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1 **Palmitoylated cysteines in Chikungunya virus nsP1 are critical for targeting to cholesterol-rich**  
2 **plasma membrane microdomains with functional consequences for viral genome replication**

3

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9 **Running head:** CHIKV nsP1 affinity for cholesterol enriched membranes

10

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14

15 **Key words:** Chikungunya virus, Methyl/guanylyltransferase, Cholesterol, Plasma membrane,  
16 Replication complexes, Alphavirus,

17

## 18 **Abstract**

19 In mammalian cells, alphavirus replication complexes are anchored to the plasma membrane. This  
20 interaction with lipid bilayers is mediated through the viral methyl/guanylyltransferase nsP1 and  
21 reinforced by palmitoylation of cysteine residue(s) in the C-terminal region of this protein. Lipid  
22 content of membranes supporting nsP1 anchoring remains poorly studied. Here, we explore the  
23 membrane binding capacity of nsP1 with regards to cholesterol. Using the medically important  
24 Chikungunya virus (CHIKV) as a model, we report that nsP1 co-segregates with cholesterol-rich  
25 detergent-resistant membrane microdomains (DRMs), also called lipid rafts. In search for critical  
26 factor for cholesterol partitioning, we identify nsP1 palmitoylated cysteines as major players in this  
27 process. In cells infected with CHIKV or transfected with CHIKV trans-replicase plasmids, nsP1,  
28 together with the other nonstructural proteins, are detected in DRMs. While the functional  
29 importance of CHIKV nsP1 preference for cholesterol-rich membrane domains remains to be  
30 determined, we observed that U18666A- and imipramine-induced sequestration of cholesterol in  
31 late endosomes redirected nsP1 to these compartments and simultaneously dramatically decreased  
32 CHIKV genome replication. A parallel study of Sindbis virus (SINV) revealed that nsP1 from this  
33 divergent alphavirus displays a low affinity for cholesterol and only moderately segregates with  
34 DRMs. Behaviors of CHIKV and SINV with regards to cholesterol therefore match with the previously  
35 reported differences in requirement for nsP1 palmitoylation, that is dispensable for SINV but strictly  
36 required for CHIKV replication. Altogether, this study highlights the functional importance of nsP1  
37 segregation with DRMs and provides new insight into the functional role of nsP1 palmitoylated  
38 cysteines during alphavirus replication.

## 39 **Importance**

40 Functional alphavirus replication complexes are anchored to the host cell membranes through the  
41 interaction of nsP1 with the lipid bilayers. In this work, we investigate the importance of cholesterol

42 for such association. We show that nsP1 has affinity for cholesterol-rich membrane microdomains  
43 formed at the plasma membrane and identify conserved palmitoylated cysteine(s) in nsP1 as the key  
44 determinant for cholesterol affinity. We demonstrate that drug-induced cholesterol sequestration in  
45 late endosomes not only redirects nsP1 to this compartment but also dramatically decreases genome  
46 replication, suggesting the functional importance of nsP1 targeting to cholesterol-rich plasma  
47 membrane microdomains. Finally, we evidence that nsP1 from Chikungunya and Sindbis viruses  
48 display different sensitivity to cholesterol sequestering agents, that parallel with their difference in  
49 the requirement for nsP1 palmitoylation for replication. This research, therefore, gives new insight  
50 into the functional role of palmitoylated cysteines in nsP1 for the assembly of functional alphavirus  
51 replication complexes in their mammalian host.

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53



## 54 **Introduction**

55 In the last decade, evidence has pointed toward the intricate relationship between host lipid  
56 metabolism and the replication of viral pathogens. Indeed, viruses can co-opt or reprogram lipid  
57 signaling, synthesis and metabolism either to generate ATP, to extend cellular membranes or to  
58 remodel membrane lipid content. These modifications will serve to create an environment that is  
59 optimal for viral replication. This need is dictated by the pivotal role played by membranes in almost  
60 all steps of the virus life cycle. Indeed, the importance of cellular lipids during the binding/entry  
61 process and assembly/budding of new infectious progeny into the extracellular space has long been  
62 appreciated (for review see (1)). However, the discovery that viruses with a positive-strand RNA  
63 genome ((+)RNA viruses) replicate in association with host cell membranes has expanded the  
64 regulatory function of lipids to genome replication. It is now well established that such viruses create  
65 membranous compartments, also called virus replication organelles, originating from the  
66 endoplasmic reticulum (ER), Golgi apparatus, mitochondria, peroxisomes, endosomes/lysosomes or  
67 from the plasma membrane (PM) (2). In these compartments, viral replication proteins bind cell  
68 membranes with an affinity for determined lipid species (3, 4).

69 Cell membranes are composed of phospholipids, glycolipids, and cholesterol. Among other lipids,  
70 cholesterol constitutes a unique type of cellular membrane building block. It is responsible for  
71 regulating fluidity and impermeability to lipid bilayers. In vertebrate cells, cholesterol homeostasis is  
72 maintained through *de novo* synthesis in the ER, with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-  
73 CoA) reductase being the rate-limiting enzyme, and through receptor-mediated intake from the  
74 extracellular medium in the form of low-density lipoproteins (LDLs) (5). In this scenario, LDLs are  
75 delivered to and hydrolyzed in late endosomes/lysosomes (LE/Ls) where free cholesterol is released.  
76 Then, cholesterol requires proper intracellular transport to exit the LE/Ls and reach its final  
77 destination mainly at the PM, in the Golgi apparatus and the ER. This is ensured by Niemann-Pick C 1  
78 and 2 (NPC1 and NPC2) proteins localized at the limiting membrane of lysosomes and in the

79 lysosomal lumen respectively (6). Once at the PM, cholesterol together with glycosphingolipids,  
80 glycoposphatidylinositol (GPI)-anchored proteins and transmembrane proteins can cluster into  
81 discrete domains. These cholesterol-enriched detergent-resistant membrane microdomains, referred  
82 to as DRMs or lipid rafts, have been identified as platforms for both endocytosis of penetrating viral  
83 particles and for progeny assembly and budding (7). More recently, cholesterol regulatory function  
84 was extended to the replication step of (+)RNA viruses. The local accumulation of cholesterol was  
85 proposed to contribute to the creation of a membrane microenvironment conducive to assembly and  
86 optimal functioning of replication complexes formed by members of the *Flaviviridae* family,  
87 including hepatitis C virus (HCV) (8–10) and West Nile virus (11), by members of *Picornaviridae*  
88 family, including Coxsackievirus and poliovirus (12) and by plant viruses from the *Bromoviridae* family  
89 (Brome mosaic virus; BMV) (13). Consistent with the idea that cholesterol accumulation may be  
90 required for optimal activity of viral replication machinery, manipulation of cholesterol metabolism  
91 was found to impair genome replication of taxonomically divergent (+)RNA viruses (8, 12, 14–18).

92 Alphaviruses are (+)RNA viruses, which are predominantly transmitted to vertebrates by mosquito  
93 vectors. Chikungunya virus (CHIKV) is an Old World alphavirus causing millions of infections in  
94 tropical and subtropical geographical areas with a potential risk of spreading to regions with a  
95 temperate climate. It has recently received significant attention as a consequence of its re-  
96 emergence in the Indian Ocean and Caribbean Islands before spreading worldwide (19). CHIKV, like  
97 other alphaviruses, replicates its genome in membranous niches derived from the host PM (20–22).  
98 The replication complex confined in these organelles, termed as spherules, contains the four non-  
99 structural proteins nsP1, nsP2, nsP3 and nsP4 encoded by the 5' ORF in the viral genome which are  
100 expressed in the form of P123 and P1234 polyprotein precursors. In this complex, membrane binding  
101 is mediated by nsP1, the viral methyltransferase (MTase) and guanylyltransferase (GTase), which  
102 catalyzes the formation of the cap structure at the 5' end of nascent positive strand viral RNAs (23,  
103 24). A putative  $\alpha$ -helix central to nsP1 was proposed to play a crucial role for membrane attachment  
104 (25, 26). S-acylated cysteines located in the C-terminal region of this protein were then proposed to

105 stabilize this interaction (27). The functional requirement for nsP1 interaction with membranes  
106 varies among alphaviruses. It is strictly required for Semliki Forest virus (SFV) nsP1 enzymatic activity  
107 (28) while it is dispensable for that of Sindbis virus (SINV) nsP1 (29). More specifically, alanine  
108 substitution of palmitoylated cysteines has only a marginal inhibitory effect on SINV replication (30,  
109 31) while leading to the acquisition of compensatory mutations for SFV (31, 32). Recently, this  
110 mutation was reported to completely abolish the replication of CHIKV (33, 34).

111 *In vitro*, SFV nsP1 has affinity for anionic phospholipids especially phosphatidylserine,  
112 phosphatidylglycerol, and cardiolipin; these lipid species significantly improve its capping activity (25,  
113 28). In cells, however, nsP1 affinity for specific lipids remains almost uninvestigated. In recent years,  
114 cholesterol metabolism was reported to be critical for the replication of alphavirus genome. Indeed,  
115 SINV RNA replication and protein synthesis is significantly decreased in fibroblasts from patients with  
116 type A Niemann-Pick disease (NPD-A) which induces cholesterol and sphingolipid storage in LE/Ls  
117 (35). More recently, we reported that U18666A, a class II cationic amphipathic steroid 3- $\beta$ -[2-  
118 (diethylamine)ethoxy]androst-5-en-17-one, and the anti-depressant drug imipramine, which both  
119 phenocopy NPD-A, are CHIKV inhibitors with potential activity against RNA replication steps (16).  
120 Here, we further question the importance of cholesterol metabolism in the alphavirus life cycle. We  
121 especially explore the outcome of cholesterol manipulation on nsP1 subcellular distribution and  
122 membrane anchoring. We show that nsP1 has affinity for cholesterol-rich membranes. Indeed,  
123 U18666A or imipramine redirect CHIKV nsP1 to Lamp2-positive endosomes where unesterified  
124 cholesterol accumulates. At the plasma membrane, nsP1 co-segregates with cholesterol-containing  
125 DRMs as attested by membrane flotation assays. Investigating the molecular basis of nsP1 targeting  
126 to DRMs revealed that the cysteines, previously reported to be palmitoylated (34), are the main  
127 determinants for association with these domains. Interestingly, when expressed transiently in the  
128 context of a P1234 polyprotein precursor or by an infectious CHIKV, nsP2, nsP3, and nsP4 were found  
129 to co-segregate with nsP1 in cholesterol-rich membrane fractions, a property that was abolished  
130 when nsP1 cysteines were mutated. In a parallel study we found that cysteine-mediated nsP1

131 association with cholesterol-rich membranes is conserved for SINV despite being less marked than  
132 for CHIKV. Moreover, SINV nsP1 was also less sensitive to U18666A-induced cholesterol  
133 sequestration. This phenotype with regard to nsP1 cholesterol partitioning parallels the reduced  
134 requirement for cysteine palmitoylation previously reported for SINV replication (30, 31). Altogether  
135 this study provides clues on the proviral role of cholesterol in alphavirus replication, suggesting its  
136 regulatory function in nsPs association with the PM and presumably in the formation of functional  
137 replication complexes.

## 138 **Results**

### 139 **Cholesterol is pivotal for alphavirus genome replication.**

140 First, we set out to study the involvement of cholesterol homeostasis in CHIKV replication.  
141 Cholesterogenesis was inhibited using lovastatin, an FDA-approved inhibitor of HMG-CoA reductase,  
142 that catalyzes the conversion of HMG-CoA to mevalonate in the cholesterol biosynthesis pathway  
143 (Fig 1A) (36). Cholesterol availability at the PM was reduced using U18666A or imipramine. By  
144 targeting NPC1 transporter, both drugs block the transfer of endocytosed cholesterol from late  
145 endosomes to different organelles, including the PM, without significant effect on other lipid species  
146 (37). Additionally, U18666A inhibits enzymes of the cholesterol synthesis pathway (38). Each drug,  
147 used in a concentration range that was controlled to have limited toxicity (Fig 1B-b,d,f), was added to  
148 HEK293T cells 30 mins before infection with CHIKV-LR-5'GFP at a multiplicity of infection (MOI) of  
149 0.5. Cells were maintained in culture with the drugs for 24 hrs and then infection was monitored  
150 measuring levels of GFP reporter expressed by this recombinant virus. In each case, CHIKV infection  
151 was significantly reduced as compared with the mock-treated condition (Fig 1B-a,c,e).

