

2 **Profound structural conservation of chemically cross-linked HIV-1 envelope**
3 **glycoprotein experimental vaccine antigens**

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5 Gregory Martin^{1*}, Rebecca A Russell^{2*}, Philip Mundsperger^{3,4}, Scarlett Harris², Lu Jovanoska²,
6 Luiza Farache Trajano², Torben Schiffner^{2,5}, Katalin Fabian⁶, Monica Tolazzi⁷, Gabriella
7 Scarlatti⁷, Leon McFarlane⁸, Hannah Cheeseman⁸, Yoann Aldon⁸, Marielle Breemen⁹, Kwinten
8 Sliepen⁹, Dietmar Katinger³, Renate Kunert⁴, Rogier W Sanders⁹, Robin Shattock⁸, Andrew B
9 Ward¹, Quentin J Sattentau²

10
11 ¹Department of Integrative Structural and Computational Biology, IAVI Neutralizing Antibody
12 Center, Collaboration for AIDS Vaccine Discovery and Center for HIV/AIDS Vaccine
13 Immunology and Immunogen Discovery, The Scripps Research Institute, La Jolla, California,
14 USA

15 ²The Sir William Dunn School of Pathology, The University of Oxford, Oxford, UK

16 ³Polymun Scientific Immunbiologische Forschung GmbH, Klosterneuburg, Austria

17 ⁴Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna,
18 Austria

19 ⁵Current address: Department of Immunology and Microbial Science, The Scripps Research
20 Institute, La Jolla, California, USA

21 ⁶Department of Immunology, National Food Chain Safety Office, Directorate of Veterinary
22 Medicinal Products, Budapest, Hungary

23 ⁷Viral Evolution and Transmission Unit, Division of Immunology, Transplantation, and
24 Infectious Diseases, IRCCS Ospedale San Raffaele, Milan, Italy

25 ⁸Imperial College London, Department of Medicine, Division of Infectious Diseases, Section of
26 Virology, Norfolk Place, London W2 1PG, UK

27 ⁹Department of Medical Microbiology, Academic Medical Centre University of Amsterdam,
28 Amsterdam, The Netherlands

29
30 *Equal contribution

31
32 Corresponding author Emails:

33 quentin.sattentau@path.ox.ac.uk (QJS) and andrew@scripps.edu (ABW)

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40

41 **Abstract**

42 Chemical cross-linking is used to stabilise protein structure with additional benefits of pathogen
43 and toxin inactivation for vaccine use, but its use is restricted by potential induction of local or
44 global structural distortion. This is of particular importance when the protein in question
45 requires a high degree of structural conservation for the purposes of understanding function,
46 or for inducing a biological outcome such as elicitation of antibodies to conformationally-
47 sensitive epitopes. The HIV-1 envelope glycoprotein (Env) trimer is metastable and shifts
48 between different conformational states, complicating its functional analysis and use as a
49 vaccine antigen. Here we have used the hetero-bifunctional zero-length reagent EDC to cross-
50 link two soluble Env trimers, selected well-folded trimers using an antibody affinity column, and
51 transferred this process to good manufacturing practice (GMP) for clinical trial use. Cross-
52 linking enhanced GMP trimer stability to biophysical and enzyme attack, and had broadly
53 beneficial effects on morphology, antigenicity and immunogenicity. Cryo-EM analysis revealed
54 that cross-linking essentially completely retained overall structure with RMSDs between
55 unmodified and cross-linked Env trimers of 0.4 - 0.5 Å. Despite this negligible distortion of
56 global trimer structure we identified individual inter-subunit, intra-subunit and intra-protomer
57 cross-links. Thus, EDC cross-linking maintains protein folding, improves stability, and is readily
58 transferred to GMP, consistent with use of this approach in probing protein structure/function
59 relationships and in the design of vaccines.

60

61 **Introduction**

62 HIV-1 vaccine design is primarily focused on eliciting neutralizing antibodies (nAb) by targeting
63 the viral envelope glycoproteins (Env), the only target of nAb [1]. Over the past decade a large
64 number of broadly neutralizing antibodies (bnAbs) that target highly conserved surfaces on
65 HIV-1 Env have been isolated from HIV-1-infected individuals, and bnAb infusion into non-
66 human primates and human immune system mice can provide sterilizing immunity [1]. This
67 provides proof of concept that if bnAbs could be elicited by active vaccination, they would be
68 protective. However, Env is metastable and can adopt different conformational states with
69 implications for bnAb binding and elicitation [2, 3]. This is particularly true for soluble forms of
70 Env, which require specific stabilizing mutations to remain in trimeric form. Currently, the two
71 leading approaches to preparing soluble, near-natively folded HIV-1 trimers for vaccine use
72 are either cleaved between gp120 and gp41 with stability maintained by engineered disulfide
73 bonds and other mutations (termed 'SOSIP'), or cleavage replaced or supplemented by the
74 use of a flexible linker (here termed Uncleaved pre-Fusion Optimized, UFO). Recently a novel
75 SOSIP trimer – ConM - based upon the sequence of all group M isolates, showed native-like
76 morphology by electron microscopy (EM), bound most bnAbs but not non-neutralizing Abs
77 (non-nAbs), and elicited antibodies in rabbits that neutralized pseudoviruses carrying the

78 autologous Env [4]. Similarly, a combined SOSIP - UFO-type design of a group M consensus
79 soluble trimer (ConSOSL.UFO, here termed ConS) was shown by EM to be well-folded in the
80 'closed' conformation and displayed bnAb and non-nAb binding similar to its membrane
81 anchored counterpart [5]. However, despite their relative stability in vitro, these trimers may
82 still sample different conformational states, and their structural and antigenic stability in vivo is
83 unknown, but likely to be detrimentally affected by enzymatic and non-enzymatic modification.

84
85 Chemical cross-linking remains widely used for pathogen and toxin inactivation, and for
86 stabilizing proteins for vaccine use and structural analysis. However, how this process affects
87 protein structure at the molecular and atomic level, and how this impacts vaccine antigenicity
88 and immunogenicity is poorly understood and remains empirical for vaccine use. Cross-linking
89 endpoints for vaccine use are generally defined as infectivity reduction for inactivated
90 pathogens [6], or depletion of enzyme activity for toxins [7], with mostly unknown effects on
91 immunogenicity by comparison with the unmodified material. Moreover, we have only a crude
92 understanding of how cross-linking influences vaccine antigenicity. Monoclonal antibody
93 (mAb) binding to hemagglutinin was reduced by formaldehyde (FA)-treatment for inactivated
94 influenza vaccines [8, 9] and to tetanus toxin (TT) to produce the toxoid [10]. Similarly, FA or
95 glutaraldehyde (GLA) treatment reduced pertussis toxin enzymatic and carbohydrate binding
96 activities [11, 12], and FA inactivation of poliovirus inhibited receptor binding [13]. Proteomic
97 and biophysical analyses demonstrated that FA treatment of TT introduced relatively subtle
98 molecular modifications without evidence for major protein conformational changes [14, 15].
99 However, aldehyde inactivation of vaccines introduces additional atoms from the cross-linking
100 agent, can reduce adaptive immune responses [16], and in some cases such as the prototypic
101 respiratory syncytial virus vaccine, can adversely bias adaptive immune responses driving
102 enhanced disease upon infection [17, 18]. Thus, the use of alternative, more modern cross-
103 linking reagents without these potential adverse effects requires exploration, with the caveat
104 that this process must be Good Manufacturing Practice (GMP) compliant for human use.
105 Moreover, understanding of the structural outcomes of chemically cross-linked vaccine
106 antigens using high-resolution structural analysis is almost completely lacking in the literature,
107 as is the effect of these modifications on the magnitude and specificity of ensuing B and T cell
108 responses. Acquisition of this knowledge is critical, as it would allow prediction of the effects
109 of cross-linking on protein structure, and more rational translation of chemical cross-linking
110 approaches to the design and development of vaccine antigens.

111
112 To further stabilize ConM and ConS Env trimers whilst avoiding the known pitfalls of aldehyde
113 cross-linking, we turned our attention to the zero-length heterobifunctional carbodiimide cross-
114 linker 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). This cross-linker
115 activates carboxyl groups on acidic amino acid (aspartic acid, glutamic acid) side chains that

116 then react to form a covalent amide bond with proximal amines on basic amino acid (lysine,
117 arginine) side chains, without addition of any additional atoms to the molecule. To our
118 knowledge this cross-linking reagent has not previously been used to stabilize vaccine
119 antigens. Here we have cross-linked ConM and ConS trimers using EDC, and produced them
120 to GMP for experimental medicine trials. We show that EDC cross-linking enhances trimer
121 stability against denaturation and protease attack, whilst preserving structural integrity to a
122 striking extent. Consistent with the preservation of structure, cross-linking had largely
123 beneficial effects on trimer antigenicity, and immunogenicity in mice and rabbits. Thus, antigen
124 stabilization by EDC cross-linking results in minimum structural and functional perturbation,
125 can be translated into HIV-1 vaccine immunogens for human use, and is an approach generally
126 applicable to other vaccines.

127

128 **Results**

129

130 ***Biophysical and antigenic characterization of trimers***

131 ConM and ConS trimers developed and characterized as previously described [4, 5], were
132 EDC (**Fig. 1A**) cross-linked using optimized conditions, which were translated to a GMP
133 workflow as outlined in **Fig. 1B**. In brief, trimers were cross-linked with EDC in a process
134 modified from that previously described for the BG505 trimer [19], and purified using an affinity
135 column made with the PGT145 bnAb manufactured to GMP, to select for well-folded forms.
136 The PGT145 apex-specific bnAb was chosen since it is a stringent probe of trimer
137 conformational integrity [20], and binds very similarly to unmodified and EDC cross-linked
138 ConM and ConS trimers (**Supplementary Fig. 1**). Analysis of unmodified primary amines (**Fig.**
139 **1C**) revealed no difference between unmodified and cross-linked ConM, whereas EDC
140 modification of ConS reduced amine content by ~15%, suggesting more extensive but still
141 relatively modest modification. Cross-linked trimers were resistant to reducing-denaturing
142 SDS-PAGE compared to their unmodified counterparts, which dissociated into their respective
143 monomeric species (**Fig. 1D**). Because the gp120-gp41 subunits are peptide-linked in ConS,
144 they have a higher molecular weight than ConM which dissociates into gp120 and truncated
145 gp41 subunits. As anticipated from the SDS-PAGE outcome, differential scanning calorimetry
146 (DSC) analysis revealed that cross-linking enhanced thermal stability by 18.7°C and 17.6°C
147 for ConS and ConM respectively (**Fig. 1E**). Cross-linked ConM yielded an overlapping series
148 of peaks suggesting a heterogeneous population of stabilized trimer forms. Trimer antigenicity
149 was probed by ELISA analysis with panels of bnAbs and non-neutralizing antibodies (non-
150 nAbs - **Fig. 1F and supplementary Fig. 1**). **Fig. 1F** summarizes the ratio of modified :
151 unmodified trimer binding, such that 1 represents no change, <1 represents reduced binding
152 to modified trimer and >1 represents increased binding to modified trimer. On average, cross-
153 linking reduced bnAb binding by <2-fold and non-nAb binding (with the exception of 19b,

154 excluded from the averaging) almost to background on both trimers. Selective conservation of
155 bnAb binding after cross-linking was observed. Binding to the V3 glycan epitope cluster
156 represented by 2G12 [21] and PGT121 [22] showed no change in binding, whereas to trimer
157 apex (represented by PGT145 [22] and PG16 [23]), CD4bs (VRC01 [24] and 3BNC117 [25])
158 and interface (35022 [26]) epitope clusters, there was no change or a modest reduction of <2-
159 fold. By contrast a striking reduction in binding was observed upon cross-linking of ConM with
160 the fusion peptide-specific bNAbs ACS202 [27] and VRC34 [28]. ConS does not bind these
161 bnAbs detectably since they preferentially engage the fusion peptide in cleaved trimers [28,
162 29]. This pattern of bnAb binding to modified and unmodified trimers was confirmed by bilayer
163 interferometry (Octet) analysis (**Supplementary Fig. 2** and **Table 1**). Interestingly, the
164 dramatic loss of VRC34 binding upon EDC modification of ConM was reflected primarily in a
165 highly reduced on-rate (**Supplementary Fig. 2** and **Table 1**), suggesting that bnAb access to
166 its epitope was limited by trimer cross-linking rather than direct EDC alteration of the epitope.
167 This is to be expected since the non-polar fusion peptide epitope for VRC34 does not contain
168 any carboxyl or free amine groups. Non-nAb binding to the cross-linked trimers was essentially
169 absent with the exception of the V3-specific 19b [30] which showed no change after cross-
170 linking (**Fig. 1F**), implying that this epitope is constitutively expressed on all trimer forms
171 regardless of stability. sCD4-IgG2 binding to unmodified trimers was weak, and was abolished
172 by cross-linking, implying that EDC cross-linked trimers will not engage CD4 on T cells, a
173 probable advantage for immunogenicity since CD4 T cell binding could sequester trimers, and
174 particularly the CD4 binding site, from B cell recognition. Similarly, weak binding of the CD4-
175 induced epitope-binding antibody 17b [31] to ConM (17b did not bind ConS) in the presence
176 of sCD4 was eliminated after cross-linking. Two-dimensional classification of trimer
177 morphology by negative stain electron microscopy (EM) revealed that unmodified ConM and
178 ConS trimers were 99% and 78% well-folded respectively prior to modification, and 94% and
179 99% well-folded subsequent to cross-linking and antibody selection (**Fig. 1G**). Thus, EDC
180 cross-linking and PGT145 affinity column processing may have subtly reduced the proportion
181 of well-folded ConM trimers, whereas the same procedure selected well-folded ConS trimers
182 from amongst a more heterogeneous unmodified population.

183
184 In animal models, intramuscular administration of adjuvanted vaccine formulations rapidly
185 recruits neutrophils (detectable within 1 h) [32], and subsequently monocytes and
186 macrophages [32-34], to the injection site where they will release bioactive mediators including
187 the major leukocyte proteolytic enzymes elastase and cathepsin G [35]. We therefore
188 hypothesized that adjuvanted protein vaccine immunogens will be subject to localized enzyme
189 attack in vivo. Given the importance of correct folding of Env trimers for promoting the induction
190 of nAb as opposed to non-nAb [36-38], proteolytic cleavage might compromise trimer integrity
191 and thereby limit induction of bnAbs. To test this hypothesis, we exposed trimers to

192 concentrations of elastase (200ng) or cathepsin G (168 ng) equivalent to that estimated to be
193 released by $\sim 10^5$ and 5×10^6 neutrophils respectively (data not shown) for 1 or 24 h, and
194 analyzed their integrity by reducing SDS-PAGE. For unmodified ConM trimers this resulted in
195 individual monomer gp120 and truncated gp41 bands, of which the gp120 band was quantified
196 by densitometry, whereas cross-linked trimers ran intact, and these bands were quantified by
197 densitometry. **Fig. 1H** reveals that unmodified ConM trimer was highly susceptible to elastase
198 cleavage, losing >80% integrity within 1 h, and was almost completely hydrolyzed by 24 h,
199 whereas cathepsin G had only modest effects at 1 h, but band density was reduced by $\sim 85\%$
200 at 24 h. By contrast, cross-linked ConM showed only $\sim 50\%$ elastase cleavage at 1 h,
201 significantly less than its unmodified counterpart ($p < 0.01$), reducing to 65% at 24 h, again
202 significantly less than the unmodified trimer ($p < 0.05$). ConS ran as a truncated gp140 band
203 due to the linker peptide between the gp120 and gp41 subunits, and showed high sensitivity
204 to elastase cleavage, with complete band degradation by 1 h, and partial sensitivity to
205 cathepsin G, with $\sim 30\%$ and $\sim 60\%$ reduction at 1 and 24 h respectively. As with ConM, cross-
206 linking significantly protected the trimers from degradation under every condition tested (1 h,
207 $p < 0.001$; 24 h, $p < 0.05$), and completely prevented cathepsin attack (1 h $p < 0.01$, 24 h $p <$
208 0.0001).

209
210 In summary, EDC cross-linking and PGT145 selection of both ConM and ConS trimers
211 enhanced thermal stability and resistance to biophysical and enzymatic denaturation, whilst
212 retaining good trimer morphology and selectively modifying antigenicity in a largely beneficial
213 manner.

214

215 **Structural analysis of trimers.**

216 Since preservation of HIV-1 Env trimer folding is important for maintaining conformational bnAb
217 epitopes, in particular those dependent on quaternary conformation, it is critical that cross-
218 linking does not adversely impact trimer structure. Moreover, high-resolution structural
219 information relating to cross-linking of highly conformation-dependent proteins would be
220 helpful additional knowledge relating to the use of such stabilizing agents for understanding
221 general principles of protein structure/function relationships. To interrogate the effect of EDC
222 cross-linking we carried out cryo-EM analysis of trimers bound to PGT122 [22] Fab fragments,
223 and solved the structures of unmodified and cross-linked GMP versions of ConS at 3.1 and
224 3.45Å resolution respectively, and unmodified and cross-linked GMP ConM at 3.4 and 3.85Å
225 respectively (**Fig. 2, Supplementary Table 1 and Supplementary Figs. 3-6**). Each Env is
226 found in the stable closed, prefusion state, and all are highly conserved in overall structure
227 compared to other SOSIP trimers, with an overall $C\alpha$ Root Mean Square Deviation (RMSD) of
228 1.25Å (ConS) and 1.2Å (ConM) compared to BG505 (PDB 4TVP; **Supplementary Fig. 7**).
229 The ConS structure is novel, and the ConM cryo-EM structure is similar to the previously

230 published [4] X-ray structure of ConM in complex with PGT124 and 35022 (1.0Å C α RMSD;
231 PDB 6IEQ), albeit at higher resolution (3.4Å cryo-EM; 3.9Å X-ray). Interaction of PGT122 with
232 ConM and ConS is very similar to other SOSIP trimers, and is driven by contacts with glycans
233 at N332 and N138 (N137 in BG505) and residues at the base of the V3 loop. In agreement
234 with the antigenicity data by ELISA of the related PGT121 bnAb (**Fig. 1F** and **Supplementary**
235 **Fig. 1**), and by Octet for PGT122 (**Supplementary Fig. 2**), EDC cross-linking has no obvious
236 effect on recognition of these epitopes (**Supplementary Fig. 8**).

237
238 ConM and ConS trimers are both based on consensus sequences of group M isolates, and
239 have ~91% sequence identity (**Supplementary Fig. 9**) and a C α RMSD between protomers
240 of 1Å (**Fig. 2B** and **Supplementary Fig. 7**). Inspection of the unmodified ConM and ConS
241 cryo-EM structures identified a number of notable features shared by these two Env designs;
242 among these are the trimer stabilizing mutations A316W and H66R (**Supplementary Fig.**
243 **S10**). W316 is found at the trimer apex within the V3 loop. In both structures the W316 side
244 chain is highly ordered and mediates a cation-pi interaction with R308, and a hydrophobic
245 stacking interaction with Y318, together stabilizing the β -hairpin structure of the V3 loop and
246 probably impeding V3 remodelling necessary for induction of the CD4 bound state[39, 40].
247 R66 is found within a loop in the gp120 inner domain, just beneath the apex. Interestingly, in
248 both ConM and ConS maps, we observe density for two rotamers of the R66 side chain, with
249 one conformation exposed to solvent and the other forming electrostatic interactions with T71
250 and S115. The equilibrium between these two states may underly the mechanism by which
251 H66R inhibits CD4-induced structural changes.

