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In situ characterization of late Chikungunya virus replication organelle

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Running title: Cryo-ET of late Chikungunya replication organelle

22 **ABSTRACT**

23 Chikungunya virus (CHIKV) is a mosquito-borne pathogen responsible for an acute musculo-
24 skeletal disease in humans. The viral RNA genome replication occurs in membrane spherules
25 named replication organelles (ROs). In this work, we investigate the native structural
26 organization of CHIKV ROs in their cellular context using *in situ* cryogenic-electron
27 microscopy approaches at late replication stage. We observed previously unreported diameter
28 heterogeneity of ROs at the plasma membrane of infected human cells. CHIKV ROs were
29 only marginally detected in cytopathic vacuoles where they are homogeneous in size,
30 suggesting a finely regulated internalization process. Our data show that ROs maintained at
31 the plasma membrane beyond the first viral cycle are dynamically active both in viral RNA
32 replication, in its export to the cell cytosol, but also in the production of viral proteins. We
33 suggest that late CHIKV ROs have an amplifying role or represent an alternative pathway in
34 the production of infectious viral particles. All these observations bring new insight into the
35 CHIKV life cycle in human cells.

36

37 **Keywords:** Alphavirus, Chikungunya, Replication organelles, nsP1, cryogenic-electron
38 microscopy, sub-tomogram averaging

39 INTRODUCTION

40 Positive-stranded RNA viruses replicating in the host cytoplasm dramatically remodel
41 intracellular membranes into specialized vesicular structures supporting viral RNA genome
42 replication and transcription^{1,21,52}. Generally separated from the cytoplasm by a proteinaceous
43 pore, these viral replication organelles (RO) provide an optimal micro-environment that
44 concentrate metabolites, and viral and host components required for genome replication. They
45 also presumably shield double-stranded RNA (dsRNA) replication intermediates from innate
46 immune sensors and antiviral effectors, and may additionally coordinate genome replication,
47 viral translation, and new particle assembly. Deciphering ROs organization, biogenesis, and
48 mechanisms of maintenance at the molecular level, therefore, represents an area of intense
49 interest with potential consequences for therapeutic intervention. The recent advance in
50 cryogenic-electron microscopy (cryo-EM) and cryogenic-electron tomography (cryo-ET),
51 sub-tomogram averaging and 3D reconstruction techniques has provided a substantial
52 advance in elucidating the 3D volume architecture of ROs assembled by a variety of
53 pathogenic RNA viruses (e.g. nodaviruses, flaviviruses, bromoviruses, tombusviruses,
54 coronaviruses)²⁻⁹. Contrasting with this significant breakthrough, our knowledge of
55 *Alphavirus* ROs is still incomplete.

56 *Alphaviruses* (Togaviridae family) are mosquito-borne viruses that can cause a severe human
57 illness including persistent arthritis and fatal encephalitis. Among these, the Chikungunya
58 virus (CHIKV), responsible for severely debilitating and often chronic rheumatic disease¹⁰⁻¹²,
59 has become a major public health issue, notably due to the rapid spreading of its mosquito
60 vectors *Aedes aegypti* and *Aedes albopictus*^{13,14}. CHIKV is an enveloped virus of about 50-
61 60 nm in diameter¹⁵. Its replication cycle has been the focus of an intense attention, resulting
62 in the following picture¹⁶⁻¹⁸. Replication is initiated when the viral genome, a positive-sense
63 single-stranded RNA molecule of 11.8kb containing a 5'-cap and a 3' polyadenylated tail is

64 loaded by the host translation machinery. The 5'-proximal two-third of the viral genome
65 corresponding to the first open reading frame (ORF) is translated into a nonstructural
66 polyprotein precursor (P1234) which associates with an RNA genome and is trafficked to the
67 plasma membrane¹⁹. P1234 sequential proteolytic processing results in the release of the four
68 nonstructural proteins (nsP1 to 4), which assemble to form the viral replicase. During this
69 reaction, this complex replicates the RNA genome through the synthesis of a negative-strand
70 RNA template ((-)RNA), resulting in the accumulation of double-stranded RNA (dsRNA)
71 replication intermediate species^{20,22}. Starting from the (-)RNA, CHIKV replicase also
72 controls the transcription of a 26S subgenomic RNA (sgRNA) corresponding to the 3' ORF,
73 translated into five structural proteins: capsid (C), envelope and surface glycoproteins (E1,
74 E2, E3), and 6K/TF, required to form the nascent viral particle.

75 Alphavirus genome replication takes place in bulb-shaped ROs, referred to as spherules first
76 assembled at the plasma membrane (PM)^{1,21,23,24}. For some alphaviruses, these compartments
77 undergo endocytosis to form intracytoplasmic vacuolar structures, positive for lysosomal and
78 endosomal markers, referred to as cytopathic vacuoles (CPV)²⁵. Membrane targeting of the
79 entire replication complex (RC), composed of nsP2, the RNA helicase and cysteine protease
80 responsible in polyprotein processing^{26,27}, nsP3, which contains an ADP-ribosyl binding and
81 hydrolase activity²⁸ and associates with a variety of proviral cell factors²⁹, and nsP4, the
82 RNA-dependent RNA polymerase³⁰, is guided by nsP1, the viral capping enzyme^{21,31}. This
83 540-long amino acid methyl/guanylyltransferase behaves as a monotopic protein with a
84 preference for anionic lipids and cholesterol-rich microdomains³²⁻³⁴. Mutagenesis
85 investigations assigned such affinity to the α -helix fold of the central part of nsP1 and to the
86 presence of palmitoylated cysteines which, despite not being required for membrane
87 anchoring, target nsP1 to lipid rafts and support nsP1's capacity to reshape the cell
88 membranes³²⁻³⁵. Membrane binding is critical for activation of nsP1 S-adenosyl-L-

89 methionine (SAM)-dependent methyltransferase (MTase) and m⁷GTP transferase (GTase)
90 activities which ensure viral RNA 5' cap synthesis ^{34,36,37}. Recent ultrastructure analysis
91 provided critical insights in the organization of recombinant nsP1 interacting with artificial
92 membranes ^{38,39}. These studies revealed nsP1 capacity to assemble into dodecameric rings of
93 14 nm in diameter with a central pore of 7.5 nm, interacting with the lipid bilayer. This
94 proteinaceous ring was proposed to contribute to the structural maintenance of replication
95 compartments and to mediate export of newly synthesized RNA to the cytosol where they are
96 translated or trafficked to assembly sites. The location of the active enzymatic site to the
97 internal face of this ring is finally thought to ensure the simultaneous capping of the multiple
98 RNA molecules trafficked through this pore. Despite this recent knowledge, the validity of
99 this model in infected cells as well as the global *in situ* spherule organization remains unclear.

100 In the present work, we have investigated CHIKV spherules structural organization in their
101 cellular context using *in situ* cryogenic-electron microscopy approaches, combined with sub-
102 tomogram averaging approach. To access a dynamic view of CHIKV replication cycle, we
103 imaged the infected cells at 17h post-infection (hpi), a time point that encompasses a second
104 and possibly a third CHIKV replication cycle that is 6-8h long. We observed CHIKV ROs as
105 single membrane vesicles in continuity with the plasma membrane, present both on the cell
106 body and filopodia-like extensions. Contrasting with previous electron microscopy
107 observations performed after one viral cycle (6-8h), we observed a wide-diameter distribution
108 of ROs. Associated with these structures, we visualized the presence of extruding filaments
109 resembling viral RNA, tightly associated with ribosome-like densities and additional
110 complexes nearby, arguing for a strong CHIKV translating activity at the plasma membrane.

111 In addition, we also observed that a part of ROs, with a smaller and more homogeneous
112 diameter, is internalized into cytosolic vacuoles. The sub-tomographic averaging of the RO
113 neck evidenced that, in infected human cells, nsP1 forms a ring compatible with that

114 assembled *in vitro*^{38,39} strongly interacting with the PM lipid bilayer and provides additional
115 details for CHIKV ROs organization in human cells.

