

# Negative-sense RNA viruses: An underexplored platform for examining virus–host lipid interactions

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**ABSTRACT** Viruses are pathogenic agents that can infect all varieties of organisms, including plants, animals, and humans. These microscopic particles are genetically simple as they encode a limited number of proteins that undertake a wide range of functions. While structurally distinct, viruses often share common characteristics that have evolved to aid in their infectious life cycles. A commonly underappreciated characteristic of many deadly viruses is a lipid envelope that surrounds their protein and genetic contents. Notably, the lipid envelope is formed from the host cell the virus infects. Lipid-enveloped viruses comprise a diverse range of pathogenic viruses, which often lead to high fatality rates and many lack effective therapeutics and/or vaccines. This perspective primarily focuses on the negative-sense RNA viruses from the order Mononegavirales, which obtain their lipid envelope from the host plasma membrane. Specifically, the perspective highlights the common themes of host cell lipid and membrane biology necessary for virus replication, assembly, and budding.

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## INTRODUCTION

Viruses have been on the world's stage with the identification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19. SARS-CoV-2 is a lipid-enveloped coronavirus that assembles and buds from the host cell ER-Golgi intermediate compartment (Boson *et al.*, 2020; Plescia *et al.*, 2020) and traffics out of the host cell through lysosomes (Ghosh *et al.*, 2020). Little is known on how coronaviruses utilize host cell lipids for their assembly and budding, but based upon the presence of a transmembrane M protein, which is essential to the formation of sites of virus assembly, lipid composition undoubtedly plays a critical role. More detailed investigations will be needed into the mechanisms by which SARS-CoV-2 (and coronaviruses as a whole) interacts and modifies host cell lipid composition or lipid metabolism

(Archambault *et al.*, 2021; Pérez-Torres *et al.*, 2021) to unveil how this family of viruses interacts with membranes. Many of the methods and strategies utilized for other lipid-enveloped viruses should be applicable to understanding and ultimately elucidating the host cell lipid characteristics critical to the spread of SARS-CoV-2. With the increase in global travel and the potential for more zoonotic transmissions of novel lipid-enveloped viruses, basic research geared toward an understanding of virus–host cell hijacking should help shed light on fundamental strategies of drug targeting and perhaps opportunities for antiviral therapies.

Mononegavirales is a diverse order of RNA viruses established in 1991, initially comprised of three viral families grouped by their structure and morphology. As of 2018, the Mononegavirales has expanded to eight families encompassing some of the most infectious and fatal viruses known to cause disease in humans and animals (Amarasinghe *et al.*, 2018). Mononegavirales include both well established and emerging viruses, such as *Rhabdoviridae* (includes rabies virus, which can lead to 95% fatality in untreated cases), *Pneumoviridae* (includes respiratory syncytial virus [RSV], one of the most common human viral infections), and the highly infectious and lethal *Filoviridae* (Ebola virus [EBOV] and Marburg virus [MARV]). This order also includes the diverse family *Paramyxoviridae* (e.g., measles *morbivirus* [MeV], mumps, and *Henipaviridae* Hendra virus [HeV] and Nipah virus [NiV]; Stallcup *et al.*, 1983; Liu, 2014; Cox and Plemper, 2017; Amarasinghe *et al.*, 2018; Emanuel *et al.*, 2018).

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Abbreviations used: EBOV, Ebola virus; MARV, Marburg virus; MeV, measles *morbivirus*; NiV, Nipah virus; RdRp, RNA-dependent RNA polymerase; VP40, viral protein 40 kDa; VSV, vesicular stomatitis virus.

