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## Molecular mechanisms regulating the pH-dependent pr/E interaction in yellow fever virus

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1           **Molecular mechanisms regulating the pH-dependent pr/E**  
2                                   **interaction in yellow fever virus.**

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21

22   **ABSTRACT**

23   Flavivirus particles bud in the ER of infected cells as immature virions composed of 180  
24   heterodimers of glycoproteins prM and E, associated as 60 (prM/E)<sub>3</sub> trimeric spikes. Exposure  
25   to the mildly acidic pH of the TGN results in dissociation of the trimeric spikes followed by re-  
26   association of the prM/E protomers into 90 dimers organized in a characteristic herringbone  
27   pattern. The furin site in prM is exposed in the dimers for maturation of prM into M and pr. For

28 flaviviruses such as the tick-borne encephalitis virus (TBEV) as well as for dengue virus, it was  
29 shown that at neutral pH pr loses affinity for E, such that it dissociates from the mature particle  
30 as soon as it reaches the external milieu, which is at neutral pH. Using a soluble recombinant  
31 form of E (sE) and pr from yellow fever virus (YFV), we show here that the affinity of pr for  
32 recombinant E protein remains high even at neutral pH. The X-ray structure of YFV pr/sE  
33 shows more extensive inter-chain hydrogen bonding than does the dengue or TBEV, and also  
34 that it retains the charge complementarity between the interacting surfaces of the two proteins  
35 even at neutral pH. We further show that pr blocks sE flotation with liposomes when exposed  
36 at low pH at a 1:1 stoichiometry, yet in the context of the virus particle, an excess of 10:1 pr:E  
37 ratio is required to block virus/liposome fusion. In aggregate, our results show that the  
38 paradigm obtained from earlier studies of other flaviviruses does not apply to yellow fever virus,  
39 the flavivirus type species. A mechanism that does not rely solely in a change in the  
40 environmental pH is thus required for the release of pr from the mature particles upon release  
41 from infected cells. These results open up new avenues to understand the activation  
42 mechanism that yields mature, infectious YFV particles.

43

## 44 INTRODUCTION

45 Enveloped viruses use membrane fusion protein (MFP) to mediate viral fusion with the  
46 host cell. The majority of MFPs belong to three structural classes, I, II, or III. Flaviviruses have  
47 class II MFPs carrying an elongated ectodomain divided into three distinct  $\beta$ -sheet rich  
48 domains (DI, DII, DIII), a stem region, and are anchored to the viral membrane by C-terminal  
49 trans-membrane (TM) domains (1). Their folding in the ER of the infected cell is assisted by  
50 an accompanying protein (AP) which acts as a chaperone. The MFP/AP heterodimer is the  
51 building block at the surface of the mature virus, with the AP positioned to protect the fusion  
52 loop (FL) of MFP, the hydrophobic region responsible for the insertion into the host membrane.  
53 Flaviviruses are the only exception. Their viral particle is indeed constituted by homodimers of  
54 the MFP envelope (E) protein tightly organized in a herringbone pattern with the FL buried at

55 the homodimer interface. The pre-membrane (prM) protein is the flaviviruses AP protein. It  
56 gets cleaved by furin during flavivirus maturation in the trans-Golgi network (TGN) into M, that  
57 remains anchored by its TM domains to the viral membrane underneath the E homodimer, and  
58 pr moiety that interacts with the FL of E to prevent premature triggering of viral fusion in the  
59 acidic environment of the TGN (2), (3). The necessity to protect the FL and, at the same time,  
60 to have a particle ready to fuse after receptor-mediated endocytosis, has pushed the  
61 flaviviruses to evolve concerted strategies based on conformational changes of the E/prM  
62 complex driven by a low pH-triggering switch (4). During viral entry, the acidic pH of the  
63 endosome triggers a dimer-to-trimer transition of the E protein resulting in an exposure of the  
64 fusion loop at the tip of the trimer for insertion into the host membrane and successive viral  
65 fusion. During virus secretion in the secretory pathway, a trimer-to-dimer transition brings the  
66 trimer of E/prM heterodimers, that form as the noninfectious immature virus buds in the ER, to  
67 an E dimer with M underneath and pr on top, associated to the fusion loop. In the TGN the  
68 furin protease cleaves pr-M but pr remains associated to the pre-mature particle. When the  
69 viral particle is released in the neutral pH extracellular environment, pr is then removed from  
70 the virus which is now infectious,(6) and ready to begin a new cycle (5),(6).

71 The mechanism regulating these transitions is not fully understood but a recent work  
72 from Vaney and coll. showed how for TBE, during the transit across the secretory pathway,  
73 the 150 loop and the N-terminal of the E protein act in coordination with the pr protein to assure  
74 protection of the FL in the transition from low to neutral pH. It is indeed the movement of the  
75 150 loop towards the N-terminal of E at neutral pH that actively expels pr from its binding site  
76 (7). These regions show structural conservation between flaviviruses suggesting a common  
77 mechanism of action, however, the determinants of their interaction may vary (i.e. the length  
78 of the 150 loop or the presence of glycosylation) and may result in differences in infectivity  
79 and/or pathogenicity (8).

80 Our work describes the interaction of pr/E for yellow fever virus (YFV) and identifies a  
81 unique interaction of pr/E at neutral pH, absent in the other flaviviruses. We show the structural

82 basis of this interaction, relying on an extensive inter-chain hydrogen bonds with interactions  
83 specific to YFV. At low pH pr prevents E insertion into membranes and blocks viral fusion as  
84 for the other flaviviruses. However, at neutral pH, the pr/E interactions are weakened but still  
85 present suggesting the necessity of additional mechanisms for the release of pr from the  
86 mature particle.

## 87 **RESULTS**

88 **Interactions of YFV pr and E proteins.** To produce correctly folded YFV sE protein,  
89 we used the same strategy that we previously adopted for the production of dengue sE protein  
90 expressing the prME region as it is in the viral polyprotein (9). This type of construct assures  
91 the secretion of soluble E (sE) while M remains membrane-anchored in the cell and pr, cleaved  
92 by furin in the TGN, dissociates from sE when the complex reaches the extracellular milieu.  
93 Similar constructs for dengue and tick-borne encephalitis (TBE) viruses resulted indeed in  
94 secretion of the soluble sE protein (9), (10). In the case of YFV instead, we obtained a stable  
95 pr/sE complex even in the absence of covalent linker, which indeed was necessary for the  
96 production of DENV2 pr/sE complex crystallized previously (11). We obtained crystals for the  
97 YFV wild type Asibi pr/sE complex that yielded a structure to 2.7Å resolution and refined to  
98 free R factors of 19% (Fig. 1A and Suppl. Table S1). This structure shows the YFV pr/sE  
99 complex as it is supposed to be in the secretory pathway after furin cleavage.

100 Although YFV E is not glycosylated, to sites of glycosylation, Asn13 and Asn29, are  
101 present in YFV pr (Fig. 1A,B). Asn13 glycan, a specific glycosylation site in YFV group, packs  
102 against Trp40. The glycan Asn29 glycan is located on  $\beta$ -strand  $\beta$ 3 in a location spatially just  
103 nearby just the DENV pr glycosylation Asn69 (YFV-Tyr66,  $\beta$ -strand  $\beta$ x). In YFV the glycan  
104 packs against Tyr66 and Arg55 stabilizing the capping loop conformation (CL, shown in bright  
105 green in Fig. 1B). The capping loop is a protruding loop that wraps around the sE fusion loop  
106 (FL, shown in orange in Fig.1). Its conformation is stabilized by a disulfide bridge between  
107 Cys49 and Cys63, and hydrophobic packing interactions with Trp64, Tyr66 and the Asn29

108 glycan chain (Fig. 1B). pr-CL makes multiple polar and hydrophobic contacts including many  
109 main-chain/main-chain interactions, some of them are conserved in DENV2 (PDB 3C5X) and  
110 TBEV (PDB 7QRE) pr/sE structures (Table 1). In particular, residue pr-Asp60 makes, among  
111 all the flaviviruses, a strictly conserved salt-bridge interaction with sE-His238 stabilizing the *ij*-  
112 loop of E-domain II (Fig. 1C). This interaction was previously described in the pr/sE complex  
113 of DENV2 (PDB code 3C5X) (11) and TBEV (PDB 7QRE, 7QRF) (7). In addition, pr and sE  
114 interaction is further stabilized by main-chain interactions and side-chains H-bonds between  
115 pr-Ser44 and sE-His67 or pr-Asn48 and sE-Asn71/Asp72 at both ends of the  $\beta$ -strands (Fig.  
116 1D).

117 Thus, the tight association of YFV pr/E complex is supported by conserved interactions,  
118 also present in DENV2 and TBEV (highlighted by the green background in Table 1 and  
119 interactions specific to YFV involving the Ile70-Asp72 region (Fig. 1D and Table 1).

120

121 **Chaperone role of pr and YFV pr/sE interactions.** To perform functional studies on  
122 YFV soluble E protein (sE) and its interactions with membranes, we had to separate the pr/E  
123 complex. A first attempt was done using anion exchange chromatography with a NaCl gradient.  
124 We were able to separate two peaks, one still containing the pr/sE complex and the other one  
125 containing sE alone (sE') (Suppl. Fig. S1). However, further functional analysis of the protein  
126 sE' eluting at 400mM NaCl, revealed that this protein was unable to insert into liposomes at  
127 acidic pH (see experimental details below) and it was probably a misfolded form of sE. We  
128 then used 8M urea for denaturation of the pr/sE complex eluting at 240mM NaCl, followed by  
129 renaturation with extensive dialysis against Tris 20mM pH 8.0 of the separated E and pr  
130 proteins. To simplify the protein preparation for the functional studies, we also decided to  
131 express in S2 cells the pr protein alone and the sE protein without prM. The yields of sE  
132 produced in absence of prM were sensibly lower and the SEC profile showed a large  
133 heterogeneity of the produced protein (Fig. 2A and B) confirming the chaperone role of prM for

134 YFV E protein. Comparison with the SEC profile of the protein produced by the prME construct  
135 identified peak 3 as the corrected folded protein (Fig. 2A). Both peak 3 and sE obtained after  
136 urea treatment of pr/sE complex were used for further functional studies.

137 Differently to previous studies with dengue virus, which had shown very weak or no  
138 pr/sE interactions at neutral pH, we observed a stable pr/sE complex at pH 8.0. We measured  
139 the affinity of YFV pr/sE interaction at pHs 6.0 and 8.0 by two different methods, isothermal  
140 calorimetry (ICT, Fig. 2C) (12) and surface plasmon resonance (SPR, Fig. 2D). The  
141 dissociation constants ( $K_D$ ) obtained by the two independent methods followed a similar trend:  
142 a  $K_D$  under 10 nM at pH 6.0 (8.5nM by ITC and 6.2nM by SPR) and about five times higher at  
143 pH 8.0 (58.8nM by ITC and 19.7nM by SPR) (Table 2), indicating a pH sensitive interaction. In  
144 previous studies of the interaction between DENV2 pr and sE, although a  $K_D$  had not been  
145 reported, SPR experiments revealed undetectable or no binding at pH 8.0 (13), whereas in the  
146 case of YFV we find an affinity still under 100 nM under these pH conditions, indicating a real  
147 difference in the two viruses.