152 We and others have demonstrated that the depletion of membrane cholesterol is deleterious for  
153 fusion of the virion and host membranes (39, 40). Because host membranes are also pivotal for  
154 replication of alphavirus genome through the creation of membranous replication organelles, we

155 investigated whether cholesterol biosynthesis is also required for the post-entry step of CHIKV  
156 infection cycle. To this end, we performed experiments in which cells were treated with cholesterol  
157 metabolism and transport inhibitors 1 hr after CHIKV infection. In these conditions, lovastatin,  
158 U18666A and imipramine decreased CHIKV genome replication (Fig 2A). To definitively omit drug  
159 effects on viral entry, we finally took advantage of a CHIKV trans-replication system that  
160 recapitulates post-entry events of CHIKV life cycle (33). Plasmids CMV-P1234 and HSPoll-Fluc-Gluc,  
161 encoding the P1234 polyprotein and a replication-competent template RNA containing firefly and  
162 *Gaussia* luciferase reporter genes under the control of genomic and subgenomic viral promoters  
163 respectively (Fig 2B), were cotransfected in HEK293T cells. Consequences of cholesterol transport  
164 inhibition were assayed by adding increasing concentrations of lovastatin, U18666A, and imipramine  
165 to transfected cells and quantification of reporter activities in cell lysates. As depicted in Figure 2C,  
166 reporter expression directed by both genomic and subgenomic promoters was decreased by the  
167 drugs when compared to control conditions. Altogether, these results indicate that cholesterol  
168 homeostasis, including ongoing biosynthesis and transport of unesterified cholesterol to the host  
169 membranes, is pivotal for CHIKV genome replication.

170  **$\alpha$ -helix and putative palmitoylated cysteines cooperate for CHIKV nsP1 binding to host**  
171 **membranes.**

172 Alphavirus replication complexes are anchored to the host membranes thanks to nsP1 membrane  
173 binding capacity. While extensively reported for nsP1 encoded by SFV and SINV (25, 30), this feature  
174 has not yet been studied functionally for CHIKV. To investigate CHIKV nsP1 behavior with regard to  
175 host cell membranes, a plasmid encoding a GFP-fused CHIKV nsP1 protein (GFP-nsP1; Fig 3A) was  
176 generated and used to transfect HEK293T cells. NsP1 membrane association was investigated by  
177 fractionation of transfected cells. Post-nuclear extract prepared from GFP-nsP1-expressing cells was  
178 separated into membranous (P25) and cytosolic (S25) samples by differential centrifugation as  
179 previously reported (41). Each fraction was resolved using SDS-PAGE and probed with anti-GFP

180 antibodies and with antibodies against Na<sup>+</sup>/K<sup>+</sup> ATPase or GAPDH, that respectively associate with  
181 membrane and cytosolic compartments. In these conditions, GFP alone was detected in the cytosolic  
182 sample together with GAPDH (Fig 3B and 3C). By contrast, GFP-nsP1 was detected in the membrane  
183 fraction also containing Na<sup>+</sup>/K<sup>+</sup> ATPase. In parallel HeLa cells expressing these proteins were analyzed  
184 using confocal microscopy. As expected, the fluorescence of individual GFP was diffuse in the  
185 cytoplasm and nucleus. However, GFP-nsP1 fluorescence overlapped with PM stained using wheat  
186 germ hemagglutinin (WGA)-conjugated with Alexa Fluor 647 (Fig 3D). As previously reported for  
187 related alphaviruses (42), expression of GFP-nsP1 generated huge membrane reshaping creating  
188 filopodia- and lamellipodia-like structures covering the entire cell surface, that stained positive for  
189 the green fluorescence. Of note, this profile was also observed for a C-terminally GFP-tagged nsP1-  
190 GFP protein as well as for an untagged nsP1 detected by mean of anti-nsP1 serum (Fig 3E), thereby  
191 supporting that GFP-nsP1 behaves as native nsP1 regarding localization and association with  
192 membranes. According to the focal plane chosen, a fraction of each of these proteins was also  
193 detected as small cytosolic aggregates as illustrated in Figure 3E.

194 Because alphavirus nsP1 was previously proposed to be trafficked to endosomes (43) and in light of  
195 our confocal microscopy analysis, we further questioned nsP1 subcellular distribution by performing  
196 fractionation assays that separated the PM from other cell membranes. The post-nuclear extract was  
197 prepared from GFP-nsP1 expressing cells and separated by isopycnic centrifugation in a self-forming  
198 linear 10-20-30% iodixanol density gradient. Twenty-four samples were collected from top to bottom  
199 and assayed by western blotting for GFP-nsP1 content. A roughly equal proportion of GFP-nsP1 was  
200 detected in the top fractions 1 and 2 and also in fractions 9 to 12 that all stained positive for the  
201 Na<sup>+</sup>/K<sup>+</sup> ATPase membrane marker (Fig 3F). These fractions also contained flotillin-1 (FLOT1) that is  
202 known to localize predominantly to the PM and endosomal compartments, *i.e.* late endosomes and  
203 recycling endosomes (44). By contrast, individual GFP segregated with fractions 17 to 24 at the  
204 bottom of the gradient that corresponded to the cytosolic compartment. Altogether, these results  
205 show that CHIKV nsP1 is a membrane-associated protein, that cofractionates equally with the PM

206 and some internal membranes, probably endosomal in nature, suggesting that this protein may  
207 traffic between the two compartments.

208 We next questioned whether the functions of membrane binding determinants previously identified  
209 in alphavirus nsP1 proteins are also conserved for CHIKV nsP1. Extensive analysis performed using  
210 SFV and SINV as models established that membrane association of nsP1 relies on a central conserved  
211 sequence that folds as an amphipathic  $\alpha$ -helix when studied as a synthetic peptide in solution (25). In  
212 this sequence, a pivotal role in membrane anchoring was attributed to tryptophan at position 259  
213 (W<sub>259</sub>) that sinks into the phospholipid bilayer (25). This interaction was proposed to be reinforced by  
214 the presence of cysteine residue at position 420 (C420) in SINV or cysteine residues 418-420 in SFV  
215 (C418-420) which are covalently palmitoylated and render the protein highly hydrophobic (27, 30).  
216 These motifs are conserved in the CHIKV nsP1 sequence where W258 and C417-419 of residues  
217 correspond to W259 and C418-420 of SFV nsP1, respectively (Fig 3A). Recently, C417-419 in CHIKV  
218 nsP1 were reported to be palmitoylated (34), therefore further attesting that nsP1 palmitoylation is a  
219 conserved feature among Old World alphaviruses. To study the contribution of each motif in CHIKV  
220 nsP1 membrane binding, we generated GFP-nsP1 mutants in which W258 (GFP-nsP1<sub>W258A</sub>) or C<sub>417-419</sub>  
221 (GFP-nsP1<sub>3A</sub>) residues in nsP1 sequence were replaced by alanine as well as a double nsP1 mutant  
222 bearing a combination of W<sub>258A</sub> or C<sub>417-419A</sub> mutations (GFP-nsP1<sub>DM</sub>). Fluorescence microscopy of cells  
223 transfected with the corresponding plasmids evidenced a diffuse green fluorescence that  
224 predominated in the cytoplasm by contrast with cells expressing GFP-nsP1 that was mainly detected  
225 at the plasma membrane (Fig 3D). Fractionation experiments confirmed that GFP-nsP1<sub>W258A</sub> and GFP-  
226 nsP1<sub>3A</sub> were more abundant in the cytosolic fraction than GFP-nsP1, with a significant amount of  
227 each mutant protein remaining associated with cell membranes (Fig 3B and 3C). The same cell  
228 extracts were then subjected to membrane flotation assay in iodixanol gradient to appreciate their  
229 capacity to associate with the internal/plasma membrane compartments. GFP-nsP1<sub>W258A</sub> and GFP-  
230 nsP1<sub>3A</sub> proteins were both detected in fractions 18-24 of the gradient thereby confirming that these  
231 proteins have a decreased membrane affinity (Fig 3F). Each mutant protein was also present at the

232 top of the gradient in fractions 1-2 corresponding to the PM and in fractions 9-12 corresponding to  
233 internal membranes. Distribution of mutant proteins in these fractions generally mimicked that of  
234 GFP-nsP1, except that GFP-nsP1<sub>W258A</sub> was somewhat more abundant in internal membrane fractions.  
235 This was also reflected in more diffuse localization of GFP fluorescence in cells expressing GFP-  
236 nsP1<sub>W258A</sub> (compare panels b and d of Fig. 3D) indicating possible role of W258 for PM affinity of  
237 CHIKV nsP1. Nevertheless, the observed differences were small suggesting that W<sub>258A</sub> and C<sub>417-419A</sub>  
238 mutations despite decreasing membrane affinity do not prominently modify nsP1 distribution  
239 between intracellular and plasma membranes or nsP1 trafficking capacity. Interestingly, membrane  
240 association was further decreased when W<sub>258A</sub> and C<sub>417-419A</sub> mutations were combined in GFP-nsP1<sub>DM</sub>  
241 (Fig 3B and 3D). Altogether these results establish that CHIKV nsP1 associates both with the plasma  
242 and internal membranes, an association dictated by the cooperation of motifs that contain C417-419  
243 and W258 amino acids in this protein.

#### 244 **Cholesterol storage defect redirects CHIKV nsP1 to late endosomes.**

245 Based on the observation that cholesterol synthesis and transport is required for CHIKV RNA  
246 replication and on the intimate relationship of CHIKV nsP1 with membranes, we next wondered  
247 whether cholesterol homeostasis has an impact on nsP1 behavior. We first investigated nsP1  
248 subcellular localization with respect to cholesterol distribution. Cells transfected with plasmid  
249 expressing GFP-nsP1 were incubated with U18666A or imipramine to generate a cholesterol  
250 entrapment in LE/Ls (45). This capacity was controlled using the antifungal antibiotic filipin III that  
251 forms a fluorescent complex upon association with unesterified cholesterol (46). In the presence of  
252 U18666A or imipramine, filipin III evidenced the formation of large fluorescent cytosolic aggregates  
253 that contrasted with the presence of cholesterol at the PM and more evenly distributed in the  
254 cytoplasm of mock-treated cells (Fig 4A and data not shown). For U18666A, these clusters colocalized  
255 with Lamp2 (Fig 4B), a LE/Ls marker, thereby confirming that this pharmacological agent stimulates  
256 the accumulation of unesterified cholesterol in late endosomes as previously reported (47). Using



257 these experimental conditions, we next assessed the impact of cholesterol storage defect on GFP-  
258 nsP1 subcellular localization and membrane affinity. Fractionation assays established that drug  
259 treatment did not increase amount of cytosolic nsP1 (Fig 4C; S25 fraction) indicating that it had no  
260 consequence on nsP1 membrane affinity. However, microscopy imaging revealed that, in the  
261 presence of U18666A, a significant part of GFP-nsP1 fluorescence was redirected from the PM to  
262 cytosolic aggregates. This signal overlapped with filipin III staining as attested by cross-sectional  
263 analysis of the fluorescent signals (Fig 4D and 4E). In similar experimental conditions, no  
264 redistribution of individual GFP fluorescence was observed. Accordingly, upon cholesterol storage  
265 condition, GFP-nsP1 colocalized with unesterified cholesterol stored in late endosomes as quantified  
266 by calculating Mander's coefficient (Fig 4F). This situation contrasted with that of mock-treated cells  
267 in which the GFP-nsP1 fluorescence colocalized with filipin III at the PM and did not overlap with  
268 Lamp 2 staining. Altogether, our results argue that the inhibition of NPC1-mediated cholesterol  
269 transport by U18666A redirects GFP-nsP1 to LE/Ls where unesterified cholesterol accumulates. Re-  
270 targeting GFP-nsP1 to these compartments has no significant impact on nsP1 membrane binding  
271 capacity.

#### 272 **C417-419 but not W258 residue determine CHIKV nsP1 sensitivity to cholesterol distribution.**

273 Palmitoylation governs protein trafficking and association with membranes. A major focus of studies  
274 on protein palmitoylation has been the role of this modification in promoting interaction with  
275 gangliosides and cholesterol, leading at certain conditions, to protein translocation to raft/caveolae  
276 membrane domains (48). Given that the C417-429 in CHIKV nsP1 required for optimal membrane  
277 association have recently been reported to be palmitoylated (34), we investigated their contribution  
278 to GFP-nsP1 cholesterol affinity. The above described experiments were repeated using cells  
279 transfected with GFP-nsP1<sub>3A</sub> plasmid. In the presence of U18666A, GFP-nsP1<sub>3A</sub> fluorescence  
280 remained diffuse in the cytoplasm whether the cells were maintained in the presence of U18666A or  
281 with an appropriate concentration of vehicle (Fig 5C). GFP-nsP1<sub>W258A</sub> fluorescence was concentrated

282 in cytosolic foci colocalized with filipin III fluorescence (Fig 5A) as observed for GFP-nsP1. These  
283 phenotypes were confirmed by cross-sectional analysis of the fluorescent signals (Fig 5B and D) and  
284 by calculation of Mander's coefficients (Fig 5E). Accordingly, W258, even if required for  
285 strengthening nsP1 membrane affinity, is not critical for cholesterol affinity. In contrast, cysteine-to-  
286 alanine substitution in mutant GFP- nsP1<sub>3A</sub> abolished sensitivity to U18666A, suggesting a critical role  
287 of palmitoylated cysteines in nsP1 cholesterol affinity.

