

1           Role of Capsid anchor in the morphogenesis of Zika virus

2   Jyoti Rana,<sup>a#</sup> José Luis Slon Campos,<sup>a\*</sup> Gabriella Leccese,<sup>a</sup> , Maura Francolini,<sup>b</sup>

3   Marco Bestagno,<sup>a‡</sup> Monica Poggianella<sup>a</sup> and Oscar R Burrone<sup>a#</sup>

4   <sup>a</sup> Molecular Immunology Group, International Centre for Genetic Engineering and  
5   Biotechnology (ICGEB), 34149 Trieste, Italy

6   <sup>b</sup> Università degli Studi di Milano - Dept. of Medical Biotechnology and Translational  
7   Medicine, Via L. Vanvitelli 32, 20129 Milan, Italy

8

9   Running title: Role of Capsid anchor in ZIKV assembly

10   <sup>#</sup> Corresponding authors: rana@icgeb.org, burrone@icgeb.org, tel: +39-040-  
11   3757314

12   <sup>\*</sup> Present address: Nuffield Department of Medicine, Wellcome Trust Centre for  
13   Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, UK

14   <sup>‡</sup> Present address: Cellular Immunology Group, International Centre for Genetic  
15   Engineering and Biotechnology (ICGEB), 34149 Trieste, Italy

16   Word count for abstract: 146

17   Word count for text: 5202

18

19 **ABSTRACT**

20 The flavivirus capsid protein (C) is separated from the downstream pre-membrane  
21 (PrM) protein by a hydrophobic sequence named capsid anchor (Ca). During  
22 polyprotein processing, Ca is sequentially cleaved by the viral NS2B/NS3 protease  
23 on the cytosolic side and by signal peptidase on the luminal side of the ER. To date,  
24 Ca is considered important mostly for directing translocation of PrM into the ER  
25 lumen. In this study, the role of Ca in the assembly and secretion of ZIKV was  
26 investigated using a pseudovirus-based approach. Our results show that, while Ca-  
27 mediated anchoring of C to the ER membrane is not needed for the production of  
28 infective particles, Ca expression *in cis* with respect to PrM is strictly required to  
29 allow proper assembly of infectious particles. Finally, we show that the presence of a  
30 heterologous, but not the homologous, Ca induces degradation of E through the  
31 autophagy/lysosomal pathway.

32 **IMPORTANCE**

33 The capsid anchor (Ca) is a single pass transmembrane domain at the C-terminus of  
34 the capsid protein (C) known to function as a signal for the translocation of PrM into  
35 the ER lumen. The objective of this study was to further understand the role of Ca in  
36 ZIKV life cycle, whether involved in the formation of nucleocapsid through  
37 association with C or in the formation of viral envelope. In this study, we show that  
38 Ca has a function beyond the one of translocation signal, controlling protein E  
39 stability and therefore its availability for assembly of infectious particles.

40

## 41 INTRODUCTION

42 Similar to other members of the Flaviviridae family which also includes significant  
43 human pathogens like West Nile virus (WNV) and Dengue virus (DENV), Zika (ZIKV)  
44 is an enveloped, single-stranded, positive-sensed RNA virus (1). The surface  
45 structure of a typical flavivirus particle is formed by 180 copies of the envelope  
46 glycoprotein (E) and the membrane protein (M), which are anchored onto the host-  
47 derived lipid bilayer through their transmembrane domains (TMDs). The viral  
48 envelope encloses a nucleocapsid core composed of the single molecule of RNA  
49 genome and several copies of the Capsid protein (C) (2-4).

50 In all flaviviruses, the viral genome encodes a single open reading frame (ORF)  
51 which is translated into a polyprotein that is co- and post-translationally cleaved into  
52 three structural (C, pre-membrane/Membrane (PrM/M) and E) and seven non-  
53 structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Cleavage into  
54 mature viral proteins is catalyzed by the virus encoded NS2B-NS3 protease on the  
55 cytosolic side and by the host signal peptidase and furin at the luminal side of the ER  
56 and Golgi network, respectively (5). Processing of the polyprotein is crucial for virus  
57 replication and regulation of gene expression in the majority of positive sense RNA  
58 viruses (6, 7). Replication and morphogenesis of flaviviruses occur in close  
59 association with intracellular membranes (8-11). Following translation, virus  
60 assembly occurs by budding of the nucleocapsid into the ER, thus acquiring the  
61 membrane envelope together with proteins E and PrM. During traffic through the  
62 Golgi network, furin cleaves PrM to generate mature and infective virions. Following  
63 its cleavage, the Pr peptide remains associated to the viral particle and dissociates  
64 when the virus reaches neutral pH upon exit from the cell (10, 12-14).

65 During translation, the structural proteins PrM and E, and the non-structural proteins  
66 NS1 and NS4B are translocated into the ER lumen by TMDs present at the C-  
67 terminus of the upstream proteins, which act concomitantly as signal peptides for ER  
68 translocation of the downstream protein and as a stop transfer signal for the  
69 upstream one. The capsid protein (C), the first one of the polypeptide, carries a  
70 single TMD, also termed as capsid anchor (Ca), which anchors C to the ER  
71 membrane before cleavage by NS2B/NS3 protease and is a translocation signal for  
72 PrM into the ER lumen. Instead, PrM and E proteins have double TMDs, in which  
73 the first one acts as a stop transfer signal while the second serves as a translocation  
74 signal for the following protein (E in the case of PrM and NS1 in the case of E) (15 ).  
75 Different studies performed to understand the role of PrM and E TMDs in viral  
76 assembly and release showed that mutations in these regions or interchange of  
77 them among flaviviruses severely impair virus assembly and release of viral particles  
78 (16-20). However, the role of Ca in the viral life cycle has not been extensively  
79 investigated.

80 The Ca region consists of three main segments and varies from 14 to 22 amino  
81 acids among the different flaviviruses. The amino-terminal region contains basic  
82 residues, which determine the orientation of the polyprotein insertion into the ER and  
83 provides the cleavage site for the viral protease, and is followed by a core formed by  
84 a series of uninterrupted hydrophobic amino acids and a carboxy-terminal cleavage  
85 region which is relatively hydrophobic but consistent with the (-3, -1) rule (21). The  
86 flavivirus Ca region has the requirement to be cleaved sequentially during  
87 processing of the polyprotein. The first cleavage takes place on the cytosolic side by  
88 the viral protease NS2B/NS3 releasing mature C in the cytosol, which is followed by  
89 cleavage by the host signal peptidase on the ER luminal side (22-25). This ordered

90 processing, controlled by the Ca sequence, was shown to be required for efficient  
91 assembly of Murray valley encephalitic virus (MVEV) and Yellow fever virus (YFV)  
92 (23, 26).

93 To date, flavivirus Ca has been considered to function mainly as a signal sequence  
94 for PrM translocation. Here, we show that Ca plays an essential role in controlling  
95 stability of downstream structural polyprotein, thus participating in the fate of virus  
96 assembly.

## 97 **RESULTS**

### 98 **Role of Ca on ZIKV packaging**

99 The role of the Capsid anchor (Ca) on the production of ZIKV was investigated using  
100 a WNV replicon-packaging approach, which is based on the production of infectious  
101 pseudoviral particles by providing *in trans* the viral structural proteins expressed from  
102 appropriate constructs (27). A schematic representation of the structural proteins  
103 topology and the sequences at the C-Pr junction are shown in Figs. 1A and 1B,  
104 respectively. For the production of pseudoviruses, a WNV replicon that harbours an  
105 EGFP reporter (WNV-rep) (27) was co-transfected in HEK293T cells with packing  
106 constructs encoding C and PrM-E derived from WNV (positive control), ZIKV or  
107 DENV2. Production of infective pseudoviral particles was analysed by infecting Vero  
108 cells and determining the number of EGFP positive cells by cytofluorimetry at 24  
109 hours post-infection (hpi) (Fig. 1C).