148

149 **pH-dependent binding of pr and sE.** We used size-exclusion chromatography (SEC)  
150 combined with multi-angle light scattering (MALS) to analyze the binding of pr to YFV sE at  
151 neutral and acid pH. At pH 8.0 and pH 5.5 both pr and YFV sE elute as monomer (Fig. 3A top  
152 and bottom panels). Although the sE monomer has a higher molecular mass than the pr  
153 monomer, it elutes from the SEC column at a later peak, corresponding to the elution of small  
154 molecules. This behavior has been described for other class II proteins (14) and it is probably  
155 due to the interaction of the exposed fusion loop with the resin of the column that delays elution.  
156 The pr/sE complex at both pH elutes as a 65-60KDa peak (Fig. 3B top and bottom panels)  
157 containing both sE and pr proteins as shown by the SDS-PAGE analysis of the peak fractions  
158 (Fig. 3C top and bottom panels). This is different from what has been observed for TBE sE  
159 protein (7) and for DENV or ZIKV sE proteins (Suppl. Fig. S2). The ZIKV sE protein is a dimer

160 at pH 8.0 (Suppl. Fig. S2Aa top panel) and associates to pr only at pH 5.5 (Suppl. Fig. S2Ab  
161 top and bottom panels and Suppl. Fig. S2Ac top and bottom panels). The DENV sE protein at  
162 pH 8.0 is a monomer (Suppl. Fig. S2Ba top panel). This monomer dissociates into two peaks  
163 at pH 5.5, that we called P1 and P2 (Suppl. Fig. S2Ba bottom panel). We further analyzed only  
164 P2 for the complex with pr because P1 was shown to be probably misfolded sE since it was  
165 not recognized by EDE neutralizing antibodies (9). Peak P2 showed a retarded elution profile  
166 (18ml elution volume, Suppl. Fig. S2Ba bottom panel), similarly to YFV sE protein. After mixing  
167 with pr at pH5.5, P2 peak shifts to 14.4ml elution volume (Suppl. Fig.S2Bb bottom panel)  
168 suggesting interaction with pr and prevention of the FL interaction with the resin of the column.  
169 At pH8.0 instead, the sE and pr peaks overlapped and it was not possible to distinguish  
170 whether pr interacts with sE or not (Suppl. Fig. S2Bc top panel).

171           Since the E protein is a dimer at the surface of the virus but the sE of YFV is a monomer,  
172 we sought to test the interaction of pr protein with a YFV E dimer. To obtain this protein in  
173 solution, we engineered a mutation to cysteine in position S253 to induce the formation of a  
174 disulfide bond and link the two sE protomers, following the same strategy previously used to  
175 stabilize the dengue E dimer (15). The S253C mutant SEC profile showed the presence of  
176 high-molecular weight aggregates and peaks corresponding to monomeric protein but a  
177 fraction of the protein was produced as a disulfide linked dimer as shown by MALS and SDS-  
178 PAGE analysis (Fig. 3D and 3E). Interaction of this dimer with pr resulted in an association  
179 only at pH 5.5 (Fig. 3F) similarly to Zika sE dimer and to a stabilized dimer construct for DENV2,  
180 mutant A259C (Suppl. Fig. S2Ca,b,c). In conclusion, from the analysis of several mosquito-  
181 borne flaviviruses, the pr binding site on the E protein is accessible at low pH on both E  
182 monomer or dimer but it becomes hidden on the dimer at neutral pH. However, in the context  
183 of the E monomer, the YFV E protein is the only one showing an interaction with pr also at  
184 neutral pH (Table 3).

185



186           **YFV pr protein blocks insertion of sE protein into membranes.** We tested the effect  
187 of the presence of pr on the interactions of sE protein with membranes by measuring co-  
188 flotation with liposomes in density gradients (Fig 4A and B). In this assay we mixed purified sE  
189 protein with liposomes (see Methods for composition) and, after incubation at neutral or low  
190 pH, we separated the complex on a density gradient. If the protein inserts in the membrane of  
191 the liposomes, it will co-float to the top fraction of the gradient. We found that at pH 8.0 sE  
192 remained at the bottom of the gradient and do not interact with the liposomes in spite of FL  
193 exposure (Fig. 4A, left column). Instead, at pH 6.0, about 45% of the sE protein floated to the  
194 top fractions (Fig. 4A, B). In the presence of pr, we found a dose-dependent inhibition of sE  
195 co-floatation, such that at a molar ratio of 1:1 pr:sE there was no sE protein found in the top  
196 fraction, in line with the  $K_D$  of 10 nM or less of the pr/sE complex at pH 6.0 (Table 2). These  
197 results are different to those obtained in the DENV2 system, where a 10-fold molar excess of  
198 pr was required to inhibit liposome insertion (13), again indicating that the interaction of pr with  
199 the E protein is much stronger in the case of YFV.

200

201           **Interaction of pr with the YFV viral particle inhibits viral fusion.**

202           Viral fusion to membranes can be measured using lipid mixing fusion assays. We used  
203 a system based on fluorescence resonance energy transfer between the fluorophores 7-nitro-  
204 2-1,3-benzoxadiazol-4-yl (NBD) and rhodamine covalently coupled to lipids. The fluorescence  
205 is quenched by a high concentration of the two fluorophores in the liposomes, and becomes  
206 de-quenched upon dilution into the lipids derived from the viral membrane upon fusion of the  
207 two lipid bilayers, allowing to follow the lipid merger reaction. The fluorescence profile observed  
208 upon mixing YFV strain 17D virus with the NBD/rhodamine labeled lipids at different pH values  
209 is displayed in Fig. 5A. Fluorescence dequenching is optimal between pH 5.6 and pH 6.2 and  
210 is negligible at neutral pH. A plot of the mean intensities reached at each pH shows a peak of  
211 lipid mixing at around pH 6.0 (Fig. 5B). We therefore used pH 6.0 to test the inhibition of lipid-

212 mixing by recombinant pr added at different pr:E stoichiometries to the virus preparation before  
213 mixing with liposomes and found a dose-dependent inhibition of the reaction by exogenous pr  
214 (Fig. 5C). For the fusion experiments, we used YFV17D virus because of safety reasons, since  
215 the vaccine strain can be manipulated under BSL2 conditions. The vaccine strain 17D carries  
216 10 amino acids mutation in the sE protein (16) but their localization does not interfere with the  
217 pr/sE binding site. We quantified the relative stoichiometry of pr:E by western blot as described  
218 in the Materials and Methods section (see Suppl. Fig. S3). Differently to the results observed  
219 on the inhibition of YFV sE protein insertion into liposomes by pr (Fig. 4A), we observed a  
220 requirement of pr in excess of at least 10 times to obtain 100% inhibition of lipid mixing (Fig.  
221 5D). This discrepancy suggests a different affinity of pr for E on virions compared to sE in  
222 solution. This is probably due to the different accessibility of the pr binding site in the context  
223 of the E dimer (present on the virus) compared to the E monomer present in solution. The pr  
224 binding site could be indeed buried in the E dimer of the viral particle at neutral pH and become  
225 available only when the dimer is opening at low pH. To test this hypothesis, we mixed pr with  
226 YFV particles in an excess of 50:1 pr:E stoichiometry at various pH values, and measured the  
227 amount of pr brought down upon pelleting of the virion by ultracentrifugation (Fig. 5E). This  
228 experiment showed very little pr co-precipitating with the virus at pH 8.0, and a maximum of  
229 co-precipitation at pH6.0, suggesting a pH dependent exposure of the pr binding site on virions  
230 (Fig. 5F).

231

232 **The YFV sE dimer.** While the YFV sE protein is mainly a monomer in solution, we  
233 were able to obtain crystals of a sE dimer using the construct without prM. This protein formed  
234 tetragonal crystals that diffracted to 3.5Å (Suppl. Table S1). The structure, determined by  
235 molecular replacement (using the 6EPK structure) and refined to a free R factor of ~ 27% (see  
236 Methods), showed the typical head-to-tail sE dimer conformation observed initially for sE of  
237 TBEV (17) and later for the DENV2 (18), JEV (19), and ZIKV (20), (21) counterparts. There  
238 are two main sE dimer interfaces, the first by the dimer axis and the second one involving the

239 fusion loop, away from the dimer axis. The first interface involves antiparallel interaction of the  
240 polypeptide chain around helix  $\alpha$ B (Fig. 6A), including several inter-protomer hydrogen bonds,  
241 some of which involving main-chain / main-chain interactions. In the second interface, the FL  
242 at the tip of domain II packs against domains III and I of the other protomer in the dimer (Fig.  
243 6B). The FL residue Trp101 has its side chain covered by that of Lys308 of domain III, while  
244 the FL main chain is partially tucked in between two short helices in domain I, the N-terminal  
245 helical turn (N-helix in Fig. 6B) and the “150-helix” (150-loop forming an  $\alpha$  helix) described in  
246 more detail below (Fig. 6B).

247       **The 150-loop.** The YFV sE dimer displays a unique organization of the 150-loop in  
248 domain I, which is highly variable in sequence across flaviviruses and connects  $\beta$ -strands  $E_0$   
249 and  $F_0$  in domain I (Fig. 6B). Most flaviviruses carry an N-linked glycan at positions 153 or 154,  
250 except for YFV, for which only a few attenuated strains are N-glycosylated (22). The “150-  
251 helix” (residues 149-155) is highly exposed at the dimer surface. A short helix in the 150-loop  
252 is present in other flaviviruses as well (MBEVs and ZIKV) albeit oriented almost at 90 degrees  
253 (7). The side chain of Trp152 appears as an important element of the “150-helix”, as it packs  
254 against the N-terminal end of the polypeptide chain, which is buried underneath. The positively  
255 charged N-terminal Ala1 is neutralized by a salt bridge and hydrogen bond with the Asp42  
256 sidechain, which is also buried. The buried N-terminal end of the protein appears to confer a  
257 specific structure to domain I, as in the structure of the pr/sE complex of dengue virus serotype  
258 2, in which a linker connected the region of prM just upstream of the trans-membrane (TM)  
259 segment to the N-terminus of sE (thereby by-passing the TM region), showed a disordered  
260 150-loop with the N-terminal helix continuing in the linker and projecting out at the top of  
261 domain I (7). The N-terminus of the wild type YFV E protein indeed participates in a network  
262 of hydrogen bonds also involving residues from domain III.