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Members of the Mononegavirales order are lipid enveloped and constructed from a 10–20-kb-long single-stranded nonsegmented RNA genome (Liu, 2014), which encodes 5–10 proteins (Latorre et al., 2018). These proteins subvert host processes during infection, leading to a wide range of diseases. The structure and morphology of viruses arise from a complex network of protein–protein, protein–RNA, and protein–lipid interactions. These interactions are dependent on multimeric complexes of thousands of viral proteins. All members of the Mononegavirales order of viruses carry along a core set of proteins encoded by their RNA genome. There are four principal units of Mononegavirales viral structure: a linear (–) sense genome, helical nucleocapsid structure, extensive matrix layer, and the lipid envelope studded with surface glycoproteins. The genome is protected by a nucleocapsid complex, comprised of the nucleoprotein (NP) and accessory proteins. The genome–nucleocapsid structure is encapsulated by an extensive grid-like scaffold comprised by the self-assembly of the matrix protein. Lastly, the virus is bound by a lipid envelope derived from the host plasma membrane, which is studded with glycoproteins to aid in attachment and fusion (Latorre et al., 2018) to continue the viral life cycle.

*Filoviruses* and *paramyxoviruses* contain several proteins with conserved structure and function. The matrix protein in *filoviruses* and *paramyxoviruses* is highly abundant, viral protein 40 (VP40) and matrix protein (M), respectively. The matrix protein, initially made up of dimers (Adu-Gyamfi et al., 2012a; Bornholdt et al., 2013; Oda et al., 2016; Liu et al., 2018; Wan et al., 2020), oligomerizes into an extensive matrix interlaced between the nucleocapsid complex and lipid envelope (Adu-Gyamfi et al., 2012a, 2013, 2015; Bornholdt et al., 2013; Oda et al., 2015; Liu et al., 2018b) as an assembly of dimers (Wan et al., 2020). The matrix layer is paramount to the structural morphology and integrity observed in both viral families. Within the nucleocapsid lies the NP, considered the main structural component of the nucleocapsid complex, which interacts with both the RNA genome and other viral proteins. Additionally, *filoviruses* and *paramyxoviruses* encode an RNA-dependent RNA polymerase (RdRP), coined the L protein. The L protein of *filoviruses* and *paramyxoviruses* is quite large (more than 2000 amino acids) and is the only viral protein known to possess enzymatic activity. An essential polymerase cofactor can be found in both *filoviruses* (viral protein 35, VP35) and *paramyxoviruses* (phosphoprotein, P). Lastly, critical to viral attachment and fusion are the glycoproteins. EBOV and MARV contain one glycoprotein (GP; Emanuel et al., 2018), while two are found within paramyxoviruses such as MeV (fusion protein, F, and attachment protein, H) and NiV (fusion protein, F, and attachment protein, G; Thibault et al., 2017).

## VIRUS REPLICATION

*Filoviruses* and *paramyxoviruses* have an ~19-kb nonsegmented genome with conserved gene order, encoding seven and six structural proteins, respectively (Liu et al., 2014; Thibault et al., 2017). Additionally, these viral families utilize a RdRP for viral transcription and replication. Due to the “stop-start” model of gene transcription by the RdRP on the nonsegmented RNA genome, a gradient of gene products is observed with genes at the 3′ end of the genome transcribed more abundantly than those at the 5′ end. Transcription and replication of *filoviruses* and *paramyxoviruses* takes place within the cytosol in inclusion bodies (Thibault et al., 2017; Emanuel et al., 2018).

Following uncoating of the viral matrix layer within the cytosol, the nucleocapsid complex proteins commence coordinating transcription of the (–) sense RNA genome. Transcription of the negative-sense genome must occur before replication, to produce the (+)