288 To further explore GFP-nsP1<sub>3A</sub> behavior with regard to cholesterol, we took advantage of CD81, a  
289 heavily palmitoylated tetraspanin, segregating mainly with DRMs, as a lipid raft biomarker (49).  
290 Indeed, in our hands, this protein was mainly detected at the PM of untreated U2OS cells (Fig 6A). It  
291 was redirected to cytosolic aggregates in cells cultured with U18666A or imipramine (Fig 6B) where it  
292 colocalized with GFP-nsP1 as attested by Mander's and Pearson's coefficient calculation (Fig 6D and  
293 6E). CD81 therefore appears as a sensitive cholesterol sensor. Using this property, we explored the  
294 importance of nsP1 palmitoylated cysteines for cholesterol dependency. Cells transfected to express  
295 GFP-nsP1<sub>3A</sub> were incubated in the presence of U18666A. Despite inducing the clustering of CD81 into  
296 intracellular compartments, this treatment did not affect GFP-nsP1<sub>3A</sub> distribution (Fig 6C). Indeed,  
297 GFP-nsP1<sub>3A</sub> remained detected as a diffuse cytoplasmic protein as observed in mock-treated cells  
298 thereby contrasting with the phenotype observed for GFP-nsP1. No colocalization of CD81 and GFP-  
299 nsP1<sub>3A</sub> fluorescence was observed (Fig 6D). Optical sectioning (z-stack) and three-dimensional (3D)  
300 volume reconstruction from image stacks confirmed that conversely to GFP-nsP1, GFP-nsP1<sub>3A</sub> poorly  
301 colocalized with CD81 in drug treated cells (Fig 6E and 6F). These phenotypes were confirmed from  
302 cells cultured with imipramine. According to these results, nsP1 behaves similarly to CD81 with  
303 regard to cholesterol storage, a property that requires the presence of palmitoylated cysteines in  
304 nsP1 C-terminus.

305 **CHIKV nsP1 partitions with DRMs.**

306 Given that nsP1 subcellular localization is sensitive to cholesterol redistribution to the PM, we  
307 questioned its capacity to segregate with cholesterol-enriched membrane microdomains. Cholesterol  
308 is not uniformly distributed in membranes. In living cells, it concentrates in nanoscale assemblies,  
309 also enriched in sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins, referred to  
310 as lipid rafts. These compartments are characterized biochemically by their insolubility in non-ionic  
311 detergents, a property reflected in their name (DRMs - detergent-resistant membrane  
312 microdomains), and by their light density on sucrose gradients. Therefore, they can be separated  
313 from non-raft membranes by centrifugation methods. Samples prepared from cells transfected with  
314 GFP-nsP1 expression plasmid were treated with Triton X-100 at 4°C, separated on a 10-80% sucrose  
315 density gradient, and then analyzed by western blot. Detergent-resistant fractions corresponding to  
316 DRMs were identified utilizing an antibody against FLOT1, a well-known raft-associated protein (50).  
317 In our experimental conditions, FLOT1 fractionated into light density fractions 1 and 2 at the top of  
318 the gradient thereby identifying DRMs (Fig 7A). In contrast, the non-raft marker Na<sup>+</sup>/K<sup>+</sup> ATPase  
319 remained associated with fractions 7-9 of heavier density corresponding to non-raft membranes and  
320 cytosolic compartment (detergent sensitive; DS). Using this protocol, more than 85% of the GFP-nsP1  
321 protein was detected in FLOT1-positive fractions at the top of the gradient supporting its capacity to  
322 associate with cholesterol-enriched DRMs (Fig 7A and 7B). To confirm GFP-nsP1 affinity for  
323 cholesterol-enriched microdomains, this experiment was repeated starting from cells cultured in the  
324 presence of the cholesterol depleting agent methyl-β-cyclodextrin (βMCD). Due to its ability to  
325 sequester cholesterol in its hydrophobic pocket, βMCD extracts cholesterol from the lipid bilayer and  
326 disrupts DRMs (51). Under these cholesterol depletion conditions, FLOT1 was redistributed from  
327 DRMs to DS fractions and sedimented at the bottom of the density gradient (Fig 7C and 7D). Analysis  
328 of the same fractions with anti-nsP1 antibodies revealed that GFP-nsP1 was barely detectable in top  
329 fractions while it accumulated in fractions of heavier density together with FLOT1. Therefore, GFP-  
330 nsP1 fractionation with DRMs is sensitive to cholesterol extraction from cell membranes. Finally, this  
331 experiment was repeated starting from cells expressing GFP-nsP1<sub>W258A</sub> or GFP-nsP1<sub>3A</sub> mutant proteins

332 that displayed different sensitivity to U18666A. As observed for GFP-nsP1, the GFP-nsP1<sub>W258A</sub> mainly  
333 segregated with DRM fractions attesting that raft association was not disrupted by mutation of  
334 W258 residue in the putative  $\alpha$ -helix of nsP1 (Fig 7E and 7F). Analyzing the behavior of the GFP-  
335 nsP1<sub>3A</sub> mutant protein revealed, in contrast, that most of this protein was absent from the top  
336 fraction and co-segregated with the DS fractions. These experiments demonstrate that the presence  
337 of GFP-nsP1 in DRMs is mainly dictated by acylated cysteines while mutation in the putative  $\alpha$ -helical  
338 peptide has only marginal impact on this phenotype.

### 339 **nsPs associate with DRM fractions in cells with CHIKV RNA replication**

340 Unlike SFV, CHIKV replication is completely abolished by nsP1<sub>3A</sub> mutation regardless of the type and  
341 growth temperature of cells (33, 34). In contrast, nsP1<sub>W258A</sub> mutation allows virus to grow efficiently  
342 in insect cells or in mammalian cells cultivated at 28°C (33, 52). Combined with our observations, this  
343 strongly indicates that the presence of nsP1 in DRMs is an absolute requirement for CHIKV genome  
344 replication. Therefore, we questioned whether nsP1 affinity for DRMs was conserved in cells  
345 containing functional CHIKV replicase complexes. To this end, HEK293T cells either transfected with a  
346 trans-replication system that reproduces CHIKV RNA replication (Fig 2B) or infected with the CHIKV-  
347 LR-5'GFP were used. DRMs isolation followed by immunoblot analysis revealed that a significant  
348 fraction of nsP1 expressed in transfected or infected cells (~85 and ~40%, respectively) was detected  
349 in cholesterol-rich fractions (Fig 8D and 8E). NsP1 is the only alphavirus nonstructural protein with  
350 membrane affinity. In the replication complex, nsP1 co-localizes with nsP2, nsP3, and nsP4 (53–56).  
351 Thus, it is suspected to play a critical role in replication complex anchoring to the PM. Therefore, we  
352 investigated whether nsP1 capacity to segregate with DRMs has an impact on the association of  
353 other nsPs with specialized membrane microdomains. Probing gradient fractions prepared from  
354 transfected and infected cells with antibodies against nsPs established that a significant part of each  
355 of them was detected in DS fractions thereby agreeing with the previously reported capacity of nsPs  
356 to be present in different cytosolic compartments (57). However, approximately 10-30 % of total

357 nsP3 and nsP4 levels were also present in raft fractions. In some, but not in all experiments, nsP2 was  
358 also detectable in DRMs; most probably reflecting the weaker interaction of nsP2 with other  
359 components of replicase complexes. In order to further assess the relevance for the presence of  
360 nsP2, nsP3 and nsP4 in DRMs fractions, these experiments were reproduced starting from cells  
361 transfected with a CHIKV trans-replicase system in which the nsP1 protein contained the C<sub>418-420</sub>A  
362 mutation. As expected, this mutation prevented nsP1 association with DRMs (Fig 8C). Concomitantly,  
363 nsP2, nsP3 and nsP4 were also excluded from these fractions. Altogether these results confirm the  
364 capacity of nsP1, expressed in the context of trans-replicase or by infectious CHIKV, to associate with  
365 DRMs, an association that dictates targeting of other nsPs, albeit at low levels, to cholesterol-  
366 enriched membrane microdomain. Furthermore, the C<sub>418-420</sub>A mutation that completely inactivates  
367 CHIKV trans-replicase (33) completely prevented association of CHIKV nsPs with DRMs.

#### 368 **Conservation of nsP1-directed cholesterol affinity among divergent Old World alphaviruses.**

369 Despite the presence of palmitoylated cysteine residues in the C-terminal region of nsP1 is a  
370 conserved feature of distantly related Old World alphaviruses (30, 34), functional studies have  
371 highlighted differences with regard to cysteine requirement for genome replication. Cysteine-to-  
372 alanine mutation are lethal for CHIKV (33, 34) but is well tolerated by SINV (30). In the light of such  
373 differences and of the herein evidenced role of the CHIKV nsP1 cysteines in lipid raft association, we  
374 next questioned whether SINV nsP1 behaves similarly with respect to cholesterol. To achieve this, a  
375 plasmid encoding a GFP-fused SINV nsP1 protein was generated (Fig 9A) and used for the  
376 transfection of HEK293T cells. Then, we tested SINV nsP1 partitioning to DRMs by membrane  
377 flotation assays. As previously observed for CHIKV nsP1, SINV nsP1 was also detected in the top  
378 fractions of the density gradient supporting its ability to partition with cholesterol-rich membrane  
379 microdomains. However, contrasting with CHIKV for which almost 85% of nsP1 associated to DRM  
380 fractions, a roughly equal proportion of the SINV nsP1 was associated with DRMs and DS (Fig 9B). We  
381 further investigated SINV GFP-nsP1 affinity for cholesterol by testing its sensitivity to U18666A in

382 confocal microscopy experiments. In control conditions, SINV GFP-nsP1 was detected at the PM,  
383 including in filopodia-like membrane protrusions that were abundantly observed as reported before  
384 for the untagged protein (42) and for CHIKV GFP-nsP1 (Fig 9C). In the presence of U18666A, SINV  
385 GFP-nsP1 was still mainly detected at the PM, while cholesterol stained with filipin was concentrated  
386 in intracellular storage compartments as expected. In these conditions, co-localization of GFP-nsP1  
387 SINV with cholesterol-enriched endosomes was unfrequently detected. This is in contrast with the  
388 results obtained for CHIKV nsP1 (Fig 4D) (Mander's coefficient of  $0.394 \pm 0.032$  and  $0.217 \pm 0.001$  for  
389 CHIKV and SINV nsP1 respectively). Next, we investigated the role of palmitoylated cysteine by  
390 repeating these experiments starting from cells expressing a cysteine-to-alanine SINV nsP1  
391 palmitoylation-negative mutant protein (GFP-nsP1<sub>C420A</sub>). As shown in Figure 9B, the DRMs association  
392 of GFP-nsP1<sub>C420A</sub> was significantly reduced when compared with that of SINV GFP-nsP1 (Fig 8A).  
393 Analyzing SINV GFP-nsP1<sub>C420A</sub> subcellular localization in U18666A treated cells confirmed that this  
394 mutant did not colocalize with filipin-labelled cholesterol (Fig 9D). Altogether these results suggest  
395 that SINV nsP1 is targeted to DRMs, a property that depends of palmitoylated cysteine as previously  
396 observed for CHIKV nsP1. However, compared with CHIKV counterpart, SINV nsP1 is equally  
397 abundant in DS compartments and displays only modest sensitivity to U18666A suggesting a reduced  
398 affinity for cholesterol.

## 399 Discussion

400 The present study identifies CHIKV nsP1 as a lipid-raft co-segregating protein with an affinity for  
401 cholesterol. We defined cysteine residues that can be palmitoylated as the molecular determinant  
402 important for this targeting. In the context of cells with ongoing CHIKV RNA replication, nsP1,  
403 together with a fraction of other nsPs, partitions with cholesterol-rich DRMs. Together with evidence  
404 that drugs reducing cholesterol availability at the PM impair CHIKV RNA replication, our results  
405 support that nsP1 targeting to cholesterol-rich PM microdomains may have a functional importance  
406 for viral genome replication.