110 As shown in Fig. 2A and 2B, pseudoviruses were efficiently produced with both WNV  
111 and ZIKV packaging constructs, but not with DENV2, in agreement with previous  
112 studies showing context-dependent activity of the WNV NS2B/NS3 protease (28). A

113 mutation in DENV2 (T101G) at the Ca cytosolic cleavage site improved proteolytic  
114 activity and partially restored pseudovirus production (Fig. 2A and 2B) (28).  
115 Production of pseudoviruses was further confirmed by western blot analysis of  
116 virions (obtained from culture supernatants by ultracentrifugation) using anti-  
117 flavivirus E protein mAb 4G2 and by detection of replicon RNA in supernatants using  
118 RT/PCR (Fig. 2C). These results show that pseudovirus infectivity corresponds to  
119 the relative levels of secreted particles.

120 We then investigated the role of Ca in ZIKV life cycle. For this purpose we generated  
121 different ZIKV chimeric packaging constructs in which the C and/or Ca region, and/or  
122 the first 5 amino acids of Pr ([5]), were substituted with the homologous regions from  
123 WNV (schematically shown in Fig. 3A). Cytofluorimetric analysis of cells infected with  
124 pseudoviruses obtained with the different packaging constructs, showed that ZIKV  
125 chimeras carrying a heterologous Ca, i.e.  $C^W-Ca^W-(5^W)PrME^Z$ ,  $C^W-Ca^W-PrME^Z$  and  
126  $C^Z-Ca^W-PrME^Z$  (Fig. 3A, constructs 2, 3 and 5, respectively), did not infect Vero  
127 cells, indicating that these replacements were lethal (Fig. 3B and 3C). In contrast,  
128 the chimera with homologous Ca, i.e.  $C^W-Ca^Z-PrME^Z$  (construct 4), produced  
129 significant levels of infective particles, as well as the chimera with substitution of the  
130 5 Pr N-terminal amino acids ( $C^Z-Ca^Z-[5^W]PrME^Z$ , construct 6) albeit in lower amounts  
131 (Fig. 3B and 3C). In both cases, the number of infected cells were significantly lower  
132 than when using the wild type ZIKV construct (construct 1). Pseudovirus production  
133 was further confirmed by western blots. Consistent with the infection assay, secreted  
134 pseudoviruses were only detected in ZIKV constructs with the homologous Ca,  
135 suggesting a role of this region in the assembly and/or secretion of virion particles  
136 (Fig. 3D). Furthermore, the intracellular E levels were higher with constructs carrying  
137 ZIKV Ca (Fig. 3D). In agreement with the infection data, the chimera with the

138 heterologous first 5 amino acids of Pr ( $C^Z$ - $Ca^Z$ -[5<sup>W</sup>]PrME<sup>Z</sup>, construct 6) showed  
139 reduced level of intracellular and secreted E and was found to significantly  
140 compromise secretion of viral particles (Fig. 3D).

141 These results indicate a key role for Ca in the morphogenesis of ZIKV infectious  
142 particles, a function that goes beyond its signal peptide activity for PrM translocation  
143 into the ER lumen.

#### 144 **Cytosolic and ER luminal Ca processing**

145 The processing of Ca is a coordinated step of cleavage by viral and host proteases.  
146 The chimeric constructs generated by replacements at the C-Pr junctions have  
147 modified cleavage site sequences, which could affect Ca processing and thus  
148 potentially affect the production of pseudoviruses. To examine the effect of these  
149 replacements on proteolytic cleavage, N-terminally SV5-tagged packaging  
150 constructs carrying the full ZIKV sequence and the Ca substitution were generated  
151 (SV5- $C^Z$ - $Ca^Z$ -PrME<sup>Z</sup> and SV5- $C^Z$ - $Ca^W$ -PrME<sup>Z</sup>, respectively) (Fig. 4A). These  
152 constructs were transfected into HEK293T cells in the presence and absence of the  
153 viral protease NS2B/NS3 provided by WNV-rep. Western blot analysis showed that  
154 in the absence of viral protease, C protein was not cleaved as only CPrM and  
155 CPrME unprocessed forms were detected at very low levels (Fig. 4B). Conversely, in  
156 the presence of NS2B/NS3, C was equally processed in both constructs, as  
157 represented by the high levels of SV5-tagged C (Fig. 4B). Similar results were  
158 obtained for E protein (detected with mAb 4G2) further confirming the downstream  
159 effect of Ca processing and the sequential requirement of cytosolic cleavage before  
160 signal peptidase luminal proteolysis (Fig. 4B). As shown in Fig. 4C, the ZIKV SV5-  
161 tagged packaging construct was able to produce infective particles although reduced

162 in comparison to the untagged version while, as expected, the one with the Ca<sup>W</sup> was  
163 not.

164 These results indicate that the lack of pseudovirus production with the heterologous  
165 Ca<sup>W</sup> was not the consequence of impaired processing on the cytosolic side by the  
166 viral protease.

167 We next investigated processing by the signal peptidase at the Ca-Pr peptide  
168 junction of chimeric constructs and the performance of the different Ca as secretion  
169 signals. For this purpose, Ca from DENV2, ZIKV and WNV with wild type or chimeric  
170 Pr N-terminal amino acids were used as signal peptides on an irrelevant secretory  
171 soluble reporter protein (a VHH-Fc $\gamma$  fusion protein). As positive control we used an  
172 efficient immunoglobulin-derived signal peptide (sec) frequently used for different  
173 secretory proteins (29, 30) (Fig. 4D). Western blot analysis of intracellular and  
174 secreted protein levels showed that all three Ca allowed secretion of the reporter  
175 protein with an efficiency comparable to sec, irrespective of the 5 amino acids  
176 immediately downstream of the cleavage site (Fig. 4E). Thus, the comparable signal  
177 peptide activity of each Ca and their ability to be cleaved by the signal peptidase,  
178 suggest that the differences observed in pseudovirus production are not a  
179 consequence of impaired translocation efficiency.

#### 180 **Effect of Ca on VLPs secretion**

181 We then analysed the role of Ca in the production of virus-like particles (VLPs),  
182 which correspond to secretory vesicles harbouring the PrME viral proteins without  
183 the nucleocapsid core. Although VLPs are not identical to viral particles, they  
184 represent structures that assemble into membranous vesicles, are secreted and  
185 show antigenic properties similar to infectious viruses. For this purpose, we used

186 constructs encoding ZIKV PrME with Ca from ZIKV or WNV as signal peptides (Fig.  
187 5A, left panel). The non-viral sec signal peptide was used as a control. Western blot  
188 analysis of cell extracts and ultracentrifuged supernatants (to concentrate secreted  
189 VLPs) were in line with the production of pseudoviruses. VLPs were produced at  
190 higher levels and secreted only in the presence of its homologous Ca<sup>Z</sup> (Fig. 5A, right  
191 panel). The vesicular nature of the secreted material was confirmed by treating  
192 supernatants with a non-ionic detergent (NP40, 0.5%) before ultracentrifugation. As  
193 expected, this treatment dissolved the membrane of the vesicles present in the  
194 supernatant, which did not sediment by ultracentrifugation. Further, we used DENV2  
195 VLP constructs with different Ca's to analyse whether these replacements had the  
196 same impact on a different flavivirus (Fig. 5B, left panel). The results showed that, in  
197 contrast to ZIKV, DENV2 VLPs were produced even when the homologous Ca<sup>D</sup> was  
198 replaced with the ones from ZIKV and WNV but not with the control sec (Fig. 5B,  
199 right panel). This indicates that, while ZIKV strictly requires its homologous Ca,  
200 DENV is more permissive to other viral derived sequences, but remains refractory to  
201 the non-viral one.

