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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)₃ 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 β -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 β -strand β 3 in a location spatially just
103 nearby just the DENV pr glycosylation Asn69 (YFV-Tyr66, β -strand β 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 β -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 α 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 α 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 β -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)₃ 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 1×10^6 cells per mL
349 with 500 μM Cu_2SO_4 for 10 days or 5 μM 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 μM CuSO_4 or 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 P4₁22. 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 Li₂SO₄. 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 (NH₄)₂SO₄ 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 N₂ 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 μ m acid washed
446 glass beads (Sigma). The resulting opaque solution, composed by multilamellar vesicles, was
447 subjected to 10 cycles of liquid N₂ 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 N₂ (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/mm²) 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 × 10⁶ 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 μ 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°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°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°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 μ l of purified virus (10^9 - 10^{10} ffu/mL) were added to 100 μ 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 μ 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 μ L total volume. Subsequently, 100 μ 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 ± 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 μ 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 μ l to 10 μ l 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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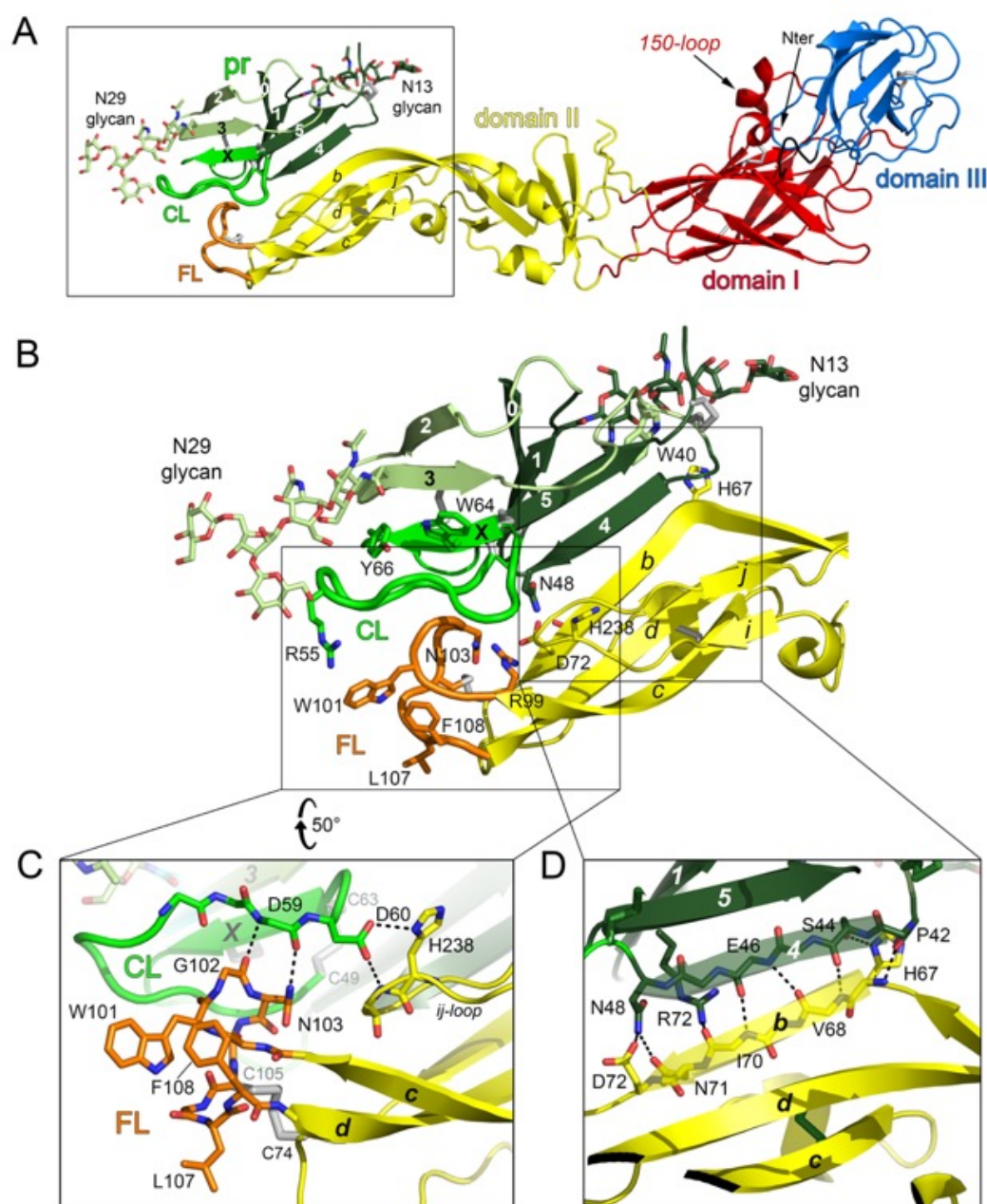
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706 FIGURES

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.