252

253 **Structural Impacts of EDC Crosslinking**

254 Since EDC is a 'zero-length' cross-linker, we did not anticipate the presence of additional
255 atoms in the cross-linked structures, but the formation of individual amide bonds between
256 proximal carboxyl and amine groups could distort local structure, and multiple cross-links might
257 deform overall protein folding. However, comparison of the unmodified and cross-linked
258 structures revealed a striking near identity, with a C α RMSD of only 0.45Å between ConS and
259 ConS-EDC, and 0.5Å between ConM and ConM-EDC (**Fig. 2D, E**). Similarly, the RMSD
260 between cross-linked ConM and ConS trimers was also only 1Å (**Fig. 2C**). Thus, within the
261 limits of resolution, EDC cross-linking had a negligible impact on trimer structure. Thus, overall,
262 these data suggest that any impacts to antigenicity and bnAb binding are not a direct result of
263 global structural or conformational changes imparted by EDC crosslinking. Moreover, this
264 highlights a more general point that EDC cross-linking may be used to stabilize
265 conformationally sensitive protein structure without substantial local or global distortion.

266

267 Throughout the ConS and ConM structures there are numerous potential EDC crosslinking
268 sites, in which an aspartate or glutamate side chain is in proximity to, or directly interacting
269 with, a lysine side chain (**Supplementary Table 2**). However, as a cryo-EM reconstruction
270 represents an ensemble of many thousands of particles, and in this case contains C3
271 symmetry, only high-frequency events will be observed in the final map. Strikingly, close
272 inspection of the ConS-EDC and ConM-EDC density maps, and careful comparison with their
273 unmodified counterparts, revealed a series of high confidence cross-links. Observed cross-
274 links are listed in **Supplementary Table 2**, and examples are summarized visually in **Fig. 3**.
275 Most were intra-subunit crosslinks within gp120, with an inter-subunit gp120-gp41 (intra-
276 protomer, **Fig. 3A**) and an intra-subunit within gp41 (**Fig. 3B**). Inter-subunit (gp120-gp41)
277 crosslinks such as K46-D632 (**Fig. 3A**) will stabilize quaternary structure of the complex
278 consistent with the SDS-PAGE analysis (**Fig. 1D**). This should increase stability and in vivo
279 lifetime of the ‘closed’ state of the trimer, thus maintaining bnAb epitopes but reducing
280 exposure of non-nAb epitopes such as the V1V2 and V3 loops and the CD4i surface [19, 41-
281 43]. Since each crosslink is in essence a “molecular staple”, even intra-subunit cross-links
282 (**Fig. 3B, C**) will act to stabilize secondary and tertiary structure, increasing overall molecular
283 stability and potentially reducing exposure to protease attack.

284
285 Structural analysis informs antibody binding, allowing comparison between unmodified and
286 cross-linked antigens. Modification of antigenicity may result from global changes in trimer
287 folding, which we have shown is not the case. Thus, changes will most probably be local, and
288 will result from introduction of cross-links within or proximal to the epitope, and potentially also
289 the change in charge at the site of cross-linking arising from amide bond formation that
290 eliminates the previous negative charge of the reacted carboxylate group [44]. However,
291 inspection of the epitope cluster most heavily antigenically modified in ConM - the fusion
292 peptide - (**Fig. 1F, Supplementary Figs. 1 and 2 and Table 1**), revealed no obvious cross-
293 links. However, E87 in gp120 that is reported to be critical for ACS202 binding [29] may be
294 modified by EDC in ConM, reducing bnAb engagement. However, this would not explain loss
295 of VRC34 binding to the same region of ConM, as this bnAb does not include E87 in its
296 epitope[28]. Therefore, it seems most likely that fusion peptide exposure is prevented by the
297 trimer cross-linking process. By contrast, whilst none of the CD4bs, apex and interface
298 epitopes analyzed contained obvious cross-links, all contained acidic residues that could form
299 heterogenous low-frequency, unobserved crosslinks with proximal lysines (**Supplementary**
300 **Fig. 11**), potentially resulting in reduced affinity which would be consistent with the modified
301 binding curves (**Supplementary Fig. 2**) and reduced k_D values (**Table 1**). An alternative is that
302 the quenching step to eliminate unreacted O-acylisourea intermediates in the absence of
303 proximal primary amines might form an amide bond with quenching agent (glycine), the added
304 glycine residues disrupting antibody epitopes. However, this seems unlikely since no extra

305 density was observed on aspartate or glutamate side chains (data not shown), suggesting that
306 if it does occur, it is likely to be a rare event. Moreover, as mentioned previously, EDC cross-
307 linking primarily influenced bnAb on-rate, strongly implying reduced epitope exposure rather
308 than direct impact on epitope chemistry.

309
310 Taken together, these data demonstrate a striking conservation of structure in cross-linked
311 trimers with highly localized and epitope-specific modulation of bNAbs binding evidenced by
312 high-resolution structural analysis.

313 314 **Adaptive immune responses to trimer immunization.**

315 Modification of antigenicity by cross-linking may influence trimer immunogenicity with respect
316 to adaptive immune recognition of B and T cell epitopes. Preclinical analysis is of particular
317 importance for GMP material as it will help to predict immune responses in experimental
318 medicine trials. To probe antigen-specific adaptive immune responses, mice were immunized
319 with 10 µg trimer/dose formulated in 10 µg/dose MPLA adjuvant at the times indicated by the
320 arrows (**Fig. 4A, B**), and antibody kinetics analysed by ELISA. Serum from each group was
321 assayed for antigen-specific IgG against either unmodified or EDC cross-linked homologous
322 trimers at the times shown. Unmodified ConM elicited an IgG response that reacted
323 equivalently with unmodified and cross-linked ConM at week 2, but which then diverged after
324 the prime showing significantly greater reactivity against unmodified compared to cross-linked
325 ConM at 6 weeks ($p < 0.0001$) and 12 weeks ($p < 0.01$), **Figure 4A**. Similar results were obtained
326 for ConS (**Fig. 4B**), with sera from ConS-immunized mice giving significantly higher titres on
327 ConS compared to EDC cross-linked ConS at week 6 ($p < 0.01$). These data suggest that
328 epitopes available on unmodified trimers, potentially as a result of trimer opening and/or
329 dissociation in vivo, are less represented on cross-linked trimers, which are unable to open
330 and dissociate into their components. By contrast, sera from mice immunized with cross-linked
331 ConM and ConS trimers gave very similar titres on both unmodified and cross-linked
332 homologous trimers, consistent with epitopes available on the cross-linked trimers being
333 equivalently presented on the unmodified trimers.

334
335 Since cross-linking might modify antigen processing and peptide presentation to T helper (Th)
336 cells, we evaluated antigen-specific responses to immunization. Spleens were harvested at
337 week 14 and splenocytes restimulated in vitro with unmodified homologous antigen for 3 days,
338 followed by analysis of cytokine release. In vitro ConM restimulated splenocytes from mice
339 immunized with unmodified or cross-linked ConM trimer showed a non-significant trend
340 towards enhanced IL-2, IL-13 and IL-17a but a significant ($p < 0.05$) increase in IFN γ release in
341 cross-linked compared to unmodified trimer-immunized mice (**Fig. 4C**). Similarly, restimulation
342 of splenocytes from mice immunized with cross-linked ConS trimer resulted in significantly

343 increased IL-2 and IFN γ ($p < 0.05$) and a trend towards increased IL13 and IL17a release. Thus
344 we anticipate that immunization with cross-linked trimer may elicit a modest enhancement of
345 Th cell responses that appear to be balanced between Th1, Th2 and Th17.

346
347 Toxicity analysis of the GMP product required immunization of rabbits with trimer (100 μ g) co-
348 formulated with MPLA adjuvant (500 μ g), according to the regimen in **Supplementary Fig.**
349 **12A, B**. IgG purified from rabbit sera were titrated onto ELISA wells coated with the antigens
350 shown, and titration curves generated (**Supplementary Fig. 12C-F**). Binding curves showed
351 no significant differences between groups in kinetics of IgG elicitation, and endpoint titers
352 determined at week 12 were almost identical between all adjuvanted groups (**Fig. 4E**). Thus,
353 differences noted with the mouse immunizations were not recapitulated in the rabbits,
354 potentially because of the much higher (20-fold) dose of antigen used may have masked
355 differences. Week 12 rabbit sera were assayed against infectious molecular clones (IMCs)
356 expressing Envs from ConM and ConS in a TZMbl assay, and results expressed as reciprocal
357 serum dilution yielding 50% inhibition (IC₅₀). Sera from rabbits immunized with unmodified
358 ConM neutralized ConM (**Fig. 4F**) and ConS (**Fig. 4G**) IMCs approximately 30-fold and 10-
359 fold more respectively than sera from animals that received cross-linked ConM, although the
360 lack of statistical significance mean that this was only a trend. Similarly, cross-linked ConS
361 trimer showed reduced autologous neutralization on ConS IMC, but again this was not
362 significant. Since both ConM and ConS Envs generate relatively neutralization sensitive Tier-
363 1-type pseudoviruses [4, 5], most of the serum neutralization activity observed is likely to be
364 against the V1V2 loops, as previously described [4]. Therefore, the reduction in serum
365 neutralization probably represents either beneficial masking of these structures by cross-
366 linking V1V2 within the trimer context, or local EDC modification of the specific epitopes
367 recognized by these Tier-1 virus neutralizing antibodies. We suspect that the first hypothesis
368 is the more likely as the animals will mount a polyclonal response to these epitopes, and so
369 epitope-specific modification seems less likely than domain masking. Heterologous Tier-2
370 virus neutralization was not tested since the short immunization regimen was designed as a
371 toxicity study and was not optimized to elicit nAb, and so we did not anticipate broadly
372 neutralizing responses.

373
374 In summary, immunization of mice and rabbits with GMP EDC cross-linked trimers formulated
375 in MPLA adjuvant revealed broadly similar kinetics of antigen-specific IgG induction in mice
376 and rabbits between unmodified and cross-linked trimers, with a modestly enhanced Th
377 cytokine response in mice against the cross-linked trimers. Trimer cross-linking reduced
378 induction of autologous virus neutralization in rabbit sera potentially via masking of V1V2
379 epitopes.

380

381

382 **Discussion**

383 The structural analysis of complex antigens has been facilitated over the past decade by the
384 use of cryo-EM single particle analysis, allowing for near-atomic resolution. This has led to
385 determination of a variety of structures such as those here, which provide a detailed snapshot
386 of the effects of chemical cross-linking on two GMP experimental vaccine immunogens
387 destined for clinical use. The most striking outcome from the present analysis is the almost
388 complete conservation of trimer structural integrity after robust trimer stabilization by cross-
389 linking with EDC, which was unexpected. Consistent with the structural conservation is the
390 linked broad conservation of antigenicity, with the only major loss of bnAb binding occurring to
391 the fusion peptide in ConM trimers. Whilst ACS202 binding may potentially be compromised
392 by EDC modification of E87, the reason for VRC34 binding is currently unclear as its epitope
393 does not contain charged residues [28]. Aside from the loss of fusion peptide bnAb binding,
394 we anticipate from the antigenic analysis of cross-linked trimers that other major bnAb epitope
395 clusters should be well conserved, and therefore have the potential to trigger bnAb-reactive B
396 cells. To our knowledge only one other study has investigated the structure of a cross-linked
397 vaccine immunogen compared to its un-crosslinked counterpart at high resolution [43]. In this
398 previous study we showed that glutaraldehyde, a 5 carbon homobifunctional amine cross-
399 linker, introduced cross-links into the prototypic SOSIP trimer BG505, which subtly modified
400 global molecular conformation, and eliminated bnAb binding to quaternary epitopes at the
401 trimer apex [43]. However, that study revealed that GLA added extra atoms to the structure
402 with the added risk of creation of immunogenic neoepitopes, and moreover did not analyze
403 GMP produced material and hence is distal from translation to human use.

404

405 Our current results reveal that chemical cross-linking of two M group consensus sequence-
406 derived Env trimers substantially enhanced biophysical stability. It is widely accepted that
407 enhancing stability of subunit immunogens, particularly those with metastable characteristics
408 such as HIV-1 Env, is likely to increase in vivo lifetime and reduce exposure of
409 immunodominant non-neutralizing epitopes such as the V1, V2 and V3 loops [39, 40, 45, 46].
410 In this respect the inter-subunit cross-link K46-D632 may be particularly relevant since as it is
411 likely to substantially restrict molecular movement. However, it is likely that intra-subunit cross-
412 links and modifications will also play a role in restricting molecular movement via secondary
413 and tertiary structure stabilization. Additional benefits of stabilization may also include
414 increased in vivo lifetime, particularly in the presence of antibodies that trigger disassembly of
415 non-cross-linked trimers [47], and resistance to proteases found at sites of inflammation such
416 as at vaccine injection sites. The local in vivo milieu after administration of vaccine formulations
417 has not been well studied, but is likely to be hostile to proteins in terms of proteolytic attack,
418 particularly when the immunogen has stimulated an inflammatory environment containing cells

419 such as neutrophils that release highly active proteases such as elastase [32-34]. Our in vitro
420 models of protease attack revealed that unmodified trimers were sensitive to cleavage by
421 elastase, and to a lesser extent by cathepsin G, but that cross-linking significantly enhanced
422 protease resistance. Measurement of antigen protease sensitivity over 1 and 24 h seems
423 relevant, since a recent study suggests that antigen may persist at the site of intramuscular
424 administration for several days in an adjuvant-dependent manner, and its elimination can
425 substantially reduce ensuing adaptive immune responses [34]. Our demonstration of
426 enhanced trimer resistance to leukocyte elastase and cathepsin G in vitro may have in vivo
427 significance that has not so far been considered. Future studies will be aimed at characterizing
428 the in vivo fate of conformationally-sensitive immunogens such as soluble Env trimers by the
429 use of highly conformation-dependent bNAb probes.

430
431 Whilst the minimal modification of trimer structure and antigenicity upon cross-linking suggests
432 that immunogenicity may not be substantially affected, the complexity of the immune system
433 is such that this is impossible to predict. We therefore carried out immunization of mice with
434 the unmodified and cross-linked trimers formulated with the TLR4 agonist adjuvant MPLA.
435 Interestingly, quantitative differences were seen in mouse T cell cytokine responses, with
436 significantly increased titres of IFN γ (ConM-EDC and ConS-EDC) and IL-2 (ConS-EDC),
437 compared to unmodified trimer, suggesting enhanced Th responses. It is unclear why cross-
438 linking might elicit these increases in cytokine response, particularly in light of the minimal
439 structural changes observed, however cross-links and other EDC modifications made to acidic
440 residues may alter antigen processing and presentation to T cells resulting in cytokine
441 modulation. These modulated cytokine responses did not appear to result in any obvious
442 differences in outcome for B cells, given the similar antigen-specific IgG responses. Small
443 differences in antigen-specific IgG responses were observed in mice, but these normalized
444 between the unmodified and cross-linked groups after the first (ConM) and second (ConS)
445 boosts respectively. Rabbit immunizations were designed to test formulation toxicity and so
446 were not optimized for eliciting neutralizing antibody responses. Nevertheless, autologous and
447 heterologous Tier-1a (ConM) and Tier-1b (ConS) pseudovirus neutralizing responses were
448 observed. Interestingly both autologous (in ConM and ConS sera) and heterologous (in ConM
449 sera) responses tended to be reduced by cross-linking, suggesting that one or more Tier-1
450 type neutralization epitopes was being masked or otherwise compromised. Since one of the
451 goals of immunogen design is to focus immune responses away from regions that may
452 compete for production of bNAbs, such reductions in Tier-1 neutralization may be helpful.
453 Future studies will address the target specificity of these responses and the mechanism of
454 their reduction in cross-linked trimers.

455

456 The increasing ease with which complex molecules such as the HIV-1 Env trimer can be
457 structurally analysed by cryo-EM allows the unprecedented high-resolution analysis of post-
458 translational modifications such as chemical cross-linking, which up to now have not been
459 interrogated in vaccine antigens. This information will enable a more rational approach to
460 immunogen stabilization, particularly for molecules that are structurally and/or conformationally
461 metastable. Equally, the proof of concept shown here for the use of EDC in manufacturing a
462 GMP immunogen, currently in experimental medicine trials, paves the way for use of this, and
463 other newer cross-linking reagents, in vaccine manufacture. Indeed, the EDC process added
464 only two additional steps to the GMP process. Since EDC adds no additional atoms to the
465 cross-links, unlike for example glutaraldehyde, there will be minimal risk of generation of
466 neoepitopes with associated concerns of antigenic modification potentially associated with
467 cross-reactivity and autoimmunity.

468

469 **Materials and methods**

470 *Antibodies and ligands*

471 Antibodies VRC01 [24], PGT145 [48], 2G12 [49], PGT122 [48], 19b [30], 35022 [26], ACS202
472 [27] and VRC34 [28] were expressed in freestyle 293F cells under serum-free conditions and
473 purified by protein A chromatography (Thermofisher) following manufacturer's instructions.
474 Soluble CD4 (sCD4) [50], CD4-IgG2 [51], 15e [30], F105 [30], 17b [31], 19b and PG16 [52]
475 were from the IAVI Neutralizing Antibody Consortium. Fragments antibody-binding (Fabs)
476 VRC01, PGT122, PGT145, and 35022, used in BLI-Octet studies, were expressed in Freestyle
477 293F cells under serum-free conditions and purified as previously described (Rantalainen et
478 al. 2020. *Cell Reports*). VRC43 Fab was a kind gift from P Kwong and the VRC. Where
479 required antibodies were biotinylated using EZ-link NHS-LC-Biotin according to the
480 manufacturer's instructions (Thermofisher), or were attached to cyanogen bromide-activated
481 agarose using the manufacturer's protocol (GE Healthcare).

482

483 *Generation of CHO cell lines for stable expression of ConM and ConS Env trimers.*

484 Stable Chinese Hamster Ovary (CHO) cell lines expressing ConM and ConS Env trimers were
485 generated by transfection of a parental CHO K1 host cell line using BAC (Bacterial Artificial
486 Chromosome) vector technology [53]. The transfection strategy for ConM (but not ConS)
487 involved co-transfection (ratio 1:1) of a second BAC vector harbouring the gene coding for the
488 human pro-protein convertase (pc) furin for efficient cleavage of the Env precursor in the trans-
489 Golgi network (TGN) [54]. Positive transfected cell pools for both trimers were selected in
490 serum-free medium (CD CHO (Gibco), supplemented with selection medium (SM, 8 mM L-
491 glutamine and phenol red, with 0.5 mg/mL G-418, Sigma). Cell pools were maintained at 37°C,

492 5% CO₂ and 80% humidity in a shaking incubator (Kuhner) at 125 rpm, and regularly checked
493 for onset of cell growth until cell viability reached >95 % with steady growth. ConM and ConS
494 expressing cell pools were expanded to 125 mL shake-flasks (Corning) and were split every 3
495 to 4 days to starting cell concentrations of 2-3 x 10⁵ cells x mL⁻¹, in SM). Cell concentrations
496 and cell viabilities were monitored using a Bioprofile CDV cell counter (Nova biomedical, US)
497 and a Multisizer 4e (Beckman Coulter, US) instruments. At each passage supernatant samples
498 were collected for quantification of ConM and ConS Env titres by PGT145 binding ELISA.

