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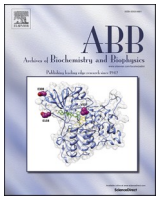

The cell edit: Looking at and beyond non-structural proteins to understand membrane rearrangement in coronaviruses

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-stranded RNA virus that sits at the centre of the recent global pandemic. As a member of the *coronaviridae* family of viruses, it shares features such as a very large genome (>30 kb) that is replicated in a purpose-built replication organelle. Biogenesis of the replication organelle requires significant and concerted rearrangement of the endoplasmic reticulum membrane, a job that is carried out by a group of integral membrane non-structural proteins (NSP3, 4 and 6) expressed by the virus along with a host of viral replication enzymes and other factors that support transcription and replication. The primary sites for RNA replication within the replication organelle are double membrane vesicles (DMVs). The small size of DMVs requires generation of high membrane curvature, as well as stabilization of a double-membrane arrangement, but the mechanisms that underlie DMV formation remain elusive. In this review, we discuss recent breakthroughs in our understanding of the molecular basis for membrane rearrangements by coronaviruses. We incorporate established models of NSP3-4 protein-protein interactions to drive double membrane formation, and recent data highlighting the roles of lipid composition and host factor proteins (e.g. reticulons) that influence membrane curvature, to propose a revised model for DMV formation in SARS-CoV-2.

1. Introduction

In recent years, the term "Coronavirus" has been used ubiquitously following the 2019 pandemic outbreak. Specifically, it refers to a positive-stranded (+) RNA virus family belonging to the order *Nidovirales* that can infect mammalian species, including humans [1]. Perhaps the most well-known coronavirus types that can cause mild to severe pathologies include *s*evere *a*cute *r*espiratory *s*yndrome-associated *c*oronavirus (SARS-CoV), *m*iddle *e*ast *r*espiratory *s*yndrome-associated coronavirus (MERS-CoV), and SARS-CoV-2. The latter put the world on hold in March 2020, and quickly reached pandemic levels [2]. Although human coronaviruses were identified almost 60 years ago and have been heavily studied over the past four years, fundamental aspects of their function still remain elusive including their mechanisms of viral replication. Given the high potential for future viral infections, in part due to the ability of coronaviruses (and many other viruses) to cross the species-barrier via zoonosis [3], enhancing our understanding of viral replication mechanisms on a molecular scale is essential for successful development of new treatment options.

1.1. The coronavirus replication cycle and its replication organelle

Coronavirus genomes can range in size from around 26 to 32 kb [4], making it up to three times larger than that of other RNA viruses. Replication of this genome is carried out by a large number of RNA synthesising and processing enzymes and coronaviruses have devised a strategy in which they utilise host cell membranes and machinery to support their replication cycle (Fig. 1). Viral entry into the host cell is facilitated by the interaction of the viral spike protein (S) and the host cell angiotensin-converting enzyme 2 (ACE2) cell surface receptor [5]. This interaction has formed the primary target of coronavirus vaccine development [6]. The viral genome is then released into the cytoplasm where it undergoes translation, producing two large polyproteins (pp1A, pp1AB) that are cleaved into a set of 16 non-structural proteins (NSPs) by virally-encoded proteinases [7]. These NSPs promote substantial remodelling of endoplasmic reticulum (ER) membranes to create a site for viral replication, specifically RNA synthesis, in the perinuclear region [8,9]. Creation of this site, called the replication organelle, is a highly conserved feature among coronaviruses, and similar replication

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structures have been reported for other viruses, including, Hepatitis C Virus (HCV), Poliovirus, and other *Picornaviridae* [10–12]. This strict conservation stems from the advantages the replication organelle affords the virus: it localises and concentrates replication machinery, host factors (host proteins and lipids), and viral species (RNA and proteins) in a microenvironment optimised for RNA synthesis and protects RNA intermediates from activating an immune response in the host.

The replication organelle is composed of three distinct but interconnected membrane compartments of varying size and curvature [13]: convoluted membrane (CM), double membrane spherules (DMS), and double membrane vesicles (DMVs). Convoluted membranes, analogous to zippered-ER, are attached to the ER and thought to provide the membrane lipids required for DMS formation [14]. These DMS structures have an average diameter of ~80 nm [15] and their function remains unknown. Interestingly, these structures do not appear to be uniformly distributed among coronavirus types, as they were reportedly lacking in murine hepatitis virus (MHV) infected cells [16]. Additionally, it has been shown that DMSs are not associated with coronaviral RNA synthesis [14]. DMVs contain the viral replication and transcription complex (RTC) and are widely accepted as the primary site of viral RNA synthesis [14,17,18]. Following replication in the DMV interior, genomic RNA and translated structural and accessory proteins are exported via a recently discovered molecular pore [19]. Together, they are assembled into virions at the ER-Golgi intermediate compartment (ERGIC) and released via exocytosis.

2. Structure and composition of DMVs

As mentioned above, the primary sites associated with viral RNA synthesis are the DMVs [20,21], composed of an outer and inner membrane, separated by a luminal space of approximately 18 nm (Fig. 2) [22]. The overall size of DMVs and the time at which they appear post-infection is influenced by the type of virus under investigation as well as other non-viral factors [16,18,22–26]. For example, in the early stages of hepatitis C infection, DMVs of 50–250 nm in diameter appear as protrusions connected to the ER membrane (Fig. 2A) [27]. Conversely, it is only in the later stages of poliovirus infection that DMVs of 100–300 nm in diameter appear, emerging from single-walled tubular structures of the replication organelle (Fig. 2B) [28]. In SARS-CoV infected cells, DMVs of diameters between 100 and 300 nm have been observed in the cytoplasm as early as 2 h post-infection in Vero E6 cells (Fig. 2C) [22], with increased concentration and clustering of DMVs appearing as the infection progresses (Fig. 2D). However, smaller DMVs with sizes ranging from 60 to 80 nm have also been observed in HeLa cells (Fig. 2E) [26]. Coronaviral DMVs are unique in that they are thought to be interconnected via their outer membranes [20], forming clusters of DMVs that are linked to ER. These variations highlight that all DMVs (and replication organelles) are not identical, and the DMV morphologies induced by different (+) RNA viruses are summarised by Zhang and co-workers in an excellent recent review [24].