407 Cholesterol is a main component of membranes. Together with sphingolipids, it segregates into  
408 discrete microdomains, referred to as lipid rafts or DRMs, present both on the inner and the outer  
409 leaflet of the PM (58, 59). These membrane domains with a size on the nm scale are highly dynamic.  
410 They accumulate a subset of membrane proteins, mainly GPI-anchored proteins, transmembrane  
411 proteins, and acylated cell components (60, 61). Based on these properties, rafts were seen as  
412 platforms that compartmentalize cellular processes with an important function in receptor-ligand  
413 interaction, signal transduction and endocytosis (60). Herein, we establish that CHIKV nsP1 associates  
414 with the PM. In this compartment, a pool of nsP1 is targeted to cholesterol-containing DRMs (Fig  
415 10a), an association that was reversed by  $\beta$ MCD cholesterol-removal agent. Affinity for cholesterol  
416 was further supported by investigating nsP1 behavior with regard to U18666A- or imipramine-  
417 mediated cholesterol sequestration in LE/Ls. We found that intracellular cholesterol storage resulted  
418 in nsP1 accumulation in endosomes without consequence on overall nsP1 membrane binding ability  
419 (Fig 10b). Altogether these results indicate that availability of cholesterol at the PM is required for  
420 appropriate targeting of CHIKV nsP1 to this compartment.

421 In the last decade, the biochemical or biophysical underpinnings that govern nsP1 association with  
422 membranes have been the focus of an intense attention. For SFV and SINV, the central  $\alpha$ -helical  
423 motif in nsP1 spanning amino acid residues 245 to 264 was proposed as the main determinant for  
424 membrane anchoring to lipid bilayers with W259 residue being critical for hydrophobic interactions  
425 with the phospholipid acyl chains (25). Conserved acylated cysteine(s) in nsP1 were proposed to  
426 tighten this membrane interaction (26, 27, 30, 34). Using cell fractionation assays, we show that both  
427 W<sub>258</sub>A substitution in the putative  $\alpha$ -helix of CHIKV nsP1 and C<sub>417-419</sub>A mutations indeed decrease  
428 nsP1 affinity for cell membranes. Interestingly, combining W<sub>258</sub>A and C<sub>417-419</sub>A mutations further  
429 reduced nsP1 membrane association thereby suggesting that in the CHIKV nsP1 the two domains  
430 may synergize for membrane interaction, a situation that was not described for other alphaviruses.  
431 Analyzing the contribution of these membrane interaction determinants in nsP1 targeting to lipid

432 rafts revealed that the W<sub>258</sub>A mutation had only a slight effect on nsP1 co-fractionation with  
433 cholesterol-rich domains. This mutation also slightly reduced PM association of nsP1 and facilitated  
434 its association with internal membranes. This may indicate that W258 residue is important for  
435 membrane/plasma membrane targeting of nsP1 but not for its palmitoylation, DRMs association and  
436 enzymatic activity. How these properties correlate with the proposed role of W258 residue as one of  
437 membrane anchors of nsP1 is currently unclear. For the proper understanding of the somewhat  
438 controversial data regarding the importance of the W258 residue, the structure of the membrane  
439 bound  $\alpha$ -helical peptide of nsP1 (25) should be compared with the structure of membrane-bound  
440 enzymatically active nsP1 which, to this date, is not available. In contrast to the W<sub>258</sub>A mutation the  
441 effects of the C<sub>417-419</sub>A mutation on CHIKV nsP1 were unambiguous. This mutation, previously  
442 reported to prevent CHIKV nsP1 palmitoylation and replication (33, 34), dramatically reduced DRMs  
443 co-fractionation (Fig 10c). Moreover, by contrast with wild-type nsP1, nsP1<sub>C417-419A</sub> sequestration with  
444 Lamp2 in endosomes could not be observed under U18666A or imipramine treatment. According to  
445 these experiments, acylation appears as critical to direct nsP1 to cholesterol-enriched membrane  
446 microdomains. This result parallels previous evidence regarding the role of palmitoylation in cellular  
447 (p59<sup>fyn</sup> and p60<sup>src</sup>) (62) or viral (influenza hemagglutinin) (63) proteins association with rafts. This  
448 observation raised the question of the functional outcome of nsP1 association with cholesterol-rich  
449 DRMs.

450 Because other nonstructural proteins of alphaviruses cannot directly associate with membranes,  
451 nsP1 plays a decisive role in proper targeting and membrane binding of other nsPs involved in the  
452 formation and functioning of alphavirus replication complex (24). Starting from cells infected with  
453 CHIKV, we found that a fraction of each of the four nsPs was associated with DRMs. These results  
454 were confirmed in cells transfected with plasmids encoding for a CHIKV trans-replication system, in  
455 which other non-structural proteins co-sedimented with DRMs depending on the integrity of nsP1  
456 C417-419 residues. Currently the functional importance of nsPs targeting to rafts is unknown.  
457 Nevertheless, we observed that in addition to nsP1 sequestration into late endosomes, U18666A and



458 imipramine also generated a significant drop in CHIKV genome replication. This raises the question of  
459 the functional consequences of nsP1 mistargeting and of its impact on replication complex assembly.

460 Over two decades, alphaviruses encoding nsP1 mutants with reduced membrane binding ability have  
461 been in the focus of different studies. They established that nsP1 palmitoylation had only a mild  
462 impact on SINV or SFV infectivity, highlighting the essential role of the W259 residue in nsP1's central  
463  $\alpha$ -helix for both membrane anchoring and genome replication (30, 31). For CHIKV, the W<sub>258</sub>A mutant  
464 is viable, albeit having a temperature-sensitive phenotype (33). By contrast, the C<sub>417-419</sub>A mutation  
465 results in complete inactivation of the CHIKV replicase, leading to non-functional enzymes unable to  
466 synthesize any viral RNAs both in mammalian and insect cells (33, 34, 52). Here, the direct  
467 comparison of SINV and CHIKV revealed that SINV nsP1 partitions with DRMs but to a lesser extent  
468 than observed for CHIKV nsP1. This phenotype was equally dependent upon conserved cysteine in  
469 nsP1. Moreover, SINV nsP1 targeting to the PM was less sensitive to cholesterol manipulation by  
470 U18666A. These discrepancies therefore parallel the differences in nsP1 palmitoylation requirement  
471 previously reported for CHIKV and SINV replication (33). However, counterintuitively, SINV genome  
472 replication was also sensitive to U18666A and cholesterol sequestration (this study and (35)) albeit  
473 less than observed for CHIKV (data not shown). Conversely to CHIKV, this might not reflect  
474 dependency on PM rafts, but instead cholesterol-induced alteration of endosomes where SINV  
475 preferentially replicates as proposed by others (35, 64).

476 In the last decade, cholesterol-rich membrane microdomains, beyond their role in virus entry and  
477 exit, have also been identified as platforms for the assembly and anchoring of replication complexes  
478 produced by a broad range of RNA viruses including HCV, picornaviruses or flaviviruses (9, 10, 12, 18,  
479 65). A dramatic reduction of viral RNA replication was observed upon membrane cholesterol  
480 extraction or in conditions reducing cholesterol availability (12, 14, 66, 67) establishing functional  
481 importance for this association. Besides changing membrane composition and fluidity, which may  
482 affect different interactions between virus-encoded replicase subunits, cholesterol partitioning in

483 membranes also attract cellular proteins, with functions in cell signaling and intracellular trafficking.  
484 We can assume that targeting CHIKV nsP1 and possibly other nsPs at these sites may favor  
485 interaction with raft-associated cellular factors indispensable for viral replication. The exact necessity  
486 for nsP1 partitioning to DRMs therefore will deserve further investigations.

487

## 488 **Materials and Methods**

489 **Antibodies and reagents:** The following antibodies/reagents and respective dilutions were used in  
490 this study: rabbit polyclonal antisera against CHIKV nsP1, nsP2, nsP3, nsP4 (all in-house, 1:1,000),  
491 monoclonal antibodies against GAPDH (1:1,000) (Santa Cruz Biotechnologies Inc.), Na<sup>+</sup> K<sup>+</sup> ATPase  
492 (1:50,000) (Ab76020, Abcam), GFP (1:1,000) (Chromotek), Lamp2 (1:1,000), CD81 (1:500) (Clone JS-  
493 81, BD Biosciences) and flotillin-1 (1:1,000) (BD Biosciences). Secondary antibodies conjugated to  
494 horseradish peroxidase or Alexa Fluor were purchased from Jackson ImmunoResearch and Thermo  
495 Fisher Scientific, respectively. Filipin III, U18666A, Lovastatin, methyl- $\beta$ -cyclodextrin were purchased  
496 from Sigma-Aldrich. Imipramine was obtained from Abcam and WGA-647 from Thermo Fisher  
497 Scientific.

498 **Cells:** HEK293T cells (ATCC # ACS-4500), HeLa cells (ATCC # CRM-CCL2), BHK21 cells (ATCC # CCL-10)  
499 and U2OS cells (ATCC # HTB-96) used for propagation and Vero cells (ATCC # CCL-81) used for  
500 titration of the CHIKV were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fischer  
501 Scientific) supplemented with penicillin and 10% fetal calf serum (FCS, Lonza) and grown at 37°C in a  
502 5% CO<sub>2</sub> atmosphere. Viability of cells incubated with drugs for 24 hrs was measured using Cell Titer  
503 96 Aqueous one solution cell proliferation assay (Promega) according to the manufacturer's protocol.

504 **Viruses:** The pCHIKV-LR-5'GFP, full-length molecular clone of CHIKV (LR2006OPY1 strain) with GFP  
505 reporter (68), was linearized and transcribed *in vitro* using the mMACHINE kit (Ambion-

506 Life Technologies). 1 µg of RNA was then transfected with lipofectamine 2000 (Thermo Fisher  
507 Scientific) into 10<sup>5</sup> HEK293T cells and the cells were incubated at 37°C for 24 hrs. Culture medium  
508 was collected, and virus stock was amplified on BHK-21 cells. After 48 hrs at 37°C, the supernatant  
509 was collected, filtered through a 0.45 µm membrane, aliquoted and stored at -80°C. Viral stocks were  
510 tittered using plaque assay as previously reported (39).

511 **Infection with CHIKV-reporter viruses:** The cells (70-80% confluence) were rinsed once with PBS  
512 before infection with CHIKV-LR-5'GFP diluted to achieve the desired MOI. For pre-infection  
513 experiments, the cells were pre-incubated with the virus for 1 hr or 2 hrs before drug addition. For  
514 post-infection experiments, the cells were incubated with drugs for 30 min before infection. After 24  
515 hrs in culture, the cells were lysed with RIPA buffer. GFP reporter fluorescence was measured directly  
516 from the cell lysate using an Infinite F200PRO fluorometer (Tecan). Values were normalized to the  
517 protein content in the sample determined using the BCA Assay (Pierce).

518 **Plasmids and transfection:** Sequence encoding for CHIKV nsP1 was amplified by PCR using pCHIKV-  
519 LR-5'GFP as a template; obtained fragment was cloned into the pEGFP-C1 plasmid as previously  
520 described (69). Sequences encoding for a nsP1<sub>W258A</sub>, nsP1<sub>C417-419A</sub> and nsP1<sub>DM</sub> were generated using  
521 the Quikchange site-directed mutagenesis kit by Agilent. SINV nsP1 and nsP1<sub>420A</sub> were obtained  
522 using PCR and pTOTO1101 (70) or its derivative pSINV-C<sub>420</sub>A1 (30) as templates. These inserts were  
523 cloned in frame with eGFP in the peGFP-C1 plasmid. Cells were transfected with obtained plasmids  
524 using JetPei reagent (Polyplus Transfection) according to manufacturer recommendations.

525 **Trans-replication assay:** For trans-replication assays, CMV-P1234 (71) or CMV-SINV-P1234 (72)  
526 plasmids encoding nonstructural polyprotein from CHIKV or SINV respectively were cotransfected  
527 together with the HSPoll-Fluc-Gluc (33) or HSPoll-SINV-tFluc-Gluc (73) template plasmids encoding  
528 for replication-competent template RNA of CHIKV or SINV containing firefly and *Gaussia* luciferase  
529 reporter sequences placed under control of genomic and subgenomic promoters respectively. Equal  
530 amounts of plasmids were transfected into HEK293T cells using JetPei transfection reagent. After 24

531 hrs in culture, cells were washed in PBS and lysed using Passive Lysis Buffer (Promega). Expression of  
532 firefly and *Gaussia* luciferase was determined using the Dual-Glo Luciferase Assay system (Promega)  
533 and a Spark luminometer (Tecan). Reporter activities were normalized to the protein content in the  
534 sample determined using the BCA Assay (Pierce).

535 **Immunoblotting:** Samples were separated by SDS-PAGE and then transferred to PVDF membrane  
536 (Hybond, Amersham). Membranes were blocked using 5% non-fat dry milk in PBS and probed with  
537 appropriate primary antibodies. After wash steps with PBS – 0.1% Tween 20, the membranes were  
538 probed with HRP-conjugated secondary antibodies. The revelation was performed by incubating the  
539 membranes with either Luminata Forte (Merck) or Clarity Max (BioRad) and then image acquisition  
540 was done using a Chemidoc (Bio-Rad).

