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Cell Insight 1 (2022) 100031

Contents lists available at ScienceDirect

Cell Insight

journal homepage: www.journals.elsevier.com/cell-insight

Review

Endomembrane remodeling in SARS-CoV-2 infection

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ARTICLE INFO

Keywords:
Coronavirus
SARS-CoV-2
Endocytosis
DMV
Autophagy

ABSTRACT

During severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, the viral proteins intimately interact with host factors to remodel the endomembrane system at various steps of the viral lifecycle. The entry of SARS-CoV-2 can be mediated by endocytosis-mediated internalization. Virus-containing endosomes then fuse with lysosomes, in which the viral S protein is cleaved to trigger membrane fusion. Double-membrane vesicles generated from the ER serve as platforms for viral replication and transcription. Virions are assembled at the ER–Golgi intermediate compartment and released through the secretory pathway and/or lysosome-mediated exocytosis. In this review, we will focus on how SARS-CoV-2 viral proteins collaborate with host factors to remodel the endomembrane system for viral entry, replication, assembly and egress. We will also describe how viral proteins hijack the host cell surveillance system—the autophagic degradation pathway—to evade destruction and benefit virus production. Finally, potential antiviral therapies targeting the host cell endomembrane system will be discussed.

1. Introduction

Coronaviruses (CoVs), belonging to the family *Coronaviridae*, are a group of spherical, enveloped, positive-sense, single-stranded RNA virus. The recently emerged highly pathogenic human coronaviruses, including SARS-CoV-2, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), belong to the *betacoronavirus* genera (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020; Woo et al., 2012). Coronaviruses consist of a ~30 kb genome and 4 structural proteins, spike (S), envelope (E), membrane (M) and nucleocapsid (N). In addition to the structural proteins, the coronavirus genomic RNA also encodes over six accessory proteins, known as ORFs, and two polyproteins, PP1a and PP1ab, which are further cleaved into 15–16 non-structural proteins (NSPs) (Cui et al., 2019). The NSPs and ORFs form the viral replication and transcription complex (RTC) for viral RNA synthesis and processing, and also modulate various host cell processes to facilitate viral replication and egress (Astuti and Ysrafil, 2020; Chen et al., 2020).

The endosomal system consists of a series of interconnected membrane compartments, mediating cargo recognition, sorting, trafficking and lysosomal degradation (Soldati and Schliwa, 2006). During endocytosis, cell-surface proteins, lipids and/or other biomacromolecules are internalized at the plasma membrane (PM) into vesicles which then fuse with early endosomes. The internalized cargos are ultimately delivered to lysosomes for degradation or retrieved by endosomal protein sorting/-trafficking complexes, such as retromer, for recycling (Naslavsky and Caplan, 2018). The coronaviral proteins intersect extensively with the endosomal network at various steps of infection (Fig. 1). The binding of the surface-anchored viral glycoprotein S with cell-membrane receptors (e.g. angiotensin converting enzyme 2 (ACE2) for SARS-CoV and SARS-CoV-2) facilitates the entry of coronaviruses into host cells via direct membrane fusion or endocytosis-mediated uptake (Bayati et al., 2021; Hoffmann et al., 2020b; Li et al., 2003). The virus-containing endosomes fuse with lysosomes, resulting in cleavage of S proteins by cathepsin proteases and subsequent viral membrane fusion to release the viral genome into the cytosol (Fig. 1). The receptor is recycled back to the PM for further rounds of infection. Double-membrane vesicles (DMVs), which are generated from the ER, act as platforms for anchoring the RTCs for viral replication (Fig. 1). Virions are assembled at the ER or the ER–Golgi intermediate compartment (ERGIC) and are thought to use smooth-wall secretory vesicles for release (Fig. 1) (Knoops et al., 2008; Reggiori et al., 2010; Snijder et al., 2020). Recent studies of SARS-CoV-2 infection, which induces extensive ER remodeling, Golgi fragmentation and mitochondrial network reshaping (Cortese et al., 2020; Scherer et al., 2022), have greatly

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https://doi.org/10.1016/j.cellin.2022.100031
Received 14 April 2022; Received in revised form 9 May 2022; Accepted 9 May 2022
Available online 17 May 2022