202 Contrary to what we observed with VLP constructs, secretion of a soluble version of  
203 ZIKV dimeric E protein (sE-cvD, which lacks its own stem and C-terminal anchor  
204 sequences, and carries a A264C mutation to induce covalent dimerization) (31) was  
205 efficient with both Ca and the control sec, independently of the presence or absence  
206 of PrM (Fig. 5C and 5D). We decided to use the cvD variant of ZIKV sE because, as  
207 we have previously shown (31), the dimeric protein is properly folded and secreted  
208 from mammalian cells and is closer to the settings in which vesicles are formed  
209 (dimerised E). Taken together these results strongly suggest that Ca is not just a  
210 signal for PrM translocation but plays a crucial role in the assembly of VLP vesicles.

211 **Ca determines E protein stability**

212 The largely reduced intracellular levels of the E protein expressed from the chimeric  
213 packaging and VLP constructs (Fig. 3B and 5A) suggested activation of a  
214 degradation pathway. This was confirmed in cells transfected with Ca<sup>W</sup>-PrME<sup>Z</sup> or  
215 Ca<sup>Z</sup>-PrME<sup>Z</sup> following Cycloheximide (CHX) treatment (Fig. 6A). While E expressed  
216 from the chimeric construct with the heterologous Ca<sup>W</sup> was actively degraded, the  
217 one produced from the construct with the homologous Ca<sup>Z</sup> was not (Fig. 6B). To  
218 analyse the degradation pathway, transfected cells were treated with a lysosomal  
219 inhibitor (Chloroquine, CQ) or a proteasome inhibitor (MG132) along with CHX. CQ  
220 treatment, completely rescued expression, while treatment with MG132, did it only  
221 partially (Fig. 6C). Further, western blot analysis of cells extracts from VLP  
222 transfected cells after CQ treatment showed accumulation of protein E and LC3-II  
223 (microtubule associated protein 1 light chain 3), a marker of autophagy (32) (Fig.  
224 6D), thus indicating that degradation occurs mainly through the autophagy-lysosomal  
225 pathway. LC3-II is known to be partially degraded with the target protein (33).  
226 Subsequently, activation of autophagy was studied by confocal microscopy with an  
227 anti-LC3 antibody recognising both non-lipidated (LC3-I) and lipidated (LC3-II) forms,  
228 and an anti-E serum specific for ZIKV domain III (DIII) produced by immunizing  
229 Balb/c mice. As shown in Fig. 6E robust expression of E and a low number of LC3  
230 puncta was observed in cells transfected with the Ca<sup>Z</sup>-PrME<sup>Z</sup> construct. In contrast,  
231 but in agreement with the activation of autophagy, in cells transfected with the  
232 chimeric Ca<sup>W</sup>-PrME<sup>Z</sup>, E was undetectable but a large increase in LC3 puncta was  
233 observed. Further evidence was obtained by siRNA silencing of Atg7. LC3 is  
234 produced as a precursor protein, which is first cleaved by Atg4 to generate LC3-I and  
235 then by Atg3 and Atg7 to generate the lipidated form LC3-II that is associated to

autophagic vesicles. Confocal microscopy showed that, while Atg7 silencing had no effect on E expressed from the wildtype construct, it did induce E accumulation when expressed from the chimeric Ca<sup>W</sup>-PrME<sup>Z</sup> when compared to cells treated with an irrelevant siRNA (Fig. 6F). These results were confirmed by western blot analysis of E and endogenous LC3 protein levels as well (Fig. 6G and Fig. S2). Furthermore, electron microscopy studies have shown that the cytoplasm of cells expressing Ca<sup>W</sup>-PrME<sup>Z</sup> was enriched in late endosome/multivesicular bodies and organelles of the degradative compartment/autolysosomes (Fig. 6H). Overall these results indicate that the single replacement of the homologous Ca with a closely related one was sufficient to induce activation of the autophagy pathway resulting in degradation of protein E and therefore impaired pseudovirus assembly.

#### **Effect of Ca on the assembly of infective particles**

We then investigated, whether in addition to the Ca role in determining stability of the packaging structural proteins, Ca-mediated anchoring of C to the ER membrane is also required for virus morphogenesis. To achieve this, we analysed production of pseudoviruses using a tripartite system: C and PrME were expressed in *trans* from two different constructs instead of the classical *cis* C-PrME and co-transfected with the WNV replicon (Fig. 7A). We used ZIKV C in two different versions: one with its own Ca<sup>Z</sup> and one without any anchor. The ZIKV PrME constructs instead, had different Ca or sec (as a non-viral control sequence) as secretion signals (Fig. 7B). Pseudoviruses were produced with PrME constructs containing Ca<sup>Z</sup>, but not with Ca<sup>W</sup> or sec, regardless of whether C was anchored to the ER membrane (C-Ca<sup>Z</sup>) or not (C) (Fig. 7C and 7D). Secretion of ZIKV pseudoviral particles was confirmed by western blot and RT/PCR (Fig. 7E). The higher efficiency of pseudovirus production when C was expressed without any anchor at its carboxy-terminus was most likely

261 due to the lower levels of soluble C produced from the C-Ca<sup>Z</sup> construct in the  
262 presence of the non-structural viral proteins (WNV-rep) because of incomplete  
263 processing (Fig. 7F, left panel). Interestingly, we observed that cleavage efficiency  
264 by the viral protease was largely improved (almost complete) in the presence of PrM  
265 (Fig. 7F, right panel).

266 Taken together our results demonstrate that i) the viral Ca is strictly required at the  
267 N-terminal side of PrM for proper assembly of infective particles, as PrM constructs  
268 with other signal peptides that allow efficient translocation into the lumen, were  
269 unable to assemble even when co-expressed with C-Ca; and ii) C anchorage to the  
270 ER membrane is not essential for the formation of nucleocapsid and virus assembly,  
271 consistent with the requirement of sequential cleavage on the cytosolic side before  
272 cleavage by the signal peptidase.

## 273 DISCUSSION

274 The topological distribution of structural and non-structural proteins is a conserved  
275 characteristic feature of flaviviruses, which have a replication and assembly strategy  
276 centred on attachment to the ER membranes. With the exception of NS1, most of the  
277 non-structural proteins, are mainly facing the cytosolic side and remain attached to  
278 the ER membrane either by their own TMDs or through interactions with other NS  
279 proteins. The two structural proteins M and E associated to the virus envelope, have  
280 both double spanning trans-membrane domains that serve as anchors to expose the  
281 proteins to the lumen of the ER. The role of their TMDs in assembly and release of  
282 infective particles have been extensively studied in the context of other flaviviruses  
283 like tick borne encephalitic virus and Yellow fever virus (16, 20). Conversely, little is  
284 known on the Ca trans-membrane domain, which has long been considered as a

285 signal peptide to allow translocation of PrM into the ER lumen. Here we show that  
286 Ca plays a significant role in ZIKV morphogenesis by controlling E protein stability,  
287 thus making it an essential determinant for assembly of infective particles.

288 In other viruses signal sequences have also been shown to play additional roles. In  
289 hepatitis C virus (HCV), a member of the *Flaviviridae* family (*Hepacivirus* genus), the  
290 signal peptide at the C terminus of the core protein (equivalent to Ca in ZIKV) allows  
291 secretion of non-enveloped nucleocapsids a phenomenon that contributes to  
292 persistent infection and escape from immune surveillance (34, 35). It has also been  
293 shown that this transmembrane signal peptide is responsible for lipid accumulation in  
294 infected cells causing steatosis (36). In alphaviruses, like Semiliki Forest Virus (SFV)  
295 and Sindbis Virus, the signal peptide of p62 is found in mature viral particles  
296 associated with the complex formed by E1-E2 glycoproteins (37), whereas in the  
297 lymphocytic choriomeningitis virus (LCMV) the long extended N-terminal sequence  
298 (58 amino acids) of glycoprotein C remains anchored to the viral membrane after  
299 cleavage by the signal peptidase and plays a role in glycoprotein maturation and  
300 virus infectivity (38).