541 **Cell fractionation and membrane flotation assays:** Cells were incubated in hypotonic buffer (10mM  
542 Tris/HCl [pH 7.4], 10 mM NaCl supplemented with protease inhibitors) for 10 min on ice and then  
543 lysed with a Dounce homogenizer (30-40 strokes). The lysates were clarified by low-speed  
544 centrifugation at 1,000 g for 10 min. Obtained post-nuclear supernatants (PNS) were then adjusted  
545 to a final concentration of 500 mM NaCl and incubated for 30 min on ice. After ultracentrifugation at  
546 25,000 g for 20 min, the cytosolic (supernatant, S25) and membrane fraction (pellet, P25) were  
547 collected. P25 samples were solubilized in lysis buffer composed of 1% Brij 96 in 20 mM Tris/HCl [pH  
548 7.5] before analysis. For membrane flotation experiments, cells were resuspended in 250 mM  
549 sucrose in PBS supplemented with protease inhibitors and then lysed with a Dounce homogenizer  
550 (30-40 strokes). Cell lysates were spun at 1,000 g for 10 min to pellet the nuclei. The supernatant  
551 referred to as crude lysate (CL) was then adjusted to 30% iodixanol concentration by mixing Optiprep  
552 (Axis-Shield). CL (4 mL) was loaded at the bottom of a centrifuge tube, and then overlaid with 4 mL  
553 20% iodixanol and then 4 mL 10% iodixanol. The gradient was then spun 200,000 g at 4°C for 16 hrs  
554 in a Beckman SW41 rotor. Finally, 24 fractions were collected from top to bottom.

555 **Detergent-resistant membrane isolation:** Cells were lysed on ice in TNE buffer (10 mM Tris/HCl [pH  
556 7.5], 100 mM NaCl, 10 mM EDTA) containing 0.5% Triton X-100 for 30 min. Lysates were then further  
557 treated with the Dounce homogenizer, and then clarified by low-speed centrifugation at 1,000 g for  
558 10 min to obtain the PNS. PNS (0.5 mL) was adjusted to 60% sucrose by adding 1.5 mL of 80% sucrose  
559 TNE (w/v). The lysates were layered over 500  $\mu$ L of 80% sucrose TNE, then covered with 2 mL of 50%  
560 sucrose TNE, 6 mL of 38% sucrose TNE, and 1.5 mL of 10% sucrose TNE. The sucrose gradients were  
561 centrifuged at 100,000 g at 4°C for 18 hrs in a Beckman SW41 rotor (Beckman Coulter). Nine fractions  
562 were then collected and analyzed by immunoblotting.

563 **Immunofluorescence microscopy and image analysis:** Cells grown on glass coverslips were washed  
564 with PBS and then fixed with 4% paraformaldehyde/PBS (Sigma Aldrich) for 10 min. For intracellular  
565 labeling, the cells were permeabilized with 0.1% Triton-X100 in PBS and blocked for 30 min with PBS  
566 containing 0.2% Bovine Serum Albumin. Incubation with primary antibody was performed for 1 hr.  
567 After washes with PBS, secondary reagents were added for 30 min. DAPI (Sigma-Aldrich) was used to  
568 stain the nuclei. Filipin or WGA-647 staining was performed by incubation at room temperature for  
569 either 1 hr or 10 min respectively. After final washes, coverslips were mounted with Prolong Gold  
570 antifade mounting media (Thermo Fisher Scientific). Images were acquired using a Leica SP5-SMD  
571 scanning confocal microscope equipped with a 63 $\times$ , 1.4 numerical aperture Leica Apochromat oil  
572 lense at the Montpellier Resources Imaging platform. Image analysis was performed utilizing Fiji  
573 ImageJ and the JACoP plugin. For Mander's analysis, the threshold and region of interest were  
574 maintained consistent between the different conditions. 3D reconstruction was performed by Imaris  
575 software.

576 **Statistical analysis:** All of the analyses (unpaired Student's t-test) were performed using GraphPad  
577 Prism version 6 (GraphPad Software Inc.). A p-value of <0.05 was considered statistically significant.  
578 Following designations are used on figures: \* p<0.05; \*\* p<0.001; \*\*\*p<0.0001; \*\*\*\* p<0.0001.

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### 583 **Authors contribution**

584 *Conceptualization:* LB, AM; *Methodology:* LB; *Funding acquisition:* LB; *Investigation-main*  
585 *experiments:* WB. *Investigations - additional experiments:* AN, EB; *Supervision:* LB; *Writing original*  
586 *draft:* LB, AM, WB.

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### 593 **Competing interests**

594 The authors have declared that no competing interests exist.

595

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783 efficiency of replicase polyprotein processing.
- 784

785 **Figure legends**

786

787 **Figure 1: Cholesterol homeostasis regulates CHIKV life cycle**

788 (A) Cholesterol metabolism and biosynthesis inhibitors. (B) Drugs affecting cholesterol metabolism  
789 inhibit CHIKV infection. HEK293T cells treated for 30 min with the indicated concentrations of (a)  
790 lovastatin, (c) U18666A, (e) imipramine were infected with CHIKV-LR-5'GFP at a MOI of 0.5. Infection  
791 was monitored after 24 hrs by quantification of GFP reporter in the cell lysates. For each drug  
792 concentration, cell viability was determined after 24 hrs using Cell Titer Glo assay (b, d, f). Values are  
793 expressed as a percentage of control condition that was taken as 100%. A representative experiment  
794 is shown. Values are means of triplicates  $\pm$  SEM. p-values are calculated by comparing each treated-  
795 condition with the mock.

796

797 **Figure 2: Cholesterol transport inhibitors regulate intracellular CHIKV replication**

798 (A) Post-infection addition of lovastatin, U18666A or imipramine reduces CHIKV replication.  
799 Increasing concentrations of drugs were added to HEK293T cells 1 hr after CHIKV infection at a MOI  
800 of 1. After 24 hrs, CHIKV replication was monitored by quantification of GFP fluorescence in the cell  
801 lysates. Values, normalized to the protein content in the samples, are expressed as a percentage of  
802 the non-infected (NI) condition. (B) Schematics of CMV-P1234 and HSPoll-Fluc-Gluc constructs,  
803 plasmid backbones are shown. (C) HEK293T cells were transfected with plasmids depicted in (B) and  
804 treated with the indicated concentrations of lovastatin, U18666A or imipramine at 2 hrs post-  
805 transfection. Firefly and Gaussia luciferase activities were determined after 24 hrs in culture. A  
806 representative experiment is shown. Values are expressed as a percentage of the untreated  
807 condition and are means of triplicates  $\pm$  SEM. NT designates non-transfected control cells. p-values  
808 are calculated by comparing each treated-condition with the mock.

809 **Figure 3: CHIKV nsP1 associates with the PM through conserved sequence motifs**

810 (A) Organization of the CHIKV nsP1. Conserved amino-acids involved in the central  $\alpha$ -helix (aa 244-  
811 263) and those required for nsP1 acylation (417-419) are indicated. Mutants used in this study are  
812 depicted. (B) Membrane affinity of GFP-nP1, GFP-nsP1<sub>3A</sub>, GFP-nsP1<sub>W258A</sub> and GFP-nsP1<sub>DM</sub> was  
813 determined by cell fractionation assays. HEK293T cells transfected to express each GFP-fused protein  
814 were fractionated to produce cytosolic (S25) and membranous (P25) fractions. Equal amounts of  
815 fractions were analyzed by SDS-PAGE and western blotting with anti-nsP1 or anti-GFP antibodies.  
816 Antibodies against Na<sup>+</sup>/K<sup>+</sup> ATPase (ATPase) and GAPDH were used to control the separation of  
817 membranes and cytosolic compartments respectively. For each experiment a sample of the  
818 unfractionated cell lysate (CL) was run in parallel to determine the relative amount of each nsP1  
819 mutant expressed. Cells expressing the GFP protein alone are shown as a control. (C) The intensity of  
820 bands in S25 and P25 samples from panel B was determined using Image J software and plotted as a  
821 histogram. (D) Subcellular localization of GFP-nsP1 and its mutant was determined by confocal  
822 imaging of transfected HeLa cells. Cell membranes are labeled with Alexa Fluor 647-conjugated  
823 wheat germ hemagglutinin (WGA). Scale bars are 5  $\mu$ m. (E) Confocal microscopy imaging of HeLa  
824 cells transfected with expression plasmids for nsP1-GFP or for an untagged nsP1. The untagged nsP1  
825 protein was detected with rabbit antiserum and Alexa-647-conjugated secondary reagents. Nuclei  
826 were stained with DAPI before analysis by confocal imaging. GFP-nsP1 at the plasma membrane and  
827 filopodia-like structures formed at the surface of transfected cells are indicated by white arrows.  
828 Cytosolic aggregates are indicated by blue arrows. Bars: 5  $\mu$ m. (F) Lysates of cells expressing GFP-  
829 nP1, GFP-nsP1<sub>3A</sub>, GFP-nsP1<sub>W258A</sub>, GFP were subjected to membrane flotation assay in iodixanol  
830 gradient. Fractions collected from top to bottom were separated on SDS-PAGE and probed with  
831 antibodies specific for nsP1. Lysates from GFP expressing cells were probed with antibodies specific  
832 for GFP, flotillin-1 (FLOT1) or ATPase. Data are representative of three separate experiments.

833 **Figure 4: Cholesterol trafficking inhibitors redirect nsP1 to endo/lysosomal compartments**

834 (A) HeLa cells cultured in the presence of 12  $\mu$ M U18666A were stained with filipin III to visualize  
835 cholesterol distribution. Cells cultured in medium containing an appropriate amount of vehicle are

836 shown as control (Mock). (B) Cells expressing the GFP-nsP1 protein were maintained for 24 hrs in the  
837 presence of 12  $\mu$ M U18666A and then co-labeled with filipin III and anti-Lamp2 antibodies to  
838 visualize cholesterol and endo/lysosomes respectively. Cells maintained with vehicle alone are  
839 shown as controls (Mock). (C) Consequences of U18666A treatment on GFP-nsP1 membrane binding  
840 capacity was determined by cell fractionation assays. Crude lysates (CL) and cytosolic fractions (S25)  
841 prepared from mock-treated or U18666A-treated HEK293T cells were separated on SDS-PAGE and  
842 probed with antibodies against GFP or GAPDH. (D) HeLa cells transfected with GFP or GFP-nsP1  
843 expression plasmid and maintained with 12  $\mu$ M U18666A or in medium alone (Mock) for 24 hrs were  
844 stained with filipin III and DAPI and then processed for confocal imaging. (E) Histograms indicate the  
845 signal intensity profile of filipin III and green fluorescence along the white lines in (D). Bars: 5  $\mu$ m. (F)  
846 Colocalization of filipin and GFP signals in U18666A-treated cells was determined by calculation of  
847 Mander's overlap coefficients ( $n \geq 20$  cells).

848 **Figure 5: GFP-nsP1<sub>W258A</sub> and GFP-nsP1<sub>3A</sub> display different sensitivity to U18666A-induced**  
849 **cholesterol storage defect.**

850 HeLa cells expressing either GFP-nsP1<sub>3A</sub> (A) or GFP-nsP1<sub>W258A</sub> (C) were incubated with 12  $\mu$ M  
851 U18666A for 24 hrs. Unesterified cholesterol was stained with filipin-III and cells were processed for  
852 confocal microscopy. (B) and (D) Histograms showing filipin III and GFP fluorescence intensity along  
853 the white lines indicated in (A) and (B) respectively. Bars: 5  $\mu$ m. (E) Colocalization of filipin and GFP  
854 signals in U18666A-treated cells was determined by calculation of Mander's overlap coefficients ( $n \geq$   
855 20 cells).

856 **Figure 6: C417-419 are key determinants for CHIKV nsP1 targeting to CD81-positive cholesterol-rich**  
857 **compartments**

858 U2OS cells expressing (A) the GFP, (B) GFP-nsP1, (C) GFP-nsP1<sub>3A</sub> were cultured in control condition or  
859 in medium supplemented with 12  $\mu$ M U18666A or 75  $\mu$ M imipramine for 24 hrs. Then, the cells were  
860 probed with anti-CD81 mAbs and Alexa Fluor-594-conjugated secondary antibodies. Nuclei were

861 stained with DAPI and the cells were processed for confocal microscopy. Scale bars are 5  $\mu\text{m}$ . (D)  
862 Quantification of the degrees of colocalization of GFP fluorescence and CD81 staining in treated cells  
863 was determined by calculation of Mander's and Pearson's overlap coefficients ( $n \geq 20$  cells). (E and F)  
864 3D image reconstruction was performed from z-stacks acquired from treated cells expressing either  
865 GFP-nsP1 or GFP-nsP1<sub>3A</sub>.

