UNIVERSITY OF CALIFORNIA SAN DIEGO

# Shared stages in early replication and membrane rearrangements in SARS-CoV and other Coronaviruses

A thesis submitted in partial satisfaction of the requirement for the degree of Master of Science

in

Biology

by

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2022

Thesis Approval Page

The thesis of Nicholas A. Wong is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Acknowledgements

I would like to acknowledge Professor Saier for his outstanding support as the chair of my committee. His fatherly like guidance led to me to numerous publication co-authorships, a first authorship, and several scholarships.

I would like to thank Dr. Arturo Medrano Sato Luis for his bioinformatic teachings, and intellectual conversations that help everyone keep their sanity.

I would also like to acknowledge my Mother and Father for their love and support.

Finally, I would like to acknowledge my Virology and Medical Microbiology Professor, Dr. Gustafson Cindy Brown whose care for student learning and public health has reverberated in my work ethic for this Master's defense and publication on the SARS Coronavirus.

The material in this thesis is a selected excerpt from the original paper, The SARS Coronavirus Infection Cycle: A Survey of Transmembrane Proteins, Their Functional Interactions and Pathogenesis published in *MDPI-International Journal of Molecular Medicine* to which I am the primary author of, along with Dr. Milton Saier, my Committee Chair.

Abstract Of The Thesis

Shared stages in early replication and membrane rearrangements in SARS-CoV and other Coronaviruses

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Master of Science in Biology

University of California San Diego, 2022

Professor Milton Saier, Chair
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The Severe Acute Respiratory Syndrome Coronavirus-2 (SARS2) is a novel epidemic strain of *Betacoronaviruses* responsible for the Covid-19 pandemic. This virus, and its corresponding disease has claimed hundreds of millions of lives and caused major obstructions to social and economic platforms around the world. Other notable *Betacoronaviruses* include the 2003 SARS1 which caused a brief epidemic in China, and the 2011 Middle East Respiratory Syndrome Virus (MERS) which affects northern Africa and central Asia to this day. Despite issuing multiple FDA emergency-use authorized vaccines for the virus, symptomatic breakthrough cases are still possible largely due to already established variants of the SARS2 virus, in particular the *delta-variant*. With such a large spread, and propensity to evolve, the *Betacoronaviruses* are likely to remain a challenge to civilization along with the seasonal flu. In any case, preparation against novel *Betacoronaviruses* is a necessity. In this review, we investigate the early replication stages of Coronaviruses, focusing on SARS1/2. We discuss the proteins associated with genome replication, and the formation of the coronaviral replication organelle, and the discovery of a novel putative coronaviral nuclear pore protein complex. Despite major differences in amino acid sequences, the proteins associated with early replication share strong structural homology. Additionally, stages of membrane rearrangements are shared amongst all the coronaviruses. Understanding the shared mechanisms of replication amongst these viruses is the first step in discovering potential drug targets for coronavirus antivirals.

## I. Introduction:

The Coronavirses are among several +ssRNA viruses in the Order of Nidovirales. Viruses within this order are known for their ability to dramatically modulate the inner membranes of the host cell. In particular, the ER/Golgi and ERGIC are stretched and contorted to form large interconnected networks of double membrane vesicles (DMVs), convoluted membranes (CMs), large vesiculations (LVs) and other structures. All +ssRNA viruses form some replication organelle to avoid host intracellular innate immune mechanisms due to RNA-dependent RNA synthesis. Foreign nucleotides and dsRNAs are hallmarks of an infection and can be efficiently detected by cytosolic or membrane bound Toll-like receptors, and RIG-I-like receptors in the cytosol. Housing and compartmentalizing viral genome/transcript synthesis by rearranging host internal membranes into a Replication Organelle (RO) is a sensible solution to protect viral synthesis from detection. It has been strongly suggested that networks of DMVs housed the (RO) of the viruses. In support of the DMV RO hypothesis, DMVs tend to be speckled with ribosomes, house dsRNAs inside their compartments and their formation parallels viral transcription levels over time. Despite understanding how DMVs are formed through electron microscopy, scanning electron microscopy and click-chemistry, no experiment could directly associate these regions with novel RNA synthesis. However, a recent discovery of a nuclear pore was discovered on the surface of DMVs strengthening the hypothesis that the RO is at least partially composed of DMVs.