301 In this work we used a pseudovirus-based system that allows production of infective  
302 particles. Infectivity was determined as a function of the expression of the EGFP  
303 reporter encoded in the WNV replicon. This approach has been previously used to  
304 study different aspects of flavivirus life cycle (39-44).

305 To understand the role of Ca in viral life cycle, different chimeras were generated by  
306 replacements of C and/or Ca on the ZIKV packaging constructs. We took advantage  
307 of the strict requirement shown by ZIKV, which only permits its own Ca to produce  
308 pseudoviruses and VLPs. This was surprising, as Ca of other viruses, like that of

309 WNV have a closely related sequence. Previous studies in other flaviviruses have  
310 shown that replacement of TBEV Ca with that of JEV, a distantly related flavivirus,  
311 did not impair virion formation or infectivity of viral particles (20), highlighting a  
312 permissiveness not present in ZIKV. In contrast, replacement of PrME from DENV2  
313 with that of ZIKV without the exchange of Ca, failed to produce infectious chimeric  
314 viruses (45). This was also true when pseudoviruses were produced in other cell  
315 lines such as HeLa and Vero (data not shown). In addition, a study on a ZIKV VLP-  
316 based vaccine showed that replacement of the signal sequence of PrM (i.e: Ca) and  
317 TMDs of E with those of JEV improved secretion of VLPs (46). These contrasting  
318 data indicate that different flaviviruses show markedly different permissiveness upon  
319 replacements of structural components.

320 To understand how the replacement of Ca in ZIKV structural polyprotein affected  
321 production of viral and subviral particles, we first ruled out impairment of the cytosolic  
322 and luminal cleavages of Ca using different packaging constructs. The signal peptide  
323 activity was tested on both the viral sE as well as on an irrelevant secretory protein  
324 and the results showed that this function was not dependent on the downstream-  
325 encoded protein. However, different Cas did show clearly distinct activities when  
326 assessing their ability to produce VLPs, which are the result of co-expression of prM-  
327 E and require incorporation of the two membrane proteins into a lipid vesicle. In this  
328 context, only the homologous Ca was permissive for assembly of VLPs.  
329 Interestingly, the non-viral leader peptide (sec) was completely incompetent in  
330 allowing production of pseudoviruses or VLPs even with the DENV2 construct, which  
331 is highly permissive to heterologous Ca sequences. This suggests that the viral-  
332 derived Ca sequences have been selected for a function that goes beyond their  
333 capacity to allow PrM translocation to the ER lumen.

334 It was clear that replacement of Ca abolished production of pseudoviral particles by  
335 strongly reducing the intracellular levels of protein E. The experiments performed in  
336 the presence of inhibitors indicated that enhanced E degradation took place  
337 preferentially through activation of the autophagy/lysosomal pathway. These results  
338 were further confirmed by electron microscopy and immunofluorescence analysis  
339 using anti-E and anti-LC3 antibodies and Atg7 silencing. Other studies have  
340 shown that accumulation of large protein aggregates in the ER triggers the  
341 autophagy dependent degradation pathway (47). One possibility is that Ca actively  
342 participates in folding of PrM and/or E. However, since proper folding of the  
343 extracellular domains of E (sE) can be efficiently achieved even in the absence of Ca  
344 and PrM (31), this putative role for Ca might be dependent on anchorage of the  
345 proteins to the ER membrane through their TM domains. In this scenario, Ca might  
346 be required to allow proper interactions and assembly of PrM-E complexes, which  
347 are otherwise actively targeted to degradation. As Ca is a short hydrophobic  
348 sequence embedded in the ER membrane, the different tolerance of exogenous Ca  
349 sequences by different flaviviruses suggest complex interactions taking place  
350 between Ca and the TM domains of M and/or E. Surprisingly, the lack of permissive  
351 interactions leads to activation of a degradation pathway. In agreement with the  
352 existence of these interactions, secretion of E with exogenous Ca in the absence of  
353 PrM and of its own TMs was also well tolerated by ZIKV.

354 Interestingly, the experiments with the tripartite system for the production of  
355 pseudoviruses showed a strict requirement for  $\text{Ca}^Z$  to be expressed in *cis* with  
356 respect to PrME. In fact, providing  $\text{Ca}^Z$  in *trans* (as when expressed fused to C in C-  
357  $\text{Ca}^Z$  construct) was not sufficient to allow assembly into vesicles. In agreement with  
358 our results, trans-complementation studies on MVEV, YFV and Kunjin viruses have

359 also shown reduced pseudovirus assembly when C-Ca and PrME proteins were  
360 translated from separate coding units (23, 41, 48, 49).

361 It was not surprising to find that protein C does not require anchorage to the ER  
362 membrane for the formation of the nucleocapsid and assembly into infectious  
363 particles, as this is consistent with the sequential cleavage requirement on the  
364 cytosolic side before processing by the signal peptidase. Moreover, soluble C was  
365 more efficient in packaging WNV-rep than the membrane anchored C, most likely  
366 because in the absence of the downstream Pr peptide, Ca processing efficiency on  
367 the cytosolic side was affected. These results are consistent with a previous report  
368 showing that in YFV efficient cleavage was dependent of the Pr peptide sequence  
369 downstream (49).

370 In conclusion, our study shows that ZIKV assembly and maturation rearrangements  
371 involve not only the transmembrane domains of the lipid embedded M and E protein,  
372 but also Ca, which determines E stability controlling activation of the  
373 autophagy/lysosomal pathway.

## 374 MATERIALS AND METHODS

375 **Cell lines, monoclonal antibodies and chemicals.** HEK293T (ATCC CRL-11268,  
376 Rockville, MD, USA) and Vero (ATCC CCL-81) cells were cultured in DMEM (Life  
377 Technologies, Paisley, UK) supplemented with 10% heat-inactivated foetal calf  
378 serum (FCS) (Life Technologies), 50 µg/ml gentamycin and 2 mM L-glutamine.  
379 Unless indicated otherwise, all cells were incubated at 37 °C in the presence of 5%  
380 CO<sub>2</sub>. For the expression of covalent E dimers, transfected cells were incubated at 28  
381 °C instead of 37 °C. Flavivirus E protein-specific mAb 4G2 was expressed as a scFv  
382 (50; NCBI accession codes KJ438784 and KJ43875) fused to avidin in an avibody

383 format (Predonzani A and Burrone OR, unpublished) and used as cultured  
384 supernatant from HEK293T transfected cells. mAbs SV5 was obtained as previously  
385 described (30). The ZIKV specific anti-DIII serum was produced by immunising  
386 Balb/c mice with a DNA construct encoding the DIII-CH3 as in reference (30)

387 For protein degradation analysis, HEK293T cells were treated with translation  
388 inhibitor cycloheximide (100 µg/ml, Sigma), proteasomal inhibitor MG132 (20 µM,  
389 Calbiochem) or autophagy inhibitor chloroquine (50 µM, Sigma) 16 h post-  
390 transfection and cells were collected at different time intervals after treatment to  
391 analyse protein levels.

392 **Plasmid DNA constructs.** The gene fragments encoding for the structural proteins  
393 (C-PrM-E) from ZIKV MR766 strain (GenBank accession number AEN75266.1) and  
394 DENV2 NGC strain (GenBank accession number AAA42941) were obtained as a  
395 synthetic, mammalian-codon optimised genes (GenScript, Piscataway, NJ, USA)  
396 and cloned into pVAX1 expression vectors (Life Technologies). The WNV packaging  
397 construct and the DNA-launched WNV sub-genomic replicon expressing EGFP  
398 (herein named WNV-rep) were kindly provided by Theodore Pierson (National  
399 Institute of Allergies and Infectious Diseases, MD, USA) (27). The chimeric  
400 packaging constructs were obtained either by cloning or site-directed mutagenesis  
401 (QuikChange XL Site-Directed Mutagenesis Kit, Agilent Technologies, La Jolla, CA,  
402 USA) following manufacturer instructions.

