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Review

Ultrastructure of the replication sites of positive-strand RNA viruses

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ABSTRACT

Positive strand RNA viruses replicate in the cytoplasm of infected cells and induce intracellular membranous compartments harboring the sites of viral RNA synthesis. These replication factories are supposed to concentrate the components of the replicase and to shield replication intermediates from the host cell innate immune defense. Virus induced membrane alterations are often generated in coordination with host factors and can be grouped into different morphotypes. Recent advances in conventional and electron microscopy have contributed greatly to our understanding of their biogenesis, but still many questions remain how viral proteins capture membranes and subvert host factors for their need. In this review, we will discuss different representatives of positive strand RNA viruses and their ways of hijacking cellular membranes to establish replication complexes. We will further focus on host cell factors that are critically involved in formation of these membranes and how they contribute to viral replication.

Contents

Introduction

Positive-strand RNA viruses replicate in the cytoplasm of an infected host cell, where they are confronted with host cell defense mechanisms and rather unfavorable conditions for genome replication. Therefore, the virus has to establish an intracellular environment that concentrates the viral proteins and allows productive replication of the viral genome, which is facilitated by induction of membranous alterations mediated by viral proteins. Host factors involved in lipid biosynthesis or vesicular trafficking are often recruited and regulated by viral proteins as well, contributing to the biogenesis of virus-induced membrane compartments. These host-derived membranes anchor the components of

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the replicase complex, protect the viral replication machinery from host cell immunity and allow creating a protected environment for RNA synthesis and packaging of the viral genome.

Despite a great diversity between the different families of positive-strand RNA viruses and between the organelles used as origin for membrane capturing, the morphology of the replication factories shares many similarities. In general, two distinct types of membrane alterations have been identified in the last decades: the double membrane vesicle (DMV) type (*Nidovirales*, *Picornaviridae* and *Hepaciviruses*) and the spherule invagination type (*Flaviviruses*, *Togaviridae*, *Bromoviridae*, *Nodaviridae*). Thus, it appears that there may be common mechanisms to modulate host membranes and lipid homeostasis. In the last years, great advances have been made in imaging and visualization of subcellular structures. Techniques like electron tomography (for a technical introduction, see McIntosh et al. (2005)) allow reconstruction of the three-dimensional architecture of viral replication complexes in nanometer-scale resolution, revealing astonishing structural details, which have not been observed in traditional transmission electron microscopy. Further developments like cryo-preservation of samples and correlative light and electron microscopy (CLEM, see Sartori et al. (2007) and Lucic et al. (2013) for technical details) allow preserving the delicate ultrastructures of replication membranes and combining the advantages of fluorescently labeled proteins with the nanometer-scale resolution of electron microscopy to visualize the localization of viral or cellular proteins. In this review, we will discuss the current knowledge of the architecture of membrane rearrangements induced by different positive-strand RNA viruses. We mainly focus on studies that revealed ultrastructural details of the viral replication complexes and give some insights into the role of host factors that are hijacked to build and maintain those viral replication factories.

DMV type

Hepaciviruses

Hepatitis C virus (HCV) is the most prominent member of the genus *Hepacivirus* in the family of *Flaviviridae*. Chronic HCV infections represent a global health burden and often lead to severe liver damage. The virus genome encompasses 9.6 kb in length and encodes for one polyprotein that is cleaved by viral and cellular proteases into 10 distinct proteins (reviewed in Bartenschlager et al. (2013)). Virus induced membrane alteration, the so-called membranous web (MW), was initially described as accumulation of vesicles of about 85 nm in diameter embedded in a membranous matrix (Egger et al., 2002), resembling the vesicles previously observed in poliovirus infected cells (Dales et al., 1965). The presence of non-structural proteins and double-stranded RNA (dsRNA) within the MW suggested these structures being the site of HCV RNA replication (Gosert et al., 2003; Ferraris et al., 2010). The MW further was found to be closely associated with cellular lipid droplets (LDs) (Egger et al., 2002; Gosert et al., 2003; Targett-Adams et al., 2008), which later were identified to play a major role in assembly of progeny virus (Boulant et al., 2007; Boulant et al., 2006; Salloum et al., 2013; Miyanari et al., 2007) (see also the reviews provided by Lindenbach and Rice (2013) and Lindenbach (2013)).

Detailed dissection of the MW architecture revealed that the main components are double membrane vesicles (DMVs, Fig. 1A left and top right), which probably originate as exvaginations from ER membranes and are heterogeneous in diameter (150–1000 nm). Most DMVs have two tightly opposed membrane layers, but some also exhibit two loosely surrounding lipid bilayers, which might reflect different stages of DMV formation (Ferraris et al., 2010; Romero-Brey et al., 2012). Other studies also revealed alternative virus-induced structures like clustered vesicles, single membrane vesicles (SMVs), contiguous vesicles (Ferraris et al., 2013) or multi-membrane vesicles (MMVs) (Ferraris et al., 2010; Reiss et al., 2011), which relevance for the HCV life cycle has not been determined so far. First DMVs are observed 16 h after infection, which correlates with increasing RNA replication, whereas MMVs occur late after around 36 h and their number further increases 48 h after infection (Romero-Brey et al., 2012). Whether MMVs play a role in late stages of the HCV life cycle or are just a by-product of a cellular response to the DMVs is still not clear. The occurrence of MMVs might be linked to autophagic processes, but a role of autophagy in MW formation is still up to debate. MMVs might arise from self-invagination of DMVs or by double membrane tubules enwrapping DMVs (Romero-Brey et al., 2012). In fact, upon treatment with the antiviral drug Silibinin the number of MMVs is strongly induced and RNA replication is inhibited in a genotype-specific manner (Esser-Nobis et al., 2013). Thus, MMVs are likely inactive and do not take part in RNA replication.

Currently, most evidence points to DMVs representing the structure where RNA replication takes place. Indeed, isolated DMVs revealed replicase activity in vitro (Paul et al., 2013). However, it still is not possible to visualize newly synthesized HCV RNA in EM micrographs, since preservation of the DMVs often is not compatible with labeling techniques and the DMVs also preclude antibodies from their inside (Romero-Brey et al., 2012). Thus, it is still under debate whether the replication complexes reside on the surface of the DMVs or are located within the vesicles. Considering the latter hypothesis, it can be expected that the vesicles must have an opening to allow entry of nucleotides or other factors and release of viral progeny RNA. Indeed, tomographic analysis of DMVs revealed that around 10% showed a small pore that connects the inside of the vesicle with the cytosol (Figs. 1A, bottom right and 2A); however, still most of the DMVs appeared as closed structures (Romero-Brey et al., 2012). Thus, it might be envisaged that just a small number of DMVs are actively engaged in replication at a given time point, or closed DMVs might still be active and use alternative ways to exchange metabolites, e.g. by using the nuclear transport machinery (Neufeldt et al., 2013). Alternatively, the replication complexes might sit on the outside and the pore of some DMVs has another function or represents an epiphenomenon during membrane biogenesis. However, in vitro replicase activity in cell extracts is highly resistant to nuclease and protease treatment and only gets sensitive upon addition of detergent, suggesting that the replication complex is located within a protecting membrane structure (Paul et al., 2013; Quinkert et al., 2005; Miyanari et al., 2003).

Although the morphology of DMVs has now been studied in great detail (Romero-Brey et al., 2012), the different steps of DMV biogenesis are still subject to speculation. One scenario similar to DENV involves membranes invaginating into the ER leading to membrane pairing, engulfing of cytosol and subsequently formation of DMVs by further wrapping of the ER membrane (Fig. 2A). Alternatively, HCV proteins might induce exvagination of ER membranes resulting in a SMV that is still connected to the ER (Fig. 2A). In this scenario, the viral proteins would reside on the outside of the vesicles. Indeed, immunolabeling revealed that SMVs are highly enriched in viral proteins, suggesting that SMVs might represent an intermediate step in DMV biogenesis driven by viral proteins (Romero-Brey et al., 2012). As a second step, a SMV then might undergo invagination and a DMV is formed keeping the viral proteins inside (Fig. 2A), which also could explain the low labeling efficiency of viral proteins at DMVs (Romero-Brey et al., 2012).