116 **RESULTS**

117 **CHIKV replication sites preferentially localize at the plasma membrane of human**
118 **epithelial cells**

119 *Alphaviruses* ROs, which concentrate the four virus-encoded nsPs and double-stranded RNA
120 (dsRNA) replication forms are first assembled at the plasma membrane (PM) of the infected
121 host cell, and later internalized and fused with endosomal compartments to produce type-1
122 cytopathic vacuoles²⁵. We first questioned the localization of CHIKV replication sites in the
123 virus permissive HEK293T epithelial cell line infected with a CHIKV reporter virus (BNI-
124 CHIKV_899 strain) containing a mCherry coding sequence in nsP3 hypervariable domain⁴⁰.
125 To increase the probability to detect replication events, we used an MOI of 50, and to access
126 the different replication steps the cells were infected for 17h, a duration that allows several
127 replication cycles (estimated for alphavirus to around 6-8h) to occur^{11,41} (Figure S1).
128 Replication sites were identified by immunostaining of the four nsPs together with dsRNA,
129 which represents a good RCs marker, and by confocal microscopy. In our experimental
130 conditions, colocalized fluorescence signal is mainly detected at the plasma membrane (PM)
131 (Figures S1A and S1B). Interestingly this signal is observed in cellular extensions detected in
132 these cells (Figure S1B, merge), suggesting that replication compartments take place both on
133 the cell body and at the PM limiting virus-induced filopodia-like extensions previously
134 reported in *alphavirus*-infected vertebrate cells⁴². Finally, a minor fraction of dsRNA
135 colocalizes with both nsP1 and nsP3 in cytosolic aggregates, supporting that CHIKV
136 replication complexes undergo some marginal endocytosis events as suggested⁴³. According
137 to these characteristics, CHIKV replication sites detected at the plasma membrane of cell
138 bodies or cellular extensions are suitable for cryo-EM investigation.

139

140 **Cryo-electron tomography of CHIKV ROs reveals their colocalization on cell body and**
141 **filopodia-like extensions plasma membrane of infected cells**

142 HEK293T cells were directly grown onto gold EM grids, infected with CHIKV, and
143 embedded in vitreous ice. Compared to confocal experiments, we increased cell density to
144 maintain sufficient cells onto grids after their freezing, still keeping a high multiplicity of
145 infection (MOI 50) and 17h of CHIKV infection. We collected more than 300 cryo-
146 tomographic tilt series of the infected cell peripheries that were processed in etomo⁴⁴ and
147 segmented using Amira software (Thermo Fisher scientific) (Figure 1). These tomograms of
148 CHIKV-infected cells are in agreement with confocal observations, revealing the presence of
149 numerous filopodia extending from HEK293T cell bodies. The ROs appear as clusters of
150 round vesicles of variable diameter covering the PM. They are delimited by a lipid bilayer
151 connected to the PM through a narrow neck (Figure3 1A and 1C). Analyzing spherules
152 content, all compartments imaged contain a compactly coiled filamentous density which
153 corresponds to dsRNA packed inside as previously observed for Nodavirus ROs³. No
154 additional inner density is observed, indicating the absence of an internal coat contributing to
155 membrane curvature. Contrasting with this appearance, CHIKV virions are observed as
156 spherical particles of about 50-60 nm in diameter, highly electron-dense, and covered by
157 protruding densities (Figure 1C). In the present study, most CHIKV replication spherules are
158 located at the PM limiting the cell body of infected cells (Figure 1A) while filopodia-like
159 extensions exhibited some ROs colocalized with most CHIKV budding particles (Figure 1C).
160 The computed 3D reconstructions granted us access to the connection of ROs with the PM
161 and other cellular structural details of ROs local cellular environment, notably revealing the
162 presence of cytoskeleton filaments or cell host factors like ribosomes. When both ice
163 thickness and defocus values used during the tilt series acquisitions are low, the level of
164 details observed in images can be high. Thus, it is possible to discriminate the double-layer

165 organization of the plasma membrane or observe the repetitive nature of actin filaments
166 present in filopodia-like extensions (Figure 1C), some good indicators of the datasets intrinsic
167 resolution. It is worth noting that CHIKV infection induces local cellular re-arrangement.
168 While filopodia normally appear as cellular extensions containing a continuous bundle of
169 parallel actin filaments in close contact with the PM, actin filaments in CHIKV-induced
170 filopodia-like extensions bearing ROs and/or CHIKV viral particles are separated from the
171 PM by a space in which cellular compounds like ribosomes accumulate (Figures 1 and 2
172 versus S2). This phase separation suggests a cellular activity that certainly relies on the
173 CHIKV viral cycle, possibly associated to the production and assembly of viral particles.
174 Indeed, as exposed in Figure 2 (blue arrows), filopodia-like extensions also display CHIKV
175 budding sites near ROs, often associated with a thickening of the PM, a characteristic recently
176 correlated to the insertion of immature envelope spikes in the lipid bilayer⁴⁵ (Figure 2, yellow
177 arrows). Furthermore, ROs from the aforementioned region bear some extra densities
178 protruding out of their membrane (Figure 2, red arrows). Considering their proximity to
179 budding sites, these densities likely correspond to CHIKV envelope glycoproteins. This
180 unexpected feature is in agreement with recent immuno-labeling experiments of CHIKV-
181 infected cells with anti-E2 antibodies reported by Jin et al.⁴⁶, revealing some signals at the
182 surface of ROs.

183

184 **CHIK ROs display variable size distribution**

185 Alphavirus ROs have been initially reported to display a standard diameter of around 50 nm
186^{1,47}. As mentioned above, CHIKV replication compartments detected in our experiments
187 primarily appeared unexpectedly heterogeneous in size (Figure 1), an observation also
188 validated by CHIKV infected cells plastic sections performed at 17 hpi at 4 MOI (Figure S3).
189 To objectify this observation, we measured the diameter of more than 3000 ROs from cryo-

190 tomograms. As ROs can be elongated perpendicularly to the PM, we considered the maximal
191 diameter of ROs parallel to the PM (Figure S4). In this context, we determined the diameters
192 of 2,083 and 950 ROs from PM of cell bodies and filopodia-like extensions respectively, and
193 plotted them as a histogram representation (Figure 3). RO diameters display a Gaussian
194 distribution ranging from 40 to 140 nm with a mean of 84.2 nm and 90 nm for ROs located at
195 the PM of cell bodies and filopodia-like extensions respectively (Figure 3A). Thus, we only
196 observed a slight shift of the mean diameter between ROs localized on the PM of cell bodies
197 and filopodia. It is worth noting that we never observed ROs with a diameter small than 40
198 nm. Moreover, the biggest ROs, having a diameter superior to 120 nm, are only found at the
199 PM of cell bodies. While these observations support that ROs assembled at the PM of cell
200 bodies can be slightly bigger than those detected on filopodia, this feature is not entirely clear,
201 especially due to the overall unexpected ROs size variability observed in our experiments.

202 In a closer analysis of our data, considering the whole ROs population, we discerned several
203 RO diameter maxima of 55, 64, 74.5, 84, 91, 100, 122, and 139 nm (Figure 3B). It is worth
204 noting that some very recent cryo-EM studies reported RO with diameters of about 50 to 70
205 nm formed 6-8h after CHIKV infection^{48,49}, a time point at which CHIKV first replication
206 cycle is admitted to be complete, resulting in the presence of mature ROs containing fully-
207 processed nsPs and dsRNA replication intermediates. Contrasting with this experimental
208 scheme, our observations were set at 17h after the CHIKV challenge. Previous molecular
209 studies established that the volume of ROs and consequently their diameter tightly correlates
210 with RNA genome length and indirectly with the size and amount of neosynthesized genetic
211 material contained in these compartments^{3,50}. Considering this information and to the late
212 time point considered in this study, we propose that ROs having a diameter superior to 80 nm
213 may correspond to ROs in which the viral RNA continues accumulating. Accordingly, the
214 idea of finite final size of ROs needs to be reconsidered according to the post-infection time.

215

216 **CHIKV internalization in cytopathic vacuoles is regulated**

217 Our confocal microscopy and electron microscopy analysis reveal that after a prolonged
218 CHIKV infection, most ROs remain at the PM. To confirm this peculiarity and investigate the
219 presence and structural organization of ROs internalized in CPVs, CHIKV-infected cells were
220 processed according to the cryo-EM method of vitreous sectioning (CEMOVIS)⁵¹. CPVs
221 were rarely detected in vitreous sections, and usually at vicinity of mitochondria (Figure 4).
222 They appear as vacuolar structures ranging from 200 to 500 nm in size, delineated by a lipid
223 bilayer and containing budded spherules. From a morphological point of view, these
224 organelles are similar to ROs located at the PM with a lipid membrane delimiting an internal
225 area full of ball-of-yarn-like densities and connected to the CPV membrane envelope via a
226 neck. No obvious additional density could be observed in the cytoplasm beneath these
227 spherules. Although we did not observed in our vitreous sections, CPVs are often associated
228 with honeycomb arrangement of capsid proteins (Figure S5) (José et al. 2017). Contrasting
229 with previous observations on related Alphavirus^{1,41,52}, CHIKV spherules appear
230 disorganized in these compartments, instead of lining the vacuole membrane, a feature that is
231 also observed in plastic sections observed by conventional electron microscopy (Figure S5).
232 Interestingly, in these compartments, ROs display a rather homogeneous diameter of about
233 50.6 nm ± 6 nm (Figure 4), therefore contrasting with the heterogeneous size detected at the
234 PM. This unexpected homogeneity in size argues for a regulated internalization mechanism
235 susceptible to occur at a defined step of CHIKV replication cycle.

