

New insights on the role of paired membrane structures in coronavirus replication

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New insights on the role of paired membrane structures in coronavirus replication

Highlights

1. The replication of coronaviruses, as in other positive-strand RNA viruses, is closely tied to the formation of membrane-bound replicative organelles (DMOs)
2. The proteins responsible for rearranging cellular membranes to form the organelles are conserved not just among the *Coronaviridae* family members, but across the order *Nidovirales*.
3. Here, we collect and interpret the recent experimental evidence about the role and importance of membrane-bound organelles in coronavirus replication.

1 **New insights on the role of paired membrane structures in coronavirus**
2 **replication**

3

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19

20

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22 organelle, virus factory

23

24

25 **Abstract**

26 The replication of coronaviruses, as in other positive-strand RNA viruses, is
27 closely tied to the formation of membrane-bound replicative organelles inside
28 infected cells. The proteins responsible for rearranging cellular membranes to
29 form the organelles are conserved not just among the *Coronaviridae* family
30 members, but across the order *Nidovirales*. Taken together, these
31 observations suggest that the coronavirus replicative organelle plays an
32 important role in viral replication, perhaps facilitating the production or
33 protection of viral RNA. However, the exact nature of this role, and the
34 specific contexts under which it is important have not been fully elucidated.
35 Here, we collect and interpret the recent experimental evidence about the role
36 and importance of membrane-bound organelles in coronavirus replication.

37

38 **Paired membranes associated with viral RNA**

39 All positive-stranded RNA viruses (+RNA) that infect eukaryotes are believed
40 to form membrane-bound replicative organelles, though this remains to be
41 formally tested for several families of viruses (1). One of the most widespread
42 membrane modifications caused by +RNA viruses results in the formation of
43 paired membranes, i.e. two closely apposed lipid bilayers. A growing body of
44 evidence, presented in Table 1 indicates that the paired membrane structures
45 are induced by the expression of viral proteins – most typically by parts of the
46 viral replicase. Table 1 lists the virus lineages for which there is evidence that
47 some form of virus-induced paired-membrane structure is associated with
48 viral replication. The wide distribution of membrane pairing in +RNA viruses
49 suggests that this is an effective strategy for successfully producing new
50 viruses, and that membrane pairing may somehow increase the competitive
51 fitness of these viruses.

52

53 While we can speculate that +RNA viruses may gain a fitness advantage by
54 replicating on the membranes of dedicated viral organelles, this has been
55 difficult to test experimentally. However, there are several lines of
56 experimental and genetic evidence that suggest that RNA synthesis is tied to
57 the formation of replicative organelles. Viral RNA accumulates in the

58 coronavirus organelles, suggesting that the organelles may be a site of RNA
59 synthesis (2-5). Furthermore, viral organelles are not formed when RNA
60 synthesis is stopped (6, 7). While it is clear that RNA synthesis is linked with
61 the organelles, it has proved difficult to directly test whether or to what extent
62 the process of organelle formation is necessary for the process of RNA
63 synthesis, because of the practical difficulty in separating the two processes in
64 an experimental setting.

65

66 **Structure of the organelles**

67 Electron tomography studies have revealed that the replicative organelles of
68 different nidoviruses are drawn from a repertoire of paired-membrane
69 structures, including (paired) convoluted membranes, pouch-like double-
70 membrane spherules, long paired membranes and double-membrane
71 vesicles (2, 5, 8), though studies of the more recently discovered
72 mesoniviruses and roniviruses remain poorly characterized (9, 10). A catalog
73 of the virus-induced membrane structures that have been observed for each
74 coronavirus is shown at right in Figure 1.

75

76 The common element in nidovirus-like membrane rearrangement is that the
77 membranes are paired, usually maintaining a consistent-sized gap between
78 the two membranes (reviewed here (11)). Since protein-induced membrane
79 pairing appears to be a consistent feature associated with nidovirus
80 replication, and in the absence of data carefully dissecting the relationship
81 between the shape and function of these different paired membrane
82 structures, it makes sense to refer to the resulting structures collectively as
83 double-membrane organelles (DMO).

