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Chapter 2

Biogenesis and architecture of arterivirus replication organelles

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ABSTRACT

All eukaryotic positive-stranded RNA (+RNA) viruses appropriate host cell membranes and transform them into replication organelles, specialized micro-environments that are thought to support viral RNA synthesis. Arteriviruses (order Nidovirales) belong to the subset of +RNA viruses that induce double-membrane vesicles (DMVs), similar to the structures induced by e.g. coronaviruses, picornaviruses and hepatitis C virus. In recent years, electron tomography has revealed substantial differences between the structures induced by these different virus groups. Arterivirus-induced DMVs appear to be closed compartments that are continuous with endoplasmic reticulum membranes, thus forming an extensive reticulovesicular network (RVN) of intriguing complexity. This RVN is remarkably similar to that described for the distantly related coronaviruses (also order Nidovirales) and sets them apart from other DMV-inducing viruses analyzed to date. This review summarizes the current knowledge and open questions regarding arterivirus replication organelles and discusses them in the light of the latest studies on other DMV-inducing viruses, particularly coronaviruses.
MEMBRANE MODIFICATIONS INDUCED TO ACCOMMODATE +RNA VIRUS REPLICATION

All positive-stranded RNA (+RNA) viruses of eukaryotes replicate their genome in the cytoplasm of the host cell using a common strategy characterized by the modification of host membranes into organelle-like structures that, for many +RNA viruses, have been directly implicated in viral RNA synthesis (reviewed in (18, 35, 36)). Although these virus-induced membrane structures, often referred to as viral replication organelles or replication structures, have been known for decades, their exact purpose remains enigmatic. Three major advantages of associating viral RNA synthesis with dedicated membranes have been proposed. Firstly, confining viral RNA synthesis in a specific compartment could generate an optimally suited micro-environment by concentrating the viral proteins and precursors necessary for the process. Furthermore, the anchoring of viral replication complexes to membranes creates a planar geometry for diffusion of metabolites and macromolecules, which can increase the efficacy of the enzymatic processes. Secondly, compartmentalization could provide a means to spatially separate and coordinate the different stages of the infectious cycle, such as genome translation, replication and packaging. Finally, during viral RNA synthesis several intermediate nucleic acid species such as double-stranded RNA (dsRNA) and 5' triphosphate-containing RNAs are formed, which are potent activators of the host cell’s innate immune response (29, 30). Insulation of these intermediates could therefore prevent or delay detection by the defense systems of the host cell (37). Clearly, these proposed functions are not mutually exclusive and, likely, +RNA viruses take advantage of multiple benefits associated with the compartmentalization of their replicative process.

Both viral and cellular factors are thought to be important for the biogenesis of +RNA viral replication organelles, although in most cases the specific players and processes involved remain poorly understood. Multiple +RNA viruses encode non-structural proteins (nsps) that include proven or predicted transmembrane domains and, for some of them, expression of a combination of such membrane-associated viral nsps has been shown to be necessary and sufficient for the induction of membrane modifications resembling those found in infected cells (23-28, 38, 39). Besides these viral proteins, a wide variety of host factors have been implicated in the formation and functioning of these structures (reviewed in (40, 41)). Their roles range from the recruitment of viral replication proteins to the induction of membrane modifications. Not surprisingly, the list of identified host factors includes proteins involved in lipid metabolism (e.g. phosphatidylinositol-4-phosphate (PI4P) kinases, fatty acid synthase), specific lipids (e.g. PI4P, sterols), and membrane-shaping proteins (e.g. reticulons and endosomal sorting complexes required for transport (ESCRT) proteins) (42-46).

Over the last decades, the replication organelles of a large number of +RNA viruses have been characterized using electron microscopy (EM). It has become apparent that, despite the large evolutionary distances between these viruses and the different cellular organelles they manipulate, all +RNA viruses infecting eukaryotic host cells seem to induce one of
<table>
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<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>DMVs</th>
<th>Membrane connections</th>
<th>Open or closed to the cytosol</th>
<th>Additional membranous structures</th>
<th>References</th>
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<tr>
<td>Nidoviruses</td>
<td>Arteriviridae</td>
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<td>SHFV</td>
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<td>PM</td>
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<td>Isolated DMVs / DMV-ER</td>
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<td>ZM, spherules</td>
<td>Maier et al. (2013)³</td>
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<td>SMT, MLV</td>
<td>Limpens et al. (2011)²</td>
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<td>SMT, MLV</td>
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<td>Hepacivirus</td>
<td>HCV</td>
<td>150</td>
<td>Isolated DMVs / DMV-ER</td>
<td>Closed / Open</td>
<td>SMV, MLV</td>
<td>Romero-Brey et al. (2012)</td>
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two basic morphotypes of membrane modifications: invaginations or double-membrane vesicles. In the last decade, the study of the architecture of viral replication organelles has been stimulated further by the introduction of electron tomography (ET). ET enables the three-dimensional (3D) characterization of biological specimens at nanometer resolution by computationally combining images collected at different tilt angles in a transmission electron microscope (47). The first +RNA virus replication organelles characterized by ET were those induced by the nodavirus Flock House virus (FHV) (48). FHV induces invaginations in the outer mitochondrial membrane and therefore belong to the first morphotype of +RNA virus replication organelles. Using immuno electron microscopy (IEM) to detect BrUTP incorporated into viral RNA, the interior of these spherules was shown to contain newly-synthesized viral RNA that is thought to be exported to the cytosol through a neck-like channel of ~10 nm in diameter, which could be clearly visualized in the 3D reconstruction. Since this first study, the membrane modifications induced by, for example, coronaviruses (21, 49), arteriviruses (20), flaviviruses (50-54), hepaciviruses (36), togaviruses (55), picornaviruses (41, 56) and tombusviruses (57) have been characterized using ET.

The flavivirus dengue virus (DENV) provides another well-characterized example of virus-induced invaginations of cellular membranes (50) that, in this case, occur at the rough endoplasmic reticulum (ER). Despite the use of a different membrane donor organelle, the DENV-induced spherules share several characteristics with those generated during FHV infection. DENV-induced membrane invaginations are also open to the cytosol through a neck-like connection (~10 nm in diameter) and contain dsRNA. Apart from these invaginations, DENV infection also induces the formation of so-called convoluted membranes, which are continuous with the spherules through their connection with ER membranes and are speculated to be a reservoir of proteins and lipids used for DENV replication (50). The formation of ER invaginations that retain a connection to the cytosol has also been shown for other flaviviruses, like tick-borne encephalitis virus (53), West Nile virus (51), and Langat virus (52).