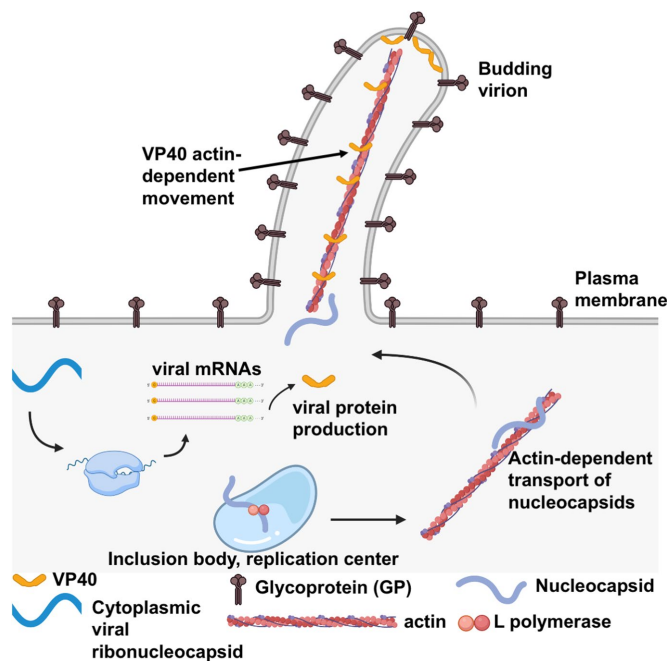
sense RNA gene products required for protein translation and the production of new (–) sense genomes. Three viral proteins have emerged as required components for replication: NP, P or VP35, and L (Conzelmann, 2004; Whelan et al., 2004; Emanuel et al., 2018). NP is an RNA-binding protein that multimerizes into a helical assembly wrapped around the genome. Upon independent expression in mammalian cells, NP forms cytoplasmic inclusion bodies, where it has been shown to interact with host RNA (Noda et al., 2010; Emanuel et al., 2018). L is a multidomain enzymatic component of the system, responsible for transcription, replication, genome capping, and polyadenylation (Latorre et al., 2018). Lastly, P/VP35 serve as a cofactor by linking the L and NP proteins (P in paramyxoviruses and VP35 in filoviruses). Moreover, it has been proposed that P/VP35 carry out chaperone functions by obstructing the self-association of NP and nonproductive associations between NP and RNA, allowing replication to complete before NP begins its helical assembly around the viral genome (Latorre et al., 2018). When expressed together, NP, P/VP35, and L colocalize in the cell and fulfill each requirement for productive viral transcription and replication.

Cytoplasmic inclusion bodies form with Mononegavirale cell infections and are thought to be the sites of virus translation and transcription (Figure 1). While the origins of such inclusion bodies (reviewed in detail in Hume and Mühlberger, 2019) are unknown for filoviruses, they resemble liquid phase-separated structures or structures enriched in membrane components. These inclusions are formed in the cytoplasm of infected cells (Hoenen et al., 2012; Dolnik et al., 2015; Kolesnikova et al., 2017) and are sites of viral RNA synthesis as well as nucleocapsid formation. EBOV nucleocapsids are transported via an actin-dependent process to sites of virus assembly at the plasma membrane inner leaflet (Schudt et al., 2013). Despite lack of mechanistic knowledge on *filovirus* inclusion body formation, a number of studies have elucidated the origins of inclusion body formation for *paramyxovirus* replication.

In the case of measles virus infection, N and P form a membraneless organelle (Guseva et al., 2020) at least in vitro. The proposed mechanism of this phase separation, which may come as a surprise, is attributed to intrinsically disordered regions in both the N and P proteins, which are sufficient to recruit RNA to the surface of the membraneless organelle (Guseva et al., 2020). The addition of RNA hexamers to N and P membraneless organelles triggered formation of nucleocapsids at a constant concentration of N. The rate at which RNA encapsulation occurred was significantly higher in phase-separated liquid droplets compared with the more dilute phase, strongly suggesting an important role for nucleocapsid assembly in the host cell cytoplasm. While the role of liquid–liquid phase separation is only beginning to unravel in Mononegavirale replication, it has become clear it likely plays a significant role in localization of viral machinery for genome replication and nucleocapsid formation. Whether or not lipids are involved in the localization and regulation of the viral replication machinery is still unknown, but warrants careful and critical further investigation into polymerase activity and localization and packaging of the viral nucleocapsid.

## PERSPECTIVES ON VIRUS ASSEMBLY, BUDDING, AND EXIT

The matrix (or M) proteins direct the trafficking of viral components to the plasma membrane. Consequently, proper trafficking of the matrix proteins to the plasma membrane is a prerequisite for efficient viral assembly and budding. Early in infection, matrix proteins are cytosolically localized, and in the case of MARV, highly associated with intracellular membranes (Kolesnikova et al., 2004).