403 Capsid, PrME, PrMsE encoding constructs were obtained by cloning the sequence  
404 of interest into pVAX1 expression vector after a PCR amplification of the fragment  
405 from the packaging constructs. PrMsE constructs were fused to a carboxy-terminal  
406 SV5 tag (GKPIPPLLGLD) for detection of the soluble protein (PrMsE-SV5) (51).

407 sE-SV5 constructs were obtained after directed deletion of PrM from PrME-SV5  
408 plasmids.

409 **Production of Pseudoviral particles and VLPs.** Pseudoviral particles were  
410 produced as previously described (52). Briefly, the WNV-rep and plasmids  
411 expressing the wild type (WT) or chimeric packaging constructs of WNV, ZIKV and  
412 DENV2 (1:3 WNV-rep: packaging construct ratio) were cotransfected into HEK293T  
413 cells using linear polyethylenimine (PEI, MW 25,000, Polysciences, Warrington, PA,  
414 USA) in a 1:3 DNA-polymer ratio. When the structural genes were provided in *trans*,  
415 the WNV-rep, PrME and C gene constructs were used in a 1:1:4 ratio, respectively.  
416 16 h post-transfection, culture medium was replaced by DMEM with 7% FCS and  
417 pseudoviruses were harvested after 24 h of incubation at 37 °C. Supernatants were  
418 clarified by centrifugation and stored at -20 °C until use. Using the same protocol,  
419 VLPs were obtained by transfecting the PrME constructs into HEK293T cells. When  
420 needed, the culture supernatant was ultra-centrifuged with Beckman coulter Airfuge  
421 centrifuge using A100/18 rotor at 149000xg for 2 h at 4 °C, and the pellet containing  
422 either pseudoviruses or VLPs, was resuspended in non-reducing sample buffer for  
423 western blot analysis.

424 **Infection of Vero cells.**  $4 \times 10^4$  Vero cells were seeded in 24-multiwell plates 24 h  
425 before infection. Culture media was removed and cells were infected with 200 µl of  
426 pseudoviral preparations for 3 h at 37 °C. Then, 0.5 ml of DMEM with 2% FBS was  
427 added and cells were cultured for 24 h. Cell infection was determined by measuring  
428 the percentage of EGFP positive cells in cytofluorimetric analysis in a FACS Calibur  
429 (BD Biosciences, San Jose, CA, USA).

430 **Expression of secretory proteins.** Standard calcium phosphate transfections were  
431 performed on HEK293T cells in 6-multiwell plates as previously described (53). After  
432 overnight incubation at 37 °C, culture medium was replaced with serum-free DMEM  
433 and cells incubated for another 24 h at 37 °C or 28 °C depending on the construct.  
434 Then, culture supernatants were cleared by centrifugation and cellular extracts were  
435 prepared in 100 µl of TNN lysis buffer (100 mM Tris-HCl, pH 8, 250 mM NaCl, 0.5%  
436 NP-40) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO,  
437 USA). Samples were stored at -20 °C until use.

438 **siRNA transfection.** For siRNA experiments,  $5 \times 10^4$  HeLa cells/well and  $1 \times 10^5$   
439 HEK293T cells were seeded in 12-multiwell plates and transfected with 0.1 nmol of  
440 annealed duplex siRNA (Sigma) using 5 µl of RNAiMAX Lipofectamine 2000 (Life  
441 technologies) following manufacturer's protocol. The following siRNA were  
442 transfected: siAtg7 5'-GGUCAAAGGAUGAAGAUAA-3' and control non-targetted  
443 siRNA named siNT 5'-UCGUCUUCUACAACGUCAATT-3'. At 48 hrs post-  
444 transfection, media was changed and cells were allowed to grow for 24 hrs followed  
445 by transfection with VLP constructs. Next day, HeLa cells were used for  
446 immunofluorescence and HEK293T cells for western analysis.

447 **Western blot.** Western blot analyses of untagged and SV5-tagged proteins were  
448 done as previously reported (30). Briefly, samples were separated by non-reducing  
449 SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore,  
450 Temecula, CA, USA) and blocked with 5% milk solution in PBS (PBS-milk).  
451 Afterwards, membranes were incubated for 1h with the 4G2 avibody or anti-SV5  
452 mAb and washed. For SV5-tagged proteins, membranes were incubated for 1 h with  
453 anti-SV5 mAb (1 µg/ml), washed, and further probed with HRP-linked anti-mouse

454 IgG goat antibodies (KPL, Gaithersburg, MA, USA, 074-1809, 1:10,000) for 1 hr. In  
455 the case of 4G2, the avibody was previously conjugated with biotinylated-HRP  
456 (ThermoFisher-Pierce, Rockford, IL, USA), incubated for 1 hr and washed. Mouse  
457 HRP-conjugated anti-actin (clone AC-15, Sigma-Aldrich, 1:30000) was used as  
458 loading control. Signals were developed by ECL (ThermoFisher-Pierce, Rockford, IL,  
459 USA).

460 **RT-PCR.** WNV Replicon-derived RNA was isolated from 100µl of pseudoviral  
461 preparations using the RNAzol-BEE solution (Tel-Test, Friendswood, TX, USA) and  
462 then treated with DNaseI (Promega, Madison, WI, USA) following manufacturer's  
463 instructions. Reverse transcription was performed using random hexamers (Sigma)  
464 and M-MLV Reverse transcriptase (Life Technologies) according to the  
465 manufacturer's protocol. cDNAs were then amplified by PCR using WNV-3'UTR  
466 specific primers (Forward, 5'-CAGTGTCAGACCACACTTTAATGT-3'; Reverse, 5'-  
467 GCTTACAGCTTCAGCCAAG-3').

468 **Immunofluorescence Microscopy.** For immunofluorescence,  $5 \times 10^4$  HeLa  
469 cells/well were seeded in 12-multiwell plate and transfected with VLP constructs  
470 using calcium phosphate. The cells were fixed with 4% paraformaldehyde (PFA) 24  
471 h post-transfection and immunofluorescence experiments were performed as  
472 described previously (54) using following antibody dilutions: mouse ZIKV anti-DIII  
473 sera (1:100), anti-LC3B rabbit antibody (1:200, Sigma), Alexa Fluor 647 conjugated  
474 anti-mouse (1:500, Life technologies), Alexa Fluor-488 conjugated anti-rabbit (1:500,  
475 Life technologies). Samples were analysed by confocal microscope Zeiss LSM510  
476 (Goettingen, Germany) equipped with a 100X NA 1.3 objective.

477 **Electron Microscopy.** HEK293T cells were fixed 20 h post-transfection as a  
478 monolayer in 2% glutaraldehyde/0.1 M cacodylate buffer for 30 min at room  
479 temperature, scraped, collected as a pellet and supplemented with fresh fixative for  
480 24 hours at room temperature. Cell pellets were further processed and embedded in  
481 epoxy resin. Thin sections were observed with a Philips CM10 transmission electron  
482 microscope and images were acquired at a final magnification of 25.000–34.000x  
483 using a Morada CDD camera (Olympus, Munster - Germany).