Table 1. (left page) Arteriviruses and other animal +RNA viruses inducing DMVs.

<table>
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<th>Arteriviruses and other animal +RNA viruses inducing DMVs.</th>
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<tr>
<td>a Equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase elevating virus (LDV), simian hemorrhagic fever virus (SHFV), human coronavirus NL63 (NL63), human coronavirus 229e (229e), severe acute respiratory syndrome coronavirus (SARS-CoV), mouse hepatitis virus (MHV), Middle East respiratory syndrome coronavirus (MERS-CoV), infectious bronchitis virus (IBV), coxsackievirus B3 (CVB3), poliovirus (PV), hepatitis C virus (HCV).</td>
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<td>b DMV connections and openings are only specified when 3D studies are available. When different DMV subpopulations have been reported, the distinct features of these subpopulations are separated by a slash.</td>
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<td>c Paired membranes (PM), single-membrane vesicle (SMV), convoluted membranes (CM), vesicle packets (VP), zipper membranes (ZM), single-membrane tubules (SMT), multilamellar vesicles (MLV).</td>
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<td>d ER, endoplasmic reticulum</td>
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<td>e Average diameter</td>
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<td>f Modal diameter</td>
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<td>g Not reported, estimate from published images</td>
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<td>h 3D characterizations by electron tomography</td>
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The formation of cytopathic vacuoles (CPVs), which are modified endosomes and lysosomes of about 600-2000 nm in diameter that accommodate invaginations of their membranes, is a hallmark for togavirus infection. ET applied to cells infected with rubella virus (RUBV) showed that these CPVs also contain interconnected small vesicles, vacuoles and stacked membranes (55). Furthermore, RUBV recruits rough ER, mitochondrial, and Golgi membranes close to the CPVs, presumably to make use of the resources present in these organelles to fuel its genome replication. Early in infection, Semliki Forest virus and Sindbis virus (genus Alphavirus) induce plasma membrane invaginations that afterwards appear to be internalized through the endosomal pathway to produce the CPVs typical of togavirus-infected cells (58-61).

The morphotype of virus-induced invaginations seems to extend to plant +RNA viruses (reviewed in (62), for an ET characterization see (57)). The striking similarities in the morphology of these invaginations across widely different virus groups strongly suggest a highly conserved mechanism behind their formation. In this regard, brome mosaic virus (Bromoviridae), which induces invaginations into the ER, is one of the best studied models for this replication organelle morphotype (27, 63). The insights obtained into the biogenesis and function of BMV-induced spherules highlighted remarkable parallels with the assembly of retrovirus capsids, which could hint to an ancestral common mechanism to alter cellular membranes that may have been preserved even more broadly among viruses (64).

The architecture of the second morphotype of replication organelles is strikingly different compared to the invagination morphotype and consists of double-membrane vesicles (DMVs). In contrast with the virus-induced invaginations characterized so far, which have the same topology among different virus groups, viral DMVs with different architectures have been found. Thanks to ET, variations in DMV connectivity and topology, not only between different virus groups but sometimes also between subpopulations of DMVs induced by a specific virus, have become apparent (Table 1). Moreover, infection with viruses in this group often results in the formation of additional types of membrane modifications, thus creating complex scenarios in which the exact role of the different virus-induced structures and their interplay remains elusive (Table 1). An example are the structures induced by the picornaviruses coxsackievirus B3 (CVB3) and poliovirus (PV), two enteroviruses that first generate single-membrane tubular structures that appear to transform into DMVs later in infection, presumably through an enwrapping mechanism (56, 65).

Notably, whereas the replication organelles of all other members of the family Flaviviridae that have been analyzed so far belong to the invagination morphotype, hepatitis C virus (HCV), the prototype of the Hepacivirus genus, predominantly induces DMVs (23, 66). Although single-membrane vesicles could also be detected in HCV-infected cells, the presence of DMVs appeared to correlate with viral RNA replication (36, 67). These HCV DMVs were described as protrusions from the ER into the cytosol and were often still connected to the ER (23).

Other viruses that, like HCV, seem to use the ER as the membrane donor organelle and also generate DMVs are viruses belonging to the order Nidovirales. This order
currently encompasses four virus families, *Coronaviridae, Arteriviridae, Roniviridae* and *Mesoniviridae*. Only a limited number of species have been assigned to the latter two families thus far and many aspects of their replication remain to be studied in detail (1, 68). In contrast, coronaviruses have been studied extensively because of their role as veterinary pathogens and the large societal impact of the zoonotic disease outbreaks caused by severe acute respiratory syndrome coronavirus (SARS-CoV; 2003) and Middle East respiratory syndrome coronavirus (MERS-CoV; since 2012). Additionally, several established human coronaviruses are widespread in the population and frequently associated with common colds. During the past decade, detailed 3D ultrastructural characterizations of virus-induced replication structures have been published for a few nidoviruses, specifically, for the arterivirus equine arteritis virus (EAV) (20) and the coronaviruses SARS-CoV and infectious bronchitis virus (IBV) (21, 22, 69). This review focuses on arterivirus replication organelles. The fundamental analogies and differences between arteriviral and coronaviral replication organelles, as revealed by recent studies, will be analyzed in parallel and also in the wider context of DMV-inducing +RNA viruses. Finally, we will discuss some of the open questions and future research directions that, in our view, will be important to unravel the role of these remarkable structures.

**ARTERIVIRUSES AND THE MEMBRANE REARRANGEMENTS THEY INDUCE**

**Arterivirus biology**

Although the arterivirus prototype EAV was discovered in 1953 (5), the family *Arteriviridae* was not established until 1996 (70). The group was long comprised of four members: EAV, porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever (SHFV) and lactase dehydrogenase-elevating virus (LDV). However, over the course of the last five years, several new (tentative) arteriviruses were discovered such as wobbly possum disease virus (WPDV), African pouched rat virus 1 and multiple new simian arteriviruses that are only distantly related to SHFV (4, 71-73). Because of their economic impact, EAV and PRRSV have been characterized most extensively and research on these viruses has provided the basis for most of our current understanding of arterivirus replication.