84

85 Despite a relative wealth of structural data, it has proved difficult to test
86 hypotheses about the role of DMOs in viral replication and fitness directly
87 because DMO formation is linked so closely to replication and expression of
88 replicase proteins. Here, we will discuss the implications of two recent studies
89 that address questions about the role of DMOs in nidovirus replication (12),
90 and characterize the effects of a new DMO-blocking drug against a variety of
91 coronaviruses (13).

92

93 **Viral proteins involved in organelle formation**

94 Further evidence of the probable importance of nidovirus replicative
95 organelles for viral RNA replication comes in the form of genetic conservation.
96 Nidoviruses, and most particularly coronaviruses, are highly genetically
97 variable and contain several genus-specific or even species-specific genes
98 (14). However, there are two clusters of genes that are conserved in all
99 known nidoviruses (11, 14). The first is a highly conserved cluster of genes
100 homologous to the Severe Acute Respiratory Syndrome Coronavirus (SARS-
101 CoV) nsp3-6 (Figure 1). Expression of the membrane-anchored proteins
102 nsp3, nsp4 and nsp6 is sufficient to induce the formation of SARS-like paired-
103 membrane replicative organelles (15). The second conserved gene cluster
104 encodes the viral RNA polymerase and superfamily 1 helicase (16). The
105 conservation of membrane-pairing genes in the context of an otherwise
106 hypervariable group of viruses is a strong argument in favour of the
107 importance of at least the membrane-pairing genes for RNA synthesis
108

109 The proteins that form SARS-CoV replicative organelles have several features
110 in common with distant homologs found throughout the *Nidovirales*. We will
111 refer to the transmembrane proteins homologous to SARS-CoV nsp3, nsp4
112 and nsp6 as TM1, TM2, and TM3, respectively. The relative genomic
113 positions and functions attributed to TM1-3 in nidoviruses are shown in Figure
114 1.

115

116 Of the three proteins involved in SARS-CoV replicative organelle formation,
117 the least conserved is TM1, which has a multidomain architecture (17). Many
118 nidovirus and all coronavirus TM1 proteins contain one or more ubiquitin-like
119 domains which may help to anchor the viral RNA to the membranes where
120 replication takes place (18). Potentially RNA-binding macrodomains (19-25),
121 papain-like proteinases (26-28), other RNA binding domains (29) and a well
122 conserved but poorly understood region known only as the Y domain (17) are
123 also commonly but not ubiquitously found in nidovirus TM1 proteins. All
124 putative TM1 proteins are predicted to contain one or more transmembrane
125 domains, as shown in Figure 1. The C-terminal region of TM1, from the first

126 transmembrane region to the end of the Y domain induces membrane
127 proliferation, which in some ways resembles an autophagy response (30).

128

129 TM2 and TM3 are recognizable because they contain four or more predicted
130 transmembrane regions, and are encoded immediately before and after the
131 viral main protease (M^{pro}). Bioinformatics generally predicts an even number
132 of transmembrane spans in these proteins, which would be necessary to
133 localize M^{pro} on the same side of the membrane as all of its predicted
134 upstream and downstream cleavage sites. However there are additional
135 hydrophobic regions that are strongly predicted to span the membrane, but
136 which do not for several viruses, including most coronaviruses (31-33).

137

138 TM2 contains two potential conserved domains located between the first and
139 second transmembrane domains in coronavirus, and after the final
140 transmembrane domain in most nidoviruses. Mutations in the first non-
141 hydrophobic domain of TM2, which is the largest part of the coronavirus
142 replicase to localize on the luminal face of the membrane, have been
143 demonstrated to disrupt RNA replication and may cause defects in membrane
144 pairing (34). Deletion of the latter conserved domain of TM2, which has been
145 structurally solved (35, 36), was surprisingly well tolerated (35, 37). TM2
146 localizes to membranes, but does not induce any recognizable change to
147 intracellular membranes in the absence of other viral proteins (30). However,
148 co-expression of TM2 with full-length TM1 results in extensive pairing of
149 perinuclear membranes in both coronavirus (30) and arterivirus (38, 39).
150 Additionally, it has recently been shown that co-expression of a fragment of
151 MHV TM1 including the transmembrane region and the C-terminus with TM2
152 induced ER membrane zippering and curvature similar to the phenotype
153 observed after SARS-CoV TM1 and TM2 co-expression (40). In that report
154 TM1 and TM2 were demonstrated to interact via protein loops on the luminal
155 face of the membrane.

