Untangling Membrane Rearrangement in the *Nidovirales*

Megan Mary Angelini,¹ Benjamin William Neuman,² and Michael J. Buchmeier^{1,3}

All known positive sense single-stranded RNA viruses induce host cell membrane rearrangement for purposes of aiding viral genome replication and transcription. Members of the *Nidovirales* order are no exception, inducing intricate regions of double membrane vesicles and convoluted membranes crucial for the production of viral progeny. Although these structures have been well studied for some members of this order, much remains unclear regarding the biogenesis of these rearranged membranes. Here, we discuss what is known about these structures and their formation, compare some of the driving viral proteins behind this process across the nidovirus order, and examine possible routes of mechanism by which membrane rearrangement may occur.

Membrane Rearrangement as a General Strategy of Positive Sense RNA Viruses

HOST CELL MEMBRANE remodeling is a tactic used by many viruses as a means of reaching their end game of using the host cell for viral production. Positive sense single-stranded RNA viruses make use of the host's internal membrane environment for viral genome replication and transcription (Ahlquist, 2006; Denison, 2008; Miller and Krijnse-Locker, 2008; Netherton and Wileman, 2011). The reasons for doing so include the need to concentrate, localize, and anchor the machinery and precursors required for transcription and aiding in shielding double-stranded RNA intermediates from activating an innate immune response. Membrane rearrangements involved in viral genome replication and transcription for some members of the positive sense RNA viruses have been well characterized and have a wide range of complexity, both of membrane involvement and numbers and types of proteins responsible for the remodeling (Kirkegaard and Jackson, 2005; Mackenzie, 2005; Novoa *et al.*, 2005; Salonen *et al.*, 2005; den Boon and Ahlquist, 2010). The typical method of designating which membranes are involved is by mapping the intracellular localization of the dsRNA replicative intermediates, nascent RNA, and replicase proteins. Once the membranous replicase structures have been identified, further characterization and ultrastructural description can occur, including the more recently applied technique of three-dimensional electron tomography (Subramaniam, 2005; Kopek *et al.*, 2007; Subramaniam *et al.*, 2007).

Although the strategy of membrane remodeling is thought to be conserved in all positive sense RNA viruses, the actual modifications and structures made and the means by which they are formed can drastically vary between viruses. Fla-

viruses induce an organized network of interconnected double-walled endoplasmic reticulum (ER)-derived membranes termed “vesicle packets” and “convoluted membranes” (Welsch *et al.*, 2009; Gillespie *et al.*, 2010; Miorin *et al.*, 2013). Picornaviruses have been shown to reorganize ER, Golgi, and lysosomes into both single and double membrane vesicles (DMVs), the latter of which are similar to autophagosomes (Suhy *et al.*, 2000; Limpens *et al.*, 2011; Belov *et al.*, 2012). Alphaviruses induce ~50 nm in diameter single membrane vesicles termed “spherules,” seen to be derived from invaginated ER, plasma membrane, and endosomes/lysosomes depending on the virus (Froshauer *et al.*, 1988; Schwartz *et al.*, 2002; Kopek *et al.*, 2007; Spuul *et al.*, 2010). Nodaviruses reorganize the mitochondrial membrane into small ~50 nm vesicles. These vesicles are single membranes and are positioned between the inner and outer mitochondrial membrane (Miller *et al.*, 2001; Kopek *et al.*, 2010).

An Introduction to Nidoviruses

The order *Nidovirales* contains families of positive sense nonsegmented single-stranded RNA viruses featuring an envelope and, notably, a mechanism of discontinuous transcription to produce nested subgenomic mRNAs for which the order is named (Latin *nidus* means nest) (González *et al.*, 2003; Gorbalenya *et al.*, 2006; Pasternak *et al.*, 2006). This order contains families capable of infecting both vertebrates (*Coronaviridae*, *Arteriviridae*, and *Roniviridae*) and invertebrates (*Mesoniviridae*) (Cowley *et al.*, 2000; Lauber *et al.*, 2013). All nidoviruses share a genome with a similar layout with the first two overlapping open reading frames (ORF1a and 1b) producing two large polyproteins (pp1a and pp1ab) that are co- and post-translationally cleaved free

¹Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California.

