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Structure of HIV-1 gp41 with its membrane anchors targeted by neutralizing antibodies

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37 **Abstract**

38
39 The HIV-1 gp120/gp41 trimer undergoes a series of conformational changes in order to catalyze
40 gp41-induced fusion of viral and cellular membranes. Here, we present the crystal structure of gp41
41 locked in a fusion intermediate state by an MPER-specific neutralizing antibody. The structure
42 illustrates the conformational plasticity of the six membrane anchors arranged asymmetrically with
43 the fusion peptides and the transmembrane regions pointing into different directions. Hinge regions
44 located adjacent to the fusion peptide and the transmembrane region facilitate the conformational
45 flexibility that allows high affinity binding of broadly neutralizing anti-MPER antibodies.
46 Molecular dynamics simulation of the MPER Ab-induced gp41 conformation reveals the transition
47 into the final post-fusion conformation with the central fusion peptides forming a hydrophobic core
48 with flanking transmembrane regions. This, thus, suggests that MPER-specific broadly neutralizing
49 antibodies can block final steps of refolding of the fusion peptide and the transmembrane region,
50 which is required for completing membrane fusion.

51
52 **Introduction**

53
54 Viral fusion proteins catalyze virus entry by fusing the viral membrane with cellular
55 membranes of the host cell, thereby establishing infection. The HIV-1 envelope glycoprotein (Env)
56 is a prototypic class I fusion protein that shares common pathways in membrane fusion with class
57 II and III viral membrane fusion proteins¹⁻⁴. HIV-1 Env is expressed as a gp160 precursor
58 glycoprotein that is cleaved into the fusion protein subunit gp41 and the receptor binding subunit
59 gp120 by host furin-like proteases. Gp41 anchors Env to the membrane and associates non-
60 covalently with gp120, thereby forming a stable trimer of heterodimers, the metastable Env
61 prefusion conformation^{5,6}. Orchestration of a series of conformational changes transforms energy-
62 rich prefusion Env into the low-energy, highly stable gp41 post-fusion conformation, which
63 provides the free energy to overcome the kinetic barriers associated with bringing two opposing
64 membranes into close enough contact to facilitate membrane fusion^{2,3}.

65 HIV-1 gp41 is composed of several functional segments that have been shown or suggested
66 to extensively refold upon fusion activation: the N-terminal fusion peptide (FP), a fusion peptide
67 proximal region (FPPR), the heptad repeat region 1 (HR1), a loop region followed by HR2, the
68 membrane proximal external region (MPER), the transmembrane region (TMR), and a cytoplasmic
69 domain. Structures of native Env trimers in complex with different broadly neutralizing antibodies
70 revealed the conformation of the gp41 ectodomain lacking MPER in the native prefusion
71 conformation⁷⁻¹². Env interaction with CD4 results in opening of the closed prefusion trimer^{13,14},

72 which includes the displacement of gp120 variable regions 1 and 2 (V1-V2) at the apex of the trimer
73 but no changes in gp41¹⁵. This is required for the formation of a stable ternary complex of Env-
74 CD4 with the co-receptor¹⁶⁻¹⁸. Co-receptor binding positions prefusion gp41 closer to the host-cell
75 membrane⁵ and induces a cascade of conformational changes in gp41. First, the fusion peptide is
76 repositioned by $\sim 70 \text{ \AA}$ ⁹ to interact with the target cell membrane, generating a 110 \AA extended
77 fusion-intermediate conformation^{19,20} that bridges the viral and the host cell membrane²¹.
78 Subsequent refolding of HR2 onto HR1 leads to the formation of the six-helix bundle core structure
79²²⁻²⁴, which pulls the viral membrane into close apposition to the host-cell membrane and, thus, sets
80 the stage for membrane fusion²².

81 Membrane fusion generates a lipid intermediate hemifusion state, that is predicted to break
82 and evolve to fusion pore opening²⁵, which is regulated by six-helical bundle formation^{26,27}.
83 Furthermore residues within FPPR, FP, MPER and TM have been as well implicated in fusion²⁸⁻³²
84 indicating that final steps in fusion are controlled by the conformational transitions of the membrane
85 anchors into the final post-fusion conformation.

86 Here, we set out to understand the conformational transitions of the gp41 membrane
87 anchors. We show that the presence of the membrane anchors increases thermostability. However,
88 complex formation with a MPER-specific neutralizing nanobody induced an asymmetric
89 conformation of the membrane anchors, which constitutes a late fusion intermediate. We show that
90 this conformation can be targeted by MPER bnAbs consistent with the possibility that MPER-
91 specific nAbs can interfere all along the fusion process until a late stage. Starting from the
92 asymmetric conformation, we used MD simulation based modelling to generate the final post-
93 fusion conformation, which reveals a tight helical interaction of FP and TM in the membrane
94 consistent with its high thermostability. Our work, thus, elucidates the structural transitions of the
95 membrane anchors that are essential for membrane fusion, which can be blocked by MPER-specific
96 bnAbs up to a late stage in fusion.

97

98 **Results**

99

100 **Gp41FP-TM interaction with 2H10.**

101 Two gp41 constructs, one containing residues 512 to 581 comprising FP, FPPR and HR1 (N-
102 terminal chain, chain N) and one coding for residues 629 to 715 including HR2, MPER and TM (C-
103 terminal chain, chain C)(**Fig. S1A**) were expressed separately, purified and assembled into the
104 monodisperse trimeric complex gp41FP-TM (**Fig. S1B**). Gp41FP-TM reveals a thermostability of
105 $> 95^\circ\text{C}$ as measured by circular dichroism (**Fig. S2A**) indicating that the presence of FP and TMR

106 increases the thermostability by $> 7^{\circ}\text{C}$ compared to gp41 lacking FP and TM³³. In order to facilitate
107 crystallization, gp41FP-TM was complexed with the llama nanobody 2H10³⁴ in β -OG buffer and
108 purified by size exclusion chromatography (SEC)(**Fig. S1C**). To determine the stoichiometry of
109 binding, we performed isothermal titration calorimetry (ITC), which indicated that gp41FP-TM and
110 2H10 form a 3:1 complex with a K_D of $2.1 \pm 0.9 \mu\text{M}$ (**Fig. S2B**). Interaction of gp41FP-TM with
111 2H10 was further confirmed by biolayer interferometry (BLI) analysis (**Fig. S2C**).

112

113 **Crystal structure of gp41 in complex with 2H10**

114 The structure of gp41FP-TM in complex with 2H10 was solved by molecular replacement to a
115 resolution of 3.8 \AA (**Table S1**). The asymmetric unit contained trimeric gp41FP-TM bound to one
116 2H10 nanobody as indicated by ITC (**Fig. S2B**). The six-helix bundle structure composed of three
117 N-terminal and three C-terminal chains is conserved from HR1 residue A541 to HR2 residue L661
118 in all three protomers, and identical to previous structures^{22,23}. However, TMR and FP do not follow
119 the three-fold symmetry and their chains point into opposite directions (**Fig. 1A**). 2H10 interacts
120 with chain C-A (**Fig. 1A and B**) and induces a partially extended MPER conformation, including
121 a kink at L669 that positions the rest of MPER and TM (N674 to V693) at a 45° angle with respect
122 to the six-helix threefold symmetry axis. The corresponding N-terminal chain A (chain N-A) has
123 its FP disordered and FPPR from G527 to A533 is flexible, while the remaining FPPR and HR1
124 form a continuous helix (**Fig. 1C**). The chain C-A 2H10 epitope spans from residues Q658 to N671,
125 which is involved in a series of polar contacts with 2H10. These include interactions of gp41FP-
126 TM E662 to 2H10 Y37, S668 and the carbonyl of D664 to R56, K665 to E95, N671 and the
127 carbonyl of A667 to R54, K655 to R97 and R93 contacts E95 to position it for interaction with
128 K665 (**Fig. 1B**). Notably, mutations of R56, R93, E95 and R97 have been shown to affect
129 interaction³⁴. Chain N-B of the second protomer forms a long continuous helix comprising FP,
130 FPPR and HR1 from residues L518 to D589 with the first six residues of FP being disordered.
131 Likewise, chain C-B folds into a continuous helix from M629 to A700 comprising HR2, MPER
132 and TM (**Fig. 1D**). $C\alpha$ superimposition of chain C-B with MPER containing gp41 structures^{33,35}
133 yields root mean-square deviations of 0.55 \AA and 0.29 \AA (**Fig. S3**), indicating that the straight
134 helical conformation is the preferred conformation in threefold symmetrical gp41. In the third
135 protomer, chain N-C has a helical FP linked by flexible residues G531 to A533 to a short helix of
136 FPPR that bends at A541 with respect to helical HR1. Its corresponding chain C-C contains helical
137 HR2 and a flexible region from N671 to N674, which stabilizes a $\sim 45^{\circ}$ rotation of the remaining
138 MPER-TM helix that extends to residue R707 (**Fig. 1E**). Thus, the structure reveals flexible regions
139 within FPPR and MPER. FPPR flexibility is supported by strictly conserved G528 and G531, while

140 MPER has no conserved glycine residues. However, the same kink within L661 to F673 has been
141 observed in the MPER peptide structure ³⁶, and in complex with bnAb 10E8 ³⁷. The N-terminal FP
142 residues 512 to 517 are disordered within the detergent micelle. Flexibility of this region in the
143 absence of membrane is consistent with NMR peptide structures that propose a flexible coil
144 structure of the N-terminal part of FP in solution followed by a helical region starting at L518 ³⁸ as
145 observed here. Based on the flexible linkage of FP and TMR, we propose that both FPPR and
146 MPER act as hinges during gp41 refolding leading to membrane fusion.