156

157 The maze-like paired-membrane structures that resulted from coexpression of
158 SARS-CoV TM1 and TM2 have not ever been reported in coronavirus-
159 infected cells, suggesting that this should be interpreted as a conditional, or

160 perhaps partial phenotype, that is not observed when the full viral replicase
161 polyprotein is expressed. This suggests that membrane pairing is caused by
162 heterotypic interactions between TM1 and TM2 on opposing membranes, but
163 that the final architecture of the paired membranes is dependent on additional
164 viral proteins.

165

166 TM3 largely consists of transmembrane regions, without the hallmarks of
167 amino acid conservation or predicted structural conservation that would be
168 expected for an enzyme. Overexpression of TM3 alone disturbs intracellular
169 membrane trafficking (41, 42), resulting in an accumulation of single-
170 membrane vesicles around the microtubule organization complex (30).
171 However, quantitative electron microscopy revealed that expression of TM2
172 with TM3 prevents the membrane disruption seen with TM3 expression alone
173 (30). When SARS-CoV TM1, TM2 and TM3 are coexpressed, membrane-
174 containing bodies which resembled authentic SARS-CoV replicative
175 organelles were formed. However, in each of the cell sections where DMV-
176 like membranes were observed, the membrane proliferation phenotype of
177 TM1, the paired membrane phenotype of TM1+TM2 and the single membrane
178 vesicle accumulation from TM3 were each visible, suggesting that these
179 proteins do not always colocalize efficiently when expressed from plasmids in
180 different parts of the cell instead of being expressed in the natural form as a
181 polyprotein (BWN, personal communication). This suggests that while TM3 is
182 not necessary for membrane pairing, TM3 may be necessary to induce the
183 formation of the double-membrane vesicles (DMVs) that are characteristic of
184 coronavirus replicative organelles.

185

186 **Interactions among DMV-making proteins**

187 The formation of large intracellular structures such as the maze-like
188 TM1+TM2 bodies and DMV-like TM1+TM2+TM3 bodies suggests that nsp3,
189 nsp4 and nsp6 may interact both homotypically and heterotypically. SARS-
190 CoV nsp3-nsp3 interactions have been detected in cells by yeast two-
191 hybridization (43) and GST pulldown (44), and in purified protein by
192 perfluorooctanoic acid polyacrylamide gel electrophoresis (17). While SARS-
193 CoV nsp4-nsp4 interactions were not found in yeast-two hybrid or mammalian

194 two-hybrid screens (43, 45) studies with another coronavirus did detect nsp4-
195 nsp4 interactions by Venus reporter fluorescence (46). To date, homotypic
196 interactions have not been demonstrated for nsp6 despite several attempts
197 (43-45).

198

199 Heterotypic interactions between coronavirus TM1-3 proteins have been
200 demonstrated biochemically: a TM1-TM2 interaction was detected by
201 mammalian two-hybridization (43) and weakly detected by Venus reporter
202 fluorescence (46). A TM2-3 interaction has been demonstrated by Venus
203 reporter fluorescence (46), though it did not appear in other hybridization
204 studies. A one-way interaction between the amino-terminal 192 amino acid
205 domain of TM1 and TM3 detected by yeast two-hybridization (44) has also
206 been reported. However, the apparent independence of TM1 and TM3
207 phenotypes after coexpression, coupled with the abrupt change in both
208 phenotypes in the presence of TM2 suggests that interactions between these
209 proteins may be largely mediated by TM2 (30).