**FIGURE 1:** Schematic of Ebola virus replication and budding. Ebola virus (EBOV) enters the host cell via fusion at the lysosomal membrane. The viral ribonucleocapsid harbors the negative-sense RNA genome and becomes cytosolic postfusion of the virus and host membranes. The negative-sense RNA genome is used as a template to produce viral mRNAs for viral protein synthesis such as that of VP40. The negative-sense genome is also used to generate a complementary strand of RNA (positive strand), which is used to generate copies of the negative-sense RNA genome that can be packaged in virions. These replication processes occur via the EBOV L polymerase in the host cell cytoplasm. Inclusion bodies (virus replication centers) have been observed in the cytosol for both EBOV and MARV and are deemed replication centers for viral RNA synthesis and nucleocapsid (NC) assembly. The biophysical and biochemical nature of these viral inclusions is still unknown and may form via liquid–liquid phase separation as shown for other virus replication centers or through selective hijacking of host membrane components. Following NC assembly, NCs are trafficked in an actin-dependent manner to sites of virus assembly where the VP40 matrix layer underlies the plasma membrane. The host cell plasma membrane and budding virions are studded with transmembrane EBOV glycoproteins. VP40 dimers are peripheral proteins that interact with the plasma membrane inner leaflet (PS and PI(4,5)P<sub>2</sub>) and a VP40 matrix layer is formed via oligomerization of VP40 dimers in an end-to-end manner. VP40 has been shown to be transported on actin in filaments extending from the plasma membrane. Virus nucleocapsids recruited to VP40 assembly sites harbor the encapsulated negative-sense RNA genome and interactions between VP40 and NP most likely stabilize the NC in filaments emanating from the plasma membrane. Following the assembly of new virions at the plasma membrane, membrane scission occurs releasing the virion (or VLP) into the extracellular space giving rise to filamentous structures with a consistent diameter (~80 nm) and variable lengths (1–14 μm). This figure was prepared in BioRender.

Interestingly, VP40 has been observed to transiently translocate into the nucleus; however, the function of nuclear import and export is not well understood in filoviruses (Del Vecchio *et al.*, 2018a). In the case of M proteins, ubiquitination and nuclear-cytoplasmic transport is required for efficient viral production of several members of the paramyxovirus family (Pohl *et al.*, 2007; Harrison *et al.*, 2010; Wang *et al.*, 2010, 2012). Additionally, there is significant

evidence of filoviral VP40 ubiquitination, suggested by pronounced interactions between VP40 and E3 ubiquitin ligases from the neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) family (Harty *et al.*, 2000; Han *et al.*, 2016, 2017; Urata and Yasuda, 2010; Zhang *et al.*, 2021).

Matrix protein-mediated viral assembly requires the spatial and temporal alignment of each viral component. Following viral replication, the ribonucleoprotein (RNP) complexes in filoviruses and paramyxoviruses traverse to the matrix protein-enriched regions of the plasma membrane via the actin network (Schudt *et al.*, 2013, 2015). In-depth live cell imaging experiments have highlighted that once at the cell periphery, filovirus RNPs are directed within filopodia by VP40 in an actin-dependent manner (Adu-Gyamfi *et al.*, 2012b; Schudt *et al.*, 2013; Takamatsu *et al.*, 2018). Additionally, VP40 itself has been shown to move in an actin-dependent manner into sites of assembly at the plasma membrane (Adu-Gyamfi *et al.*, 2012b) before RNP transport. However, the role of actin in M transport is not as clearly defined. Pharmacological inhibition of actin polymerization perturbs the assembly and release of MeV particles (Dietzel *et al.*, 2013) and a recent proteomics analysis of NiV-infected cells suggested a role for actin in NiV trafficking and release (Johnston *et al.*, 2019). Furthermore, the endocytic pathway and specifically Rab-11-positive recycling endosomes have also been implicated in MeV and NiV RNP transport (Nakatsu *et al.*, 2013; Johnston *et al.*, 2019) as well as in EBOV budding (Nanbo and Ohba, 2018; Nanbo *et al.*, 2018).