Studies of the MW are greatly facilitated by the fact that these membrane structures were not only observed in infected cells (Romero-Brey et al., 2012) or in cells harboring stably replicating subgenomic replicons (Gosert et al., 2003), but also upon expression of viral proteins (Romero-Brey et al., 2012; Reiss et al., 2011; Berger et al., 2014). Indeed, expression of the NS3-5B polyprotein induces the same membrane structures as observed in infection with cell culture derived virus (Romero-Brey et al., 2012). This

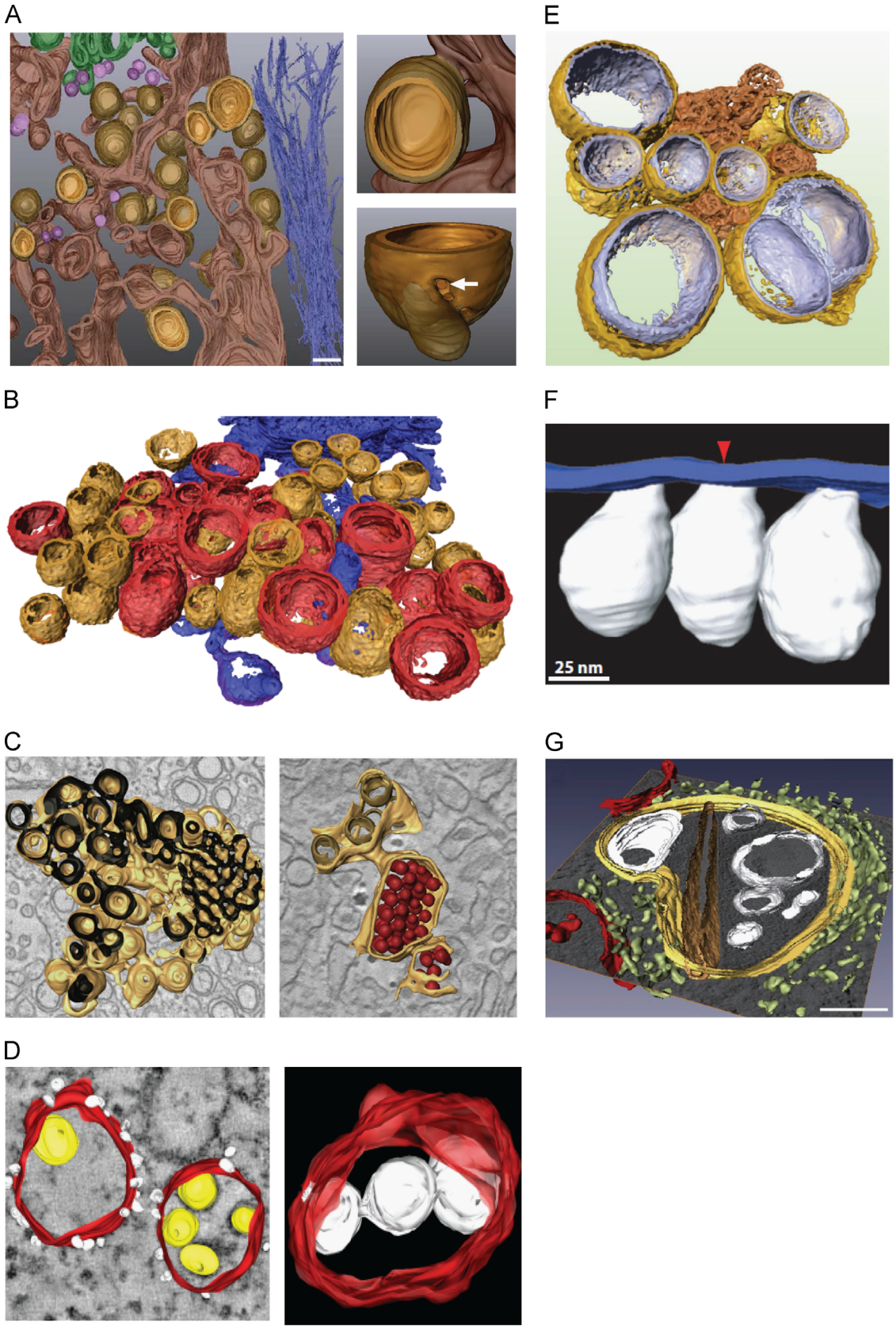

Fig. 1. Ultrastructure of membrane alterations induced by different positive strand RNA viruses. (A) Left: Membranous web induced by HCV. The ER is shown in dark brown, the inner membrane of DMVs and double membrane tubules as yellow brown and their outer membrane as semi-transparent light brown. Top right: View into the lumen of a DMV connected to ER membranes. Bottom right: View of a pore (white arrow) connecting the DMV lumen to the cytosol (Romero-Brey et al., 2012). (B) Late replication complexes of CVB3-infected cells. DMVs are shown in orange, multilamellar structures in red and parts of the neighboring ER in blue (Limpens et al., 2011). (C) Left: Interconnected reticular network induced by DENV infection. The cytosolic face of the membrane network is shown in brown, the ER lumen in black. Right: Viral particles were found in continuous ER cisternae and are depicted in red. ER membranes are colored in light bown and inner vesicle membranes in dark brown (Welsch et al., 2009). (D) Left: Surface model of replication complexes induced by Kunjin virus showing ER membranes in red, ribosomes in white and viral RNA in yellow. Right: Vesicles (white) were found to be connected to each other and to ER membranes (red) (Gillespie et al., 2010). (E) Cluster of heterogeneous DMVs induced by SARS-CoV infection. The outer DMV membrane is shown in gold, the inner membrane in silver and CMs in bronze (Knoops et al., 2008). (F) Surface rendering of FHV replication complexes. Virally-induced spherules into the mitochondrial lumen are shown in white, mitochondrial membranes in blue. A red arrow depicts an opening of a spherule towards the cytosol (Kopek et al., 2007). (G) 3D model of a tomographic slice of Rubella virus replication factories. Invaginated vesicles are shown in white, rigid membrane sheets in dark brown, the cytopathic vacuole in yellow, the ER in light-green and mitochondria in red (Fontana et al., 2010). Figures were reproduced with permission from the respective journals.