236 **Late CHIK ROs exhibit heterogeneous patterns**

237 Considering the above-discussed ROs size heterogeneity, the functionality of compartments
238 with an unexpected diameter may be questioned. In this context, we performed a thorough
239 inspection of our cryo-tomograms intending to report all different types of molecular patterns

240 of ROs, taking into account the size of these organelles and the presence or absence of
241 densities inside and immediately below spherules. In Figure 5, we display different views of
242 ROs according to their diameter, which ranges from 50 to more than 120 nm. This exploration
243 reveal that whatever the size of ROs, the lumen of spherules is full of ball-of-yarn-like
244 densities, with a state of compaction that seems similar, suggesting a high RNA replication
245 activity inside these compartments. It is worth noting that all ROs display a clear density at
246 the base of spherules which separates the inner space of spherules from the cell cytoplasm and
247 that seems in continuity with the PM. Thus, spherules seem to stick on the PM. Exploration of
248 cytoplasmic areas immediately beneath ROs revealed variable patterns, varying from the
249 absence of clear associated densities below spherules (Fig 5A; thumbnails 1 to 5) to the
250 presence of compact or filamentous densities extruding from ROs (Fig 5B; thumbnails 6 to
251 12). Therefore, elongated densities can be observed just beneath spherules, displaying various
252 length ranging from 10 nm (Fig 5, thumbnail 6) to more than 100 nm (Fig 5, thumbnail 8-10,
253 12). Some of these filamentous objects are covered by small globular densities, forming a sort
254 of pearl necklace (Thumbnail 8). This pattern reminiscent of RNA covered with translating
255 ribosomes suggests a local translating activity⁵³, which is in agreement with the local
256 presence of CHIKV budding particles and immature envelope spikes accumulation in close
257 proximity as in Figure 2. It is important to note that these long exported viral RNA molecules
258 were only observed for ROs larger than 90 nm in diameter. Just beneath some spherules, two
259 elongated, short and parallel densities can be observed, perpendicular to the PM plane and
260 located on either side of the neck as in thumbnails 7 to 9 (in dark blue in the schematic
261 representations). Finally, additional isolated globular densities are observed, possibly attesting
262 to the presence of translated viral proteins and/or cell host factors recruited to replication sites.
263 Interestingly, no clear correlation could be made between these various ROs profiles and
264 compartment size. Altogether, these different patterns of ROs deviating from the canonical 50

265 -70 nm size, strongly suggest that ROs maintained at the PM after the first viral cycle display
266 viral RNA replication, its export and translating activities.

267

268 **The CHIK ROs display a crown-like structural organization of the spherule neck**

269 ROs assembled by (+)RNA viruses are thought to be connected to the cytoplasm by a pore
270 complex localized at the spherule neck, allowing the import of metabolites and cell factors,
271 and facilitating the export of newly synthesized viral RNA to the cytoplasm for translation
272 and packaging. Two recent cryo-EM single particle analysis studies revealed that recombinant
273 alphavirus nsP1 assembles into a dodecameric ring that is proposed to take place at the
274 spherule neck^{38,39}. We investigated the possibility of transposing this model to our infected
275 cultures at late replication stages. As previously mentioned above, we noticed the presence of
276 a strong density at the base of each spherule neck, sometimes giving the impression that the
277 PM of the infected cell is continuous and that ROs are just sitting on top of it. To decipher the
278 molecular organization of the connecting region, we developed a sub-tomogram averaging
279 (STA) approach focused on ROs neck. The STA workflow is described in details in Figure
280 S6. Briefly, a total of 463 sub-volumes were extracted and subjected to iterative alignment,
281 averaging and classification steps, using full rotational symmetry, resulting in a sub-
282 tomogram average with an estimated resolution of 25 Å (gold standard FSC, FSC=0.143
283 threshold) from a final subset of 98 spherules. The dedicated use of masking and classification
284 was instrumental in sorting out spherule necks morphological heterogeneity, such as
285 variations in spherules diameter, neck length, curvature of the membrane at the base of the
286 neck, as well as perpendicularity of spherules main axis to the PM plane. The bilayer
287 organization of the membrane is clearly revealed in a central slice through the final
288 reconstruction (Figure 6A) with two linear densities corresponding to the hydrophilic lipid
289 polar heads encompassing a dark region corresponding to the hydrophobic core of the plasma

290 membrane. The continuity of the lipid membrane bearing two clearly defined leaflets indicate
291 that our STA strategy was able to successfully sort out morphological heterogeneity. The
292 reconstruction reveals a high negative curvature of the PM at the base of the neck, with an
293 average angle of 65 degrees. Additional protein densities are tightly associated with the lipid
294 bilayer at the neck base and extend to the cytoplasmic side. This neck complex is composed
295 of three regions: a membrane-bound ring, a barrel-like density composed of three stacked
296 rings present in the cytoplasmic moiety and a central elongated density passing throughout all
297 the rings. The first membrane-bound ring has an outer and inner diameter of 18 nm and 8 nm
298 respectively and is formed by a density showing a tilted “C-like” shape, where the opening of
299 the “C” points toward the PM. Recently, dodecameric cryo-EM structures assembled from the
300 recombinant nsP1 have been resolved and proposed to form a connecting pore separating the
301 inner part of the spherule from the cytoplasm^{38,39,49}. The atomic structure of this nsP1 ring
302 perfectly fits into the density of the membrane-bound ring evidenced in our reconstruction
303 (Figures 6B-D). The conical shape of the ring exhibiting a larger, flared base is related to the
304 tilted arrangement of nsP1 in the dodecamer. It is worth noting that while the PM outer layer
305 has a continuous organization, the inner layer engages extended contact with nsP1. This is in
306 agreement with the role depicted for MBO loop 1 and 2, corresponding to nsP1 amino-acids
307 200-238 and 405-430 respectively in the recombinant nsP1 ring, that forms amphipathic
308 membrane-binding spikes penetrating by about 10 Å into detergent FC12 micelles. This tight
309 interaction is proposed to be reinforced by a triad of palmitoylated cysteines in MBO loop 2
310³⁹. Nevertheless, our observations support additional interactions of nsP1 residues on the
311 outermost domains of the nsP1 ring with PM phospholipids. Going throughout the nsP1 ring,
312 a second central and elongated complex displays two prominent densities, one located on the
313 spherule side and the other more pronounced located in the cytoplasmic part, is observed. As
314 we applied a full rotational symmetry, we cannot accurately determine the shape and the

315 stoichiometry of individual compounds involved in this elongated density. Finally,
316 surrounding the cytoplasmic part of this central density, the additional barrel-like assembly
317 composed of three stacked rings does not show obvious direct interaction with the nsP1 ring
318 or the central elongated complex.
319

320 **Discussion:**

321 In the present study, with the aim at providing a new insight into the CHIKV life cycle in
322 human cells, we investigated the native structural organization of CHIKV ROs in their
323 cellular context using *in situ* cryogenic-electron microscopy approaches. This study
324 considered late infection time to access the dynamic of ROs through the study of multiple
325 infectious cycles in a single cell. Microscopy analysis of cells infected with Sindbis or
326 Semliki Forest alphaviruses previously revealed that after initial assembly at the PM²¹, ROs
327 initially visualized as bulb-shaped organelles in continuity with the PM are generally rapidly
328 internalized and fused with endosomes to form large vesicles with a diameter up to 2 microns,
329 named type I cytoplasmic vacuoles (CPV-I) where RNA synthesis continues^{43,52}. These
330 modified endosomes formed after 3-6h of infection also support the translation of viral
331 proteins and nucleocapsids^{52,54}. Here, we investigated the outcome of CHIKV ROs after 17 h
332 of infection. This strategy revealed that CHIKV ROs are mainly located at the PM of
333 HEK293T cells and attesting of the poor internalization capacity of these compartments as
334 previously reported by others⁴³. We detected CHIKV ROs at the surface of the cell body and,
335 also contrasting with SINV (José et al. 2017), on filopodia-like membrane protrusions were
336 they colocalize with budding viral particles and with patches of membrane-anchored densities
337 attributed to CHIKV envelope glycoproteins. This colocalization indicates the absence of
338 exclusion process between spherules formation and particles budding sites. Interestingly,
339 additional densities were found inserted in spherules delimiting membranes. While
340 unexpected, recent immunolabelling studies revealed the presence of anti-E2 antibody
341 staining at the surface of CHIKV spherules⁴⁶. Together with this observation, our results raise
342 the question of the possible functional consequences of such incorporation. A direct cell-to-
343 cell transport of budded particles to uninfected mosquito cells, involving the E2 envelope
344 glycoprotein was previously reported⁵⁵. Whether it is tempting to speculate it could be a

345 direct way for cell-to-cell transmission of CHIKV genetic material through filopodia, a recent
346 hypothesis on the incomplete maturation state of these glycoproteins rather argues for a side
347 effect resulting from the co-localization of replication and budding events, with no associated
348 functional role. This question is therefore still open.