210

211 **Virus-host interactions**

212 Molecular interactions between host and viral factors are observed in virtually
213 every step of the viral life cycle. Viruses rely on and manipulate established
214 cellular pathways to accommodate their needs during replication and to
215 counteract host innate immune signalling. Replication of coronaviruses is no
216 exception; while some host factors have been described in the context of viral
217 RNA replication and transcription (47), few studies have looked closely at the
218 complex interplay of host pathways in the establishment of virus-induced
219 membrane-bound replication complexes.

220

221 To date, the precise origin of DMO membranes remains elusive. DMO
222 membranes were initially suggested to derive from the early secretory
223 pathway, although the absence of conventional ER, ERGIC and Golgi protein
224 markers on viral replicative membranes argues against this hypothesis (48,
225 49). Since DMVs are reminiscent of the double-membranes of
226 autophagosomes, several lines of controversial evidence hypothesized a
227 diversion of Atg (autophagy-related) proteins and autophagosome function

228 during coronavirus replication, as it is the case for other +RNA viruses (50-
229 54). The involvement of autophagy was recently investigated in the context of
230 the avian CoV Infectious Bronchitis Virus (IBV) infections (41). The authors
231 conclude that the presence of exogenous, individually expressed IBV nsp6,
232 which localizes to the ER, induces the formation of autophagosomes in
233 contrast to other IBV replicase proteins. Additionally, although
234 autophagosomes induced by IBV nsp6 or IBV infection appeared smaller than
235 conventional autophagosomes observed after starvation of cells, they were
236 similar in size to DMVs (42). However, the data reported here do not appear
237 to support the assumption that there is a functional link between IBV nsp6 and
238 autophagosomes, and a role of the autophagy in the formation of IBV
239 replicative structures can hereby not be demonstrated. Moreover, neither
240 induction nor inhibition of autophagy seems to affect IBV replication (55).

241

242 New evidence concerning the source of membranes for CoV-induced DMOs
243 was proposed, in which Mouse Hepatitis Virus (MHV) probably co-opts a
244 cellular degradation pathway of ER-associated degradation (ERAD)
245 regulators, known as the ERAD tuning pathway (56). The ERAD pathway is
246 responsible for the turnover of folding-defective polypeptides in the ER and is
247 modulated by stress-inducible positive regulators of ERAD-mediated protein
248 disposal such as EDEM1 (ER degradation-enhancing alpha mannosidase-like
249 1) and OS-9 (osteosarcoma amplified 9). The latter assist in transporting
250 misfolded proteins into the cytosol for subsequent degradation by the
251 proteasomal system. Under physiological conditions, however, low
252 concentrations of EDEM1 and OS-9 are maintained in the ER lumen in order
253 to avoid premature degradation of proteins that are undergoing folding
254 programs (57). In this case, EDEM1 and OS-9 are selectively confined by
255 interacting with the transmembrane-anchored cargo receptor SEL1L
256 (suppressor of lin-12-like protein 1) and later released from the ER lumen in
257 small short-lived vesicles, called EDEMosomes, which rapidly fuse with the
258 endolysosomal compartments (58). This steady-state disposal of EDEM1 and
259 OS-9 is known as ERAD tuning pathway. While not relying on the coat protein
260 complex II (COPII) or Atg7, it critically depends on the non-lipidated form of
261 LC3 (LC3-I), which is recruited to EDEMosomes. However, the specific

262 autophagosomal marker GFP-LC3 does not associate with EDEMosomes,
263 which are therefore distinct structures (59).

264

265 The coronavirus MHV is hypothesized to divert the ERAD tuning machinery
266 for the generation of DMOs. Similarly to EDEMosomes, colocalization of
267 EDEM1, OS-9, SEL1L, LC3-I and double-stranded (ds) RNA is observed
268 upon MHV infection. Moreover, replication of MHV, which does not require an
269 intact autophagy pathway, is impaired upon knockdown of LC3 or SEL1L (58).
270 DMVs furthermore lack conventional ER markers and do not associate with
271 GFP-LC3 (56). Altogether, the evidence from this study strongly suggests that
272 MHV exploits the ERAD-tuning machinery to establish its replicative
273 structures.