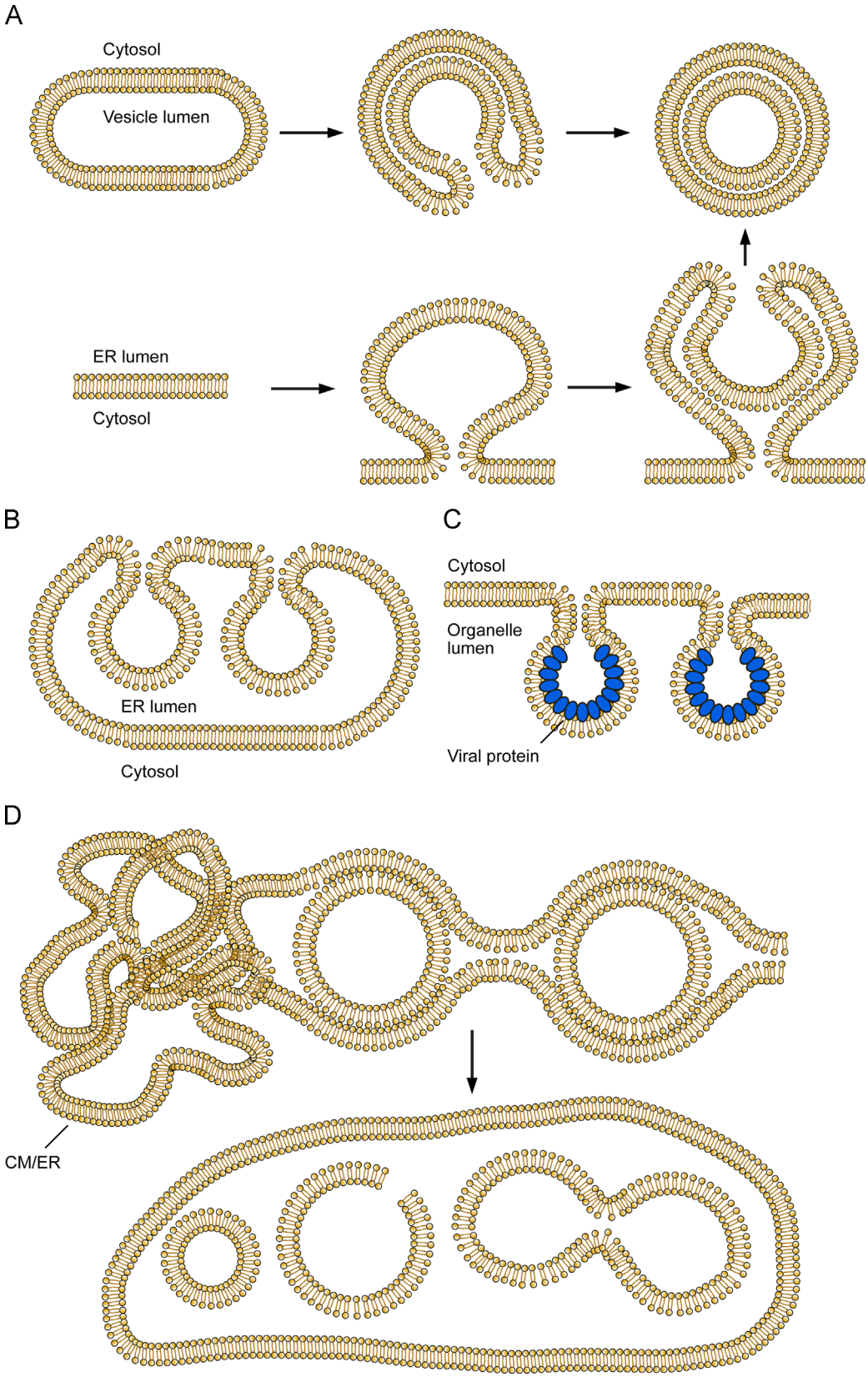

Fig. 2. Schematic illustration depicting the diversity of membrane alterations caused by a selection of different viruses. (A) Hypothetical model of double membrane vesicle (DMV) biogenesis originating from a single membrane vesicle (SMV, top), typically seen for Enteroviruses. The DMV is generated by an invagination event engulfing cytosol and creating a confined protected luminal space that can be connected to the cytosol by a narrow channel. Upon membrane merging, a closed DMV is created. Alternatively, DMVs can arise from exvagination of membranes into the cytosol accompanied by a secondary invagination event (bottom), as it has been suggested for HCV. (B) Replication complexes induced by DENV. An interconnected network is formed by ER membranes including SMVs that originate from invaginations into the ER. (C) Invaginated spherules as observed for BMV or FHV. A single oligomerizing viral protein (blue) is responsible for membrane alterations and forms a protein shell inside the spherules keeping them in shape. (D) Complexity of membrane alterations induced by SARS-CoV. Early stages of infection show DMVs interconnected by their outer membrane to each other as well as to convoluted membranes (CM) or ER membranes (top). Later stages of infection show membranous vesicle packets containing one or several SMVs, which can be fused to each other or exhibit membrane discontinuities (bottom) (Fontana et al., 2010).

expression model allows investigating the mechanisms of MW biogenesis also in conditions unfavorable for replication, e.g. using mutants devoid of replication or treatment with replication inhibitors.

Previously, it was suggested that nonstructural protein (NS)4B is the main driver of MW biogenesis, since expression of NS4B alone was sufficient to induce MW-like structures (Egger et al., 2002). NS4B is a highly hydrophobic integral membrane protein harboring two N-terminal amphipathic helices, four putative transmembrane domains and a highly conserved C-terminal domain. The topology of the different domains as well as oligomerization of NS4B seems to be critical for the morphology of the MW (Gouttenoire et al., 2010, 2014; Paul et al., 2011). However, recent studies revealed that expression of NS4B alone induces only the formation of SMVs but not DMVs. In fact, all nonstructural proteins induce membrane alterations when expressed alone, mostly SMVs or single membrane tubules. Only NS5A seems to be capable of inducing DMVs upon sole expression (Romero-Brey et al., 2012). NS5A is a multifunctional phosphoprotein harboring an N-terminal amphipathic helix associated with ER membranes, a structured domain I and two largely unstructured domains II and III (reviewed in Bartenschlager et al. (2013)). Domain I mediates formation of different homodimeric forms and it is also suggested to form oligomers as well (Tellinghuisen et al., 2005; Love et al., 2009; Lambert et al., 2014). Further, NS5A exists in two different phosphorylated forms, which distinct roles in HCV replication are still elusive (reviewed in Huang et al. (2007)). To date, it is unclear if NS5A dimerization, oligomerization or the phosphorylation status might contribute to formation of the MW. Interestingly, the recently approved NS5A-specific inhibitor Daclatasvir can completely block MW formation (Berger et al., 2014), suggesting that viral membrane alterations are indeed an attractive target for antiviral therapy. Further, NS5A interacts with a variety of host factors, which also contribute to MW architecture including PI4KIIIα (Reiss et al., 2011) and Cyclophilin A (Madan et al., 2014). In any case, although NS5A expressed alone can induce DMV formation, a single protein is still not able to induce MW structures comparable to authentic infection. All nonstructural proteins expressed alone mediate vesicle formation, but the membrane modulating functions of all nonstructural proteins together are required for proper formation of the MW, demonstrating various complementary functions of the different proteins to generate functional replication complexes (Romero-Brey et al., 2012). Still, we are far off understanding the molecular details of the biogenesis of the HCV replication sites.

Picornaviridae

Picornaviruses comprise a diverse group of animal and human pathogens like poliovirus (PV), Hepatitis A virus (HAV), coxsackievirus (CV), foot-and-mouth-disease virus (FMDV), rhinoviruses and others. The genome of Picornaviruses comprises ca. 6.5–8 kb and encodes for a polyprotein translated by an internal ribosome entry site, which is processed by viral proteases, while the cleavage intermediates often have their own function in the viral life cycle (reviewed in Whitton et al. (2005)).

Only a few representatives of this virus family, mostly of the genus *Enterovirus*, have been analyzed regarding the question how Picornaviruses modulate membrane homeostasis. Today, PV is probably one of the best-studied human viruses and indeed the earliest electron microscopy images depicting membrane alterations caused by viral infection were taken from PV-infected cells (Dales et al., 1965; Kallman et al., 1958). Later studies on the ultrastructure of PV replication complexes revealed occurrence of either SMVs (Bienz et al., 1980, 1983) or DMVs (Schlegel et al., 1996), but a more recent study demonstrated that indeed both structures appeared in infected cells in a time-dependent manner (Belov et al., 2012). First membrane alterations occur 2–3 h after PV infection and were observed as elongated tubular SMVs originating from positive curvature of cellular membranes. These membrane structures were probably arising from Golgi membranes as several studies suggested (Bienz et al., 1983; Belov et al., 2012; Hsu et al., 2010). The Golgi origin was confirmed by the sensitivity of PV replication to brefeldin A (Irurzun et al., 1992; Maynell et al., 1992). However, fractionation analyses revealed that the replication complexes were associated with various other organelle membranes as well, arguing for different membrane sources used by the virus (Schlegel et al., 1996). After 4 h, first DMVs of about 100–300 nm in diameter were visible and their number increased during the course of infection, while at the same time the SMVs disappeared (Belov et al., 2012). This conversion from SMVs to DMVs probably occurs through membrane wrapping and might be due to autophagic processes (Belov et al., 2012; Limpens et al., 2011; Suhy et al., 2000). Thus, the formation of replication vesicles seems to be a multi-step process originating from single membrane compartments to formation of DMVs. Despite the presence of dsRNA and viral proteins on both single membrane and double membrane compartments, RNA replication correlates best with the emergence of SMVs (Belov et al., 2012). Therefore, the function of DMVs for PV is not clear, but it has been suggested that DMVs play a role in virion maturation and the non-lytic release of progeny virus (Kirkegaard and Jackson, 2005; Richards and Jackson, 2012). Thus, PV might be able to control different steps of its life cycle by modulating the membranes of the replication complexes to either favor replication or assembly and release of virions.

A three-dimensional electron tomographic study by Limpens et al. (2011) shed light on the replication complex architecture of the related Coxsackievirus B3 (CVB3) (Fig. 1B). Similar transitions from single membrane tubules to DMVs were observed as for PV, which number greatly increased over the course of infection. About 20% of the DMVs exhibited a pore-like opening connecting the lumen to the cytosol, as it was observed for HCV (Romero-Brey et al., 2012). The vesicles induced by CVB3 are mostly isolated compartments, which implied that the vesicles originate from exvagination of cellular compartments (Limpens et al., 2011). Late in infection, an increasing number of DMVs were enwrapped by other membranes resulting in multilamellar vesicles occupying large areas in the cytoplasm (Fig. 1B), reminiscent to MMVs occurring in late HCV infection (Romero-Brey et al., 2012). In contrast to the MMVs in HCV infection, the multilamellar vesicles are enwrapped DMVs and always remain open to the cytoplasm (Limpens et al., 2011). It still is not clear whether these structures contribute to the viral life cycle or if they are just the result of a cellular response to the DMVs.

Of the seven nonstructural proteins of Enteroviruses, only three harbor hydrophobic domains (2B, 2C and 3A). 2B and 2C both have amphipathic helices that might contribute to membrane modulation (van Kuppeveld et al., 1996; Echeverri and Dasgupta, 1995; Paul et al., 1994; Teterina et al., 1997). For the 2B protein, a model has been suggested where 2B acts as a pore integrating into Golgi-derived membranes modulating membrane permeability (de Jong et al., 2003), a function that has been described also by other groups and is linked to increased cytoplasmic calcium levels (Aldabe et al., 1997; van Kuppeveld et al., 1997). However, a membrane modulating function has been mainly assigned to the 2BC precursor protein (Barco and Carrasco, 1995; Cho et al., 1994), but only when coexpressed with 3A, similar membrane structures arise as observed in authentic infection (Suhy et al., 2000). Conclusively, CVB3 seems to use only a subset of its proteins to generate the replication complexes, in contrast to HCV, which requires all of its nonstructural proteins (Romero-Brey et al., 2012).