349 Surprisingly, CHIKV spherules imaged in the current study, whatever their localization on the
350 PM, are heterogeneous in size, with a mean diameter of 90 nm, therefore contrasting with
351 previous observations mainly performed from other model *alphaviruses* (i.e. SFV and SINV)
352 or even for CHIKV for which ROs diameter was reported ranging from 50 to 70 nm at 6-8 hpi
353 ⁴⁸, a time corresponding to the end of the first viral cycle. Analyzing more closely CHIKV
354 ROs size distribution revealed at least three main categories. ROs with maximal diameter of
355 84-100 nm represent >60% of total events, while compartments with a size of 40-83 nm or
356 101-152 nm were more rarely detected. As the size of ROs was correlated with RNA genome
357 length contained in these compartments ^{3,50}, and that the spherules of 50-70 nm were proposed
358 to contained a single copy of the viral genome in double-stranded form ⁴⁸, it signifies that the
359 vast majority of CHIKV ROs observed at the PM at 17 hpi, corresponds to ROs maintained at
360 the PM after the first viral cycle, continuing to replicate and accumulate viral RNA in their
361 lumen. The variation of ROs diameters at the PM contrasts with homogeneity of spherule
362 diameters in CPVs (50-60 nm). As the size of ROs in CPVs is very close to that of CHIKV
363 ROs detected by others at the PM after 6-8 hpi ^{48,49}, this argues for a regulated internalization
364 mechanism, appearing at the end of the replication cycle, it means in the 6-8h after CHIKV
365 infection. Consequently, we suppose that during the first 6-8h of infection, the spherules
366 replicate RNA, growing up to the optimal size of about 50-70 nm. Then, a small part of these
367 spherules is internalized in CPV, supporting the translation of viral proteins and
368 nucleocapsids, likely explaining the presence of honeycomb arrangement capsid protein
369 structures at vicinity of CPV (Figure S5). Therefore, the ROs internalization likely represents

370 one pathway leading to the production of new infectious particles. However, it has also been
371 shown that CHIKV can still replicate even if internalization of ROs is blocked ⁵⁶.
372 Consequently, this raises the question of the fate and possible role of CHIKV spherules
373 staying at the PM after the first viral cycle.

374 As previously mentioned, the ROs observed at the PM at 17 hpi, corresponds to ROs
375 maintained at the PM after the first viral cycle, whose RO diameters distribution superior to
376 80 nm reflects the continuous transcription of the various CHIKV RNA species in these
377 compartments (i.e. (-)RNA, (+)RNA and subgenomic RNA). Determining the RNA species
378 and copy number packaged in these various spherules remains a challenging issue to solve.
379 Since SFV, SINV and CHIKV genomes are comparable in size, enlarged CHIKV ROs may
380 reflect subtle differences in replication mechanisms. In this context, it would be also very
381 interesting to explore the size of SFV or SINV ROs at longer infection times.

382 The presence of filamentous densities resembling RNA or ribosome-decorated RNA only
383 extruded from ROs was observed only for ROs with diameters larger than 90 nm. Hence, this
384 observation reinforces the idea of sequential models in which spherules would reach an
385 optimal size, and therefore complete (-)RNA synthesis, before the replication complex
386 switches its activity from (-)RNA synthesis to transcription and export of (+) stranded
387 genomic and subgenomic RNAs ^{25,43}.

388 Now, focusing on the architecture of ROs remained at the PM after the first viral cycle, cryo-
389 EM and cryo-ET both revealed a density that separating the RO lumen from the cell
390 cytoplasmic compartment. The sub-tomogram approach allowed us to compute a 3D
391 reconstruction of the spherule neck, which forms a crown, a structural feature also found for
392 FHV ^{3,4} or SARS-Cov-2 ⁹ replication organelles. It is worth noting that the fully symmetrical
393 volume of the crown is not compatible with the fit of nsP1 and its partners (nsP2, nsP3 and
394 nsP4) according to a 1:1 molecular ratio (nsP1: partner). This was confirmed by the cryo-EM

395 single particle analysis of complexes assembled using recombinant CHIKV nsP1, nsP2 and
396 nsP4 encoded by the related O'nyong-nyong *Alphavirus*, which assigned the central disk at
397 the base of the neck crossed by an elongated density to the dodecameric nsP1 in interaction
398 with the viral polymerase nsP4 facing the RO lumen and the nsP2 helicase-protease facing the
399 cell cytoplasm⁴⁹ (Figure S7). The second ring of densities is supposed to correspond to nsP3
400 and associated cellular factors, including G3BP.

401 Thus, the structural organization of RO neck revealed in our study is in agreement with that
402 described recently from infected cells after 6-8 hpi^{48,49}, meaning that the crown-like
403 organization of the replication complex persists in ROs maintained at the PM even after 17 h
404 of infection, arguing again for the functionality of ROs in our experimental conditions.
405 Interestingly, the close inspection of ROs revealed that the crown organization of the complex
406 is often difficult to see in individual sub-tomograms. Indeed, several patterns are mainly
407 observed where all densities present in the tomographic reconstruction are not simultaneously
408 present. Hence, the sub-tomogram averaging method provides an overview of the CHIKV
409 replication complex at the base of the neck of spherules that is not completely realistic if the
410 dynamical of the complex is not considered.

411 In conclusion of this study, we show that late CHIKV ROs are maintained at the PM beyond
412 the first viral cycle, exhibiting a normally folded replication complex at the base of the neck,
413 displaying an unexpectedly range of large RO diameters that reflects the role of these
414 organelles in the viral RNA replication, with the ability also to export the viral RNA to the
415 cytoplasm of cells where it is seemingly translated. All these points indicate that the late ROs
416 are still active. This picture was not detected for ROs with a size below 50-70 nm. The
417 observation of extruded RNA filamentous densities exclusively beneath ROs with a diameter
418 greater than 90 nm supposes a sequential model in which spherules would reach an optimal
419 final size before the replication complex switches its activity to (+) stranded genomic and

420 subgenomic RNA transcription and export. In this dynamic picture, at the end of the first viral
421 cycle, a few spherules of about 50-70 nm in diameter could be marginally internalized in
422 CPVs according to a regulated process. At this time, spherules activity is switched from RNA
423 transcription to its export and translation, resulting in the viral protein synthesis revealed by
424 the presence of honeycomb arrangement of capsid proteins at vicinity of CPVs. Meanwhile,
425 ROs maintained at the PM are actively involved in the production of new RNA genomes and
426 new viral proteins required for assembly of nascent infectious viral particles, then also
427 representing an alternative pathway if RO internalization failed. All these observations bring
428 new insight into the CHIKV life cycle in human cells, highlighting the functional role of late
429 ROs.

430

431

432 **EXPERIMENTAL PROCEDURES**

433 ***Materials***

434 **Cells.** Human Embryonic kidney (HEK293T, ATCC #ACS-4500) and BHK-21 cells (ATCC
435 #CCL-10) were used for viral propagation. African green monkey (Vero ATCC #CCL-81)
436 cells were used for viral titration. All cells were cultured in Dulbecco modified Eagle medium
437 (DMEM, Thermo Fisher Scientific) supplemented with penicillin, 10% foetal calf serum
438 (FCS, Lonza) and grown at 37°C, in a 5% CO₂ atmosphere.

