic virus Brome mosaic virus | Vacuole Endoplasmic reticulum | [17,18] |
| <i>Coronaviridae</i> | SARS-coronavirus | Endoplasmic reticulum/Golgi | [19-21] |
| <i>Flaviviridae</i> | Hepatitis C virus Dengue virus West Nile virus | Endoplasmic reticulum Endoplasmic reticulum Endoplasmic reticulum/Golgi | [22-24] |
| <i>Nodaviridae</i> | Flock House virus | Mitochondria | [25,26] |
| <i>Picornaviridae</i> | Poliovirus | Endoplasmic reticulum/Golgi | [27-29] |
| <i>Togaviridae</i> | Rubella virus Semliki Forest virus | Lysosomes Endosomes/lysosomes | [30-32] |
| <i>Tombusviridae</i> | Carnation Italian ringspot virus Cucumber necrosis virus Tomato bushy stunt virus | Mitochondria Endoplasmic reticulum Peroxisome | [33-36] |
| <i>Tymoviridae</i> | Turnip yellow mosaic virus | Chloroplast | [37] |

The structural changes that must occur within lipid bilayers to produce the virus-induced alterations in intracellular membranes are substantial. For example, the outer mitochondrial membrane invaginations induced by Flock House virus [38] (see also Figure 2) and the endoplasmic reticulum changes induced by dengue virus [23] and SARS-coronavirus [20] require regions of negative and positive curvature, as depicted schematically in Figure 3A. Membrane curvature can be induced by both protein and lipid modifications [77], which are depicted in Figures 3B and 3C, respectively. Protein modifications that induce membrane curvature include internal and external protein scaffolding by peripheral membrane proteins, such as cellular clathrin and calveolin, insertion of proteins with amphipathic helices, such as cellular amphiphysin and endophilins, and insertion or oligomerization of transmembrane proteins, for which there are numerous cellular examples. For positive-sense RNA viruses, replicase proteins with amphipathic helices [44,78,79] or transmembrane domains [68,70] with demonstrated membrane-binding characteristics have also been described. In addition, expression of specific viral proteins alone, in the absence of active viral RNA replication, can frequently induce intracellular membrane structures reminiscent of those induced by viral infections [16,24,29].

Figure 2. Membrane alterations induced by positive-sense RNA virus replication. Transmission electron micrographs of mitochondria isolated from mock (left) and Flock House virus-infected (right) *Drosophila* cells. Note the normal matrix (M) and cristae (C) in mock mitochondria, whereas the matrix is compacted in mitochondria from infected cells. Furthermore, the outer mitochondrial membrane is studded with spherules (black arrows), which represent viral RNA replication factories.

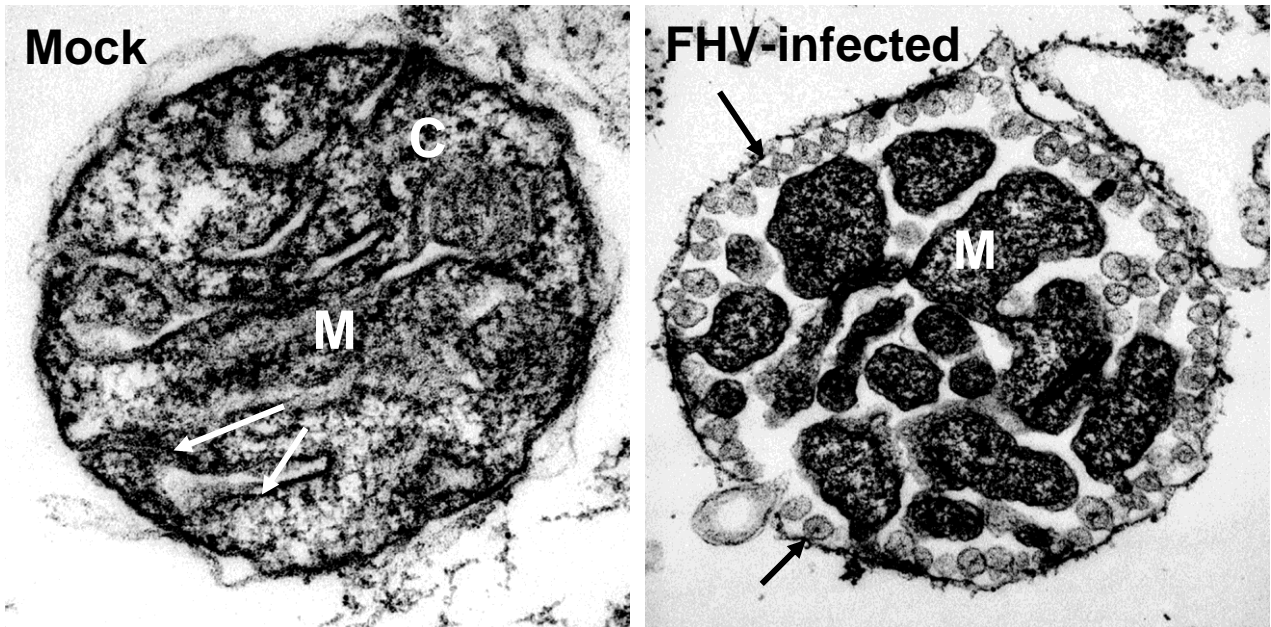
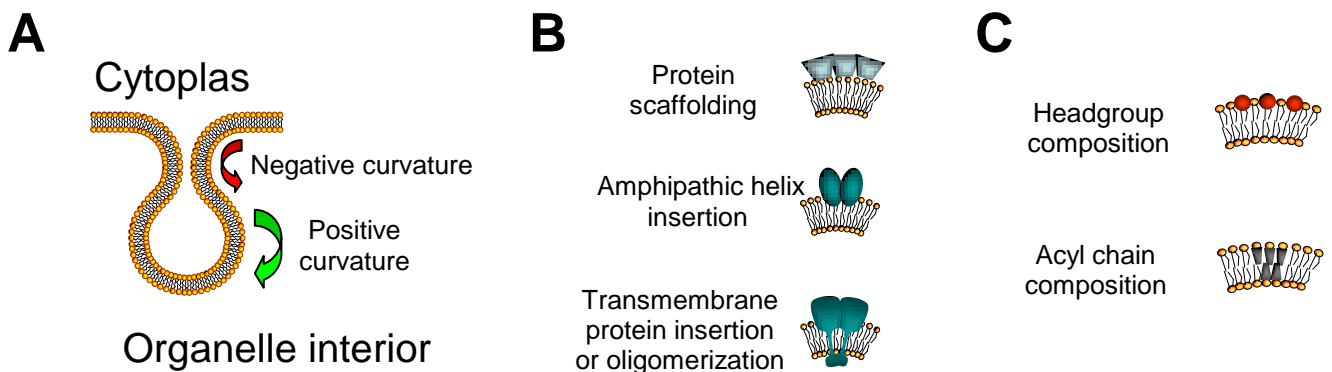