263       The first helical turn of the “150-helix” is somewhat distorted, but the second turn is  
264 further constrained by a hydrogen bond between the side chains of the consecutive Thr154  
265 and Asp155 (Fig. 6B). Importantly, one of the virulence determinants of YFV in a hamster

266 model was found to map to position 154, which was identified as conferring virulence when  
267 Thr154 was replaced by Ala (23). In that study, mutating Asp155 to Ala resulted in a variant  
268 with the same virulence phenotype even when Thr154 was maintained, suggesting that the  
269 hydrogen bond between these two adjacent side chains residues is important for stabilizing  
270 the relevant conformation required for interactions with the host that affect virulence. In  
271 summary, the “150-helix” is highly exposed and structured at the dimer surface, in a region  
272 important for stabilizing interactions between domains I and III and the fusion loop on the  
273 adjacent dimer subunit.

274         To understand the interactions of pr with the sE dimer, we modeled the pr binding site  
275 (as determined in the pr/sE monomer (6EPK)) on one subunit of the sE dimer and identified a  
276 clash between the pr capping loop and the E 150-loop. This clash suggests an impaired  
277 binding unless the 150-loop moves out of the way in an “open” position as it has been shown  
278 for TBE dimer at low pH (7). To understand which interactions would allow the pH-dependent  
279 movement of the 150-loop and release of pr at neutral pH, we analyze the electrostatic  
280 potential of E and pr surfaces at their binding site at pH 8.0 and pH 6.0 (Fig. 6D). We could  
281 still detect a fair charge complementarity at pH 8.0 which can explain why the affinity of pr for  
282 the E monomer is still high at neutral pH. It remains to be determined if, in the context of the  
283 dimer, these interactions are sufficient to expel pr at neutral pH or if additional re-arrangements  
284 of the envelope proteins are required.

285

## 286 **DISCUSSION**

287         Our data provide a structural and functional analysis of the interaction between pr and  
288 E protein of yellow fever virus. Comparison of these data to other flaviviruses, such dengue  
289 and Zika viruses, show a general mechanism of action of pr in protecting the FL at low pH, a  
290 critical step of virus maturation. We show that pr associates to the E dimer at low pH for YFV,  
291 DENV and ZIKV but this interaction is lost at neutral pH. However, only the YFV E monomeric

292 protein showed an interaction with pr also at neutral pH. This interaction is stabilized by several  
293 inter chain contacts that are absent in the other flaviviruses (see Table 1). We show, as  
294 previously reported in the literature, that exogenous addition of purified pr to sE interferes with  
295 its insertion into liposomes at low pH in a floatation assay, an assay mimicking the dimer-to-  
296 trimer transition occurring during viral fusion (4), (13). Differently from what it has been shown  
297 for DENV, where high concentration of pr were required to inhibit sE co-floatation with the  
298 liposomes, a 1:1 pr:sE molar ratio was sufficient for YFV sE to block membrane insertion,  
299 confirming the high affinity of these two proteins. Moreover, we were able to show, using  
300 infectious virus in a fusion assay, that this interaction actively blocks viral fusion. In contrast to  
301 the results obtained with the purified protein, we needed a 10-fold excess of pr protein to  
302 completely inhibit fusion of infectious virus. This is due to the fact that the E protein at the  
303 surface of the virus is present as a dimer and, at neutral pH, the FL is not accessible to pr as  
304 it is on the monomeric purified protein. In both experiments, the pr/E complex was generated  
305 at neutral pH and after addition of the liposomes the pH was lowered to the chosen acidic  
306 value. Virus and purified sE protein at low pH, in absence of membranes, would indeed  
307 aggregate interfering with the read-out of the assay. These results confirm what we have  
308 observed in our SEC-MALS analysis that showed pr/sE binding at neutral pH only for the  
309 monomeric form of sE and not for the dimer. Our MALS analysis revealed also some difference  
310 in the way flaviviruses handle the pr/E interaction. The FL is protected by the pr interaction at  
311 low pH but while ZIKV dimer dissociates at acidic pH, TBE remains dimeric. DENV and YFV  
312 instead are monomeric at both neutral and acidic pHs (Table 3). These data support how  
313 different flavivirus sE proteins vary regarding their pH sensitivity to dimerization/dissociation,  
314 while the molecular mechanism dictating pr binding/unbinding and thus flavivirus maturation,  
315 is common to all flaviviruses.

316           During maturation there are three critical steps in which is mandatory for flaviviruses to  
317 protect the fusion loop from premature membrane insertion. First, after budding in the ER, the  
318 immature virus carries pr bound to the FL on top of the trimeric (prM/E)<sub>3</sub> spikes; second, during

319 the transit through the acidic TGN, the pH-induced trimer-to-dimer transition generates  
320 immature smooth particles carrying pr on top of the FL exposing the furin cleavage site; third,  
321 after pr-M cleavage, at the neutral pH of the extracellular milieu, pr is displaced from the FL by  
322 the snap-lock movement of the 150loop (7). While pr binding to FL is the key interaction  
323 throughout these steps, its regulation occurs via combined action of several regions of the pr/E  
324 complex, identifying the pr-binding site as a leading character in the flavivirus maturation  
325 process. Not surprisingly this region is targeted by highly cross-neutralizing antibodies (20).  
326 Our structure of the YFV sE dimer confirmed the folding previously described (24) and showed  
327 how the 150 loop at neutral pH is in closed conformation and would clash with pr binding, a  
328 mechanism previously described for TBE (7). This explains the higher ratio of pr required to  
329 block viral fusion in our lipid mixing experiments.

330 In conclusion, we describe the molecular interactions regulating a crucial process in  
331 flavivirus maturation. Interestingly, while the basic organization of the interactions is common  
332 to all flaviviruses, each virus seems to modulate them differently. In particular, we found for  
333 yellow fever a stable association with pr also at neutral pH suggesting that its release from the  
334 mature particle cannot occur exclusively by a passive pH-dependent change of charges but it  
335 will require an active reorganization involving the viral particle in its whole.

336

## 337 **METHODS**

338

### 339 **Recombinant pr/sE protein production.**

340 The YFV Asibi pr/sE and sE constructs were cloned onto a pMT-derived vector (25).  
341 This vector allows expression of the gene of interest downstream an insect signal peptide BiP  
342 and in frame with an enterokinase or a thrombin cleavage site followed by a StrepTag, for  
343 purification purpose. The sequence encoding for prM and the ectodomain of E for Asibi strain

344 (NCBI AY640589) was taken from pACNR-113.16 (Rice and Barba-Spaeth, unpublished).  
345 Single cysteine mutation S253C was introduced to generate a disulfide stabilized E dimer  
346 protein. All the constructs were restricted to residues 1 to 392 for *E. D. melanogaster* S2  
347 pseudo-clonal pools were generated by co-transfection with a pCoPURO (26) by Effectene  
348 transfection (QIAGEN). For expression, cells were induced at a density of  $1 \times 10^6$  cells per mL  
349 with  $500 \mu\text{M}$   $\text{Cu}_2\text{SO}_4$  for 10 days or  $5 \mu\text{M}$   $\text{CdCl}_2$  for 7 days. The supernatant was then  
350 harvested, concentrated on a Vivaflow 200 concentration system with a 10 kDa-cutoff  
351 membrane (Sartorius). The pH of the concentrated supernatant was adjusted to 8.0 with 100  
352 mM Tris HCl and avidin was added to a final concentration of  $1 \mu\text{g}/\text{mL}$ . Soluble YFV sE protein  
353 was then captured on a StrepTactin column, washed and eluted with binding buffer (100 mM  
354 Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) supplemented with 2.5 mM desthiobiotin. The peak  
355 obtained by affinity chromatography was further purified by a size-exclusion chromatography,  
356 using a Superose6 16/300 column (GE Healthcare) with 20 mM Tris HCl pH 8.0 and 150 mM  
357 NaCl. The purified protein was then dialyzed against 10 mM Tris HCl pH 8.0 and loaded on  
358 MonoQ 5/15 column (GE Healthcare) to be eluted using a step gradient of 240 mM and 400  
359 mM NaCl in the same buffer.

360 To denature the complex pr/sE under non-reducing conditions, 8 M urea was added to the  
361 solution and the two proteins (47KDa and 10Kda) were separated by a size exclusion  
362 chromatography (SEC) in 10 mM Tris-HCl pH 8, 6 M urea and 1 M KSCN. Samples were  
363 collected and dialyzed overnight against 20 mM Tris-HCl pH 8 to remove any trace of urea. A  
364 final purification on a Superdex 200 16/60 in 20mM Tris-HCl pH 8.0 and 150mM NaCl was  
365 done to obtain pure and refolded E protein and pr peptide.

366 ZIKV sE (strain PF13), DENV2 sE (SG strain) and DENV2 sE A259C mutant (16681 strain)  
367 were produced as described earlier. Briefly, sE genes with a tandem C-terminal strep-tag in  
368 pMT/BIP/V5 plasmid were expressed in *Drosophila* S2 cells (Invitrogen) as described  
369 previously (20), (9). Protein expression was induced by the addition of  $5 \mu\text{M}$   $\text{CuSO}_4$  or  $\text{CdCl}_2$ .  
370 Supernatants were harvested 8–10 days post-induction, and sE were purified using Streptactin



371 columns (GE) according to manufacturer's instructions. This affinity chromatography step was  
372 followed by size exclusion chromatography using Superdex 200 10/300 GL column  
373 equilibrated in 50 mM Tris (pH 8) and 500 mM NaCl. Pr proteins from YFV, ZIKV (PF13 strain)  
374 and DENV2 (16681 strain) were expressed similar to sE proteins, using same pMT/BIP/V5  
375 plasmid with double C-terminal strep tag in were expressed in *Drosophila* S2. Pr proteins were  
376 purified using a streptactin columns based affinity step and followed by a single SEC step  
377 using Superdex 75 10/300 GL column equilibrated in 50 mM Tris (pH 8) and 300 mM NaCl.

### 378 **Crystallization.**

379 **pr/sE Asibi crystallization.** After optimization, crystals diffracted up to 3Å resolution  
380 but an analysis of the intensity distribution revealed that the datasets was perfectly and  
381 merohedral twinned with apparent space group P4<sub>1</sub>22. To overcome the problem an additional  
382 purification step using denaturation / renaturation of the heterodimer under non-reducing  
383 conditions was introduced. The reassembled pr/sE complex was concentrated to 3 mg/mL in  
384 20 mM Tris-HCl pH 8 and 150 mM NaCl and crystallized into 100 mM Tris HCl pH 8 and a  
385 range of 1.2-1.8 M Li<sub>2</sub>SO<sub>4</sub>. For cryoprotection, crystals were soaked in the precipitation solution  
386 plus 25% glycerol and flash-frozen under liquid nitrogen.

387 **sE Asibi dimer crystallization.** Asibi sE, produced without co-expression of prM, and  
388 purified by SEC in 20 mM Tris pH 8, 150 mM NaCl, was adjusted to a concentration of 3.2  
389 mg/mL, and formed highly regular crystals in 1.26 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 0.1 M HEPES pH 7.5.  
390 The crystals diffracted at very low resolution and optimized conditions allowed to grow bigger  
391 crystals which gave diffractions ranging from 6 Å to 3.7 Å.