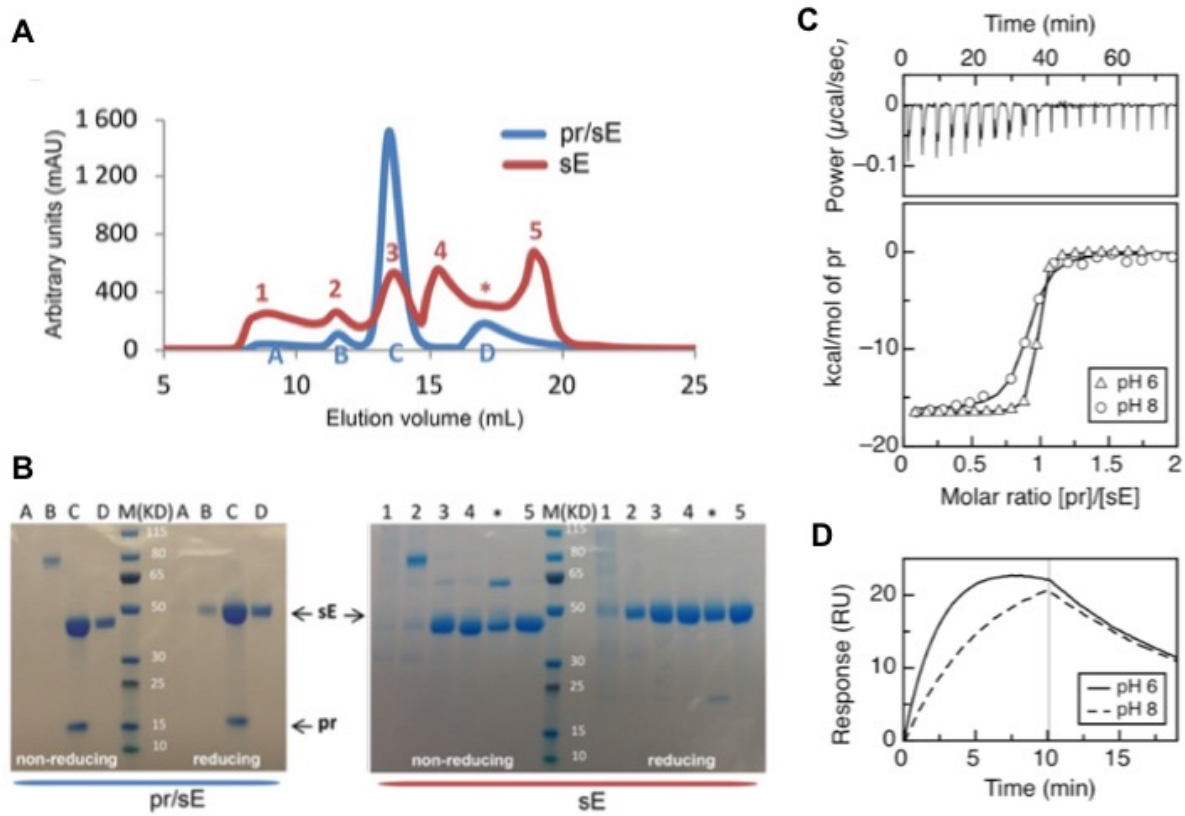
(B) The framed region in (A) is shown. Residues of the fusion loop (FL) are displayed as sticks. E β -strands of domain II are labeled. The pr molecule is displayed with three shades of green, with the two beta-sheets sandwich colored dark and light green and with the capping-loop (CL) displayed thicker 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 β -strand β_4 of pr (in dark green) and the β -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.