Figure 3. Schematics of membrane curvature necessary to form virus-induced membrane structures (A), and potential protein (B) or lipid (C) modification that may induce membrane curvature.



An alternative mechanism to induce membrane curvature is modification of lipid structure, either through changes in the polar headgroup or acyl chain composition. For example, lysophospholipids, which contain only one acyl chain per phospholipid molecule, and special lipids such as cholesterol or cardiolipin, which has four acyl chains attached to diphosphatidylglycerol, can have profound impacts

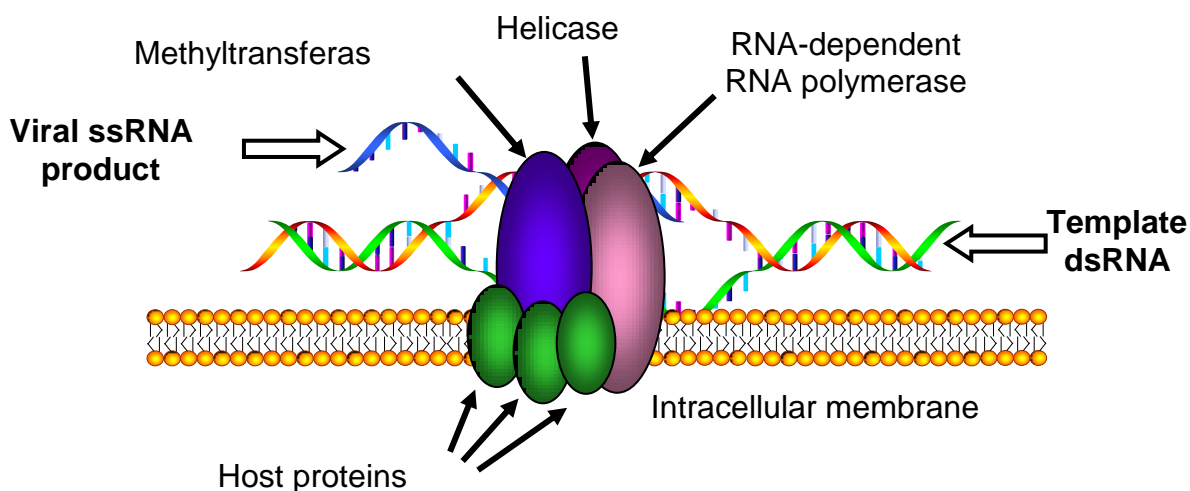
on membrane curvature and plasticity [80]. Acyl chain length and saturation can also impact membrane curvature [81]. Although the currently available data on the potential phospholipid alterations that are induced by and/or required for positive-sense RNA virus replication are limited, there are suggestive reports that specific and functionally important changes do occur. Brome mosaic virus RNA replication is suppressed in cells that lack $\Delta 9$ fatty acid desaturase and hence contain reduced levels of unsaturated fatty acids [49], Flock House virus induces a preferential increase in lipid molecules with longer and unsaturated acyl chains [54], and West Nile virus redistributes cholesterol to sites of active viral replication [56]. We anticipate that continued advances in cell fractionation, membrane isolation, and lipidomics techniques will begin to address this substantial gap in our understanding of positive-sense RNA virus biology.