The 5’ end of the ssRNA coronaviral genome is ORF1a/ORF1ab which consists of the replication-transcription complex (RTC) genes, the RNA-dependent RNA polymerase, and proteins associated with membrane rearrangements. ORF1a includes proteins nsp1-10, and ORF1ab includes nsp1-16. A ribosomal slip site exists in this region that can extend ORF1a into ORF1ab. Proteins are directly expressed from the ORF1a/ORF1ab into polyproteins pp1a/pp1ab and are autocleaved by a papain-like proteases in nsp3 ($PL^{pro}$) and nsp5. Proteins nsp2-6 are membrane bound and critical for membrane rearrangements in coronaviruses, while nsp7-10,12-16 are directly associated with the amplification of the genome. However, nsps3 has DNA binding sites and may be a necessary protein in the replication organelle. Nearly all nsps have a secondary function associated with immune evasion and pathology (Table 1). In SARS1/2, MERS, and Murine Hepatitis Virus (MHV), nsps3-6 are not only solely responsible for the hallmark coronavirus membrane rearrangements, but also viral genome replication. RTC

transcripts are disproportionately amplified over subgenomic transcripts due to discontinuous transcription, and may indicate the importance of nonstructural proteins in Coronavirus replication (Wong & Saier, 2021).

**Table 1.** Functions and properties of corona virus nonstructural proteins nsp3-14 and structural protein Nucelocapsid protein N.

| | | | |
|---|---|---|---|
| Nsp3 | Membrane rearrangements for replication organelle formation. Viral proteolytic activity. Membrane anchoring of other viral proteins to perinuclear membranes; potential role in genome packaging. IFN antagonism. | Nsp8 | RNA binding, RNA polymerase activity and essential RdRp complex cofactor protein [74]. |
| Nsp4 | Membrane rearrangements for replication organelle formation. | Nsp9 | Novel ssRNA binding protein. May participate in RNA processing [77]. |
| Nsp5 | Viral proteolytic activity. | Nsp10 | Replicative cofactor to nsp14 [78]. |
| Nsp6 | Essential for membrane rearrangements for SARS-CoV. May induce autophagy. | Nsp12 | RNA-dependent RNA polymerase (RdRp) [79]. |
| | | Nsp14 | S-adenosyl methionine-dependent (N7-guanine)-methyl transferase, assembling cap1 structure at 5' end of viral mRNA to promote translation and avoid antiviral detection. Proofreading of viral RNA transcripts [78]. |
| Nsp7 | Cofactor for RdRp complex [74]. | N | Nucleocapsid protein, binds to viral RNAs. Necessary for packaging genome and protection from host RNAases. |

## II. Understanding the membrane rearrangements:

Membrane rearrangements in coronavirus infections begin as early as 2 hours post infection (hpi) and are indicated by a variety of structures spawned from the endoplasmic reticulum (ER), marked by a CoV induced zipper shape. Of these structures include DMVs which extend into vast interconnected networks during mid-late infection merging with the golgi. Other structures include CMs which appear as a nest of inter-folded membranes from the

ER and connect with the network of DMVs. Late stage membrane rearrangements include large vesiculations, (LVs), Vesicle Packets (VPs), Double Membrane Spheres (DMSs), Large Virion Containing Vesicles (LVCV), occasional Membrane Whorls (MWs), Tubular Bodies (TBs) and cubic membrane structures (CMSs). See Table 2 for comparisons between important membrane rearrangements amongst different Coronaviruses.