866 **Figure 7: CHIKV nsP1 partitioning with lipid rafts in human cells requires C417-419**

867 (A) HEK293T cells were transfected with plasmids encoding GFP-nsP1 and processed for raft  
868 isolation. Nine fractions of the density gradients, numbered from top to bottom, were collected and  
869 analyzed using SDS-PAGE. Proteins were probed with antibodies against nsP1, FLOT1 and Na<sup>+</sup>/K<sup>+</sup>  
870 ATPase. Fractions containing detergent-resistant membranes (DRMs) and detergent soluble  
871 membranes (DS) are indicated. (B) The amount of GFP-nsP1 and FLOT1 associated with each fraction  
872 was determined by densitometry scanning of immunoblots and expressed as a percentage of total  
873 protein expression level. The diagram is representative of three experiments. (C, D) A similar  
874 experiment was performed starting from cells incubated for 30 minutes in the presence of 10 mM  
875  $\beta\text{MCD}$ . (E) Cells expressing the GFP-nsP1<sub>3A</sub> or GFP-nsP1<sub>W258A</sub> mutants were processed as in (A). (F)  
876 Amounts of GFP-nsP1, GFP-nsP1<sub>3A</sub> or GFP-nsP1<sub>W258A</sub> in DRM and DS fractions were determined by  
877 densitometry scanning of the immunoblots shown in (A) and (E) and expressed as a percentage of  
878 total protein signal.

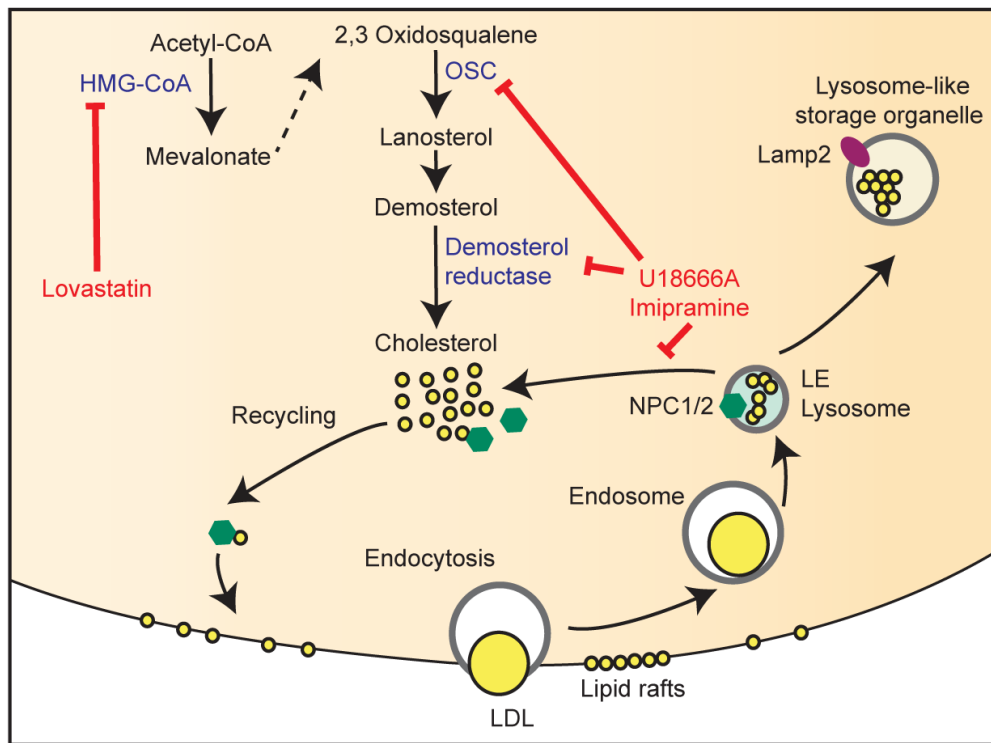
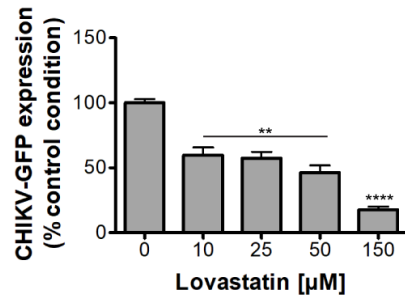
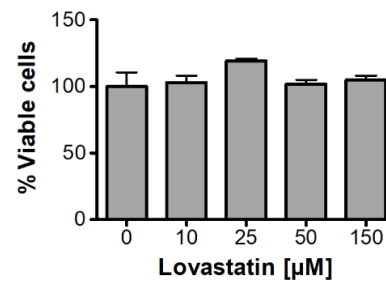
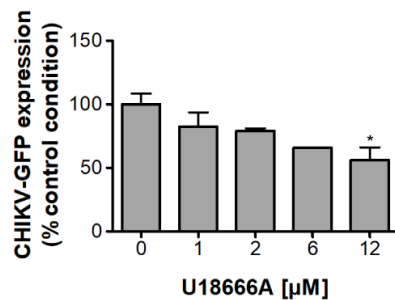
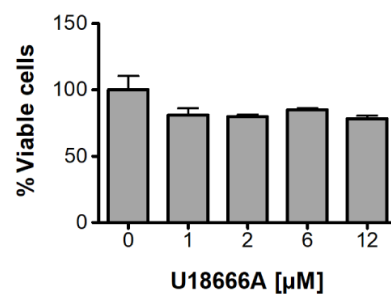
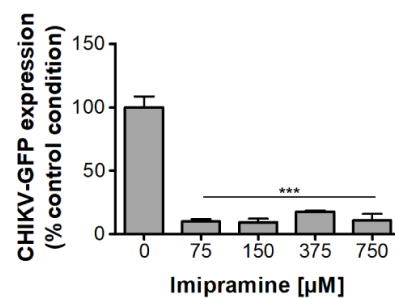
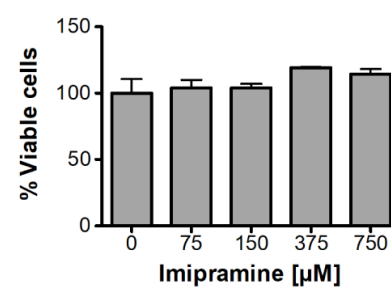
879 **Figure 8: nsPs expressed by a CHIKV trans-replicase or an infectious CHIKV co-segregate with DRMs**

880 **depending of nsP1 C417-419.** DRMs were isolated from (A) HEK293T cells transfected with CMV-  
881 P1234 and HSPoll-Fluc-Gluc plasmids (depicted in Fig 2B); (B) cells infected with CHIKV-LR-5'GFP  
882 (MOI 0.1); (C) cells transfected with CMV-P1<sub>3A</sub>234 and HSPoll-Fluc-Gluc plasmids. After 24 hrs,  
883 fractions were prepared and analyzed as described for Fig 7A, immunoblots were probed with  
884 antibodies against each CHIKV nonstructural protein as indicated. (D and E) Amounts of nsPs in DRM

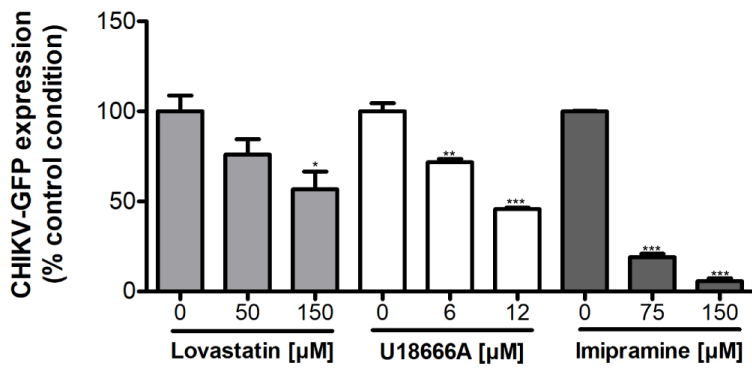
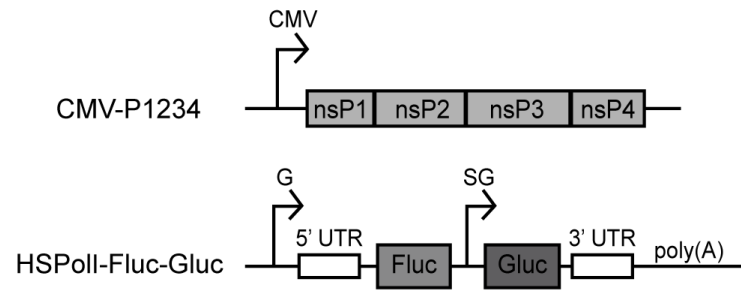
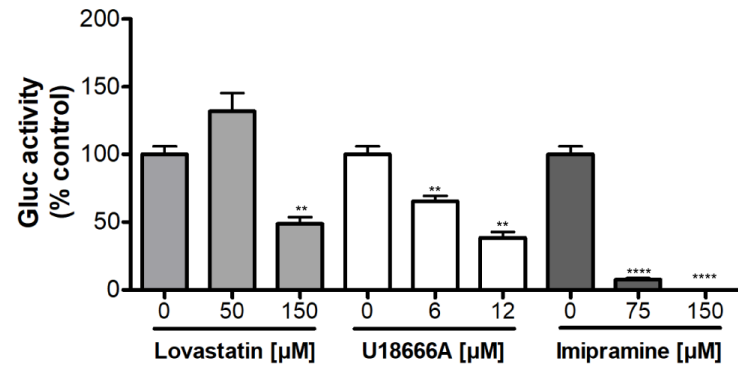
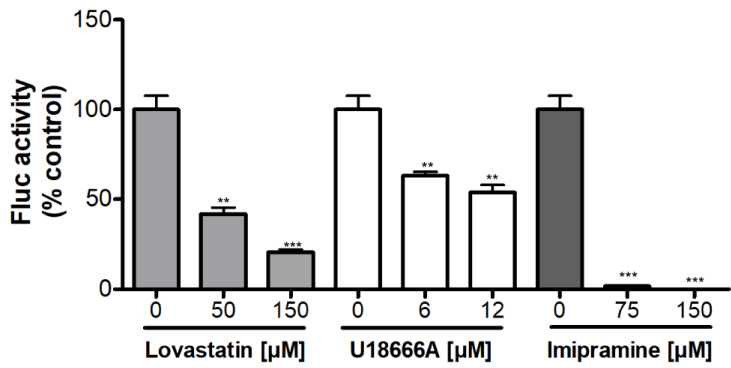
885 and DS fractions were determined by densitometry scanning of the immunoblots shown in (A) and  
886 (B) respectively and expressed as a percentage of total protein signal.

887 **Figure 9: SINV nsP1 affinity for cholesterol.** (A) Organization of the SINV nsP1 and mutant studied.  
888 Conserved amino-acids involved in the central  $\alpha$ -helix (aa 245-264) and palmitoylated cysteine (C420)  
889 are indicated. Mutant used in this study is depicted. (B) HEK293T cells transfected with either SINV  
890 GFP-nsP1 or GFP-nsP1<sub>C420A</sub> plasmids were processed for DRMs isolation as described for Fig 7.  
891 Fractions (numbered from top to bottom) were probed in immunoblot with antibodies against GFP.  
892 (C) HeLa cells were transfected with a plasmid encoding a SINV GFP-nsP1 protein for 4 hrs and  
893 cultured for an additional 24 hrs in the presence of 12  $\mu$ M U18666A. Cells were labeled with filipin III  
894 to visualize cholesterol. Controls cells (Mock) were maintained with vehicle. (D) HeLa cells  
895 transfected to express the GFP-nsP1<sub>C420A</sub> mutant were processed as in (C). Bars: 5  $\mu$ m.