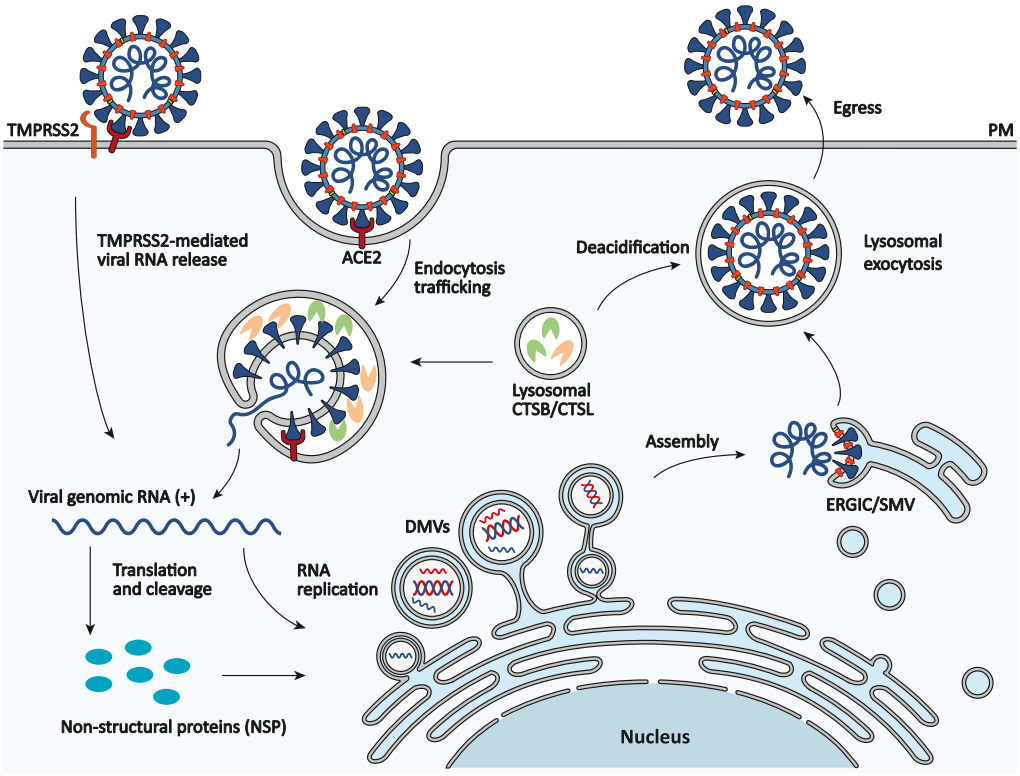

Fig. 1. The SARS-CoV-2 infection cycle

SARS-CoV-2 virions attach to the host cell through the interaction between viral spike (S) protein and the plasma membrane receptor ACE2. The cell-surface protease TMPRSS2 mediates the cleavage of S protein, which further triggers fusion of the viral envelope with the plasma membrane and subsequent entry of viral genomic RNA. SARS-CoV-2 can also enter into the host cell by the endocytosis pathway, in which S protein is cleaved by lysosomal cysteine proteases (cathepsins; CTSB and CTSL). The viral positive-strand (+) RNA genome is translated to polyproteins PP1a and PP1ab, which are further processed into 16 non-structural proteins (NSP1-16). NSPs participate in the formation of viral transcription replication complexes (RTCs) in DMVs to facilitate viral RNA synthesis and processing. The DMVs serve as a platform for RNA synthesis and the storage of negative-stranded templates/double-stranded viral RNAs. Nascent viral RNA is transported to the ERGIC or single-membrane vesicles (SMVs) for assembly. Viral particles egress through the secretory pathway and/or the lysosomal exocytosis pathway.

advanced our understanding of the mechanism and function of endomembrane remodeling in coronavirus infection. For example, double-membrane-spanning pores in DMVs transport the viral RNA products from the interior of DMVs to the cytosol for translation or viral assembly (Wolff et al., 2020). The secretory pathway and/or the late endosome/lysosome exocytic pathway are involved in the release of coronaviruses such as SARS-CoV-2 and MHV (Chen et al., 2021; Ghosh et al., 2020). SARS-CoV-2 also employs a novel mechanism to modulate the autophagic machinery in order to evade destruction (Miao et al., 2021). Genome-wide CRISPR/Cas9 screens identified numerous host factors that are involved in endomembrane remodeling in the infection cycle of SARS-CoV-2 (Daniloski et al., 2021; Hoffmann et al., 2021a; Morita et al., 2018; Schneider et al., 2021; Wang et al., 2021). In this review, we will summarize the most recent progress in understanding the involvement of endomembrane remodeling in the entry, replication, assembly and egress of SARS-CoV-2. Finally, we will offer insights into the possibility of targeting the endomembrane system as a therapeutic strategy.

2. Endocytosis-mediated entry of SARS-CoV-2

Entry of SARS-CoV-2 is triggered by interaction of the viral S protein with its receptor ACE2 on the host cell surface. ACE2 is expressed in multiple tissues such as the respiratory tract and the cardiovascular and renal systems (Hou et al., 2020). It was previously identified as a cell surface-localized transmembrane carboxypeptidase which acts in the renin-angiotensin system, an important hormonal axis for blood pressure maintenance and cardiovascular functions (Crackower et al., 2002). SARS-CoV or SARS-CoV-2 infection requires a suitable cell-surface level of ACE2 but not its catalytic activities (Gordon et al., 2020; Li et al., 2005; Schneider et al., 2021; Wang et al., 2008; Zhang et al., 2021d). Newly synthesized ACE2 is N-glycosylated in the ER and then transported to the *cis* Golgi apparatus, where it is further modified, packaged and translocated to the PM via the vesicular trafficking route (Hirschberg et al., 1998; Lumangtad and Bell, 2020; Vincent et al., 2005). S glycoprotein forms homotrimers on the virus surface. Each monomer consists of an S1 subunit (extracellular subunit) and an S2 subunit (transmembrane subunit) (Walls et al., 2020; Watanabe et al., 2020; Wrapp et al., 2020). During its biosynthesis, the S protein is cleaved in the Golgi apparatus by the proprotein convertase Furin at the S1–S2 boundary, where the polybasic cleavage site (PRRAR) is located (Hoffmann et al., 2020a; Shang et al., 2020; Walls et al., 2020). The S1 subunit remains non-covalently associated with S2. Furin-like cleavage sites are present in the entry glycoproteins of other human viruses, such as HIV, HCoV-OC43 and MERS-CoV (Binley et al., 2002; Le Coupanec et al., 2015; Millet and Whittaker, 2014). The Furin target site (PRRAR) is absent from the SARS-CoV S protein, even though it displays ~76% amino acid identity and shares a similar entry process with SARS-CoV-2 (Hartenian et al., 2020; Hoffmann et al., 2020a; Zhang et al., 2021a). The Furin cleavage site is an important determinant of the increased transmissibility of SARS-CoV-2 compared to SARS-CoV (Shang et al., 2020). At the initial step of SARS-CoV-2 infection, the receptor-binding domain (RBD) of the S1 subunit specifically binds to ACE2, resulting in stable association of the virion with the PM (Hartenian et al., 2020). The interaction induces conformational changes and subsequent exposure of an internal site in the S2 subunit (S2′ cleavage site), which can be cleaved by cell surface-localized transmembrane protease serine 2 (TMPRSS2) (Hoffmann et al., 2020a; Tang et al., 2021). In cells expressing TMPRSS2, such as human respiratory epithelium, S2' cleavage usually occurs at the host cell surface. This process subsequently induces S1/ACE2 shedding and dramatic conformational changes of the S2 subunit (Fig. 2A). The exposed hydrophobic fusion peptide of S2 inserts into the target cell membrane and mediates membrane fusion to release the viral genome into the cytosol for transcription and replication (Hoffmann et al., 2021b; Zhang et al., 2020). TMPRSS2 plays important roles in the entry of respiratory viruses, such as SARS-CoV-2, SARS-CoV and Avian Influenza Viruses (Bestle et al., 2021; Glowacka et al., 2011; Song et al., 2020). Inhibition of TMPRSS2 significantly prevents SARS-CoV and SARS-CoV-2 from entering into target cells (Hoffmann et al., 2021b; Kawase et al., 2012). Human coronaviruses, such as SARS-CoV-2, SARS-CoV, MERS-CoV and HCoV-229E, also exploit the endocytosis pathway for viral entry (Fig. 2B) (Bayati et al., 2021; Inoue et al., 2007; Lu et al., 2013; Nomura et al., 2004; Wang et al., 2008).