**Table 2.** Membrane structures found in various coronaviruses.

| Membrane Structures | SARS | MERS | MHV | HCoV-229E | PEDV | PDCov | IBV |
|---|---|---|---|---|---|---|---|
| **DMV** | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| **DMS** | ✓ | ✓ | ✓ | ✓ | | ✓ | ✓ |
| **CM** | ✓ | ✓ | ✓ | ✓ | ✓ | | |
| **VP** | ✓ | ✓ | ✓ | | | | |
| **Zippered ER** | ✓ | ✓ | ✓ | ✓ | | ✓ | ✓ |
| **LVCV** | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | |
| **GVP** | ✓ | ✓ | ✓ | | | | |
| **TB** | ✓ | ✓ | ✓ | | ✓ | | |
| **Interconnections** | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ (Perinuclear DMVs) | ✓ (Perinuclear DMVs) |

Approximately at the time of early RNA synthesis and expression of RTC genes, viral replication complexes begin to form within the cytoplasmic membranes. Abundant transmembrane nsps (SARS1-2 and MERS nsp3, 4 and 6 or MHV nsps2-3) being to accumulate in the ER membranes inducing unusual rearrangements. After 1-2 hpi for Beta-COVs infected cells, the ER begins taking on a zippered shape, and isolated DMVs can be seen forming in the cytoplasm in proximity with the ER. RNA synthesis becomes detectable in MERS, SARS, MHV and IBV. Large CMs (0.2-2μm) and reticular inclusions begin to form interconnected to the DMVs and ER. By 4 hpi, DMVs numbers have increased dramatically and begin to cluster in the perinuclear space. Eventually, the golgi membrane is incorporated into the now vast DMV-CM-ER reticulovesicular network. By 5-7 hpi, newly assembled and budding virus particles appear in the Golgi cisternae. Additionally, large virion containing vesicles (LVCVs) derived from the Golgi form. After 7 hpi, there may be 200-300 interconnected DMVs clustered within the cells infected with SARS. The outermembranes of these DMVs fuse together to create a system of interconnected single membrane vesicles contained in a singular outer membrane. At least 95% of these DMVs have at least one neck like connection (~8nm) to another coronavirus induced membrane structure (DMVs, CMs, zippered-ER, LVCV). This interconnected system, which connects the ER to viral induced membrane structures to the golgi and perinuclear

space indicates that late stage membrane rearrangements may be facilitating viral replication, particle manufacturing and secretion. By 10 hpi, assembly of new virions are budding into and from the modified ERGIC lumen (Wong & Saier, 2021).

As mentioned before, β-CoVs require nsp3 and nps4 for modification of cell membranes. Exactly how these nsps are capable of modifying the ER membrane to produce zippering and vesiculations remains uncertain, but indicated to be performed through nsp3 binding with nsp4 in the lumen of the ER. Disruption of nsp3 and nsp4 expression greatly inhibits RVN formation and viral genomic replication. Co-expression of nsp3, nsp4 and nsp6 (for SARS) induces clustered DMVs and disorganized double membraned CM-like structures sprouting from the ER. It is thus proposed that accessory structures other than DMVs are induced by other viral proteins or viral replication.

**II.1 Structure and Importance of Nsp3**

Although the RdRp of CoVs is composed of nsps12-16, it has become more apparent that the other nsps are more directly involved in the viral replication complex than previously understood. Nsp3 is the most multi-faceted versatile protein in the coronavirus genome. It includes several RNA binding domains, a papain-like protease, protein-protein interacting domains, a transmembrane region, and a luminal 3-ecto domain, yet its primary function seems to be forming membrane rearrangements. When attempting to pinpoint the driver of the replication organelle, nsp3 plays a pivotal role in RNA synthesis. From the N-terminal side, nsp3 contains a Ubiquitin-like domain (UBl-1) which plays a role in ssRNA binding, and possible association with N protein. Next to this domain are 3 Macrodomains (Mac1-3) which participate in poly-G nucleotide and polyA nucleotide binding. Following is a crucial 'Domain Preceding Macrodomains and Papaine-like protease' (DPUP) which folds into antiparallel beta-sheets and participates in ssRNA binding as well. Next is a second UBl-2 domain, and then the Papaine-like protease ($PL2^{pro}$) which cleaves nsps1-4 from pp1A or pp1AB. The final domain on the N-terminal side is a Nucleic Acid Binding (NAB) domain which binds to ssRNA and unwinding dsRNA. The C-terminal end spans two transmembrane regions flanking a 3-Ecto domain which lies inside the lumen of the Endoplasmic Reticulum. This 3-Ecto domain directly binds to Nsp4 luminal loops to form membrane rearranging scaffolds, and may associate with other host/coronaviral proteins in the ER. Finally, at the very end is a an amphiphatic helix (AH1) that sits on the outermembrane of the ER for stability (Lei, Kusov, & Hilgenfeld, 2018).