Regardless of the mechanism whereby membrane curvature occurs, the function of these curious virus-induced structures remains enigmatic. One interesting possibility is that virus-induced membrane structures shield viral products that have the potential to activate cellular innate immune pathways. The replication of positive-sense RNA viruses involves the formation of dsRNA intermediates that serve as templates for genome amplification (see below, Figure 4). These replication intermediates, or other “foreign” chemical moieties such as 5' triphosphorylated RNAs, are potent stimuli for inducing innate antiviral immune responses [82]. Since positive-sense RNA viruses replicate almost exclusively within the cytoplasm of cells, they likely employ mechanisms to suppress or modify activation or amplification of these responses. Positive-sense RNA viruses possess multiple mechanisms to evade innate immune system activation, including the production of proteases that cleave, degrade, or inhibit essential innate immune signaling pathway components or the use of viral proteins to sequester viral dsRNA (reviewed in ref [83,84]). It is intriguing to speculate that virus-induced membrane structures may also play a role in sequestering or shielding viral products from the innate immune system, thus providing an additional level of protection.

1.3. Lipids and replication complex function

One major goal of positive-sense RNA virus research is the complete isolation, characterization, and *de novo* synthesis of fully functional RNA replication complexes, a generic example of which is shown schematically in Figure 4. The achievement of this goal has been hampered in part by the essential membrane-bound nature of these complexes. Substantial hurdles include the frequent inability to obtain soluble viral replicase proteins for detailed biochemical or structural studies, the inherent difficulties in interpreting protein co-purification results when detergents are required to solubilize viral RNA replication complexes, and the presence of three different types of molecular structures (proteins, lipids, and nucleic acids) that are all components of the RNA replication complex yet have different chemical characteristics. However, despite these difficulties some progress has been made. For example, *de novo* synthesis of a functional RNA replication complex in cell-free extracts has been accomplished for both poliovirus [85] and several related plant viruses [86], and this important step will have to be achieved for other viruses as well to provide additional systems in which to investigate the cellular components needed to assemble these viral genome production factories.

Figure 4. Schematic of positive-sense RNA virus replication complex. Virus-encoded proteins with known or hypothesized enzymatic functions are labeled at the top.



A substantial focus in the field of positive-sense RNA virus research has been the identification and characterization of the protein components necessary for replication complex function. The impact of lipids on this process has received far less attention in the past, in part due to some of the technical difficulties in lipid research noted above. However, there is both circumstantial and direct evidence that lipids can serve as functional co-factors for viral replicase proteins [18,26,28,40-45]. For example, although individual viruses typically assemble replication complexes on particular intracellular membranes (see Table 1), this specific targeting can be disrupted by altering either virus-encoded targeting signals [26,35] or host membrane-specific signals [71]. Furthermore, these “retargeted” replication complexes retain functional activity, suggesting that any necessary host components are widely present in multiple intracellular membranes. Although the exact lipid composition of intracellular membranes varies between organelles, phospholipids represent a prominent component of all intracellular membranes [76]. Positive-sense RNA virus replication complexes can be recovered from infected cells by density gradient centrifugation and the isolation of membrane fractions, which retain detergent-sensitive enzymatic activity. Although for many viruses this enzymatic activity is limited to primer-independent synthesis of a complementary negative strand, fully functional replication complexes from Flock House virus-infected cells that produce single-stranded positive-sense products have been described [42], where replicase activity is stimulated by specific phospholipids [45]. In addition, phospholipids have also been shown to influence alphavirus replicase protein activity [44,87]. These observations suggest that in addition to the potential structural roles of lipids in positive-sense RNA virus replication complex assembly described above, lipids may also play important functional roles to maximize replication complex activity.

2. Conclusions

Despite the axiom that cellular membranes are essential host factors for positive-sense RNA virus replication, the specific role of individual membrane components, and in particular lipids, represents a vastly understudied area of virus biology and pathogenesis. We have discussed three potential structural or functional roles that cellular membrane-resident lipids may play in the assembly and function of positive-sense RNA virus replication complexes. These roles are not mutually exclusive, and it is possible that lipids contribute to several steps in the virus life cycle via multiple mechanisms, some of which we currently recognize, and others that remain to be discovered. The recent increase in targeted and genome-wide screens to identify host factors that impact positive-sense RNA virus replication [54-63], several of which have highlighted the importance of lipid metabolism-associated genes, provides an exciting foundation for these discoveries.

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