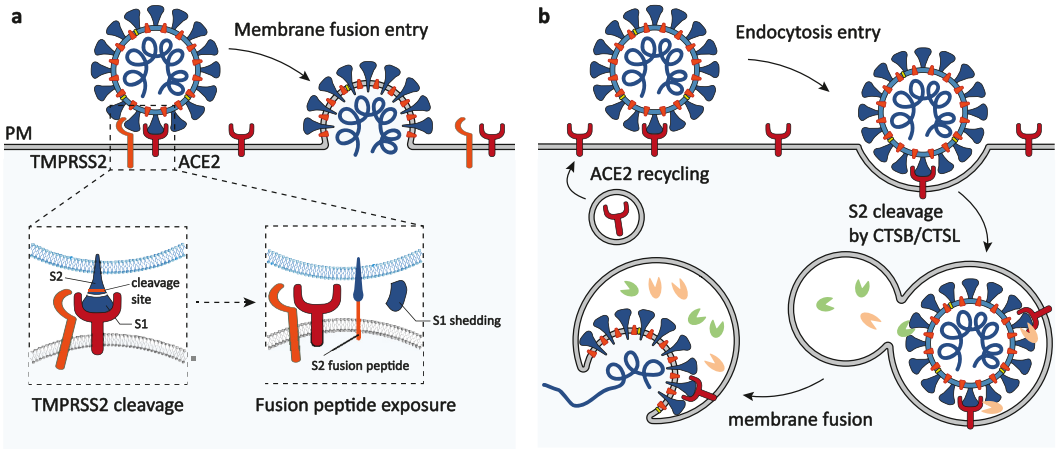

Fig. 2. The entry of SARS-CoV-2

SARS-CoV-2 enters into the host cell through direct membrane fusion or the endocytosis pathway. After the S protein binds to ACE2, a site is revealed in the S2 subunit which can be recognized and cleaved by the cell surface-localized protease TMPRSS2. TMPRSS2 cleavage leads to dramatic conformational changes of the S protein and shedding of the S1 subunit. This exposes the S2 fusion peptide, which induces the fusion of viral and host plasma membranes so that the viral genomic RNA is released into the host cell. When TMPRSS2 is absent, ACE2-bound virions are internalized through endocytosis. Upon fusion of the virion-containing endosomes with late endosomes/lysosomes, S protein is cleaved by CTSB/CTSL. The exposed S2 fusion peptide facilitates fusion of the viral membrane with the endocytic vesicle and release of the viral genome.

Coronaviruses can enter host cells via clathrin-mediated endocytosis and other endocytic processes. For example, SARS-CoV uses both clathrin-mediated and clathrin- and caveola-independent endocytic pathways for viral entry (Inoue et al., 2007; Wang et al., 2008), while lipid raft/caveola-mediated endocytosis is involved in HCoV-229E entry and probably in mouse hepatitis virus (MHV) entry (Choi et al., 2005; Nomura et al., 2004). In the absence of TMPRSS2, the ACE2-engaged SARS-CoV-2 virion is internalized into the cytosol through clathrin-mediated endocytosis (Bayati et al., 2021; Yang et al., 2022). SARS-CoV-2 pseudovirus infection assays suggest that caveolar/lipid raft- and cytoskeleton-mediated endocytosis are also involved in viral infection (Li et al., 2021a; Zhou et al., 2022). After fusion of virion-containing endosomes with lysosomes, S2 subunit is cleaved by cathepsin cysteine proteases (e.g. CTSB and CTSL), resulting in the exposure of the fusion peptide to promote fusion between endosomal and viral membranes for release of the viral genome (Hoffmann et al., 2020b; Mellott et al., 2021). The S protein of the recently emerged SARS-CoV-2 Omicron variant shows less efficient TMPRSS2-mediated S1/S2 cleavage (Meng et al., 2022; Zhao et al., 2022). The entry of Omicron mainly depends on the endocytic pathway, and compared to the Delta variant it thus exhibits lower infectivity in cells expressing high levels of TMPRSS2, and less sensitivity to the TMPRSS2 inhibitor Camostat (Meng et al., 2022; Zhao et al., 2022). The different entry routes exploited by Omicron and other variants may contribute to the distinct clinical manifestations.

Genome-wide screens have revealed that host factors involved in endocytic cargo internalization and endosomal trafficking/recycling are essential for SARS-CoV-2 entry (Daniloski et al., 2021; Zhu et al., 2021). These host factors include endosomal cargo-sorting nexin-27 (SNX27), the retromer complex, the COMMD/CCDC22/CCDC93 (CCC) complex, the actin-related protein 2/3 (ARP2/3) complex, the ARP2/3 activator WASH complex, and the late-endosomal/lysosomal GTPase Rab7a (Daniloski et al., 2021; Zhu et al., 2021). The ARP2/3 complex acts as an actin-polymerization/organization factor, initiating actin filament branches (Welch et al., 1997). During endocytic cargo internalization, the ARP2/3 complex and type I myosin motor provide the force to extend vesicle invagination (Sun et al., 2006). Inhibiting ARP2/3-mediated actin polymerization blocks endocytic uptake of cell membrane proteins (Park et al., 2013; Rocca et al., 2008). The endosomal protein sorting and trafficking complex retromer, which consists of vacuolar protein sorting proteins (VPS26, VPS29 and VPS35), mediates the recycling of transmembrane proteins to various compartments. In concert with sorting nexins, retromer recruits the WASH complex to activate ARP2/3-dependent actin polymerization, and facilitates the formation of endosomal actin-enriched subdomains for selective cargo recycling (Gomez and Billadeau, 2009; Puthenveedu et al., 2010). SNX27 acts as a cargo-sorting nexin of retromer, mediating the recycling of transmembrane receptors from the endosome to the PM (Gallon et al., 2014; Lauffer et al., 2010). During SARS-CoV infection, the internalized ACE2 is recycled from endosomes back to the PM (Wang et al., 2008). ACE2 binds to the PDZ domain of SNX27 through its short linear motifs (SLiMs) (Kliche et al., 2021), which suggests that the internalized ACE2 might be rapidly recycled back to the PM via the SNX27-retromer complex. Levels of surface ACE2 are dramatically reduced in cells with knockout (KO) of *COMMD3*, *CCDC53* (encoding a component of WASH complex), *VPS29* and *VPS35* (Zhu et al., 2021). Interestingly, the internalized ACE2 is transported to lysosomes for degradation during angiotensin II (Ang-II) treatment (Deshotels et al., 2014). The mechanism specifying ACE2 degradation or recycling has yet to be determined. SNX27 also interacts with the SARS-CoV/SARS-CoV-2 S protein, facilitating its intracellular trafficking and cell surface expression and consequently promoting S protein-mediated cell-to-cell fusion (Petit et al., 2005; Zhao et al., 2021a). Depletion of SNX27, retromer, WASH or components of the ARP2/3 complex (i.e. ACTR2, ACTR3, ARPC3 and ARPC4) effectively prevents SARS-CoV-2 pseudovirus entry and SARS-CoV-2 infection (Daniloski et al., 2021; Zhu et al., 2021). Rab7a is involved in trafficking and degradation of cell membrane receptors through the endo-lysosome pathway (Shinde and Maddika, 2016). Rab7a also recruits the retromer complex to late endosomes through interaction with VPS35, thus contributing to endosome maturation and cargo export (Rojas et al., 2008). Depletion of Rab7a reduces viral entry by sequestering the ACE2 receptor in endosomes (Daniloski et al., 2021). The SARS-CoV-2 NSP7 interacts with Rab7a, which suggests that NSP7 may manipulate Rab7a to facilitate viral infection (Gordon et al., 2020). Thus, endocytic trafficking/recycling plays multiple roles in the entry of coronaviruses.