148 **MD simulation of gp41FP-TM in a lipid bilayer**

149 In order to test whether the structure is influenced by the presence of the detergent, we probed its
150 stability by MD simulation in a bilayer having the lipid composition of the HIV-1 envelope. This
151 confirmed that the structure is stable in a membrane environment during a 1 μ s simulation as only
152 the flexibly linked FP of chain N-C moves within the bilayer during the simulation (**Fig. S4A and**
153 **B**). The tip of the 2H10 CDR3 dips into the bilayer (**Fig. S4B**), hence confirming the membrane-
154 anchoring role of W100 for neutralization ³⁴.

156 **Neutralization activity of 2H10 depends on membrane interaction**

157 The structure suggests that 2H10 induced the asymmetry within the membrane anchors. Crystal
158 packing effects on the conformation can be excluded, because only the C-terminus of the chain C-
159 C helix is involved in crystal lattice contacts (**Fig. S5**). We therefore further evaluated 2H10 as a
160 neutralizing nanobody, which showed modest neutralization as a bi-head (bi-2H10), whereas
161 neutralization depended on W100 located at the tip of CDR3 ³⁴, a hall mark of MPER-specific
162 bnAbs ³⁹. In order to improve the breadth and potency of monovalent 2H10, we increased its
163 membrane interaction capacity by changing CDR3 S100d to F (2H10-F) alone and in combination
164 with additional basic residues S27R, S30K and S74R (2H10-RKRF) within the putative 2H10
165 membrane-binding interface suggested by MD simulation (**Fig. S4C**). Wild type 2H10 did not show
166 significant neutralization against a panel of 10 clade B pseudo-viruses as shown previously ³⁴, with
167 the exception of some weak neutralization of NL4-3 and SF163P3. However, both 2H10-F and
168 2H10-RKRF show improved potency and breadth neutralizing six and eight pseudo-viruses,
169 respectively, albeit with less potency than wild-type bi-2H10 and bnAb 2F5, the latter recognizing
170 an overlapping epitope (**Table 1**). This result, thus, confirms monovalent 2H10 as a modest anti-
171 MPER Ab that neutralizes by engaging MPER and the membrane.

173 **2H10 blocks fusion before the stage of lipid mixing**

174 The efficacy of bi-2H10 and 2H10-RKRF for blocking membrane merging was further assessed in
175 peptide-induced lipid-mixing assays, whereas a vesicle population is primed for fusion by addition
176 of the N-MPER peptide containing the 2H10 epitope, which produces a fluorescence intensity spark
177 at time 20 s (**Fig. 2A**)⁴⁰. Under these experimental conditions, incorporation of the peptide into the
178 vesicles takes less than 10 sec⁴⁰. After 120 sec, the mixture was supplemented with target vesicles
179 fluorescently labeled with N-NBD-PE/N-Rh-PE (indicated by the arrow in **Fig. 2A**). The increase
180 in NBD intensity as a function of time followed the mixing of the target vesicle lipids with those of
181 the unlabeled vesicles (kinetic trace labeled '+N-MPER'). The increase in NBD fluorescence was
182 not observed when labeled target vesicles were injected in a cuvette containing unlabeled vesicles
183 not primed with peptide ('no peptide' trace). Lipid mixing was strongly attenuated when the
184 vesicles primed for fusion with N-MPER were incubated with bi-2H10 before addition of the target
185 vesicles (**Fig. 2A**, +N-MPER/+bi-2H10, dotted trace). Thus, the N-MPER-induced membrane
186 perturbations, which can induce fusion with target membranes, were inhibited by incubation with
187 bi-2H10. Comparison of the kinetics of the lipid-mixing blocking effect of 2H10-RKRF, bi-2H10
188 and the 2F5 Fab showed that the three antibodies inhibited both the initial rates and final extents of
189 lipid mixing induced by N-MPER (**Fig. 2B**). Using a control MPER peptide lacking the 2H10 and
190 2F5 epitopes for vesicle priming no inhibition of lipid mixing by 2H10-RKRF, bi-2H10 and 2F5
191 Fab was observed (**Fig. 2C**), indicating that the inhibitory effects depend on epitope recognition.
192 Fusion inhibition levels estimated as a function of the antibody concentration further confirmed the
193 apparent higher potency exhibited by the bi-2H10 (**Fig. 2D**). Lower concentrations of bi-2H10
194 compared to 2H10-RKRF were required to attain full blocking of the lipid-mixing process when
195 measured 20 sec (initial rates) or 300 sec (final extents) after target-vesicle injection (**Fig. 2D and**
196 **E**). The higher inhibitory potency of bi-2H10 indicates an avidity effect, which was also evident
197 when the concentration of the epitope-binding fragments was plotted (**Fig. 2D and E**, empty
198 squares and dotted line). Moreover, bi-2H10 appeared to block efficiently the process even at
199 2H10:N-MPER ratios below 1:3 (mol:mol), consistent with the involvement of peptide oligomers
200 in the promotion of membrane fusion. Based on these data, we suggest that both 2H10-RKRF and
201 bi-2H10 neutralize HIV-1 at the stage of lipid mixing.

202

203 **GP41FP-TM interaction with MPER bnAbs**

204 Although the 2H10 epitope overlaps with the 2F5 MPER epitope⁴¹, the 2F5-bound peptide
205 structure⁴¹, cannot be superimposed without major clashes with adjacent gp41 protomers. In
206 contrast, Ca superposition of the structures of 10E8 and LN01 in complex with MPER peptides
207 demonstrated possible binding to gp41FP-TM chain C-C (**Fig. 3A and B**). Furthermore, HCDR3

208 of both 10E8 and LN01 could make additional hydrophobic contacts with adjacent FP in this
209 binding mode. To confirm 10E8 and LN01 interaction, we performed immunoprecipitation of
210 gp41FP-TM with both bnAbs, which confirmed their interaction *in vitro* (**Fig. S2D**). We next
211 validated binding by bio-layer interferometry (BLI) using gp41FP-TM as analyte. This revealed
212 K_{DS} of 0,2 nM for 10E8 and 34 nM for LN01 (**Fig. 3C and D**). We conclude that bnAbs 10E8 and
213 LN01 interact with gp41FP-TM with high affinity likely by inducing and stabilizing an asymmetric
214 gp41 conformation similar to the one observed in complex with 2H10 as suggested by the structural
215 modeling (**Fig. 3 and B**).

216

217 **Building a post fusion conformation by MD simulation**

218 In order to follow the final refolding of the membrane anchors we modeled the post fusion
219 conformation employing MD simulation. Assuming that the final post-fusion conformation shows
220 a straight symmetric rod-like structure we constructed a model of gp41 from the protomer
221 composed of the straight helical chains N-B and C-B (**Fig. S6A and B**). This conformation is also
222 present in the symmetric six-helix bundle structures containing either MPER³⁵ or FPPR and MPER
223³³ (**Fig. S3**). In this model, FP and TM do not interact tightly (**Fig. S6B**), which, however, does not
224 explain the increased thermostability induced by FP and TM (**Fig. S2A**). 1- μ s MD simulation of
225 this model (**Fig. S6B**) in solution, rearranges the membrane anchors such that they adopt a compact
226 structure with trimeric FP interacting with adjacent TMs. Furthermore, the TMs kink at the
227 conserved Gly positions 690 and 691, as observed previously⁴² (**Fig. S6C**). In order to recapitulate
228 the stability of the model in the membrane, we performed an additional 1- μ s MD simulation of the
229 model (**Fig. S6C**) in a bilayer resembling the HIV-1 lipid composition, which relaxed the TM to its
230 straight conformation (**Fig. 4A**). The final structural model reveals tight packing of trimeric FP
231 flexibly linked to HR1 by FPPR G525 to G527 (**Fig. 4B**). HR2-MPER and TMR form continuous
232 helices with the TMRs packing against trimeric FP (**Fig. 4A and C**), which spans one monolayer
233 (**Fig. 4A**). As conserved tryptophan residues within MPER have been previously implicated in
234 fusion^{28,30}, we analyzed their structural role in the post fusion model. This reveals that the indole
235 ring of W666 is sandwiched between Leu669 and T536 and packs against L537. W670 makes a
236 coiled-coil interaction with S534, while W672 is partially exposed and packs against L669 and
237 T676. W678 binds into a hydrophobic pocket defined by I675, L679, I682 and adjacent FP/FPPR
238 residues F522 and A526. W680 is partially exposed, but reaches into a pocket created by the flexible
239 FPPR coil (**Fig. S7**). Thus, most of the tryptophan residues have structural roles in the post-fusion
240 conformation, hence providing an explanation for their functional role in fusion²⁸. The MPER
241 epitopes recognized by 10E8 and LN01 are exposed in the post-fusion model, but antibody docking

242 to this conformation produced major clashes, consistent with no expected binding to the final post
243 fusion conformation.