499

500 *Isolation of ConM and ConS Env trimer clonal lines*

501 Clonal lines of ConM and ConS transfected cell pools were obtained through limiting-dilution
502 single-cell-cloning. Cell pools were diluted to a limiting cell concentration of 1 cell per 40 µL
503 well aliquot in conditioned single-cell-cloning medium (1:1) mixture of SM, and 0.2 µm filtered
504 CHO medium from a 3-day passaging harvest from cultivation of the parental CHO K1 host
505 cell line, supplemented with rhAlbumin (Sigma Aldrich), rEGF (Repligen) and rTransferrin
506 (Merck). Diluted cell suspensions were plated into 384-well plates (Corning), centrifuged at
507 200 x g for 5 min, and imaged with a CellMetric (Solentim) imaging device to capture single-
508 cell plating events, and to follow outgrowth of clonal cell populations. Clonal ConM and ConS
509 cell populations were then expanded to 96-well plates (Thermo Scientific) and evaluated for
510 cell growth using the CellMetric imaging system, and expression of ConM and ConS Env
511 trimers was measured by PGT145 ELISA at the end-point of two consecutive passages. Based
512 on expression levels and cell growth 20 ConM and ConS lead clones were selected, expanded
513 into 25 cm² tissue culture flasks (Greiner) and cultivated for three consecutive passages.
514 Sampling at each passage end-point (every 3rd to 4th day) included measurement of cell
515 concentration and viability. Further, cell-free supernatant was collected for analysis of Env
516 trimer expression by PGT145 ELISA. Additionally, cell pellets of the 20 ConM lead clones were
517 analyzed for the co-transfected hFurin construct by standard end-point PCR and hfurin specific
518 primers. Based on cell growth and PGT145 ELISA expression data, ConM and ConS Env cell-
519 specific productivity was calculated on the basis of which lead clone number was further
520 reduced to eight, which were then cryo-preserved and stored in liquid nitrogen.

521

522 *Evaluation of ConM and ConS Env trimer expressing CHO lead clone candidates.*

523 In order to identify optimal clones to enter the GMP manufacturing pipeline, small-scale shake-
524 flask fed-batch experiments were performed. Fed-batches were performed in ActiCHO P
525 medium (GE Healthcare) supplemented with 8 mM L-glutamine and phenol red in 125 mL
526 shake flasks (Corning), at a working volume of 45 mL and a seeding cell density of 0.3 × 10⁶
527 cells × mL⁻¹ and a target stop criterion of culture viabilities ≤ 80%. Addition of feed media,
528 ActiCHO Feed A and ActiCHO Feed B (both GE Healthcare) started on day four based on a
529 glucose-controlled feeding regimen. Feed A was added in order to meet a target glucose

530 concentration of $6.5 \text{ g} \times \text{L}^{-1}$. Feed B was added as a constant 0.28% (v/v) feed based on the
531 actual culture volume. Sampling of fed-batch cultures for monitoring of cell concentrations and
532 culture viability, as well as HIV Env trimer concentration and levels of glucose, lactate,
533 ammonium, L-glutamine and L-glutamate were performed on the day of seeding, and
534 continued on day 4 until the end of the process (culture viability $\leq 80\%$). Further, ConM and
535 ConS lead clones were monitored for their ability to maintain cell specific growth rates $[\mu]$ and
536 specific productivities $[q_p]$ of trimer expression over a period of ≥ 70 population doublings (PDL)
537 under routine cultivation conditions. Following small-scale fed-batch evaluation, stability
538 monitoring, and selection of the final ConM and ConS lead clones to enter the GMP pipeline,
539 master cell banks (MCBs) were prepared and cryo-preserved in liquid nitrogen under GMP
540 conditions.

541

542 *Scale-up of ConM and ConS expressing CHO lead clones for GMP manufacture.*

543 Scale-up and process development for trimer production in fed-batch mode was performed in
544 a ReadyToProcess WAVE 25 bioreactor system using ActiCHO P medium (GE Healthcare)
545 and the respective feed media ActiCHO Feed a and ActiCHO Feed B (both GE Healthcare).
546 Inoculation cultures were obtained by expansion of ConM and ConS lead clones from MCBs
547 in production medium (ActiCHO P medium supplemented with 8 mM L-glutamine) in 1000 mL
548 shake flasks (Corning) in a shaking incubator (Kuhner) (125 rpm, 37 °C, 5% CO₂, 80% relative
549 humidity) to support a starting cell density of $0.3 \times 10^6 \text{ cells} \times \text{mL}^{-1}$ in a starting volume of 8 L.
550 WAVE 25 bioreactor runs were operated at 37°C, dissolved oxygen was set to 30%, with no
551 additional pH control by base addition. Sampling of fed-batch cultures was performed in 24-h
552 intervals for monitoring of cell concentration, cell viability, levels of glucose, lactate,
553 ammonium, l-glutamine and l-glutamate. Additionally, cell-free supernatant was collected to
554 measure product concentration by PGT145 ELISA. Feeding of WAVE fed-batch cultures was
555 performed as previously outlined for small-scale fed-batches experiments. At harvest, cells
556 were removed by centrifugation and cell-free supernatants were passed through a positively
557 charged 3M™ Zeta Plus™ depth filter (3M) for removal of negatively-charged contaminants.
558 As a final step, depth filtered supernatants were sterile filtered through a Sartopore 2
559 (Sartorius) filter unit and stored until further processing at 2-8 °C.

560

561 *GMP production of ConM and ConS Env trimers in fed-batch mode.*

562 GMP manufacture of ConM and ConS Env trimers was based on previously established
563 master cell banks of ConM and ConS expressing lead clones and cultivation in fed-batch mode
564 using a ReadyToProcess WAVE50 Bioreactor system (GE Healthcare) and purification of well-
565 folded Env trimers from harvested culture supernatant by PGT145 affinity chromatography and
566 subsequent polishing steps. In a nutshell, inoculum cultures were prepared from cryo-
567 preserved master cell banks and cultures were expanded in production medium (ActiCHO P

568 medium, supplemented with 8 mM L-glutamine) to support starting cell densities of $\geq 3 \times 10^5$
569 cells $\times \text{mL}^{-1}$ at a starting volume of 17 L. WAVE GMP fed-batch production runs were operated
570 at 37 °C, pH was set to 7.0 (controlled via base addition and purging with CO₂), 20 rpm rocking
571 speed (6° angle) and 30% dissolved oxygen (DO). Daily sampling was performed for analysis
572 of cell concentration, viability, ConM and ConS Env concentration, glucose levels and key
573 metabolites. Addition of nutrient feeds ActiCHO Feed A and ActiCHO Feed B (both GE
574 Healthcare) started when glucose levels dropped below $\leq 4 \text{ g} \times \text{L}^{-1}$ and was continued daily
575 thereafter. ActiCHO Feed A was added to a final glucose concentration of $6.5 \text{ g} \times \text{L}^{-1}$, ActiCHO
576 Feed B was added as a 0.28% (v/v) volume feed based on the working volume in the
577 bioreactor. Once viabilities dropped below 80% ($\geq 60\%$) fed-batch cultures were terminated
578 and culture supernatants were harvested by 2-stage depth filtration using 3M™ ZetaPlus™
579 filters for cell removal, followed by removal of host cell proteins (HCP) and DNA by anion-
580 exchange (AEX) membrane absorption using 3M™ Emphaze™ Hybrid Purifier filters, followed
581 by a final 0.2 μm sterile filtration step using Polyethersulfone (PES) filters (PALL).

582

583 *Purification of ConM and ConS Env trimers*

584 Fed-batch supernatants were concentrated (≤ 14 -times) and dia-filtered (≥ 6 -times buffer
585 exchange) into 20 mM Tris / 500 mM NaCl / pH 8.3 using Sartocan ECO (30 kDa MWCO, 0.70
586 m²) PES membranes (Sartorius). Buffer exchanged supernatants were filtered using 0.8 + 0.45
587 μm Sartopore 2 filters (Sartorius) particulate contaminant removal and further sterile filtered
588 using 0.2 μm Sartopore 2 filters (Sartorius), then divided into four parts for further processing.
589 Virus inactivation was performed by addition of 1% (w/w) Triton X-100 and incubation at RT
590 for 60-120 mins and solutions sterile filtered using 0.45 μm Sartopore 2 filters (Sartorius). Triton
591 X-100 inactivated solutions were batch-wise loaded onto a MAb PGT145 immunoaffinity
592 column for capture of ConM and ConS Env trimers. The affinity columns were prepared from
593 an in-house prepared GMP stock of MAb PGT145 by coupling of the antibody to Toyopearl
594 650M chromatography resin (Tosoh). Loaded columns were washed with $\geq 10 \text{ GV}$ of 20 mM
595 Tris / 500 mM NaCl / pH 8.3 and bound ConM and ConS trimers were eluted from the columns
596 with 50 mM histidine / 2 M MgCl₂ / pH 6.0. Trimer elution fractions were concentrated (≤ 5 -
597 times) and dia-filtered (≥ 10 -times buffer exchange) to 0.1 M Glycine / 10 mM Tris / pH 7.5
598 using a Sartocan Slice ECO (30 kDa MWCO, 0.14 m²) PES membrane (Sartorius). Trimer-
599 containing fractions were pooled and passed over a pre-equilibrated MAbSelect SuRe Protein
600 A (ProA) affinity column (GE Healthcare) to remove leached residual PGT145-affinity ligand
601 from the previous column. The trimer containing ProA flow through fractions were loaded onto
602 a Q-Sepharose FF anion-exchange column (GE Healthcare), and columns washed with ≥ 5
603 column volumes of 0.1 M Glycine / 0.01 M Tris / pH 7.5. Trimers were then actively eluted with
604 0.1 M Glycine / 0.01 M Tris / 0.25 M NaCl / pH 7.5 (injection grade). AEX eluate fractions were
605 sterile filtered using 0.45 + 0.2 μm Sartobran 300 filters (Sartorius), concentrated and dia-

606 filtered to 20 mM Tris / 150 mM NaCl / pH 7.5 (injection grade drug substance/product buffer)
607 using a Sartoclon Slice ECO (30 kDa MWCO, 0.14 m²), diluted to a final Env trimer
608 concentration of 2 mg/mL and filtered using 0.45 + 0.2 µm Sartobran 300 filter capsules
609 (Sartorius). As final steps, nano-filtration of formulated ConM and ConS drug substance
610 batches was performed using Planova™ 20N (0.3 m²) (Asahi Kasei) filters for virus removal,
611 and drug substance batches were further diluted to concentrations of ~1 mg/mL in drug
612 substance/drug product buffer (20 mM Tris / 150 mM NaCl / pH 7.5) and stored for further
613 processing at 2-8°C.

614
615 *EDC cross-linking of GMP produced trimers and PGT145 immunoaffinity purification.*
616 ConM and ConS trimer preparations manufactured under GMP conditions were cross-linked
617 by EDC / sulfo-NHS chemistry. As a preparatory step, ConM and ConS bulk drug substance
618 (1 mg x mL⁻¹; 20 mM Tris / 150 mM NaCl / pH 7.5; injection grade) were concentrated and dia-
619 filtered into their optimal cross-linking buffer environments. ConM bulk drug substance (DS)
620 preparations were buffer exchanged to 20 mM HEPES / 150 mM NaCl / pH 7.5; injection grade,
621 and ConS DS preparations were buffer exchanged to phosphate buffered saline (PBS), pH
622 7.4; injection grade (≥ 10 volume changes) using a Sartoclon Slice ECO (30 kDa MWCO, 0.14
623 m²) PES membrane (Sartorius). Buffer exchanged DS preparations were further adjusted to a
624 concentration of ~1 mg x mL⁻¹ in 20 mM Tris / 150 mM NaCl / pH 7.5; injection grade. EDC
625 cross-linking conditions were different for ConM and ConS trimers. For both trimers, EDC and
626 NHS stock solutions were diluted in the trimer specific buffers and mixed with equal amounts
627 (w/w) of buffer exchanged trimer solution (1 mg x mL⁻¹). Cross-linking reactions for ConM were
628 performed at RT for 30 mins, and ConS was incubated for 10 mins. Optimal molar
629 concentrations for cross-linking of ConM and ConS were 1 M EDC / 10 mM NHS and 0.25 M
630 EDC / 10 mM NHS respectively. Cross-linking reactions were quenched by addition of equal
631 amounts (w/w) of a 1 M Glycine, pH 7.4 stock solution and incubation at RT for ≥ 10 mins.
632 Further, cross-linked ConM (ConM-EDC) and ConS (ConS-EDC) preparations were
633 concentrated and dia-filtered (≥ 7-times buffer exchange) with 20 mM Tris / 500 mM NaCl / pH
634 8.3; injection grade and passed through a 0.45 + 0.2 µm Sartopore 2 300 (Sartorius) filter for
635 sterile filtration. ConM-EDC and ConS-EDC trimer preparations were loaded onto a GMP
636 grade MAb PGT145 immunoaffinity column for positive selection of well-folded / cross-linked
637 ConM and ConS Env trimers as previously described for purification of non-cross-linked trimer
638 preparations. Immediately after, ConM-EDC and ConS-EDC MAb PGT145 immunoaffinity
639 eluates were concentrated (≤ 5-times) and dia-filtered (≥ 14-times buffer exchange) into drug
640 substance (DS) formulation buffer 20 mM Tris / 150 mM NaCl / pH 7.5; injection grade. As a
641 last step, formulated ConM-EDC and ConS-EDC solutions were 0.2 µm filtered using a PES
642 beaker filter (Millipore), concentrations were adjusted to ~1 mg x mL⁻¹ and the final DS
643 preparation were stored at 2-8 °C.

644

645 *PGT145 ELISA for quantification of ConM and ConS from supernatant samples.*

646 96-well plates (Nunc, Maxisorp) were coated with $1 \mu\text{g} \times \text{mL}^{-1}$ of MAb 2G12 (Polymun
647 Scientific) in carbonate/bicarbonate coating buffer, pH 9.5 at 4°C overnight. Before use, plates
648 were blocked with PBS + 0.1% Tween and 1% BSA (PBS-T, 1% BSA) at RT for 1h.
649 Supernatant samples and reference standards were diluted performing a 1:2 serial dilution
650 series in PBS-T, 1% BSA. Assay plates (pre-coated) were washed (4-times) with PBS-T and
651 aliquots of serially-diluted samples and standards were transferred to pre-coated assay plates
652 and incubated at RT for 1h. After washing (4-times with PBS-T), biotinylated MAb PGT145 at
653 $1 \mu\text{g}/\text{mL}$ was applied for 1h. Following incubation with MAb PGT145, plates were washed and
654 incubated with streptavidin-HRP conjugate (Roche) for 30 mins. Color development was
655 started by addition of o-phenylenediamine dihydrochloride (OPD) substrate, the reaction
656 stopped by addition of 25% H_2SO_4 (Merck) and OD492 was measured with a Synergy 2 plate
657 reader (BioTek). Evaluation of unknown supernatant samples by interpolation from standard
658 curves of ConM and ConS reference material was performed using the Gen5 Microplate
659 Reader and Imager Software package, version 3.05 (BioTek).

660

661 *Characterization of GMP-produced ConM, ConM-EDC, ConS and ConS-EDC trimer*
662 *preparations.*

663 Trimer integrity and stability of GMP produced ConM and ConS trimer preparations and their
664 EDC cross-linked versions were subject to long-term stability monitoring of up to 60 months.
665 Trimer preparations were evaluated by BN PAGE (Native PAGE Novex 3–12% Bis-Tris Gels)
666 and Size Exclusion HPLC (SE-HPLC) using 2 consecutive Acquity UPLC protein columns
667 (Waters) with 200 Å and 450 Å to assess integrity and purity of GMP trimer preparations.
668 Reducing and non-reducing SDS PAGE (NuPAGE 4 to 12%, Bis-Tris gels) was performed
669 together with immunoblotting and probing with anti-Env specific antibodies (5F3, 447-52D,
670 2G12). GMP produced preparations were routinely monitored for the presence of particulate
671 contaminants, pH, endotoxin levels and osmolality.

672 *DSC analysis*

673 Thermal denaturation of unmodified and cross-linked GMP batches of ConM and ConS was
674 studied using a nano-DSC calorimeter (TA instruments, Etten-Leur, The Netherlands)[39].
675 Briefly, trimers were first dialyzed against PBS and concentration adjusted to ~0.25 or
676 ~1.0 mg/mL, respectively. After sample loading, thermal denaturation was probed at a scan
677 rate of 60 °C/h. Buffer correction, normalization, and baseline subtraction procedures were
678 applied and data were analyzed using the NanoAnalyze Software v.3.3.0 (TA Instruments).
679 The data were fitted using a non-two-state model, as the asymmetry of some of the peaks
680 suggested that unfolding intermediates were present. DSC experiments were performed with

681 a D7324-tagged trimer, but the presence of the D7324-tag did not alter the T_m values
682 compared to the corresponding non-tagged trimers [39].

683 *Free amine assay*

684 Unmodified or cross-linked trimer (5 μ g) in 20 μ L PBS was added to 30 μ L of 0.1 M
685 NaHCO_3 , pH 8.5. 25 μ L of 5% 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) diluted 1/500 in
686 0.1 M NaHCO_3 pH 8.5 was added to the samples for 2 h at 37°C, followed by 25 μ L of 10%
687 SDS and 12.5 μ L of 1M HCl. Samples were vortexed and the optical density read at 335 nm.
688 The relative quantity of free amines was calculated as $(\text{OD}_{335}(\text{GLA-SOSIP trimer}) -$
689 $\text{OD}_{335}(\text{blank})) / (\text{OD}_{335}(\text{SOSIP trimer}) - \text{OD}_{335}(\text{blank}))$.