3. Double-membrane vesicles in SARS-CoV-2 replication/transcription

Positive-stranded RNA viruses remodel the endomembrane system to form viral replication organelles (ROs) for RNA synthesis. ROs serve as platforms for docking cellular and viral factors for viral genome replication, and also shield viral RNAs from cellular innate immune defenses

or degradation (Harak and Lohmann, 2015; Scutigliani and Kikkert, 2017; Snijder et al., 2020). The architecture of ROs varies among different virus families. Alphaviruses, flaviviruses, bromoviruses and nodaviruses generate small invaginations, called spherules/vesicles, in the membrane of specific organelles, such as the ER, mitochondria and endolysosomes. The invaginated spherules/vesicles are often tethered to the endomembrane and possess a narrow channel connecting the spherule interior with the cytosol (Ertel et al., 2017; Fernández de Castro et al., 2017; Gillespie et al., 2010; Kallio et al., 2013; Kujala et al., 2001; Schwartz et al., 2004; Welsch et al., 2009). In contrast, coronaviruses, noroviruses, hepacivirus, arteriviruses and picornaviruses induce the protrusion of DMVs from the ER (Belov et al., 2012; Doerflinger et al., 2017; Gosert et al., 2003; Knoops et al., 2008, 2012; Wolff et al., 2020). DMVs create a microenvironment for RNA replication and subgenomic mRNA synthesis, and also serve as storage sites for negative-stranded templates and double-stranded viral RNAs. Infection by *betacoronaviruses*, including SARS-CoV-2, SARS-CoV, MERS-CoV and MHV, generates a reticulovesicular network (RVN) consisting of DMVs (200–300 nm in diameter) that connect to a complex of convoluted membranes (CMs) derived from the ER (Knoops et al., 2008; Ogando et al., 2020; Snijder et al., 2020; Wolff et al., 2020). DMV biogenesis involves multiple membrane rearrangements, including membrane pairing, curvature induction and membrane fission. The paired ER membrane forms CMs, which are reticular inclusion structures (Angelini et al., 2013; de Wilde et al., 2013; Knoops et al., 2008; Ulasli et al., 2010). Electron tomography analysis revealed that DMV outer membranes are often connected to the ER or CMs (Angelini et al., 2013; Oudshoorn et al., 2017; van der Hoeven et al., 2016). A majority of DMVs connect with the RVN, while about 20% of DMVs are free-floating (Knoops et al., 2008; Snijder et al., 2020; Wolff et al., 2020). The number of DMVs increases at early stages of MHV infection but dramatically decreases at late stages (Mihelc et al., 2021). Some DMVs are separated from the RVN and trafficked to lysosomes for degradation or exocytosis during MHV infection (Ghosh et al., 2020).

Unlike invaginated spherules/vesicles, whose "neck-like" channels export nascent positive-sense viral RNAs to the cytosol for translation or virion assembly, DMVs are closed compartments that lack visible connections to the cytosol (Ertel et al., 2017; Knoops et al., 2008). Cryo-electron microscopy analysis revealed that RNAs of MHV and SARS-CoV-2 are transported from the interior of DMVs to the cytosol via double-membrane-spanning pores (Wolff et al., 2020). The pore complex in the MHV-induced DMV is a structure with sixfold symmetry. It consists of a crown-like structure extending ~13 nm into the cytosol, a ~24-nm-wide platform embedded in the double membranes, and a ~6-nm-wide channel connecting the DMV interior to the cytosol (Wolff et al., 2020). NSP3 of MHV is the major component of the cytosolic crown-like structure of the DMV pore complex (Wolff et al., 2020). Pore-like complexes are also observed in SARS-CoV-2-induced DMVs, but the role of SARS-CoV-2 NSP3 in DMV pore formation is still obscure. Further investigations will be required to identify the constituents of the inter-membrane platform and also the cytosolic/luminal partners associated with the pore complex that mediate viral RNA synthesis, transcription, import/export, RNA packaging and transport.

The concerted actions of positive-stranded RNA viral proteins and host factors drive the generation of ROs. The formation of dengue virus (DENV) ROs, which consist of invaginated small vesicles in the ER, requires the non-structural proteins NS4A and NS4B, both of which are embedded in the ER membrane. NS4A and NS4B induce negative membrane curvature. Other viral proteins, including the ER transmembrane protein NS2A and the ER lumen-localized NS1, and host factors such as the endosomal sorting complex required for transport (ESCRT) also participate in the formation of invaginated vesicles (Neufeldt et al., 2018). Several viral proteins have been reported to be responsible for coronavirus DMV induction. The multi-spanning transmembrane coronaviral proteins NSP3, NSP4, and NSP6 are localized on the ER (Snijder et al., 2020). Co-expression of SARS-CoV NSP3, NSP4 and NSP6 induces the formation of DMVs that are similar to those in SARS-CoV-infected cells (Angelini et al., 2013). NSP3 and NSP4 are required for ER membrane pairing and formation of the maze-like single-membrane structures (Angelini et al., 2013; Hagemeijer et al., 2014). Depletion or mutation of NSP3 and NSP4 causes a defect in DMV formation and dramatically decreases SARS-CoV or MHV replication (Beachboard et al., 2015; Clementz et al., 2008; Sakai et al., 2017; Stokes et al., 2010). Co-expression of MERS-CoV or SARS-CoV-2 NSP3 and NSP4 is required and sufficient to induce DMVs, while NSP6 is dispensable (Oudshoorn et al., 2017; Twu et al., 2021; Ji et al., 2022). NSP3 and NSP4 form homotypic and heterotypic interactions through their intraluminal domains (Hagemeijer et al., 2011; Neuman et al., 2008; Ji et al., 2022). NSP3 and NSP4 are located in the outer and inner membrane of DMVs, respectively, and this distinct localization is necessary for DMV formation (Ji et al., 2022). Other NSPs, such as NSP2/3, NSP8 and NSP12, are also enriched in DMVs or CMs (Bost et al., 2000; Hagemeijer et al., 2012; van der Meer et al., 1999), but their functions during DMV biogenesis are still unknown.