Another difference between DMVs is the mechanism via which newly synthesised viral RNA and proteins are exported from the interior of the DMV. Unlike DMVs formed upon infection by hepatitis C virus, which

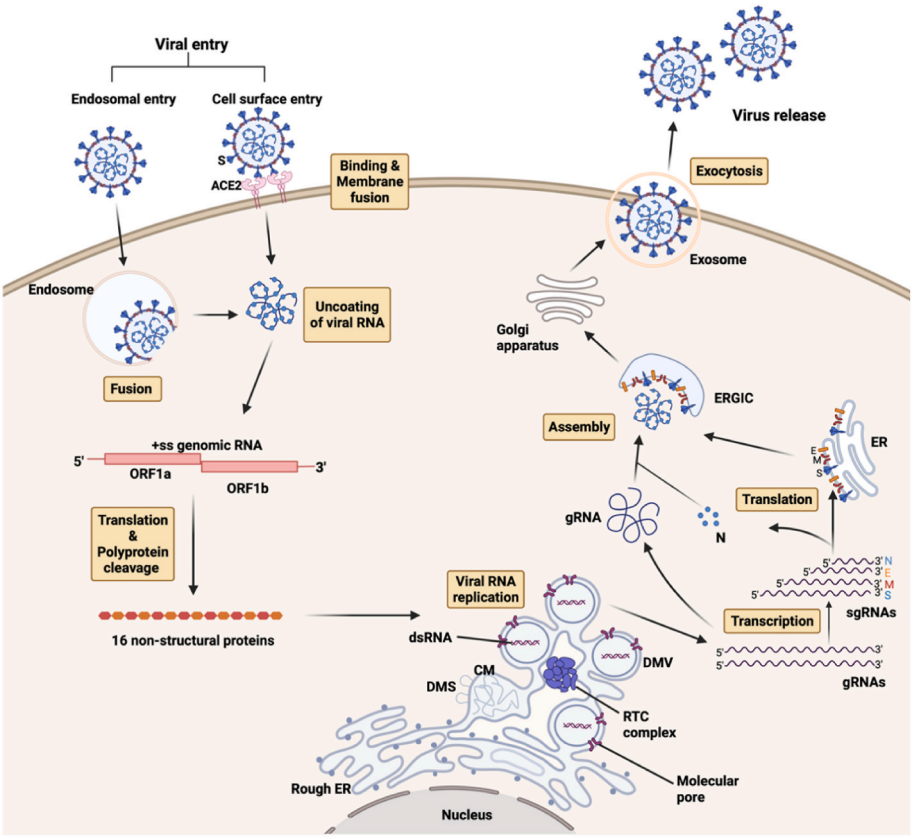

Fig. 1. Coronavirus replication cycle on the example for SARS-CoV-2. Viral entry via interaction between the spike protein (S) and the ACE cells surface receptor. Once in the cell, the viral RNA is released and translated into open reading frame (ORF) 1a and 1b. The resulting polyproteins are translationally cleaved into 16 non-structural proteins (NSPs), which compose the viral replication and transcription complex (RTC). The RTC encompasses various membranous structures; convoluted membranes (CM), double membrane spherules (DMS) and double membrane vesicles (DMV) The RNA replication occurs within DMVs, which are ER-derived structures induced by the virus. Genomic RNA (gRNA) is synthesised and transcribed into a set of subgenomic RNAs (sgRNAs). In turn, these are translated into structural and accessory proteins. Together with the new viral RNA, virions are assembled at the ER-Golgi intermediate compartment (ERGIC) and subsequently release from the cell. Created with BioRender.com.

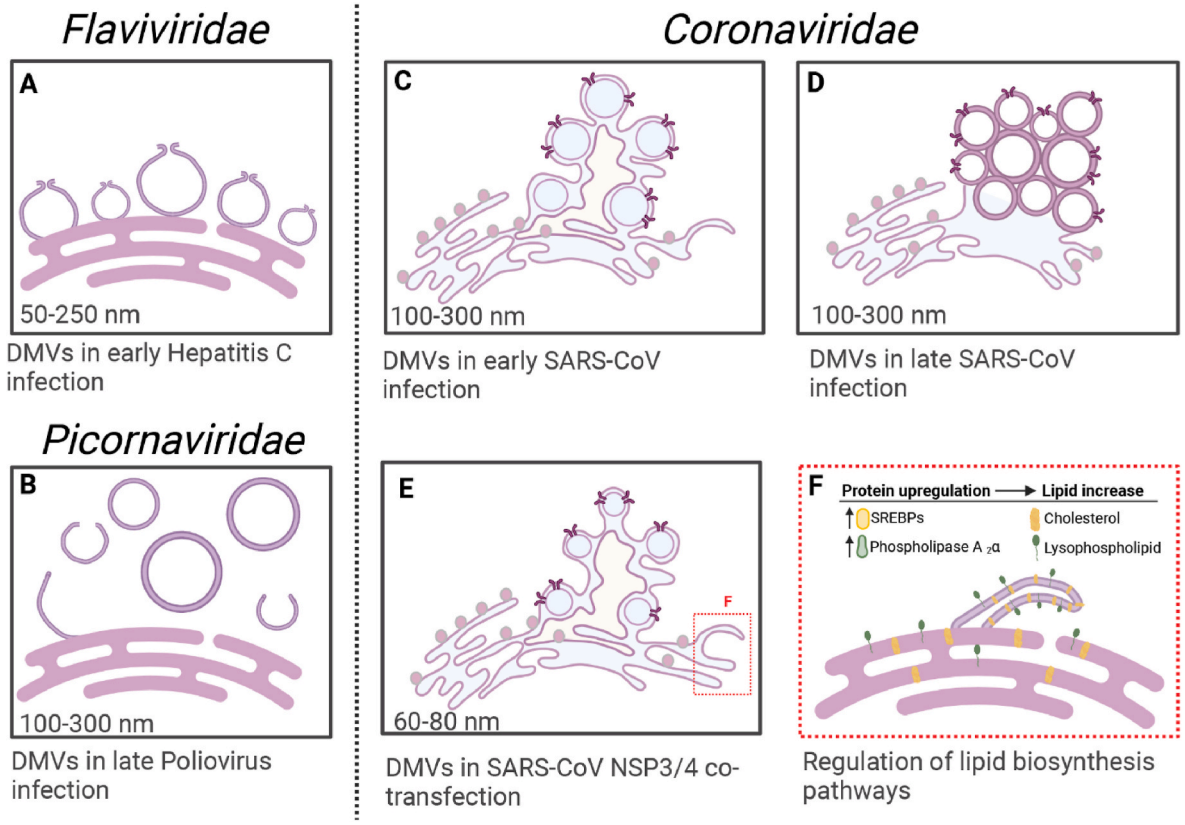

Fig. 2. Virus-induced double membrane vesicle structures as primary sites of RNA synthesis. A DMVs with openings into the cytosol during early Hepatitis C infection in size range of 50–250 nm. B DMVs induced in late stages of Poliovirus infection from single walled membrane structures. Sizes range from 100 to 300 nm. C Early stage coronavirus induced DMVs with size range of 100–300 nm interconnected to the ER. D Late stage development of DMV vesicle packets. E DMVs induced by co-transfection of NSP3 and NSP4 *in vitro*, yielding 60–80 nm DMVs. F Coronavirus modulated lipid biosynthesis pathways during infection to facilitate induction of positive membrane curvature. Created with BioRender.com.

contain openings between the cytoplasm and the DMV interior (Fig. 2A), no such opening was observed in tomography data of SARS-CoV or MERS-CoV-induced DMVs [20]. In 2020, Wolff and co-workers discovered a molecular pore which spans the coronaviral DMV double-membrane, thus connecting the interior to the cytosol (Fig. 2C) [19]. These researchers proposed that the newly synthesised coronaviral RNA is exported into the cytosol via this crown-shaped pore to facilitate further processing. A more in-depth examination of the pore revealed that an average of 11 pores was present in DMVs induced by NSP3-NSP4 co-transfection [29]. On a structural level, the pores were clearly discernible in the DMV membrane, whereby the "crown" spans across 25 nm, providing a cytosolic exit platform to the underlying 2–3 nm channel connected to the DMV interior.