484 **Statistical analysis.** Data were obtained from at least six independent experiments  
485 done in duplicate or triplicate. Unless indicated otherwise, arithmetic means  $\pm$   
486 standard deviations were calculated. Non-parametric Mann-Whitney test for median  
487 comparison was used when required (GraphPad Prism 6.0, GraphPad Software Inc.,  
488 La Jolla, CA, USA); in all cases  $p$  values  $<0.005$  were considered significant. Sample  
489 size was not statistically assessed and no randomization was done. Researchers  
490 involved were not blind for data collection or analysis.

#### 491 **Acknowledgements**

492 We are very grateful to Dr. Theodore Pierson (National Institute of Allergies and  
493 Infectious Diseases, MD, USA) for kindly providing us the WNV packaging construct  
494 and the DNA-launched WNV sub-genomic replicon expressing EGFP. J.R. and  
495 J.L.S.C. were supported by Arturo Falaschi ICGEB post-doctoral fellowships.

#### 496 **References**

- 497 1. Pierson TC, Diamond MS. 2013. Flaviviruses, p 747–794. *In* Knipe DM,  
498 Howley PM, Cohen JI, Griffin DE, Lamb RA, Martin MA, Racaniello VR,  
499 Roizman B. (ed), Fields virology, 6th ed Lippincott Williams & Wilkins,  
500 Philadelphia, PA.

- 501 2. Zhang Y, Corver J, Chipman PR, Zhang W, Pletnev SV, Sedlak D, Baker TS,  
502 Strauss JH, Kuhn RJ, Rossmann MG. 2003. Structures of immature flavivirus  
503 particles. EMBO J 22:2604–2613.
- 504 3. Kostyuchenko VA, Zhang Q, Tan JL, Ng TS, Lok SM. 2013. Immature and  
505 mature dengue serotype 1 virus structures provide insight into the maturation  
506 process. J Virol 87:7700–7707.
- 507 4. Apte-Sengupta S, Sirohi D, Kuhn RJ. 2014. Coupling of replication and  
508 assembly in flaviviruses. Curr Opin Virol 9:134–142.
- 509 5. Lindenbach BD, Rice CM. 2001. *Flaviviridae*: the viruses and their replication  
510 D.M. Knipe, P.M. Howley (Eds.), Fields Virology (4th ed), Lippincott Williams &  
511 Wilkins, Philadelphia, PA, pp. 991-1042
- 512 6. Hellen CUT, Kräusslich HG, Wimmer E. 1989. Proteolytic processing of  
513 polyproteins in the replication of RNA viruses. Biochem 28:9881–9890.
- 514 7. Yamshchikov VF, Compans RW. 1993. Regulation of the late events in  
515 flavivirus protein processing and maturation. Virol 192:38–51.
- 516 8. Gillespie LK, Hoenen A, Morgan G, Mackenzie JM. 2010. The endoplasmic  
517 reticulum provides the membrane platform for biogenesis of the Flavivirus  
518 replication complex. J Virol 84:10438–10447.
- 519 9. Westaway EG, Mackenzie JM, Kenney MT, Jones MK, Khromykh AA. 1997.  
520 Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3  
521 with double-stranded RNA, and of NS2B with NS3, in virus-induced  
522 membrane structures. J Virol 71:6650–6661.
- 523 10. Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CKE, Walther P, Fuller SD,  
524 Antony C, Krijnse-Locker J, Bartenschlager R. 2009. Composition and three-

- 525 dimensional architecture of the Dengue virus replication and assembly  
526 sites. *Cell Host & Microbe* 5:365–375.
- 527 11. Junjhon J, Pennington JG, Edwards TJ, Perera R, Lanman J, Kuhn RJ. 2014.  
528 Ultrastructural characterization and three-dimensional architecture of  
529 replication sites in Dengue virus-infected mosquito cells. *J Virol* 88:4687–  
530 4697.
- 531 12. Stadler K, Allison SL, Schalich J, Heinz FX. 1997. Proteolytic activation of  
532 tick-borne encephalitis virus by furin. *J Virol* 71 (11): 8475-8481.
- 533 13. Chambers TJ, Weir RC, Grakoui A, McCourt DW, Bazan JF, Fletterick RJ,  
534 Rice CM. 1990. Evidence that the N-terminal domain of nonstructural protein  
535 NS3 from yellow fever virus is a serine protease responsible for site-specific  
536 cleavages in the viral polyprotein. *PNAS* 87:8898–8902.
- 537 14. Ivanyi-Nagy R, Darlix JL. 2010. Intrinsic disorder in the core proteins of  
538 flaviviruses. *Protein Pept Lett* 17:1019–1025.
- 539 15. Lindenbach BD, Murray CL, Thiel HJ, Rice CM. 2013. *Flaviviridae*. *Fields*  
540 *Virology*, Sixth Edition, Lippincott Williams & Wilkins. Vol. I, Chapter 25:713–  
541 746.
- 542 16. Op De Beeck A, Molenkamp R, Caron M, Ben Younes A, Bredenbeek  
543 P, Dubuisson J. 2003. Role of the transmembrane domains of prM and E  
544 proteins in the formation of yellow fever virus envelope. *J Virol* 77:813–820
- 545 17. Orlinger KK, Hoenninger VM, Kofler RM, Mandl CW. 2006. Construction and  
546 mutagenesis of an artificial bicistronic tick-borne encephalitis virus genome  
547 reveals an essential function of the second transmembrane region of protein  
548 E in flavivirus assembly. *J Virol* 80:12197–12208.

- 549 18. Hsieh SC, Tsai WY, Wang WK. 2010. The length of and nonhydrophobic  
550 residues in the transmembrane domain of dengue virus envelope protein are  
551 critical for its retention and assembly in the endoplasmic reticulum. *J Virol*  
552 84:4782–4797.
- 553 19. Fritz R, Blazevic J, Taucher C, Pangerl K, Heinz FX, Stiasny K. 2011. The  
554 Unique transmembrane hairpin of flavivirus fusion protein E is essential for  
555 membrane fusion. *J Virol* 85(9): 4377–4385.
- 556 20. Blazevic J, Rouha H, Bradt V, Heinz FX, Stiasny K. 2016. Membrane anchors  
557 of the structural flavivirus proteins and their role in virus assembly. *J Virol*  
558 90(14): 6365–6378.
- 559 21. von Heijne G. 1990. The signal peptide. *J Membr Biol* 115:195–201.
- 560 22. Amberg SM, Nestorowicz A, McCourt DW, Rice CM. 1994. NS2B-3  
561 proteinase-mediated processing in the yellow fever virus structural region: in  
562 vitro and in vivo studies. *J Virol* 68:3794–3802.
- 563 23. Lobigs M, Lee E, Ng ML, Pavy M, Lobigs P. 2010. A flavivirus signal peptide  
564 balances the catalytic activity of two proteases and thereby facilitates virus  
565 morphogenesis. *Virol* 401:80–89.
- 566 24. Lobigs M. 1993. Flavivirus premembrane protein cleavage and spike  
567 heterodimer secretion require the function of the viral proteinase NS3. *PNAS*  
568 90:6218–6222.
- 569 25. Stocks CE, Lobigs M. 1998. Signal peptidase cleavage at the flavivirus C-prM  
570 junction: dependence on the viral NS2B-3 protease for efficient processing  
571 requires determinants in C, the signal peptide, and prM. *J Virol* 72(3):2141-  
572 2149.