Arteriviruses have polycistronic +RNA genomes with sizes ranging from 13-16 kb (3). The two large 5’-proximal open reading frames (ORF1a and ORF1b), which encompass more than 60% of the genome space, encode the replicase or non-structural (poly)proteins (Fig. 1A). The 3’-proximal part of the genome contains an array of smaller ORFs encoding the structural proteins, which are expressed from a nested set of subgenomic (sg) mRNAs (Fig. 1A) (4). After virus entry, the genome is released into the cytosol and ORF1a can be directly translated into polyprotein 1a (pp1a). Genome translation can be extended using the downstream ORF1b reading frame when a -1 ribosomal frameshift (RFS) occurs just before the end of ORF1a. This event, which results in the synthesis of pp1ab, is promoted by a specific heptanucleotide shift site and an RNA pseudoknot structure (74, 75).
Fig. 1. Comparison of transmembrane non-structural proteins of arteri- and coronaviruses. (A) Overview of the EAV genome organization with several conserved domains encoded in ORF1a and ORF1b indicated (see main text for details). The structural protein genes, such as those encoding the viral glycoproteins (GP), envelope (E), membrane (M) and nucleocapsid (N) proteins, are located in the 3’-proximal quarter of the genome (shown in orange). (B) The top panel presents a scaled schematic of EAV pp1a. The three proteases (PLP1β, PLP2 and Mpro) are indicated with colored boxes and their corresponding cleavage sites in pp1a with triangles. The middle panel shows a model of the membrane topology of EAV ORF1a based on a Kyte & Doolittle hydropathy plot (blue line) and TMHMM predictions (red line; the percentage on the right represents the probability for that part of the protein to be cytosolic), which are both shown in the lower panel. (C) SARS-CoV nsp3-7 region, represented as in B. The region encompassing the TM domains is shown using the same scale as used for EAV pp1a (amino acids 2110 – 3836 of SARS-CoV pp1a). SARS-CoV nsp3 has been truncated internally to include the protease corresponding to the nsp3/4 cleavage site. Kyte & Doolittle hydropathy plots were generated on web.expasy.org and TMHMM predictions using the Geneious software package both based on database sequences of EAV (NC_002532.2) and SARS-CoV (NC_004718.3).
polyproteins pp1a and pp1ab contain the viral nsps, which are released by multiple internal proteases (76, 77). Together they are often referred to as the replicase, as they either are part of or support the functions of the so-called replication and transcription complex (RTC), the aggregate enzymes that replicate the viral genome and produce the sg mRNAs used to express the structural proteins.

The ORF1b-encoded subunits include the main enzymatic activities required for RNA replication, such as the RNA-dependent RNA polymerase (RdRp) and the helicase (4, 75, 78-81). The pp1a subunits seem to support the viral RNA replication more indirectly. Their functions include modulation of host gene expression, proteolytic activity required for polyprotein maturation, and modification of host cell membranes to accommodate viral RNA synthesis.

When considering nidoviruses at large, a conserved array of replicase domains can be defined, consisting of the viral main protease (M\textsuperscript{pro}) flanked by two transmembrane (TM) domains, the ORF1a/1b ribosomal frameshift site, a nidovirus RdRp-associated nucleotidyltransferase (NiRAN), the RdRp, a zinc-binding domain (ZBD), a superfamily 1 helicase (HEL) and, only in vertebrate nidoviruses, an endoribonuclease (NendoU) (3, 78, 81, 82). In all arteri- and coronavirus replicases, a third TM domain (TM1) is present upstream of the hydrophobic regions that flank M\textsuperscript{pro} (TM2 and TM3; Fig. 1A). Although many other virus groups contain some or most of these elements, the array of (TM1-)TM2-M\textsuperscript{pro}-TM3-RFS-NiRAN-RdRp-ZBD-HEL is nidovirus specific.

Arteriviruses encode multiple papain-like proteases (PLPs) and a 3C-like or chymotrypsin-like protease that is their M\textsuperscript{pro} (reviewed in (4, 76, 79)). The number of active PLP domains varies per virus species (e.g. 2 for EAV, 3 for PRRSV and LDV, and 4 for SHFV) and these proteases are all located upstream of TM1 in pp1a/pp1ab, where each of them processes a single downstream cleavage site (83). In the case of EAV, the PLPs located in nsp1 and nsp2 (called PLP1β and PLP2) both swiftly process a single site in the polyprotein (the nsp1/nsp2 and nsp2/nsp3 junctions, respectively; Fig. 1B). M\textsuperscript{pro} is located in nsp4 and processes the remainder of pp1a/pp1ab (the nsp3-8/nsp3-12 region; Fig. 1B). The C-terminal half of pp1a encompassing nsp3-8 can be processed following two different pathways, each yielding specific end products. The pathway used for nsp3-8 processing depends on the presence or absence of cleaved nsp2 (84). When free nsp2 is present, the nsp4/nsp5 junction can be cleaved, which yields the nsp5-7 and nsp5-8 cleavage products that, apart from the slow processing of the nsp7/nsp8 junction, do not seem to undergo further processing. In the absence of nsp2, the nsp4/5 junction remains uncleaved, but instead the nsp5/6, nsp6/7 and possibly the nsp7α/7β sites are processed (77, 84-86). In infected cells, the nsp4/5 junction is mostly cleaved, resulting in the majority of nsp5 being present in nsp5-7 and nsp5-8 cleavage products; however, cleavage of sites in nsp5-7, even though occurring in a minority of cases, is critical for virus replication (86).

For the three TM regions in pp1a of arteriviruses, located in nsp2, nsp3 and nsp5, the exact membrane topology has not been established in full experimental detail, but all of them are predicted to span the membrane multiple times (87, 88). Furthermore, it is assumed that both termini of each of these nsps must face the cytosol, where the viral proteases that
must have access to the cleavage sites are present (Fig. 1B) (89). Nevertheless, PRRSV TM1 in nsp2 was suggested to span the membrane 5 times after performing in vitro translation of nsp2 alone in the presence of artificial membranes (90). Such a topology, however, would appear to pose a major problem for polyprotein processing since PLP2 and the nsp2/3 cleavage site would reside on opposite sides of the membrane. The transmembrane nature of EAV TM2 in nsp3 was confirmed by engineering a site for N-linked glycosylation in the first predicted luminal loop, which was indeed glycosylated when pp1a was expressed transiently (89). This result strongly supports the nsp3 topology model that places the nsp2/3 cleavage site at the cytoplasmic side of the membrane. Translocation of the nsp2 C-terminal domain following polyprotein cleavage would be compatible with all these observations; however, evidence supporting this hypothesis remains to be obtained.