In addition to the RNPs, the glycoproteins must convene with the matrix proteins at sites of viral assembly. During viral infection, GP travels independently of other viral proteins to the plasma membrane where they are randomly distributed. Filovirus GP hijacks the secretory pathway to arrive at the plasma membrane; however, detailed reports of this observation are lacking (Becker *et al.*, 1996; Bavari *et al.*, 2002). In the case of *paramyxoviruses*, the glycoproteins (F, G, H) travel to the basolateral membrane when expressed independently in polarized cells. However, upon coexpression with the M protein, the glycoproteins are redistributed to the apical side of the cell where budding occurs (Maisner *et al.*, 1998; Lamp *et al.*, 2013). This phenomenon is also observed in MARV budding within polarized cells; however, in the opposite direction. In MARV budding, GP will localize to the apical membrane, and upon coexpression with mVP40 will redistribute to the basolateral membrane where budding will occur (Kolesnikova *et al.*, 2007).

A direct interaction between matrix proteins and glycoproteins has been established for HIV-1 (Cosson, 1996), influenza (Jin *et al.*, 1997), as well as respiratory syncytial virus (Ghildyal *et al.*, 2005). Biochemical and cryo-electron tomography (cryo-ET) studies have shown MeV and NiV M proteins directly interact with the cytoplasmic tails of F and H proteins, respectively (Rima and Duprex, 2006; Tahara *et al.*, 2007; Johnston *et al.*, 2017; Ke *et al.*, 2018). However, the function of this interaction is not well understood. Specifically, recent cryo-ET and superresolution imaging of MeV and NiV proteins has provided conflicting evidence as to whether M redistributes the glycoproteins within the apical membrane to sites of viral assembly or if glycoprotein localization is stochastic (Ke *et al.*, 2018; Liu *et al.*, 2018). Unlike paramyxoviruses, a direct filovirus matrix protein VP40 and GP interaction has not been established. However, a VP40-GP relationship is suggested by the finding that viral budding is enhanced when both proteins are expressed compared with VP40 alone (Licata *et al.*, 2004). Whether through a direct or indirect interaction, glycoproteins play an imperative role in successful viral budding.

Viral assembly is the penultimate step of the viral life cycle, followed by membrane scission and release (Figure 1). Upon congregation of the glycoproteins, matrix proteins, and RNPs within the plasma membrane inner leaflet, progeny virions mature that must be released to propagate the viral life cycle. A required step for viral assembly and scission is the generation of significant plasma membrane deformation, or negative curvature generation (Rossman *et al.*, 2010; Rossman and Lamb, 2013; Soni and Stahelin, 2014). The movement of the plasma membrane (i.e., pushing out away from the cytosol) is believed to be attributed to several factors including accumulation of viral proteins at the plasma membrane, rearrangement of the actin network, and the hijacking of host machinery such as the ESCRT proteins (Rossman and Lamb, 2013; Lee *et al.*, 2015).

Irrespective of viral morphology, *filovirus* and *paramyxovirus* virions pinch off from the plasma membrane, ready to disseminate throughout the host. The intimate role between plasma membrane lipids and Mononegavirale components has not been studied in great detail. This interface of viral assembly remains an underexplored platform for examining virus–host interactions as well as targets of drug development. An in-depth understanding of assembly processes between the plasma membrane, host proteins, and viral matrix proteins may provide the framework for a panviral therapy.