Genome-wide CRISPR/Cas9 screens, proteomics and SARS-CoV-2-human interactomics have identified host factors that interact with viral proteins for DMV formation (Daniloski et al., 2021; Gordon et al., 2020; Stukalov et al., 2021; Wang et al., 2021). The lipid transport protein sigma nonopioid intracellular receptor 1 (SIGMAR1) contributes to viral replication (Hayashi and Su, 2004, 2007; Yang et al., 2019). Depletion or inhibition of SIGMAR1 effectively reduces SARS-CoV-2 infectivity (Daniloski et al., 2021; Gordon et al., 2020). Additionally, SIGMAR1 mediates the early steps of viral RNA replication during hepatitis C virus (HCV) infection, which also involves the formation of DMVs (Friesland et al., 2013). As a cholesterol- and sphingolipid-binding protein, SIGMAR1 primarily resides in lipid-rich areas of the ER (Hayashi and Su, 2003). It is possible that viral proteins bind to SIGMAR1 and use constituents (such as cholesterol) of the ER as platforms for the initial steps of DMV formation. Consistent with this, genes involved in cholesterol or lipid synthesis, such as SCAP, MBPTS1 and MBPTS2, have also been identified as critical factors for SARS-CoV-2 infection (Schneider et al., 2021; Wang et al., 2021). The viral proteins NS4B and NS5A, which induce formation of DMVs in HCV-infected cells, interact with host proteins in the positive membrane curvature-inducing reticulon (RTN) family and phosphatidylinositol 4-kinase (PI4K), respectively (Arakawa and Morita, 2019; Neufeldt et al., 2018). The role of RTNs and PI(4)P in DMV formation in SARS-CoV-2-infected cells has yet to be determined. In general, generation of DMVs requires interactions between viral transmembrane NSPs and host proteins to trigger ER membrane pairing and curvature.

For coronaviral particle assembly, the genomic RNAs exported from the DMV are recognized by the dimeric nucleocapsid phosphoprotein (N) and tightly packaged into higher-order supercoiled ribonucleoprotein complexes (RNPs) (Dinesh et al., 2020; Gui et al., 2017). The M protein interacts with RNPs and S glycoproteins at the budding site to promote assembly and to control the viral particle shape (Neuman et al., 2011). Budding occurs at the ERGIC (Ogando et al., 2020; Stertz et al., 2007; Wolff et al., 2020). A recent study showed that SARS-CoV-2 induces the formation of single-membrane vesicles (SMVs) as virion assembly and budding sites (Mendonça et al., 2021). Transport vesicles containing S proteins fuse with the SMVs. The S proteins on the SMV membrane form clusters with viral RNPs to initiate the assembly of viral particles and their subsequent budding and release into the SMV (Mendonça et al., 2021). Elucidating the nature of the endomembrane structures that facilitate virion assembly requires detailed dynamic analysis of characteristic markers for various compartments.

4. Shared components for the formation of SARS-CoV-2-induced DMVs and double-membrane autophagosomes

Autophagy is a cellular metabolic process in which cytoplasmic contents are sequestered within a double-membrane autophagosome,

which eventually fuses with lysosomes for degradation (Zhao et al., 2021b). The core step in autophagy is the generation of the double-membrane autophagosome from a precursor called an isolation membrane (IM). Upon autophagy induction, the FIP200/ULK1/ATG13/ATG101 complex and vesicles carrying the multispanning membrane protein Atg9 are targeted to the ER. This is followed by recruitment of the class III VPS34 phosphatidylinositol 3-kinase (PI3K) complex to generate PI(3)P-enriched ER subdomains, which serve as platforms for subsequent recruitment of other ATG proteins for autophagosome formation (Ktistakis and Tooze, 2016). The integral ER membrane proteins VAPA/B (VAPs) and Atlastin 2/3 (ATLs) facilitate and stabilize the association of the FIP200/ULK1 complex with the ER (Zhao et al., 2018; Liu et al., 2021). The origin of the initial membrane seed for the IM remains unknown. The phospholipid transfer activity of ATG2, which transports lipids from the ER, and the lipid scramblase activity of ATG9 are involved in IM expansion (Matoba et al., 2020; Maeda et al., 2020; Zhang, 2020). Two ubiquitin-like conjugation systems (conjugation of Atg8 to phosphatidylethanolamine (PE) by the E1-like enzyme ATG7 and the E2-like enzyme ATG3; and conjugation of ATG12 to ATG5 by ATG7 and the E2-like enzyme ATG10, which further interacts with ATG16) act at several steps of autophagosome formation, including IM expansion and closure (Mizushima, 2020; Nakatogawa, 2020). During expansion, the IM forms dynamic contacts with the ER. The ER transmembrane autophagy protein VMP1 controls the dynamics of the IM-ER contact (Tian et al., 2010; Zhao et al., 2017).

DMVs and autophagosomes are both double-membrane structures, but the membrane remodeling processes are distinct. The DMV is directly generated via remodeling of the ER, while autophagosomes require the ER as a platform and also as a lipid source for IM expansion (Snijder et al., 2020; Zhao and Zhang, 2019). The ER does not directly constitute the membrane of autophagosomes (Zhao and Zhang, 2019). DMVs in cells infected with coronaviruses, such as MHV, SARS-CoV and SARS-CoV-2, are not generated via the canonical autophagic machinery (Prentice et al., 2004; Reggiori et al., 2010; Shang et al., 2021). In the canonical autophagy pathway, the mammalian Atg8 family protein LC3 is conjugated to PE in autophagosome membranes. Interestingly, non-lipidated LC3 is colocalized with MHV-induced DMVs and is required for MHV infection; the machinery for LC3 lipidation is not required for the replication of MHV or SARS-CoV (Reggiori et al., 2010; Schneider et al., 2012; Zhao et al., 2007). Instead, components which modulate the ER-associated degradation (ERAD) pathway are involved in MHV-induced DMV formation and viral replication (Reggiori et al., 2010). To tightly control ERAD activity, effectors, including mannosidase alpha-like 1 (EDEM1), osteosarcoma amplified 9 (OS9), and ERAD component SEL1, are delivered from the ER to late endosomes/lysosomes through ER-derived vesicles named EDEMosomes (Noack et al., 2014). The non-lipidated form of LC3 (LC3-I) interacts with SEL1 and is recruited to EDEMosomes. MHV-induced DMVs colocalize with the ERAD components EDEM1, OS-9 and LC3-I (Reggiori et al., 2010). Thus, coronaviruses probably hijack the LC3-I-coated EDEMosome formation machinery for DMV biogenesis.