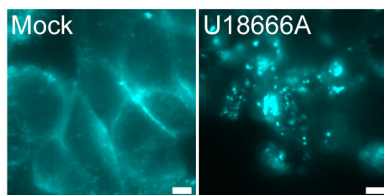
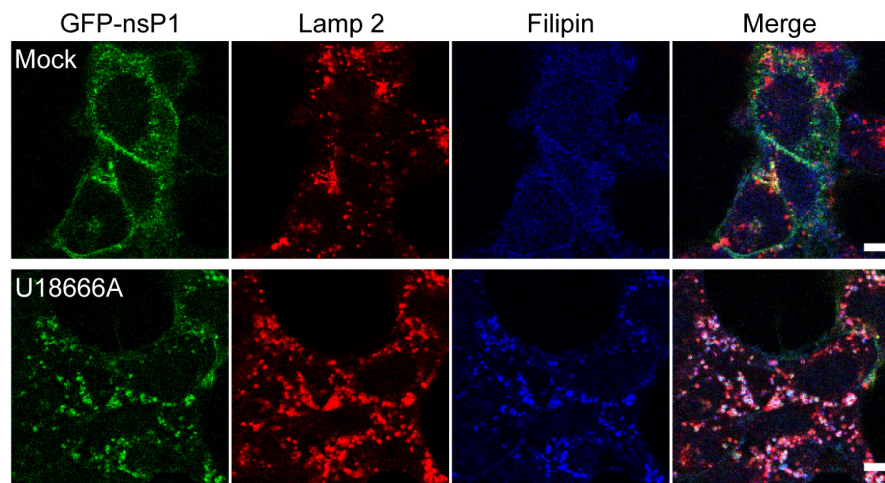
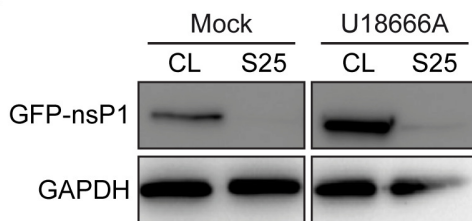
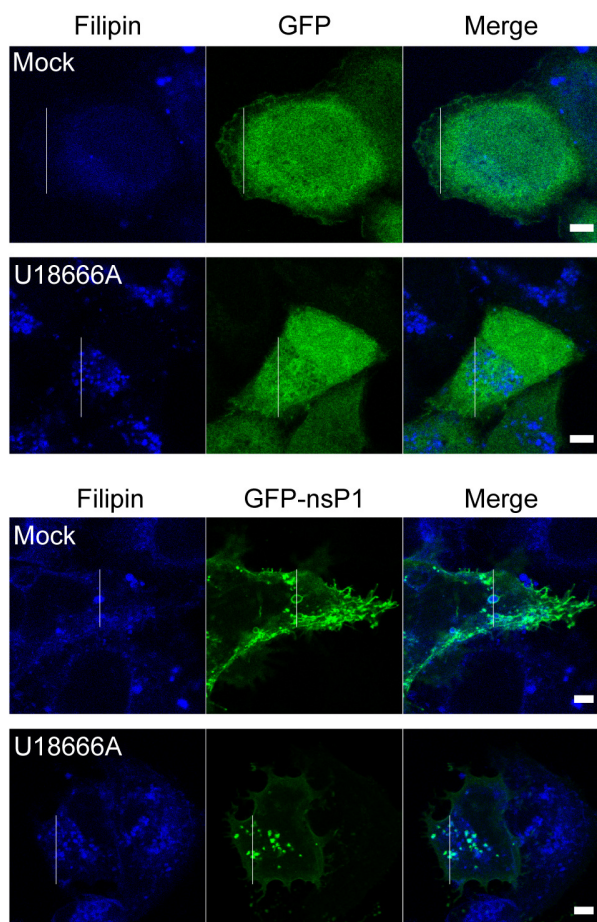
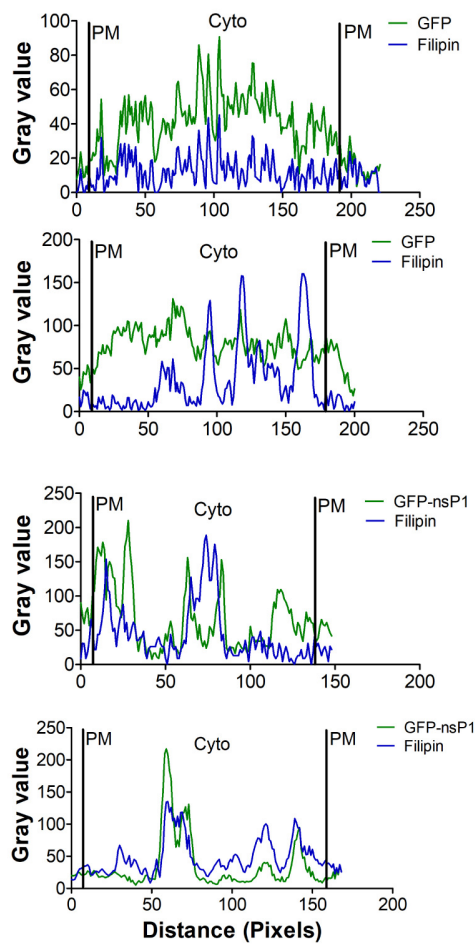
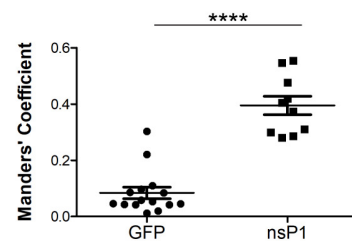
896 **Figure 10: Model for nsP1/cholesterol interplay in transfected and CHIKV-infected cells.** a)  
897 Palmitoylated cysteines in wild type nsP1 are critical for targeting to cholesterol-rich membranes  
898 microdomains. In infected cells, the preferential association of nsP1 with DRMs increases its local  
899 concentration which may facilitate its oligomerization and dictates the localization of a fraction of  
900 other nsPs to lipid rafts. In this scenario CHIKV efficiently replicates its genome. b) Imipramine and  
901 U18666A, by inhibiting cholesterol trafficking to the PM, generate nsP1 accumulation in Lamp2-  
902 positive late endosomes/lysosomes. Association of nsP2, nsP3 and nsP4 to cholesterol rich  
903 microdomains is no more observed. Under these conditions, CHIKV genome replication is abolished  
904 by a mechanism that remains to be fully elucidated. c) nsP1<sub>3A</sub> that weakly interacts with the plasma  
905 membrane is not targeted to DRMs. In this situation CHIKV genomes replication is impaired. For  
906 simplicity, only mature nsPs are shown in this model. In natural infection, the membrane association  
907 begins by modification and membrane targeting of polyprotein precursors.

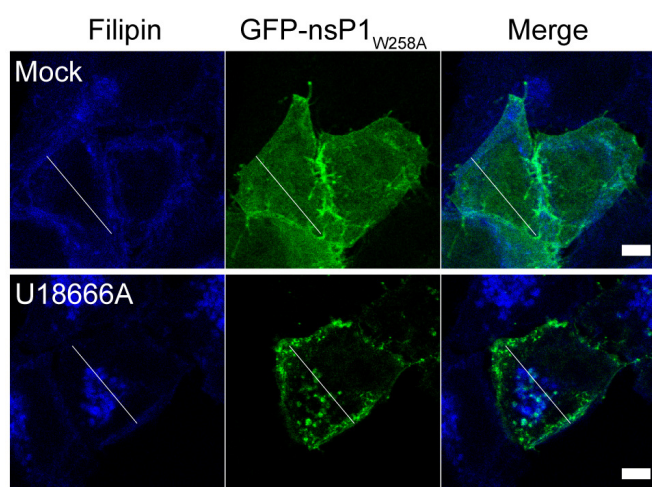
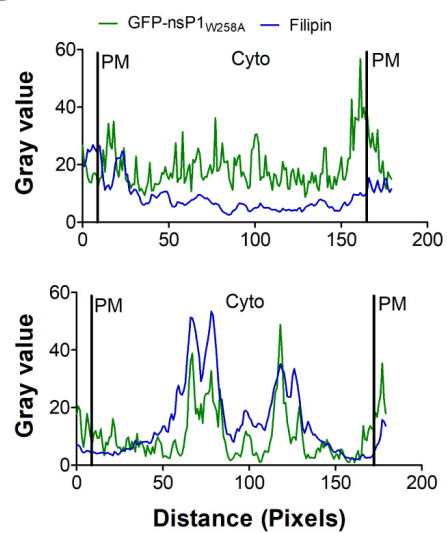
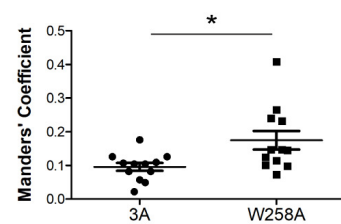
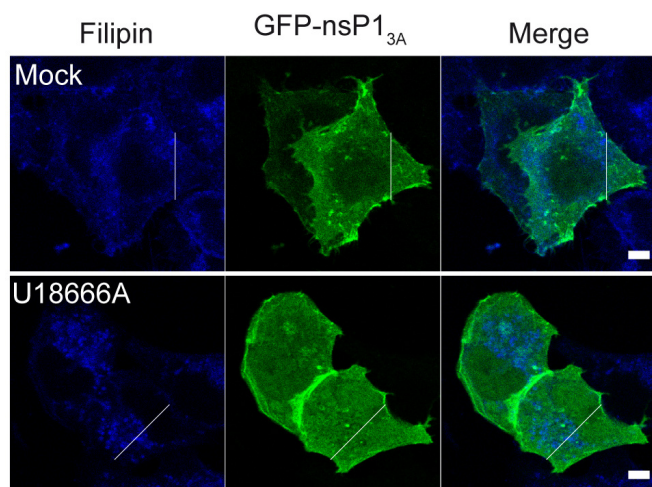
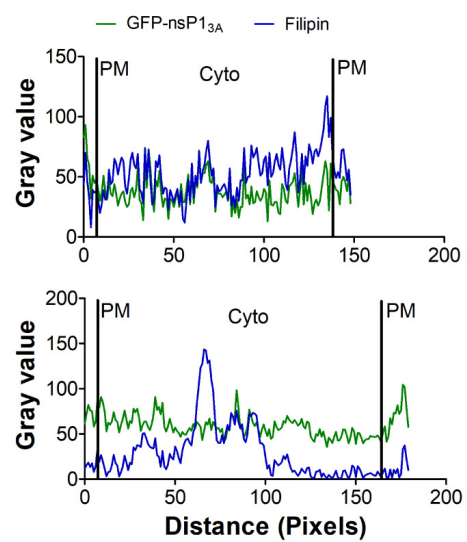
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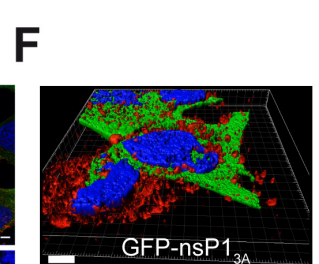
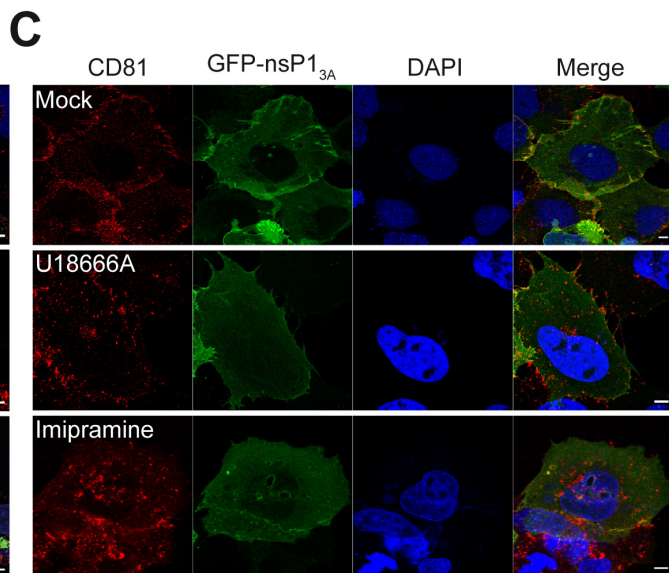
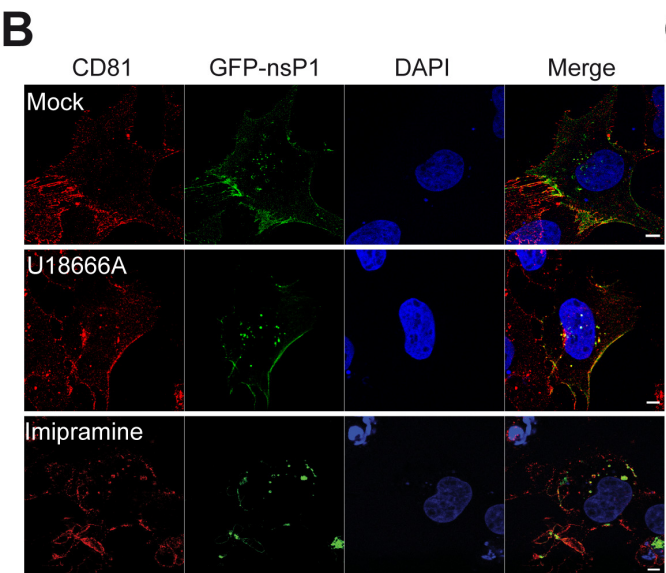
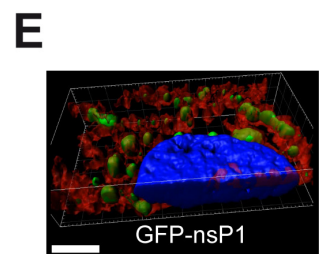
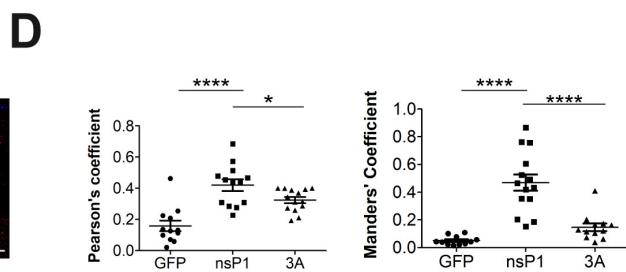
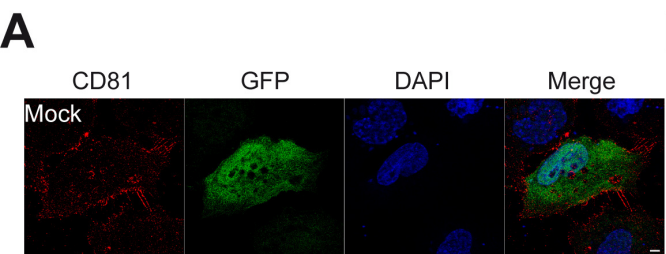
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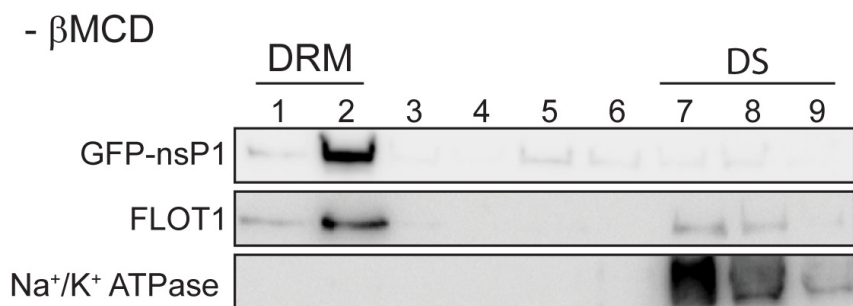
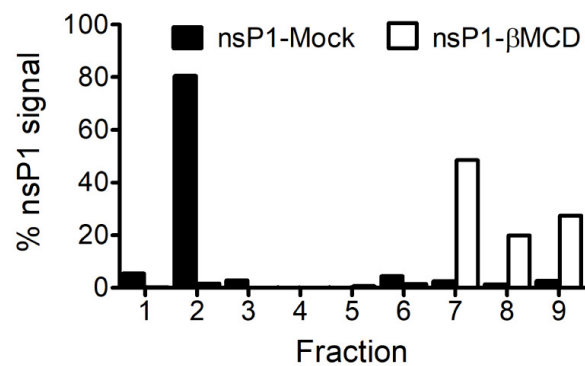
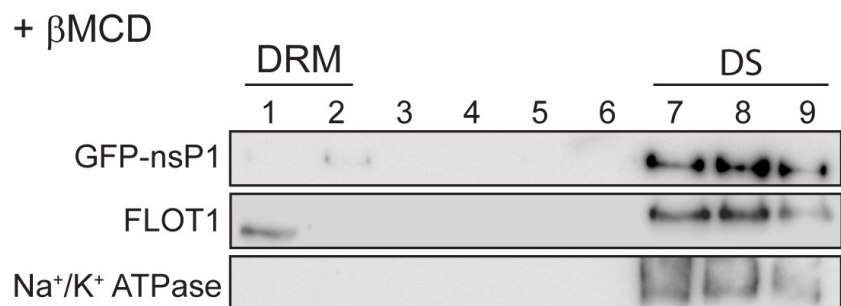
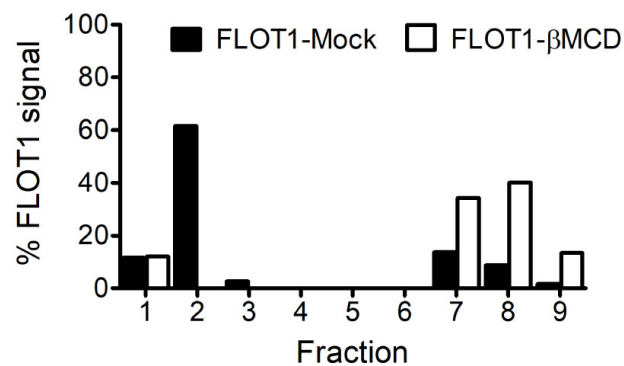
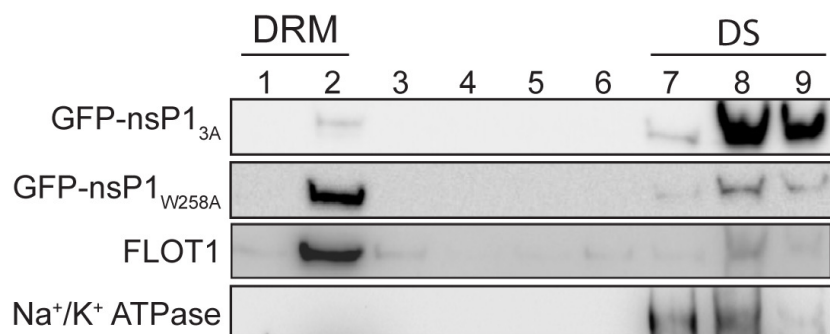
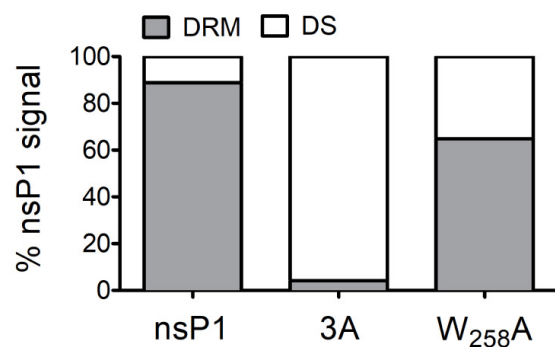