²School of Biological Sciences, University of Reading, Reading, Berkshire, United Kingdom.

³Division of Infectious Disease, Department of Medicine, University of California, Irvine, Irvine, California.

into the nonstructural proteins (nsps) (Brian and Baric, 2005; Britton and Cavanagh, 2008). Processing of these polyproteins is directed by nsp encoded proteinases, which vary among families and species of these viruses (Ziebuhr, 2006; Snijder *et al.*, 2013).

These nsps are part of the viral replication transcription complex machinery necessary for viral genome replication and transcription, in association with cellular membranes (van Hemert *et al.*, 2008; Hagemeijer *et al.*, 2010). ORFs downstream from ORF1a and 1b encode varying structural and accessory proteins. These structural and accessory proteins are produced from nested 3' co-terminal subgenomic mRNAs via a discontinuous transcription process (Faaberg, 2008; Hogue and Machamer, 2008). While nidovirus genomes range significantly in sequence and size, from 12.7 kb for the smaller arteriviruses to 31.7 kb for the large coronaviruses, they share some commonalities (Gorbalenya *et al.*, 2006). Figure 1 highlights the conservation across the *Nidovirales* in the pp1a-based nsps involved in membrane rearrangement. Coronaviruses and arteriviruses remain the best studied and characterized nidoviruses.

Membrane Rearrangement in the Nidoviruses

Arteriviruses and coronaviruses both induce characteristic DMVs in host cells (David-Ferreira and Manaker, 1965; van der Meer *et al.*, 1998; Pedersen *et al.*, 1999; Gosert *et al.*, 2002; Snijder *et al.*, 2006; Knoop *et al.*, 2008, 2012; Ulasli *et al.*, 2010; de Wilde *et al.*, 2013). The DMVs, so named for their distinctive two-layer delineated membranes, are interconnected with regions of convoluted membranes (CMs). Despite the fact that DMVs and CMs often appear in close and/or continuous proximity to the ER and their evidenced partial colocalization with the ER marker protein disulfide isomerase, the precise progenitor pathway from

which they are formed remains unclear (Goldsmith *et al.*, 2004; Snijder *et al.*, 2006; Knoop *et al.*, 2008).

For both the coronaviruses and arteriviruses, members of the cellular autophagy machinery have been implicated in DMV formation (Maier and Britton, 2012). The autophagosomes themselves are double-membrane vesicles, which initially suggested the possibility of this pathway's involvement. Multiple studies have shown activation of autophagy machinery upon coronavirus infection or in the presence of coronavirus proteins (Prentice *et al.*, 2004; de Haan and Reggiori, 2008; Cottam *et al.*, 2011). Additionally, microtubule-associated protein light chain 3 (LC3) has been shown to associate with the DMVs of both arteriviruses and coronaviruses and loss of LC3 had an overall negative effect on DMV formation (Prentice *et al.*, 2004; Reggiori *et al.*, 2010; Monastyrskaya *et al.*, 2013). During autophagy, cytoplasmic LC3-I becomes lipidated and studs the autophagosome, serving as a marker for these vesicles. This is in contrast to the LC3 that has been shown to decorate the DMVs, which is the nonlipidated LC3-I form, which has also been implicated in the ER-associated degradation (ERAD) pathway. Additionally, chaperone members of the ERAD machinery were shown to be present in the DMVs, suggesting a role for ERAD in DMV formation (Cali *et al.*, 2008; Reggiori *et al.*, 2010). Absence of Atg5, a critical member of the autophagy machinery, did not cause a defect in DMV formation in coronavirus-infected cells, showing that DMV formation does not require an intact autophagy pathway (Zhao *et al.*, 2007).