The small size of DMVs requires bending of the ER membrane, the primary donor organelle in the host, to generate positive membrane curvature. Given the intrinsic link between lipid composition and membrane morphology, this implies that DMV formation depends, at least in part, on the lipid composition of the donor membrane. In order to impact the lipid composition, coronaviruses employ a number of approaches targeting lipid biosynthesis and lipid processing pathways (Fig. 2F). One approach is the activation of cholesterol and fatty acid biosynthesis via a group of transcription factors called sterol regulator element-binding protein (or SREBPs). It was shown that inhibition of SREBPs, and subsequent down-regulation of cholesterol and fatty acid production, lead to inhibition of viral replication and DMV formation upon MERS-CoV infection [30]. Another approach employed by coronaviruses to affect lipid composition is the upregulation of cytosolic phospholipase $A_2\alpha$, an enzyme that catalyses the hydrolysis of membrane phospholipids to produce lysophospholipids [31,32]. In this way, coronaviruses appear to be increasing the concentration of cholesterol and lysophospholipids, both of which have been shown to exert a large influence on membrane curvature. Lysophospholipids have long been known induce positive membrane curvature, with the degree of curvature depending on the chain length and identity of the head group (i.e. PC-lysolipids induce higher curvatures than PE-lysolipids) [33]. Cholesterol has also been shown to facilitate membrane bending and fusion events [34,35] and is known to spontaneously induce negative curvature [36]. This may seem counterintuitive, however, a recent study has shown that cholesterol-rich regions can coincide with positive membrane curvature in an asymmetric membrane, where cholesterol may be present in a higher concentration in a single leaflet [35].

Alterations in the lipid composition to establish formation of the replication organelle are also supported by the observation of larger DMV sizes associated with the use of infectious virus (SARS-CoV-2), while smaller sizes were reported as a result of co-transfection with coronavirus NSPs 3 and 4 [37]. This suggests that other factors modulate DMV size, or indeed that cell type could have a pronounced effect. While similar DMV size distributions were shown across SARS-CoV-2 infected A549-ACE2, Vero E6, and Calu3 cell lines [22], varying sizes were reported in MHV-A59 infected L929 and DBT cell lines [38]. Variations in the experimental designs make a direct comparison difficult, however, lipidomic analysis has outlined differences in cellular lipid profiles [39], which in turn can be a modulator of membrane re-arrangements [40,41]. For example, it has been shown that charged lipids, which are also encountered in cells, influence the bending rigidity of a membrane [42]. Therefore, native variations between cell lines could be a contributing factor to the observed size divergence of coronavirus-induced DMVs, which, as a concept, warrants further investigation.

3. DMV biogenesis

3.1. Current models of DMV formation and organisation

The generation of DMVs in the cell upon coronavirus infection is a subject of great interest, as elimination of these structures represents a powerful approach in which to target progression of infection. In early seminal work by Knoops and co-workers [20], electron tomography images revealed DMVs connected to the rough ER as part of a continuous reticulovesicular membrane network in SARS-CoV infected cells. This network of modified membranes contained an abundance of DMVs linked either directly to the ER or linked via the CM, indicating that DMVs were not "free floating" vesicles as initially assumed (Fig. 3A). Images also showed that DMVs were linked to one another via their outer membranes, with 95 % of the DMVs containing at least one inter-DMV connection and over half of the DMVs showing multiple connections. This ER-linked "web" of CM and DMVs also contained the viral replicase subunits and dsRNA. Data from this study were used to create a temporal model of the biogenesis of the replication organelle, with early viral proteins triggering ER membrane modifications that link the ER to interconnected CM and DMVs, and ultimately to what was referred to as "vesicle packets" and membrane compartments used for virus assembly and budding [20].

In parallel to this model of DMV biogenesis is a second model involving the hijack of ER-associated degradation (ERAD) tuning vesicles, or EDEMosomes, by coronaviruses for use in viral replication [43]. EDEMosomes form part of the ERAD pathway, a quality control system that ensures only correctly-folded proteins leave the ER [44], and they are enriched in the ERAD regulator proteins EDEM1 and OS-9 in the lumen of the vesicle (Fig. 3B) [45]. In a study of the coronavirus mouse hepatitis virus (MHV), Reggiori and co-workers found that the virus induced formation of DMVs carrying LC3-I [45]. It was also shown that the other EDEMosome markers EDEM1 and OS-9 colocalised to vesicles containing viral RNA and other NSPs [43], indicating that MHV captures EDEMosomes for use in viral replication. Although no direct interactions between EDEM1, OS-9 or LC3-1 and viral proteins have been reported, it has been shown that knockdown of LC3 inhibited coronaviral replication by causing a defect in DMV biogenesis [45].

The two models discussed above describe mechanisms for DMV biogenesis that are supported by a wealth of data and could be occurring in parallel in the same cell, but neither model describes the molecular basis for large-scale membrane rearrangements. From a biophysical perspective, DMV biogenesis involves ER membrane proliferation and expansion, membrane pairing, and positive curvature induction [25,