392

### 393 **Data collection, Refinement and Model building.**

394 Diffraction data were collected at the beamlines Proxima-1 and Proxima-2 at the  
395 SOLEIL synchrotron and ID23-1 at the ESRF synchrotron, were processed using XDS  
396 package (27) and scaled with AIMLESS (28). Only the diffraction data of the sE dimer crystal



397 shown significant anisotropy. Therefore, this data was elliptically truncated and corrected using  
398 the DEBYE and STARANISO programs (developed by Global Phasing Ltd) using the  
399 STARANISO server (29). The unmerged protocol applied to this data produced a best-  
400 resolution limit of 3.48Å and a worst-resolution limit of 4.87Å with a surface threshold of 1.2 of  
401 the local  $I/\sigma(I)$ . This corrected data was used for refinement of the sE dimer structure. The  
402 structure of Asibi pr/sE (6EPK) was first determined by molecular replacement with the  
403 program AMoRe (30) using the atomic models TBEV sE protein (PDB entry 1SVB, 43.4%  
404 sequence identity, (17) and DENV pr protein (PDB entry 3C5X, 34.6 % sequence identity, (11).  
405 Then, the Asibi sE protein from the pr/sE structure was used as a template for molecular  
406 replacement for solving the sE dimer structure. The two models were subsequently modified  
407 manually with COOT (31) and refined with BUSTER-TNT (32), (33) or PHENIX.REFINE (34).  
408 Refinement was constrained to respect non-crystallographic symmetry and target restraints  
409 (35) using high resolution structures of parts of the complexes, as detailed in the Table SUPP  
410 1. TLS refinement (36) (parameterization describing translation, liberation and screw-motion  
411 to model anisotropic displacements) was done depending on the resolution of the crystal. The  
412 final models of pr/sE (PDB 6EPK) and sE dimer contain all amino acids of YFV sE (1-392)  
413 and residues 1 to 80 of pr. Data collection and refinement statistics as well as the MolProbity  
414 (37) validation statistics for all the two structures are presented in the Table SUPP 1. The  
415 figures of the structures were prepared using the PyMOL molecular graphics system  
416 (Schrodinger)(pymol.sourceforge.net).

#### 417 **Multi-angle static light scattering-Size exclusion chromatography.**

418 MALS studies were performed using a SEC Superdex 200 column (GE Healthcare)  
419 previously equilibrated with the corresponding buffer, see below. SEC runs were performed at  
420 25 °C with a flow rate of 0.4 mL/min, protein injection concentration was 100 µg. Online MALS  
421 detection was performed with a DAWN-HELEOS II detector (Wyatt Technology, Santa  
422 Barbara, CA, USA) using a laser emitting at 690 nm. Online differential refractive index  
423 measurement was performed with an Optilab T-rEX detector (Wyatt Technology). Data were

424 analyzed, and weight-averaged molecular masses ( $M_w$ ) and mass distributions  
425 (polydispersity) for each sample were calculated using the ASTRA software (Wyatt  
426 Technology). For each virus, equilibration buffers for addressing the effect of pH for sE, pr and  
427 the sE:pr complex were the three-component buffers, 100 mM Tris-HCl, 50 mM MES, 50 mM  
428 sodium acetate and 150 mM NaCl, at pH 5.5 or pH 8.0. The sE:pr complex, in 1:2 molar ratio  
429 (monomer:monomer molar ratio), were prepared by incubation in the corresponding three-  
430 component buffers. Buffer exchange was performed by extensive dialysis of the sample, 12 h  
431 stirring at 4 °C and two 500 mL buffer replacement in 10 kDa molecular weight cut-off dialysis  
432 membranes (Spectrum). SEC fractions of sE:pr complexes at pH 5.5 or 8.0 were further  
433 analyzed by Coomassie blue or Silver nitrate SDS-PAGE or by western blot using an anti-strep  
434 antibody for simultaneously detection of both E and pr proteins.

435

#### 436 **Liposomes preparation.**

437 Liposomes used for lipid mixing and co-flotation assays were prepared by following a  
438 modified film-hydration protocol (38). Briefly, chloroform solutions of DOPC, DOPE, SM,  
439 Cholesterol, NBD-PE and Rho-PE, were pooled using glass graduated syringes (Hamilton) in  
440 borosilicate tubes at a molar ratio of 1:1:1:3:0.1:0.1, respectively, and a total lipid concentration  
441 of 10 mM. The fluorescent lipids (NBD-PE and Rho-PE) were omitted in the preparation of  
442 liposomes for co-flotation assays. The organic solvent was evaporated in the tube under a  
443 steam of N<sub>2</sub> gas yielding a thin lipid film which was further dried by Speed-Vac (Thermo  
444 Electron, RVT400), 1 hour at room temperature. The lipid film was resuspended in 20 mM  
445 HEPES pH 7, 50 mM NaCl degassed buffer, by vortexing in presence of 180  $\mu$ m acid washed  
446 glass beads (Sigma). The resulting opaque solution, composed by multilamellar vesicles, was  
447 subjected to 10 cycles of liquid N<sub>2</sub> flash freeze-thaw and extruded using a polycarbonate filter  
448 of 100 nm pore size until translucency, more than 20 extrusion cycles. The hydrodynamic  
449 diameter and homogeneity of the sample was controlled by dynamic light scattering. The final  
450 lipid concentration was determined by a using NBD-PE absorbance at 460 nM and a standard

451 curve. The liposomes were stored under N<sub>2</sub> (gas) for up to three weeks at 4°C. All the lipids  
452 as well as the extrusion system were purchased from AVANTI Polar Lipids (USA).  
453 Abbreviations: DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE: 1,2-dioleoyl-sn-  
454 glycero-3-phosphoethanolamine; SM: Sphingomyelin (brain, porcine); NBD-PE: 2-dioleoyl-sn-  
455 glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); Rho-PE: 1,2-dioleoyl-  
456 sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt).

457

#### 458 **sE-liposomes co-floatation assay.**

459 Renatured sE and pr proteins were mixed at different molar ratio and incubated for 10  
460 min at RT before addition of liposomes. The mixture was further incubated for 10 min at RT  
461 before overnight incubation at 30°C under acidic conditions. The liposomes were then  
462 separated by ultracentrifugation on an Optiprep (Proteogenix 1114542) continuous 0-30%  
463 gradient. Aliquots from top and bottom fractions were analyzed by Coomassie gel or by  
464 western blot gels using in house produced anti-YFV E (E21.3) mouse monoclonal antibody. At  
465 least two and up to nine experiments were performed for the different molar ratios tested, the  
466 bands intensity from top and bottom fractions were analyzed by ImageJ software and plotted  
467 as ratio to total protein present in each floatation assay.

468

#### 469 **Isothermal titration calorimetry (ITC).**

470 We titrated 10 μM of E in the cell with several injections of 100 μM pr. The injection  
471 volume was 2 μL. We continued the injections beyond saturation to determine the heat of  
472 ligand dilution, which was subtracted from the data prior to fitting with a single site binding  
473 model. We used Microcal ITC200 from Microcal and the associated Origin software for fitting  
474 of the data. The two-component buffer was prepared by dissolving appropriate weights of each  
475 component in water (39). The resulting solution had a pH of 8.3, which was taken to the desired  
476 value with concentrated HCl. The pH was measured in a Sartorius PB11 pH-meter. The protein

477 samples were extensively dialyzed prior the titrations. ITC measurements were performed in  
478 50 mM Tris, 50 mM MES (pH 6, 7 and 8) and 150 mM NaCl at 25°C (Table 2).

479

#### 480 **Surface plasmon resonance (SPR).**

481 The affinity of the sE protein for the pr peptide was measured by SPR using a Biacore  
482 T200 system (GE Healthcare Life Sciences) equilibrated at 25°C. The carboxylic groups of a  
483 Series S CM5 sensor chip were activated for 10 min using a mix of N-Hydroxysuccinimide  
484 (NHS, 50 mM) and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC, 200 mM). The  
485 Strep-Tactin XT (IBA lifesciences) at 2 µg/mL in acetate pH 5 was injected for 20 min, followed  
486 by deactivation with 1 M ethanolamine for 7 min, reaching a density of 800 resonance units (1  
487 RU corresponds to about 1 pg/mm<sup>2</sup>) of amine coupled Strep-Tactin XT. At the start of each  
488 cycle, double strep tagged sE protein was captured on a Strep-Tactin XT surface for 3 min at  
489 5 µg/mL. Eight concentrations of pr peptide (2-fold dilutions ranging from 100 nM to 0.78 nM)  
490 were then injected at 30 µl/min for 600s. At the end of each cycle, the surfaces were  
491 regenerated by sequential 15s injections of Gly-HCl pH 1.5 and 10 mM NaOH. Experiments  
492 were performed in duplicate, using 3 different running buffers, 50 mM MES, 50 mM Tris (pH 6,  
493 7 and 8) with 150 mM NaCl and 0.2 mg/mL BSA at 25°C (Table 2). The association and  
494 dissociation profiles were fitted globally using the Biacore T200 evaluation software (GE  
495 Healthcare) assuming a 1:1 interaction between sE and pr.

496

#### 497 **Virus stocks.**

498 Yellow fever 17D (YF17D) viral stocks were derived from pACNR/FLYF plasmid (40)  
499 containing the full length infectious YF17D-204 genome under a SP6 promoter, after  
500 electroporation of in vitro-generated RNA transcripts in SW13 cells as previously described  
501 (41). Briefly, 3 µg of RNA were mixed with 4 × 10<sup>6</sup> SW-13 cells in PBS and pulsed in 2-mm-  
502 gap electroporation cuvettes (BTX) with an electroporator (BTX Electro Square Porator model

503 T820) set for 3 pulses at 800 V with a pulse length of 60  $\mu$ s. After a 10-min recovery phase at  
504 room temperature, cells were plated in a p75 flask in complete medium (Minimum Essential  
505 Medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM  
506 Glutamax and 0.1 mM MEM non-essential amino acids). Virus stocks were harvested 48h  
507 post-transfection with typical yields of  $10^7$ - $10^8$  FFU/mL as determined by focus forming assay  
508 on SW13. Single use aliquots were stored frozen at  $-80^{\circ}\text{C}$  until use.