Nonstructural Proteins Involved in Membrane Rearrangement: Similarities and Differences

The identity of the specific viral proteins responsible for inducing membrane rearrangement in both arterivirus and coronavirus-infected cells has long been of interest. Both

FIG. 1. Conservation of double membrane vesicle (DMV)-making proteins in the *Nidovirales*. Domain annotations were based on conserved amino acid sequences (solid colors) or secondary structure patterns (diagonal stripes). Positions of transmembrane and hydrophobic nontransmembrane regions were predicted by TMHMM 2.0 (Sonnhammer *et al.*, 1998; Krogh *et al.*, 2001) and amended to reflect known topologies (Baker nsp3, Oostra nsp4, Oostra nsp3 + nsp6) wherever possible. Virus names are abbreviated as follows: Human coronavirus 229E (HCoV-229E), severe acute respiratory syndrome coronavirus (SARS-CoV), infectious bronchitis virus (IBV), muna coronavirus HKU13 (MuCoV), equine torovirus (EToV), white bream virus (WBV), fathead minnow virus (FHMV), equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), cavally virus (CAVV), and gill-associated virus (GAV). The amino-terminal region of the polyprotein is shown for CAVV and GAV because no obvious homolog of nonstructural protein (nsp)3 was detected. A jagged line denotes the uncertain position of the amino termini of EToV, WBV, FHMV, and GAV.

families have three nsps that feature putative transmembrane (TM) domains (Oostra *et al.*, 2007, 2008; Snijder *et al.*, 2013). Arteriviruses have TM domains within nsp2, nsp3, and nsp5, which appear to be homologous to coronavirus TM proteins nsp3, nsp4, and nsp6. These three proteins share conserved features between families and across the *Nidovirales*, as shown in Figure 1. Due to their presence at the DMVs and CMs and their TM domains, these three nsps were proposed as being important for membrane rearrangement. It was shown that the arterivirus equivalents of coronavirus nsp3 and nsp4 were sufficient for inducing membrane modifications similar to those seen in arterivirus-infected cells (Snijder *et al.*, 2001). Recently, we showed that coronavirus nsp3, nsp4, and nsp6 are all required to induce DMVs resembling those of virus-infected cells (Angelini *et al.*, 2013). Coronavirus nsp3 and nsp4 alone were incapable of forming full DMVs, but they were capable of creating maze-like patterns of paired membrane, agreeing with previously published immunofluorescence studies using expressed nsp4 and a truncated version of nsp3 (Hagemeijer *et al.*, 2011; Angelini *et al.*, 2013). Mutation in coronavirus nsp3 has been shown to lead to virus having a defect in DMV-formation ability, suggesting an important role for nsp5 and polyprotein processing in DMV formation (Stokes *et al.*, 2010). Coronavirus nsp4 and its arterivirus homolog have both been shown to be important for DMV formation through mutagenesis approaches (Posthuma *et al.*, 2008; Gadlage *et al.*, 2010). MHV nsp4 has been shown to be important for DMV formation. As mentioned earlier, nsp6 of coronavirus and nsp5–7 of arterivirus have been shown to induce autophagy when expressed in the absence of virus (Cottam *et al.*, 2011).

Theoretical Mechanism

As the identity of the viral proteins important for membrane rearrangement has become clear and the cellular