Genome-wide CRISPR/Cas9 screens have identified a subset of autophagy genes essential for SARS-CoV-2 infection. PI(3)P synthesis is required for DMV formation and viral replication of HCV and SARS-CoV-2, while the essential autophagy proteins ATG5 and ATG7 are dispensable for SARS-CoV-2 infection (Daniloski et al., 2021; Kapadia and Chisari, 2005; Shang et al., 2021; Twu et al., 2021; Zhu et al., 2021). Class III PI3K inhibitors inhibit SARS-CoV-2 replication (Yuen et al., 2021; Zhu et al., 2021). The ER-localized transmembrane autophagy proteins TMEM41B and VMP1 are essential for a post-entry step in the infection cycle of SARS-CoV-2 (Morita et al., 2018; Zhao et al., 2017; Hoffmann et al., 2021a; Shoemaker et al., 2019). Depletion of VMP1 or TMEM41B greatly impairs DMV formation induced by MHV infection or expression of SARS-CoV-2 NSP3/4 (Ji et al., 2022). VMP1 and TMEM41B interact with the NSP3/4 complex and are recruited to DMV formation sites (Fig. 3). They function at different steps during DMV generation: TMEM41B facilitates NSP3/4 binding, while VMP1 is required for curvature of paired ER membranes into closed DMVs (Ji et al., 2022). TMEM41B and VMP1 have recently been shown to act as ER scramblases for normal cross-membrane distribution of phospholipids, such as cholesterol and phosphatidylserine (Ghanbarpour et al., 2021; Li et al., 2021b; Huang et al., 2021a). Depletion of PS synthetase PTDSS1 partially suppresses the DMV defects in *VMP1* KO cells (Ji et al., 2022), which indicates that VMP1 may participate in DMV formation by regulating PS levels. During autophagosome formation, VMP1 promotes the activity of the ER-localized Ca^{2+} pump SERCA to control ER-IM contacts (Zhao et al., 2017). However, inhibition of SERCA activity by thapsigargin has no effect on DMV formation (Ji et al., 2022), which indicates that VMP1 functions in autophagosome and DMV formation via distinct mechanisms. Thus, SARS-CoV-2 exploits a subset of autophagy machinery components to facilitate viral replication.

5. The late endosome/lysosome exocytic pathway mediates SARS-CoV-2 release

Similar to other enveloped RNA viruses, such as HCV and DENV, coronaviruses use smooth-wall secretory vesicles for release. In this process, trans-Golgi network (TGN)-localized viral particles are trafficked to the PM for egress (Machamer, 2013; Robinson et al., 2018). SARS-CoV-2 modulates the Golgi apparatus to facilitate viral trafficking. SARS-CoV-2 infection or expression of certain viral proteins induces Golgi fragmentation by modulating the expression level of GRASP55 and TGN46 (Zhang et al., 2022). Interference with the dysregulated GRASP55/TGN46 expression reduces SARS-CoV-2 infection in Huh7-ACE2 cells (Scherer et al., 2022; Zhang et al., 2022). Transmission electron microscopy analysis revealed the presence of a large number of small secretory vesicles containing a single virion in SARS-CoV-2-infected Vero cells (Eymieux et al., 2021). Consistent with this, multiple subunits of the exocyst, an octameric protein complex that interacts with the ARP2/3 complex and facilitates the tethering of secretory vesicles to the PM, have been isolated as host factors essential for the SARS-CoV-2 infection cycle (Schneider et al., 2021; Wang et al., 2021). Coronaviruses such as SARS-CoV-2 and MHV also appear to use the late endosome/lysosome exocytic pathway for release (Ghosh et al., 2020). SARS-CoV-2 and MHV virions are enriched in endosomes labeled by the late endosomal/lysosomal marker LAMP1. The level of LAMP1 is dramatically increased after SARS-CoV-2 infection (Chen et al., 2021; Ghosh et al., 2020). The Rab7a inhibitor CID1067700, which inhibits the maturation of endo-lysosomes, but not the biosynthetic secretory inhibitor Brefeldin A (BFA), significantly reduces *betacoronavirus* egress (Ghosh et al., 2020). Late endosome/lysosome-localized SARS-CoV-2 ORF3a promotes the recruitment of BORC-ARL8b complex and exocytosis-related SNARE proteins to facilitate retrograde transport of lysosomes and subsequent fusion with the PM (Chen et al., 2021). Overexpression of SARS-CoV-2 ORF3a promotes egress of MHV, which itself does not contain an ORF3a counterpart (Chen et al., 2021). During SARS-CoV-2 infection, lysosomes are deacidified (Ghosh et al., 2020). Lysosomal neutralization promotes their exocytosis (Carnell et al., 2011; Miao et al., 2015). SARS-CoV-2 ORF3a also causes a certain amount of lysosomal damage and deacidification. The number of Galectin 3-labeled damaged lysosomal structures is slightly increased and the fluorescence intensities of puncta labeled by LysoTracker, a pH-sensitive acid dye, are weaker in ORF3a-expressing cells (Chen et al., 2021; Miao et al., 2021). ORF7a expression also causes deacidification of lysosomes (Hayn et al., 2021). Indirect causes of lysosomal deacidification may include excessive loading with viral particles or impaired function of lysosomal proton pumps or ion channels (Westerbeck and Machamer, 2019). The lysosomal membrane protein TMEM106B, components of the vacuolar ATPase proton pump, and cathepsins are essential host factors for SARS-CoV-2 infection (Baggen et al., 2021; Daniloski et al., 2021; Wang et al., 2021). Lysosomal exocytosis requires lysosomal Ca^{2+} release, which is mediated by lysosomal/endosomal membrane-localized Ca^{2+}

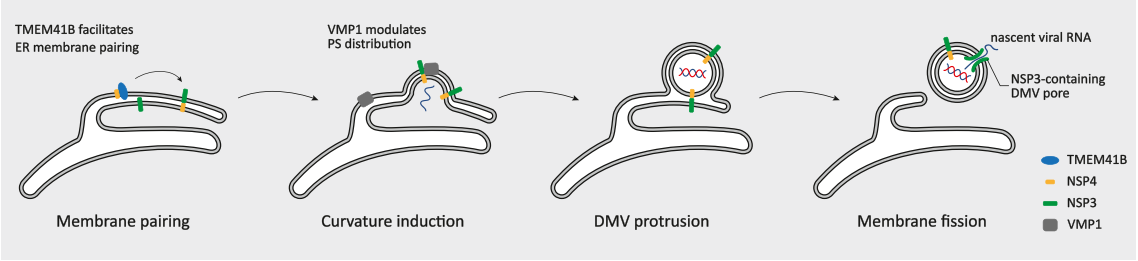

Fig. 3. DMV formation in SARS-CoV-2-infected cells
At the initial step of biogenesis of double-membrane vesicles (DMVs), viral nonstructural proteins NSP3 and NSP4 interact to induce pairing of ER membranes. This is facilitated by the ER transmembrane protein TMEM41B. Upon interaction, NSP3 is distributed on the side of the ER which will become the cytoplasmic face of the DMV, while NSP4 is mainly located on the side of the ER which will become the luminal face of the DMV. The segregation of NSP3/4 occurs via unknown mechanisms. NSP3/4 interaction and segregation promote membrane curving and protrusion of DMVs from the ER, which is probably regulated by VMP1-modulated PS distribution. In most DMVs, the outer membranes are connected to the ER, while some DMVs undergo membrane fission and are free-floating. The nascent viral RNAs are transported to the cytosol through membrane-spanning DMV pores, which contain NSP3.

channel mucolipins (TRPML1, TRPML2 and TRPML3) (Rosato et al., 2021). Depletion of TRPML3 effectively inhibits ORF3a-mediated lysosomal exocytosis (Chen et al., 2021). Knocking down TRPMLs prevents SARS-CoV-2 invasion (Huang et al., 2021b). Berbamine inhibits SARS-CoV-2 infection by inhibiting TRPML-mediated lysosomal Ca^{2+} release (Huang et al., 2021b). Berbamine treatment inhibits endolysosomal retrograde trafficking and fusion with the PM, and also causes an increase in alternative secretion of ACE2 via exosomes, resulting in the reduction of cell surface-localized ACE2 (Huang et al., 2021b).