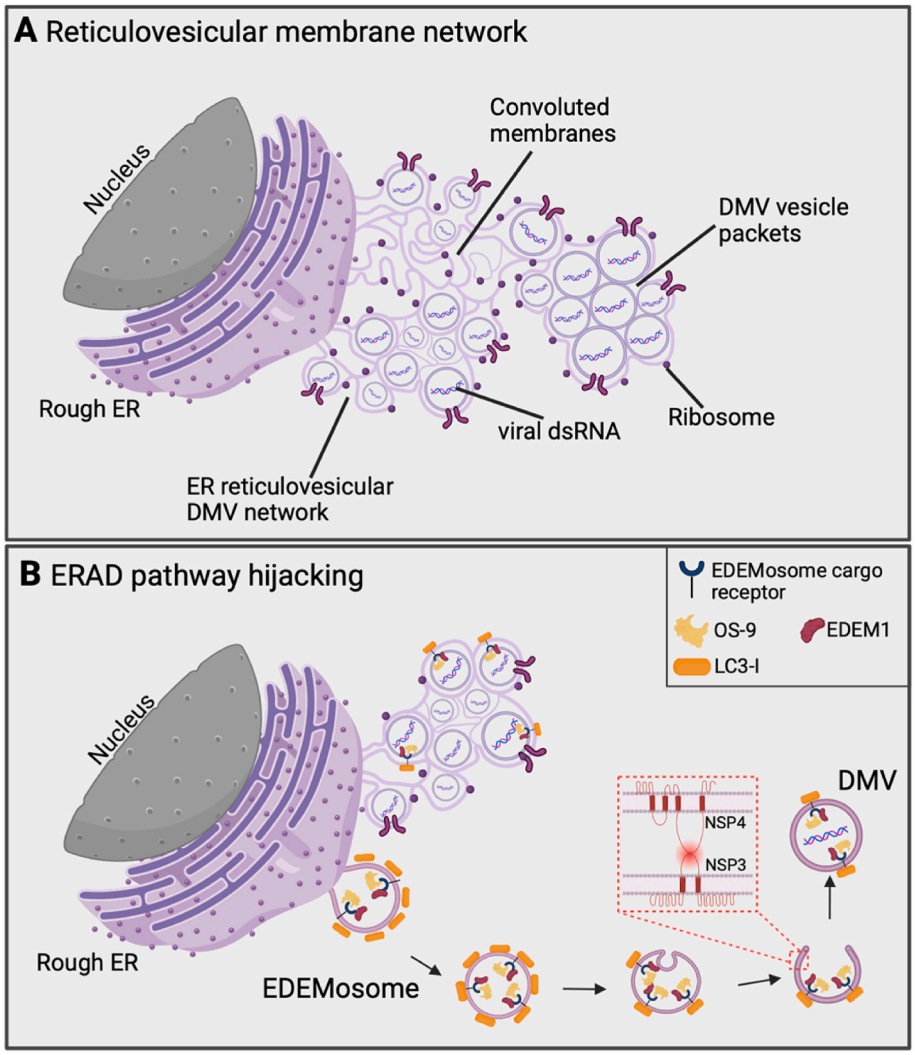

Fig. 3. Models of DMV biogenesis. A Formation of a reticulovesicular membrane network, including ER-interconnected DMVs, convoluted membranes, and vesicle packets observed in later stages of infection. B ERAD pathway hijacking by coronavirus, utilising EDEMosomes containing ERAD regulator proteins OS-9 and EDEM1in the vesicle lumen. Cytoplasmic autophagosome marker LC3-I has been associated with DMVs. Non-structural proteins NSP3/4 can be found in the vesicle membrane. Created with BioRender.com.

46]. Given that DMVs have been reported as early as 2 h post infection [20], biogenesis occurs at a remarkably fast rate. The first model describing how coronaviral proteins orchestrate formation of the small, highly curved DMVs was proposed by Hagemeijer and co-workers in 2014 [8], and remains the most widely accepted model to date. However, before describing this model, the relevant NSPs produced by coronavirus must be described in more detail.

3.2. Viral non-structural proteins required for DMV formation

Translation of the viral genome yields two large polyproteins that are cleaved into a set of 16 separate NSPs by virally-encoded proteinases [7], but only three of these proteins have been attributed to induction of DMVs during coronavirus replication – NSP3, NSP4, and NSP6. While these proteins have been established as key players for more than a decade, their molecular structures remain unknown to date and their functional mechanisms remains elusive [8,12,24,47,48]. However, they set themselves apart from the remaining NSPs by being the only transmembrane proteins in the set.

NSP3 is the largest multidomain protein [25,46], which localises to the ER [20] and is predicted to possess two transmembrane domains with an N-endo/C-endo topology (Fig. 4A) [49]. The nearly 1400 amino acid residue N-terminus of the protein contains a variety of sub-domains, whose functionality has been attributed to RNA binding as well as interference with the host cell immune response [48]. Recently, the N-terminal Ubl1/2 domains have also been suggested to play a role in the generation of membrane curvature [29]. Overall, NSP3 is classed as a scaffolding protein that is capable of initiating membrane rearrangements inside the host cell, as well as interacting with other viral proteins such as the N protein [50,51] or NSP12 [52] and host cell ubiquitin ligase RCHY1 [53]. Combined, this highlights the key role that NSP3 plays in coronavirus DMV formation, which has been extensively corroborated [8,13,29,48].

NSP4 is predicted to be a tetra-spanning transmembrane protein (TM), which also localises to the ER in a reticular pattern when expressed singly [8] with its N- and C-termini on the cytoplasmic side of the bilayer (Fig. 4B) [47]. To date, the primary function attributed to NSP4 is its involvement in DMV formation as part of the coronaviral replication and transcription complex, though it has been suggested that the N-terminal TM domain is involved in cleavable signalling via a PLpro domain [47]. Additionally, it has been observed that NSP4 causes structural changes in mitochondria that lead to dysfunction and the release of mitochondrial DNA [54]. Like NSP3, NSP4 is a limiting protein for coronaviral DMV formation [25,55,56]. In the absence of NSP4, membrane rearrangement via NSP3 is initiated, however no double membrane or closed structures are formed [25], thus speaks to its importance.

NSP6 is the third transmembrane NSP and, like NSP3 and NSP4, it localises to the ER [17]. While the protein structure is not yet solved, cytosolic processing of the N-/C-termini by NSP5 implies an even number of TM domains [57]. Based on experimental observation and bioinformatic predictions, the proposed NSP6 topology comprises six TM domains, with an N-endo/C-endo orientation (Fig. 4C) [58]. Over the years, there have been conflicting reports regarding the requirement of NSP6 to form DMVs, but recent work has attributed an ER zippering and DMV organisational capacity to NSP6 [26]. Although the protein is not a main driver of DMV formation, it exhibits membrane interaction and modulation capabilities. Indeed, it has been suggested that the NSP6-induced ER constriction observed in DMV connectors is mediated by the formation of a NSP6 homodimer via an N-terminal 157-residue interface [26]. This action appears to be stabilised by a C-terminal amphipathic helix (APH), which aligns with previously reported functions [59]. While NSP6 is not limiting for DMV formation, there is evidence to suggest that NSP6 has a contributing effect on DMV size, as the frequency of smaller diameter DMVs increased significantly compared to DMVs formed in the absence of NSP6 [26].

3.3. NSP3-NSP4 interaction and a model for double membrane formation

The co-expression of NSP3 and NSP4 has been shown to be vital for the formation of DMVs during replication [26,37]. As outlined above, singular expression of NSP3 or NSP4 results in reticular staining patterns that correlate with the ER. However, if the proteins are co-expressed, a re-distribution into perinuclear foci is reported, whereby NSP3 and NSP4 co-localise [8]. There are several studies that subsequently investigated direct interactions between NSP3 and NSP4 further, aiming to identify the contributing interface on each protein. In NSP3, the luminal loop domain was shown to be critical for an interaction to occur [8]; however, no additional investigation has been done since to narrow down the exact location within the 70 amino acid stretch. In contrast, the interaction domain located in NSP4 has received much more attention. While initially the large luminal domain located between the first two TM domains was implicated in the interaction with NSP3 [8], it was later proposed that specifically residues H120 and F121 were responsible for establishing an interaction with the loop in NSP3 [55]. Interestingly, the phenylalanine in position 121 was fully conserved between coronavirus types, thus supporting its importance. Yet, recent results from Alphafold2 modelling suggest that the interaction site is located further downstream in the NSP4 loop at residues 224–228 of the protein [60]. Together, these studies provide the basis for an intriguing concept, in which substitution of F121 could lead to changes in the proteins' tertiary structure that impede interaction with NSP3. A similar observation has previously been made in a cystic fibrosis transmembrane conductance regulator, where the deletion of a key phenylalanine residue destabilised the protein structure, resulting in a lack of dimerization ability [61].