244 **Structural transitions of gp41**

245 A number of Env SOSIP structures revealed the native conformation of gp41 (**Fig. 5A and**
246 **C**)^{9,43,44}. The gp41FP-TM crystal structure and the model of its post fusion conformation provide
247 further insight into the path of conformational changes that native gp41 must undergo to adopt its
248 final lowest energy state conformation. The first major conformational changes in gp41 that take
249 place upon receptor binding are extension of HR1, FPPR and FP into a triple stranded coiled coil
250 with flexible linkers between FPPR and FP that projects FP ~115 Å away from its starting position
251 (**Fig. 5D**). Notably, such an early intermediate fusion conformation structure has been reported for
252 influenza hemagglutinin (HA)⁴⁵. This is likely followed by an extension and rearrangement of HR2
253 and MPER producing 11-15-nm long intermediates that connect the viral and cellular membranes
254 ^{20,46}. Gp41 refolding into the six-helix bundle structure then produces flexibly linked asymmetric
255 conformations of FPPR-FP and MPER-TMR anchored in the cellular and viral membranes,
256 respectively, as indicated by the gp41FP-TM structure. This intermediate conformation may bring
257 viral and cellular membranes into close proximity (**Fig. 5E**) or may act at the subsequent stage of
258 hemifusion (**Fig. 5F**). Further refolding and interaction of FP-FPPR and MPER-TM will generate
259 the stable post fusion conformation (**Fig. 5G**), a process that completes membrane fusion.

260 **Discussion**

261 Membrane fusion is an essential step of infection for enveloped viruses such as HIV-1, and requires
262 extensive conformational rearrangements of the Env prefusion conformation⁷⁻⁹ into the final
263 inactive post-fusion conformation^{2,3}. The fusion model predicts that six-helix bundle formation
264 apposes viral and cellular membranes with FP and TM inserted asymmetrically in the cellular
265 membrane and the viral membrane, respectively²¹. Here, we show that gp41 containing its
266 membrane anchors can adopt this predicted conformation, which is facilitated by flexible hinges
267 present in FPPR and MPER, thus corroborating their essential roles in membrane fusion^{3,5,47}. The
268 asymmetric conformation of the membrane anchors suggest further that bundle formation occurs
269 before pore formation as suggested previously^{26,27}. The membrane-fusion model proposes further
270 that final steps in fusion involves rearrangement and interaction of TM and FP²¹, which is
271 confirmed by the MD-simulation model of the post-fusion conformation. Furthermore, the length
272 of the rod-like post-fusion structure of 13 nm lacking the C-C loop is consistent with the gp41
273 structure lacking FP and TM⁴⁸.

276 FP is helical in the gp41FP-TM-2H10 complex and the MD-based post-fusion
277 conformation, in agreement with NMR-based helical FP peptide models^{49,50}, although β -strand
278 structures of FP have been implicated in fusion as well⁵¹. In comparison, in native Env
279 conformations, FP adopts multiple dynamic conformations that are recognized by broadly
280 neutralizing antibodies^{43,44,52,53}. In the post-fusion conformation, FP spans one monolayer of the
281 membrane, in contrast to suggested amphipathic helix-like interaction of FP with the outer layer of
282 the target cell membrane^{54,55}.

283 Furthermore, the coiled-coil interactions within FP and with TM in the post fusion model
284 explain the increased thermostability of gp41FP-TM compared to gp41 lacking FP and TM³³. We
285 propose that refolding of FP and TM can liberate additional free energy to catalyze final steps of
286 fusion. Hence, replacement of TM by a phosphatidylinositol (PI) anchor inhibits membrane fusion
287^{56,57}, akin to the GPI-anchored HA inhibition of influenza virus membrane fusion at the stage of
288 hemifusion⁵⁸.

289 Mutations in MPER and FPPR interfere with fusion^{28,30,59,60}, and mutations in TM block
290 fusion³¹ or reduce fusion efficiency³². Our structural model of the post-fusion conformation
291 predicts that most of these mutations affect the final post-fusion conformation, in agreement with
292 proposed interactions of FPPR and MPER, as well as FP and TM^{61,62}, thereby corroborating their
293 essential roles at late stages of membrane fusion.

294 Gp41FP-TM interaction with the 2H10 MPER-specific nanobody induces the asymmetric
295 conformation of the membrane anchors. In order to confirm that 2H10 is, indeed, a neutralizing
296 MPER-specific nanobody, we engineered increased 2H10 membrane binding, which improved
297 breadth and potency of 2H10 neutralization, in agreement with enlarged potency by increasing
298 membrane-binding of 10E8⁶³⁻⁶⁵. This result, thus confirmed 2H10 as a modest anti-MPER
299 neutralizing antibody that recognizes both its linear epitope and membrane³⁴. Consistent with its
300 neutralization capacity, 2H10 inhibits membrane fusion at the stage of lipid mixing like 2F5 and
301 other anti-MPER bnAbs^{40,66}. Moreover, gp41FP-TM interacts with MPER bnAbs 10E8³⁷ and
302 LN01⁴² in agreement with docking both structures onto the kinked chain C-C MPER epitope.
303 Notably, the kink in the MPER peptide in complex with 10E8³⁷ is similar to the chain C-C kink
304 and present in MPER peptide NMR structures^{36,50}. Furthermore, Ala mutations in the kink (671-
305 674) affect cell-cell fusion and lower virus infectivity⁶⁷ corroborating the physiological relevance
306 of the kinked conformation. We therefore propose that 10E8 and LN01 binding to gp41FP-TM
307 induces similar asymmetry, as observed in the gp41FP-TM-2H10 structure by sampling the
308 dynamic states of the membrane anchors.

309 Our data, thus, indicate that MPER antibodies can act all along the gp41 refolding pathway
310 from blocking initial conformations of close to native Env⁶⁸⁻⁷⁰ up to a late fusion intermediate state
311 that has already pulled viral and cellular membranes into close apposition. This thus, opens a long
312 temporal window of action for MPER bnAbs consistent with the findings that the half-life of
313 neutralization of MPER bnAbs is up to 30 minutes post virus exposure to target cells^{71,72}.
314 Furthermore, only one Ab per trimer may suffice to block final refolding of the membrane anchors
315 required for fusion. Finally, the presence of dynamic linkers connecting the core of viral fusion
316 proteins with their membrane anchors FP and TM must be a general feature of all viral membrane
317 fusion proteins.

318

319 **Materials and Methods**

320

321 **Cell Lines**

322 TZM-bl cells were obtained from NIH-AIDS Research and Reference Reagent Program (ARRRP)
323 and used for neutralization assays. TZM-bl cells were maintained in Dulbecco's modified Eagle's
324 medium supplemented with 10% fetal bovine serum, 100 units of Penicillin and 0.1 mg/ml of
325 Streptomycin while TZM-bl expressing the FcγRI cells were maintained in Dulbecco's modified
326 Eagle's medium supplemented with 10% fetal bovine serum, 0.025M HEPES, 50 μg/ml of
327 Gentamicin, 20 μg/ml of Blasticidin.

328

329 **HIV-1 Primary Viruses**

330 Env-pseudotyped viruses were prepared by co-transfection of HEK 293-T cells with plasmids
331 encoding the respective *env* genes and the luciferase reporter HIV vector pNLluc-AM as described
332⁷³. A full list of Env pseudotyped viruses generated with corresponding gene bank entry, subtype
333 and Tier information is provided in Table S2.