439 **Viruses.** The pCHIKV-LR-5'GFP (LR-OPY1 isolate) and the CHIKV-377-mCherry (BNI-
440 CHIKV_899 isolate) full-length molecular clones containing GFP and m-cherry reporters
441 respectively ^{33,40,57} were transcribed *in vitro* using the mMMESSAGE mMACHINE kit
442 (Invitrogen, AM1344) following manufacturer's instructions. RNA (1µg) was then
443 transfected with Lipofectamine 2000 (Thermo Fisher Scientific) into 105 HEK293T cells and
444 incubated at 37°C 5% CO₂ for 24h. Supernatant was harvested, and virus stock was amplified
445 using BHK-21 cells. After 48 hours culture supernatant was collected, filtered through
446 0.45µm, aliquoted and stored at -80°C. Viral stocks were titrated using Vero cell plaque
447 assays as previously described (Bernard et al., 2010). The virus assays were performed under
448 BSL-3 safety conditions.

449 **Immunofluorescence assay.** CHIKV infected HEK293T cells grown on a glass coverslip
450 were fixed with 4% formaldehyde/PBS (Sigma-Aldrich), permeabilized with 0.1% Triton X-
451 100 in PBS, and blocked in PBS–2% FCS. Incubation with primary antibody was performed
452 at 37°C for 1h at room temperature, and secondary reagents were added for 30 min at 37°C.
453 Nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole; Sigma-Aldrich). After the
454 final washes, coverslips were mounted with ProLong Gold antifade reagent (Invitrogen).
455 Images were acquired using an Ayriscan superresolution microscope (Confocal Zeiss

456 LSM880 Airyscan) at the Montpellier Resources Imaging (MRI) platform. Image processing
457 and colocalization analysis were performed using ImageJ software ⁵⁸.

458 **Sample preparation - cryo EM.** Cells were cultured directly on gold grids (R2/1, carbon
459 film, Quantifoil) after being glow-discharged using a Pelco GD system. HEK293T cells were
460 infected at the MOI of 50 for 17h in the growing medium and then 4% PFA fixed. After a
461 brief wash with PBS, grids were loaded on the Mark IV Vitrobot system tweezers (FEI).
462 Before 22 seconds of Whatman paper blotting at a -5-force offset, 3 μ L of 10 nm BSA-treated
463 fiducial gold particles (BBI solutions) were added on both sides of the grid. The chamber was
464 kept under 100% humidity and at room temperature conditions. Grids were then rapidly
465 plunged frozen in nitrogen-cooled liquid ethane and stored in liquid nitrogen waiting to be
466 imaged.

467 **Electron microscope setup – tilt series acquisition.** For cryo-data acquisition, we used a
468 Titan Krios cryo-TEM equipped with a field emission gun, operating at 300 kV (IRBA,
469 Institut de Recherche Biomédicale des Armées, Brétigny-sur-Orge, France). Images were
470 recorded on a Falcon III direct electron detector (Thermo Fisher Scientific). Regions of
471 interest were recorded using MAPS software (Thermo Fisher Scientific). Tomographic tilt
472 series were acquired with Tomo4 (Thermo Fisher Scientific) at a magnification of 29,000 \times
473 and exposed for 1.3-1.55 seconds, with a physical pixel size of 0.2931 \AA and continuous
474 focusing and tracking controls. Tomograms were collected with 2° tilt increment for a range
475 spanning from -60° to $+60^\circ$ according to a dose symmetric acquisition tilt scheme ⁵⁹, using
476 defocus values ranging from -3 to -12 μ m. The total electron dose per tomogram was between
477 120 and 140 electrons/ \AA^2 .

478 **Tomographic reconstruction.** In general, tomograms were reconstructed similarly as
479 described elsewhere ⁶⁰. Movies were firstly averaged and corrected using the *MotionCorr*

480 software ⁶¹. Tomogram reconstruction was then performed with Etomo from IMOD ⁴⁴. The
481 tilt series were coarsely aligned using cross correlation. CTF curves were estimated with
482 *CtfPlotter* implemented in IMOD ^{62,63}. To remove noise coming from high frequencies due to
483 beam damages during tilt series acquisition, dose filtration was performed ⁶⁴ and antialiasing
484 filter was used. To this end, the *MtfFilter* function of IMOD was used, considering the
485 cumulative dose of each image taken to apply increasing lowpass filters to consecutive
486 images. The tomograms were reconstructed alternatively by weighted back-projection (WBP)
487 and by SIRT (simultaneous iterations reconstruction technique) using 8 iterations and
488 subsequently binned by 2, resulting in pixel sizes of 0.2931 nm (un-binned). To further
489 facilitate visualization, a non-linear anisotropic diffusion (NAD) filter provided by IMOD is
490 applied to the 2 times binned tomograms. Segmentation and animation were done using
491 Amira software (Thermo Fisher Scientific).

492 **Sub-Tomogram averaging.** All sub-tomogram averaging steps were performed using the
493 EMAN2 package ^{65,66} in version 2.9.9 unless stated otherwise. Masks were created with the
494 EMAN2 *e2filtertool.py* and *e2proc3d.py* programs.

495 *Pre-processing.* Movie fractions were motion-corrected using MotionCor2 to compensate for
496 beam-induced sample motion. A custom python script was used to automate that process and
497 build a motion-corrected tilt-series stack, relying on the *newstack* and *clip* programs from the
498 IMOD package ⁴⁴.

499 *Tilt-series alignment.* A total of 38 tilt-series were aligned using EMAN2 landmark-based
500 iterative approach and CTF estimation was performed. Low-pass filtered, binned by 4
501 tomograms were reconstructed for visualization and particle picking only.

502 *Particle picking and extraction.* We repurposed the *e2tomo_drawcurve.py* *boxer* program,
503 originally designed to pick particles along filaments to pick orientation aware spherule
504 particles on bin 4 tomograms. Two points (3D coordinates) were assigned to each particle

505 deemed suitable for analysis. One point was placed at the estimated center of the spherule
506 neck and designates the center of the particle to be extracted. A second point was set inside
507 the spherule compartment, towards its main axis as a way to manually assign an approximate
508 orientation to the particle. Those coordinates were stored in .json files. Stacks of per-particle
509 CTF corrected 2D sub-tilt-series were extracted and corresponding 3D sub-tomograms
510 reconstructed at binning 4 using `e2spt_extract.py`. The `-curves` option was set so as to read the
511 particles orientation metadata and encode it in the extracted particle stacks header. The
512 `e2spt_extract.py` script was modified so that it accepts particles with only 2 points defined and
513 avoids doing any coordinates interpolation. A total of 463 binning 4 particles, 384 unbinned
514 pixels in size were extracted from 38 tilt-series.

515 *Initial model generation.* A low-resolution initial model was generated at binning 8 using the
516 iterative stochastic gradient descent (SGD) approach implemented in `e2spt_sgd.py`. Initial
517 particle orientations read from their header were used as a seed for refinement. C57 symmetry
518 was used during alignments and averaging as a way to improve the signal to noise ratio and
519 force any rotational symmetry around the spherule main axis without applying any specific
520 symmetry order. C1 refinements did not reveal obvious symmetry in the data, therefore we
521 carried on using C57 symmetry for the downstream processing steps.

522 *Model refinement and classification.* Gold-standard single model refinements were done using
523 the `e2spt_refine_new.py` program while multi-reference refinements employed
524 `e2spt_refinemulti_new.py`. A first refined model was generated at binning 4 taking the initial
525 model as a reference and using a large soft spherical mask, 192 unbinned pixels in diameter,
526 for alignment. Particles orientations were refined within a search range of 16 degrees around
527 their initial orientations determined at picking time. A first round of classification by iterative
528 multi-reference refinement was performed using the same mask. Three references were
529 initialized by randomly splitting particles in three sub-sets and averaging them. One class

530 containing 166 particles was selected for further refinement. Particles belonging to that class
531 were re-extracted at binning 2, with a size of 256 unbinned pixels, and a new model was
532 refined from them. To tackle remaining heterogeneity in the data, a second round of
533 classification without alignment was performed inside a tighter soft threshold based mask.
534 Out of the four classes, the major class containing 98 particles was further refined inside a
535 similar tight mask. For that last round of iterative refinement, both traditional iterations of 3D
536 particles alignment refinement and 2D sub-tilt refinement including translation and rotation
537 were performed.

538 *Validation and post-processing.* The gold-standard Fourier shell correlation (FSC) curve
539 calculated from two independently refined half-maps using the same mask as the one used for
540 refinement indicates an estimated resolution of 25 Å at FSC=0.143. The final map was
541 Wiener filtered by the masked FSC.