Another member of the *Picornaviridae* family, FMDV, causes accumulation of the majority of organelles to perinuclear regions and fragmentation of the rough ER (Monaghan et al., 2004). Ribosomes accumulate in long chains in vicinity of the replication sites represented by SMVs and DMVs. In mid-phase and late infection, the Golgi is mostly dispersed and the number of vesicles increased, although the overall number and the proportion of DMVs were less than for other Picornaviruses. The origin of these vesicles is still not fully understood, although it is thought that they are derived from vesicles trafficking between the ER and the Golgi (Monaghan et al., 2004), similar to the mechanism suggested for PV (Rust et al., 2001). Despite some differences in the intracellular organization of membrane compartments between FMDV and other Picornaviruses, the same proteins (2B, 2C and 3A) seem to be of relevance. Also for FMDV, the 2BC precursor plays a major role as it is able to modulate ER membranes, in contrast to 2B or 2C expressed individually (Moffat et al., 2005). 2B and 2C also block the secretory pathway (Moffat et al., 2007), probably allowing the virus to use membranes involved in this process, which otherwise would be unavailable. Another recent study also demonstrated that the 3C protease is capable of blocking this pathway by inducing Golgi fragmentation (Zhou et al., 2013).

In summary, for Picornaviruses the replication most probably takes place at SMVs, which are visible early in infection and are later converted into DMVs (Fig. 2A, top), which might fulfill functions in the late viral life cycle like assembly of viral particles.

Nidovirales

The Nidovirales are an order of positive strand RNA viruses containing, among others, the families of *Coronaviridae* and *Arteriviridae*. Coronaviruses harbor a polycistronic RNA genome with around 27–31 kb in size, which represents the largest RNA genome known to date. Besides the production of new RNA genomes from full-length negative strand RNA, they also produce subgenomic template RNAs, which are then used for production of mRNAs used for translation of structural and accessory proteins (reviewed in Masters (2006)). Coronaviruses can infect a wide range of mammals and birds and some members impose a serious health threat to humans like the Middle East respiratory syndrome coronavirus (MERS-CoV) or the severe acute respiratory syndrom coronavirus (SARS-CoV).

In infected cells, both viruses induce similar formation of paired membranes as well as ER-derived DMVs located mostly in perinuclear areas, which are also reminiscent to structures induced by mouse hepatitis coronavirus (MHV) (Goldsmith et al., 2004; Snijder et al., 2006; Knoops et al., 2008; de Wilde et al., 2013a). First DMVs appear about 2 h after infection with a diameter of 150–300 nm. After 4 h, the number of clustered DMVs greatly increases and the vesicles are often connected to reticular membranes. Most DMVs show a tightly connected bilayer and only occasionally the membranes are loosely surrounding each other. From 3 h after infection, also CMs appear in close proximity of DMVs (David-Ferreira and Manaker, 1965; Krijnse-Locker et al., 1994), which closely resemble the CMs observed for Flavivirus infection (Welsch et al., 2009; Westaway et al., 1997) (Fig. 1E). The CMs induced by SARS-CoV appear largely as paired membranes having the same intermembrane distance as the DMVs (Angelini et al., 2013) and sometimes are connected to the outer membrane of DMVs and ER cisternae (Knoops et al., 2008). Interestingly, the viral replicase proteins nsp3, nsp5 and nsp8 are located mostly in the CMs and much less in the DMVs (Goldsmith et al., 2004; Knoops et al., 2008), similar to observations made for Kunjin virus (Westaway et al., 1997). The DMVs in turn are largely positive for dsRNA, while the CMs are almost devoid of dsRNA (Knoops et al., 2008), arguing for a physical segregation of viral proteins and replication intermediates. Later in infection, packets of SMVs were observed, which are surrounded by a common membrane and contained viral particles. Since many DMVs disappear during the course of infection, it is suggested that the vesicle packets might arise from merged DMVs. Electron tomographic studies confirmed that almost all DMVs are interconnected to each other by their outer membrane and are part of a continuous network with the ER (Figs. 1E and 2D). A single DMV could be connected to one or more other DMVs, to the ER or to CMs via a narrow 8 nm neck. Surprisingly, there is no connection to the cytosol, which segregates SARS-CoV from many other viruses and suggests another strategy to transport metabolites to the replication sites and to release viral RNA (Knoops et al., 2008).

The viral proteins nsp3, nsp4 and nsp6 harboring hydrophobic domains were identified to be responsible for membrane alterations (Angelini et al., 2013), with nsp4 and nsp6 being highly conserved among the Nidovirales (Neuman et al., 2014). Nsp3 is able to disorder and proliferate membranes. Together with nsp4, it also mediates membrane pairing. Nsp6 also has membrane proliferation activity and can induce perinuclear vesicles. All three proteins expressed together are able to induce vesicular structures very similar to those seen in authentic infection (Angelini et al., 2013). For Arteriviruses, very similar membrane alterations have been identified consisting of an interconnected network of DMVs (Knoops et al., 2012) and nsp2 and nsp3 of the related equine arteritis virus (EAV) of the *Arteriviridae* family are sufficient to induce DMVs reminiscent of viral infection (Snijder et al., 2001). Still, it is not fully understood how the viral proteins modulate host membranes, since no clear functions in membrane alterations could yet be assigned to nsp4 and nsp6 (Angelini et al., 2013). Critical host factors involved in the biogenesis of the replication sites or the lipid composition of the replication complexes have also not been identified so far. It further remains to be determined why SARS-CoV does not require its replication vesicles being connected to the cytoplasm and why the DMVs form an interconnected network, which is an interesting and unique feature of SARS-CoV replication complexes.

Spherule/invagination type

Flaviviruses

The genus *Flavivirus* includes various important human-pathogenic members like Denge virus (DENV), West Nile virus (WNV), Yellow Fever virus (YFV) or tick-borne encephalitis virus (TBEV). Flaviviruses have a capped genome of about 10–11 kb that encodes for a polyprotein processed by viral and cellular proteases to generate the mature structural and nonstructural proteins (reviewed in Chambers et al. (1990)).

DENV is an enveloped arthropod-borne pathogen and infects about 50–100 million people worldwide. Molecular aspects of DENV replication are elaborated in other reviews (Bartenschlager and Miller, 2008; Acosta et al., 2014). First visualizations of membrane rearrangements within DENV-infected cells showed cytoplasmic vacuoles and accumulation of one or several virions in crystalloid arrays, which are surrounded by host membranes (Stohlman et al., 1975; Matsumura et al., 1971). Later studies focused more on infected mosquito cells and visualized different steps of the viral life cycle (Hase et al., 1987; Tu et al., 1998). Finally, the use of cryosectioning and high-pressure freezing allowed better preservation of ultrastructures and visualization of a variety of different membrane alterations (Welsch et al., 2009; Mackenzie et al., 1996a; Grief et al., 1997).

Eventually, with the use of electron tomography it was possible to image the DENV replication vesicles in three dimensions providing very detailed insights into the diverse membrane structures induced by the virus. A sophisticated study by Welsch et al. (2009) demonstrated the variety of ER-derived membrane alterations induced by

DENV. Essentially, they discovered the presence of vesicle packets (VP), membrane tubules and convoluted membranes (CM). The latter ones usually were surrounded by spherical SMVs of 80–90 nm in diameter located within the lumen of the rough ER or appearing as DMV associated with ER cisternae (Fig. 1C, left). Virus particles were observed in dilated cisternae of the rough ER in close association with the CMs and VPs (Fig. 1C, right) and could be labeled for glycoprotein E, which was not present in the VPs, suggesting distinct sites of viral RNA replication and particle assembly.

Three-dimensional imaging of the DENV-induced vesicular structures using electron tomography revealed that the vesicles previously appearing as DMVs are in fact part of an interconnected network via their outer membrane (Welsch et al., 2009) (Figs. 1C and 2B). Virion-filled ER cisternae were found to be always in near vicinity of virus-induced vesicles and very often being connected to ER structures containing these vesicles. More than half of the vesicles were connected to the outer ER membrane, which leaves a pore-like opening connecting the vesicle lumen to the cytosol. These pores have a diameter of about 11 nm and could allow both entry of metabolites required for replication and exit of viral RNA for translation or viral assembly. Further, this finding argues for an invagination event of ER membranes into the ER lumen giving rise to these vesicular structures. Additionally, Welsch et al. could demonstrate that the intraluminal virions have budded from the ER and that virions are transported to the Golgi complex by secretory vesicles originating from peripheral ER cisternae. Putative viral budding sites were identified very close to the pores of the replication vesicles, which supports the idea that the pores represent an exit site for viral RNA, which then can be efficiently packaged into viral particles (Welsch et al., 2009).