274

275 In order to learn whether this mechanism might be common to other
276 nidoviruses, other viruses that use a similar replication strategy to MHV were
277 examined. One of these, the arterivirus Equine Arteritis Virus (EAV) has been
278 shown to require the same subset of ERAD tuning factors as MHV to ensure
279 replication (60). Recently, investigations of the even more distantly-related
280 Japanese Encephalitis Virus (JEV), which belongs to the *Flaviviridae* family,
281 revealed that it may usurp the same components of the ERAD-tuning pathway
282 as well (61). Consistent with this hypothesis, both viruses were shown to
283 replicate independently of a functional autophagy pathway. The non-lipidated
284 LC3 marker protein, which is essential for the replication of EAV and JEV,
285 associated with their replication complexes together with EDEM1 whereas
286 GFP-LC3 did not label these structures. These observations parallel the ones
287 seen for MHV but raise further questions whether this feature is even more
288 widespread amongst +RNA viruses.

289

290 Despite the resemblance of MHV, EAV and JEV in the requirement of host
291 factors for efficient replication, diversion of the ERAD tuning pathway cannot
292 be considered as a generic way of inducing replicative membranes by these
293 viral families. Probable variations within families have to be kept in mind as
294 exemplified by the comparison of DMOs from two different coronavirus genus
295 members. Indeed, IBV's recently described spherules derived from paired ER

296 membranes significantly differ from the DMO structures observed upon alpha-
297 and beta-coronaviruses infections (8, 62) and their generation might require a
298 different set of factors. Furthermore, the morphology of DMOs induced by
299 flaviviruses such as Hepatitis C Virus, Dengue virus or West Nile Virus is
300 highly heterogeneous and the identification of a common, conserved
301 membrane diversion strategy seems unlikely (63). However, it is possible that
302 the diversion of one pathway could lead to the generation of the different
303 arrangements of membrane that we collectively refer to as the DMO.

304

305 Importantly, it has been shown that, in contrast to what is observed during
306 EAV infection, endogenous LC3 does not colocalize with membrane puncta
307 induced by expression of EAV nsp2 and nsp3, and the membrane
308 modifications induced by the latter are not affected by LC3 knockdown (60).
309 Similarly, LC3 and EDEM1 were not recruited to rearranged membranes
310 induced by co-expression of MHV TM1 and TM2 (40). While this still has to be
311 proven in the context of CoV TM1, TM2 and TM3 expression, it raises the
312 questions whether LC3 participates to the biological function of DMVs rather
313 than its generation. A novel hypothesis has been recently suggested for
314 Poliovirus, according to which the virus might not only co-opt a host pathway,
315 but also divert the functional network of individual proteins (64). Host factors
316 could therefore have a proviral function during infection, distinct from the
317 function for which they have been initially described. Accordingly, this is
318 reminiscent with novel functions attributed to LC3 during cellular homeostasis,
319 cytoprotection against invading pathogens or during *Chlamydia trachomatis*'
320 intracellular life cycle (65).

321

322 **Natural variation in DMV structure**

323 The DMOs of the model coronavirus MHV take the form of perinuclear DMVs
324 which appear either singly, or grouped around and interconnected with a
325 region of paired, convoluted membrane (CM;). A recent study examined DMV
326 formation by *wild-type* MHV-inf-1 (*wt*) and five *temperature-sensitive* (*ts*) MHV
327 mutants, each of which differed from *wt* by a single amino acid substitution.
328 The panel of *ts* viruses chosen contained mutations in an interdomain linker of
329 nsp3 (TM1), M^{PRO}, the viral RNA polymerase, cap N-methyltransferase and

330 cap O-methyltransferase, respectively (6, 12, 66). With the exception of the
331 polymerase mutant, which was attenuated tenfold, these viruses produced the
332 same amount of infectious progeny as *wt* (12).