membranes and pathways involved begin to be distinguished, the mechanism of membrane deformation can be deciphered. DMVs and CMs are comprised of highly curved membranes and presumably this curvature is induced via the TM domains located in coronavirus nsp3, nsp4, and nsp6 (nsp2, nsp3, and nsp5 for arteriviruses) and protein–protein interactions of these nsps with each other, with other viral proteins, and with host cellular proteins. TM domains that feature conical shapes, as a product of this shape, will induce membrane curvature by forcing the membrane to accommodate and deform around the conical region in a wedge-like manner (McMahon and Gallop, 2005; Shibata *et al.*, 2009). Additionally, suites of proteins may form together as a scaffold that has the ability to deform lipid bilayers and steric effects of these membrane-interacting proteins may also aid in deformation (Schley *et al.*, 2013). Since nsp3, nsp4, and nsp6 (and their *Nidovirales* homologs) contain TM domains and work together in a scaffold-like fashion, it is possible that both of these approaches work together to induce DMVs and CMs. As we propose in Figure 2, following initial genome polyprotein translation and proteolytic processing, nsp3 (dark blue), nsp4 (teal), and nsp6 (green) remain inserted in the lipid bilayer (Figure 2–upper left inset).

The intracellular phenotypes of cells expressing both nsp3 and nsp6 suggest that these proteins may promote membrane curvature, inducing proliferated membranes and vesiculation respectively on their own. Nsp4 alone appears incapable of inducing membrane curvature but, in conjunction with nsp3, is able to produce paired membranes, suggesting that some combination of homotypic and/or heterotypic interactions is driving this pairing (Figure 2–lower right inset). Nsp3–nsp3, nsp4–nsp4, and nsp3–nsp4 interactions have all been previously identified by mass spectrometry-based approaches, yeast two-hybrid assays, and co-immunoprecipitation studies (von Brunn *et al.*, 2007;

FIG. 2. Theoretical mechanism for DMV and convoluted membrane (CM) formation using coronavirus nsps as examples. Polyprotein translation occurs from genome, featuring co- and post-translational cleavage of nsps, including nsp3 (dark blue), nsp4 (teal), nsp5 (dark green), and nsp6 (light green). Nsp3 alone produces proliferated membrane, nsp4 alone has no membrane phenotype, nsp6 alone produces vesiculation. Nsp4 has a negative effect on nsp6's vesiculation. Nsp3 and nsp4 together induce paired membranes. Nsp3, nsp4, and nsp6 together induce DMVs.

Imbert *et al.*, 2008; Neuman *et al.*, 2008; Hagemeijer *et al.*, 2011). Since this membrane pairing was not observed when using a C-terminal region of nsp3, it is likely that the scaffolding function relies on interaction of nsp4 with some region of nsp3 N-terminal to its TM domain. Addition of nsp6 changes the organization of paired membranes from remarkably consistent maze-like swirls to a mixture of heterogeneous DMVs and CMs that resembles the viral replicative organelles found in infected cells. This suggests that nsp6 modifies the long-range organization of paired membranes without disrupting membrane pairing, possibly by inducing deep curvature into the nsp3+nsp4 scaffold. Nsp4–nsp6 heterotypic interaction has been previously shown via co-immunoprecipitation and Venus reporter protein complementation assay (Hagemeijer *et al.*, 2011) (Figure 2-upper right inset). Due to the high homology of these three TM regions across the *Nidovirales*, this proposed mechanism may apply generally (Fig. 1).

The lipid environment may also be affected in nidovirus-infected cells for the purposes of membrane rearrangement. This is the case for dengue virus, another positive sense RNA virus featuring extensive membrane rearrangement (Perera *et al.*, 2012). The virus may induce changes in the lipid composition of the progenitor membranes to better facilitate DMV and CM formation as increased amounts of certain species of lipids may aid in certain types of membrane deformation (Baumgart *et al.*, 2003; Bacia *et al.*, 2005; McMahon and Gallop, 2005).

Overall, the study of membrane rearrangement in the *Nidovirales* still has many unanswered questions and a precise understanding of the mechanisms behind this process will aid in future research and potential therapies for these viruses and possibly for other positive sense RNA viruses as well.

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Disclosure Statement

The authors have no conflicts of interest.

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Address correspondence to:
Michael J. Buchmeier, PhD
Division of Infectious Disease
Department of Medicine
University of California, Irvine
3205 McGaugh Hall
Irvine, CA 92697-3900
E-mail: m.buchmeier@uci.edu

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