334

335 **GP41 expression and purification**

336 DNA fragments encoding HIV-1 Env glycoprotein amino acids 512 to 581 (N-terminal chain, chain
337 N) and residues 629 to 715 (C-terminal chain, chain C) were cloned into vectors pETM20 and
338 pETM11 (PEPcore facility-EMBL), respectively. Both constructs contain an N-terminal Flag-tag
339 (DDDDK sequence) and chain C contains additional two C-terminal arginine residues (Fig. S1A).
340 Proteins were expressed separately in *E. coli* strain C41(DE3)(Lucigen). Bacteria were grown at
341 37°C to an OD_{600nm} of 0.9. Cultures were induced with 1mM IPTG at 37°C for 3h for gp41 chain
342 N and at 25°C for 20h for gp41 chain C. Cells were lysed by sonication in buffer A containing 20

343 mM Tris pH 8, 100 mM NaCl and 1% CHAPS (3-[(3-cholamidopropyl) diméthylammonio]-1-
344 propanesulfonate (Euromedex). The supernatant was cleared by centrifugation at 53 000 g for 30
345 min. Gp41 chain N supernatant was loaded on a Ni²⁺-sepharose column, washed successively with
346 Buffer A containing 1M NaCl and 1M KCl, then Buffer A containing 50 mM imidazole. Gp41
347 chain N was eluted in Buffer A containing 500 mM imidazole. Gp41 chain C was purified
348 employing the same protocol as for gp41 chain N. Gp41 chain N was subsequently cleaved with
349 TEV (Tobacco Etch Virus) protease for 2h at 20°C and then overnight at 4°C. After buffer exchange
350 with a mono Q column using buffer B (Buffer A with 0,5 M NaCl), uncleaved material and cleaved
351 His-tags were removed by a second Ni²⁺-sepharose column in buffer A. TEV-cleaved gp41 chain
352 C and chain N were then mixed in a molar ratio 4:1 and incubated overnight. To remove the excess
353 of gp41 chain C, the gp41 complex was loaded on a 3rd Ni²⁺-sepharose column in buffer A, washed
354 with buffer A containing 50 mM imidazole and eluted with buffer A containing 500 mM imidazole.
355 Subsequently the gp41 chain N TrxA-His-tag was removed by TEV digestion for 2h at 20°C and
356 overnight at 4°C. After buffer exchange with a mono Q column in buffer B uncleaved complex and
357 the TrxA-His-tag fusion were removed by a 4th Ni²⁺-sepharose column. The final gp41FP-TM
358 complex was concentrated and loaded onto a Superdex 200 size exclusion column (SEC) in buffer
359 C containing 20 mM Tris pH 8,0, 100 mM NaCl and 1% n-octyl β-D-glucopyranoside (Anatrace).

360

361 **Nanobody 2H10 expression**

362 2H10 encoding DNA was cloned into the vector pAX51³⁴ and expressed in the *E. coli* BL21(DE3)
363 strain (Invitrogen). Bacteria were grown at 37°C to an OD_{600nm} of 0,7 and induced with 1mM IPTG
364 at 20°C for 20h. After harvesting by centrifugation, bacteria were resuspended in lysis buffer
365 containing 20 mM Hepes pH 7,5 and 100 mM NaCl. Bacteria were lysed by sonication and
366 centrifuged at 48 000g for 30 min. Cleared supernatant was loaded on Protein A sepharose column,
367 washed with lysis buffer and eluted with 0,1 M glycine pH 2,9. Eluted fractions were immediately
368 mixed with 1/5 volume of 1M Tris pH 9,0. 2H10 was then further purified by SEC on a superdex
369 75 column in PBS buffer. Mutants of 2H10, 2H10-F (S100d) and 2H10-RKRF (S27R, S30K, S74R
370 and S100d) were synthesized (Biomatik) and purified as described for the wild type. The 2H10 bi-
371 head was purified as described³⁴.

372

373 **Circular dichroism**

374 CD measurements were performed using a JASCO Spectropolarimeter equipped with a
375 thermoelectric temperature controller. Spectra of gp41-TM were recorded at 20 °C in 1 nm steps
376 from 190 to 260 nm in a buffer containing PBS supplemented with 1% n-octyl β-D-

377 glucopyranoside. For thermal denaturation experiments, the ellipticity was recorded at 222 nm with
378 1°C steps from 20° to 95°C with an increment of 80°C h⁻¹, and an averaging time of 30 s/step. For
379 data analysis, raw ellipticity values recorded at 222 nm were converted to mean residue ellipticity.

381 **Isothermal Titration Calorimetry (ITC)**

382 The stoichiometry and binding constants of 2H10 binding to gp41 FP-TM was measured by ITC200
383 (MicroCal Inc.). All samples used in the ITC experiments were purified by SEC in a buffer
384 containing 20 mM Tris pH 8.0, 100 mM NaCl and 1 % n-octyl β-D glucopyranoside and used
385 without further concentration. Samples and were equilibrated at 25 °C before the start of the
386 experiment. The ITC measurements were performed at 25 °C by making 20 2-μl injections of 267
387 μM 2H10 to 0.2 ml of 19.5 μM gp41FP-TM. Curve fitting was performed with MicroCal Origin
388 software. Three experiments were performed, with an average stoichiometry N = 1.1 +/- 0.2 2H10
389 binds to gp41FP-TM with a KD of 2.1 μM +/- 0.9.

391 **Bio-layer Interferometry Binding Analysis**

392 Binding measurements between antibodies (10E8 IgG, LN01 IgG and 2H10) were carried out on
393 an Octet Red instrument (ForteBio). For the determination of the binding between antibodies and
394 gp41FP-TM, 10E8 IgG or LN01 IgG or 2H10 were labelled with biotin (EZ-Link NHS-PEG4-
395 Biotin) and bound to Streptavidin (SA) biosensors (ForteBio). The biosensors loaded with the
396 antibodies were equilibrated in the kinetic buffer (20 mM Tris pH 8.0, 100 mM NaCl and 1 % n-
397 octyl β-D glucopyranoside) for 200-500 sec prior to measuring association with different
398 concentrations of gp41FP- for 100-200 seconds at 25 °C. Data were analyzed using the ForteBio
399 analysis software version 11.1.0.25 (ForteBio). For 10E8 the kinetic parameters were calculated
400 using a global fit 1:1 model and 2:1 model. For the determination of the binding of LN01 IgG and
401 2H10, KDs were estimated by steady state analysis. All bio-layer interferometry experiments were
402 conducted at least three times.

404 **Immunoprecipitation of gp41FP-TM by bnAbs 10E8 and LN01**

405 220 μg of Gp41FP-TM were incubated alone or with 50μg of 10E8 or LN01 antibodies for 10 h at
406 20°C in buffer C. The complex was loaded on Protein A sepharose affinity resin and incubated for
407 1h. The resin was subsequently washed 3 times with buffer C and eluted with SDS gel loading
408 buffer and boiling at 95°C for 5 min. Samples were separated on a 15% SDS-PAGE and stained
409 with Coomassie brilliant blue.

411 **Neutralization assay**

412 The neutralization activity of the 2H10 variants and mAbs was evaluated using TZM-bl cells and
413 Env pseudotyped viruses as described⁷³. Briefly, serial dilutions of inhibitor were prepared in cell
414 culture medium (DMEM with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml
415 streptomycin (all from Gibco)) and added at a 1:1 volume ratio to pseudovirus suspension in 384
416 well plates (aiming for 500'000–5'000'000 relative light units (RLU) per well in the absence of
417 inhibitors). After one-hour incubation at 37°C, 30 µl of virus-inhibitor mixture was transferred to
418 TZM-bl cells in 384 well plates (6000 cells/well in 30µl cell culture medium supplemented with
419 20µg/ml DEAE-Dextran seeded the previous day). The plates were further incubated for 48 hours
420 at 37°C before readout of luciferase reporter gene expression on a Perkin Elmer EnVision
421 Multilabel Reader using the Bright-Glo Luciferase Assay System (Promega).

422 The inhibitor concentration (referring to the mix with cells, virus and inhibitor) causing 50%
423 reduction in luciferase signal with respect to a reference well without inhibitor (inhibitory
424 concentration IC50) was calculated by fitting a non-linear regression curve (variable slope) to data
425 from two independent experiments using Prism (GraphPad Software). If 50% inhibition was not
426 achieved at the highest inhibitor concentration tested, a greater than value was recorded. To control
427 for unspecific effects all inhibitors were tested for activity against MuLV envelope pseudotyped
428 virus.

430 **Fusion assay.**

431 The peptides used in the fusion inhibition experiments, NEQELLELDKWASLW
432 NWFNITNWLWYIK (N-MPER) and KKK-NWFDITNWLWYIKLFIMIVGGLV-KK (C-MPER),
433 were synthesized in C-terminal carboxamide form by solid-phase methods using Fmoc chemistry,
434 purified by reverse phase HPLC, and characterized by matrix-assisted time-of-flight (MALDI-
435 TOF) mass spectrometry (purity >95%). Peptides were routinely dissolved in dimethylsulfoxide
436 (DMSO, spectroscopy grade) and their concentration determined by the Biscinchoninic Acid
437 microassay (Pierce, Rockford, IL, USA).