In 2014, Hagemeijer and co-workers proposed the first model describing the molecular details of double membrane formation and membrane deformation by viral NSPs [8], both of which would facilitate DMV biogenesis. In this study, NSP4 and a truncated version of NSP3 containing the C-terminal region and both TM domains ($NSP3_C$) from both MHV and SARS-CoV were shown to colocalise to highly curved ER membranes. Based on their experimental observation that the luminal domains of NSP3 and NSP4 form an interaction, they proposed the

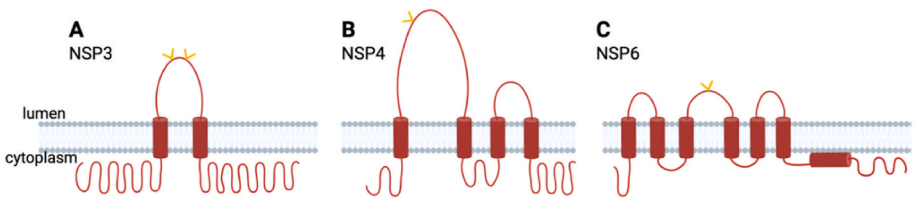

Fig. 4. Illustration of predicted topologies of NSP3, NSP4, and NSP6. A NSP3 consists of two transmembrane domains and a luminal loop with two N-glycosylation sites (yellow arrowhead) in SARS-CoV. B NSP4 consists of four transmembrane domains and two luminal loop domains, whereby the first is the largest. In SARS-CoV, the large loop contains one N-glycosylation site (yellow arrowhead). C NSP6 consists of 6 TM domains and a C-terminal APH. The second luminal domains contains one O-glycosylation site (yellow arrowhead) in SARS-CoV-2. All three proteins have an N-endo/C-endo topology. NSP3-NSP4 interaction is mediated via the luminal domains while NSP6 has a more organisational capacity. Created with BioRender.com.

following: the two proteins are inserted into the ER membrane (Fig. 5A) where they induce membrane rearrangements that start with ER tubule elongation (Fig. 5B), followed by membrane "zippering" to form the double membrane via interactions between the luminal loops of NSP3/4 proteins on opposite ER membranes (Fig. 5C) [8].

While this NSP-based model provides a solid molecular basis for understanding double membrane formation and stabilization, there is little in this model to explain the ability of NSPs to direct membrane rearrangements such as the generation of high curvature. A well-known mechanism for membrane remodelling by integral membrane proteins (IMPs) is the "wedging and scaffolding" mechanism used by reticulons [62–65], involving the interaction of multiple copies of a wedge-shaped IMP to generate a curved region of the bilayer. This scaffolding mechanism is often employed alongside the insertion of an amphipathic helical (APH) region, thought to act as a membrane wedge that can both create curvature as well as stabilise lipid packing defects in highly curved membranes [66–68].

Similar to IMPs, NSP3 has been described as a scaffolding protein [48] and the papain-like protease (PLpro) and deubiquitinating (DU) domains in the N-terminus reportedly self-associate [69]. Sole expression of NSP3 *in vitro* lead to the formation of disordered membrane regions as well as vesiculation [25], which indicates membrane altering and curvature inducing capabilities. Interestingly, NSP3 has been proposed to contain a membrane-proximal amphipathic helix [49]. However, deletion of this region (along with TM2) did not abolish co-localization of NSP3 with NSP4 into discrete perinuclear foci [8], suggesting this region of is not critical for NSP3-NSP4 interaction. Yet, the effect of this deletion on the ability to form DMVs remains to be investigated.

NSP4 does not appear to carry a membrane-proximal APH [8], but the soluble C-terminal domain has been reported to form a disulphide-linked dimer [70]. However, deletion of the entire C-terminus along with as well as TM4 did not impact co-localization with NSP3 in discrete perinuclear foci [8]. Moreover, in single NSP4 expression experiments *in vitro,* no phenotypic changes in membrane architecture were observed [25].

Taken together, these results suggest that NSP3-NSP4 interaction is mediated via their luminal domains, and that NSP3 possesses some membrane deformation potential that remains elusive. Therefore, to better understand these virally induced membrane rearrangements, we must also look to host cell factors.

4. Role of host cell factors

4.1. Reticulons 3 and 4

When considering ER membrane remodelling and curvature generation by viruses, an obvious family of proteins to consider are the reticulons. Reticulons are a family of native ER membrane proteins, whose primary function is reshaping of the ER into tubules [71]. This action is mediated by its characteristic "W" shape (reticulon homology domain, RHD), and a wedging and scaffolding mechanism (Fig. 6A) that results in the induction of positive membrane curvature [59,68]. It is this feature that, in the past, led to an investigation of reticulon involvement in the context of virus-induced ER replicative structures [72]. There, it was observed that the reticulon homology proteins RTN1p or Yop1 were crucial in the formation of double membrane structures and spherules in brome mosaic virus.

Over a decade later, the contribution of host cell factors to the formation of viral replication organelles still remains poorly understood. However, the concept of the viruses utilising host cell constituents to form replicative structures from host membranes led to the consideration of reticulons once more. A recent investigation highlighted the importance of mammalian reticulons (RTN) 3 and 4 in the formation of SARS-CoV-2 induced DMVs via interaction with NSP3 and NSP4 [73].

By utilising a small interfering RNA (siRNA) knock down approach, it was demonstrated that RTN3 and RTN4 are important factors in DMV biogenesis. More specifically, the membrane embedded RHDs of the proteins were implicated in direct interactions with NSP3 and NSP4 individually. The knock down of either RTN3 or RTN4 lead to a dramatic decrease or even the complete absence of DMVs in cells, yet reportedly did not interfere with early genome replication [73]. This raises the question of whether there is a DMV-independent genome replication pathway in coronaviruses, a concept that has been previously proposed,

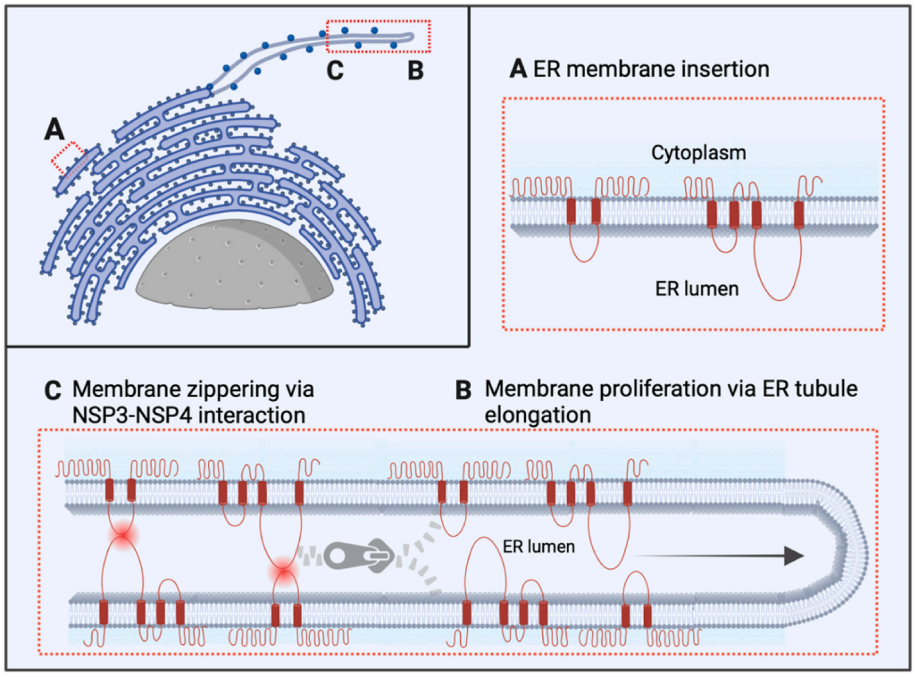

Fig. 5. NSP3-NSP4 interaction model proposed by Hagemeijer and colleagues. Schematic shows roughs ER tubule elongation mediated by NSP3/4 proteins. A NSP3/4 ER membrane insertion. B ER membrane expansion and tubule elongation driven by NSP proteins. C NSP3-NSP4 interaction via their luminal domains leads to membrane zippering and the formation of a double membrane. Created with BioRender.com.