333

334 All of the mutants produced significantly smaller DMVs than *wt* virus, varying
335 from almost *wt* size to 17% smaller (Table 2). In two of the mutants that
336 produced normal amounts of infectious progeny, not only were the DMVs
337 smaller, there were only about half as many DMVs per visibly infected cell
338 compared to *wt* (Table 2). Examination of the size and number of intracellular
339 virus particles from the same samples did not reveal corresponding changes,
340 suggesting that the observed DMV phenotypes were not an artifact of sample
341 preparation. The number of CMs remained in a constant ratio to the number
342 of DMVs present, suggesting that the mutations affected production of the
343 entire DMO.

344

345 **Induced variation in DMVs**

346 The DMOs of human coronavirus 229E (HCoV-229E) include DMVs similar to
347 those observed after MHV infection (13). In testing a new antiviral called K22,
348 it was observed that infectivity, viral RNA, and DMV formation were all
349 blocked by treatment with 4 μ M K22. A time of addition study revealed that
350 K22 did not block viral entry, and had the greatest antiviral effects after virus
351 entry during the first few hours of infection, leading to the interpretation that
352 K22 inhibits a cellular or viral component involved in a post-entry, early stage
353 of viral replication.

354

355 After serial passage of the virus in the presence of K22, resistant mutants
356 were selected. Surprisingly, two independently isolated resistance mutations
357 mapped to opposite ends of transmembrane helices in TM3 (nsp6) at
358 positions H121L and M159V. The resistant viruses released similar amounts
359 of new progeny compared to *wt*, but produced only about half as many DMVs
360 per infected cell. In addition, the DMVs induced by resistance mutants
361 appeared structurally impaired. Similarly to MHV nsp4 mutants) K22 escape
362 mutants induced DMV with partially collapsed inner membranes, even when
363 K22 was not present. Moreover, the specific infectivity of those newly

364 released virions was about ten-fold lower for TM3 mutants than for *wt*. This
365 suggested that the mutations in *nsp6* conferred resistance to K22 at a cost of
366 impairing an early intracellular step in the establishment of infection.

367

368 **Fitness consequences**

369 From these experiments it was clear that HCoV-229E viruses with K22
370 resistance mutations in TM3 incurred a steep fitness cost, in the form of
371 decreased specific infectivity. There were also indications of a similar
372 decrease in efficiency in the MHV *nsp3* mutant *Brts31*, which produced
373 significantly more intracellular RNA than *wt*, but without a corresponding
374 increase in infectious progeny.

375

376 To find out if the MHV mutants also incurred a fitness cost associated with
377 producing smaller and fewer DMVs, competitive fitness assays were carried
378 out. To do this, equal infectivities of two viruses were added to the same flask
379 at a temperature where both viruses could grow normally. After 24h in direct
380 competition, the amount of each virus was quantified either by sequencing to
381 look for the *ts* mutation, or by phenotypically screening for *ts* and non-*ts* virus.
382 None of the MHV mutants tested was significantly less fit than *wt* in
383 continuous or primary fibroblasts, and two mutants were significantly fitter
384 than *wt* under the assay conditions. One of the viruses with increased fitness
385 compared to wild-type was the N-methyltransferase mutant *Brts105*, which
386 produced only half as many DMVs as *wt*. These results demonstrated that at
387 least under these experimental conditions, producing larger or more
388 numerous DMVs did not confer a corresponding fitness advantage.

389

390 **Implications for coronavirus replication**

391 When interpreting these findings, it is important to consider that none of the
392 HCoV-229E or MHV mutants tested to date has been able to replicate entirely
393 without DMOs. And while some of these tests were carried out in primary
394 cells, work in animal models was not possible because of the lack of a small
395 animal model for HCoV-229E, and because the mutations restricted the
396 growth of MHV mutants at physiological temperatures. These two studies do
397 not disprove the fundamental connectedness between coronavirus RNA

398 replication and DMO formation, but together, they reveal an unexpected
399 plasticity in the size and number of DMVs that are needed to carry out wild-
400 type amounts of RNA synthesis.