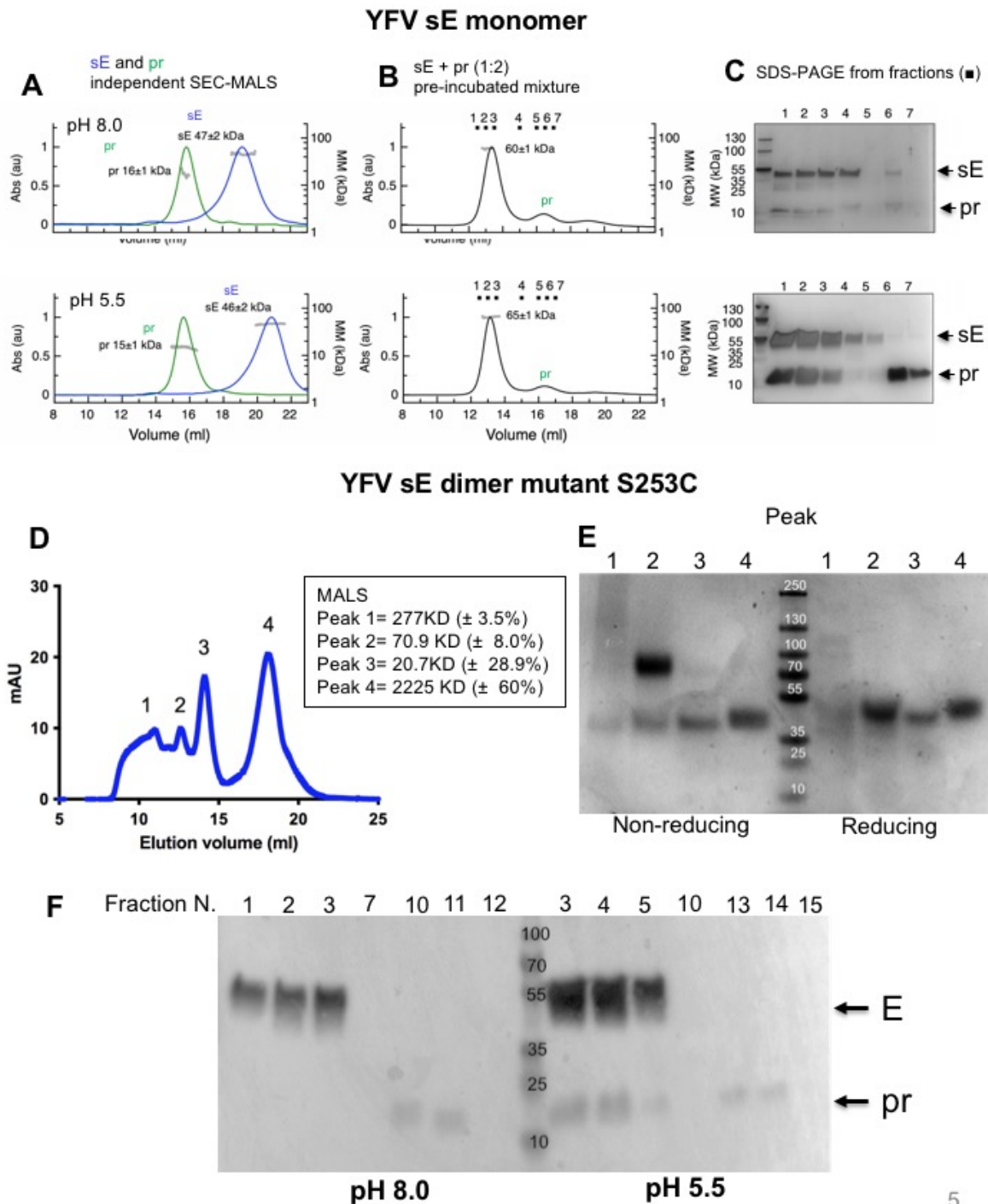


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Figure 2 legend. **(A)** SEC profiles of YFV sE expressed with prM (in blue), showing 4 peaks, labelled A, B, C and D, and of sE expressed without prM (in red), showing 6 peaks labelled 1, 2, 3, 4, * and 5. Proteins were produced in S2 cells and first 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.