438 Large unilamellar vesicles (LUV) were prepared following the extrusion method of Hope et al.⁷⁴.
439 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and cholesterol (Chol) (Avanti Polar Lipids,
440 Birmingham, AL, USA) were mixed in chloroform at a 2:1 mol:mol ratio and dried under a N₂
441 stream. Traces of organic solvent were removed by vacuum pumping. Subsequently, the dried lipid
442 films were dispersed in 5 mM Hepes and 100 mM NaCl (pH 7.4) buffer, and subjected to 10 freeze-
443 thaw cycles prior to extrusion 10 times through 2 stacked polycarbonate membranes (Nuclepore,
444 Inc., Pleasanton, CA, USA). Lipid mixing with fusion-committed vesicles was monitored based on

445 the resonance energy transfer assay described by Struck et al.⁷⁵, with the modifications introduced
446 by Apellaniz et al.⁴⁰. The assay is based on the dilution of co-mixed N-(7-nitro-benz-2-oxa-1,3-
447 diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and N-(lissamine Rhodamine B
448 sulfonyl)phosphatidylethanolamine (N-Rh-PE) (Molecular Probes, Eugene, OR, USA), whereby
449 dilution due to membrane mixing results in increased N-NBD-PE fluorescence. Vesicles containing
450 0.6 mol % of each probe (target vesicles) were added at 1:9 ratio to unlabeled vesicles (MPER
451 peptide-primed vesicles). The final lipid concentration in the mixture was 100 μ M. The increase in
452 NBD emission upon mixing of target-labeled and primed-unlabeled lipid bilayers was monitored
453 at 530 nm with the excitation wavelength set at 465 nm. A cutoff filter at 515 nm was used between
454 the sample and the emission monochromator to avoid scattering interferences. The fluorescence
455 scale was calibrated such that the zero level corresponded to the initial residual fluorescence of the
456 labeled vesicles and the 100 % value to complete mixing of all the lipids in the system (i.e., the
457 fluorescence intensity of vesicles containing 0.06 mol % of each probe). Fusion inhibition was
458 performed with bi-2H10, 2H10-RKRF and 2F5 Fabs at concentrations of 10 μ g/ml and 20 μ g/ml
459 as indicated.

460

461 **Crystallization, data collection and structure determination**

462 For crystallization, 1 mg of gp41FP-TM was mixed with 1.5 mg of 2H10. The complex was purified
463 by SEC on a Superdex 200 column in a buffer containing 100 mM NaCl, 20 mM Tris pH 8,0 and
464 1% n-octyl β -D-glucopyranoside and concentrated to 7-10 mg/ml. Crystal screening was performed
465 at the EMBL High Throughput Crystallization Laboratory (HTX lab, Grenoble) in 96-well sitting
466 drop vapor diffusion plates (Greiner). Following manual refinement of crystallization conditions,
467 crystals of gp41FP-TM in complex with 2H10 were grown by mixing 1 μ l of protein with 1 μ l of
468 reservoir buffer containing 0,1 M sodium citrate pH 6,0, 0,2 M ammonium sulfate, 20%
469 polyethylene glycol 2000 and 0,1 M NaCl at 20°C (293 K) in hanging drop vapor diffusion plates.
470 Before data collection, crystals were flash frozen at 100K in reservoir solution supplemented with
471 1% n-octyl β -D-glucopyranoside and 25 % ethylene glycol for cryo-protection.

472 Data were collected on the ESRF beamline ID30b at a wavelength of 0.9730 Å. Data were
473 processed with the program XDS⁷⁶. The data from two crystals were merged with Aimless⁷⁷. The
474 data set displayed strong anisotropy in its diffraction limits and was submitted to the STARANISO
475 Web server⁷⁸. The merged STARANISO protocol produced a best-resolution limit of 3.28 Å and
476 a worst-resolution limit of 4.74 Å at a cutoff level of 1.2 for the local $I_{\text{mean}} / \sigma(I_{\text{mean}})$ (note that
477 STARANISO does not employ ellipsoidal truncations coincident with the crystal axes). The
478 gp41FP-TM-2H10 crystals belong to space group C 2 2 2₁ and the structure was solved by

479 molecular replacement using the program Phaser⁷⁹ and pdb entries 1env and 4b50. The model was
480 rebuilt using COOT⁸⁰ and refined using Phenix⁸¹. Data up to 3.28 Å were initially used for model
481 building but were finally truncated to 3.8 Å. Statistics for data reduction and structure refinement
482 are presented in Table S1.

483 One copy of gp41FP-TM in complex with 2H10 are present in the asymmetric unit. Numbering of
484 the nanobody 2H10 was performed according to Kabat. The gp41FP-TM-2H10 complex was
485 refined to 3.8 Å data with an R/Rfree of 26.7 / 31.1 %. 99.6 % of the residues are within the most
486 favored and allowed regions of a Ramachandran plot⁷⁷. Some of the crystallographic software used
487 were compiled by SBGrid⁸². Atomic coordinates and structure factors of the reported crystal
488 structures have been deposited in the Protein Data Bank (<https://www.rcsb.org>; PDB: 7AEJ).

489

490 **Figure Generation**

491 Molecular graphics figures were generated with PyMOL (W. Delano; The PyMOL Molecular
492 Graphics System, Version 1.8 Schrödinger, LLC, <http://www.pymol.org>).

493

494 **Molecular Dynamics (MD) simulation**

495 **Molecular assays.** Starting from the crystal structure determined herein, we built two molecular
496 assays based (i) on the structure of the entire Gp41FP-TM/2H10 complex, and (ii) based on a gp41
497 model generated by a three-fold symmetrization of the straight helical chains N-B and C-B.
498 Electron density for FP and TM is partially absent in the crystal structure and the missing parts have
499 been built as helical extensions; FP from residue 512 to 518 and TM from residues 700 to 709 based
500 on TM structures (6SNE and 6B3U). All residues were taken in their standard protonation state.
501 The first assay included a fully hydrated membrane composed of 190 cholesterol, 40 1-palmitoyl-
502 2-oleoyl-glycero-3-phosphocholine (POPC), 88 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-
503 ethanolamine (POPE), 36 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and 56 N-
504 stearoyl sphingomyelin, present in the HIV-1 lipid envelope⁸³, using the CHARMM-GUI interface
505^{84,85}. The resulting molecular assembly consisted of about 178,000 atoms in a rhomboidal cell of
506 106 x 106 x 169 Å³. The second computational assay featured a water bath of 91 x 91 x 114 Å³,
507 representing a total of nearly 95,700 atoms. Both assays were electrically neutral, with a NaCl
508 concentration set to 150 mM.

509 **Molecular Dynamics.** All simulations were performed using the NAMD 2.14 program⁸⁶. Proteins,
510 cholesterol, lipids and ions were described using the CHARMM forcefield⁸⁷⁻⁸⁹ and the TIP3P
511 model⁹⁰ was used for water. MD trajectories were generated in the isobaric-isothermal ensemble
512 at a temperature of 300 K and a pressure of 1 atm. Pressure and temperature were kept constant

513 using the Langevin thermostat and the Langevin piston method ⁹¹, respectively. Long-range
514 electrostatic interactions were evaluated by the particle-mesh Ewald (PME) algorithm ⁹². Hydrogen
515 mass repartitioning ⁹³ was employed for all simulations, allowing for using a time step of 4 fs.
516 Integration was performed with a time step of 8 and 4 fs for long- and short-range interactions,
517 respectively, employing the r-RESPA multiple time-stepping algorithm ⁹⁴. The SHAKE/RATTLE
518 ^{95,96} was used to constrain covalent bonds involving hydrogen atoms to their experimental lengths,
519 and the SETTLE algorithm ⁹⁷ was utilized for water.

520 The computational assay formed by gp41 in an aqueous environment was simulated for a period of
521 1 μ s, following a thermalization of 40 ns. For the gp41FP-TM/2H10 complex, the lipid bilayer was
522 first thermalized during 200 ns using soft harmonic restraints on every dihedral angle of the protein
523 backbones, allowing the complex to align optimally with its membrane environment. Following the
524 equilibration step, a production run of 1 μ s was performed.

525 The final structure of the hydrated gp41 was also embedded in the HIV-1-like envelope membrane
526 employed for the gp41FP-TM/2H10 complex. The same 200 ns equilibration protocol was applied
527 followed by a production run of 1 μ s.

528

529 **References**

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531

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775 mixing experiments performed by J.T. A.T. supervised neutralization assays performed by N.F.
776 C.C. and F.D. performed molecular dynamics simulation experiments. D.G. produced proteins,
777 mutants and performed crystallization and pull-down experiments. C.Ca. performed the structural
778 studies and interaction studies. W.W. wrote the manuscript with input from all authors.