Nonstructural proteins NS2B, NS3, NS4A and NS4B as well as dsRNA were located in the VPs, suggesting that RNA replication probably takes place within these structures. Notably, only a subset of the vesicles were stained positive for dsRNA, which was located at the cytosolic side or inside of the vesicles, suggesting that only a part of the vesicles are participating in active replication at a given time point, similar to observations made for HCV (Romero-Brey et al., 2012).

The biogenesis of the membrane alterations and the function of the CMs are still unclear, though. The viral protease complex NS2B/NS3 mainly locates to the CMs, which is thought to be the location for protein translation and polyprotein processing for the related Kunjin virus, a subtype of WNV (Westaway et al., 1997). In expression experiments, NS4A but not NS4A-2K was able to induce CMs underlining the importance of proteolytic processing of NS4A for membrane modulation and induction of CMs. It was suggested that NS4A acts via a central peripheral domain intercalating into the luminal leaflet of the ER (Miller et al., 2007), probably via its membranotropic regions (Nemesio et al., 2012). NS4B also might play a role, since it is a highly hydrophobic protein that integrates into ER membranes (Miller et al., 2006). However, since CMs were only found in mammalian but not in mosquito cells, it is still up to debate whether they play an important role in the viral life cycle (Junjhon et al., 2014). Also for TBEV, clear differences in membrane alterations between insect and mammalian cells were observed. A comparative study showed that infected insect cells do not show any viral particles and the extent of membrane expansion and number of vesicles was lower as compared to mammalian cells (Offerdahl et al., 2012). These differences in membrane alteration generally might reflect certain viral strategies to propagate in the different organisms, with a specific outcome according to the steps of the viral life cycle in alternate hosts.

A similar membranous network was observed for Kunjin virus, where the replication sites harboring dsRNA colocalize with markers of the trans-Golgi network (Mackenzie et al., 1999) and are closely associated with the rough ER (Gillespie et al., 2010) (Fig. 1D, left). Similar to DENV, pores were found connecting the inner part of the vesicles to the cytosol, but also to each other (Fig. 1D, right). The presence of dsRNA within the vesicles argued that these vesicles are the site of RNA replication (Gillespie et al., 2010; Mackenzie et al., 1996b, 1998). Many parallels were also observed for TBEV like the occurrence of membrane-connected vesicular structures, which partly displayed pores and seem to be Golgi-derived due to the sensitivity to brefeldin A (Lorenz et al., 2003), a drug disrupting Golgi integrity (Fujiwara et al., 1988). Remarkably, dsRNA was only detected in vesicles found inside the ER and it was speculated that this mechanism prevents interferon induction by shielding the viral RNA from pathogen recognition receptors (Overby et al., 2010; Pichlmair, 2007). Electron tomography on TBEV-infected cells revealed that the vesicles originate from invaginations into the ER lumen (Miorin et al., 2013). Again, about half of the vesicles displayed a pore connected to the cytoplasm, but although the vesicles were tightly opposed to each other, they were not interconnected in contrast to vesicles induced by WNV (Gillespie et al., 2010).

Taken together, although the different Flavivirus members show some variety in the ultrastructural morphology of their replication complexes, they share many similarities: their replication sites appear as single membrane invaginations into the ER, connected to the cytoplasm by a pore and all of them induce a system of convoluted membranes, with yet to be defined functions. However, it remains to be elucidated how these different membrane compartments are generated by the viral proteins. A protein expression system would be desired to reconstitute the membrane rearrangements seen in infection in a replication-independent setup to further study the determinants of replication complex formation.

Bromoviridae

Brome mosaic virus (BMV) is a well-studied plant pathogen of the family *Bromoviridae* and the superfamily of alphalike-viruses. BMV harbors three genomic RNAs, each encoding for a single nonstructural protein, while the structural coat protein is translated from a subgenomic RNA (Noueiry and Ahlquist, 2003). Most aspects of viral replication can be reconstituted in yeast cells greatly facilitating research of this virus (Ishikawa et al., 1997; Janda and Ahlquist, 1993). The replication complexes induced by BMV are of the invaginated spherule type and occur on ER membranes (Restrepo-Hartwig and Ahlquist, 1999; Restrepo-Hartwig and Ahlquist, 1996; Schwartz et al., 2002). The main driver of membrane alterations is the multi-functional viral protein 1a, which localizes to the ER and recruits the RNA polymerase 2a (Chen and Ahlquist, 2000) as well as viral RNA templates (Chen et al., 2001; Wang et al., 2005). These processes are probably timely regulated, since interaction of 1a with the polymerase 2a occurs preferentially before 1a induces spherule formation, thereby efficiently recruiting the polymerase into membranous replication complexes (Chen et al., 2003; Liu et al., 2009). A recent report demonstrated that ectopic expression of the capsid protein also induces ER-derived vesicles reminiscent of those observed in infection, but the relevance of this mechanism for the viral life cycle is not clear yet (Bamunusinghe et al., 2011). The spherules usually are between 30 and 70 nm in diameter and are often connected to the outer ER membrane, in some cases forming an invagination connected by a neck. Similar structures also have been observed for other plant viruses (Grimley et al., 1972; Hrsel and Brcak, 1964). The spherules can contain one or several genomic RNA intermediates, and a comparably high number of 1a molecules seems to be required to maintain those structures, while the amount of 2a RNA polymerase within the spherules is comparably low (Schwartz et al., 2002). It is proposed that 1a forms a protein shell in the vesicle interior by its strong membrane

association and self-interaction (O'Reilly et al., 1995; Kao and Ahlquist, 1992; Diaz et al., 2012) (Fig. 2C).

Thus, it is remarkable that a single protein is able to fulfill a variety of tasks including membrane association, induction of curvature and maintenance of these membranes as well as recruiting other viral proteins and RNA to the replication complexes, but so far, no three-dimensional reconstruction of the replication vesicles has been published. It will be interesting to further unravel the architecture of the BMV-induced spherules, since there appear to be many parallels between the biogenesis and architecture of BMV-induced spherules and retrovirus virion budding and genome encapsidation, suggesting that positive-strand RNA viruses, reverse-transcribing viruses and dsRNA viruses might share common ancestors (reviewed in Ahlquist (2006)).

Nodaviridae

Flockhouse virus (FHV) is a non-enveloped insect virus belonging to the family of *Nodaviridae*. It harbors two capped genomic RNAs encoding for protein A or the coat protein, respectively, while a third subgenomic RNA is produced by protein A and encodes for two further proteins taking part in suppression of RNA silencing in host cells (reviewed in Venter and Schneemann (2008)).

FHV was the first virus, which replication complexes have been revealed three-dimensionally in detail by electron tomography (Kopek et al., 2007). FHV replicates within spherules of 50–70 nm in diameter, which, in contrast to BMV, invaginate into the outer mitochondrial membrane (Miller and Ahlquist, 2002) (Fig. 1F), although the virus can also be productively retargeted to ER membranes (Miller et al., 2003), demonstrating a certain structural flexibility of the replication compartments. Early in infection, affected mitochondria show compressed matrices due to the formation of spherules into the mitochondrial lumen. At later timepoints, the mitochondria suffer from dissolution of cristae and swelling of the matrices resulting in severe morphological changes (Kopek et al., 2007; Miller et al., 2001). Similar to other viral replication vesicles, all spherules contain a pore of about 10 nm connecting the lumen of the vesicle to the cytoplasm allowing diffusion of metabolites (Kopek et al., 2007) (Fig. 1F, red arrow). As for BMV, a single protein is required for inducing spherule formation, which is protein A that also functions as RNA polymerase. It localizes to outer mitochondria membrane and acts as an integral membrane protein (Miller and Ahlquist, 2002; Miller et al., 2001), similar to RNA polymerases of other related viruses (Gant et al., 2014). Similar to BMV protein 1a, it self-interacts (Dye et al., 2005) and is able to recruit RNA templates for replication (Van Wynsberghe et al., 2007). A single spherule was quantified to contain about 100 membrane spanning protein A molecules as well as 2–4 genomic RNA intermediates (Kopek et al., 2007). Thus, it is likely that protein A forms a shell around the spherules keeping them in their shape (Fig. 2C). Such a high protein to RNA ratio seems not to be uncommon in viral replication, since similar observations have been made for BMV (Schwartz et al., 2002) and for HCV (Quinkert et al., 2005). It generally seems favorable to restrict the number of genome equivalents per replication vesicle, either due to space limitations in the replication vesicles, to facilitate timely coordination of replication and assembly events by having many replication vesicles work in parallel, or to limit the presence of potential activators of host cell immunity. Interestingly, for formation of spherules, protein A has to retain its replicative function and also requires the presence of a replication-competent RNA template, arguing for active viral replication as a prerequisite for spherule formation. It is not clear yet why viral replication is required for spherule formation, but is speculated that protein A might undergo conformational changes or posttranslational modifications upon RNA replication, or that newly synthesized negative strand or dsRNA then might recruit other host factors or act as initiation site for assembly of the protein A shell (Kopek et al., 2010). Deciphering this mechanism might clarify why for some viruses it is not possible to reconstitute membrane alterations seen in infection by expressing the viral proteins alone without an RNA template.