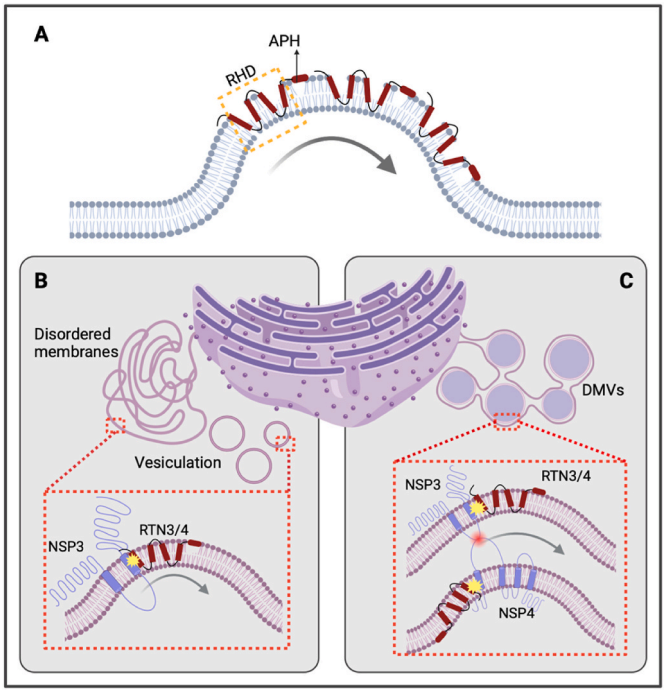

Fig. 6. Reticulon mediated curvature induction. A characteristic wedging of the reticulon homology domain (RHD; highlighted in yellow) inside the membrane alongside insertion of the C-terminal amphipathic helix (APH) followed by scaffolding to induce membrane curvature. B Membrane rearrangements induced by NSP3, mediated by binding of RTN3/4. C DMV formation induced by expression of NSP3 and NSP4 bound to RTN3/4. Created with BioRender.com.

involving lipid droplets (LDs) [74,75] and convoluted membranes (CMs) [20]. Interestingly, the effects of knocking down either RTN3 or RTN4 could be rescued by overexpression of the other [73], which suggested that the RHD present in both proteins, and not any unique feature of their soluble domains, was the key requirement for DMV formation. That being said, Williams and co-workers did reveal some functional selectivity since the RHD domain of FAM134B, an ER-phagy receptor found in ER sheets, did not promote formation of SARS-CoV 2 DMVs [73].

Consequently, the question to be asked is: "What does the interaction between RTN3/4 and NSP3/4 look like in a three-dimensional space?". Recent *in situ* electron tomography data has shown that DMVs induced by NSP3-NSP4 co-expression frequently remained interconnected to ER cisternae decorated by ribosomes [29]. This is in agreement with previous by work by Knoops and co-workers [20] and highlights ER cisternae as primary sites of DMV biogenesis. Given the flattened 3D structure of the cisternae, the induction of curvature driven by reticulons would explain the observation of vesicular structures as a result. Conversely, reticulons are often describes as being primarily associated with ER tubules and the edges of ER cisternae [65], however, imaging data has shown that RTN3 is ubiquitously expressed in the ER [13]. Consequently, the interaction between RTN3/4 and NSP3/4 in ER cisternae regions is conceivable. Since the RHD is embedded in the lipid bilayer, it is likely an interaction that occurs via interaction of the TM domains [76], and it has been proposed that the inherent function of RTN3/4 when bound to NSP3/4 is to drive formation of membrane curvature during DMV biogenesis [73]. While this evidence-based theory may provide an important new piece of information for our understanding of DMV formation, it raises additional questions.

If endogenous RTN3/4 provide the driving force for membrane curvature generation via binding to NSP3 and NSP4, this would explain experimental observations reported for both NSP3-NSP4 complexes [8,26,73] and NSP3 *in vitro* [25,77]. In singular NSP3 expression experiments, areas of disordered membrane and vesiculation were reported in the ER region [25]. Prior to the consideration of reticulons, it was believed that NSP3 itself induced these changes. However, with the newly revealed involvement of reticulons, it is highly likely that the previously observed membrane rearrangements were instead driven by endogenous RTN3/4 bound to NSP3 (Fig. 6B) [73]. Likewise, is has been shown that expression of both NSP3 and NSP4 lead to the formation of fully formed DMVs [26]. While NSP3-4 complex formation results in the formation of a double membrane, the previously unexplained induction of curvature in order to form a DMV can now likely be attributed to RTN3/4 bound to NSP3/4 (Fig. 6C), thus reconciling DMV formation from a mechanistic perspective. Conversely, involvement of the RTNs cannot explain results involving the sole expression of NSP4, which did not yield an altered phenotypic appearance of the ER membrane when examined by electron microscopy [25], despite the interaction between RTN3/4 and NSP4 being established [73]. Thus, this stands in direct contrast with the proposed role of RTN3/4 in DMV biogenesis.

Protein-mediated curvature generation is a complex process that is intrinsically linked to the structure and physical characteristics of the proteins involved. One such characteristic is the intrinsic protein rigidity required to overcome the native state of the membrane (i.e. planar) and induce high curvature [78]. Considering the structural and topological differences between NSP3 and NSP4 (Fig. 4A and B), it is reasonable to assume their rigidity would differ. The first luminal "loop" domain in NSP4 is 247 AA residues in length (UniProt KB ID: P0DTD1), making it over three times longer than that found in NSP3, and likely imparting an increased intrinsic flexibility to NSP4 compared to NSP3. This increased flexibility for NSP4 could counteract the induction of curvature when bound to RTN3/4, potentially reconciling the previously observed absence of ER membrane rearrangement *in vitro*. The differences in physical properties in NSP3 combined with the formation of a double membrane via NSP3-NSP4 interaction may impose additional physical constraints, which balance out NSP4 flexibility and thus facilitate the formation of DMVs.

4.2. *TMEM41B and VMP1*

Transmembrane protein 41B (TMEM41B) and vacuole membrane protein 1 (VMP1) are native ER membrane proteins that have been implicated in the formation of autophagosomes via complex formation [79,80]. More recently, they have been associated with calcium-independent phospholipid scramblase activity, whereby they modulate intracellular regulation of phosphatidylserine (PS) and cholesterol across the lipid bilayer membrane [81,82]. It has previously been established that cholesterol plays an important role in coronavirus infection, with functions involving viral entry [83] and the formation of DMVs [84]. Cellular cholesterol is in part synthesised by the aforementioned SREBPs [30], and thus it comes as no surprise that there is a connection between SREBP-mediated biosynthesis and TMEM41B-regulated intracellular distribution of cholesterol [85].