6. SARS-CoV-2 proteins inhibit the autophagy pathway

Autophagy acts as a surveillance system by selectively removing damaged/obsolete organelles, protein aggregates, and invading pathogens (Deretic et al., 2013; Sharma et al., 2018). Autophagy has different and even opposite functions during the infection of different viruses. Autophagy mediates viral clearance by degrading virions or nascent viral proteins/genomes and also enhances the immune response through antigen presentation (Deretic et al., 2013). Many viruses, such as MERS-CoV, Sindbis virus and herpes simplex virus 1 (HSV-1), have evolved various mechanisms to inhibit autophagy and even to exploit autophagy components to benefit viral replication (English et al., 2009; Orvedahl et al., 2010). MERS-CoV infection leads to a reduced level of the autophagy protein BECN1 and blocks the fusion of autophagosomes with lysosomes (Gassen et al., 2019). Inhibition of SKP2, which increases the BECN1 level and induces autophagy, effectively inhibits MERS-CoV replication (Gassen et al., 2019). The autophagy pathway has also been shown to positively regulate infection of many positive-stranded RNA viruses, including poliovirus (Taylor and Kirkegaard, 2008), coxsackievirus (Yoon et al., 2008), enterovirus (Huang et al., 2009), HCV (Dreux et al., 2009), and coronaviruses (Prentice et al., 2004; Shang et al., 2021). Autophagy can facilitate viral egress. For example, the release of poliovirus and other enteroviruses, including human rhinovirus (HRV) and coxsackievirus B3 (CVB3), depends on non-lytic secretory autophagy, in which autophagosomes envelop the virions and fuse with the PM instead of lysosomes (Bird et al., 2014; Chen et al., 2015; Robinson et al., 2014). Depletion of autophagy genes, such as those encoding LC3 and Beclin1, leads to significantly decreased viral release (Bird et al., 2014; Chen et al., 2015; Robinson et al., 2014). Autophagy also inhibits immune surveillance in SARS-CoV-2-infected cells. The SARS-CoV-2 ORF8 interacts with major histocompatibility complex class I (MHC-I) proteins and leads to their autophagic degradation, protecting SARS-CoV-2-infected cells from being recognized and lysed by cytotoxic T lymphocytes (Zhang et al., 2021b). Inhibiting autophagy restores MHC-I expression in ORF8-expressing cells (Zhang et al., 2021b). NSP13 promotes autophagic degradation of TANK-binding kinase 1 (TBK1) by targeting it to the autophagy substrate p62 (Sui et al., 2022). Removal of TBK1 inhibits type I interferon (IFN–I) production (Sui et al., 2022).

SARS-CoV-2 hijacks the autophagic machinery at multiple steps of the autophagy pathway to benefit viral infection (Fig. 4). SARS-CoV-2 infection promotes autophagosome generation but inhibits autophagic degradation by preventing formation of degradative autolysosomes (Miao et al., 2021). Nascent autophagosomes undergo a maturation process by fusing with early endosomes, late endosomes and lysosomes to generate single-membrane amphisomes, which eventually form degradative autolysosomes (Zhao et al., 2021b; Zhao and Zhang, 2019). SARS-CoV-2 blocks fusion of autophagosomes/amphisomes with lysosomes by sequestering the homotypic fusion and protein sorting complex (HOPS) component VPS39 on late endosomes (Mendonça et al., 2021; Miao et al., 2021). SARS-CoV-2 ORF3a interacts with and sequesters VPS39 on late endosomes/lysosomes and subsequently prevents the assembly of the STX17-SNAP29-VAMP8 SNARE complex that mediates autolysosome formation (Fig. 4) (Miao et al., 2021). SARS-CoV-2 ORF3a also sequesters the autophagy regulator UVRAG to facilitate autophagosome formation but inhibit autophagosome maturation (Qu et al., 2021). Meanwhile, ORF3a impairs lysosomal degradation (Miao et al., 2021; Zhang et al., 2021c). Other SARS-CoV-2 viral proteins also modulate autophagy. NSP15 of SARS-CoV-2 blocks autophagy induction, while ORF7a reduces the acidity of lysosomes, resulting in inhibition of autolysosome degradation (Fig. 4) (Hayn et al., 2021; Miao et al., 2021). The viral M and E proteins also inhibit formation of acidified autolysosomes (Hayn et al., 2021; Miao et al., 2021). The viral particles are detected in amphisomes in SARS-CoV-2-infected cells (Miao et al., 2021). Amphisomes also participate in unconventional secretion (Ganesan and Cai, 2021). Therefore, multiple SARS-CoV-2 viral proteins causing accumulation of amphisomes may simultaneously facilitate exocytosis for viral release.

Autophagosomes formed in SARS-CoV-2-infected cells are smaller than autophagosomes induced by starvation in uninfected cells (Miao et al., 2021). The NSP6 proteins of coronaviruses, including IBV, MHV, and SARS-CoV, have been shown to induce generation of ER-derived vesicles positive for ATG5 and lipidated LC3 (LC3-II). These vesicles possess properties of nascent autophagosomes but with a smaller diameter than starvation-induced autophagosomes (Cottam et al., 2011, 2014). Autophagosome biogenesis involves the formation of a hybrid pre-autophagosomal structure (HyPAS), which is derived from the fusion of *cis* Golgi and endosomal membranes (Kumar et al., 2021). The SARS-CoV-2 NSP6 has been shown to effectively inhibit HyPAS formation (Kumar et al., 2021). The small autophagosomes may degrade viral elements less efficiently (Cottam et al., 2014; Gassen et al., 2019). NSP6 also impairs lysosomal acidification through interaction with the lysosomal ATPase component ATP6AP1, which inhibits its activation (Sun et al., 2022). The autophagy pathway appears to be differentially modulated during different stages of virus infection due to the interaction of viral proteins with distinct host factors (Fig. 4). At early stages of infection, autophagy induction mediates degradation of MHC-I to reduce