## THE HOST PLASMA MEMBRANE AND APOPTOTIC MIMICRY

Extensive experimental evidence has confirmed that viral pathogens mimic apoptotic “eat-me” signals as a mechanism to enhance cellular entry of virus in a process known as apoptotic mimicry (Moller-Tank and Maury, 2014; Amara and Mercer, 2015; Nanbo and Kawoaka, 2019). The exploitation of apoptosis has been experimentally substantiated for a diverse set of lipid-enveloped viruses, including (+) sense RNA-enveloped viruses (*alphaviridae*, *flaviviridae*) and (–) sense RNA-enveloped viruses (*arenaviruses*, and the Mononegavirales *Filoviridae* and *Rhabdoviridae*). There are currently no reports of apoptotic mimicry strategies employed by members of the *Paramyxoviridae* family; however, it is important to note that during entry of *paramyxoviruses*, the viral envelope is thought to directly fuse with the host cell, therefore no true engulfment mechanism occurs.

*Filoviruses* are one family of Mononegavirales known to exploit apoptotic mimicry to gain entry into host cells. During EBOV entry, host cell T-cell immunoglobulin and mucin domain protein 1 (TIM-1) receptors recognize phosphatidylserine (PS) residing in the outer viral envelope (Kondratowicz *et al.*, 2011; Liu, 2014). During infection, eVP40 is the main viral component postulated to initiate exposure of PS to the outer leaflet during budding (Adu-Gyamfi *et al.*, 2015). However, recent evidence reports that GP and eVP40 accomplish this mechanism through activation of Xkr8 activation (Nanbo *et al.*, 2018). In contrast, another study performed with authentic EBOV found that TMEM16F and not Xkr8 was the main host scramblase required for PS incorporation and exposure in virions (Younan *et al.*, 2018). Future investigations will need to be performed to distill a thorough understanding of PS movement across the plasma membrane in the viral budding process and if there are any cell type–specific differences.

## LIPID-INDUCED MATRIX PROTEIN ASSEMBLY AND MEMBRANE REMODELING

The structural plasticity of matrix proteins was first documented in 1982 when it was observed that the matrix protein of Sendai virus (SeV-M) self-assembled into ordered tubes and sheets in vitro

(Heggeness *et al.*, 1982). The in vitro self-assembly of matrix proteins upon association with lipids has also been widely reported across the Mononegavirales order. In the presence of PC, the *Pneumoviridae* human metapneumovirus calcium-binding matrix protein assembles into flexible tubes (Leyrat *et al.*, 2014). Further, atomic force microscopy was employed to investigate the assembly of the *paramyxovirus* Newcastle disease virus (NDV-M). Upon incubation with a negatively charged mica surface (representative of the negative charge of the plasma membrane inner leaflet), NDV-M assembled into an extensive scaffold (Shytikova *et al.*, 2018).

Similar observations have been reported outside of the Mononegavirales order using high-resolution imaging analysis. The interaction of the influenza virus matrix protein (M1; *Orthomyxoviridae* family) with anionic lipids has been long established (Baudin *et al.*, 2001). Recent investigations have aimed to delineate which lipids M1 interacts with and which M1 processes are influenced by lipid binding (Hilsch *et al.*, 2014; Bobone *et al.*, 2017). Fluorescence scanning microscopy experiments were performed utilizing fluorescently labeled M1 proteins and supported lipid bilayers containing PS, which confirmed that M1-membrane binding is mediated by PS (Hilsch *et al.*, 2014). To understand the molecular implications of this interaction, the oligomerization of M1 was monitored in the presence of PS-containing supported lipid bilayers. M1 multimerized extensively upon incubation with supported lipid bilayers containing 40% PS (Hilsch *et al.*, 2014), which is analogous to the observed self-assembly of other matrix proteins from the aforementioned EM and atomic force microscopy experiments.

During membrane remodeling, membrane shape and curvature are altered as the membrane yields new shapes, such as tubes and vesicles. This is observed throughout viral budding, as negative curvature occurs upon bending of the plasma membrane as a new virion is formed from the plasma membrane inner leaflet. Eventually, membrane scission occurs to release the new virions. Compelling evidence to support a role for matrix proteins in facilitating membrane remodeling during viral budding has been shown via the in vitro deformation of membranes in the absence of other viral components (Solon *et al.*, 2005; Soni and Stahelin, 2014; Saletti *et al.*, 2017; Dahmani *et al.*, 2019).