690 *Capture ELISA for Env trimer binding by human mAbs*

691 ELISA plates (Greiner Bio-One) were coated with 4 μ g/mL of capture mAb 2G12 at 4°C
692 overnight in PBS. After blocking with 2% BSA/PBS + 0.05% Tween, trimer (0.2 μ g/mL) were
693 captured, labelled with a titration series of biotinylated human mAbs followed by peroxidase-
694 conjugated streptavidin detection reagent (Jackson ImmunoResearch). The colorimetric
695 endpoint was obtained using the one-step ultra TMB substrate (ThermoFisher). MAb were
696 developed until a signal of approximately 1–2 optical density (OD_{450}) units was generated for
697 each antibody, leading to longer incubation periods for non-Nabs, and colour development was
698 stopped with sulfuric acid (0.5 M) and the $\text{OD}_{450-570}$ measured. All ELISA signals were
699 corrected by subtracting the background signal obtained in the absence of primary antibody
700 and the resulting data were plotted against the \log_{10} of the antibody concentration using
701 GraphPad Prism V7.0. To generate binding indices from ELISA titration curves, an area under
702 the curve (AUC) analysis of ligand-trimer binding was performed; the binding index represents
703 the ratio of cross-linked trimer value to the value of the matched unmodified SOSIP trimer that
704 was used for cross-linking. Binding indices were calculated as $(\text{AUC}(\text{GLA-SOSIP trimer}) -$
705 $\text{AUC}(\text{blank})) / (\text{AUC}(\text{SOSIP trimer}) - \text{AUC}(\text{blank}))$, where blank = negative control curve of the
706 respective mAb without antigen. Indices <1 indicate reduced binding to the cross-linked trimer
707 compared to its unmodified counterpart, and the converse for values >1.

708 *Env trimer ELISA for mouse sera*

709 Detection of mouse trimer-specific serum antibodies was performed using endpoint titer
710 ELISA. 2G12 antibody (4 μ g/mL, 50 μ L/well) was captured overnight at 4°C onto high-protein-
711 binding ELISA plates (Spectraplate 96HB, Perkin Elmer). Plates were washed in PBS/Tween
712 (0.05% v/v) and wells blocked using BSA (2% w/v; 200 μ L/well) for 1 h at RT, and washed.
713 Unmodified trimer (0.2 μ g/mL, 50 μ L/well) was added for 2 h at RT and washed as before.
714 Mouse serum samples diluted in PBS/BSA (1% w/v) starting at 1:100 then stepwise 5-fold

715 were added to the ELISA plates (50 μ L/well) and incubated overnight at 4°C. Plates were
716 washed and Peroxidase-conjugated rabbit anti-mouse IgG antibody (1:5000; 50 μ L/well,
717 Jackson Immunoresearch) added to all wells for 1 hr at RT. Plates were washed and TMB
718 substrate (50 μ L/well, Thermofisher Scientific) added to all wells. Colour development was
719 monitored and terminated after 10 mins using sulphuric acid (0.5 M; 50 μ L/well). Optical density
720 (OD) values for each well were calculated as OD_{450-570nm}. Background values (OD_{no serum}) were
721 subtracted from sample readings. Endpoint titers were calculated using non-linear regression
722 curve fitting and interpolated values transformed into log₁₀ endpoint titers (Graphpad Prism 7
723 for Mac).

724 *Biolayer interferometry (BLI) trimer antigenicity analysis*

725 The GMP trimer constructs in this study are devoid of tags, therefore Fab fragments were
726 loaded onto anti-Human Fab CHI (FAB2G) biosensors and dipped into varying concentrations
727 (in nM: 2000, 1000, 500, 250, 125, 62.5, 31.25) of trimer using an OctetRed 96 instrument
728 (ForteBio). Trimers and Fabs were diluted into 1X kinetics buffer (PBS pH 7.4 + 0.01% BSA,
729 0.002% Tween-20), and allowed to reach room temperature before beginning the assay.
730 Association was measured for 600 seconds, followed by dissociation for 1200 seconds in 1X
731 kinetics buffer. A reference well containing kinetics buffer was subtracted from each dataset,
732 curves were aligned on the y axis using the baseline step, and an inter-step correction was
733 applied between the association and the dissociation curves. A 1:1 binding model, which
734 assumes first-order kinetics and each binding site on the SOSIP binds to immobilized Fab at
735 an equal rate, was fitted to the data and used to determine kinetic parameters.

736 *Analysis of elastase and CathepsinG activity on trimers*

737 To test Env stability in the presence of neutrophil-released proteases, 0.01 Units of elastase
738 or cathepsinG (Sigma) was added to 1 μ g of each trimer in a final volume of 11.1 μ L PBS and
739 incubated at 37° C for 1 or 24 h. A negative control, containing no enzyme, also underwent a
740 24 h incubation period at 37° C. After protease exposure the samples were analysed by
741 reducing and denaturing polyacrylamide gel electrophoresis on 4-12% bis-tris gels (Life
742 Technologies). Protein bands were developed with Simply Blue Safe Stain (Life Technologies)
743 and band intensity analysed on Biorad GelDoc XR with Lab Image software. Band intensity of
744 the untreated control was set at 100% and all other samples were normalised to that.

745

746 *TZM-bl neutralization assay*

747 Infectious molecular clones (IMC) ConM and ConS [4] were produced in HEK293T cells, titered
748 and used in TZM-bl assay to determine nAb responses as previously described[55]. Briefly,

749 duplicates of six steps of 3-fold dilution, starting with 1:20 of each serum, were incubated with
750 viral supernatant (at relative luminescence units (RLU) between 150.000 and 200.000) for 1 h.
751 Thereafter, 10^4 TZM-bl cells were added, and plates incubated for 48 h at 37°C, after which
752 Bright-Glo Luciferase assay system (Promega, Madison, Wisconsin, USA) was added to
753 measure luciferase activity with a Mithras luminometer (Berthold, Germany). Positive controls
754 were sera of HIV-1-infected individuals and monoclonal antibodies with known neutralizing
755 titers. Neutralization titers were defined as the sample dilution at which RLU were reduced by
756 50% compared to virus control wells after subtraction of background RLU in control wells with
757 only cells. Inhibitory concentrations (IC) 50 were calculated with a linear interpolation method
758 using the mean of the duplicate responses [55].

759 *T cell cytokine responses*

760 Spleens were harvested from mice 4 weeks after the final boost, dissected using aseptic
761 technique, and single cells isolated by passing through a 100 μ m filter. Splenocytes were
762 resuspended in supplemented RPMI (10% fetal bovine serum, 10 mM HEPES, 2 mM
763 Glutamax, 1x Penicillin-Streptomycin, 50 μ M 2-ME) and plated at 500,000 splenocytes per well
764 in 96 U-bottomed plates in a final volume of 200 μ L. Splenocytes were either pulsed with
765 relevant antigen at 50 μ g/mL or no antigen as a control. On day 3, 100 μ L supernatant was
766 harvested and frozen at -80 °C for cytokine analysis. The concentration of IL-2 in 2x diluted
767 supernatants was measured using an IL-2 mouse ELISA kit (Thermo Fisher Scientific) as per
768 the manufacturer's instructions. The concentration of IL-2, IFN γ , IL-5, IL-13 and IL-17a in
769 supernatants was measured using a Luminex multiplex assay (R&D) performed according to
770 the manufacturer's instructions. Briefly, supernatant was diluted 1:2 in RD1W buffer, whilst the
771 standards were prepared as instructed. 50 μ L of standard or diluted supernatant was pipetted
772 into the wells of a black 96-well plate and 50 μ L of magnetic-bead cocktail was added to each
773 well. The plates were then incubated for 2 h on a shaker set to 800 rpm. The supernatant was
774 removed and wells washed 3x with 300 μ L of wash buffer using a magnetic plate washer. 50
775 μ L of biotin-labelled secondary antibody, diluted as per the instructions was added to the wells
776 and the plate returned to the shaker for 1 h. The plates were washed again before a 30 min
777 incubation with streptavidin-PE, washed and the plates read on a Luminex Bio-Plex (Bio-Rad).

778 *Negative Stain EM*

779 GMP SOSIPs were diluted in TBS pH 7.4 to ~0.02 mg/mL, applied to glow-discharged copper
780 EM grids containing a continuous carbon film, then negatively stained with 2% uranyl formate
781 for ~10 seconds. Micrographs were collected on a Talos 200C transmission electron
782 microscope (ThermoScientific) operated at 200 keV, at a nominal magnification of 73,000X
783 resulting in a pixel size of 1.98Å. CTF estimation was performed with CTFFIND4 [56], and then

784 all subsequent processing steps were carried out in RELION-3 [57]. Particles were picked with
785 the RELION Gaussian picker, and an initial 2D classification was performed to eliminate noise
786 and non-protein particle picks. A second round of classification was then used to determine
787 the percent of trimeric and properly folded SOSIPs within each GMP sample. The percent well-
788 folded trimers was reported as the number of particles contained within 2D classes of trimeric
789 SOSIPs, divided by the total number of particles in the particle stack.

790 *Cryo-electron microscopy sample preparation*

791 Unmodified and cross-linked GMP SOSIP trimers were incubated with a 6-fold molar excess
792 of PGT122 Fab overnight at 4°C. Excess Fab was removed by ultrafiltration in TBS pH 7.4
793 with a 100 kDa cutoff Centricon filter (Amicon Ultra, Millipore), and the sample was
794 concentrated to ~5 mg/mL for cryo grid freezing. Immediately before vitrification, *n*-Dodecyl β -
795 D-maltoside (DDM) was added to a final concentration of 0.25 mM, which improved the angular
796 distribution of the protein particles in vitreous ice. 3 μ L of this mixture was applied to Quantifoil
797 R1.2/1.3 holey carbon Cu 400 mesh grids, and blotted and plunge-frozen in liquid ethane with
798 the Vitrobot Mark IV.

799 *Cryo-electron microscopy data collection*

800 Micrographs were collected on a Titan Krios (ThermoScientific) operating at 300 keV coupled
801 with a Gatan K2 direct electron detector via the Legion interface[58]. Each exposure image
802 was collected in counting mode at 29000X nominal magnification resulting in a pixel size of
803 1.03 Å/pixel, using a dose rate of ~5.5 e-/pix/sec, and 200 ms exposure per frame. The total
804 dose on the sample for each movie micrograph was 50 e-/Å², and the nominal defocus range
805 used was -1.0 to -2.2 μ m. These imaging conditions were kept consistent for each of the four
806 samples (ConS, ConS-EDC, ConM, and ConM-EDC).

807 *Cryo-electron microscopy data processing*

808 Movie micrograph frames were aligned and dose-weighted using MotionCorr2 [59], and
809 imported into cryoSPARC v2 [60]. CTF models were calculated using CTFFIND4. The
810 cryoSPARC blob picker was used on a subset of micrographs to generate an initial set of
811 particle picks, which were subjected to 3 rounds of 2D classification. High quality and
812 representative classes were selected and used as 2D templates for template picking on the
813 entire dataset. The resulting particle images were subjected to multiple rounds of 2D
814 classification, followed by one round of *ab initio* reconstruction to generate an initial model.
815 This map was used as an initial model for one round of heterogeneous refinement, specifying
816 two or four classes. For each data set there was no observable heterogeneity, but this step
817 served to further eliminate low resolution particles. Particles from the dominant class(es) were

818 re-extracted with a box size of 352 pixels, and subjected to homogeneous refinement. Next,
819 iterative rounds of global CTF refinement followed by homogenous refinement were performed
820 until the resolution had converged, followed by a final round of non-uniform refinement. The
821 ConS data showed potential to reach higher resolution, therefore these data were first
822 processed in RELION according to standard single particle workflows in order to perform
823 Bayesian polishing. This map reached a resolution of 3.3Å, but PGT122 Fab density was
824 poorly resolved. Polished particles were extracted and imported into cryoSPARC v2, and the
825 same workflow as described above was performed for this particle stack. This procedure led
826 to a very high-quality reconstruction throughout the complex, with a final resolution of 3.1Å.
827 For unmodified ConM, higher resolution was achieved by importing frames into cryoSPARC2
828 and using the native patch motion correction utility. This allowed for local (single particle)
829 motion to be corrected, which improved the resolution and led to the final 3.4Å reconstruction.
830 For EDC cross-linked ConS and ConM, these procedures led to no further improvements, thus
831 for these data pre-aligned and dose-weighted micrographs were imported, as described
832 above. All resolutions are reported according to the “gold standard” 0.143 FSC cut-off [61].

833 *Model building*

834 The initial model for ConM-PGT122 comprised the ConM SOSIP coordinates from the ConM-
835 PGT122-35022 crystal structure (PDB 6IEQ), and the PGT122 Fab coordinates from a crystal
836 structure of BG505-PGT122-35022 (PDB 4TVP). These coordinates served as the template
837 for complete rebuilding and all-atom refinement with RosettaCM [62], which couples the
838 fragment-based *de novo* Rosetta builder with sequence and structural constraints from the
839 template(s), as well as a user-defined electron density weight. The lowest energy model was
840 selected and glycans were added to the model in Coot [63], which was then iteratively refined
841 in real-space with Coot and PHENIX [64]. Once the model had converged, disordered regions
842 were removed and a final high resolution, all-atom refinement with Rosetta Relax [65] was
843 performed. This ConM-PGT122 cryo-EM structure then served as the initial model for both
844 ConM-EDC and unmodified ConS, which followed a nearly identical procedure: full model
845 rebuilding with RosettaCM, real-space refinement in Coot and PHENIX, and final refinement
846 with Rosetta Relax. This same procedure was also used for the ConS-EDC structure, except
847 that the unmodified ConS structure was used as the initial model. Structure validation was
848 performed with MolProbity [66], PHENIX, and EMRinger [67].

849 *RMSD analysis*

850 Alpha carbon ($C\alpha$) root-mean-square deviation (RMSD) was calculated by superimposition of
851 structures in UCSF Chimera [68]. This was performed globally and locally, in order to
852 differentiate global conformational changes from local changes in structure of specific
853 epitopes. Values for global RMSD are listed in Figures 2B-E, and Supplementary Figure 7.

854 The local C α RMSD was mapped onto the cryo-EM structures in Figure 2B-E using UCSF
855 Chimera.

856

857 *Mouse immunizations*

858 All experiments used 8–12 week old female BALB/c mice (Charles River) maintained under
859 specific pathogen-free conditions in the Sir William Dunn school facility. In two independent
860 experiments 4 or 5 mice per group were immunized by subcutaneous administration with 10
861 μ g trimer formulated in 100 μ L PBS/MPLA (10 μ g/dose) formulation on weeks 0, 4 and 8. Mice
862 were monitored for adverse symptoms throughout. Blood was collected by tail bleed on weeks
863 -1, 2, 6, 10 and 12, serum separated and stored at -20°C.

864 *Rabbit immunizations, IgG purification and ELISA*

865 A total of 30 New Zealand White Specific Pathogen Free (SPF) rabbits (23 males and 23
866 females (were nulliparous and non-pregnant)), 11 weeks old and weighing approximately 2 kg,
867 were immunised intramuscularly at the Research Toxicology Centre S.p.A, Italy. Each study
868 group (detailed in Supplementary Table 3) consisted of 3 male and 3 female animals. The
869 rabbits received a total of 4 administrations (Weeks 0, 3, 6 and 9) into the lateral surface of the
870 quadriceps muscle of both legs. Rabbit serum was isolated from blood at 0, 3, 6, 9- and 12-
871 weeks first post-vaccination, heat inactivated, aliquoted and stored at -20°C prior to
872 assessment for antigen-specific IgG. Antigen-specific gp140 binding antibodies were
873 measured using standardised ELISA platforms. In serum samples, antigen-specific IgG was
874 measured. In brief, 96-well medium binding plates (Griener, Kremsmunster, Austria) were
875 coated with either recombinant ConM, ConM-edc, ConS or ConS-edc gp140 (Polymun
876 Scientific) at 1 μ g/mL in PBS for 1 h 37°C. As reference material, standard immunoglobulins
877 (Serotec, UK) were captured with anti-Rabbit specific goat antibodies (Millipore, UK). After
878 blocking with assay buffer (5% bovine serum albumin; Sigma–Aldrich; 0.05% Tween
879 ThermoFisher Scientific, Pittsburgh, PA), samples were initially screened at 1:100 dilution
880 (then titrated to optimal dilutions) on antigen-coated wells, and serial dilutions of
881 immunoglobulin standards were added to the anti-Rabbit capture antibody coated wells and
882 incubated for 1 h at 37°C. Secondary antibody HRP-conjugated anti-rabbit IgG was added at
883 1:20,000 dilution and incubated for 1 h at 37°C. Plates were developed with SureBlue TMB
884 substrate (KPL, Insight Biotechnology, London, United Kingdom). The reaction was stopped
885 after 5 min by adding TMB stop solution (KPL, Insight Biotechnology), and the absorbance
886 was read at 450 nm on a VersaMax 96-well microplate reader (Molecular Devices, Sunnyvale,
887 CA). The ELISA data are expressed as positive if the blank subtracted OD450 nm was above
888 the predetermined cut-off of OD 0.2 nm and values are on the linear range of the curve. To

889 ensure assay sensitivity, a positive control composed of gp140 positive pooled rabbit serum
890 samples and a negative control composed of anti-Ad4 Rabbit Hypersera (PAXVAX, San Diego,
891 US) were used. Analyses of the data were performed using SoftMax Pro GxP software v6.5
892 (Molecular Devices).

893 *Ethics statement*

894 Animal research using rabbits and mice was carried out in full accordance with local and
895 national ethical guidelines. All protocols for breeding and procedures with mice were approved
896 by the Home Office UK, under the Animals (Scientific Procedures) Act 1986 and Home Office
897 license PPL3003421. Rabbit studies were carried out at Covance Inc.

898 *Statistical analyses*

899 Statistical analysis was performed in Prism using the tests described in the corresponding
900 figure legends. Briefly, one-way ANOVA of log-transformed data with Sidak's post-test
901 correction to account for multiple comparisons were used to analyze normally-distributed data
902 including log-transformed endpoint titers. Non-parametric analysis (not assuming a Gaussian
903 distribution) between two independent groups was performed using a two-tailed Mann-Whitney
904 U test and an unmatched, unpaired Kruskal-Wallis test with Dunn's multiple comparison test
905 was used to compare non-normally distributed data with more than one comparison.

906

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920

921 **Author contributions**

922 Gregory Martin, Conceptualization of the work, Data collection, Data analysis and
923 interpretation, Writing – original draft, writing - review and editing, Final approval of the
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925
926 Rebecca A Russell, Conceptualization of the work, Data collection, Data analysis and
927 interpretation, Supervision, Writing – review and editing, Final approval of the version to be
928 published.
929
930 Philip Mundsperger, Data collection, Data analysis and interpretation, Writing – review and
931 editing, Final approval of the version to be published.
932
933 Scarlett Harris, Data collection, Data analysis and interpretation, Writing – review and editing,
934 Final approval of the version to be published.
935
936 Lu Jovanoska, Data collection, Data analysis and interpretation, Writing – review and editing,
937 Final approval of the version to be published.
938
939 Luiza Farache Trajano - data collection, data analysis and interpretation
940
941 Leon Macfarlane - Data collection, Data analysis and interpretation, Final approval of the
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943
944 Hannah Cheeseman, Data collection, Data analysis and interpretation, Writing – review and
945 editing, Final approval of the version to be published.
946
947 Katalin Fabian, Data collection, Data analysis and interpretation, Writing – review and
948 editing, Final approval of the version to be published.
949
950 Monica Tolazzi, Data collection, Data analysis and interpretation, Writing – review and
951 editing, Final approval of the version to be published.
952
953 Marielle Breemen, Data collection, Data analysis and interpretation, Writing – review and
954 editing, Final approval of the version to be published.
955
956 Torben Schiffner, Conceptualization of the work, Writing – review and editing, Final approval
957 of the version to be published.
958

959 Kwinten Sliepen, Conceptualization of the work, Data collection, Data analysis and
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962
963 Yoann Aldon, Conceptualization of the work, Writing – review and editing, Final approval of
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965
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967 Supervision, Writing – review and editing, Final approval of the version to be published.