542 **High-Pressure Freezing and vitreous sectioning.** The CHIKV-infected HEK293T cells
543 were inactivated in 2,5% glutaraldehyde and 20% dextran-sodium cacodylate 0,1M. Pellets
544 were frozen on copper tube carriers using the EM ICE high-pressure freezer (HPF, Leica).
545 After HPF, the sample was processed by CEMOVIS (Cryo-Electron Microscopy of Vitreous
546 Sections). Vitreous sectioning was performed on infected cells tubes containing the vitrified
547 sample pre-cooled at -140°C on an EM UC6/ FC7 cryo-ultramicrotome (Leica Microsystems,
548 Vienna). The sample was trimmed to a pyramidal shaped block of 140 µm base and
549 approximately 50 µm height using a 45° cryo trimming diamond knife (CT441; Diatome,
550 Biel, Switzerland). A cryo immuno diamond knife (MT15692; Diatome, Biel, Switzerland)
551 with a clearance angle of 6° was used to get ribbons of cryo-sections at a nominal cutting feed
552 of 50 nm and at cutting speeds of 40 mm/s. Ribbons of cryo-sections were attached to pre-
553 cooled copper grids (hexagonal, 100 mesh with carbon and formwar), ready to be analysed
554 under the Titan Krios.

555 **Figure legends**

556 **Figure 1: The CHIKV replication organelles are observed at the plasma membrane of**
557 **limiting cell body and filopodia-like extensions of infected cells. A and C.** Sections in
558 electron tomogram of a HEK293T cell infected for 17h at MOI 50. Spherules are connected to
559 the PM through a narrow neck (white arrows). Some cytoskeleton filaments present beneath
560 the PM are indicated by black stars. **B and D.** Segmentation of (A) and (C) showing the
561 presence of CHIKV ROs (white arrows) and budding CHIKV viral particle (black arrows)
562 onto the same cell filopodia-like extension. The bilayer organization of the PM (black star)
563 and the repetitive nature of actin filaments (low-pass filtered inset) are clearly visible. Scale
564 bar; 50 nm.

565

566 **Figure 2: Tomogram section of CHIKV-induced filopodia-like extension.**

567 **A.** Electron tomogram of a CHIKV-induced filopodia-like extension. While CHIKV particle
568 are detected at the PM (blue arrows), CHIKV ROs appear less dense and larger (black stars).
569 Some additional densities are anchored into ROs limiting membrane (red arrows). A
570 thickening of the PM can appear locally (yellow arrows). Scale bar, 50 nm. **B.** Schematic
571 representation of A. Actin filaments are represented in light purple, ribosomes in green, cell
572 PM in brown, viral particles in red, CHIKV ROs in light brown, the dsRNA in light blue,
573 immature spikes in yellow, nsP1 putative ring in pink, and some replication partners in dark
574 blue.

575

576 **Figure 3: CHIKV replication organelles diameter distribution.**

577 **A.** Histogram distribution of ROs diameters computed from 2,083 measurements for ROs
578 located on cell bodies (red) and 950 measurements for ROs located on cell filopodia-like
579 extensions (green). Both populations fit with a Gaussian distribution with a mean diameter of
580 84.2 and 90 nm for cell body and filopodia-like extension ROs respectively. **B.** Histogram
581 distribution of ROs diameters considering all ROs, corresponding to 3033 measurements.
582 Curve fitting deconvolution unveils eight Gaussian sub-populations whose mean diameters
583 are indicated.

584

585 **Figure 4: Cryo-EM vitreous sections of CHIKV infected cells reveal the presence of**
586 **cytopathic vacuoles.**

587 **A and B.** Some RO-filled cytopathic vacuoles (CPV) (black arrows), 200 to 500 nm in
588 diameter are observed in vitreous sections. They are delineated by a lipid bilayer and often
589 found at vicinity of mitochondria (M). Scale bar, 50 nm.

590

591 **Figure 5: CHIK ROs display numerous patterns**

592 ROs can be sorted according to their diameter, ranging from 40 to 140 nm and to the absence
593 (A) or presence (B) of clear associated density patterns, just beneath the PM. A schematic
594 representation of each pattern is also presented. The PM is drawn in black, the spherule
595 membrane in brown, the envelope spikes decorating ROs membrane in yellow, the base of
596 ROs neck in red, the viral RNA in blue, ribosomes in green, intracellular filaments in purple
597 and viral or cell host partners in deep blue.

598

599 **Figure 6: Structural organization of CHIKV RO connection to the plasma membrane**
600 **revealed by sub-tomogram averaging**

601 **A.** Central section of the 3D reconstruction. As a visual aid, the location of the spherule
602 membrane is drawn as a white dotted line. The PM appears as two parallel white linear
603 densities encompassing a dark region. Several densities highlighted by the white and black
604 arrows are resolved at the level of the RO neck, as well as a C-shaped density (star)
605 corresponding to the nsP1 ring snugly interacting with the PM at the base of the neck.

606 **B.** Central section of the surface representation of the RO connection to the PM. The
607 dodecameric nsP1 ring atomic model (PDB: 6ZOV) fits the C-shaped ring density. The
608 central channel is plugged by an elongated central density protruding toward the cell
609 cytoplasm (white arrow) while a second tall barrel-like density composed of three stacked
610 ring (black arrows) show no apparent connection with others densities.

611 **C.** Surface representation of the map viewed from the interior of the spherule. A ring is
612 present at the base of the spherule in which the dodecameric nsP1 atomic model fits perfectly.

613 **D.** Model of nsP1 ring insertion in the PM internal layer that supports a tight interaction of
614 nsP1 MBO loops with the PM. The insertion of the palmitoyl moieties into the PM is indicated
615 in red.

616

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623

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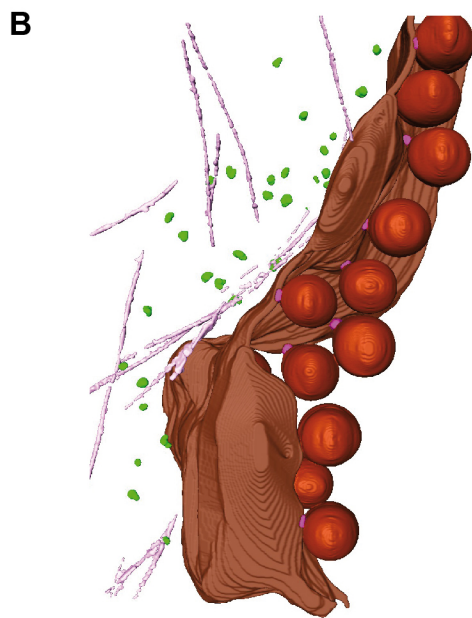
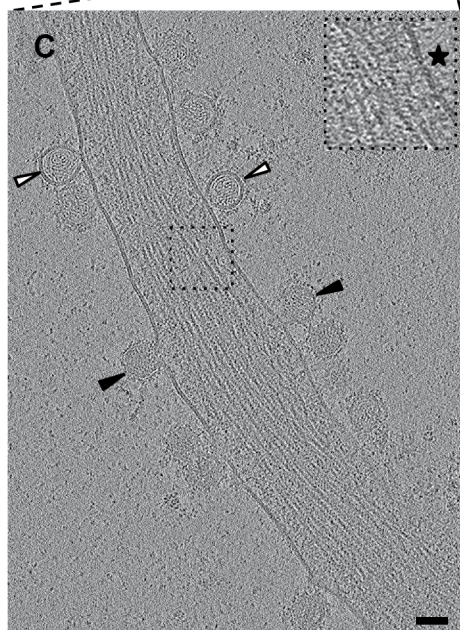
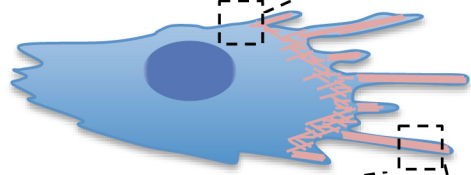
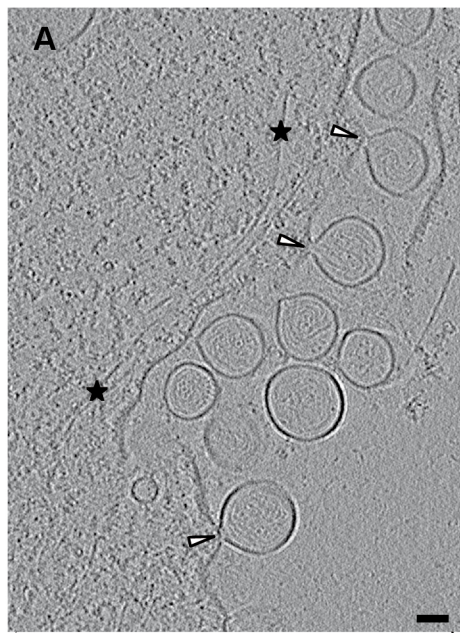
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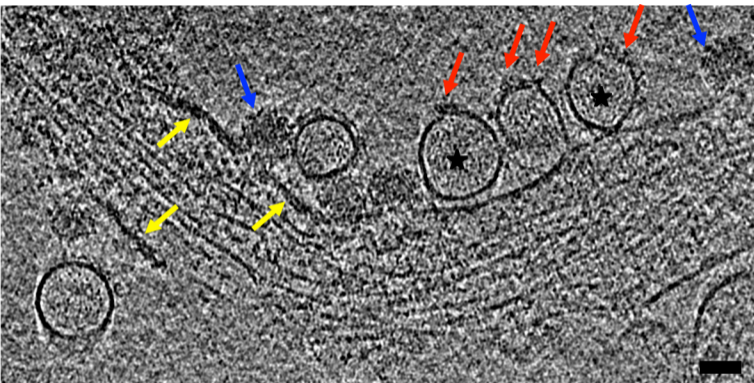
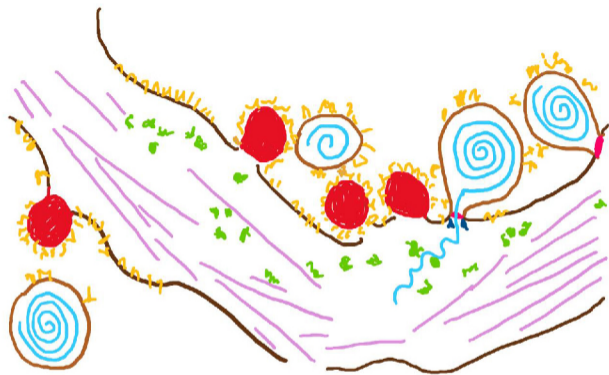
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- 846