779

780 **Competing interests:** The authors declare no competing interests.

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782 **Figures and Tables**

783

784 **Table 1.** Pseudovirus neutralization by 2H10, 2H10-F, 2H10-RKRF and bi-2H10. IC50s are

785 indicated in $\mu\text{g/ml}$.

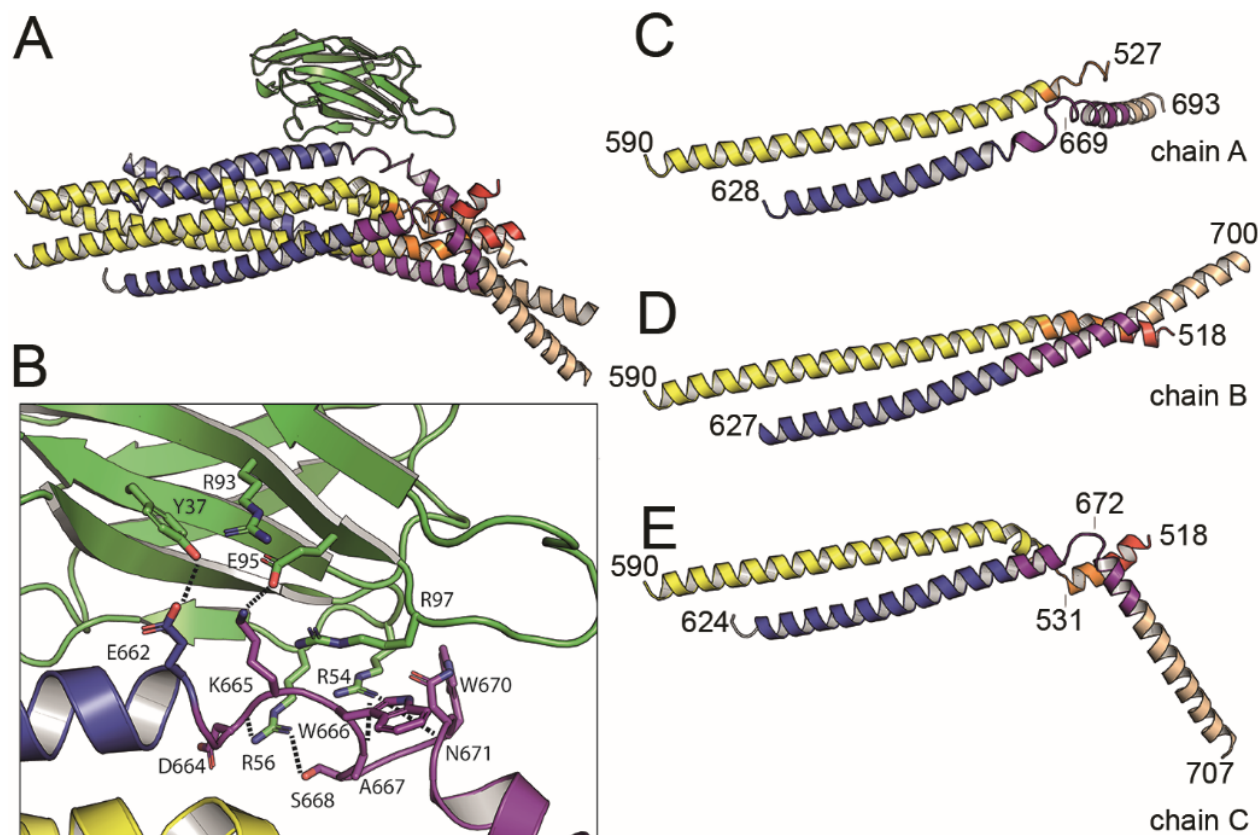
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	Tier	2H10 wt	2H10-F	2H10-RKRF	Bi-2H10	2F5	VRC01
NL4-3	1	25.20	18.68	9.15	1.84	0.16	0.20
MN-3	1	>50.00	30.38	9.36	1.39	0.03	0.06
BaL.26	1	>50.00	19.38	9.63	6.05	1.21	0.13
SF162	1a	>50.00	>50.00	25.19	6.14	1.22	0.39
SF162P3	2	22.04	13.14	6.76	1.32	1.96	0.24
SC422661.8	2	>50.00	>50.00	27.93	3.79	1.00	0.27
JR-FL	2	44.65	16.93	6.95	1.49	0.97	0.11
JR-CSF	2	>50.00	21.66	10.85	2.85	1.24	0.37
QH0692.42	2	>50.00	>50.00	>50.00	>50.00	1.20	1.21
THRO4156.18	2	>50.00	>50.00	>50.00	>50.00	>50.00	3.84

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791 **Fig. 1. Crystal structure of gp41FP-TM in complex with 2H10.**

792 **A**, Ribbon presentation of gp41TM-FP in complex with 2H10. Color-coding of the different
793 segments is as indicated in the gp41 scheme (Fig. S1A), the 2H10 nanobody is colored in green.

794 **B**, Close-up of the interaction of gp41FP-TM with 2H10. Residues in close enough contact to make
795 polar interactions are shown as sticks.

796 **C, D, E**, Ribbon diagram of the individual protomers named chain A, B and C. Residues within the
797 FPPR and MPER hinge regions are indicated.

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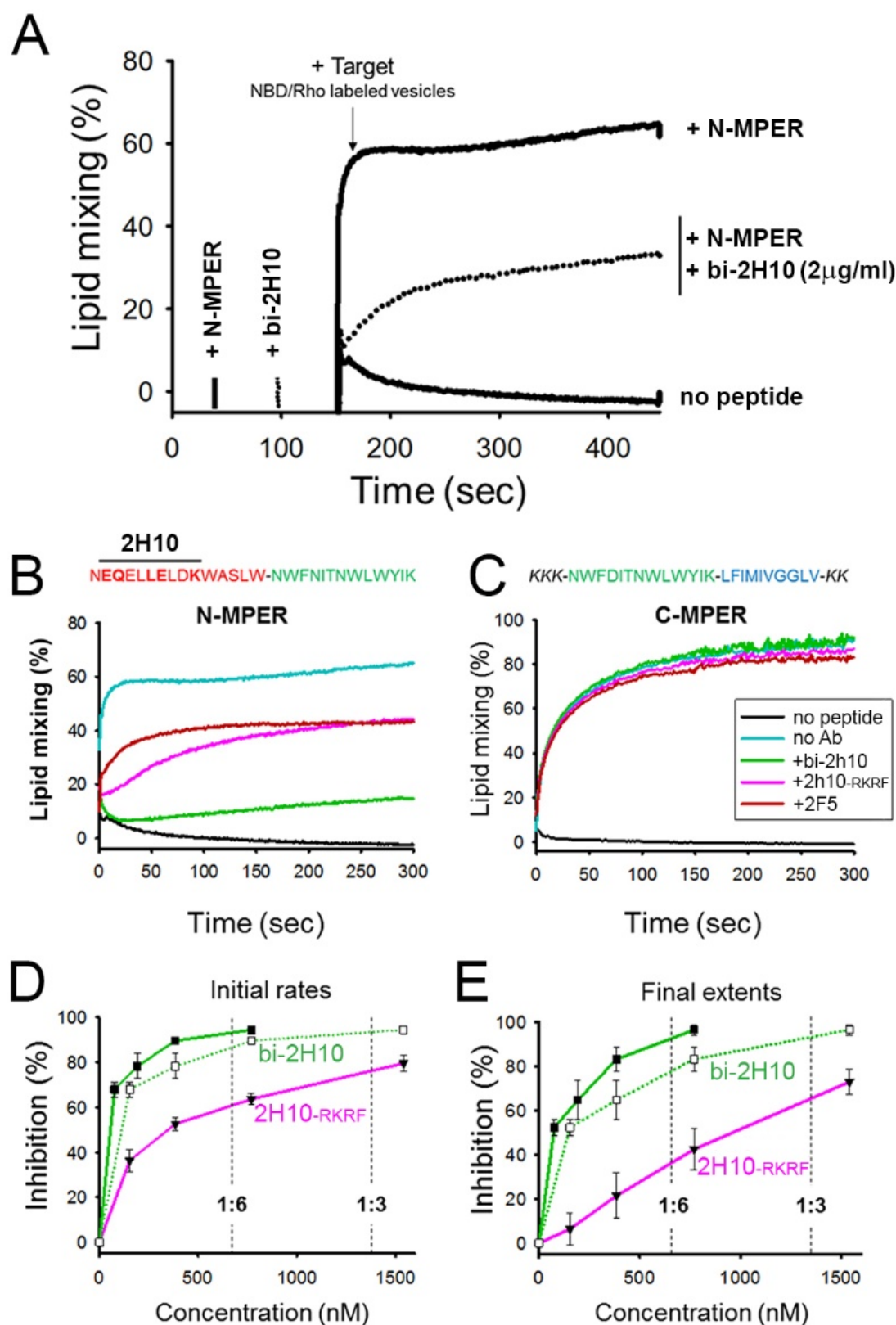


Fig. 2. Vesicle-vesicle fusion inhibition by 2H10, bi-2H10 and 2F5.