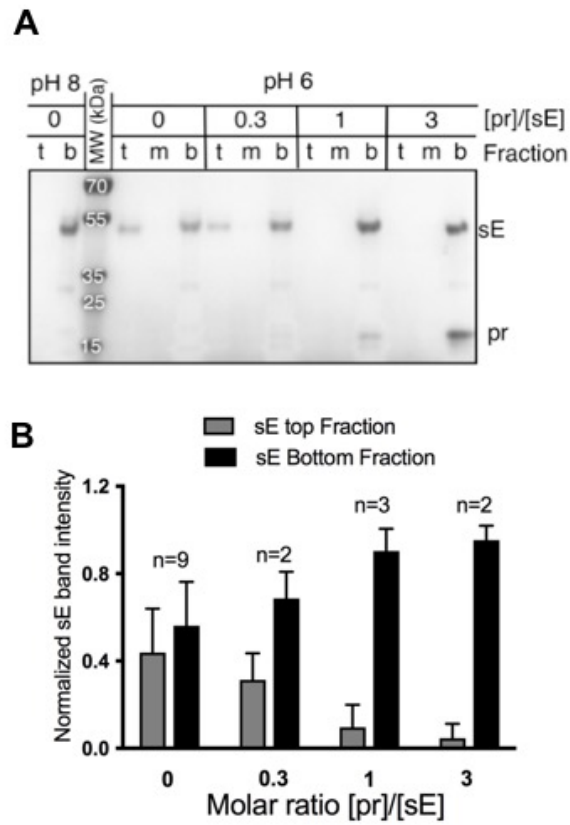
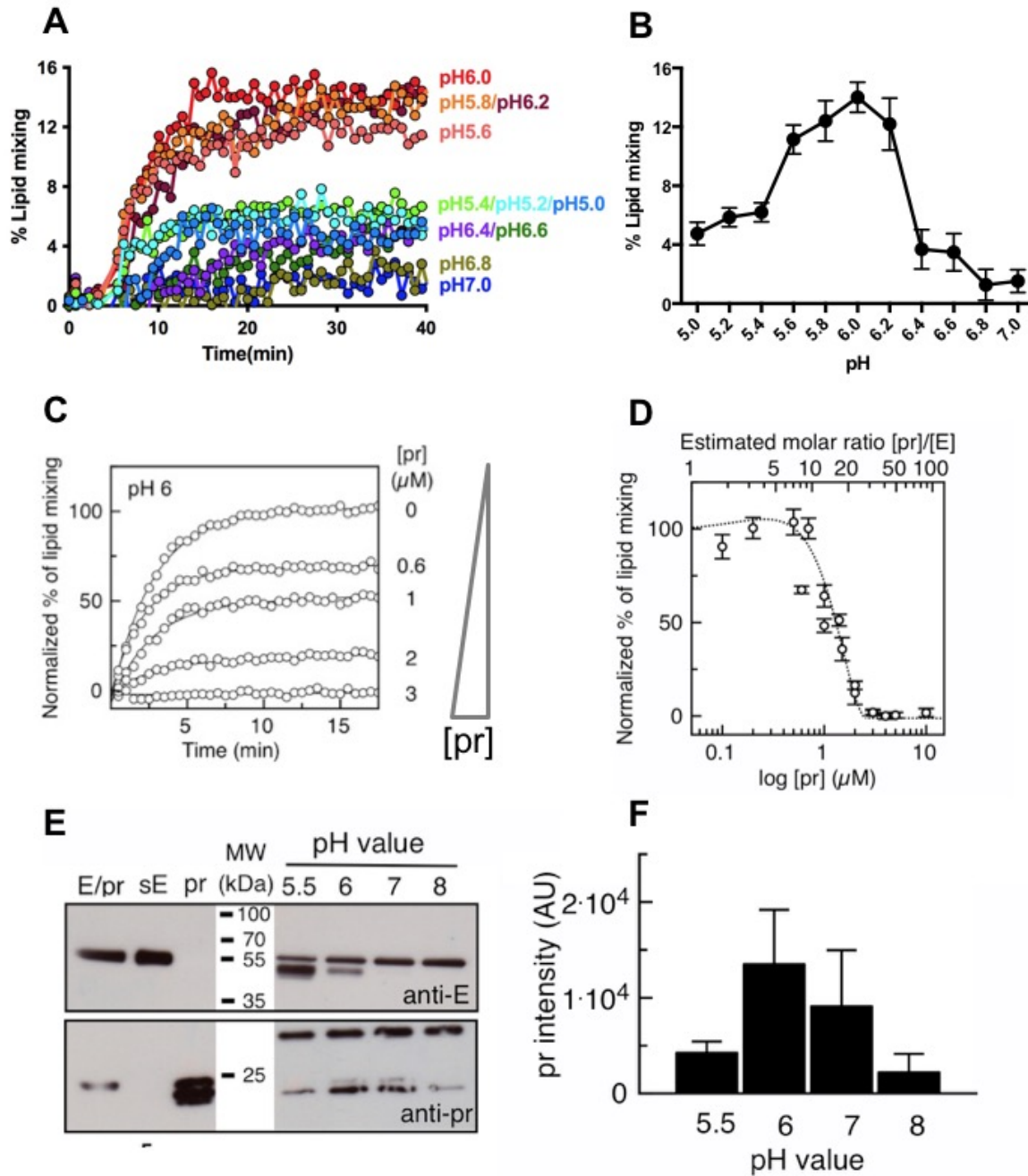


Figure 4 legend. (A) Co-floatation assay. Five μg of purified sE protein was mixed with different amounts of purified pr protein and with liposomes (refer to Methods for lipid composition). After addition of buffer at the indicated pH and over-night incubation at 30°C , the protein-liposomes mixture 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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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.