Recently, it has been shown that TMEM41B and VMP1 are key factors in DMV formation during coronavirus infection, including SARS-CoV-2 [37]. A CRISPR/Cas9-mediated knockout (KO) of VMP1 or TMEM41B dramatically decreased the number of observed DMVs as confirmed by transmission electron microscopy images. However, KO of VMP1 did not abolish NSP3/4 co-localization in perinuclear foci, and indeed paired ER membranes could still be observed, leading to the suggestion that VMP1 interferes with DMV formation, but not with initial NSP3-4 interaction and the associated membrane pairing. Interestingly, VMP1 established an interaction with both NSP3 and NSP4 independently, and it has been suggested that the VMP1 luminal domain forms the binding interface with the NSP proteins (Fig. 7B) [37]. This was based on the observation that its deletion resulted in failure to capture NSP3 or NSP4 during immunoprecipitation. From this, we can derive an intriguing premise, in which VMP1 may compete with NSP3/4

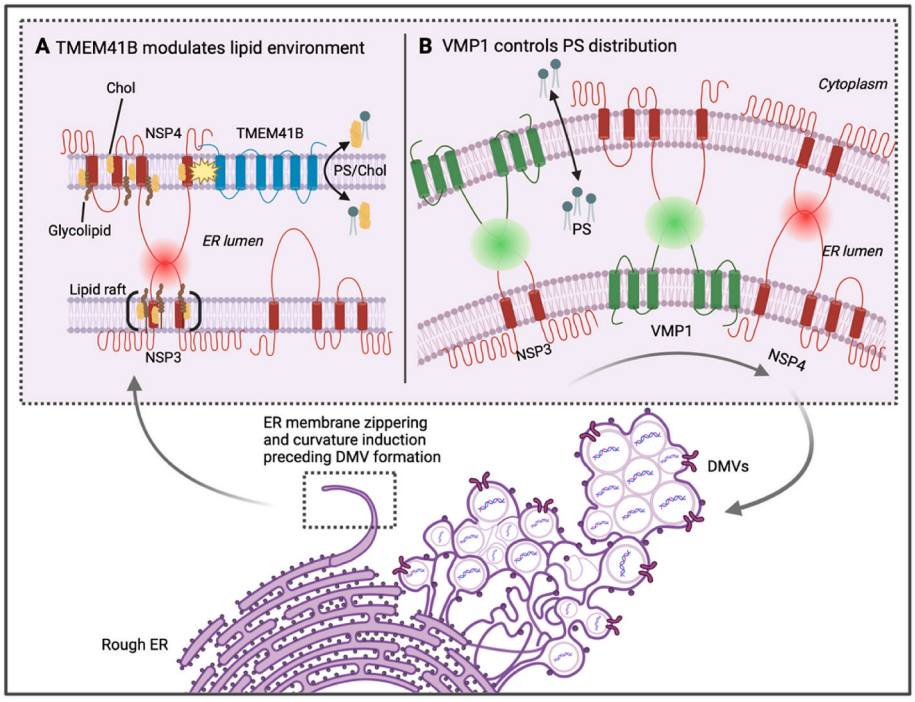

Fig. 7. Role of TMEM41B and VMP1 in coronavirus mediated membrane rearrangement during replication. A Modulation of local PS/Cholesterol levels by TMEM41B to promote NSP3-NSP4 interaction. TMEM41B establishes a weak interaction with NSP4, but no interaction with NSP3. B Control of PS distribution across the bilayer membrane to facilitate membrane rearrangement, leading to DMV formation. VMP1 establishes an interaction with both NSP3 and NSP4 via its luminal domain. Created with BioRender.com.

for binding. Given the transmembrane nature of the proteins, it is likely that this luminal domain interaction would occur on opposite membranes, similar to NSP3 and NSP4. While it could be argued that theoretically such an interaction could also lead to double membrane formation, no such observations have been reported in earlier studies [8,25], where NSP3 and NSP4 were investigated individually with EM, and endogenous VMP1 would have been present. Naturally, this leads to the question of what this interaction looks like, and additional work is required to elucidate this.

In contrast to the above observations, KO of TMEM41B reduced the number of perinuclear NSP3/4 foci and a lack of paired membranes was reported, indicating that the NSP3-4 interaction was at least partially disrupted by the absence of TMEM41B. In line with this, co-immunoprecipitation experiments in TMEM41B KO cells showed impaired NSP3-4 binding. However, upon closer investigation of the binding of TMEM41B to NSP3/4, Ji and co-workers showed that the protein does not bind to NSP3 and only weakly to NSP4 (Fig. 7A) [37]. Therefore, TMEM41B likely interferes with NSP3-4 complex formation in a secondary manner, such as lipid dysregulation [82]. It is well established that lipid environments play a major role in protein structure and function [86,87], thus disruption of the cholesterol/PS equilibrium in TMEM41B KO cells could have led to structural changes in NSP3 and or NSP4, preventing interaction. Secondary to this, cholesterol is known to be involved in the formation of lipid rafts [88], which have previously been implicated in coronaviral infection [89]. Though normally associated with endosomal fusion [90] during virus entry, lipid rafts also facilitate protein-protein interactions [91] and are crucial in the formation of various types of vesicles [92]. Given the role of TMEM41B in the regulation of cholesterol and the observation that its depletion interferes with NSP3-4 binding, it could be conceptualised that TMEM41B additionally facilitates the formation of lipid raft microdomains that promote NSP3-4 interaction (Fig. 7A).

Given that both TMEM41B and VMP1 are scramblases that regulate cellular cholesterol and PS levels [82], it follows that similar effects should be seen in cells where either protein was depleted. While some similarities in their functionality have been reported in the context of autophagy, where a defect seen in TMEM41B KO can be rescued by VMP1 overexpression [79], many of the molecular functions of these proteins remain unknown [81], particularly in the context of viral infection. Interestingly, the accumulation of PS in the cytoplasm, caused by the absence of TMEM41B or VMP1 [82], which resulted in the observed DMV defects, could be partially rescued by targeted interference with cellular PS biosynthesis in VMP1 KO cells [37]. This is in agreement with previous reports, whereby high levels of PS in a membrane significantly increased its bending rigidity [42]. Consequently, the energy required to overcome this and to deform a membrane into a DMV, for instance, becomes much greater, potentially rendering the existing NSP3/4 complex insufficient. Taken together, it emphasizes the importance of intracellular phospholipid regulation during DMV formation and attributes a lipid organisational role to VMP1 and TMEM41B in the context of coronavirus infection.

Based on this, the following can be proposed (Fig. 7): TMEM41B creates an optimal lipid environment to ensure correct folding of protein domains and establish lipid rafts to facilitate NSP3-NSP4 interaction (Fig. 7A). Meanwhile, VMP1 modulates ER membrane properties via regulating the distribution of PS to achieve favourable conditions for curvature induction, mediated by NSP3/4 (Fig. 7B). Lastly, the observed binding between NSP3/4 and VMP1/TMEM41B secures their recruitment to the sites of DMV formation, for the local exertion of scramblase activity and facilitation of membrane pairing and rearrangement.