When the TM1-TM2-Mpro-TM3 regions of the arterivirus and coronavirus pp1a polyproteins are compared (Fig. 1), their general organization is strikingly similar, despite the 2.5-fold overall pp1a size difference between the two families. In both virus groups, the junction between the nsps containing TM1 and TM2 is cleaved by a PLP located in the TM1-containing nsp (PLP2 in nsp2 for arteriviruses and PLpro in nsp3 for coronaviruses).
The other TM-containing nsps (nsp3 and nsp5 for arteriviruses and nsp4 and nsp6 for coronaviruses) are both released from the polyprotein by the Mpro situated between TM2 and TM3 (76). Moreover, the predicted tetraspanning membrane topology of TM2 is very similar, with two ER luminal domains of which the N-terminal one is the largest (89, 95). The fact that the general arrangement of hydrophobic domains in pp1a is conserved between arteriviruses and coronaviruses probably points to a common, and important, function in virus replication.

**Host membrane remodeling in arterivirus infection**

The non-structural proteins containing the predicted TM domains (Fig. 1B,C) appear to be the main players in the modification of host cell intracellular membranes (24, 28, 88, 96). These proteins, together with several other nsps like the RdRp-containing nsp9 and the helicase-containing nsp10, were shown by immunofluorescence and IEM to localize to the same membrane structures in the perinuclear region of EAV-infected cells (88, 97, 98). This suggested that arterivirus RNA synthesis is associated with these intracellular membranes. When a crude membrane fraction was isolated from EAV-infected cells, the viral RTC was
retained and RNA synthesis could be reconstituted in vitro, which further supported the concept of membrane-associated viral RNA synthesis (99). Interestingly, this in vitro RTC activity was lost after detergent treatment, strongly suggesting that the RTC’s functionality depends on the integrity of the membranes (99). Similar data has also been published for coronaviruses (94, 100-107).

The remarkable ability of arteriviruses to induce the formation of DMVs in the cytoplasm of infected cells was already observed by electron microscopy in the 1970’s (108, 109) and has been noticed for all family members analyzed by EM to date (88, 110-114). These DMVs, with a diameter of about 100 nm (Table 1), appear in the perinuclear region of the cell and then proliferate in number giving rise to cytoplasmic clusters that often also show an increased presence of free ribosomes (20, 109, 110).

A significant breakthrough in our understanding of the complex architecture and spatial arrangement of arteriviral replication organelles was brought about by applying electron tomography to EAV-infected Vero E6 cells (20) (Fig. 2A, Supplementary movie S1). More recently these 3D imaging studies were expanded to HuH-7 cells in our laboratory (for experimental details, see Chapter 3) showing that the original observations were not unique for a single cell line (Fig. 2). Occasionally, neck-like connections between the endoplasmic reticulum and the outer membrane of DMVs had been observed in 2D images (88). However, the 3D analysis showed that these narrow connections are actually rather common. This type of connections (Fig. 2B, white arrows) pointed directly to the ER as the most likely membrane donor organelle, a notion further supported by the fact that ribosomes decorating the outer membrane of the DMVs were regularly observed in the tomograms (Fig. 2C, black arrowheads). Although DMVs are the most prominent and abundant EAV-induced membrane structures, other modified membranes in the form of short stretches of paired membranes or small complex membrane arrangements connecting DMVs to one another or to the ER, were also apparent in the tomograms (Fig. 2D-F). The large majority of the DMVs seemed to be connected to the ER, modified membranes, other DMVs or a combination of the above (Fig. 2B,D-G, white arrows). It should be pointed out that not all of these associations appeared as clear membranous connections but, instead, as tight membrane-membrane interactions that were particularly frequent between DMVs (Fig. 2C,G, black arrows). Only about 10% of the DMVs in the tomograms appeared as separate, free-floating vesicles and this may even be an overestimation since narrow connections in the top or bottom regions of the DMVs cannot be detected due to the intrinsically lower resolution of the tomograms along the Z axis (47). Therefore, the general concept that emerges from the 3D analysis is that of a large reticulovesicular network (RVN), consisting of DMVs and modified ER.

As an additional complexity, a few hours after the appearance of the first DMVs, a rather different EAV-induced structure could be observed between the clusters of DMVs. ET revealed that it consists of a network of branching tubular structures, with an average diameter of approximately 43 nm, (Fig. 2A, right panel; in green; Fig. 2H-I) lacking the typical trilaminar profile of lipid bilayers in EM, and thus most probably representing a proteinaceous structure. Using IEM, these tubules were found to abundantly label for the
arteriviral N protein (20). Similar tubules had been detected before in EAV-infected BHK-21 cells, both upon infection (111) and upon transfection with EAV RNA replicons (115). The formation of these tubules seems to depend on expression of the arteriviral N protein as they were absent when the transfected viral RNA lacked the N protein gene. Nevertheless, additional viral components are likely required in the process as N protein expression by itself appeared to be insufficient to generate tubules (115).

Together with the previously reported observation that similar tubules extended into budding virus particles (111), the data suggests that these structures are involved in virus assembly, a process that is still largely uncharacterized for arteriviruses. The fact that this tubular network appears entwined with the RVN could reflect an efficient way of spatially linking the synthesis and packaging of genomic RNA.

Our detailed 3D characterization of the ultrastructure of the EAV-induced RVN revealed additional interesting details with intriguing functional implications. For example, in the DMV interior a previously unreported electron dense core could clearly be distinguished (Fig. 2A, blue; Fig. 2B-I, asterisks). The detection of this core depended on the use of high-pressure freezing (HPF) followed by freeze substitution (FS) for EM sample preparation (20). In chemically fixed samples, the conventional technique used in all previous studies on arterivirus-induced membrane modifications, the DMV interior appeared mainly electron translucent. Using plunge-freezing followed by FS, a granular content became apparent but a dense core was only visible in HPF-FS samples. HPF-FS is considered the gold standard for the ultrastructural preservation of resin-embedded specimens as it has several advantages over alternative methods (116). In our comparison of different protocols for fixation of EAV-infected cells, it was evident that HPF-FS offered superior preservation, not only of the RVN but also of the cytosolic content and various cellular organelles (20).