On the C-terminal end of Nsp3, it becomes obvious this protein has a direct involvement in RNA replication despite not being a primary component in the RdRp. UBl-1, Mac1-3, DPUP, and NAB all directly bind or associate with ssRNAs and even dsRNAs (Wong & Saier, 2021). Of importance is Mac2-3 and DPUP, which forms a complex shown to bind to polyG and polyA nucleotides indicating a possible mechanism in binding with viral or host mRNAs via polyA tails of transcripts. In SARS, deleting Mac3 prevents viral replication in viral-cDNA transfected cell studies. NAB is able to bind to both ssRNAs and dsRNAs and has dsDNA unwinding activity. This indicates nsp3's possible involvement in newly synthesized RNAs, and association with dsRNA intermediates during viral transcript replication. Lastly, UBl-1 binds not only to ssRNAs, but also associates with N protein (Lei, Kusov, & Hilgenfeld, 2018). Protein binding of N protein with nsp3 has already been proven, and disrupting this interaction proves fatal to coronaviral replication. More specifically, deleting UBl-1 from nsp3 produces a dead mutant, preventing coronaviral replication altogether. Hence, there is direct evidence that nsp3 may be facilitating not only the RdRp, but also packaging of newly synthesized genomes with nucelocapsids. Aside from the $PL2^{pro}$, UBl-1 and Mac1-3 are indicated to have secondary enzymatic properties for immune evasion.

In contrast to the diverse and enzymatic N-terminal is the less obvious transmembrane C-terminal end's role in the replication organelle. If nsp3 is not able to properly bind to nsp4, replication is also halted. Yet, the C-terminal end has no association with RNAs, nor does it have any enzymatic properties. These results indicate that the association of nsp3 and nsp4 is crucial to form membrane rearrangements and are essential to coronavirus replication and RNA synthesis. Truncating the SARS nsp3 protein to just the transmembrane region, TM1-3Ecto-Tm2-AH1 (nsp3C) and lone expression of it along with nsp4 on a plasmid still elicits infection like membrane rearrangements, zippered ERs, CMs and DMVs. However, expression of nsp3C, nsp4 and nsp6 together are necessary to produce membrane rearrangements most identical to infection-like morphologies for SARS (Wong & Saier, 2021). Unfortunately, the transmembrane region of nsp3 has not yet been confirmed.

### II.2 Structure of Nsp4

Despite nsp3 being very versatile, membrane rearrangements and replication are impossible without nsp4. As stated above, nsp3 and nsp4 associate with each other via transmembrane regions in the lumen of the ER. Nsp4 has 4 transmembrane regions and forms two luminal loops through the ER. The N-terminal $1^{st}$ and $2^{nd}$ TMSs, residues 13-35 and 280-302 respectively, form a large luminal loop while the $3^{rd}$ and $4^{th}$ TMSs, residues 315-337

and 365-387, form a smaller luminal loop (Wong & Saier 2021). However, deleting TMS1, or TMS2-4 completely prevents localization with nsp3 and nsp3C. Even swapping luminal loops between coronaviruses prevents localization indicating these loops are coronavirus specific. Within the first large luminal loop reside cysteines in residues 4-10, as well as strongly conserved glycosylation sites. Deletion of these glycosylated regions produces aberrant DMVs with large luminal spaces and increased CMs (Wong & Saier, 2021). More crucially, replacing the cysteine residues produces low levels of localization with nsp3 suggesting possible disulfide bridge amongst nsps during membrane pairing. Regions 112-164 and 220-234 within the first large luminal loop of nsp4 prevent localization with nsp3C. Moreover, two highly conserved amino acids H120 and F121 are shared amongst all beta-CoVs. Mutating these two residues completely prevents localization with nsp3C, replicon formation and thus viral replication (Wong & Saier, 2021).