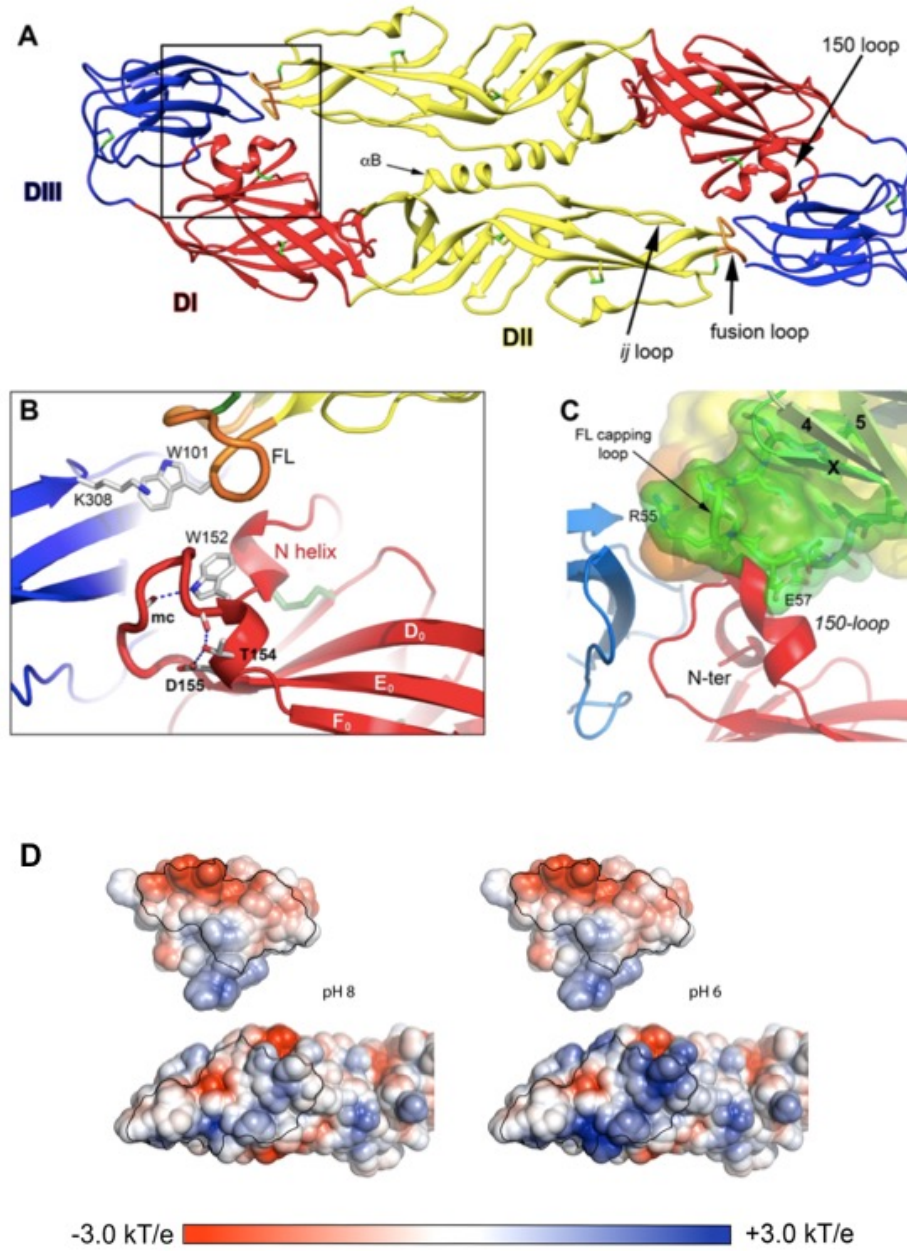


Figure 6 legend. (A) **Ribbon view** of the crystallographic YFV sE dimer colored according to definition described in figure 1. The fusion loop in orange is buried at the dimer interface against 150-loop and *ij*-loop, as indicated. (B) **Close-view** showing the proximity of the 150-loop with the N-terminal 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 (β -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	
		VAL 68 [O]	GLU 46 [N]	2.7	2.8	THR 68 [O]	THR 50 [N]	2.7	3.0	THR 68 [O]	SER 47 [N]	2.8	
		ILE 70 [N]	GLU 46 [O]	2.8	2.8	THR 70 [N]	THR 50 [O]	2.9	3.0	VAL 70 [N]	SER 47 [O]	2.9	
		ILE 70 [O]	ARG 72 [NH2]	2.3	2.3								
		ASN 71 [OD1]	ASN 48 [ND2]	3.0	2.8								
	ASP 72 [O]	ASN 48 [ND2]	3.7*	3.7*					ALA 72 [N]	GLU 49 [OE1]	3.1		
	ASP 72 [OD1]	ASN 48 [ND2]	2.6	2.8									
	η1	No interaction				GLU 84 [OE2]	ARG 6 [NH2]	3.9	3.0	No interaction			
	domain II fusion loop (FL)	GLY 102 [O]	ASP 59 [N]	3.0	2.8	GLY 102 [O]	GLU 62 [N]	2.9	2.92	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	ASN 103 [ND2]	GLU 62 [O]	3.0		ASN 103 [ND2]	VAL 60 [O]	3.3	
		ASN 103 [ND2]	ASP 59 [O]	3.3	3.6*	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	
	β-loop	HIS 238 [ND1]	ASP 60 [OD1]	2.7	3.0	HIS 244 [ND1]	ASP 63 [OD1]	3.5	2.7	HIS 248 [ND1]	ASP 61 [OD1]	2.8	
		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 249 [N]	ASP 61 [O]	3.7*	