968
969 Rogier W Sanders, Conceptualization of the work, Funding acquisition, Writing – review and
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971
972 Robin Shattock, Conceptualization of the work, Funding acquisition, Writing – review and
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974
975 Dietmar Katinger, Funding acquisition, Project administration, Supervision, Writing – review
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977
978 Andrew B Ward, Funding acquisition, Project administration, Supervision, Writing – review &
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980
981 Quentin J. Sattentau, Conceptualization, Formal analysis, Funding acquisition, Project
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984
985 **Data availability**
986 The final cryo-EM reconstructions and the resulting structural models have been deposited
987 into the Protein Data Bank (PDB) the Electron Microscopy Data Bank (EMDB) under the
988 following accession codes: **ConS**: PDB 7LX2, EMDB 23564; **ConS-EDC**: PDB 7LX3, EMDB
989 23565; **ConM**: PDB 7LXM, EMDB 23571; **ConM-EDC**: PDB 7LXN, EMDB 23572.

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1263 **Figure legends**

1264 **Figure 1. Biophysical and antigenic characterization of GMP EDC cross-linked ConM** 1265 **and ConS.**

1266 **A)** EDC cross-linker. **B)** Work-flow to GMP product. **C)** Free amine assay representing the
1267 ratio of free amines in EDC-modified : unmodified trimer. **D)** SDS-PAGE reducing gel. **E)** DSC
1268 analysis of trimer thermal stability. **F)** Antigenicity of trimers using panels of bnAbs and non-
1269 nAbs, where results represent ratio of binding modified : unmodified trimer and bars represent
1270 ± 1 SD (note 19b excluded from mean). **G)** Negative stain EM 2D classification analyses. **H)**
1271 Sensitivity of unmodified and EDC cross-linked trimers to neutrophil elastase and Cathepsin
1272 G (Cg) attack, n = 3. ns = not significant; * p<0.05; ** p<0.01; **** p<0.0001, Mann Whitney U.

1273

1274 **Figure 2. Cryo-EM analysis of ConM, ConM-EDC, ConS and ConS-EDC**

1275 **A)** Side views of unmodified ConS (red) and ConM (blue) in complex with PGT122 Fab
1276 (grey) at 3.1 Å and 3.4 Å respectively. Each cryo-EM map is shown at high (colored map)
1277 and low (light outline) threshold to highlight the PGT122 constant domain and N-linked
1278 glycans. **(B)** ConM and ConS structures were superimposed, and local $C\alpha$ RMSD was
1279 rendered onto the ConM structure with Chimera, according to color and to the thickness of
1280 the main chain cartoon representation. **(C)** Same as in **(B)** but comparing ConS-EDC and
1281 ConM-EDC structures rendered onto the ConS-EDC structure. **(D)** Same as in **(B)** but
1282 comparing ConS and ConS-EDC structures, rendered onto the ConS structure. **(E)** Same as
1283 in **(B)** but comparing ConM and ConM-EDC rendered onto the ConM structure.

1284

1285 **Figure 3. High resolution analysis of EDC-introduced cross-links**

1286 **A – C**, selected EDC crosslinks observed through comparison of modified and unmodified
1287 cryo-EM reconstructions. Each map is filtered to the same resolution (lower of the two maps)
1288 and the same B-factor is applied, and then visualized at the same contour level (σ ; see
1289 methods). The overall structure is shown in ribbon representation, and the side chains of every
1290 crosslink identified are shown as green spheres. **(A)** K46-D632 crosslink in ConS-EDC, which
1291 is a gp120-gp41 inter-subunit crosslink within the same protomer. The insets show a zoomed-
1292 in view of the K46-D632 crosslink, with cryo-EM density displayed as a mesh. **(B)** Same as in
1293 **(A)**, for the K617-E634 intra-subunit crosslink in ConS-EDC gp41. Note that the ribbon
1294 structure at left is rotated $\sim 90^\circ$ counterclockwise (as viewed from the top) from **(A)**. **(C)** K340-
1295 E290 intra-subunit crosslink in ConM-EDC gp120.

1296

1297 **Figure 4. Immunogenicity of unmodified and cross-linked trimers.**

1298 **A, B**) Mouse serum IgG responses to immunization with **(A)** ConM (M) or ConM-EDC (M-
1299 EDC), or **(B)** ConS (S) or ConS-EDC (S-EDC), assayed against unmodified (M or S) or
1300 crosslinked (M-EDC or S-EDC) versions of the protein, where $n=8$ mice, comprised of 4
1301 mice/group from 2 pooled independent experiments. $**p<0.01$, $****p<0.001$, One-way ANOVA
1302 with Sidak's multiple comparison correction. **C, D**) Mouse splenocyte antigen-specific cytokine
1303 release measured by multiplex bead array after unmodified ConM **(C)** or ConS **(D)** in vitro
1304 restimulation with homologous unmodified antigen. **E**) Endpoint ELISA titers of rabbit
1305 antiserum at week 12. **F, G**) Neutralization activity against homologous and heterologous
1306 ConM **(F)** and ConS **(G)** env pseudoviruses represented by the reciprocal serum dilution giving
1307 50% inhibition (IC_{50}). $*p<0.05$, $**p<0.01$, $****p<0.0001$, Mann Whitney U.
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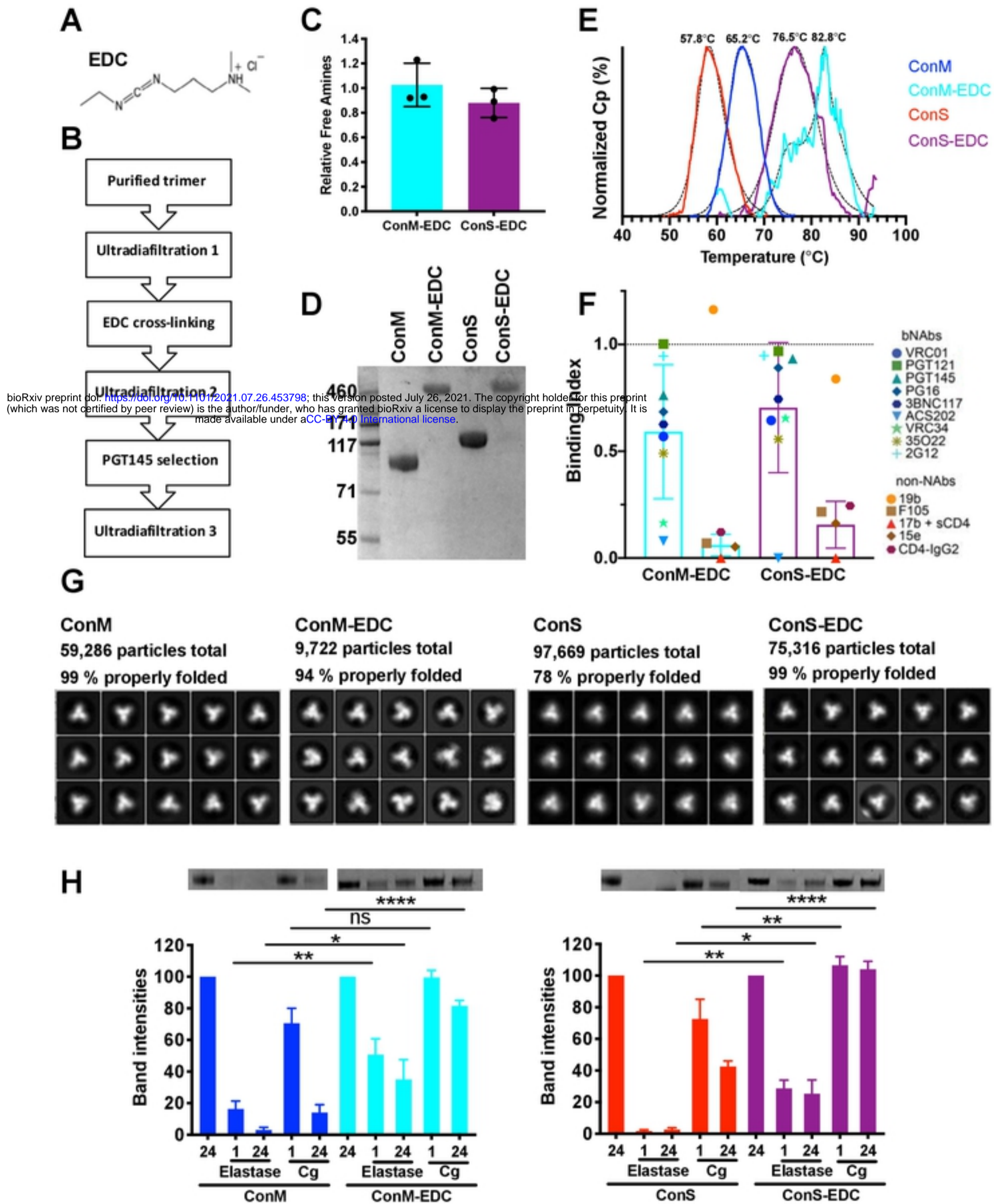


Figure 1. Biophysical and antigenic characterization of cross-linked GMP ConM and ConS. **A)** EDC cross-linker. **B)** Work-flow to GMP product. **C)** Free amine assay representing the ratio of free amines in EDC-modified : unmodified trimer. **D)** SDS-PAGE reducing gel. **E)** DSC analysis of trimer thermal stability. **F)** Antigenicity of trimers using panels of bnAbs and non-nAbs, where results represent ratio of binding modified : unmodified trimer. **G)** Negative stain EM 2D classification analyses. **H)** Sensitivity of unmodified and EDC cross-linked trimers to neutrophil elastase and Cathepsin G (Cg) attack, $n = 3$. ns = not significant; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, Mann Whitney U.

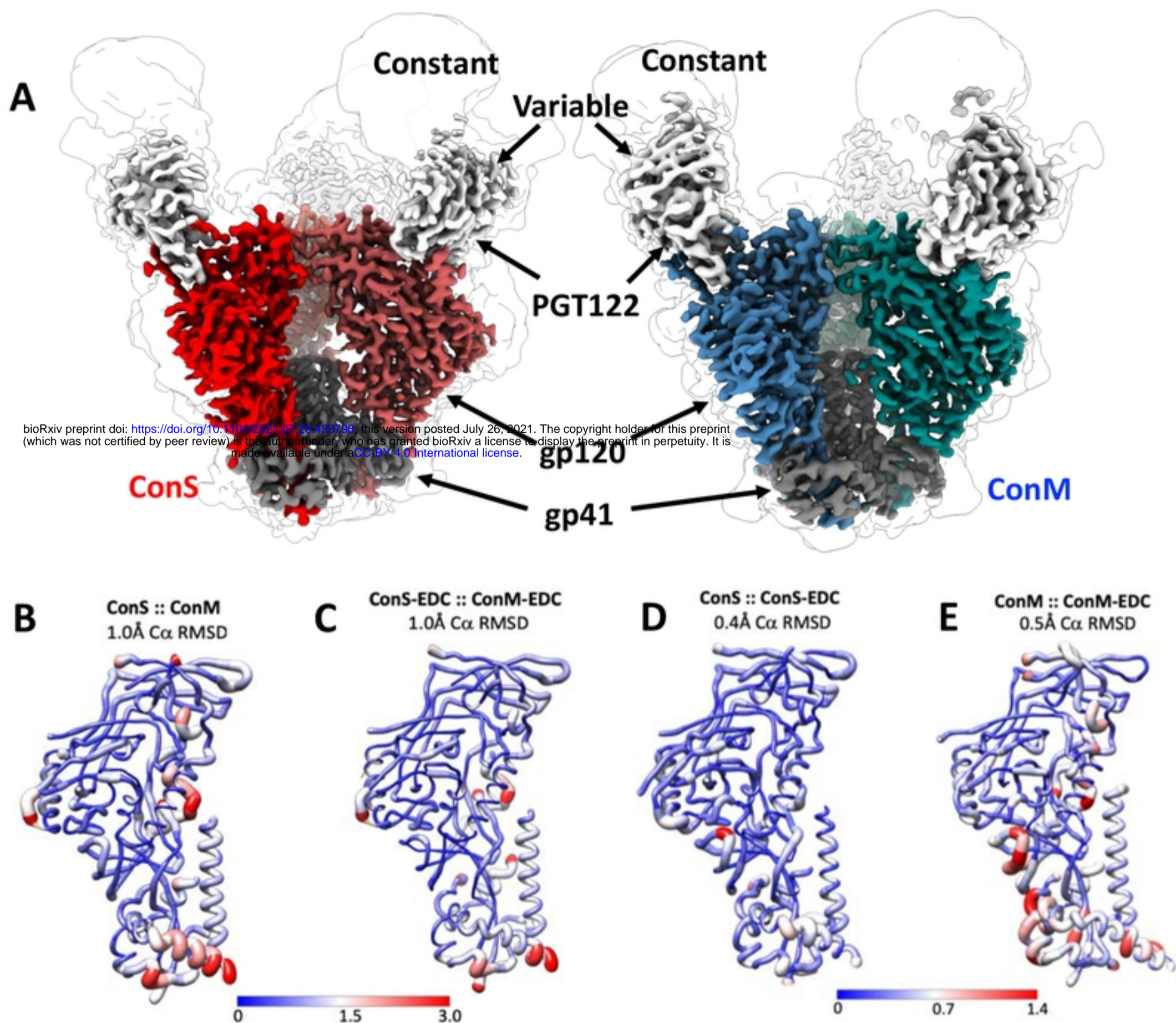
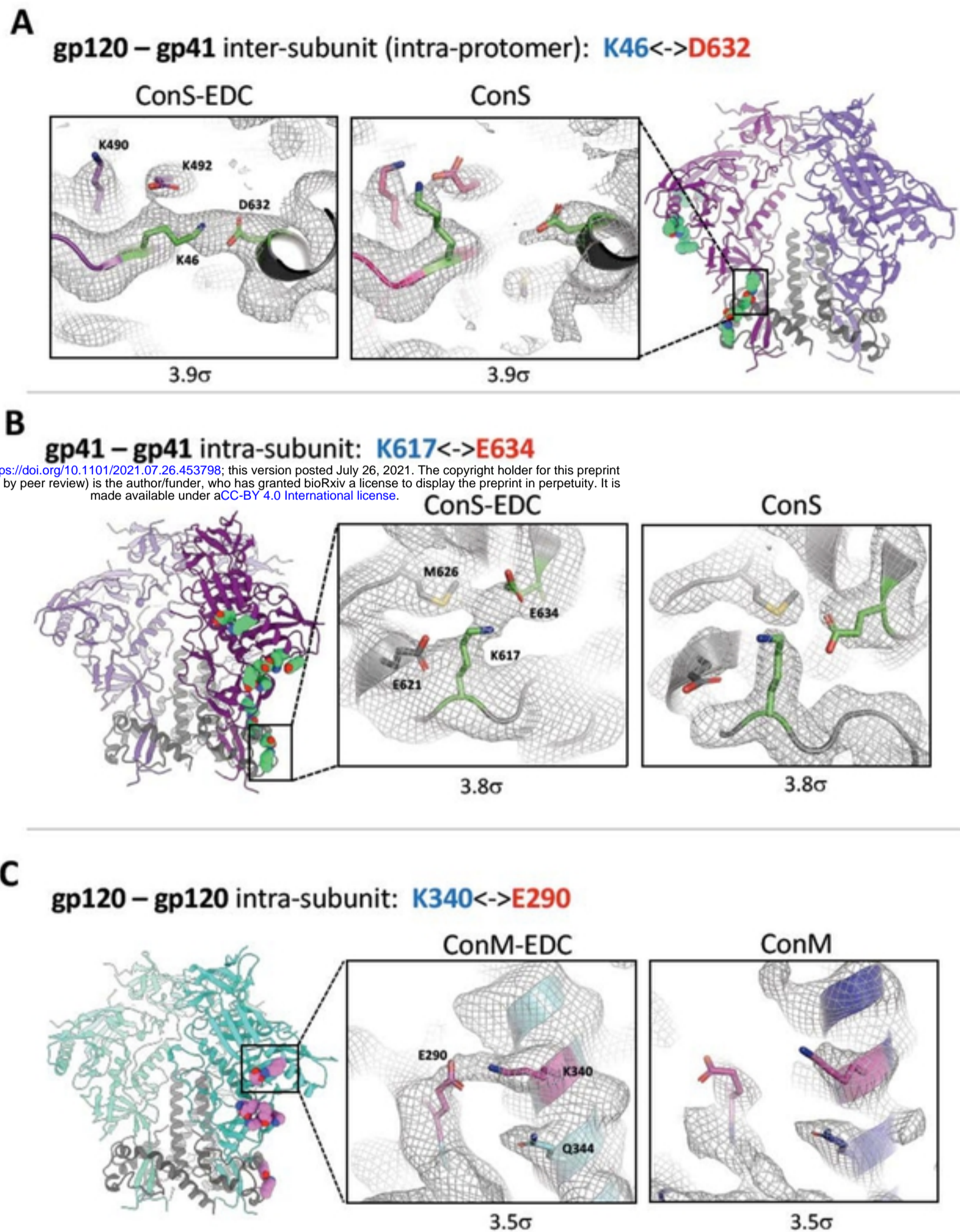