- 573 26. Lee E, Stocks CE, Amberg SM, Rice CE, Lobigs M. 2000. Mutagenesis of the  
574 signal sequence of yellow fever virus prM protein: enhancement of signalase  
575 cleavage In vitro is lethal for virus production J Virol 74:24-32
- 576 27. Pierson TC, Sanchez MD, Puffer BA, Ahmed AA, Geiss BJ, Valentine LE,  
577 Altamura LA, Diamond MS, Doms RW. 2006. A rapid and quantitative assay  
578 for measuring antibody-mediated neutralization of West Nile virus infection.  
579 Virol 346:53-65.
- 580 28. VanBlargan LA, Davis KA, Dowd KA, Akey DL, Smith JL, Pierson TC. 2015.  
581 Context-Dependent cleavage of the capsid protein by the West Nile virus  
582 protease modulates the efficiency of virus assembly. J Virol 89(16): 8632-  
583 8642.
- 584 29. Casini A, Olivieri M, Vecchi L, Burrone OR, Cereseto A. 2015. Reduction of  
585 HIV-1 infectivity through endoplasmic reticulum associated degradation-  
586 mediated Env depletion. J Virol 89(5):2966-2971.
- 587 30. Poggianella M, Slon Campos JL, Chan KR, Tan HC, Bestagno M, Ooi EE,  
588 Burrone OR. 2015. Dengue E protein domain III-based DNA immunisation  
589 induces strong antibody responses to all four viral serotypes. PLoS neg trop  
590 dis 9:e0003947.
- 591 31. Slon Campos JL, Marchese S, Rana J, Mossenta M, Poggianella  
592 M, Bestagno M, Burrone OR. 2017. Temperature-dependent folding allows  
593 stable dimerization of secretory and virus-associated E proteins of Dengue  
594 and Zika viruses in mammalian cells. Sci Rep 7: 966.
- 595 32. Kabeya YY, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T,  
596 Kominami E, Ohsumi Y, Yoshimori T. 2000. LC3, a mammalian homologue of

- 597 yeast Apg8p, is localized in autophagosome membranes after processing.  
598 EMBO J 19:5720–5728.
- 599 33. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. 2005. Lysosomal  
600 turnover, but not a cellular level, of endogenous LC3 is a marker for  
601 autophagy. *Autophagy* 1(2):84–91.
- 602 34. Maillard P, Krawczynski K, Nitkiewicz J, Bronnert C, Sidorkiewicz M, Gounon  
603 P, Dubuisson J, Faure G, Crainic R, Budkowska A. 2001. Nonenveloped  
604 nucleocapsids of Hepatitis C Virus in the serum of infected patients. *J Virol*  
605 75(17):8240-8250.
- 606 35. Choi SH, Park KJ, Kim SY, Choi DH, Park JM, Hwang SB. 2005. C-terminal  
607 domain of hepatitis C virus core protein is essential for secretion. *WJG*  
608 11(25):3887-3892.
- 609 36. Jhaveri R, Qiang G, Diehl AM. 2009. Domain 3 of Hepatitis C virus core  
610 protein is sufficient for intracellular lipid accumulation. *J Infect Dis* 200(11):  
611 1781–1788.
- 612 37. Lobigs M, Zhao HX, Garoff H. 1990. Function of Semliki forest virus E3  
613 peptide in virus assembly: replacement of E3 with an artificial signal peptide  
614 abolishes spike heterodimerization and surface expression of E1. *J Virol*  
615 64:4346–55.
- 616 38. Froeschke M, Basler M, Groettrup M, Dobberstein B. 2003. Long-lived signal  
617 peptide of lymphocytic choriomeningitis virus glycoprotein pGP-C. *J Biol*  
618 *Chem* 278:41914-41920.
- 619 39. Davis CW, Mattei LM, Nguyen HY, Ansarah-Sobrinho C, Doms RW, Pierson  
620 TC. 2006. The Location of asparagine-linked glycans on West Nile virions

- 621 controls their interactions with CD209 (dendritic cell-specific ICAM-3 grabbing  
622 nonintegrin). J Biol Chem 281(48):37183–37194.
- 623 40. Goto A, Yoshii K, Obara M, Ueki T, Mizutani T, Kariwa H, Takashima I. 2005.  
624 Role of the N-linked glycans of the prM and E envelope proteins in tick-borne  
625 encephalitis virus particle secretion. Vaccine 23:3043-3052.
- 626 41. Khromykh AA, Westaway EG. 1997. Subgenomic replicons of the flavivirus  
627 Kunjin: construction and applications. J Virol 71(2):1497–1505
- 628 42. Scholle F, Girard YA, Zhao Q, Higgs S, Mason PW. 2004. *trans*-Packaged  
629 West Nile virus-like particles: infectious properties in vitro and in infected  
630 mosquito vectors. J Virol 78:11605-11614.
- 631 43. Whitby K, Pierson TC, Geiss B, Lane K, Engle M, Zhou Y, Doms RW,  
632 Diamond MS. 2005. Castanospermine, a potent inhibitor of dengue virus  
633 infection in vitro and in vivo. J Virol 79:8698-8706.
- 634 44. Yoshii K, Goto A, Kawakami K, Kariwa H, Takashima I. 2008. Construction  
635 and application of chimeric virus-like particles of tick-borne encephalitis virus  
636 and mosquito-borne Japanese encephalitis virus. J Gen Virol 89:200–211.
- 637 45. Xie X, Yang Y, Muruato AE, Zou J, Shan C, Nunes BT, Medeiros DBA,  
638 Vasconcelos PFC, Weaver SC, Rossi SL, Shi PY. 2017. Understanding Zika  
639 virus stability and developing a chimeric vaccine through functional analysis.  
640 mBio 8(1): e02134-16.
- 641 46. Dowd KA, KO SY, Morabito KM, Yang ES, Pelc RS, DeMaso CR, Castilho  
642 LR, Abbink P, Boyd M, Nityanandam R et al. 2016. Rapid development of a  
643 DNA vaccine for Zika virus. Science 354:237-240.
- 644 47. Yorimitsu T, Nair U, Yang Z, Klionsky DJ. 2006. Endoplasmic reticulum stress  
645 triggers autophagy. J Biol Chem 281:30299–30304.

- 646 48. Harvey TJ, Liu WJ, Wang XJ, Linedale R, Jacobs M, Davidson A, Le TT,  
647 Anraku I, Suhrbier A, Shi PY, Khromykh AA. 2004. Tetracycline inducible  
648 packaging cell line for production of flavivirus replicon particles. *J Virol*  
649 78:531–538.
- 650 49. Mason PW, Shustov AV, Frolov I. 2006. Production and characterization of  
651 vaccines based on flaviviruses defective in replication. *Virology* 351:432-443.
- 652 50. Bennett KM, Gorham RD Jr., Gusti V, Trinh L, Morikis D, Lo DD. 2015. Hybrid  
653 flagellin as a T cell independent vaccine scaffold. *BMC Biotechnol* 15:71.
- 654 51. Hanke T, Szawlowski P, Randall RE. 1992. Construction of solid matrix-  
655 antibody-antigen complexes containing simian immunodeficiency virus p27  
656 using tag-specific monoclonal antibody and tag-linked antigen. *J Gen Virol* 73:  
657 653-660.
- 658 52. Mossenta M, Marchese S, Poggianella M, Slon Campos JL, Burrone OR.  
659 2017. Role of N-glycosylation on Zika virus E protein secretion, viral assembly  
660 and infectivity. *Biochem Biophys Res Commun* 492:579-586.
- 661 53. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory  
662 manual. New York: Cold Spring Harbor Laboratory Press.
- 663 54. Eichwald C, Rodriguez JF, Burrone OR. 2004. Characterisation of rotavirus  
664 NSP2/NSP5 interaction and dynamics of viroplasms formation. *J Gen Virol*  
665 85: 625–634.
- 666

## 667 **FIGURE LEGENDS**

668 **Fig. 1.** A) Schematic representation of the genomic organisation of flavivirus  
669 structural proteins (upper panel), membrane topology and cleavage sites for  
670 NS2B/NS3, furin and signal peptidase, indicated with red, blue and black arrows,

671 respectively (lower panel). B) Sequence of Capsid anchor region (Ca; in red) for  
672 WNV, ZIKV and DENV2. The first 5 amino acids of the downstream PrM used in  
673 some of the constructs are indicated in blue. The NS2B/NS3 viral protease and  
674 signal peptidase cleavage sites are indicated with arrows. C) Schematic  
675 representation of the protocol used for the production and detection of  
676 pseudoviruses.