The average diameter of the DMV cores (~50 nm) appears relatively constant during the course of infection and their individual size seems directly proportional to the size of the surrounding DMV. Interestingly, in IEM samples, these cores labeled strongly for dsRNA, a presumed intermediate in viral RNA replication (Fig. 3A). Consistent with this observation, these structures were shown by 2D and 3D electron spectroscopic imaging (ESI) to have a high phosphorus content (Fig. 3B-D). ESI is an EM technique that takes advantage of the fact that electrons that are inelastically scattered by different elements in the sample lose different amounts of energy. Using this principle, and by collecting images using only electrons that have suffered specific energy losses, images that reveal the location and concentration of a certain element, known as elemental maps, can be obtained (117). In this manner, the average phosphorous content of the cores was determined to be equivalent to about a dozen copies of the EAV RNA genome (20).

In view of the presence of dsRNA inside DMVs, it is tempting to speculate that the DMV interior is the site of viral RNA synthesis as this could provide a propitious environment that would also hide dsRNA replication intermediates from the innate immune sensors of the host. In analogy, for the other morphotype of +RNA virus-induced membrane alterations, the interior of the invaginated spherules seems to be the site of viral RNA synthesis (27, 48, 58, 60, 118, 119). However, these invaginations have openings to the cytosol that permit
the necessary import of substrates and export of RNA products, whereas in the case of EAV DMVs the inner membrane defines a closed compartment, apparently sealed from the cytosol, thus creating a topological conundrum with respect to RNA synthesis.

The situation is further complicated by the fact that, according to immunolabeling experiments, key replicase subunits, like nsp3 and the RdRp-containing nsp9, localize to DMVs and surrounding RVN membranes but only rarely to the core of DMVs (20). At this point it is important to note that the presence of neither dsRNA nor nsps may be bona fide markers for the localization of the active site of viral RNA synthesis. For example, only a small part of the abundantly expressed nsps is likely to be involved in active RNA-synthesizing complexes, as previously calculated for HCV (120). Likewise, abundant labeling of DMV cores could reflect accumulation of dsRNA intermediates rather than active RNA synthesis. A less ambiguous alternative to pinpoint viral RNA synthesis is by metabolic labeling of nascent viral RNA followed by EM detection. This latter approach was followed in the past for EAV (88) by transfecting cells with Br-UTP, after which labeling with a Br-U-specific antibody revealed some signal associated with DMV membranes. However, the size of the DMVs, relatively small when considering the distance that separates the gold markers from

**Fig. 3. Analysis of the cores in EAV-induced DMVs.** (A) Immunogold labeling for dsRNA in EAV-infected Vero E6 cells (7 h p.i.). The gallery of DMVs illustrates the strong labeling for dsRNA of the DMV core. (B-D) Analysis of the phosphorous content in EAV-infected Vero E6 cells (8 h p.i.) by 3D electron spectroscopy imaging (ESI). A tilt series of elemental map images for P was obtained using the jump-ratio approach as described in (20) from a 75-nm thick section of an infected cell. (B) Tomographic slice (~5 nm thick) extracted from the final ESI reconstruction, showing the high P content (higher intensity) of the DMV cores (boxed). The smaller brighter structures between DMV cores correspond to free ribosomes, also rich in P, which are always particularly abundant in the areas containing DMVs. (C) Direct volume rendering of the ESI tomogram showing the P-content in 3D. (D) Close-up of one of the DMV cores (discontinuous box in B and C), which is highlighted in yellow in isosurface display mode. The resulting isosurface, generated at an intensity threshold level that revealed isolated ribosomes, suggests a thread-like structure of the core RNA content. Scale bars, 100 nm. Adapted from (20) with permission.
the original epitopes (up to ~30 nm), the limited sample preservation and the qualitative nature of the analysis precluded an accurate localization of the active RTC. Furthermore, signal in the cytosol was also observed, probably reflecting the fact that the labeling times may have been too long to prevent the migration of newly-synthesized RNA.

Comparison with coronavirus-induced membrane modifications
In the wider context of the order Nidovirales, coronaviruses constitute the only other family for which the putative replication organelles have been analyzed in some detail. Coronavirus-induced membrane alterations were first described in the 1960’s (121, 122) and have been further investigated in the past decade. In all cases examined so far, also coronaviruses were shown to induce the formation of DMVs. This includes members of three of the four genera of the coronavirus family: alphacoronaviruses (123, 124), betacoronaviruses (21, 106, 125-128), and gammacoronaviruses (22). Considering the pervasiveness of DMVs across the coronavirus and arterivirus families, it seems plausible that this is a shared trait that would extend to other nidoviral families not yet characterized. A striking difference between arterivirus and coronaviruses concerns their size: coronaviruses induce DMVs that are, on average, around twice the diameter of those in arterivirus-infected cells (Table 1, Fig. 4A), which corresponds to a ~8 fold difference in DMV volume.

The betacoronavirus SARS-CoV was the second +RNA virus for which the virus-induced membrane modifications were characterized in 3D by electron tomography (Fig. 4B) (21). This study uncovered DMVs that were connected through their outer membrane with other DMVs and the ER, together forming an RVN of modified ER membranes that is topologically comparable to that described for EAV in the previous section (20). The remarkable similarities between these two distantly-related nidoviruses suggested that the RVN architecture may be a universal feature among nidoviruses. This idea, however, has been challenged by a recent tomographic study of the structures formed upon infection of primary cells with the gammacoronavirus IBV (Fig. 4C) (22). Remarkably, in these tomographic studies, most of the IBV-induced DMVs were found to be isolated vesicles: no DMV-DMV connections were observed and only a small proportion of DMVs appeared to be connected to the ER. Additionally, and in contrast to the case of EAV- and SARS-CoV-induced DMVs, ribosomes were not found to be associated with the outer membrane of IBV-induced DMVs. Whether these features represent specific differences for gammacoronaviruses or also apply to some of the other nidovirus (sub)groups remains to be analyzed. In any case, the RVNs generated by SARS-CoV and EAV appear to be a unique example of highly-interconnected membrane modifications among the +RNA viruses that induce DMVs (Table 1). The overall DMV connectivity is decidedly more limited in the case of HCV, for which direct membranous connections between DMVs were not observed (23). In terms of connectivity, enterovirus-induced modifications appear to be at the opposite end of the spectrum relative to nidoviral RVNs, as all of them seem to be isolated compartments with no membranous connections between them or any cellular organelle (56, 65).