509

### 510 **Virus purification.**

511 SW13 cell monolayers were infected at low MOI (0.1 ffu/cell) and supernatants were  
512 collected 48h post-infection. YF17D virus was recovered by precipitation with 8%(w/v) PEG  
513 8000 for 1h at  $4^{\circ}\text{C}$  and purified on a step tartrate-glycerol gradient (40-10%(w/v) tartrate - 5-  
514 30%(w/v) glycerol) by over-night ultracentrifugation in SW41 at 30 Krpm. Virus band was  
515 recovered by needle puncture at the side of the tube and virus titers were determined by focus  
516 forming assay. The total amount of virus present in the preparation was quantified by  
517 comparison against known amount of purified sE protein and western blot with YF E-specific  
518 antibody E21.3. The virus band buffer corresponded to about 25%(w/v) tartrate, 15%(w/v)  
519 glycerol and 0.02%(w/v) BSA. The virus preparation was kept at  $4^{\circ}\text{C}$  until use. Buffer-alone  
520 gradients were run and collected in parallel to each virus preparation to be used as blank in  
521 the functional assays.

522

### 523 **Focus-forming assay.**

524 Serial dilutions of the virus preparations (1/10) were prepared in 1% FBS / PBS. Each  
525 dilution was added to SW13 cells and foci were developed in the presence of 1,5%  
526 methylcellulose for 2 days in 96 well plates. Foci development was stopped by fixation with 4%  
527 formaldehyde and foci were then stained using a mouse-anti-NS1 antibody (1A5) (gift from

528 Jacob Schlesinger, Rochester University) and a horseradish peroxidase (HRP) conjugated  
529 secondary anti-mouse antibody (ThermoFisher 31430). The foci were visualized by  
530 diaminobenzidine (DAB) (Sigma D5905) staining and imaged using the ImmunoSpot S6  
531 Analyser (Cellular Technology Limited).

532

### 533 **pH triggered lipid mixing, pH and pr titrations.**

534 We adapted, from standard lipid mixing assays protocols using the pair of probes NBD-  
535 PE and Rh-PE (42), (43), a pH-triggered assay to monitor the effect of pH or pr on the extent  
536 of lipid mixing between YF 17D virus and labelled liposomes. Mixture reaction for pH titrations:  
537 10  $\mu$ l of purified virus ( $10^9$ - $10^{10}$  ffu/mL) were added to 100  $\mu$ l of 500 nM labelled liposomes  
538 diluted into 300 mM citrate-phosphate buffer at pH 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8  
539 or 7.0. Mixture reaction for pr titrations: 10  $\mu$ L of purified virus ( $10^9$ - $10^{10}$  ffu/mL) were incubated  
540 in a multi-well plate (Greiner) with increasing amounts of purified pr protein for 30 min at 37°C  
541 in 100 mM Tris HCl pH 7.5 and 150 mM NaCl, 50  $\mu$ L total volume. Subsequently, 100  $\mu$ l of 200  
542 nM NBD-PE and Rho-PE labelled liposomes in 50 mM MES pH 5.5, was added to the virus/pr  
543 complex using a multichannel pipette and gently mixed three times prior data collection  
544 (average dead time 40s). The pH after the mixture was  $6.0 \pm 0.2$ . For both titration assays, the  
545 emission fluorescence of NBD was recorded in a multi-plate reader fluorimeter (Tecan M1000),  
546 with an excitation and emission wavelength of 460 nm and 539 nm and slits widths of 10 nm  
547 and 20 nm, respectively, during more than 3 times the end of the lipid mixing reaction (~10  
548 minutes) at 25°C. The maximum NBD emission signal was recorded by addition of 10  $\mu$ l of  
549 2.5% C13E8 (Polyoxyethylene(8)tridecyl Ether, Anatrace) for 10 minutes. For pr titrations, a  
550 mock reaction (no virus) was performed for each pr concentration by using the same virus  
551 buffer and used as reference signal. The extent of lipid mixing was calculated from the  
552 recorded intensities (I) by  $(I-I_0)/(I_{100}-I_0)$ , with  $I_0$  the initial intensity and  $(I_{100})$  the maximum  
553 NBD emission signal recorded upon addition of detergent. The % of lipid mixing was calculated  
554 by the end point parameter of the fitting of the data to a mono exponential equation using

555 ProFit software (QuantumSoft). For pr titrations, we normalized all the curves to the % of lipid  
556 mixing measured in absence of pr. The concentration of viral E protein in the final volume of  
557 the assay was quantified by western blot. Shortly, a range of 25 to 200 ng of recombinant E  
558 was used as a standard curve and in the same SDS PAGE gel we loaded 0.15 µl to 10 µL of  
559 purified virus. The western blot was revealed with the E21.3 antibody. Bands intensities were  
560 calculated in ImageJ software (44) and used to interpolate the amount of E in the virus to a  
561 standard curve of purified E protein (25-200ng) by linear regression. The concentration of viral  
562 E of 50-80 nM was used to refer the titrated concentrations of pr as a pr/E molar ratio.

563

#### 564 **Co-precipitation of purified pr with YF 17D virus.**

565 Cell culture supernatant containing  $10^8$  total particles of YF 17D virus was pelleted over  
566 a 20% sucrose cushion and resuspended in 100ul of TNE buffer (10 mM Tris pH8, 150 mM  
567 NaCl, 1mM EDTA). An excess of purified pr peptide was added to the virus (ratio 1:50) and  
568 the pH was changed with phosphate/citrate buffer to pH5.5-6-7-8. After 30 min incubation at  
569 37°C the complex was pelleted in a SW55 rotor at 100 Kg for 1 hour and loaded on a 12%  
570 SDS gel. Antibody E21.3 was used in western blot to detect the viral E protein and antibody  
571 A3.2 was used to detect the pr protein. Band intensities were calculated in Image J software.

572

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582

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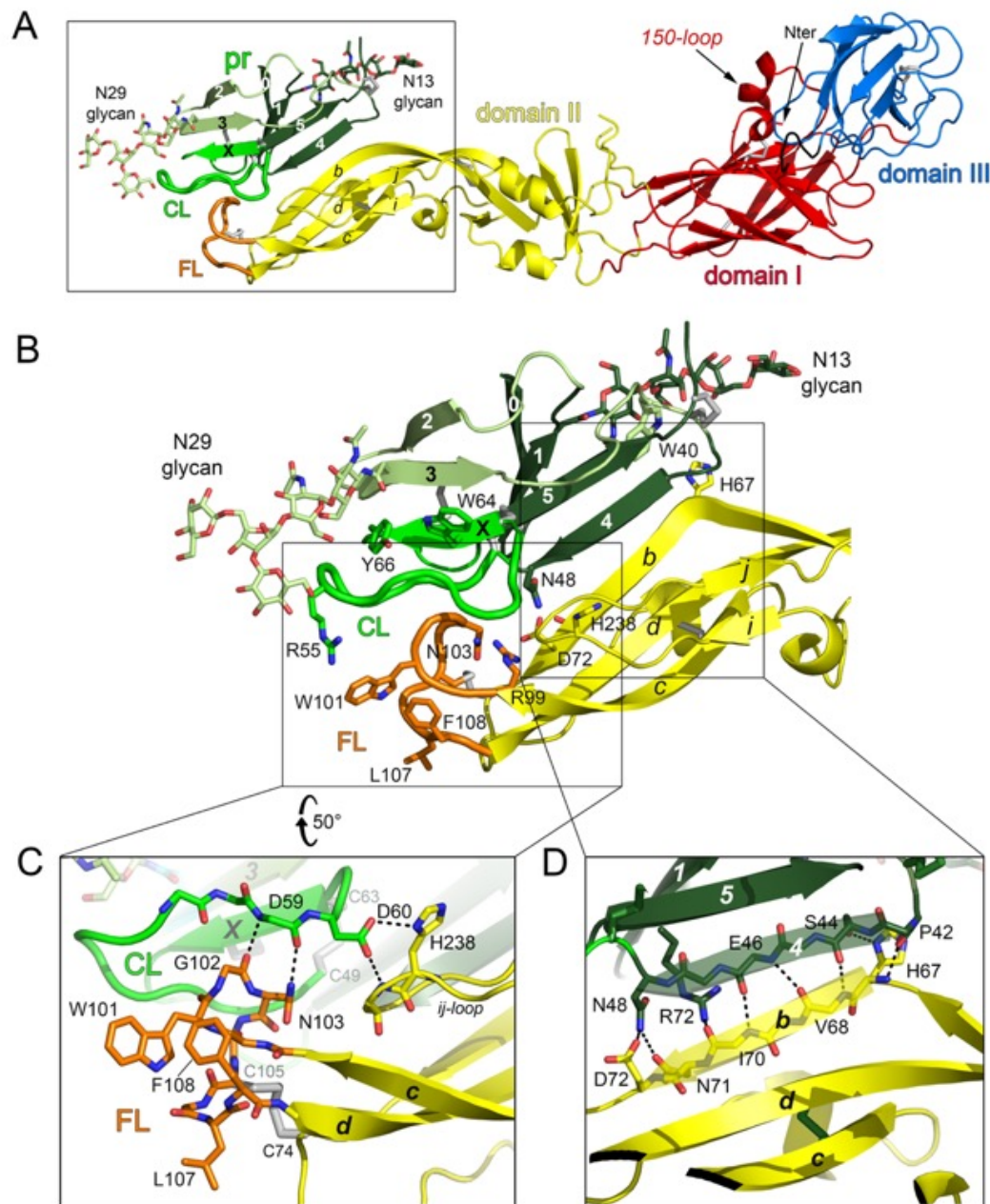
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706 FIGURES

Figure 1: Structure of the pr/sE complex



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**Figure 1 legend. (A) Structure of the pr/sE complex.** E is colored according to the classical flavivirus E domains DI, DII and DIII in red, yellow and blue respectively. The fusion loop (FL) is in orange. pr is in green and is glycosylated in positions Asn13 and Asn29 as indicated. The black arrows point the 150-loop and the N-terminal residue.

**(B) The framed region in (A) is shown.** Residues of the fusion loop (FL) are displayed as sticks. E  $\beta$ -strands of domain II are labeled. The pr molecule is displayed with three shades of green, with the two beta-sheets sandwich colored dark and light green and with the capping-loop (CL) displayed thicker in bright green. pr is glycosylated in positions Asn13 and Asn29 as indicated. Residues discussed in the text are labelled and displayed as sticks and atom color-coded. In brief, Trp40 packs against Asn13 while Tyr66 and Arg55 pack against Asn29, stabilizing CL.