(A) Time course of the lipid-mixing assay using fusion-committed vesicles. At time 30 sec ('+N-MPER'), peptide (4 μ M) was added to a stirring solution of unlabeled vesicles (90 μ M lipid), and,

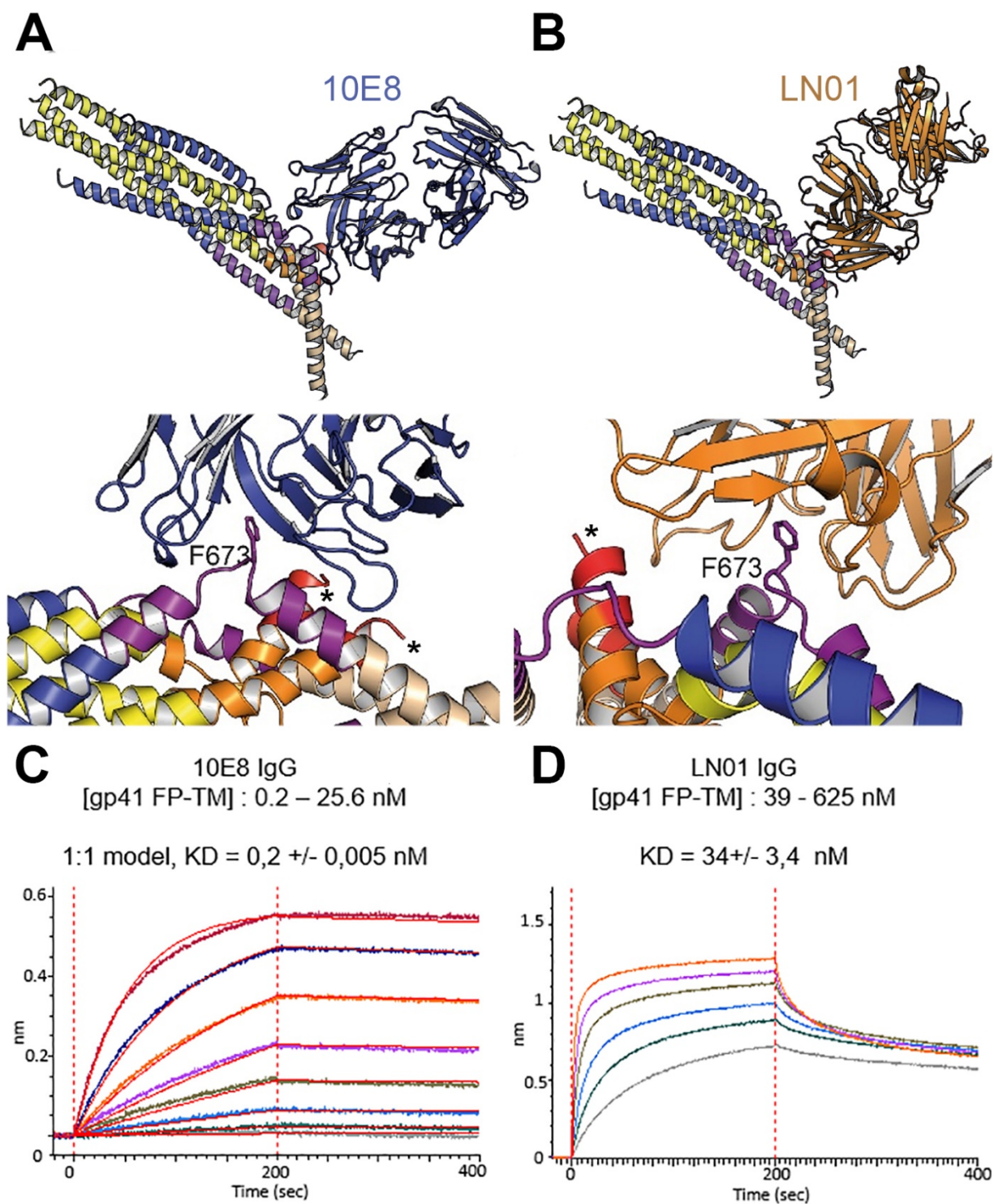
804 after 120 sec (indicated by the arrow), the mixture was supplemented with N-NBD-PE/N-Rh-PE-
805 labeled vesicles (10 μ M lipid). The increase in NBD fluorescence over time follows the dilution of
806 the probes upon mixing of lipids of target and primed vesicles (+N-MPER trace). NBD increase
807 was substantially diminished in samples incubated with bi-2H10 (2 μ g/ml) prior to the addition of
808 the target vesicles (+bi-2H10, dotted trace), and totally absent if unlabeled vesicles were devoid of
809 peptide ('no peptide' trace).

810 **(B)** Left: Kinetic traces of N-MPER-induced lipid-mixing comparing the blocking effects of 2H10-
811 RKRF, bi-2H10 and Fab 2F5.

812 **(C)** Absence of effects on lipid-mixing when vesicles were primed for fusion with the C-MPER
813 peptide, devoid of 2H10 and 2F5 epitope sequences. Antibody concentrations were 20 μ g/ml in
814 these assays.

815 **(D)** Dose-response plots comparing the inhibitory capacities of 2H10-RKRF and bi-2H10 (purple
816 and green traces, respectively). Levels of lipid-mixing 20 or 300 sec after target vesicle injection
817 were measured (initial rates **D** and final extents, **E**) and percentages of inhibition calculated as a
818 function of the Ab concentration. The dotted line and empty symbols correspond to the effect of bi-
819 2H10 when the concentration of the component 2H10 was plotted. The slashed vertical lines mark
820 the 2H10-to-peptide ratios of 1:6 and 1:3. Plotted values are means \pm SD of three independent
821 experiments.

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824 **Fig. 3. Gp41FP-TM interaction with bnAbs LN01 and 10E8**

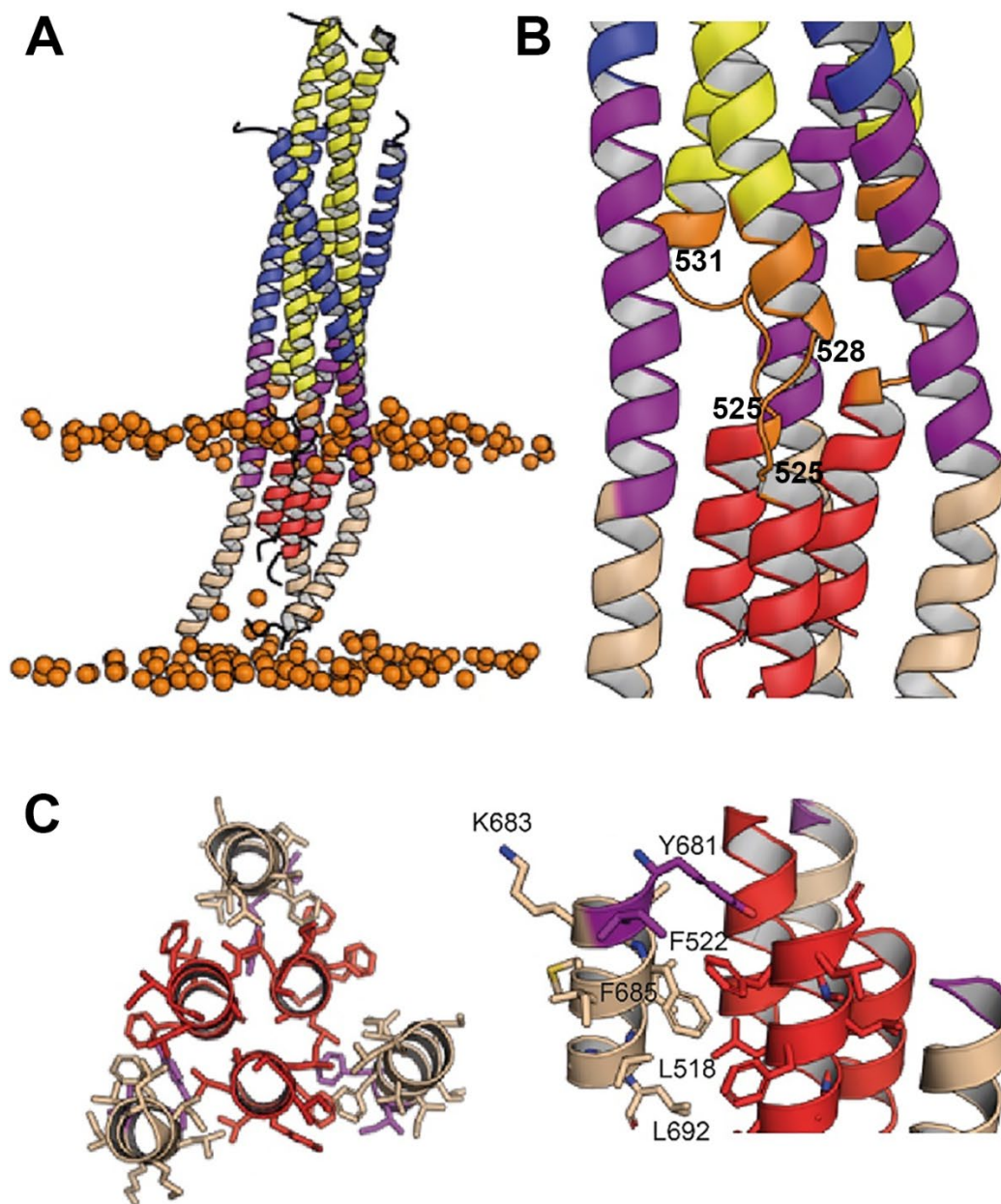
825 **A**, *Ca* superposition of the MPER peptide structure in complex with LN01 (pdb 6snd) onto chain
826 C-C of gp41FP-TM-2H10 structure. The lower panel shows a close-up of the interaction oriented
827 with respect to gp41 F673.