A common characteristic of coronavirus-infected cells, which is absent in cells infected with arteriviruses, is the formation of relatively disorganized assemblies of tangled, partially
double membranes. These structures, now mostly referred to as convoluted membranes (CM), were already described for mouse hepatitis virus (MHV) in 1965 as ‘reticular inclusions’ (122). Since then, they have been further characterized for several betacoronaviruses including MHV (127), SARS-CoV (21) and the recently emerged Middle East Respiratory Syndrome coronavirus (MERS-CoV) (128). DMV formation seems to precede CM formation, since the latter has been observed predominantly at later time points after infection (21, 127, 128). CM are found in close proximity to the DMVs, but only their 3D characterization in SARS-CoV-infected cells unambiguously showed the presence of neck-like membranous connections between the CM and surrounding DMVs and ER cisternae (21), which make the
CM also an integral part of the coronavirus-induced RVN. Notably, CM have been described so far only for cells infected with betacoronaviruses and could be a feature restricted to this genus. In the few ultrastructural studies of cells infected with alphacoronaviruses, CM were not reported (123, 124); however, these studies focused on virus morphogenesis and DMVs, respectively, and therefore it is unclear whether CM may have been present. For the gammacoronavirus IBV, a thorough search for this structure was reported to be unsuccessful (22).

Interestingly, in cells infected with the gammacoronavirus IBV, a distinct and novel membranous structure was described consisting of double-membrane spherules that were continuous with zippered ER cisternae and had a channel of 4.4 nm in diameter connecting them to the cytosol (Fig. 4C) (22). Although these spherules are delineated by two membranes instead of one, their size (~60 nm) and overall configuration with an opening to the cytosol was very reminiscent of the single-membrane invaginations induced by a large number of +RNA viruses (see section 1). Based on this similarity, the authors proposed that viral RNA synthesis would take place inside these spherules. This possibility, which requires further experimental corroboration, is particularly attractive in view of the closed configuration of the DMVs found in coronavirus infection. Both for IBV- and SARS-CoV-infected cells, the tomograms revealed DMVs in which the inner membrane seems to be fully sealed with no apparent openings to the cytosol. This makes it difficult to envision how viral RNA synthesis could take place (at least exclusively) inside these vesicles. This situation is completely analogous to the case of the EAV-induced DMVs discussed in the previous section and the parallels extend to the localization of different viral markers by IEM. Most of the dsRNA signal in SARS-CoV-infected cells was shown to map to the inner compartment of the DMVs, whereas the bulk of the labeling for several replicase proteins was located predominantly in the surrounding CM (21). The CM in MHV-infected cells also abundantly labeled for several nsps (127). The experiments using metabolic labeling of newly-synthesized viral RNA, which could be crucial to pinpoint the actual site of viral RNA synthesis, are restricted so far to MHV. IEM detection of newly-synthesized RNA using BrUTP as a probe revealed signal associated to the regions of DMV clusters, but the results are difficult to interpret due to the limited sample preservation that resulted in a clear loss of the DMV content and, possibly, also of the CM (125). In a recent study, using incorporation of ethynyl uridine into RNA and its detection through click-chemistry followed by light microscopy (129), a loss of colocalization between the ethynyl-uridine and the dsRNA signal was observed as infection progressed. The ethynyl uridine-containing foci, which early in infection overlapped with the dsRNA signal, became dispersed in the cytoplasm late in MHV infection. This prompted the idea that RNA synthesis could take place, at least during these later stages, in regions of the cell distant from those containing DMVs. The interpretation of these results at the ultrastructural level, however, is not possible in the absence of a parallel EM characterization.

Overall, the question of the actual site of viral RNA synthesis for arteriviruses and coronaviruses appears more enigmatic than ever and it is certainly a key issue to be addressed in the future. New studies that meet the challenge of achieving shorter labeling
times without critically reducing signal levels, and that combine improved ultrastructural preservation with quantitative analyses, will be crucial to accurately determine the location of the active RTC. Meanwhile, multiple scenarios remain possible, as originally discussed in (21). If viral RNA synthesis does take place exclusively inside DMVs, a yet unknown mechanism of material exchange with the cytosol should exist, perhaps in the form of a proteinaceous channel spanning the two membranes, similar to the TIM/TOM export complex in mitochondria (130). The visualization of such a channel may be possible using advanced techniques like cryo-EM and cryo-ET, which preserve molecular resolution (47). On the other hand, if such an exchange mechanism is not present, alternative locations for anchoring the RTC appear necessary. These locations could include the cytosolic face of the DMV outer membrane, but also other membrane structures induced by nidoviruses (e.g. the CM in betacoronaviruses, the open double-membrane spherules of the gammacoronavirus IBV, paired membranes in arteriviruses), and/or even other subcellular membrane structures. In this alternative scenario the DMVs may not even play a critical role in viral RNA synthesis at all, a possibility that may be supported by recent observations with temperature-sensitive MHV mutants. This study could not establish a direct correlation between the number of DMVs and MHV RNA synthesis (131), which would have been expected if DMVs would be the sole site of viral RNA synthesis. The fact that DMVs have been detected by EM a few hours later than the onset of viral RNA synthesis could also argue in the same direction; however, this could also simply be due to EM limitations. Incipient modifications, which would likely be rare, would be even less frequent in the cell sections prepared for EM and could easily go undetected.