Early evidence of membrane deformation induced by a matrix protein was from vesicular stomatitis virus (VSV-M), a member of the *Rhabdoviridae* family of Mononegavirales. VSV-M associates with membranes through basic patches on its N-terminal domain (Solon *et al.*, 2005; Liu *et al.*, 2014; Amarasinghe *et al.*, 2018). Upon incubation of fluorescently labeled giant unilamellar vesicles (GUVs) with VSV-M, confocal microscopy revealed VSV-M induced significant membrane deformation when PS was incorporated into the GUVs. Moreover, VSV-M was shown to coalesce with fluorescently labeled PS on the surface of membranes, which resulted in invaginations of the membrane in PS-VSV-M-enriched regions (Solon *et al.*, 2005).

Membrane deformations (e.g., vesiculation and tubulation) on GUVs have been observed for a number of matrix proteins of (–) sense RNA viruses. Both *filovirus* and *paramyxovirus* matrix proteins have demonstrated the capacity to remodel membranes. When incubated with fluorescently labeled GUVs, NDV-M transformed regions of the spherical GUVs into filamentous budding-like structures (Shnyrova *et al.*, 2007). Notably, this observation was found using GUVs consisting of PC and PE (Shnyrova *et al.*, 2007) and therefore no conclusions could be drawn on how an anionic lipid such as PS may contribute to NDV-M-mediated membrane deformation. Conversely, when incubated with fluorescently labeled GUVs, eVP40 selectively induced vesiculation from PS-containing membranes (Soni and Stahelin, 2014), which was corroborated by ultrastructural

transmission electron microscopy (TEM) studies (Soni *et al.*, 2013). Furthermore, eVP40 capacity to remodel membranes was abrogated when point mutations were made in a hydrophobic loop suggested to penetrate the plasma membrane and contribute to eVP40-induced membrane remodeling (Soni *et al.*, 2013). The selectivity of PS in this process has been attributed to selective binding of PS to a cationic patch in the VP40 C-terminal domain (Del Vecchio *et al.*, 2018). VP40 was also able to cluster PS in membranes, which promoted viral budding and could be dampened by treatment with an FDA-approved drug that lowered levels of PS in cells (Husby *et al.*, 2021).

Membrane deformation was also observed in (–) sense RNA viruses outside of the Mononegavirales order. The ability of M1 of influenza virus A to deform membranes was investigated using GUVs and confocal microscopy, cryo-TEM, and fluorescence correlation spectroscopy (FCS). GUV studies highlighted that M1 binding to and deformation of GUVs was PS dependent. Moreover, FCS was used to show that M1 binding was insufficient to induce deformation, but that multimerization of M1 was responsible for deforming the membrane (Dahmani *et al.*, 2019). A similar relationship between M1 and PS has also been shown through similar techniques for influenza C virus (Saletti *et al.*, 2017).

How virus protein crowding/oligomerization on membranes induces membrane structural changes is still poorly understood; however, other related cell biology studies of the plasma membrane may give insights into some possibilities. For instance, Hirama *et al.* investigated the indirect effects of extracting cholesterol from the plasma membrane with methyl- $\beta$ -cyclodextrin (M $\beta$ CD; Hirama *et al.*, 2017). Following treatment of cells with M $\beta$ CD, the high-affinity PS-binding protein lactadherin C2 (LactC2) was significantly redistributed from the plasma membrane to intracellular membranes (Hirama *et al.*, 2017). In vitro analysis of the affinity of LactC2 to liposomes confirmed that the presence of cholesterol did not alter the affinity of LactC2 to PS (Del Vecchio and Stahelin, 2018), ruling out that the displacement of LactC2 from the plasma membrane was a result of decreased LactC2 affinity to PS when cholesterol was depleted. Moreover, M $\beta$ CD treatment resulted in an increase in the negative charge density within the inner leaflet of red blood cells (Hirama *et al.*, 2017). An increase in anionic surface charge density has been reported to lead to the generation of positive curvature to relieve electrostatic repulsion between anionic lipids (Fuller *et al.*, 2003; Kooijman *et al.*, 2003). Taken together, Hirama *et al.* presented a model where cholesterol extraction results in an increase in the anionic surface charge density of the inner leaflet, which is relieved by the subsequent generation of spontaneous positive curvature.