828 **B**, *Ca* superposition of the MPER peptide structure in complex with 10E8 (pdb 5iq7) onto the
829 corresponding chain C-C of gp41FP-TM. The lower panel shows a close-up of the interaction in
830 the same orientation as in A.

831 **C**, Bio-layer interferometry (BLI) binding of gp41FP-TM to 10E8 and **D**, to LN01. 10E8 binding
832 was fit to 1:1 model and for LN01 a steady state model was employed for fitting the data. For 10E8
833 binding, gp41FP-TM was used at concentrations from 0.2 to 25,6 nM and for LN01 binding
834 gp41FP-TM concentrations ranged from 39 to 625 nM.

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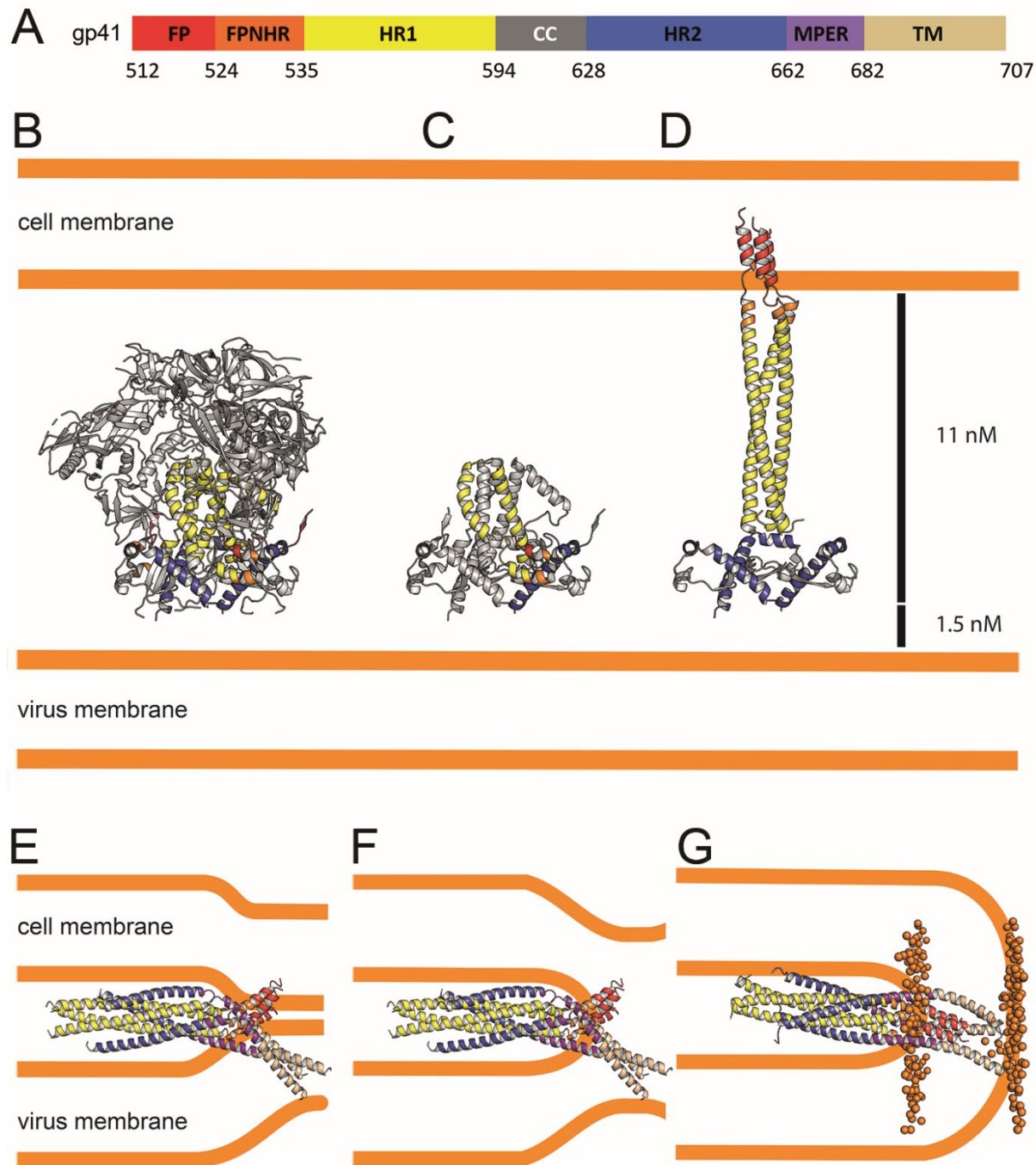
838 **Fig. 4. Interactions within the final post fusion conformation of gp41FP-TM modeled by MD.**

839 **A**, Model of gp41FP-TM (Fig. S7C) after 1 μ s MD simulation in a bilayer. Phosphate groups of the
840 phospholipids are shown as orange spheres to delineate the membrane boundaries.

841 **B**, Close up on the MPER and FPPR flexible regions.

842 **C**, Close-up of the interaction of FP (residues 514-524) and TM (residues 681-692) viewed along
843 the three-fold axis from the N-terminus indicating an intricate network of hydrophobic interactions
844 (left panel) and from the side (right panel). Interacting side chains are labeled and shown as sticks.

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Fig. 5. Conformational transitions of gp41 that lead to membrane apposition and membrane fusion.

A, Representation of the different domains of gp41 with the residue numbers delimiting each domain as indicated. The same color code has been used in all the figures.

B, Ribbon presentation of the Env prefusion conformation (pdb 5fuu), gp41 is constrained by gp120 in its native conformation. The structure of native gp41 lacks the MPER and TM regions. MPER is spanning a distance of 1.5 nm⁹⁸.

854 **C**, Ribbon of native gp41, one chain is colored according to the scheme in A and the other two chains
855 are shown in grey.

856 **D**, Binding to cellular receptors CD4 and subsequently to CXCR4/CCR5 induces a series of
857 conformational changes that eventually leads to the dissociation of gp120. During this process,
858 HR1, FPPR and FP will form a long triple stranded coiled coil extending 11 nm towards the target
859 cell membrane. In a first step HR2 may keep its prefusion conformation in analogy to a similar
860 intermediate, activated influenza virus HA structure⁴⁵. Alternatively, HR2 may dissociate and form
861 a more extended conformation in agreement with locked gp41 structures bridging viral and cellular
862 membranes that bridge distances of 11 to 15 nm⁴⁶.

863 **E**, Bending of HR1 and HR2 will result in the six-helical bundle core structure bringing cellular
864 and viral membranes into close apposition with the 3 FPs anchored in the cellular membrane and
865 the 3 TMs anchored in the viral membrane, the gp41 conformation represented by the gp41FP-TM
866 structure. This intermediate gp41 conformation may have brought both membranes into close
867 apposition or may have already induced hemifusion as indicated in F.

868 **G**, Further reordering of FPPR-FP and MPER-TM results in the final extremely stable post fusion
869 conformation. This thus suggests that rearrangement of the membrane anchors plays crucial roles in
870 lipid mixing, breaking the hemifusion diaphragm to allow fusion pore opening. Boundaries of the
871 lipid layers are shown with orange spheres representing the phosphate atoms of the lipids present
872 in the MD simulation (snapshot taken after 1 μ s MD simulation).