## III. O' Replication Organelle, Where Art Thou?

While membrane rearrangements are the most probable sites of RNA replication in any +ssRNA viruses, the replication organelle (RO) of coronaviruses has remained elusive until now. Despite confirming that DMVs house dsRNAs, showing membrane rearranging proteins nps3 and nsp4 are critical to viral replication, and that nsp3 binds to N protein, confirming the actual location of RNA synthesis seemed to defy existing evidence of the RO. The best way to image membrane rearrangements along with RNA synthesis is to stain appropriate molecules, chemically or cryogenically fixate infected cells and image them with electron microscopy (EM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Imaging through this method provides the best resolution of inner membranes, stained molecules of interest (radioactively stained protein or radioactive uridine for nucleotides). Additionally, EM allows scientists to penetrate different depths of the cell to pear into layers of the membrane rearrangements and see inside DMVs. As imaging technology improved over the past 20 years, scientists are able to better discern and navigate cell images with greater precision than before, which may indicate why the site of RNA synthesis has escaped EM in the past (Courtland, 2018). Alternative methods to indicate localization of RNA synthesis and viral proteins is with Click-Chemistry and fluorescent imaging. However such method lacks the resolution, scale and depth that empowers EM imaging in finding the site of RNA synthesis.

Until the Covid-19 pandemic, research on coronaviruses remained sparse for the past 13 years since the arise of the 2003 epidemic strain of SARS. Despite the few researchers focusing on beta-CoVs, scientists still found

breakthroughs in imaging the Coronavirus membrane rearrangements and discovering the mechanistic drivers behind them. DMVs were found to originate from the ER, be speckled with ribosomes on the outer membrane and housed dsRNAs. However, the resolution of EM at the time could not discern any nuclear pore at the surface of DMVs which confounded virologists on how and why dsRNAs were inside the lumen of DMVs. The persisting and most intuitive explanation is DMVs are the site of RNA synthesis, but without a confirmed pore the hypothesis remained incomplete.

Fluorescent imaging of cells with radiolabeled RNA via Click-Chemistry 5-ethynyluridine uridine substitute dubbed clickU allows for rapid and clean tagging of novel RNA synthesis in cells (Hagemeijer , Annelotte, Monastyrska, Rottier, & de Haan, 2012). They tagged dsRNAs and nsps independently with unique fluorescent antibodies. In combination, the authors silenced host RNA production with actinomycin D so that only viral RNAs would be labeled. In addition, the authors tagged membrane rearranging proteins nsp3, nsp4 and RdRp protein nsp12. Since images were taken at a cell-wide scale, organelles and membrane rearrangements are undetectable so DMVs were proxy detected with nsp3,4 foci. The results were unexpected and unintuitive in respect to the RO hypothesis. Their resulting images taken at mid to late infection did not correlate novel RNA synthesis with nsp3,4 nor dsRNAs. Rather nsp12 and clickU localized in the periphery regions of cells away from nps3,4 loci [source]. Analogous studies that used classical BrU RNA labelling also noted RNAs associated with nsp8 and nsp12 first localizes with dsRAs and TM nsps, but migrates to perinuclear regions later in infection (Hagemeijer , Annelotte, Monastyrska, Rottier, & de Haan, 2012). In all cases, dsRNAs never incorporated BrU nor clickU implying that dsRNA intermediates were not catalytically active and did not participate in RNA synthesis at the time of labelling (Wong & Saier, 2021). Similar findings were found in gamma-CoVs showing less than 1.5% of RdRp nsps colocalize with dsRNAs [source]. Authors who doubted the DMV as the site of RNA synthesis considered the possibility that DMVs formed around dsRNAs to prevent detection from the host innate immune system. Yet this hypothesis disregards the supporting evidence that DMVs are essential for viral replication, the established importance of nsp3-4, and in general contradicts the common theme of +ssRNA viruses.