The biogenesis of arterivirus and coronavirus DMVs

A crucial aspect for better understanding virus-induced replication organelles, and possibly for the design of antiviral strategies that target them, is to uncover the steps leading to their formation and, ultimately, to determine the exact nature of the molecular interactions of the viral and host players involved. Compared to DMVs, the +RNA virus-induced invaginations are much better understood in this respect. Mechanistically, their formation entails the induction of negative curvature (i.e. a concave membrane, bending away from the cytosol) on the host membrane-donor organelle to yield a vesicle budding into the luminal space. This negative curvature is compensated by positive curvature (i.e. convex membrane, bent towards the cytosol) at the membranous neck that keeps the vesicle attached to the organelle and connects its interior to the cytosol. Detailed studies on BMV replication organelles have greatly helped to establish a model system for the molecular players and mechanisms involved in molding the membranes to form this type of invaginations (63, 132, 133). While the BMV case illustrates the significant progress in understanding the mechanisms involved in producing virus-induced invaginations, our insight into the generation of viral DMVs lags well behind. This is in part due to the more complex architecture of a DMV compared to a single-membrane structure, which implies a more elaborate formation process that would encompass several membrane remodeling steps including fission events. In the context of nidovirus-induced DMV formation, two
alternatives have been proposed: enwrapping (also known as protrusion and detachment) and double budding (88) (Fig. 5).

In the enwrapping model (Fig. 5A) the first crucial step is the pairing of two lipids bilayers in the membrane donor organelle (likely the ER for nidoviruses). These paired membranes would then progressively curve to form a DMV that is still connected to the cytosol in a vase-like configuration. In this step, the outer and inner membranes acquire opposite curvature (positive and negative, respectively). As curvature increases, the opening would get narrower and, eventually, the DMV may be sealed by fission to give rise to an inner compartment containing cytosolic material and an outer lipid bilayer that is still continuous with the ER. The alternative mechanism, the double-budding model (Fig. 5B), would start with the budding of a single-membrane vesicle (SMV) into the lumen of the membrane donor organelle and, therefore, would involve the induction of negative curvature in the ER and
fission to release the SMV into the lumen. A second budding event, this time towards the cytosol, would make this vesicle emerge from the ER while acquiring its outer membrane. In this second step, the SMV and the ER would establish membrane-pairing interactions and positive curvature would be induced. This process would result in a DMV outer membrane that is still continuous with the ER, essentially identical to that formed in the enwrapping model. In both models, an additional fission event could lead to the pinching off of a free DMV.

Membrane pairing, fission and induction of both positive and negative membrane curvature are key features of both models, and in fact they only differ in the order in which these events occur. Membrane pairing could be mediated by protein-protein interactions across the lumen. For EAV, this could be potentially achieved by the predicted luminal domains of the transmembrane proteins nsp2 and nsp3, two non-structural proteins that have been shown to engage in a strong interaction (89, 134). Regarding curvature, several processes that can drive bending of cellular membranes have been described (reviewed in (135)). One of them is the generation of asymmetry in the bilayer by differential insertion of proteins or irregularly-shaped lipids, which act as wedges that deform the membrane. Self-oligomerization of transmembrane or soluble scaffold proteins can also result in the induction of membrane curvature. While we currently do not know how arteriviruses and coronaviruses accomplish membrane bending, it seems likely that the transmembrane viral nsps play a prominent role. Other factors could also be involved, for example, a change in lipid composition or homeostasis, as shown for other DMV-inducing viruses. Enteroviruses and HCV alter the lipid composition of the membranes recruited for the formation of replication organelles, which become enriched in phosphatidylinositol-4-phosphate (PIP4) by the specific recruitment of PI4P-kinases (PI4KIIIβ in enteroviruses, PI4KIIIα in HCV), which seems essential for viral replication (42, 44). Whether any of the PI4K variants play a role during arterivirus and coronavirus infection would be highly informative and is currently under investigation in our group. A recent siRNA library screen targeting the human kinome in fact identified some proteins involved in the metabolism of complex lipids as factors relevant for SARS-CoV replication. Interestingly, the PI4P-kinases, which appear essential for the replication of other DMV-inducing viruses, did not show an effect on SARS-CoV replication in this study (136).

A link with autophagy?
Double-membrane structures are not without precedent in the cell. Organelles delimited by a double membrane in eukaryotic cells include the nucleus, mitochondria and autophagosomes. Although much larger in size (0.5-1.5 µm in diameter), autophagosomes have a similar appearance as virus-induced DMVs. Autophagosome formation (reviewed in (137)) is a central process in the macroautophagy pathway (hereafter autophagy) (138) and involves the formation of a double-membrane sheet, known as the phagophore, which wraps and sequesters cytoplasmic material, sometimes even whole organelles or bacteria. Recently, it has become clear that the cargo for degradation in autophagosomes is selected specifically by autophagy receptors which bind to the selected cellular components and
recruit them to the growing autophagosomal membranes (139). Closure of the phagophore yields the autophagosome, which fuses with late endosomes or lysosomes after maturation to deliver its cargo for degradation and recycling of the engulfed macromolecular components.

Autophagy can be triggered by stress, starvation and infection. While autophagy can act as a cellular defense mechanism for virus clearance in response to infection, several viruses have evolved strategies to avoid or even subvert the autophagy pathway for their own benefit (140-142). The parallels between autophagosomes and virus-induced DMVs elicited the hypothesis of a link between autophagy and DMV biogenesis, postulating that DMVs may represent modified autophagosomes.

Infection with arteriviruses and coronaviruses can induce autophagy (143-148) but conflicting results on the relationship between autophagy and nidovirus replication have been published, possibly due in part to the differences in the cell lines used. Knock-down of Atg5, an essential protein in the autophagy pathway, was first reported to inhibit MHV replication and preclude the formation of DMVs in embryonic stem cells (143), but later these effects were not observed in bone marrow-derived macrophages and primary mouse embryonic fibroblasts (MEF) (149), or MEF cells lacking another key autophagy protein, Atg7 (150). For the avian coronavirus IBV, it was shown that viral replication is not affected by either inhibition or activation of autophagy in monkey kidney cells (Vero) (49, 144). Of note, IBV infection induced autophagy in mammalian cells but not in avian cell lines (49), which emphasizes the potential influence of the cell type on the study of the interplay between viral infection and autophagy and, possibly, the consequences of a virus-host mismatch. Likewise, the replication of the arterivirus EAV in Vero cells did not seem to be affected by either Atg7-dependent autophagy or the alternative Atg5/Atg7-independent autophagy pathway (148). However, for the arterivirus PRRSV, progeny titers were moderately reduced in another monkey kidney cell line (MARC-145) treated with the autophagy inhibitor 3-methyladenine or when autophagy genes were silenced, whereas activation of autophagy with rapamycin had the opposite effect (145-147). Whether these results are dependent on the cell line used or reflect a particularity of PRRSV remains to be investigated. Overall, the data gathered in the last decade seems to strongly suggest that autophagy, or at least the canonical autophagy pathway, is not essential for arterivirus and coronavirus replication and whether it plays a role in DMV biogenesis is therefore unclear. Such a role has not been established for other DMV-inducing viruses either. Whereas in the case of HCV conflicting reports make the influence of autophagy in viral replication rather unclear, for enteroviruses, autophagy appears to have only limited impact on replication. However, autophagy seems to be critically linked to virus assembly, maturation and the non-lytic release of new enterovirus particles (151-153).