5. Extending the model of coronavirus DMV formation

The processes surrounding membrane rearrangements and the formation of coronaviral DMVs are incredibly complex, involving several viral proteins as well as host cell factors, and until recently, the involvement of host cell factors was not very well understood. However, the recent work by Williams and co-workers [73] on the role of reticulons, as well as the work by Ji and co-workers [37] on TMEM41B and VMP1 discussed here has greatly advanced our understanding of DMV

formation and highlighted the importance to look beyond viral factors to understand the underlying mechanisms.

In light of this, we propose a hypothetical model that extends the prevailing model for DMV formation and accounts for both long-established and new findings (Fig. 8). Preceding any membrane altering events, viral NSP3 and NSP4 are integrated into the ER membrane following polyprotein cleavage [8], joining the endogenous reticulons 3 and 4 [62] (Fig. 8A). In anticipation of the subsequent membrane proliferation, the virus manipulates the ER lipid composition by activating cholesterol biosynthesis via SREBPs [30] and upregulating cytosolic phospholipase $A_2\alpha$, which regulates the conversion of phospholipids to lysophospholipids [32]. Both cholesterol and lysophospholipids are modulators of membrane properties to facilitate curvature generation [33,35] and have previously been established as important during coronavirus infection [24,31]. Consequently, their increased presence allows the next step in the DMV formation model: ER membrane proliferation (Fig. 8B). This process is driven by the interaction of RTN3/4 with the NSPs [73], whereby the established NSP3-RTN3/4 complex is likely the main driver, given the higher intrinsic flexibility of NSP4 due to its large luminal domain. Simultaneously, ER resident TMEM41B is tethered to the local site of DMV formation via weak transmembrane interaction with NSP4 [37] (Fig. 8C), where it exerts scramblase activity to regulate the distribution of PS and cholesterol across the bilayer membrane [82]. The resulting microenvironment may cause formation of cholesterol-rich lipid rafts [88] that incorporate the previously established protein complexes and support NSP3-4 interaction in the ER lumen to achieve membrane "zippering" [8]. In the next step, VMP1 is sequestered via competitive interaction with NSP3 and NSP4 in the ER lumen (Fig. 8D). There, VMP1 locally modulates PS levels to optimise the properties of the ER membrane for subsequent curvature induction [82]. The curvature is induced by NSP3/4-RTN3/4 complexes, whereby the inherent flexibility of NSP4 is balanced out by the physical constraints of the double membrane as well as the presence of the lipid raft microdomain, in which the high cholesterol levels reduce membrane fluidity [90], placing NSP4 in a more constrained environment. Consequently, the bending energy exerted by NSP3/4-RTN3/4 is sufficient to overcome the native membrane rigidity, leading to curvature induction and the formation of DMVs.

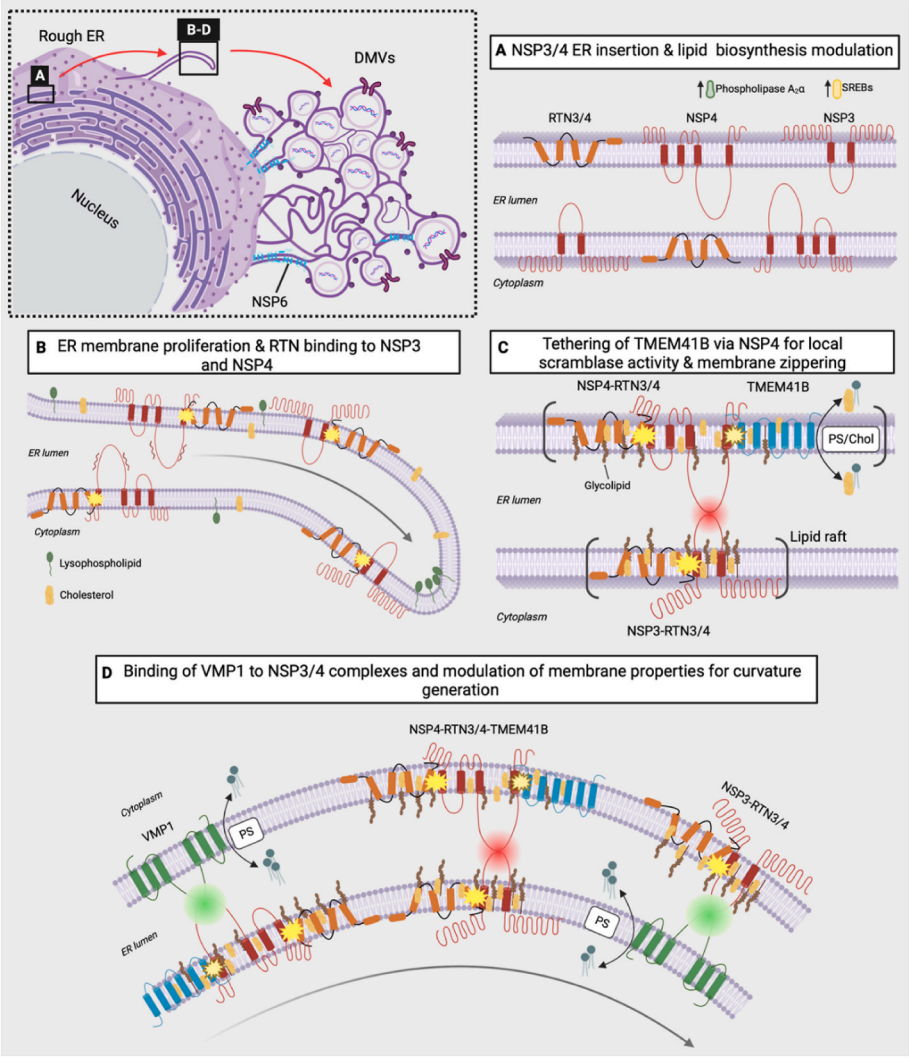

Fig. 8. Extended model of coronavirus-mediated membrane rearrangement and DMV formation involving essential host cell factors. Schematic illustrating steps required for DMV formation. A Insertion of NSP3 and NSP4 into the ER membrane where RTN3/4 are already present. Virus-induced modulation of host lipid biosynthesis pathways via upregulation of SREBs and Phospholipase A2α. B Upregulation of Cholesterol and lysophospholipids to facilitate ER membrane proliferation. This is mediated by the binding of RTN3/4 homology domains particularly to NSP3, as NSP4 possesses an inherent flexibility that interferes with active membrane deformation. C Tethering of TMEM41B scramblase protein via weak NSP4 binding for local lipid raft formation to facilitate NSP3-4 interaction and membrane "zippering". D VMP1 is sequestered to sites of DMV formation via binding to NSP3 and NSP4 via luminal interface. Modulation of ER membrane properties via PS distribution to induce membrane curvature leading to DMV formation. DMV organisation and connector establishment via NSP6. Created with BioRender.com.

While this model reconciles the observations from a wide range of studies, forming a more comprehensive view of how DMVs are formed, additional work is required to validate it experimentally.

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CRediT authorship contribution statement

Lea Denker: Conceptualization, Formal analysis, Investigation, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ann M. Dixon:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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