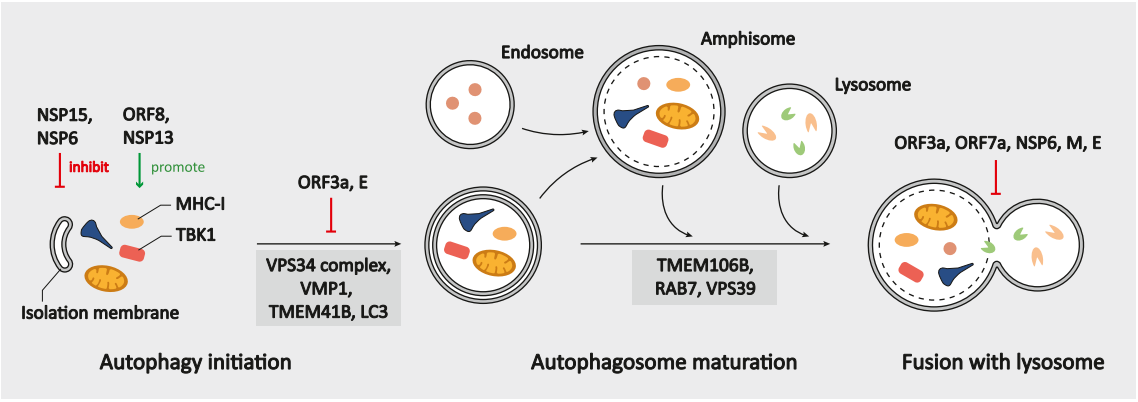

Fig. 4. Modulation of autophagy by the SARS-CoV-2 viral proteins
The SARS-CoV-2 viral proteins intersect with the autophagy pathway at multiple steps. SARS-CoV-2 promotes autophagosome initiation but blocks maturation of autophagosomes into degradative autolysosomes. SARS-CoV-2 ORF8 and NSP13 interact with MHC-I and TBK1, respectively, and promote their autophagic degradation to protect SARS-CoV-2-infected cells from immune attack. The NSP6 protein impairs autophagosome formation and also prevents lysosome acidification to inhibit autophagic degradation. SARS-CoV-2 ORF3a sequesters the HOPS component VPS39 to prevent the fusion of autophagosomes/amphisomes and lysosomes. NSP15 blocks autophagy induction, while ORF7a causes lysosome deacidification and thus inhibits auto-lysosomal degradation. M and E also inhibit the formation of acidified autolysosomes. Host factors which are both involved in autophagy and essential for SARS-CoV-2 replication are listed in the grey boxes.

the detection of SARS-CoV-2 (Zhang et al., 2021b); in contrast, at late stages, abundant viral proteins may promote viral egress by inhibiting formation of degradative autolysosomes.

7. Conclusions and perspectives

During SARS-CoV-2 infection, viral proteins interact with host factors to extensively remodel host cell endomembranes for various steps of the viral cycle. For example, the interaction of the viral S protein and host ACE2 is critical for SARS-CoV-2 entry. NSP3/NSP4 recruit the ER transmembrane proteins VMP1 and TMEM41B for RO formation and viral RNA replication, while ORF3a hijacks the HOPS component VPS39 to inhibit autophagic defense mechanisms and promote lysosomal exocytosis-mediated viral egress. Many questions still remain. How do viral proteins interact with host factors to remodel the ER for generation of DMVs? How do different viral proteins coordinate with the autophagy machinery to differentially modulate different steps of autophagy at different stages of viral infection? Given that the endomembrane system is extensively reorganized during virus infection, what is the nature of the vesicles that mediate viral release?

Our improved understanding of endomembrane system remodeling during SARS-CoV-2 infection also provides potential drug targets and diagnostic approaches. The current strategies for preventing SARS-CoV-2 infection mainly focus on blocking virus entry by therapeutic antibodies, inhibiting the viral RNA polymerase and protease, and enhancing the immune system. The emergence of SARS-CoV-2 variants, such as Omicron, with enhanced transmissibility and evasion of neutralizing antibodies (Hoffmann et al., 2022; Yin et al., 2022), highlights the importance of developing approaches to explore cellular mechanisms for treatment. Several inhibitors targeting the endomembrane system have been shown to effectively prevent SARS-CoV-2 infection. For example, FDA-approved drugs such as Haloperidol target the interaction between SIGMAR1 and NSP6 (Gordon et al., 2020). Chloroquine, which inhibits autolysosomal/endolysosomal protease activities by increasing the lysosomal pH, exerts antivirus function in SARS-CoV-2-infected Vero E6 cells (Wang et al., 2020). Chloroquine fails to block SARS-CoV-2 infection in the TMPRSS2-expressing human lung cell line Calu-3 (Hoffmann et al., 2020c). Inhibitors of PI3K, which effectively prevent DMV formation and viral replication, are also promising antiviral candidates (Twu et al., 2021). More importantly, the interaction between viral spike proteins and receptors appears to be virus-specific. Endomembrane remodeling, such as generation of DMVs for viral replication and modulation of the secretory and lysosome pathways for egress, is generally required for coronavirus infection. The endomembrane system can be targeted as a therapeutic strategy to combat SARS-CoV-2, its yet-to-emerge variants, and even new coronaviruses.

Acknowledgements

We are grateful to Dr. Isabel Hanson for editing work. This article was supported by the following: Ministry of Science and Technology of China (grant 2021YFA1300800 to H.Z.) and National Natural Science Foundation of China (grants 82188101, 92054301, and 31790403 to H.Z.).

Abbreviations

ACE2	Angiotensin converting enzyme 2
ARP2/3	Actin-related protein 2/3
CM	Convoluted membrane
CVB3	Coxsackievirus B3
DMV	Double-membrane vesicle
ERAD	ER-associated degradation
ERGIC	ER–Golgi intermediate compartment
ESCRT	Endosomal Sorting Complexes Required for Transport
HCV	Hepatitis C virus
HOPS	Homotypic fusion and protein sorting complex
HRV	Human rhinovirus
HSV-1	Herpes simplex virus 1
HyPAS	Hybrid pre-autophagosomal structure
IFN-I	Type I interferon
IM	Isolation membrane
MERS-CoV	Middle East respiratory syndrome coronavirus
MHC-I	Histocompatibility complex class I
MHV	Mouse hepatitis virus
NSP	Non-structural protein
PE	Phosphatidylethanolamine;
PI3K	Phosphatidylinositol 3-kinase
PI4K	Phosphatidylinositol 4-kinase
PI(3)P	Phosphatidylinositol 3-phosphate
PI(4)P	Phosphatidylinositol 4-phosphate
PM	Plasma membrane
RNP	Ribonucleoprotein complex
RO	Replication organelle
RTN	Reticulon
RVN	Reticulovesicular network
SARS-CoV	Severe acute respiratory syndrome coronavirus

SARS-CoV-2 Severe acute respiratory syndrome coronavirus 2
SIGMAR1 Sigma nonopioid intracellular receptor 1
SMV Single-membrane vesicle
SNX27 Sorting nexin-27
TBK1 TANK-binding kinase 1
TGN Trans-Golgi network
TMPRSS2 Transmembrane protease serine 2

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