Togaviridae

The family of *Togaviridae* comprises the genera Rubivirus and Alphavirus. The only member of the genus Rubivirus is Rubella virus (RUBV), which harbors a 10 kb RNA genome containing two open-reading-frames encoding the two non-structural replicase components P90 and P150 or the structural proteins capsid and envelope glycoproteins E1 and E2, respectively (Frey, 1994). The other genus Alphavirus comprises various members like Semliki Forest virus (SFV) or Sindbis virus (SV). Alphaviruses are arthropod-borne viruses and are usually transmitted by mosquitoes between avian and mammalian hosts, where infection can lead to febrile diseases like encephalitis or arthritis (reviewed in Suhrbier et al. (2012)). The genome comprising ca. 11.5 kb has two open-reading-frames encoding for a polyprotein precursor that is cleaved into the nonstructural proteins nsP1–nsP4 or for the structural proteins, which are expressed via a subgenomic mRNA, respectively (Kaariainen and Ahola, 2002).

All these viruses induce very similar membrane alterations in infected cells, which are designated as cytopathic vacuoles (CPV) derived from modified endosomes and lysosomes. These unique virally-induced organelles can reach very large diameters ranging from 600 to 2000 nm and contain numerous smaller invaginated spherules or vesicles of about 50 nm, as well as large vacuoles, stacked membranes and rigid membrane sheets (Fontana et al., 2007; Lee et al., 1994; Magliano et al., 1998; Froshauer et al., 1988; Grimley et al., 1968) (Fig. 1G). The formation of RUBV CPVs is induced by the viral replicase components P90 and P150, which are located within the CPVs together with dsRNA (Fontana et al., 2007). dsRNA also locates to the cytosolic side of the CPVs indicating that the replicase components located inside the CPVs probably have a connection to the cytosol. It has been suggested that the rigid membrane sheets might function as connector, since they reach into the cytosol and were labeled positive for dsRNA and viral proteins (Fontana et al., 2010). These membrane sheets also enwrap the large vacuoles present in many CPVs and in some cases are connected to the periphery of the vacuole. Further, tightly packed membranes were found in some cases, which show numerous openings to the cytosol and might take part in metabolite exchange between the CPVs and the surrounding environment (Fontana et al., 2010).

CPVs also recruit other organelles like rough ER cisternae, Golgi membranes or mitochondria (Fontana et al., 2010). Rough ER membranes connect to the CPVs either via closely apposed membranes or protein bridges, while Golgi stacks connect via small peripheral vesicles contacting the CPV membrane. Mitochondria are located in near vicinity, but do not show a direct connection to CPVs. The CPVs also are connected with the endo-lysosomal pathway as demonstrated by incorporation of BSA-gold particles (Fontana et al., 2010), which are rapidly endocytosed by the cell after addition to the culture medium. Thus, the endolysosomal function of the membranes constituting the CPVs is still active and might contribute to the formation of the CPVs.

Interestingly, although Alphaviruses induce similar structures, the requirements for CPV formation are different. Expression and cleavage of the whole P1234 polyprotein are required for replication complex formation. Biogenesis of spherules takes place at the plasma membrane (Peranen and Kaariainen, 1991; Kujala et al., 2001), where nsp1 specifically associates to the cytosolic side of the plasma membrane via an amphipathic helix (Ahola et al., 1999; Lampio et al., 2000). The spherules are then uptaken by the endolysosomal pathway via a mechanism dependent on phosphatidylinositol 3-

Table 1
Host factors involved in replication complex biogenesis of positive-strand RNA viruses covered in this review.

	Host factor	Cellular function	Virus	Function for the virus
Lipids	Sphingo-lipids	Signal transmission, cell recognition (Brown and London, 2000)	HCV	Stimulation of polymerase activity (Hirata et al., 2012)
	PI4P	Golgi recruitment of adapter proteins, membrane identity (Clayton et al., 2013)	HCV	Recruitment of OSBP (Wang et al., 2014) or FAPP2 (Khan et al., 2014)
			Rhinoviruses	Recruitment of OSBP (Roulin et al., 2014)
			Enteroviruses	Recruitment of 3D polymerase (Hsu et al., 2010)
	Cholesterol	Lipid rafts, detergent-resistant membranes (Silvius, 2003)	WNV	Immune response perturbation (Mackenzie et al., 2007)
			Enteroviruses	Impacts replication membrane architecture (Ilnytska et al., 2013)
			HCV	Impacts replication membrane architecture (Paul et al., 2013; Wang et al., 2014)
	Saturated fatty acids	Membrane component	RUBV	Unknown, enriched in infected cells (Williams et al., 1994)
	Phospho-lipids	Membrane component	FHV	Facilitates membrane association of polymerase (Castorena et al., 2010; Stapleford et al., 2009)
Lipid metabo-lism and transport	PI4KIIIα	PI4P synthesis at the ER and plasma membrane (Clayton et al., 2013)	HCV	Interacts with NS5A, impacts replication membrane architecture (Reiss et al., 2011, 2013), induces PI4P synthesis for recruitment of OSBP (Wang et al., 2014) or FAPP2 (Khan et al., 2014)
	PI4KIIIβ	PI4P synthesis at Golgi membranes (Clayton et al., 2013)	Enteroviruses	PI4P enrichment for recruitment of 3D polymerase (Hsu et al., 2010)
			HCV	Unknown, possibly PI4P enrichment and connected with GBF1 & ARF1 functions (Zhang et al., 2012)
			Rhinoviruses	PI4P/Cholesterol shuttling via OSBP (Roulin et al., 2014)
	OSBP	Sterol transport from ER to Golgi (Mesmin et al., 2013)	HCV	Impacts replication membrane architecture (Wang et al., 2014)
	FAS	Synthesis of fatty acids at the ER (Smith et al., 2003)	DENV	Recruitment and stimulation by NS3 (Heaton et al., 2010)
			WNV	Unknown, required for replication (Martin-Acebes et al., 2011)
	Sphingosin kinase 2	Lipid peroxidation (Maceyka et al., 2005)	HCV	Interferes with membrane rearrangements (Yamane et al., 2014)
	FAPP2	Glycosphingolipid transport (D'Angelo et al., 2007)	HCV	Sphingolipid transport to replication membranes (Khan et al., 2014)
Vesicle transport	VAP-A	Binding of SNARE proteins (Weir et al., 2001), ER/Golgi transport, OSBP binding (Wyles et al., 2002)	HCV	Interacts with a specific NS5A phosphoform (Evans et al., 2004)
	VAP-B	Similar to VAP-A, forms heterodimers with VAP-A (Hamamoto et al., 2005)	HCV	Interacts with NS5A and NS5B (Hamamoto et al., 2005)
	COPI (including ARF1, GBF1, ARFGAP1, ACBD3)	Retrograde Golgi/ER transport, phospholipase D activation (Mitchell et al., 2003)	Enteroviruses	Blocked by 3A interaction (Wessels et al., 2006)
			Coronaviruses	Interaction with NS4 (Oostra et al., 2007) and spike protein (McBride et al., 2007)
			HCV	Unknown, probably connected to PI4KIIIβ functions (Zhang et al., 2012)
	COPII	Vesicular ER/Golgi transport (D'Arcangelo et al., 2013)	PV	Recruitment on 2BC-induced vesicles (Rust et al., 2001), increased membrane recruitment of Sec16A (Trahey et al., 2012)
Cyclophilins	CypA	Peptidyl-prolyl *cis/trans* isomerase (Wang and Heitman, 2005)	HCV	Interacts with NS5A, impacts replication membrane architecture (Madan et al., 2014)
			Coronaviruses	Involved in RNA synthesis (Carbajo-Lozoya et al., 2014), binds to nucleocapsid (Luo et al., 2004)
			Arteriviruses	Unknown, required for replication (de Wilde et al., 2013b)
			Tombusviruses	Interacts with p33 and viral RNA (Kovalev and Nagy, 2013)
	FKBP	Peptidyl-prolyl *cis/trans* isomerase (Wang and Heitman, 2005)	Coronaviruses	Unknown, required for replication (Carbajo-Lozoya et al., 2014)
Membrane curvature	PSTPIP2	Actin-associated in macrophages (Yeung et al., 1998)	HCV	Impacts replication membrane formation, probably by membrane bending (Chao et al., 2012)
	Amphi-physins	Membrane bending via its BAR-domain (Arkhipov et al., 2009)	HCV	Impacts NS5A phosphorylation and replication membrane formation (Masumi et al., 2005)
			Alphaviruses	Recruitment by nsP3 (Neuvonen et al., 2011)
	Reticulon proteins	ER membrane shaping (Voeltz et al., 2006)	BMV	Impacts replication membrane formation (Diaz et al., 2010)
			Picornaviruses	Interacts with 2C (Tang et al., 2007)
Autophagy	LC3, ATG4B, ATG5, ATG7, ATG12, Beclin-1	Delivery of cytoplasmic components to the lysosome (Mizushima, 2007)	PV	LC3 required for replication (Taylor and Kirkegaard, 2007)
			Coronaviruses	ATG5/12 required for replication (Prentice et al., 2004)
			DENV	ATG5 required for replication (Lee et al., 2008), manipulates lipid metabolism (Heaton and Randall, 2010)
			HCV	ATG4B/5/12, Beclin-1 required for translation and replication (Dreux et al., 2009), autophagosome formation by inducing LC3 lipidation (Sir et al., 2008), ATG7 involved in particle production (Tanida et al., 2009), ATG5 interacts with NS5B (Guevin et al., 2010), NS4B-induced autophagy connected with Rab5 and Vps34 (Su et al., 2011)