401

402 For these reasons, along with the observation that RNA replication is
403 detectable before the first appearance of organelles (67), we favour an
404 interpretation in which the organelles are a late manifestation of accumulated
405 viral proteins resulting from abundant RNA expression. In this interpretation,
406 DMOs could still play an obligate role in viral replication under specific
407 conditions or in specific cell types, but the primary role for DMOs would be to
408 increase the efficiency of either RNA production, delivery of newly
409 synthesized RNA to sites where it could be translated or packaged, and/or
410 shielding abundantly synthesized viral RNA from host cell innate immune
411 sensing pathways. These studies also suggest that at least half of the DMVs
412 present in infected cells may be in excess of what is strictly needed to sustain
413 normal levels of RNA synthesis, given that both MHV and HCoV-229E
414 mutants replicated normally despite producing only half the normal
415 complement of DMVs.

416

417 Before these studies, very little was known about the potential for natural and
418 induced variation in intracellular membrane rearrangement. The viruses
419 described in these studies all produced normal amounts of progeny virus
420 particles, and were all selected for analysis for reasons unrelated to DMO
421 formation. These represent only a handful of the available nidovirus replicase
422 mutants that have been published. From this work we can hypothesize that
423 other MHV ts mutants, or K22-resistant HCoV-229E mutants with replicase
424 defects would probably make either smaller or fewer DMVs, and a larger
425 collection of such mutants will like be highly informative to further our
426 understanding on the pivotal role(s) of DMOs in the coronavirus life cycle.
427 Hopefully the unique insight provided by these results, together with the
428 relative ease of analysis will make quantitative electron microscopy a routine
429 part of the characterization of new virus mutants. In addition, the accumulated
430 knowledge on the nature of coronavirus DMOs and the possibility to
431 experimentally interfere with DMO formation by using small compound

432 inhibitors, such as K22, will allow us to dissect similarities and differences
433 between viral DMOs and related cellular organelles.

434

435

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437

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439 project 149784; VT and PV).

440

441 **Table 1.** Evidence paired membrane structures in +RNA virus infection.
 442

| Order | Family | Host ^a | Origin ^b | Type ^c | Proteins ^d | References |
|-----------------------|-------------------------|-------------------|---------------------|-------------------|------------------------|-----------------|
| <i>Nidovirales</i> | <i>Arteriviridae</i> | A | ER | V,Z | nsp2, 3 | (38, 39, 443) |
| | <i>Coronaviridae</i> | A | ER | V,Z,S,C | nsp3+4+6 | (2, 8, 30, 445) |
| | <i>Mesoniviridae</i> | A | ER | V? | <i>nr</i> ^e | (71) 446 |
| <i>Picornavirales</i> | <i>Picornaviridae</i> | A | ER | V | 2BC, 3A | (72-75) 447 |
| | <i>Secoviridae</i> | P | ER | V? | <i>nr</i> | (76) 448 |
| <i>Tymovirales</i> | <i>Betaflexiviridae</i> | P | ER | V | <i>nr</i> | (77, 78) 449 |
| | <i>Tymoviridae</i> | P | Cp, Mt | V | <i>nr</i> | (79) 450 |
| Unclassified | <i>Astroviridae</i> | A | ER | V | nsp1a | (80, 81) 451 |
| | <i>Bromoviridae</i> | P | ER | Z,S | 1a+2a ^{pol} | (82-84) 452 |
| | <i>Closteroviridae</i> | P | <i>nr</i> | V | <i>nr</i> | (85) 453 |
| | <i>Flaviviridae</i> | A | ER | V,S,C | NS4A+4B | (86-91) 454 |
| | <i>Nodaviridae</i> | A | Mito | S | pA+RNA | (92, 93) 455 |
| | <i>Togaviridae</i> | A | Ly, ER | V,S? | P123 | (94-96) 454 |
| | <i>Tombusviridae</i> | P | Px | S | <i>nr</i> | (97) 455 |