Figure 2. Cryo-EM analysis of ConM, ConM-EDC, ConS and ConS-EDC

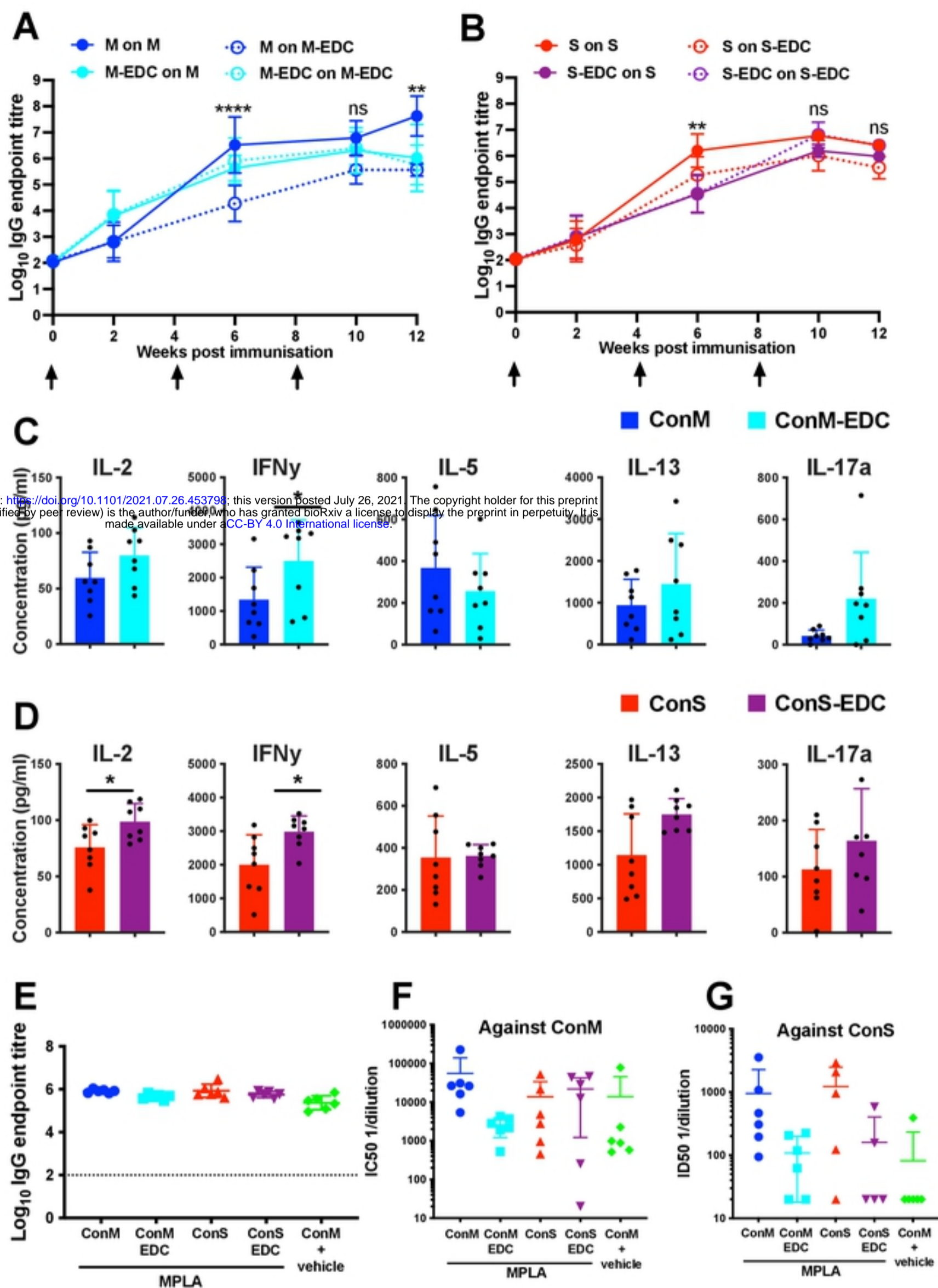
A) Side views of unmodified ConS (red) and ConM (blue) in complex with PGT122 Fab (grey) at 3.1 Å and 3.4 Å respectively. Each cryo-EM map is shown at high (colored map) and low (light outline) threshold to highlight the PGT122 constant domain and N-linked glycans. **(B)** ConM and ConS structures were superimposed, and local C α RMSD was rendered onto the ConM structure with Chimera, according to color and to the thickness of the main chain cartoon representation. **(C)** Same as in **(B)** but comparing ConS-EDC and ConM-EDC structures rendered onto the ConS-EDC structure. **(D)** Same as in **(B)** but comparing ConS and ConS-EDC structures, rendered onto the ConS structure. **(E)** Same as in **(B)** but comparing ConM and ConM-EDC rendered onto the ConM structure.



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Figure 3. Analysis of EDC-introduced cross-links.

Selected EDC crosslinks observed through comparison of modified and unmodified cryo-EM reconstructions. In **A-C** each map is filtered to the same resolution and the same B-factor is applied, and then visualized at the same contour level (σ ; see methods). The overall structure is shown in ribbon representation, and the side chains of every crosslink identified are shown as green spheres. **(A)** K46-D632 crosslink in ConS-EDC, which is a gp120-gp41 inter-subunit crosslink within the same protomer. The insets show a zoomed-in view of the K46-D632 crosslink, with cryo-EM density displayed as a mesh. **(B)** Same as in **(A)**, for the K617-E634 intra-subunit crosslink in ConS-EDC gp41. Note that the ribbon structure at left is rotated $\sim 90^\circ$ counterclockwise (as viewed from the top) from **(A)**. **(C)** K340-E290 intra-subunit crosslink in ConM-EDC gp120.



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Figure 4. Immunogenicity of unmodified and cross-linked trimers.

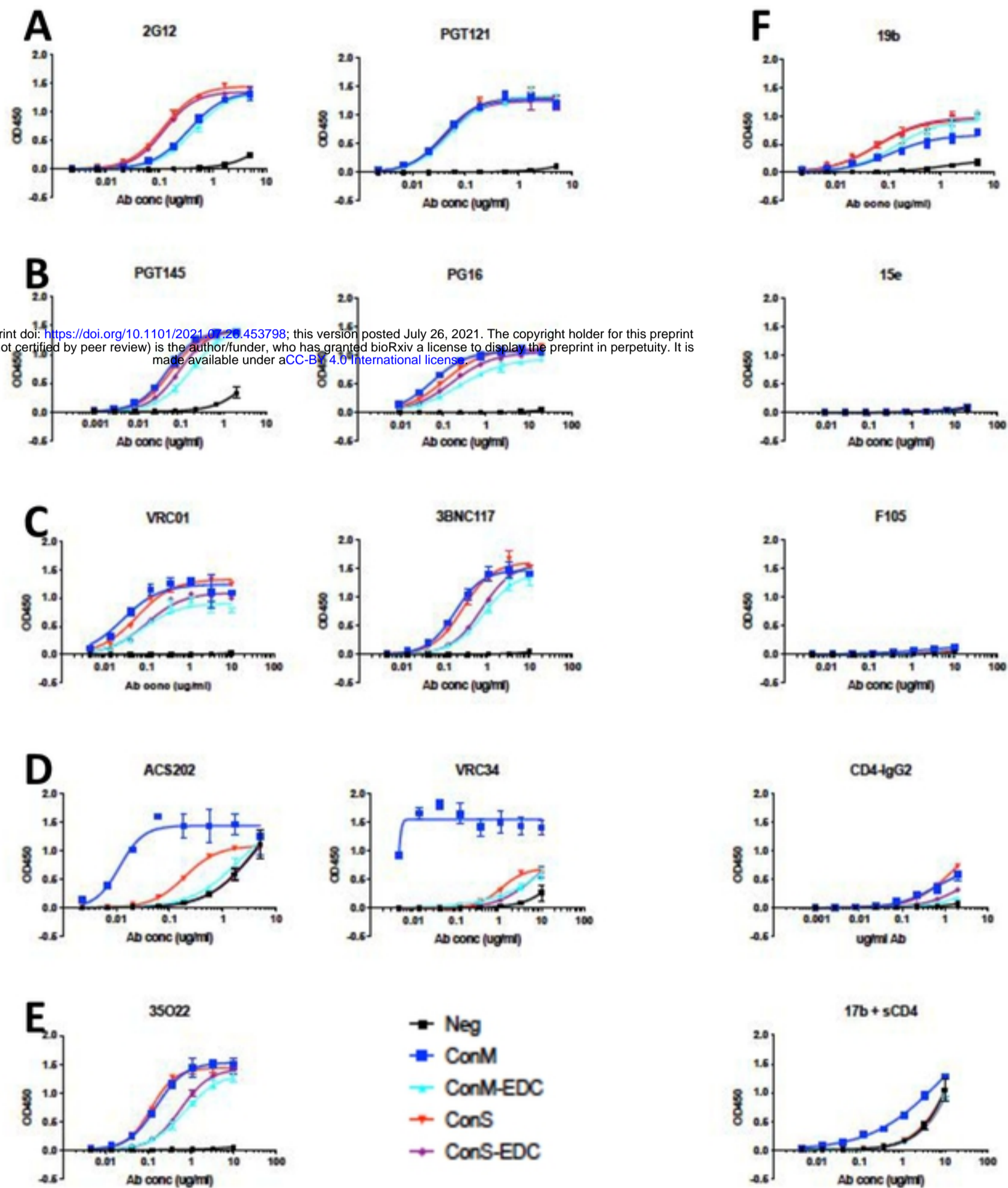
A, B) Mouse serum IgG responses to immunization with **(A)** ConM (M) or ConM-EDC (M-EDC), or **(B)** ConS (S) or ConS-EDC (S-EDC), assayed against unmodified (M or S) or crosslinked (M-EDC or S-EDC) versions of the protein, where $n=8$ mice, 4 mice/group from $n=2$ pooled independent experiments. Error bars = SD, ** $p<0.01$, **** $p<0.001$, one-way ANOVA with Sidak's multiple comparison correction. **C, D**) Mouse splenocyte antigen-specific cytokine release measured by multiplex bead array after homologous unmodified ConM **(C)** or ConS **(D)** in vitro restimulation. Data pooled from $n=2$ experiments, datum points represent individual mice. * $p<0.05$, Mann Whitney U. Error bars = SD. **E**) Endpoint ELISA titers of rabbit antiserum at week 12. **F, G**) Neutralization activity against homologous and heterologous ConM **(F)** and ConS **(G)** env pseudoviruses represented by the reciprocal serum dilution giving 50% inhibition (IC₅₀).

bnAb	trimer	KD (M)	Kon (1/ms)	Koff (1/s)
35022	ConM	3.9×10^{-7}	4.3×10^3	8.6×10^{-5}
	ConM-EDC	2.0×10^{-6}	7.4×10^1	8.3×10^{-5}
PGT122	ConM	$<1 \times 10^{-12}$	7.3×10^4	$<1 \times 10^{-7}$
	ConM-EDC	$<1 \times 10^{-12}$	7.8×10^4	$<1 \times 10^{-7}$
PGT145	ConM	3.0×10^{-8}	1.8×10^4	3.1×10^{-4}
	ConM-EDC	1.8×10^{-7}	3.7×10^3	5.1×10^{-4}
VRC01	ConM	1.5×10^{-7}	3.3×10^3	4.2×10^{-4}
	ConM-EDC	ND	ND	ND
VRC34	ConM	1.2×10^{-8}	1.9×10^5	2.2×10^{-3}
	ConM-EDC	3.2×10^{-6}	3.1×10^2	2.9×10^{-4}

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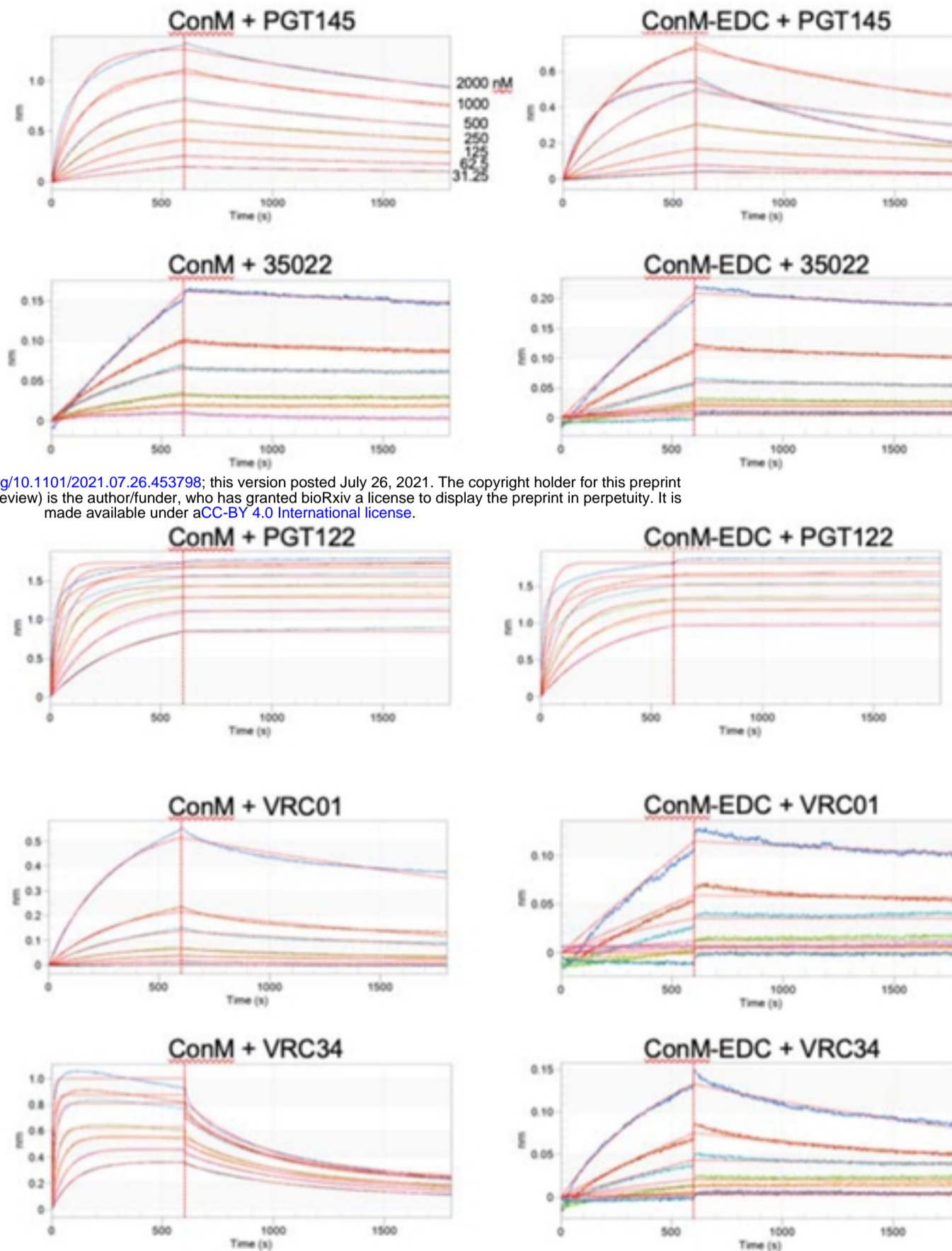
Table 1. Octet analysis of bnAb affinity for unmodified and EDC cross-linked trimers

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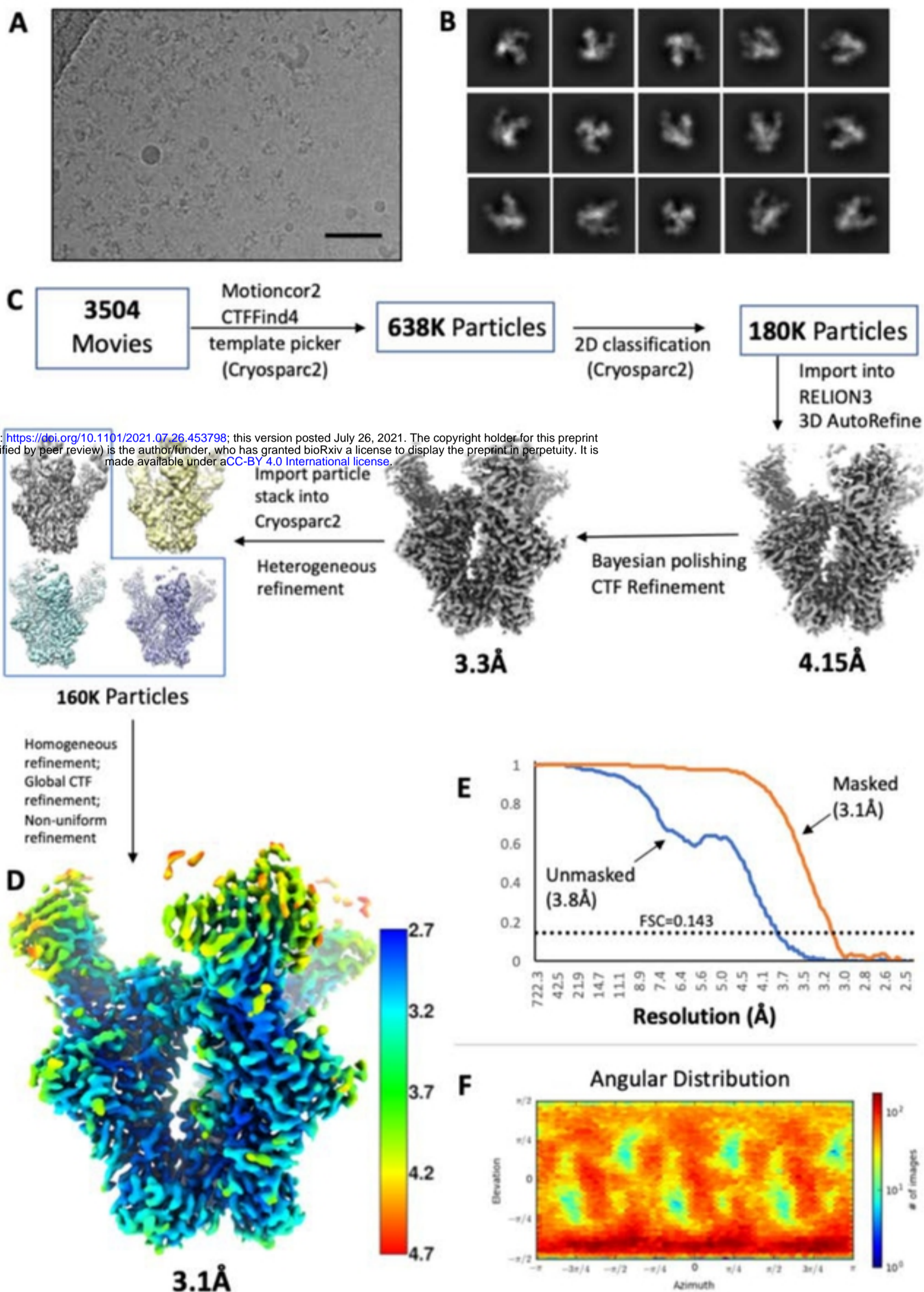
Supplementary Figure 1. ELISA binding curves associated with Figure 1F.

A) V3 glycan bnAbs; B) Apex quaternary bnAbs; C) CD4bs bnAbs; D) fusion peptide bnAbs; E) gp120-gp41 interface bnAb; F) non-nAbs and CD4-IgG2.