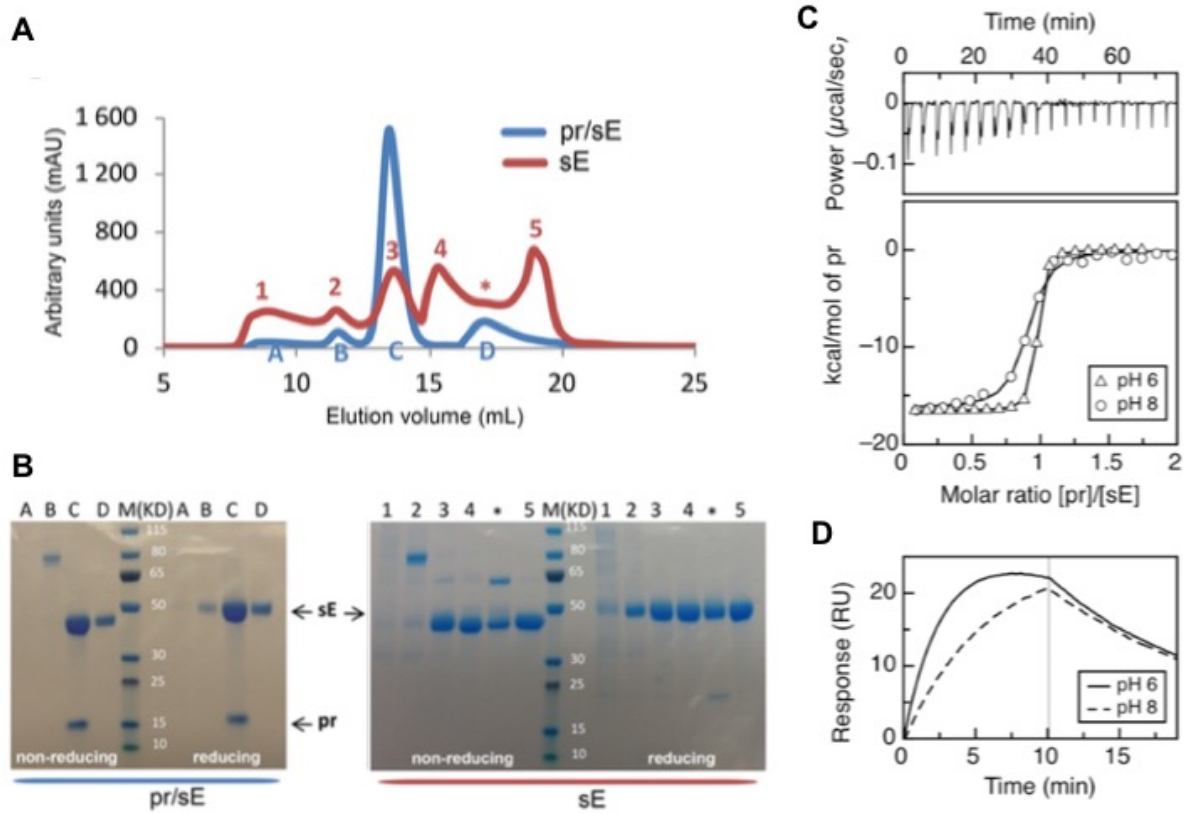
**(C-D) The framed regions in (B) show close-views of the pr/sE interactions.** The residues that interact between pr and E are displayed as sticks and atom color-coded.

**(C)** Interactions of residues of *cd*-loop (FL, in orange) and *ij*-loop (in yellow) with the residues of the pr-CL (in green). The residues that interact between pr and E are displayed as sticks and atom color-coded. The residues E-His238 and pr-Asp60 are strictly conserved among all the flaviviruses.

**(D)** Hydrogen bonded network between the  $\beta$ -strand  $\beta_4$  of pr (in dark green) and the  $\beta$ -strand *b* of E (in yellow) involving main-chain and side-chain residues. The residues are labelled, and the directions of the strands are shown in transparency.

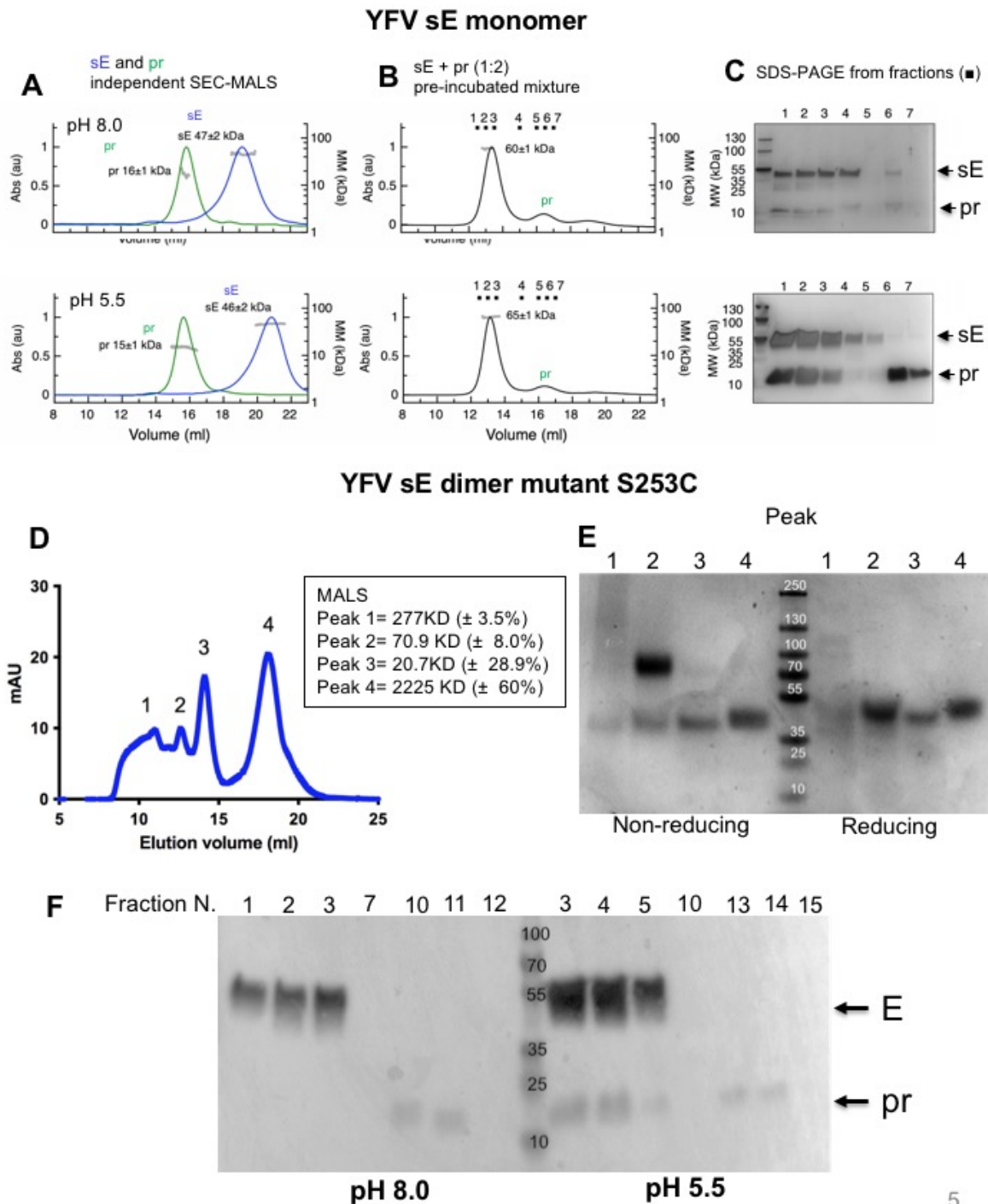


## Figure 2: Chaperone activity of pr on the E protein and biophysical characterizations of the pr/sE interaction.



**Figure 2 legend.** **(A)** SEC profiles of YFV sE expressed with prM (in blue), showing 4 peaks, labelled A, B, C and D, and of sE expressed without prM (in red), showing 6 peaks labelled 1, 2, 3, 4, \* and 5. Proteins were produced in S2 cells and first purified by affinity chromatography and then analyzed by size-exclusion chromatography (SEC). **(B)** SDS-PAGE analysis of the protein present in each peak under reducing and non-reducing conditions, as indicated. Aliquots from each peak were run under non-reducing (without DTT) or reducing (+DTT) in an SDS-PAGE and stained with Coomassie blue. The asterisk in the profile of sE indicates a shoulder of peak 4 that was treated separately. Molecular masses of marker proteins are listed in kilodaltons. **(C) Isothermal titration calorimetry.** sE:pr binding isotherms (bottom panel), recorded at pH 6.0 and pH 8.0, shown as triangles and circles, respectively, resulting from integration of the specific heats with respect to time as shown for pH 8.0, top panel. **(D) Surface plasmon resonance.** Example of sE:pr association and dissociation kinetics corresponding to injections of pr at 12.5 nM over immobilized sE, respectively at pH 6.0 (solid line) and pH 8.0 (dashed line).

**Figure 3: YFV pr protein binds to the E protein monomer at both neutral and acid pH while binding to the E protein dimer is impaired at neutral pH.**



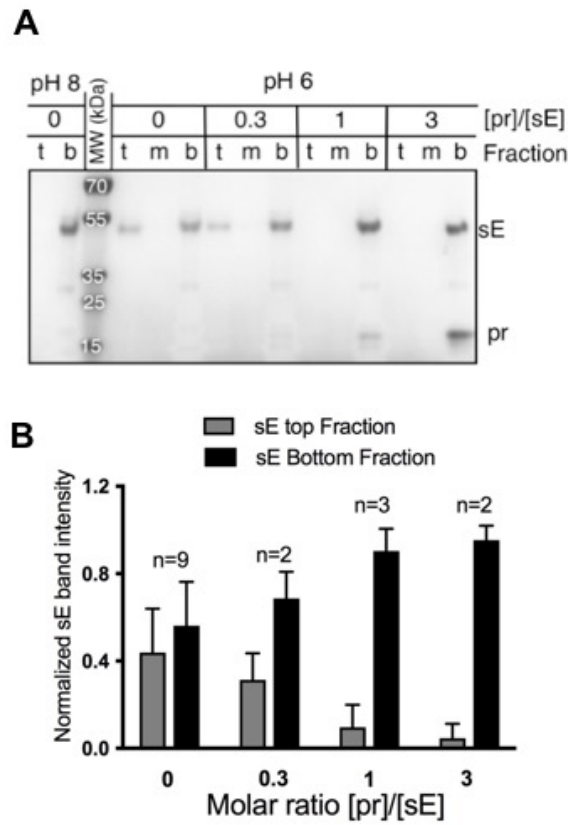
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**Figure 3 legend. (A-B)** SEC-MALS elution volume profiles. Left y axis: the ultraviolet absorbance normalized by setting the highest peak to 1. Right y axis: molecular mass (kDa) determined by MALS, with the values for each species indicated on the corresponding peak. **(A)** Equilibrated SEC-MALS elution profiles of isolated sE (in blue curves) and isolated pr (in green curves) equilibrated at pH 8.0 (top panel) and pH 5.5 (bottom panel). **(B)** SEC-MALS elution profiles of a mixture of sE with pr in excess (1:2 sE:pr monomer:monomer molar ratio) at pH 8.0 (top panel) and pH 5.5 (bottom panel). The fractions analyzed by SDS-PAGE in (C) are indicated (1–7). **(C)** SDS-PAGE and silver nitrate staining of the SEC fractions indicated in (B) at the corresponding pH. **(D)** SEC elution volume profile of a YFV single cysteine mutant E dimer (S253C) at pH 8.0. The four peaks have been run independently to determine the molecular mass (kDa) by MALS. The MALS results for each peak are listed in the inset. **(E)** SDS-PAGE Coomassie staining of the four peaks indicated in (D) under reducing and non-reducing conditions. Peak 2 contains the stabilized sE dimer confirming the molecular mass calculated by MALS (70.9 kDa in (D)). **(F)** Western blot of an SDS-PAGE in reducing conditions probed with an anti-Strep antibody of SEC fractions from sE dimer in complex with pr at pH 8.0 and pH 5.5 as indicated and as described in Methods.