456 ^aAnimals (A) or Plants (P)

457 ^bMembranes from the endoplasmic reticulum (ER), chloroplast (Cp), mitochondria (Mt),
 458 lysosome (Ly) or peroxisome (Px)

459 ^cPaired membranes in the form of double-membrane vesicles (V), zippered ER (Z), open-
 460 necked spherules (S), or convoluted membranes (C)

461 ^dProteins implicated in membrane rearrangements

462 ^eNot reported (*nr*)

463

464

465
466

Table 2. Differences in size and prevalence of MHV DMVs and intracellular virions (IV).

| Virus | Condition s | ts | Cells | Prevalence | | | | Size (nm) | | | |
|----------------------|----------------|-------|-------|------------|----------------------|-----|--------------------|-----------|----------------------|--------|---------|
| | | | | DMV | P value ^a | IV | P value | DMV | P value ^b | IV | P value |
| Wild-type | DBT 33°C | -- | n=323 | 6% | -- | 7% | -- | 228 ± 45 | -- | 69 ± 8 | -- |
| Brts31 | 5.5 hpi | nsp3 | n=753 | 2% | 8×10 ⁻⁴ | 7% | ns ^c | 195 ± 38 | 2×10 ⁻⁶ | 69 ± 9 | ns |
| Wild-type | | -- | n=161 | 40% | -- | 29% | -- | 228±36 | -- | 68±10 | -- |
| Brts31 | | nsp3 | n=238 | 24% | 4×10 ⁻⁴ | 25% | ns | 208±34 | 5×10 ⁻¹⁹ | 68±10 | ns |
| Albts16 | 17CI-1 | nsp5 | n=120 | 37% | ns | 19% | ns | 189±33 | 8×10 ⁻⁶⁶ | 70±8 | ns |
| Wüts18 | 33°C | nsp16 | n=140 | 36% | ns | 20% | ns | 211±35 | 2×10 ⁻¹⁵ | 67±12 | ns |
| Brts105 | 10 hpi | nsp14 | n=230 | 22% | 1×10 ⁻⁴ | 32% | ns | 220±36 | 2×10 ⁻⁴ | 69±10 | ns |
| Albts22 ^d | | nsp12 | n=320 | 13% | 1×10 ⁻⁵ | 9% | 1×10 ⁻⁵ | 204±43 | 2×10 ⁻¹³ | 68±11 | ns |

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^aCalculated by two-tailed Fisher's exact test

^bCalculated by two-tailed Mann-Whitney test

^cNot significantly different from the appropriate wild-type control

^dAttenuated growth at 33°C compared to wild-type

472 **Figure Legend**

473

474 **Figure 1.** Conservation and functional organization of the carboxyl-terminal
475 region of nidovirus polyprotein 1a. Domains that are homologous at the
476 amino acid level are shown at left in solid colors. More distantly related
477 potential homologs identified by genome position and comparison of predicted
478 secondary structures are marked with stripes. Positions of transmembrane
479 regions (black bars) and hydrophobic non-transmembrane regions (striped
480 bars) were predicted by TMHMM 2.0 (98) and amended to reflect known
481 topologies (31-33) wherever possible. Clusters of conserved cysteine and
482 histidine residues that may bind metal ions are marked with white ovals. A
483 jagged line denotes the uncertain position of the amino terminus. Regions
484 that induce membrane pairing, proliferation or vesiculation in betacoronavirus
485 SARS-CoV and arterivirus EAV are shown above and below the domain
486 annotation, respectively, and all annotations come from the references listed
487 for Table 1. Double-membrane organelles observed (x) or uncertainly
488 observed (?) in infected cells are marked at right. Virus names are
489 abbreviated as follows: white bream virus (WBV), fathead minnow nidovirus
490 (FHMNV), equine arteritis virus (EAV), lactate dehydrogenase elevating virus
491 (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), simian
492 hemorrhagic fever virus (SHFV) and wobbly possum nidovirus (WPNV).

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Figure 1
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