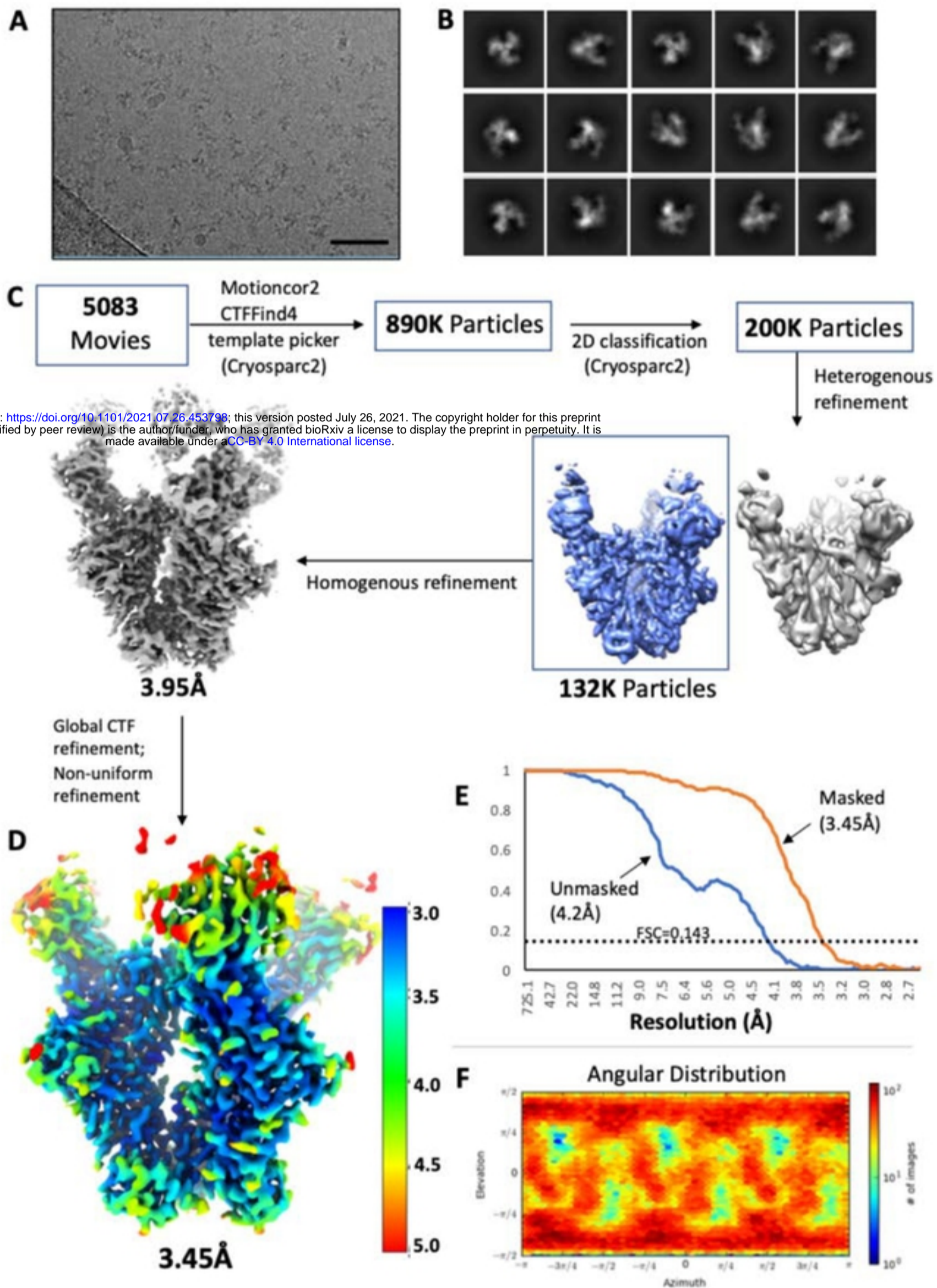
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Supplementary figure 2. Impact of EDC on binding of bNABs to ConM and ConM-EDC trimers assayed by biolayer interferometry, associated with Table 1. The GMP trimers in this study are untagged, thus bnAb Fab fragments were immobilized to anti-human Fab-CHI biosensors, and binding was analyzed at a range of SOSIP concentrations (2000, 1000, 500, 250, 125, 62.5, 31.25 nM). The aligned and reference-subtracted binding response (colored traces) was fitted to a 1:1 binding model (red lines) to determine kinetic parameters. SOSIP concentrations which gave poor fits ($R^2 < 0.95$) were not used for the kinetic analysis. In each case the final values were the average of at least three trimer concentrations.



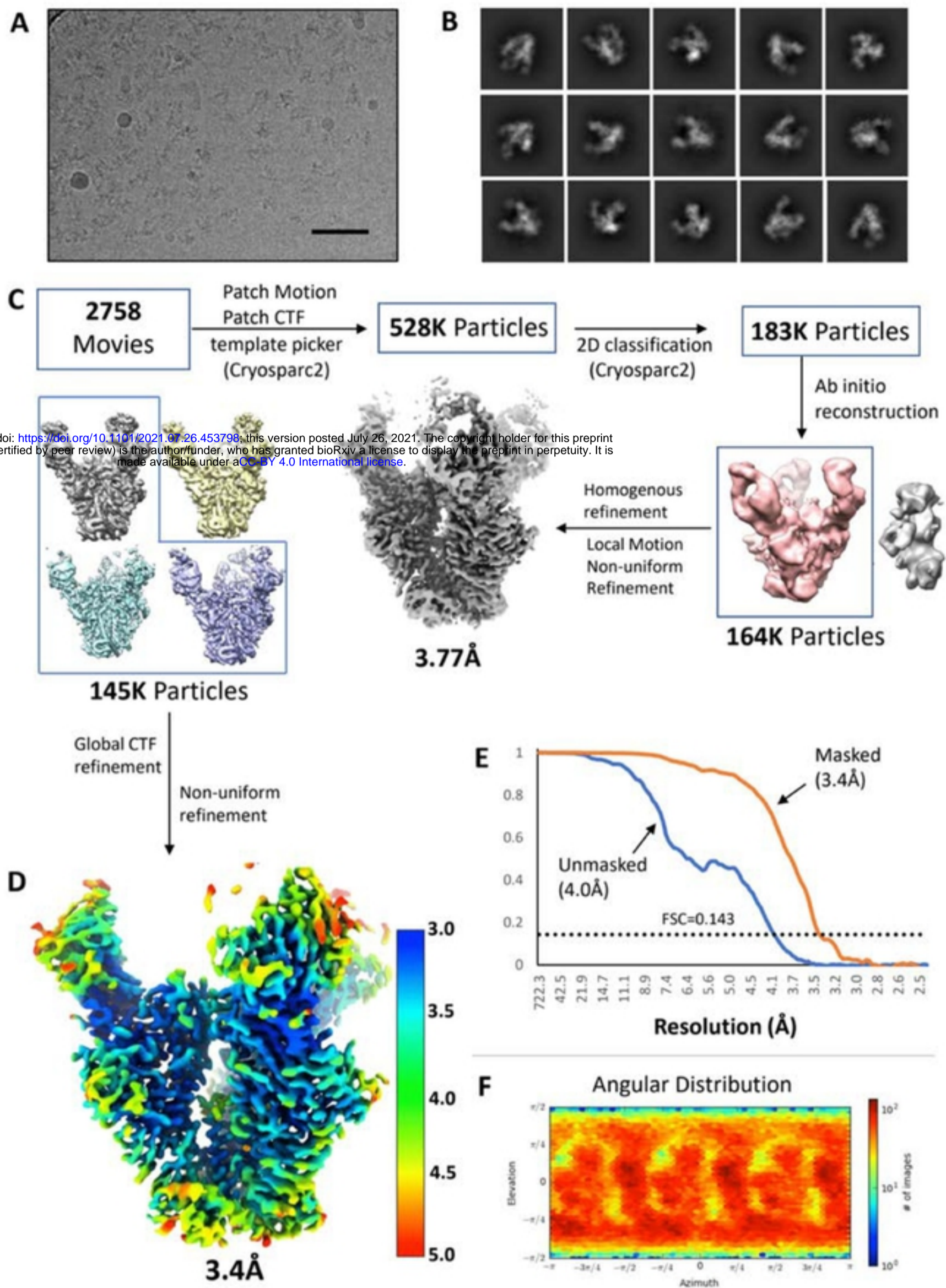
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Supplementary figure 3. Cryo-EM data processing workflow and reconstruction for unmodified ConS. (A) Motion-corrected micrograph from Titan Krios (1.03 Å/pixel). **(B)** Select 2D classes from Cryosparc v2. **(C)** Data processing workflow. **(D)** Final reconstruction and local resolution, calculated in Cryosparc v2. **(E)** Fourier shell correlation. Reported resolutions are according the 0.143 FSC gold standard. **(F)** Angular distribution plot of final reconstruction, from Cryosparc v2.



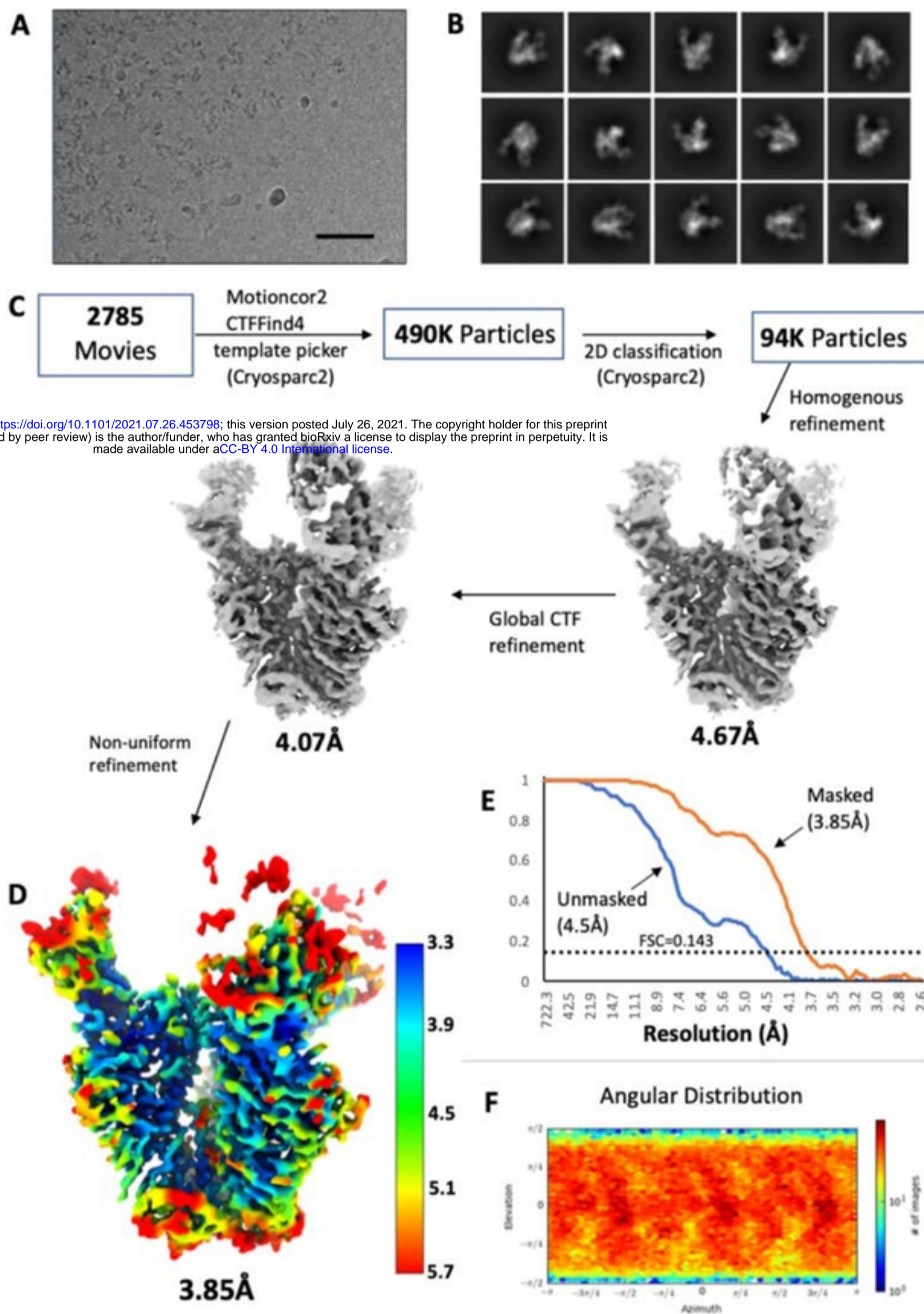
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Supplementary figure 4. Cryo-EM data processing workflow and reconstruction for EDC-crosslinked ConS. (A) Motion-corrected micrograph from Titan Krios (1.03 Å/pixel). (B) Select 2D classes from Cryosparc v2. (C) Data processing workflow. (D) Final reconstruction and local resolution, calculated in Cryosparc v2. (E) Fourier shell correlation. Reported resolutions are according the 0.143 FSC gold standard. (F) Angular distribution plot of final reconstruction, from Cryosparc v2.



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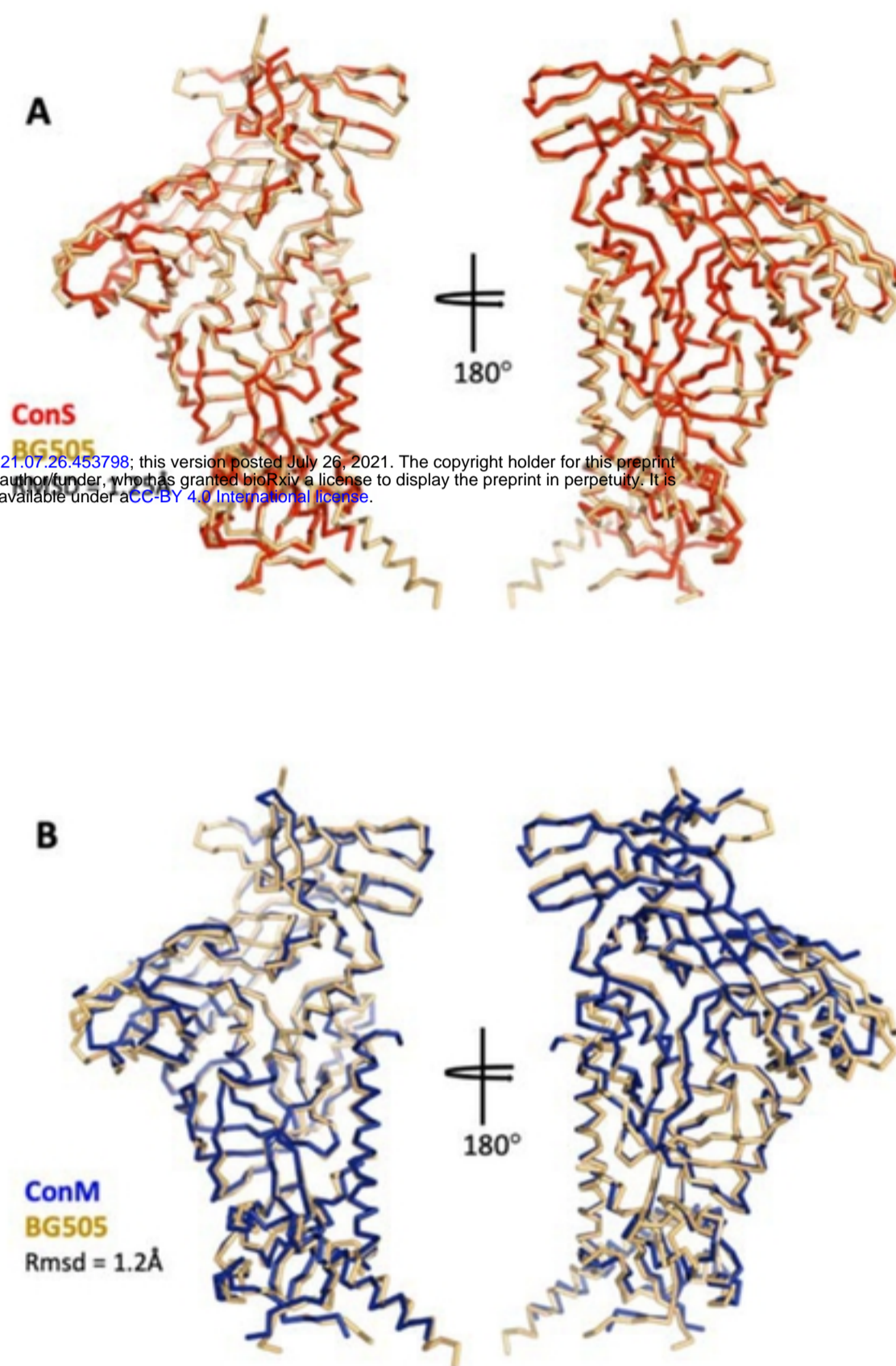
Supplementary figure 5. Cryo-EM data processing workflow and reconstruction for ConM. (A) Motion-corrected micrograph from Titan Krios (1.03 Å/pixel). (B) Select 2D classes from Cryosparc v2. (C) Data processing workflow. (D) Final reconstruction and local resolution, calculated in Cryosparc v2. (E) Fourier shell correlation. Reported resolutions are according the 0.143 FSC gold standard. (F) Angular distribution plot of final reconstruction, from Cryosparc v2.



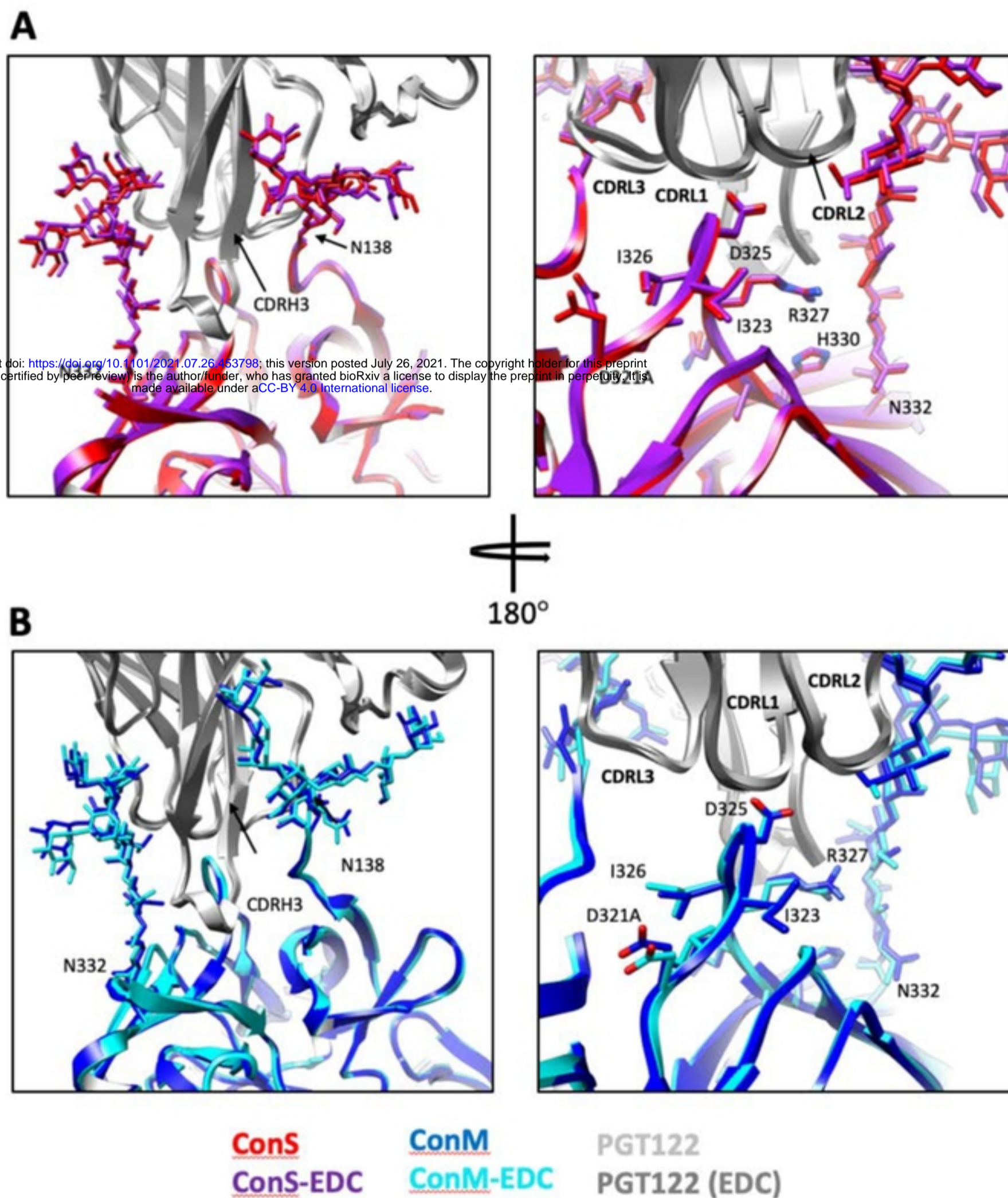
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Supplementary figure 6. Cryo-EM data processing workflow and reconstruction for EDC cross-linked ConM. (A) Motion-corrected micrograph from Titan Krios (1.03 Å/pixel). **(B)** Select 2D classes from Cryosparc v2. **(C)** Data processing workflow. **(D)** Final reconstruction and local resolution, calculated in Cryosparc v2. **(E)** Fourier shell correlation. Reported resolutions are according the 0.143 FSC gold standard. **(F)** Angular distribution plot of final reconstruction, from Cryosparc v2.