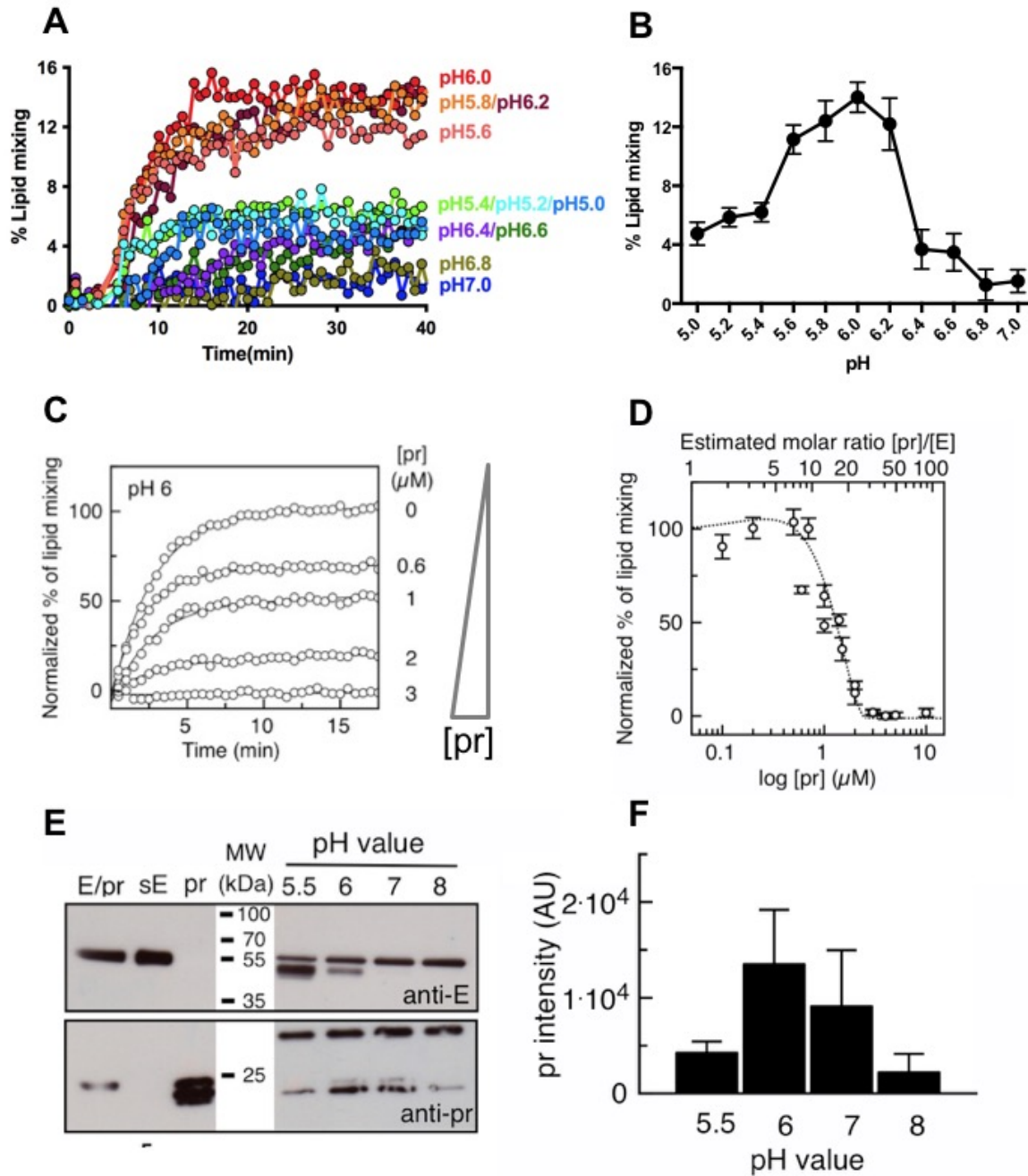


**Figure 4: Binding of pr prevents sE insertion into membranes.**



**Figure 4 legend. (A) Co-floatation assay.** Five  $\mu\text{g}$  of purified sE protein was mixed with different amounts of purified pr protein and with liposomes (refer to Methods for lipid composition). After addition of buffer at the indicated pH and over-night incubation at  $30^\circ\text{C}$ , the protein-liposomes mixture was separated on an Optiprep gradient. Coomassie stained SDS-PAGE of top (t), medium (m) and bottom (b) fractions is shown. sE protein-liposome co-floatation was performed at pH 8.0 (left columns) and at pH 6.0 in presence of pr at sE:pr molar ratios 0.3, 1 and 3 (right columns). **(B) Histogram** of normalized sE band intensity from top and bottom fractions to the amount of sE present in the bottom fraction at pH 8.0. Several floatation assays were included in the calculation using Image J software. Errors are standard deviation calculated from at least two experiments.

**Figure 5: Effect of pr binding to the E protein of the viral particle.**



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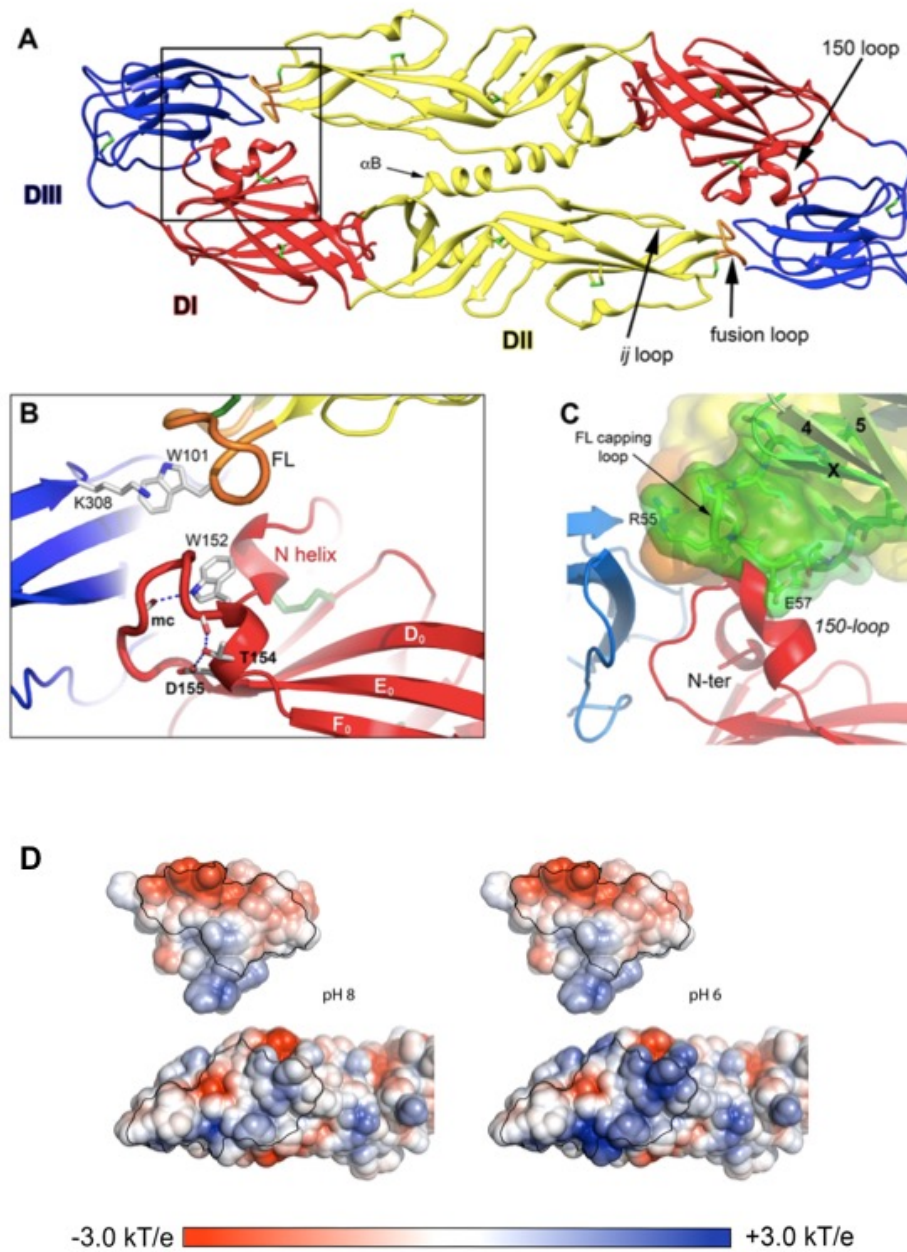
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**Figure 5. (A) Lipid mixing assays** between YF17D virus and NDB/Rho-labeled liposomes recorded from pH 5.0 to pH 7.0 at every 0.2 pH units. About  $10^7$ - $10^8$  ffu of purified virus was mixed with 500nM labeled liposomes resuspended in buffer at different pH. Fluorescence emission was recorded for 40min in a multiplate reader fluorimeter (Tecan M1000) and the reaction was stopped by addition of detergent to measure the maximal (100%) signal. The represented extent of lipid mixing was related for each pH to the maximum signal recorded upon lipid dilution by detergent addition (see Methods). **(B) Plot of mean fluorescence signal** registered at min. 7-37 for each pH. **(C) Representative curves of normalized NBD fluorescence intensity** recorded at 535 nm as a function of pr concentration, lines are mono-exponential fits to the data. Sample without pr was considered 100%. About  $10^7$ - $10^8$  ffu of purified virus was mixed with increasing amount of purified pr and incubated at 37°C for 30min. Virus/pr mixture was then added to 200nM liposomes in MES buffer pH 5.5. The final pH of the mixture was pH 6.0. Fluorescence emission was recorded for 30min in a plate reader fluorimeter (Tecan M1000) and the reaction was stopped by addition of detergent to measure the maximal signal. **(D) Percentage of lipid mixing** as a function of pr concentration. Top x-axis corresponds to the [pr]/[E] molar ratio as estimated by western blot (see Methods and Suppl. Fig.S4). The dashed line is a guide to eye. **(E) Binding of exogenous pr to YFV viral particle.** Western blot with E- or pr-specific antibodies. About  $10^8$  ffu of virus was mixed with an excess (1:50) of exogenous purified pr protein and incubated in buffer at different pHs. The complex was then pelleted by ultracentrifugation and analyzed by SDS-PAGE and western blot. **(F) Histogram** representing the values of pr band intensity from two experiments.