		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 [OD2]	3.2	
					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					
	ARG 243 [NH2]	GLU 36 [OE2]	3.8	2.4	LYS 247 [NZ]	ASP 40 [OD1]	3.9	4.0	LYS 251 [NZ]	ASP 37 [OD1]	3.9		
	ARG 243 [NE]	GLU 36 [OE2]	3.5										
	No interaction				LYS 247 [NZ]	TYR 77 [OH]	3.0	2.9	LYS 251 [NZ]	TYR 76 [OH]	3.3		
	No interaction				LYS 247 [NZ]	TYR 51 [OH]		2.9	ASP 253 [OD1]	ARG 78 [NH1]	3.1		
	No interaction								ASP 253 [OD2]	ARG 78 [NH1]	3.9		
Opposite subunit E	domain I	N-ter	NA				NA				ARG 2 [N]	GLU 58 [OE1]	3.5
		150-loop	NA				NA				GLU 155 [OE1]	ARG 67 [NE]	2.9
	domain III A-strand	EO-FD loop	NA				NA				GLU 155 [OE2]	ARG 67 [NE]	3.4
			NA				NA				GLU 155 [OE2]	ARG 67 [NH2]	3.0
	NA				NA				HIS 157 [ND1]	GLU 58 [OE1]	3.6		
	NA				NA				HIS 157 [ND1]	GLU 58 [OE2]	3.2		
	NA				NA				ARG 160 [NH2]	GLU 58 [OE1]	3.4		
	NA				NA				LYS 315 [NZ]	GLN 55 [O]	3.7*		
	NA				NA				LYS 315 [NZ]	GLY 56 [O]	3.9*		
	NA				NA				ARG 316 [NH2]	ASP 54 [OD1]	3.2		
	NA				NA				ARG 316 [NH1]	GLU 57 [OE2]	3.4		
	NA				NA				GLU 329 [OE1]	GLU 57 [OE2]	3.1		
	NA				NA				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)

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
	ΔG (kcal/mol)	-11.0 ± 0.4	-9.9 ± 0.2
	ΔH (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
	ΔG (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)