677 **Fig. 2.** Production of WNV, DENV2 and ZIKV pseudoviruses. A) Cytofluorimetry  
678 profiles of Vero cells infected with pseudoviruses produced with the different  
679 packaging constructs shown in Fig. 1. The profiles are representative of several  
680 experiments. B) Quantification of cytofluorimetry data shown in A, (n= 12, 9, 15 and  
681 11 for WNV, DENV2, DENV2 *T101G* mutant and ZIKV packaging constructs,  
682 respectively). Non-reducing western blot (upper panels) of cell extracts and pellets of  
683 ultracentrifuged supernatants of HEK293T cells co-transfected with the WNV-rep  
684 and the indicated packaging constructs. Protein E was detected with the flavivirus-  
685 specific mAb 4G2. Actin was used as a loading control. Bottom panel, agarose gel  
686 analysis of RT/PCR products of the same supernatants. DNA stained with Ethidium  
687 bromide.

688 **Fig. 3.** Generation and characterisation of Zika pseudoviruses A) Scheme and  
689 sequence at the C-Pr junction of different C-PrME ZIKV/WNV chimeras used as  
690 packaging constructs, and numbered 1-6. WNV derived sequences are shown in  
691 red. Superscripts, Z, W indicate sequences derived from ZIKV or WNV, respectively.  
692 B) Representative cytofluorimetry profiles of Vero cells infected with the indicated  
693 ZIKV/WNV chimeric pseudoviruses. C) Quantification of pseudovirus production  
694 determined by cytofluorimetry and expressed as percentage of EGFP expressing  
695 Vero cells at 24 hpi (n=11, 7, 5, 6, 7 and 7, for packaging constructs 1 thru 6,

696 respectively). A multiple t test was used to analyse statistic differences between wild  
697 type and chimeric pseudoviruses. D) Western blot analysis of intracellular protein  
698 levels and secreted virions in cell lysates and pellets of ultracentrifuged supernatants  
699 of HEK293T cells transfected with the indicated packaging constructs. E protein was  
700 detected with the flavivirus-specific mAb 4G2. Actin was used as loading control.

701 **Fig. 4.** Cleavage by NS2B/NS3 viral protease and signal peptidase. A) Schematic  
702 representation of packaging construct carrying the SV5-tag at the C N-terminus. Ca  
703 sequence derived from WNV ( $Ca^W$ ) is shown in red. B) Western blot analysis of  
704 cellular extracts of HEK293T cells transfected with the constructs shown in A, in the  
705 absence (-) or presence (+) of the WNV-rep. Migration of C, CPrM and C-PrME are  
706 indicated. C) Representative cytofluorimetry profile of cells infected with  
707 pseudoviruses produced with the packaging constructs shown in A. D) Scheme of  
708 the Ca from ZIKV, WNV and DENV2 ( $Ca^Z$ , blue;  $Ca^W$  red;  $Ca^D$  black) used as signal  
709 peptides on an irrelevant secretory reporter protein. The non-viral signal peptide sec  
710 (grey) is also shown. The vertical line indicates signal peptidase cleavage site. All  
711 constructs contain the first 5 amino acids of viral Pr indicated as [5] with the same  
712 colour code. E) Western blot of cell extracts (E) and culture supernatants (S) of cells  
713 transfected with constructs shown in D. Reporter protein developed with anti-mouse  
714 IgG. Actin was used as loading control.

715 **Fig. 5.** Secretion of VLPs and soluble E (sE). A) and B) Left panels, schemes of  
716 VLPs encoding constructs (PrME) of ZIKV (A) and DENV2 (B), with  $Ca^Z$  (blue),  $Ca^W$   
717 (red),  $Ca^D$  (black) or the non-viral sec (grey), as signal peptides. Right panels, non-  
718 reducing western blots of cellular extracts (E), culture supernatants (S) and  
719 ultracentrifuged pellet fraction (P, shown with (+) and without (-) 0.5% NP40) of  
720 HEK293T cells transfected with the ZIKV (A) or DENV2 (B) VLP constructs. Protein

721 E was detected with mAb 4G2. C) and D) Left panels, schemes of constructs  
722 encoding the covalent dimer (cvD) format of ZIKV PrME<sup>Z</sup> (C) or sE (D) with Ca<sup>Z</sup>  
723 (blue), Ca<sup>W</sup> (red) or the non-viral sec (grey), as signal peptides. Right panels, non-  
724 reducing western blots of cellular extracts (E) and culture supernatants (S) of  
725 transfected HEK293T cells developed with anti-SV5. Arrows indicate position of  
726 dimeric E.

727 **Fig. 6.** Degradation of ZIKV E protein. A) Schematic representation of ZIKV VLP  
728 encoding constructs. B) Western blot analysis at different time points of cellular  
729 extracts after CHX (+, 100 µg/ml) treatment at 16 hrs post transfection. C) CHX  
730 treatment of Ca<sup>W</sup>-PrME<sup>Z</sup> transfected cells in the presence of chloroquine (CQ; 100  
731 µM) or MG132 (20 µM). D) Western blots of Ca<sup>Z</sup>-PrME<sup>Z</sup> and Ca<sup>W</sup>-PrME<sup>Z</sup> transfected  
732 cells treated (+) or not (-) with CQ (50 µM); arrowhead indicates LC3-II. E-F)  
733 Confocal immunofluorescence of HeLa cells transfected with ZIKV VLP (wt and  
734 chimeric) using anti-ZIKV DIII sera and anti-LC3. In F, HeLa cells were transfected  
735 with the indicated siRNAs 48 hrs before transfection with ZIKV VLP constructs. siNT  
736 is a control non-targeting siRNA. Scale bar, 20 µm. G) Western blot analysis of  
737 proteins E and LC3 in HEK293T cellular extracts treated with indicated siRNAs and  
738 transfected with the wt and chimeric ZIKV VLP constructs. H) Representative  
739 electron microscopy image of a cell co-transfected with WNV-rep and the C<sup>Z</sup>- Ca<sup>W</sup>-  
740 PrME<sup>Z</sup> packaging construct. Arrowheads indicate autolysosomes/degradative  
741 compartments. PM, plasma membrane; N, nucleus; mv, multivesicular bodies. Scale  
742 bar, 500 nm.

743 **Fig. 7.** Tripartite production of pseudoviruses. A) Schematic representation of the  
744 tripartite system of transfection for the production of pseudoviruses. B) Packaging

745 constructs used. ZIKV C was expressed alone (C) or fused to its own anchor  $\text{Ca}^Z$   
746 (blue, C- $\text{Ca}^Z$ ) while ZIKV PrME was expressed with the indicated Ca (or sec), as  
747 signal peptides. C) Representative cytofluorimetry profiles of Vero cells infected with  
748 ZIKV pseudoviruses produced with the indicated set of *trans* packaging constructs.  
749 D) Quantification of ZIKV pseudoviruses production determined by cytofluorimetry of  
750 infected Vero cells at 24 hpi (n=6 in all cases). E) Non-reducing western blot (upper  
751 panels) of cell extracts and pellets of ultracentrifuged supernatants of HEK293T  
752 cells, transfected with the indicated packaging constructs and developed with mAb  
753 4G2. Bottom panel, agarose gel of RT/PCR products of the same supernatants  
754 stained with Ethidium bromide. F) Western blot analysis of cellular extracts of  
755 HEK293T cells co-transfected with ZIKV SV5-tagged constructs C and C-Ca (left  
756 panel) or C-Ca and C-PrME (right panel) and without (-) or with (+) WNV-rep.  
757 Cleaved and un-cleaved C are indicated with red and black arrows, respectively.