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**Figure 6: YFV sE dimer and structural analysis of its interaction with pr protein.**



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**Figure 6 legend.** (A) **Ribbon view** of the crystallographic YFV sE dimer colored according to definition described in figure 1. The fusion loop in orange is buried at the dimer interface against 150-loop and *ij*-loop, as indicated. (B) **Close-view** showing the proximity of the 150-loop with the N-terminal of E. (C) **Close-view** of modeled binding of pr protein on the sE dimer showing the clash of the pr capping loop with the 150-loop and N-terminal of E. The superposition of pr/sE structure on sE dimer was done using the tips of E-domain II containing the fusion loop ( $\beta$ -strands *b,c,d* and *i,j*; see Figure 1) of pr/sE monomer (6EPK). (D) **Electrostatic potential surfaces of pr/sE complex.** The electrostatic potential surfaces are displayed as an open book representation of the pr/sE interaction computed at two pH: pH 8.0 (left) and pH 6.0 (right). The potential of pr (top views) is not dependent of the pH while the potential of E (bottom views) appears to direct the change of the electrostatic at the interaction surface with pr.

**Table 1**  
**Comparison of polar pr/sE interactions in YFV - DENV - TBEV**

This should be Asibi pr/sE-GFP

sE domains	YFV Asibi pr/sE PDB 6EPK (2 HD in au)				DENV-2 prMe-sE PDB 3C5X (pH 5.5) and 3C6E (pH 7)				TBEV (pr/sE) dimer PDB 7QRE				
	sE	pr	dist (Å) HD1 HD2		sE	pr	dist (Å) 3C5X 3C6E		sE	pr	dist (Å)	pr	
Reference subunit E	b-strand				LYS 64 [NZ]	GLU 46 [OE2]	2.5	2.9	LYS 64 [NZ]	ASP 43 [OD2]	3.2		
					LYS 64 [NZ]	ASP 47 [OD1]		4.0					
		HIS 67 [N]	PRO 42 [O]	2.7	2.6	ASN 67 [N]	GLU 46 [O]	3.9		ASP 67 [N]	ASP 43 [O]	3.1	
		HIS 67 [ND1]	SER 44 [OG]		3.3	ASN 67 [OD1]	THR 48 [OG1]		2.6	ASP 67 [OD1]	SER 45 [OG]	3.6*	
		VAL 68 [N]	SER 44 [O]	3.2	3.2	THR 68 [N]	THR 48 [O]	3.4	3.5	THR 68 [N]	SER 45 [O]	3.3	
										THR 68 [OG1]	SER 45 [O]	3.1	
	η1					THR 68 [O]	THR 50 [N]	2.7	3.0	THR 68 [O]	SER 47 [N]	2.8	
						THR 70 [N]	THR 50 [O]	2.9	3.0	VAL 70 [N]	SER 47 [O]	2.9	
		VAL 68 [O]	GLU 46 [N]	2.7	2.8					ALA 72 [N]	GLU 49 [OE1]	3.1	
		ILE 70 [N]	GLU 46 [O]	2.8	2.8								
		ILE 70 [O]	ARG 72 [NH2]	2.3	2.3								
		ASN 71 [OD1]	ASN 48 [ND2]	3.0	2.8								
	fusion loop (FL)					ASP 72 [O]	ASN 48 [ND2]	3.7*	3.7*				
						ASP 72 [OD1]	ASN 48 [ND2]	2.6	2.8				
						GLU 84 [OE2]	ARG 6 [NH2]	3.9	3.0				
					GLY 102 [O]	ASP 59 [N]	3.0	2.8	GLY 102 [O]	VAL 60 [N]	2.8		
					GLY 102 [N]	GLU 56 [OE1]	2.4	2.5					
					ASN 103 [N]	GLU 56 [OE1]	3.2	3.3					
β-loop					ASN 103 [ND2]	ASP 59 [O]	3.3	3.6*	ASN 103 [ND2]	VAL 60 [O]	3.3		
					ASN 103 [ND2]	GLU 62 [O]	3.0		ASN 103 [ND2]	VAL 60 [O]	3.3		
					ASN 103 [ND2]	GLU 62 [OE1]	3.9*						
					GLY 104 [N]	GLU 56 [OE1]	2.7	2.7	HIS 104 [ND1]	ASP 54 [OD1]	3.4		
									HIS 104 [ND1]	THR 52 [O]	2.8		
									HIS 248 [ND1]	ASP 61 [OD1]	2.8		
4-X loop					HIS 238 [ND1]	ASP 60 [OD1]	2.7	3.0	HIS 244 [ND1]	ASP 63 [OD1]	3.5	2.7	
					HIS 238 [ND1]	ASP 60 [OD2]	3.1	3.3	HIS 244 [ND1]	ASP 63 [OD2]	2.8	3.6	
					ALA 239 [N]	ASP 60 [O]	3.5	3.5	ALA 245 [N]	ASP 63 [O]	3.7*	3.8*	
					ALA 239 [N]	ASP 60 [OD2]	3.2	3.1	ALA 245 [N]	ASP 63 [OD1]	3.0	2.8	
									ALA 249 [N]	ASP 61 [O]	3.7*		
									ALA 249 [N]	ASP 61 [OD2]	3.2		
3-4 loop					LYS 247 [NZ]	ASP 65 [OD1]	2.9	2.9	LYS 251 [NZ]	ASP 63 [OD2]	2.8		
					LYS 247 [NZ]	ASP 65 [OD2]	3.8	3.7					
					LYS 247 [NZ]	ASP 40 [OD1]	3.9	4.0	LYS 251 [NZ]	ASP 37 [OD1]	3.9		
					ARG 243 [NH2]	GLU 36 [OE2]	3.8	2.4					
					ARG 243 [NE]	GLU 36 [OE2]	3.5						
									LYS 251 [NZ]	TYR 76 [OH]	3.3		
5-strand					LYS 247 [NZ]	TYR 77 [OH]	3.0	2.9	ASP 253 [OD1]	ARG 78 [NH1]	3.1		
					LYS 247 [NZ]	TYR 51 [OH]		2.9	ASP 253 [OD2]	ARG 78 [NH1]	3.9		
Opposite subunit E	domain I	N-ter							ARG 2 [N]	GLU 58 [OE1]	3.5		
		150-loop							GLU 155 [OE1]	ARG 67 [NE]	2.9		
	domain III	EO-FD loop							GLU 155 [OE2]	ARG 67 [NE]	3.4		
		A-strand							GLU 155 [OE2]	ARG 67 [NH2]	3.0		
								HIS 157 [ND1]	GLU 58 [OE1]	3.6			
								HIS 157 [ND1]	GLU 58 [OE2]	3.2			
								ARG 160 [NH2]	GLU 58 [OE1]	3.4			
								LYS 315 [NZ]	GLN 55 [O]	3.7*			
								LYS 315 [NZ]	GLY 56 [O]	3.9*			
								ARG 316 [NH2]	ASP 54 [OD1]	3.2			
								ARG 316 [NH1]	GLU 57 [OE2]	3.4			
								GLU 329 [OE1]	GLU 57 [OE2]	3.1			
								GLU 329 [OE2]	GLU 57 [OE2]	3.0			

Polar contacts computed with PISA 'Protein interfaces, surfaces and assemblies' at EBI ([https://www.ebi.ac.uk/msd-srv/prot\\_int/cgi-bin/piserver](https://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver))

Conserved interactions are on green background.

In red: main chain atoms involved in H-bonds; In bold black: salt bridges; In blue and bold: acidic interactions.

Hydrogen bonds distances cut-off: 3.5Å; Salt bridges distances cut-off: 4Å. \* indicates H-bonds weaker with distances between 3.5Å and 4.1Å.

NA: non applicable; HD: heterodimer; au: asymmetric unit

## Table 2

**Table 2. pr – E binding parameters.**

Technique	Parameter	Condition	
		pH 6	pH 8
ITC			58.8 ±
	$K_D$ (nM)	8.5 ± 3.4	10.8
	$\Delta G$ (kcal/mol)	-11.0 ± 0.4	-9.9 ± 0.2
	$\Delta H$ (kcal/mol)	-16.6 ± 0.2	-16.3 ± 0.2
	$T\Delta S$ (kcal/mol)	-5.6 ± 0.3	-9.4 ± 0.2
	N	0.9 ± 0.1	0.9 ± 0.1
	C value	1324	124
SPR	$K_D$ (nM)	6.2 ± 1.8	19.7 ± 5.1
	$\Delta G$ (kcal/mol)	-11.2 ± 0.3	-10.5 ± 0.3
	$k_{on}$ ( $10^5 M^{-1}s^{-1}$ )	3.8 ± 1.7	0.7 ± 0.3
	$k_{off}$ ( $10^{-4} s^{-1}$ )	23.6 ± 4.1	13.4 ± 0.5

The fit of the ITC raw data yields the change in enthalpy upon binding and the dissociation constant.  $G$  is calculated as  $RT\ln(K_D)$ ,  $-TS$  is calculated as  $G$  minus  $H$ .  $N$  is the stoichiometry of the reaction and  $C$  is the ratio of the ligand concentration and the dissociation constant (12). Errors are fitting errors given by the Microcal Origin software (Microcal software, Northampton, MA, USA) for ITC and by the Biacore T200 software. ITC and SPR measurements were performed in 50 mM Tris, 50 mM MES (pH 6, 7 and 8) and 150 mM NaCl at 25°C (38).



**Table 3**

**Table 3.** Summary of sE oligomerization states and pr binding for YFV sE, YFV sE S253C (noted as YFVd), ZIKV, DENV2 sE A259C (noted as DENV2d) and TBEV sE, at pH 5.5 and pH 8.0. Values for tick-borne encephalitis virus (TBEV) are extracted from (7).

sE variant	pH 5.5	pH 5.5 + pr		pH 8.0	pH 8.0 + pr	
	sE Oligomer /MM (kDa)	sE Oligomer /MM (kDa)	sE-pr Binding	sE Oligomer	sE Oligomer /MM (kDa)	sE-pr Binding
YFV	Mon. / 46±2	Mon. / 65±1	Yes	Mon. / 47±2	Mon. / 60±1	Yes
YFVd	nd	nd	Yes*	Dim. / 71±8	nd	No*
ZIKV	Aggregation	Mon. / 54±3	Yes	Dim. / 91±2	Dim. / 91±1	No
DENV2	Mon. / 45±1	Mon. / 48±1	Yes	Mon. / 51±4	Mon. / 49±1	nd
DENV2d	Dim. / 92±3	Dim. / 135±7	Yes	Dim. / 91±4	Dim. / 91±2	No
TBEV	Mon. / 52±1	Dim. / 93±1	Yes	Dim. / 95±1	Dim. / nd	No*

nd, not determined

YFVd is for YFV sE S253C mutant; DENV2d is for DENV2 sE A259C mutant

\*binding determined only by SEC and SDS-PAGE

(see Fig.3 for YFVd and (7) for TBEV)