The observed positive curvature facilitates endocytic events and the concomitant loss of the abundant anionic lipid, PS, from within the plasma membrane (Hirama *et al.*, 2017). One could envision a similar mechanism whereby PS exposure on the outer leaflet of the plasma membrane during viral budding leads to an abrupt change in the lipid and electrostatics per surface area of the plasma membrane inner and outer leaflets with more favorable spontaneous positive curvature generation on the outer plasma membrane. More detailed mechanistic experiments are necessary on the process of viral budding to envision how and when PS, cholesterol, and other plasma membrane lipid abundancies change to fully understand the host plasma membrane contribution to the viral budding process. One distinct possibility is the formation of PS and cholesterol-enriched nanodomain formation at sites of VP40 or M assembly where transbilayer interactions can form between saturated PS with long acyl chains and cholesterol (Raghupathy *et al.*, 2015). Transbilayer

interactions can be attributed to cholesterol stabilization and ordered phase formation with PS with long acyl chains on the opposite leaflet (Raghupathy *et al.*, 2015). These types of transbilayer interactions may lead to enhanced liquid-ordered phase on the plasma membrane inner leaflet as PS becomes more significantly enriched on the outer plasma membrane during viral budding.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Viral budding by lipid-enveloped viruses is an exemplary illustration of how viruses hijack their host to support their own replication. Budding is predominately facilitated by the multifunctional properties of their matrix proteins, some of which are sufficient to form virus-like particles in the absence of other viral proteins. Moreover, the pleomorphic structure of lipid-enveloped viruses highlights the high conformational plasticity and multifunctionality of matrix proteins.

Matrix proteins transform into different higher ordered structures to execute necessary tasks. As the central organizer of viral budding, matrix proteins assemble into an extensive scaffold underneath the plasma membrane. This scaffold serves as a bridge between the viral envelope (derived from the plasma membrane) and the internal nucleocapsid-containing genome. Moreover, matrix proteins are responsible for actively recruiting viral components to viral budding sites (Baudin *et al.*, 2001; Kolesnikova *et al.*, 2007, 2012; Nanbo *et al.*, 2013). Matrix proteins may also cluster or restrict certain lipid formations to the sites of assembly and budding. For instance, EBOV VP40 clustered PI(4,5)P<sub>2</sub> beneath oligomers due to the abundance of multiple cationic basic patches (Gc *et al.*, 2016) and HIV-1 gag restricted cholesterol and PI(4,5)P<sub>2</sub> (Favard *et al.*, 2019). Although these lipid restrictions seem crucial to the viral assembly and budding process, the molecular details as to how these processes contribute to effective viral egress and infectivity is still not well understood. There are a plethora of critical questions regarding how these viruses, and especially the matrix protein, utilize the host cell lipid network for their infection and replication scheme. For instance:

1. How do matrix proteins utilize host cell lipids and/or vesicular pathways for plasma membrane trafficking?
2. Do these viruses, or their matrix proteins alone, induce lipid metabolic changes to favor lipid synthesis necessary for building new virions from the plasma membrane?
3. What cues of matrix protein assembly prompt changes in PS distribution across the plasma membrane?
4. What cofactors play a role in the initiation of membrane curvature changes in the plasma membrane at sites of assembly?

With the increasingly available technologies to image at high resolution and manipulate host cell lipid composition and trafficking pathways, many of these questions should be answered in the coming decade. While it seems likely that the cellular and biophysical details of how one virus family commandeers the host cell for virus assembly and spread will be elucidated, there are thousands of viruses in nature still unknown to humans. Thus, maintaining a keen understanding of virus–host cell targets and technologies that can be used to elucidate virus–host interactions will help prepare scientists to tackle new viruses if and when they emerge.

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