- Cell membrane
- Actin filament
- replication organelles (RO)
- Additional densities on RO
- Viral particles
- dsRNA
- nsP1 ring
- Ribosomes



Fig. 1

A**B****Fig. 2**

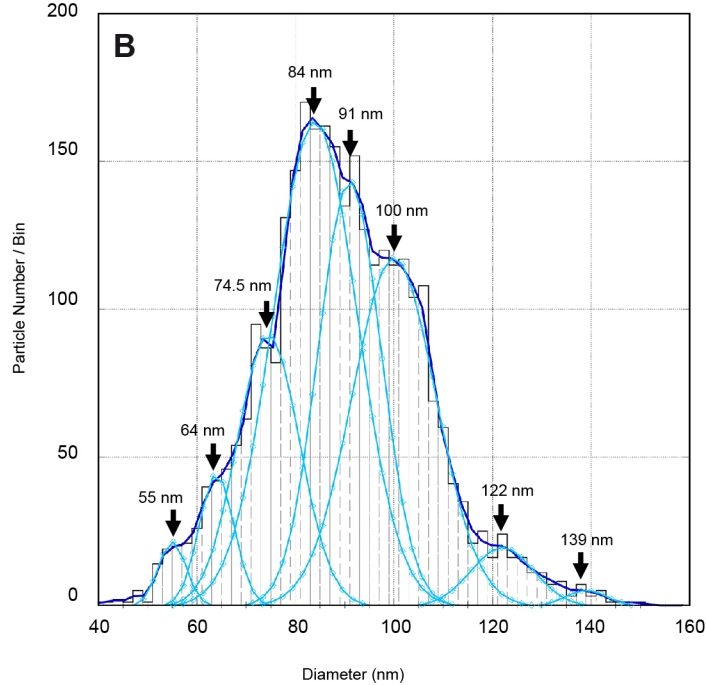
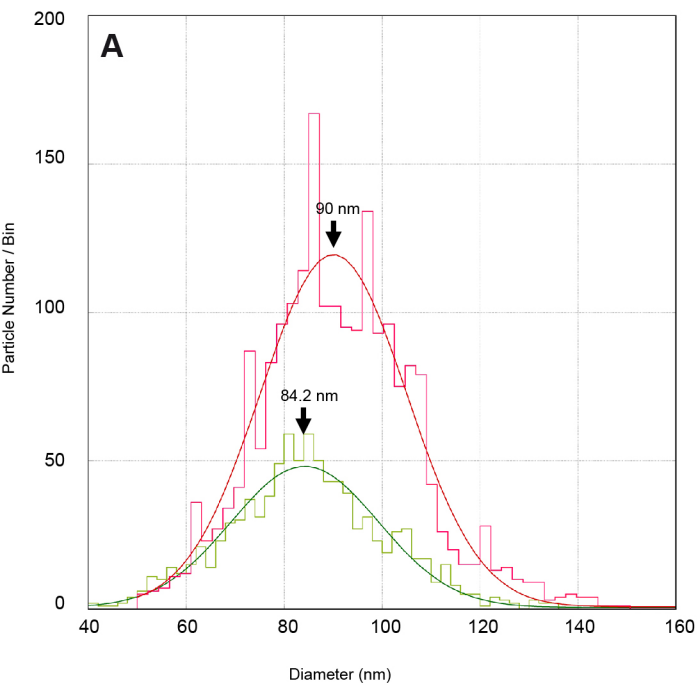


Fig. 3

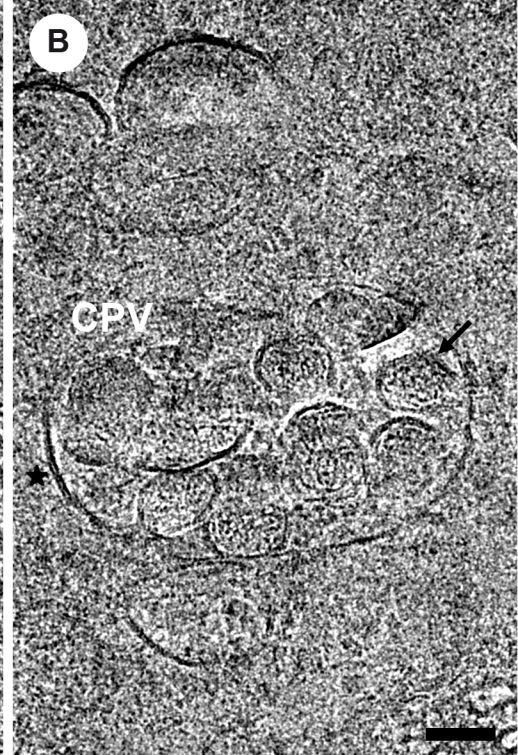
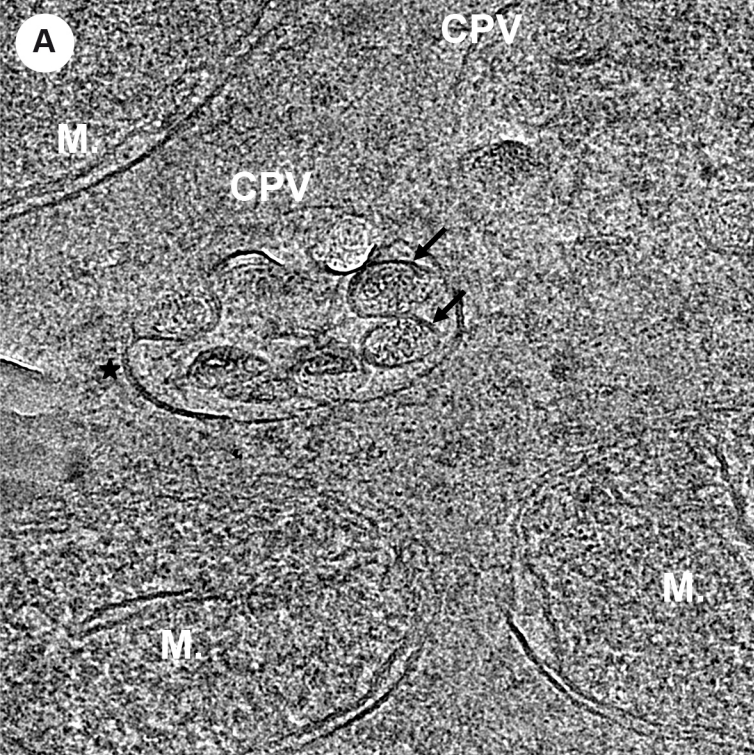
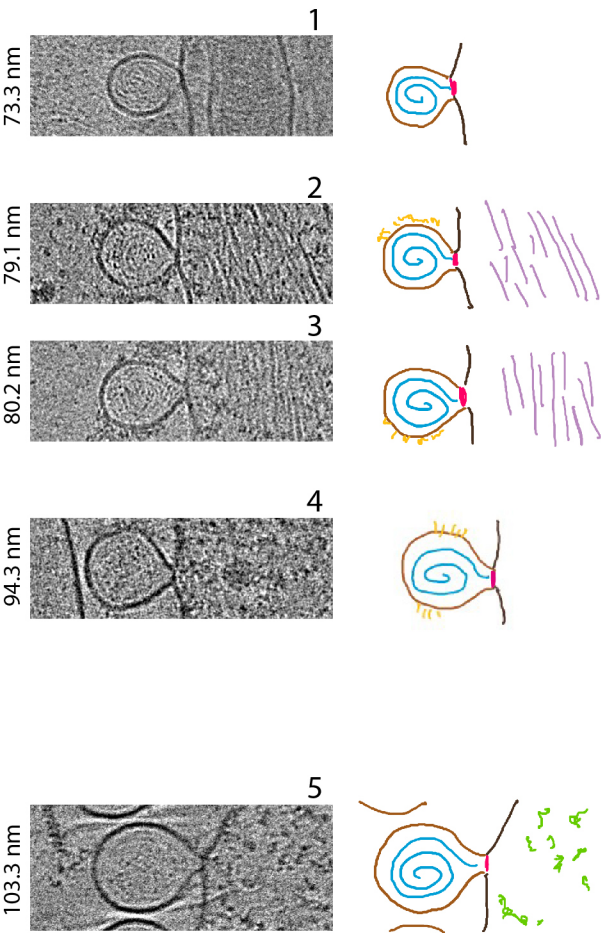