kinase and the cytoskeleton (Spuul et al., 2010), which then leads to the formation of the CPVs. Similar to FHV, the biogenesis of Alphavirus spherules is strictly dependent on presence of viral RNA, since expression of the viral proteins alone does not induce spherule formation (Spuul et al., 2011). In contrast to FHV, the diameter of the invaginations of SFV is directly dependent on the size of the viral template RNA (Kallio et al., 2013). This implies that biogenesis of the spherules is dynamic and regulated during RNA synthesis.

Host factors contributing to replication complex biogenesis

In the first part, we described the morphology of membrane alterations induced positive strand RNA viruses and the contribution of viral proteins to their biogenesis, as far as known. However, host factors are absolutely essential to form and maintain these membranous replication complexes and our knowledge on this issue is still limited but constantly increasing. In fact, the viral replication organelles often require massive expansions of particular membrane compartments and exploit cellular machineries for vesicle formation, since viruses are limited in their genome size and simply do not have the capacities to encode for all these functions. Thus, they have to develop strategies to exploit host pathways involved in lipid synthesis and membrane curvature. However, a comprehensive overview on this topic goes beyond the scope of this review and we can just put a few spotlights on commonly emerging themes. A summary of the host factors discussed in this review is listed in Table 1.

Cyclophilins, COPI/II and autophagy

Various cellular proteins involved in membrane curvature have been reported to play a role in biogenesis of viral replication complexes like amphiphysins for alphaviruses (Neuvonen et al., 2011) and HCV (Chao et al., 2012), or reticulons for Picornaviruses (Tang et al., 2007) and BMV (Diaz et al., 2010). The peptidyl-prolyl *cis/trans* isomerase cyclophilin A (CypA) has been shown to be essential for various steps in the replication cycle of very different viruses. However, in case of HCV, it was recently shown that CypA indeed plays a role in early events of MW biogenesis (Madan et al., 2014), probably by interacting with NS5A, which is one of the main drivers of MW formation (Romero-Brey et al., 2012). CypA also is involved in entry and disassembly of enterovirus particles by interacting with the VP1 protein (Qing et al., 2014) and plays an important role for replication of other viruses as well, such as HIV, HBV or influenza virus (reviewed in Zhou et al. (2012)). For example, CypA was shown to interact with p33 and viral RNA of Tombusvirus (Kovalev and Nagy, 2013), thereby contributing to viral replication, but also binds to the nucleocapsid of coronaviruses (Luo et al., 2004). For the human coronaviruses NL63, also another peptidyl-prolyl *cis*/*trans* isomerase, the FK506-binding protein (FKBP) was identified to play a role in replication (Carbajo-Lozoya et al., 2014). In contrast, the feline infectious peritonitis virus, another member of the coronavirus family, only depends on CypA, but not on FKBP (Tanaka et al., 2012). Conclusively, the functions of peptidyl-prolyl *cis*/*trans* isomerases in viral replication seem to be very diverse between different viruses, and although it probably does not directly contribute to membrane bending, it might assist to fold and translocate viral proteins or other host factors into membranes.

Different members of the *Picornaviridae* utilize components of the secretory pathway like the COPII- (Rust et al., 2001; Trahey et al., 2012) or COPI-machinery via Arf1-GTPase and its effector GBF1 for replication complex formation (Hsu et al., 2010; Belov et al., 2007, 2008). Different inhibitors of GBF1 have indeed been shown to inhibit enteroviral replication (van der Linden et al., 2010). HCV also has been shown to depend on ARF1, GBF1 or ARFGAP1 (Goueslain et al., 2010; Li et al., 2014; Zhang et al., 2012), but the exact mechanism has not been clarified yet. Interestingly, ARF1 possesses an N-terminal amphipathic helix that is able to bend membranes (Lee et al., 2005), which renders ARF1 as potential cellular candidate taking part in replication vesicle formation, although direct evidence is lacking. Still, there are profound differences between different members of the *Picornaviridae* in terms of COPI-dependent replication complex biogenesis. COPI has been shown to be important for Enterovirus 71 (Wang et al., 2012), echovirus 11 and parechovirus 1 replication (Gazina et al., 2002). However, while the COPI-component β-COP is specifically distributed to replication sites of echovirus 11 and partly of parechovirus 1, it is not detected in the replication membranes of the related encephalomyocarditis virus. The differences in COPI-dependence are indeed reflected in the sensitivity of these viruses towards Brefeldin A (Wang et al., 2012; Gazina et al., 2002). SARS-CoV also interacts with COPI components and is sensitive towards Brefeldin A treatment (Knoops et al., 2010; Oostra et al., 2007; McBride et al., 2007), but integrity of the early secretory pathway seems not to be essential for SARS-CoV to remodel ER membranes (Knoops et al., 2010), arguing for differential roles of the COPI machinery among different viruses.

Autophagy plays a role for various different viruses like PV (Taylor and Kirkegaard, 2007), coronavirus (Prentice et al., 2004) or DENV (Lee et al., 2008). HCV replication also relies on autophagic processes (Dreux et al., 2009; Sir et al., 2008; Tanida et al., 2009). It is currently not clear, whether only individual factors of the autophagic pathway might be involved in formation of the MW (Romero-Brey et al., 2012; Guevin et al., 2010; Su et al., 2011), whether DMVs are in fact autophagosomes (Sir et al., 2008), or whether autophagy contributes to the conversion of DMVs to MMVs (Romero-Brey et al., 2012). For a more comprehensive report on the role of autophagy in viral replication, the reader is referred to another review (Chiramel et al., 2013).

Host cell lipids

Viral infections can severely impact lipid homeostasis of the infected cell by inducing de novo biosynthesis of membranes. For almost all of the examples in this review, lipidomic profiles of infected cells have been published, often showing altered levels of sterols, sphingolipids or phospholipids (Castorena et al., 2010; Lee and Ahlquist, 2003; Heaton et al., 2010; Perera et al., 2012; Martin-Acebes et al., 2011; Diamond et al., 2010; Tam et al., 2013; Roe et al., 2011). For example, HCV interferes with lipogenesis pathways via the core protein or NS4B by inducing cleavage of sterol regulatory element binding proteins (SREBP), which are major transcription factors for the expression of genes involved in lipid biosynthesis (Eberle et al., 2004). Cleavage of SREBP leads to enhanced transcription of proteins involved in lipogenesis such as fatty acid synthetase (FAS) (Waris et al., 2007; Park et al., 2009). FAS itself interacts with the HCV polymerase NS5B and stimulates its activity (Huang et al., 2013) and also plays a role in DENV and WNV replication (Heaton et al., 2010; Martin-Acebes et al., 2011). Also, sphingolipids have been reported to be involved in HCV replication by contributing to the formation of detergent-resistant membranes harboring the replication complexes (Hirata et al., 2012; Shi et al., 2003; Aizaki et al., 2004). Enhanced sphingolipid synthesis was found to be beneficial for HCV replication (Hirata et al., 2012) and also stimulates polymerase activity in a genotype-specific manner (Weng et al., 2010). Further, the replication complexes are highly enriched in cholesterol (Paul et al., 2013), which is probably recruited via the oxysterol-binding protein (OSBP) (Wang et al., 2014). Enhanced cholesterol levels at the replication sites were also observed for WNV (Mackenzie et al., 2007) and enteroviruses. For enteroviruses, the cholesterol recruitment is mediated by the clathrin-mediated endocytosis

pathway, which is subverted by the virus to transport cholesterol from the plasma membrane and the extracellular environment to the replication sites (Ilnytska et al., 2013).