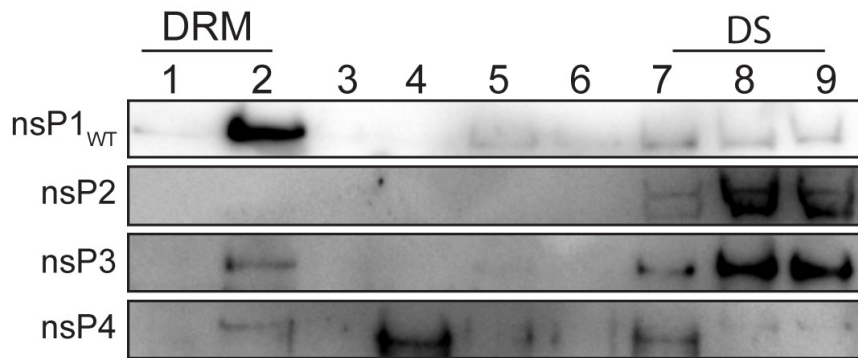
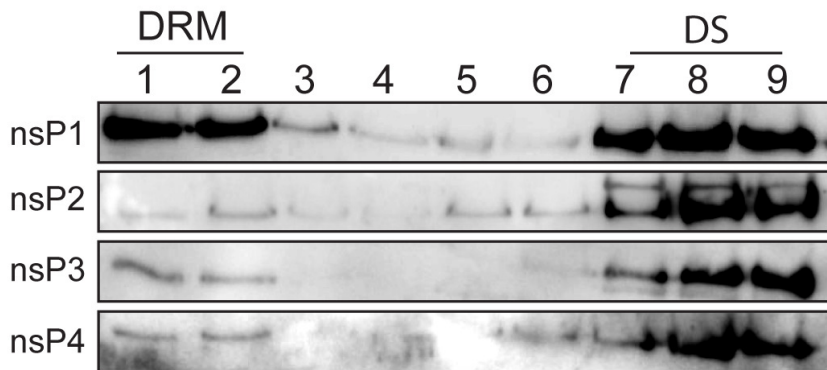
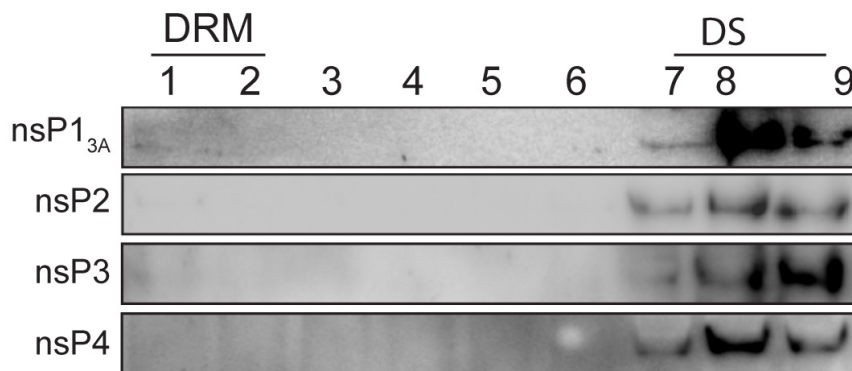
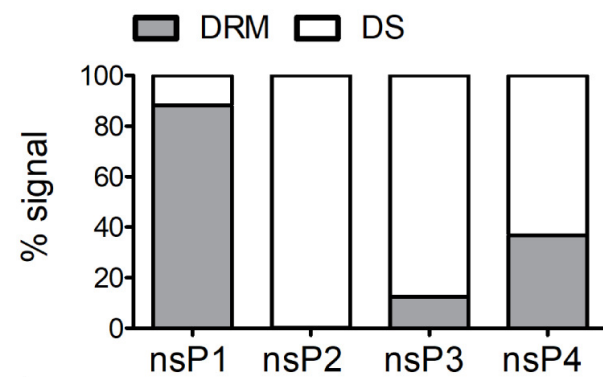
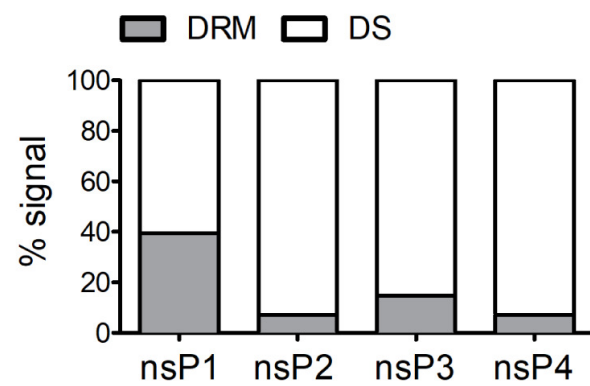
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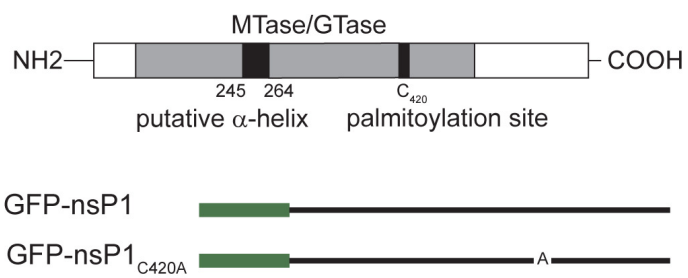
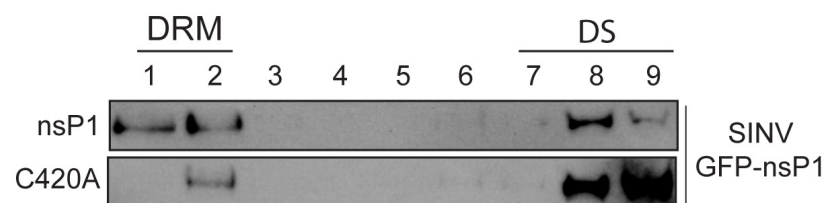
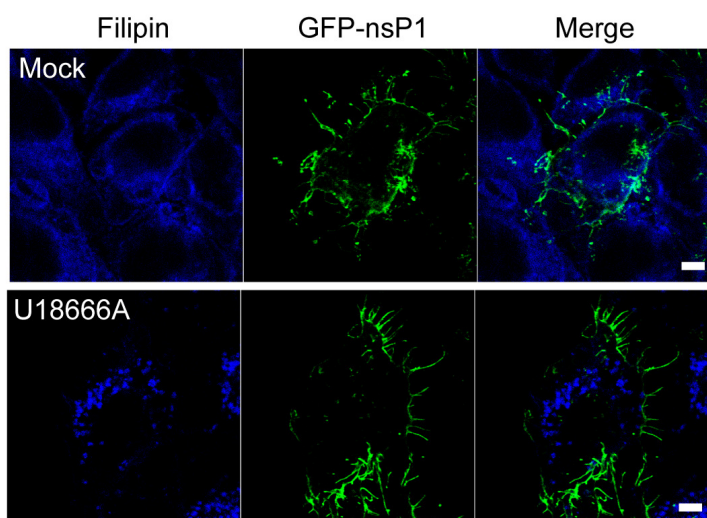
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