Fig. 4

A Without intra-cellular densities beneath ROs



B With intra-cellular densities beneath ROs

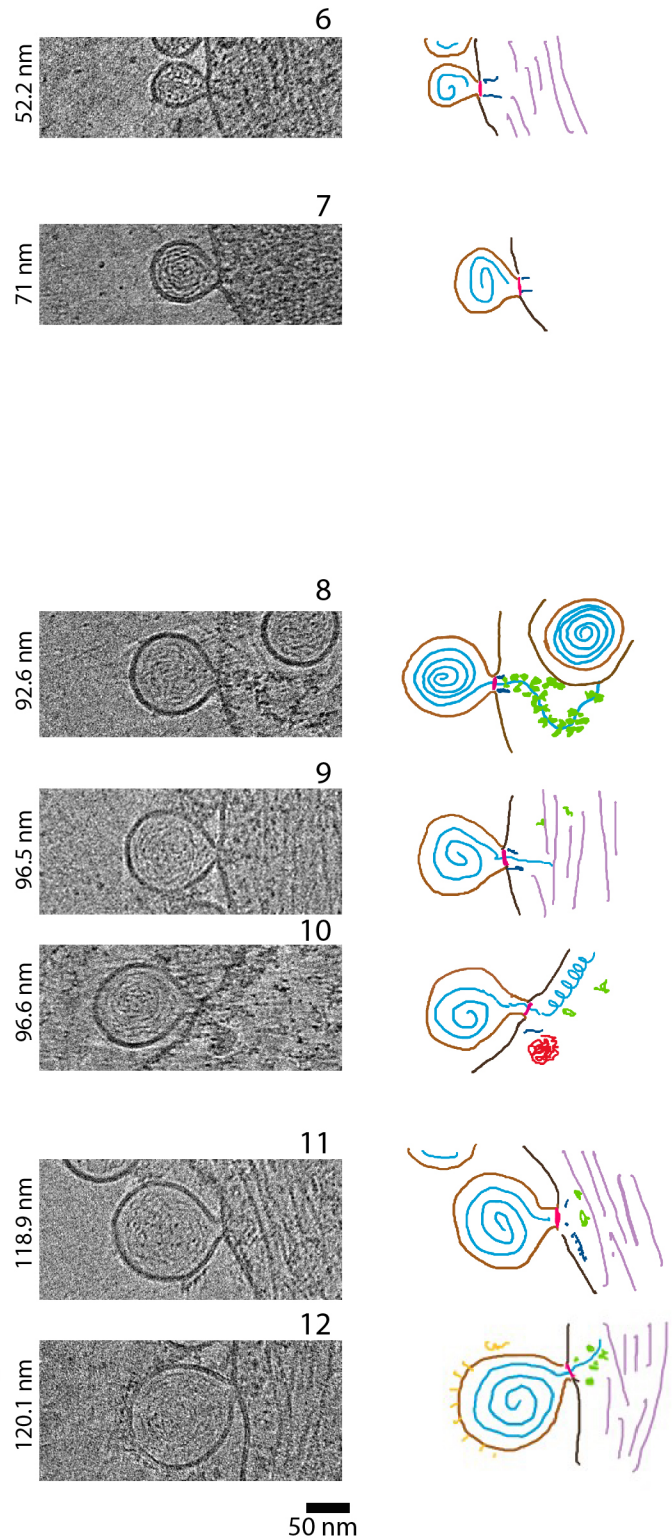


Fig. 5

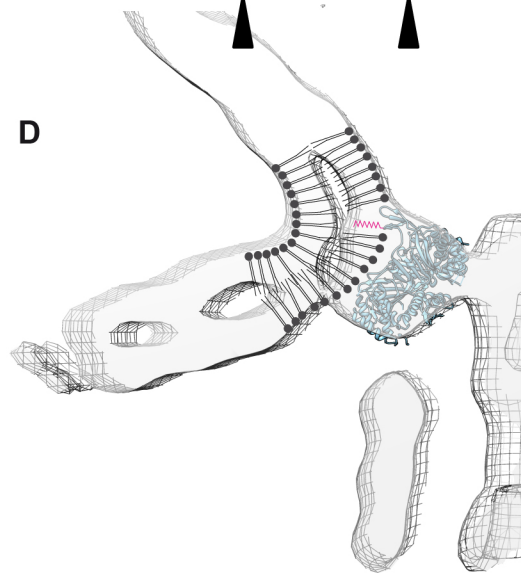
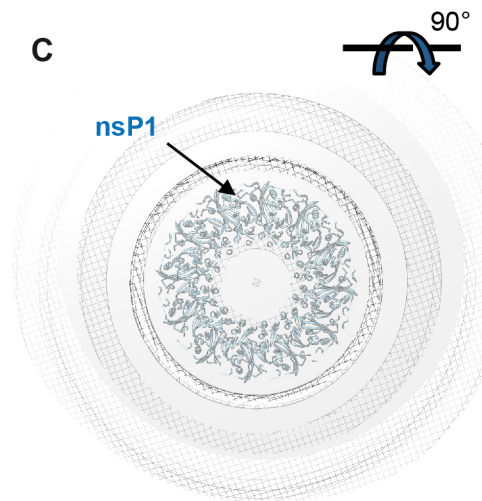
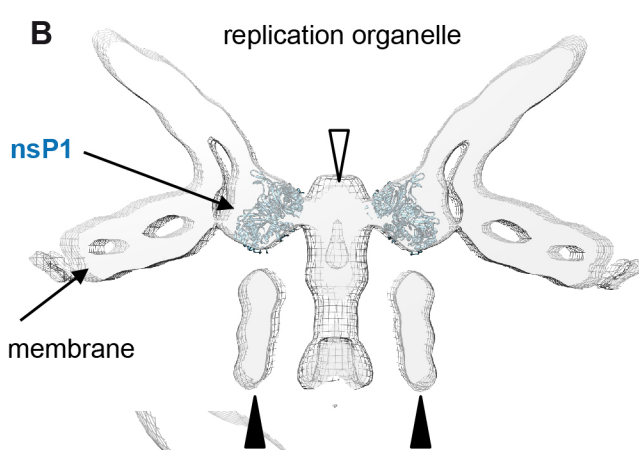
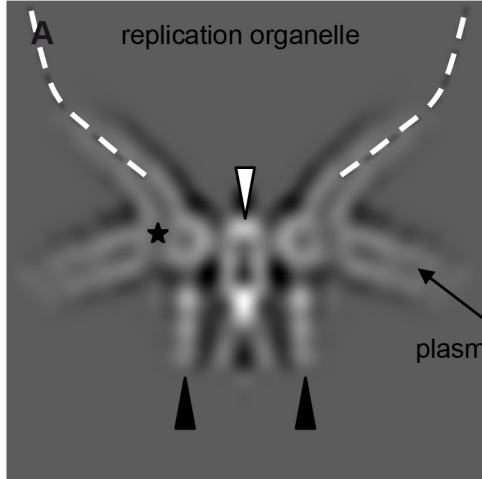


Fig. 6