Overall, the requirements for distinct lipid species vary widely among different viruses, e.g. Rubella virus profits from enhanced levels of saturated fatty acids (Williams et al., 1994), while FHV and tombusviruses require phospholipids for productive replication (Castorena et al., 2010; Stapleford et al., 2009). It would be interesting to see whether similar vesicle morphologies of different viruses are reflected by a comparable lipid composition, but so far comparative lipidomic profiles of isolated replication membranes are lacking.

Lipid-modifying enzymes and transporters

Modification of lipids and the enzymes involved therein represents another way of viruses to shape the lipid environment of their replication sites. Recently, it was suggested that lipid peroxidation mediated by sphingosin kinases is a process interfering with HCV replication and that blocking these processes or alleviating the effects of lipid peroxidation in the cell stimulates RNA replication of almost all genotypes (Yamane et al., 2014). Another interesting example of lipid-modifying enzymes contributing to viral replication involves a shared feature of Picornaviruses and HCV, which is the dependency on cellular type III phosphatidylinositol 4-kinases (PI4KIIIs) and their product PI4P. PI4P plays a role as precursor for synthesis of other phosphoinositide species and itself is involved in constituting membrane identity, signaling events and protein recruitment (reviewed in Clayton et al. (2013)), but can also induce membrane curvature (Furse et al., 2012). PI4Ks exists as two subtypes (II and III) each with two isoforms (α and β) located in distinct organelles. The alpha-isoform of PI4KIII (PI4KIIIα) is located at the ER and is an essential host factor for HCV replication (Reiss et al., 2011; Berger et al., 2009; Borawski et al., 2009; Tai and Salloum, 2011; Trotard et al., 2009). The beta-isoform (PI4KIIIβ) is located at Golgi membranes (reviewed in Clayton et al. (2013)) and plays a role for certain HCV genotypes (Reiss et al., 2011; Borawski et al., 2009) and for Picornaviruses (Hsu et al., 2010; Belov et al., 2007, 2008). PI4P was found in increased amounts at intracellular membranes as a consequence of viral replication in cell culture (Reiss et al., 2011; Berger et al., 2011) and in liver biopsies of HCV-infected patients (Reiss et al., 2011). Induction of PI4P synthesis is dependent on PI4KIIIα and probably occurs due to stimulation of the kinase by NS5A, which functionally interacts with PI4KIIIα via seven amino acids in domain 1 (Reiss et al., 2013). The kinase interacts with NS5A via different domains and only the C-terminal domains constituting the catalytic core are required for HCV replication (Harak et al., 2014). This virus–host interaction is not only essential for HCV replication, but also is required for PI4P accumulation and MW biogenesis (Reiss et al., 2013). Knockdown of PI4KIIIα or interfering with PI4KIIIα-NS5A interaction results in loss of PI4P induction and in a disturbed MW structure, which appears as clustered accumulation of DMVs with reduced diameter (Reiss et al., 2011, 2013; Berger et al., 2011), demonstrating an important role of PI4KIIIα in the morphology of the replication complexes. The specific role of PI4P in HCV replication is not clear yet and also different degrees of colocalization between PI4P and viral proteins have been reported by independent groups (Reiss et al., 2011; Wang et al., 2014; Berger et al., 2011). Dramatically increased PI4P levels seem not to be a prerequisite for replication (Reiss et al., 2013), but it can be envisaged that locally increased PI4P levels play an important role in MW biogenesis. In support of that, PI4P was shown to contribute to recruitment of OSBP, which is involved in HCV replication and assembly (Wang et al., 2014; Amako et al., 2009). OSBP was shown to transfer cholesterol to the HCV replication sites and inhibition or knockdown resulted in a similar MW phenotype as depletion of PI4P from the replication sites by directly targeting PI4KIIIα (Wang et al., 2014). This supports the idea that cholesterol plays a role in DMV morphology, as it has been suggested before (Paul et al., 2013). Also other PI4P-binding proteins were found being involved in the HCV life cycle like the Golgi-localized protein GOLPH3 for particle assembly (Bishe et al., 2012) or FAPP2 for RNA replication. FAPP2 was suggested to bind to PI4P-enriched membranes via its PH domain and thereby transporting glycosphingolipids to the replication sites (Khan et al., 2014). Also, a possible role of ceramide transport protein (CERT) and OSBP has been implicated for HCV particle assembly (Amako et al., 2011). Thus, it appears that locally increased PI4P pools can serve various purposes, from inducing membrane curvature (Furse et al., 2012), generating a lipid concentration gradient between different membranes (Mesmin et al., 2013) to recruiting lipid-binding proteins and thereby shaping the lipid composition of the replication sites (Wang et al., 2014; Bishe et al., 2012; Khan et al., 2014).

Picornaviruses, on the other hand, seem to rely more on the PI4KIIIβ isoform, since inhibition of PI4KIIIβ leads to effective block of CVB3 and PV RNA synthesis (Hsu et al., 2010; Greninger et al., 2012; Arita et al., 2011; Sasaki et al., 2012). This is consistent with the fact that PI4KIIIβ resides in the Golgi, where the Picornavirus replication sites mainly originate from. PI4KIIIβ is highly enriched at enteroviral replication sites and the recruitment is mediated by 3A–GBF1 interaction, which then activates ARF1 and leads to sequestration of PI4KIIIβ to viral membranes (Arita et al., 2011). The Golgi adapter protein ACBD3 might play an additional role for recruitment of PI4KIIIβ by enteroviral protein 3A (Greninger et al., 2012), but a recent study showed that PI4KIIIβ recruitment to CVB3 replication complexes can also occur independently of ARF1, GBF1 or ACBD3 (Dorobantu et al., 2014). However, ACBD3 still seems to be of importance, since together with the viral proteins 3A and 3B it stimulates PI4KIIIβ activity in vitro, which might account for the accumulation of PI4P at the replication sites (Ishikawa-Sasaki et al., 2014). PI4P then might function as adapter for the viral polymerase 3D, which preferentially associates with PI4P (Hsu et al., 2010). Like for HCV the cholesterol-transporting function of OSBP also seems to play a role for other viruses like PV and argues for similar requirements of cholesterol at the replication membranes (Arita, 2014) and for a similar role of lipid transport proteins in composing the lipids of the replication site.

Interestingly, a single point mutation in the viral protein 3A can bypass the need of CVB3 for PI4KIIIβ without affecting its virulence (Thibaut et al., 2014; van der Schaar et al., 2012). This mutation has been identified earlier as resistance mutation against enviroxime, a small molecule drug inhibiting replication of different positive-strand RNA viruses like PV, CVB3 and rhinoviruses (Heinz and Vance, 1996). However, it also confers cross-resistance to other inhibitors like PIK93, which efficiently targets PI4KIIIβ (Borawski et al., 2009), or to direct knockdown of PI4KIIIβ. This finding argues for a functional relationship between 3A and PI4P or other functions of PI4KIIIβ that can be overcome by changing a single amino acid residue. The identification of a virus mutant that circumvents the need for an essential host factor might facilitate our understanding of the role of PI4P in the viral life cycle (van der Schaar et al., 2012).

Closing remarks

In this review, we covered some selected examples of virally-induced membrane alterations demonstrating, on one hand, the diversity of different ultrastructures, but on the other hand, also many similarities between vesicle morphologies of related or even unrelated viruses. We certainly could only cover a small selection of positive-strand RNA viruses due to space constraints, but the reader is referred to more comprehensive reviews covering the

ultrastructures of additional viruses (Romero-Brey and Bartenschlager, 2014; Paul and Bartenschlager 2013; Miller and Krijnse-Locker, 2008).

Conclusively, immense progress in visualization of cellular ultrastructure by electron microscopy and tomography allowed researchers to explore membrane morphologies in three dimensions, revealing astonishing details of membranous networks caused by viral infections. However, we are far from understanding the viral and host factors involved in their biogenesis and it is still enigmatic, how these structures are linked to RNA synthesis. In the future, it will be important to connect microscopic techniques with elaborate mutagenesis and host factor studies to further dissect the different steps of replication complex biogenesis, and ultimately to understand the contribution of viral and cellular players taking part in these massive rearrangements of host membranes on the mechanistic level. Since lipid metabolism is tightly connected with the membrane alterations, visualization and localization of single lipid species will be as important as deciphering the specific lipid composition of isolated replication membranes. This knowledge will also help to identify novel antiviral drugs that interfere with replication complex formation, either by directly targeting a viral protein, as it has been shown for the recently approved Daclatasvir interfering with a membrane modulating function of HCV NS5A, or by targeting host factors like the COP machinery or lipid-modifying enzymes, as it is investigated for treatment of enteroviral infections. By targeting the host–virus interface it is possible to design drugs with low risk of viral resistance, but interfering with host processes also harbors the risk of severe side effects as well. It surely is desirable to develop treatments preventing the virus to use host cell processes without affecting the cellular function of those. Understanding the mechanisms behind viral replication complex biogenesis and the host factors involved will help us to pursue this direction.

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