In 2020 a revolutionary discovery by Wolff et al. finally resolved the replication organelle conundrum. EM imaging technology finally reached the resolution to discern a small putative nuclear pore on the surface of DMVs opening the lumen to the cytosol in MHV, and SARS-CoV-2 infected cells (Courtland, 2018). This pore, dubbed the

Coronavirus crown protein, has 6-fold symmetry beginning with a 6 nm wide opening that faces the cytosol. Extending outwards from the crown opening are 6 prongs extending 13 nm outward and 14 nm away from the central axis of the opening. The pore has been stated to be analogous to the reoviridae genome packaging pore (Wolff, et al., 2020). While RNA export has not yet been confirmed, the pore is primarily composed of nsp3. The luminal side of the pore complex appears denser, and is speculated that other DMV associated proteins such as N, and nsp12 RdRp complex associates with the pore. In support of the replication organelle hypothesis, this complex could rapidly transcribe RNAs, export them through the pore, and efficiently package the transcripts with N protein. If translation of transcripts is required, ribosomes docked on the outer membranes of DMVs can quickly express viral proteins before the innate immune system can detect and react. RdRp RNA synthesis is protected and housed within DMVs, while transcriptionally dead dsRNAs remain inside the lumen of DMVs, unable to pass through the pore due to the size limitation. No mechanisms have been discovered yet, so it remains unclear how the pore could export RNAs. Other viruses such as bacteriophages, Reoviridae and Herpes have pores with catalytic activity that physically force or pump nucleotides into a capsid. The complete RdRp structure is comprised of nsp 7, 8 and 12, and forms a transcription tunnel that untangles RNAs, and polymerizes a daughter transcript. These RdRp cofactors may also associate with the pore to act as a motor, pushing RNAs from the lumen to the cytosol. Considering the functional domains of nsp3, the NAB domain may facilitate the untangling of newly synthesized RNAs by untangling daughter transcripts from the parent strand as they pass through the pore. The Mac1-3 domains bind to polyA in transcripts which may grab new RNAs as they leave the pore while UBL-1 domain binds to N protein. As transcripts feed through the pore, they may be held on at the lip or prongs of the pore long enough for N protein to encapsulate the transcripts and polymerize (Wong & Saier, 2021).

While these crowns have not been imaged outside, it is likely that these structures exist in alpha, gamma and delta coronaviruses. A second comprehensive study by Snijder, et al., 2020 also confirmed DMVs are the loci of novel RNA synthesis in contrast to the BrU and ClickU studies. In this unifying study, Snijder et al. shows the shared membrane rearrangements amongst all families of Coronaviruses, and the foci of RNA synthesis indicated by radioactive Uridine. Similar to the Click-chemistry and BrU RNA staining, they halted host DNA-dependent RNA synthesis with actinomycin D. Stained RNAs localize closest to DMV structures as opposed to CMs, DMSs and perinuclear regions of the cell. These findings together with the findings of the nuclear pore show DMVs are most likely the elusive replication organelle of Coronaviruses.

## IV. Conclusion

The reemergence of epidemic strains of Coronaviruses has occurred 3 times now in human history, and due to its worldwide spread, a new epidemic beta-CoV is sure to arise in the future. Already the delta variant complicates community immunity attempts as it can break through both Pfizer and Moderna vaccines. Hence, this necessitates a deep understanding of the shared and conserved mechanisms of the viral pathogenesis and replication cycle. In this thesis, we have reviewed the unifying membrane rearrangements that form the replication organelle, and corresponding nonstructural proteins. While evidence linking the vast network of coronaviral membrane rearrangements to the site of RNA synthesis has eluded virologists, a wealth of supporting information pointed to DMVs as the site of coronaviral replication. Primarily, nsp3's activity is evidently core to viral replication and provides researchers a plethora of potential drug targets due to its multiple domains. Amongst all CoVs, DMVs are crucial to their replication. Deleting UBL-1, Mac3, the 3-Ecto domain completely prevent viral replication, and it becomes clear these domains are likely active players in the putative nuclear pore. As the structure of nsp3 is strongly conserved amongst all CoVs, nsp3 should be considered a potential drug target for antivirals.

The material in this thesis is a selected excerpt from the original paper, The SARS Coronavirus Infection Cycle: A Survey of Transmembrane Proteins, Their Functional Interactions and Pathogenesis published in *MDPI-International Journal of Molecular Medicine* to which I am the primary author of, along with Dr. Milton Saier, my Committee Chair.

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