Induction of autophagy is often monitored using the autophagy marker LC3 (microtubule-associated protein 1 light chain 3), a key player in autophagosome formation and recruitment of specific cargo for degradation. Upon exposure to autophagy stimuli, the cytosolic form (LC3-I) is converted into a lipidated form (LC3-II) by covalent binding to phosphatidylethanolamine, which is present in the autophagosomal membranes (138).
In light microscopy images, this LC3 relocation is reflected in the formation of puncta of fluorescently tagged LC3. Using this approach, the IBV nsp6 replicase protein (but not other IBV nsps) was shown to be capable of inducing autophagosome formation when individually expressed (49, 144). This effect was also described for nsp6 of MHV and SARS-CoV, as well as the arteriviral PRRSV nsp5-7 (144). The autophagosomes induced by these proteins appeared to be smaller in size than normal autophagosomes, as judged from light-microscopy images, and therefore closer to viral DMVs (154). However, in contrast with individually expressed nsp6, IBV infection did not induce LC3 relocalization and autophagic signaling in avian cells (49). Therefore the relevance of this nsp6 feature in the context of infection remains unclear.

One of the reasons to relate autophagy to nidovirus replication was the reported colocalization of LC3 with viral replicase proteins or dsRNA in light microscopy images of cells infected with MHV (143, 150), SARS-CoV (105, 149), PRRSV (147) and EAV (148), although some other studies with SARS-CoV and IBV could not confirm this finding (106, 144). Without accompanying EM data, extrapolating this colocalization, which appears to be partial in confocal images, to the specific ultrastructural location of LC3 is problematic, even more so in the case of coronaviruses considering the variety of membrane structures that they induce during infection (see section 2.3). Ultrastructural studies using IEM, which are limited so far in the case of EAV to chemically-fixed BHK-21 cells at late time points in infection (148), would be essential to establish whether LC3 is associated with virus-induced modifications and, specifically, with DMVs.

Interestingly, data obtained using EAV- and MHV-infected cells indicated that the non-lipidated form of LC3 (LC3-I), which is inactive in autophagosome formation, would be the one that is recruited from the cytosol and that partially colocalizes with viral nsps (148, 150). Silencing of LC3 was reported to reduce EAV and MHV replication and this effect could be reversed by transfecting a non-lipidable form of LC3 in MHV-infected cells (150). This was considered reminiscent of EDEMosomes, a class of short-lived small vesicles that appear to depend on LC3-I recruitment (155, 156). EDEMosomes have been proposed to serve as a mechanism to tune down the ER-associated protein degradation (ERAD) machinery, which targets misfolded proteins, by transporting some ERAD regulators, like EDEM1 (ER degradation-enhancing α-mannosidase-like protein 1) or OS-9 (osteosarcoma amplified 9), away from the ER for their disposal (155). Exploring these analogies further, EDEM1 and OS-9 were found to partially colocalize with dsRNA in MHV-infected cells, which was interpreted as recruitment of these proteins to DMVs (150). Similarly, dsRNA and EDEM-1 showed some degree of colocalization in EAV-infected cells (148). It was therefore proposed that the ERAD-tuning pathway would supply the membranes for the biogenesis of viral DMVs (150). The direct conversion of EDEMosomes into DMVs, however, seems difficult to envision, as it would entail the transformation of smooth single-membrane vesicles of about 150 nm in diameter (155) into DMVs that can reach 300 nm in diameter in the case of coronaviruses and whose outer membranes are decorated with ribosomes. The recruitment to virus-induced modifications of some components of the ERAD machinery during arterivirus and coronavirus infection is, nevertheless, plausible, but its functional
significance may well be completely unrelated to DMV biogenesis and needs to be further investigated using in-depth EM analysis and molecular biology approaches.

CONCLUSIONS AND FUTURE PERSPECTIVES

The replication organelles induced by +RNA viruses are examples of the remarkable level of sophistication in host membrane manipulation that viruses can achieve. Their complex architecture, revealed by electron tomography, poses additional challenges, for example concerning DMV structure-function interpretation. In this respect, arteriviruses and coronaviruses represent perhaps the most intriguing case (Table 1). The RVNs that EAV and SARS-CoV induce are intricate labyrinths of modified membranes that have no parallel in the +RNA virus world thus far. Additionally, and in contrast with other +RNA viruses that induce mixed populations of open and closed DMVs, the closed configuration of nidovirus-induced DMVs defies the straightforward concept of viral RNA synthesis occurring inside these compartments, and puts at the forefront the open question of the location of the viral enzyme complexes actively engaged in replication/transcription. Until this question is unambiguously answered experimentally, multiple possibilities remain open, from a scenario in which RNA synthesis would take place solely inside DMVs while a still undetected macromolecular channel would accomplish the necessary import/export processes, to a situation in which the active nidoviral RTC would be located in alternative sites and the DMVs would not even play any direct role in viral RNA synthesis. Along these lines, the possibility of DMVs simply being a by-product of the high expression levels of the viral non-structural proteins cannot be completely discarded. However, even if the DMVs are not critically involved in viral RNA synthesis, less passive roles seem also plausible. These RNA-containing structures could perhaps be used to shield an excess of viral RNA from the cellular innate immune sensors and/or be the result of the subversion of cellular pathways for the benefit of virus replication. Clearly, additional experimental data are required to provide key information on the precise role of nidovirus-induced DMVs, which may well extend to other +RNA viruses.

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