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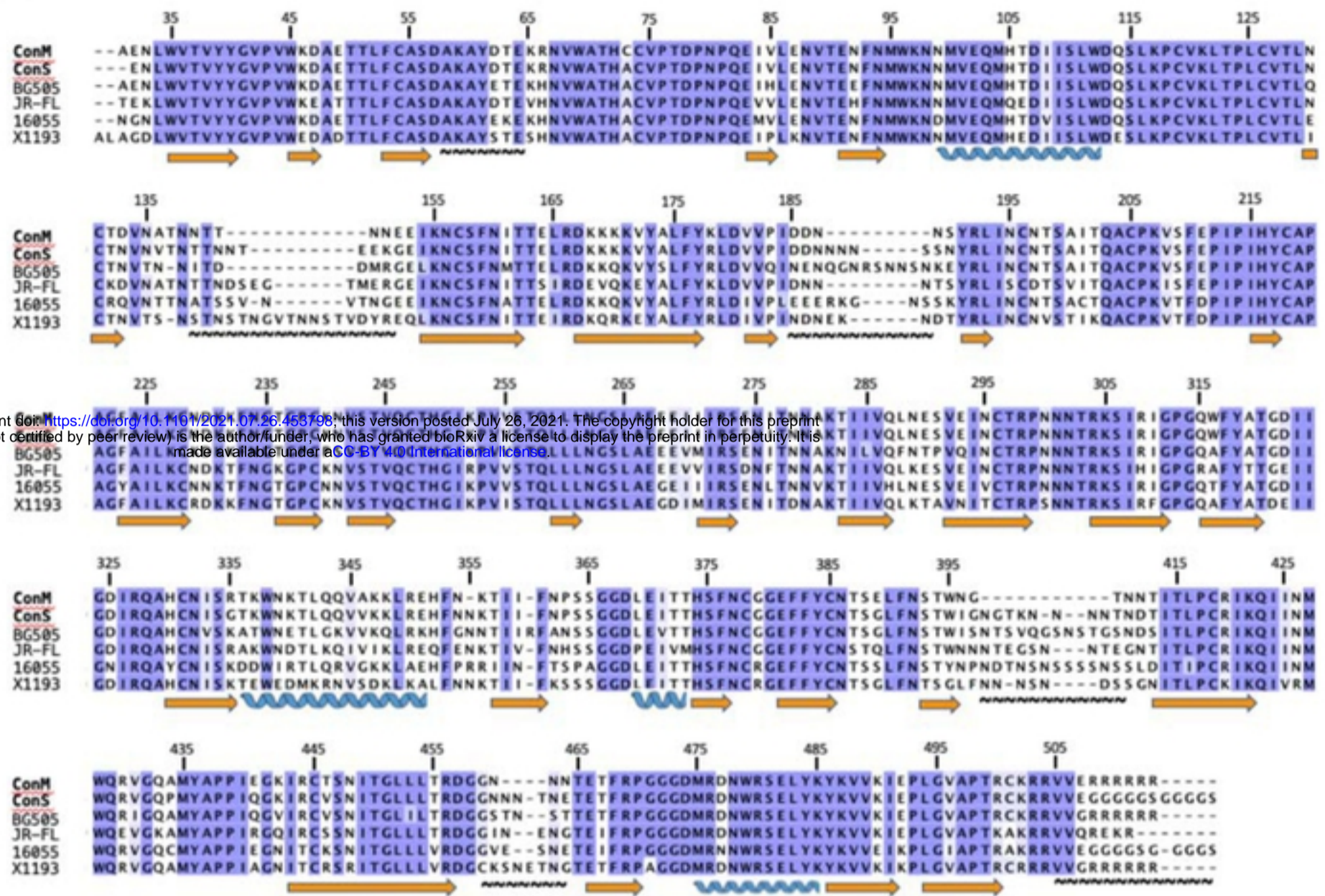


Supplementary Figure 7. Structural comparisons of ConM and ConS with BG505. (A) Superimposition of a single protomer of the ConS-PGT122 cryo-EM structure with the BG505-PGT122-35022 X-ray structure (PDB 4TVP). Shown is a ribbon representation of the C α chain trace. The C α RMSD is 1.25Å. **(B)** Same as in **(A)** but comparing BG505 with the ConM-PGT122 cryo-EM structure. The C α RMSD is 1.2Å.

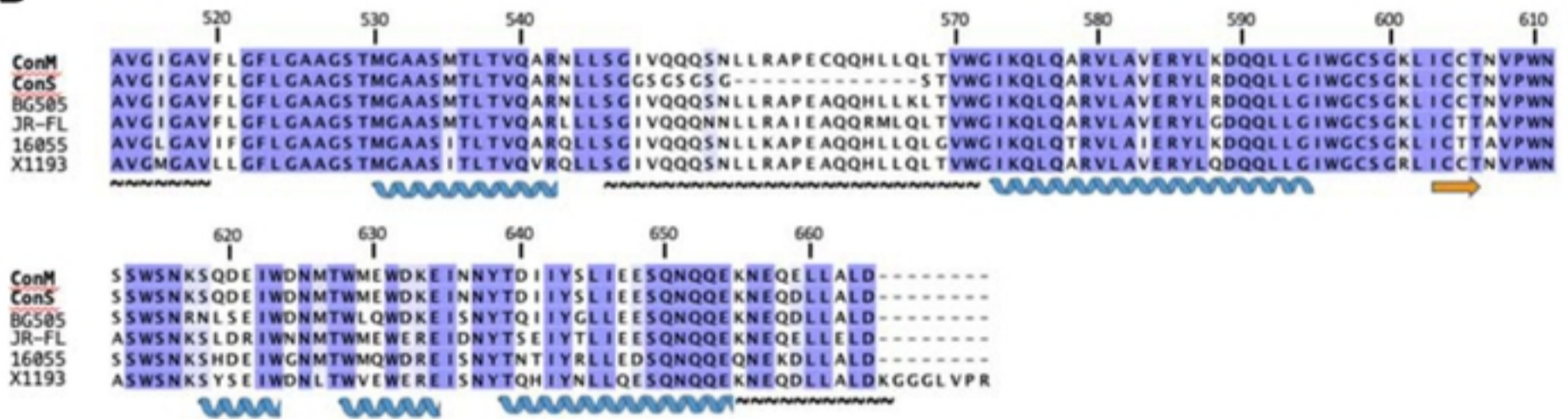


Supplementary Figure 8. Impact of cross-linking on the PGT122 epitope. (A) Superimposition of ConS (red) and ConS-EDC (purple), showing zoomed-in view of PGT122 (grey) binding site. Glycans at N332 and N138 are also shown, which were highly-ordered in the cryo-EM structure. At right the view is rotated 180° and side chains of residues directly in contact with antibody are shown as sticks, including the $G_{324}DIR_{327}$ motif. **(B)** Same as in (A), but for ConM and ConM-EDC.

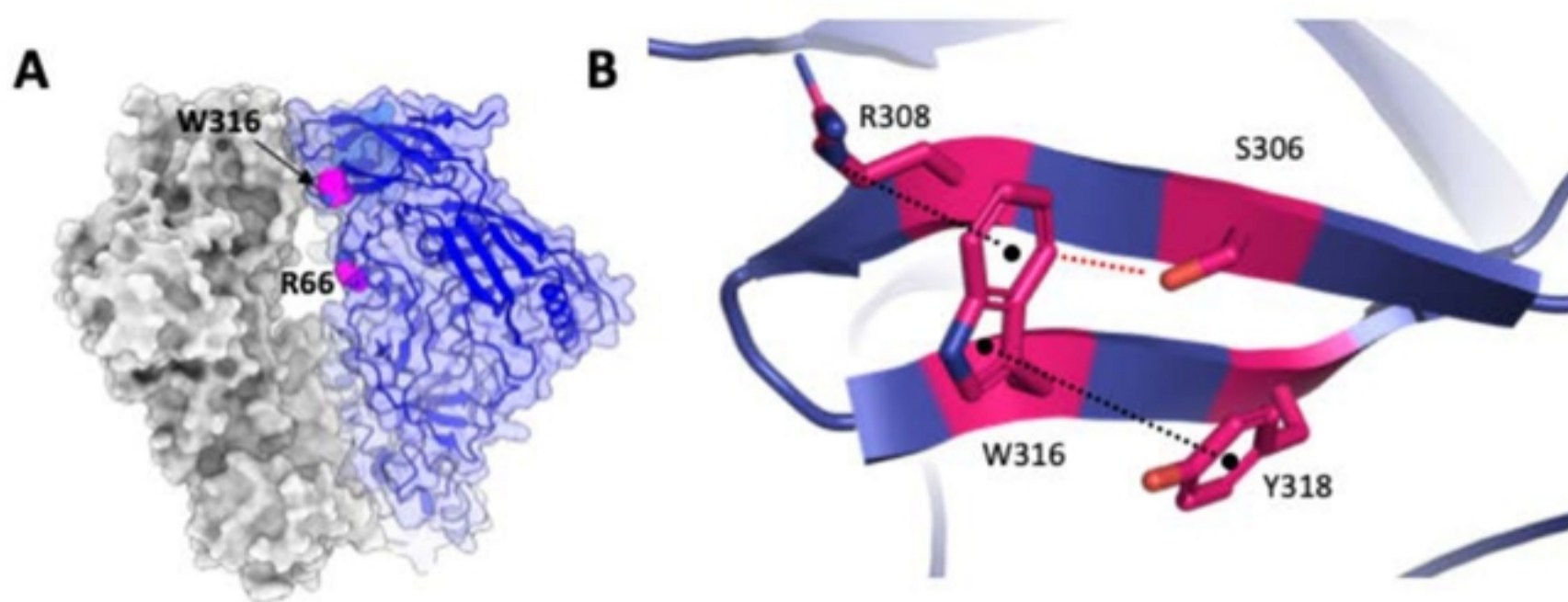
A



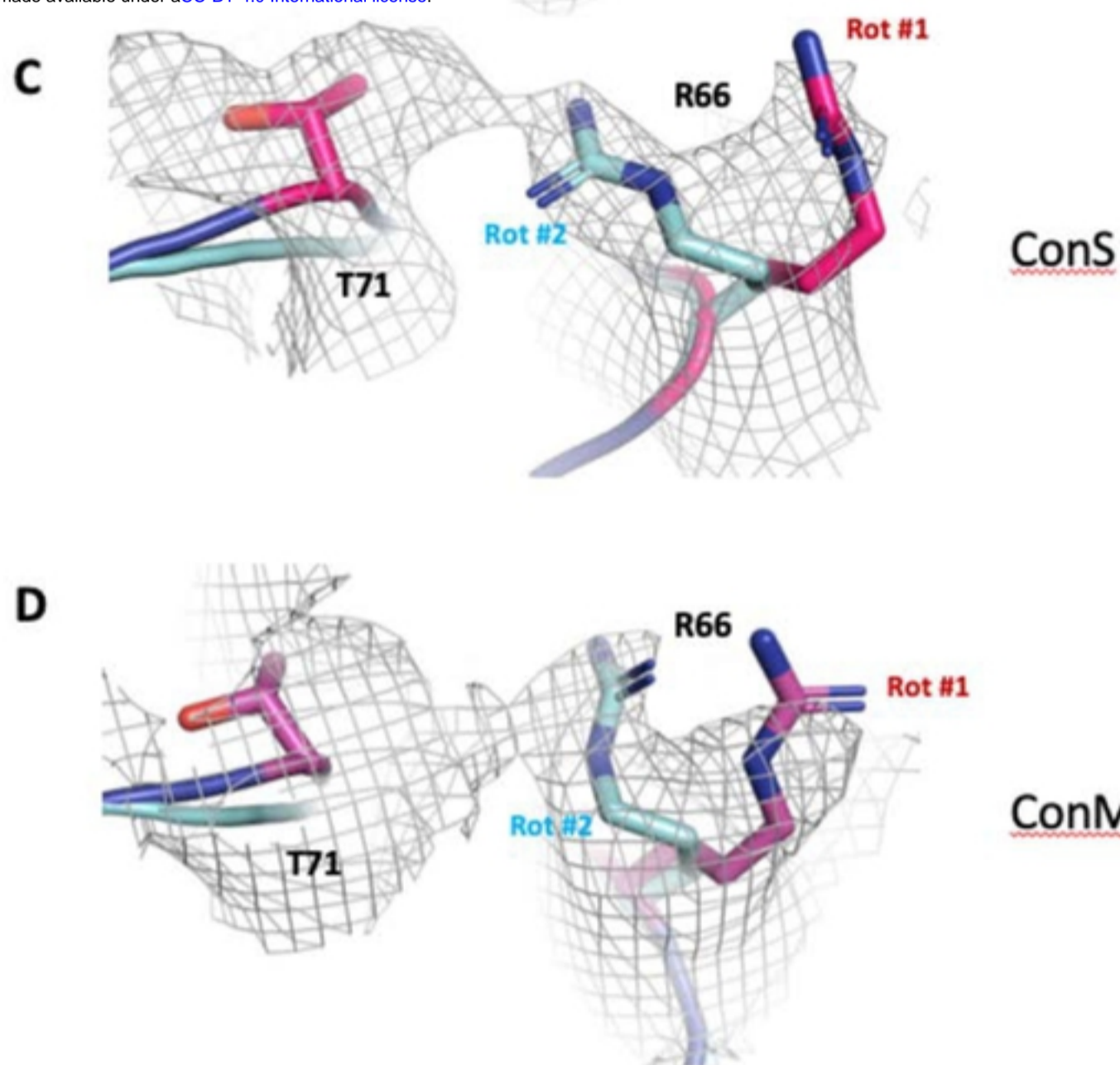
B



Supplementary Figure 9. Sequence alignment of ConM and ConS with Env sequences from various clades. BG505, clade A; JR-FL, clade B; 16055 NFL TD, clade C; X1193.c1 SOSIP.664, clade G. **(A)** gp120 alignment, **(B)** gp41 alignment. Orange arrows denote beta strands, blue coils denote alpha helices, and tilde strings are regions mostly disordered in the ConM and ConS structures. Numbering is according to the HXB2 system.

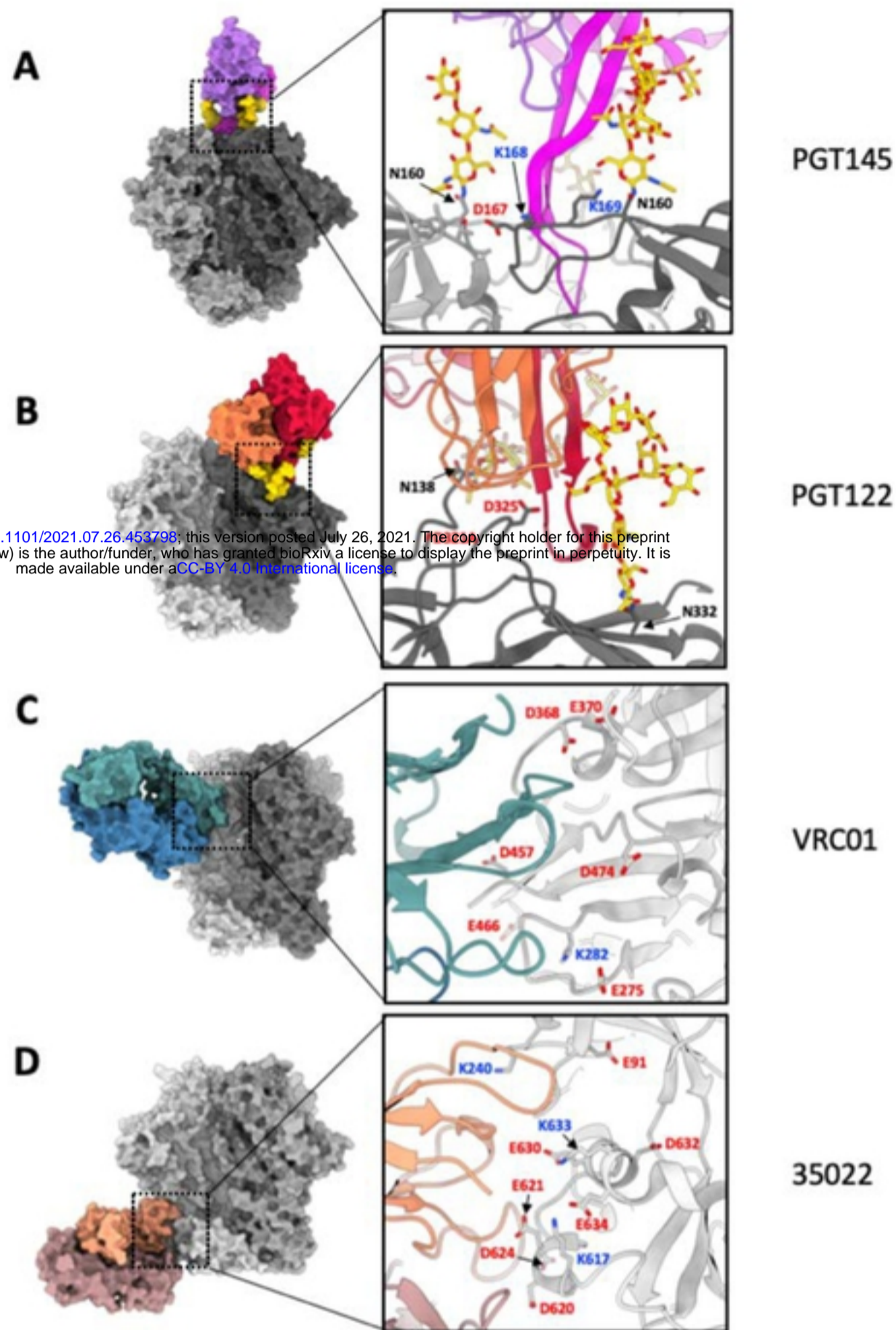


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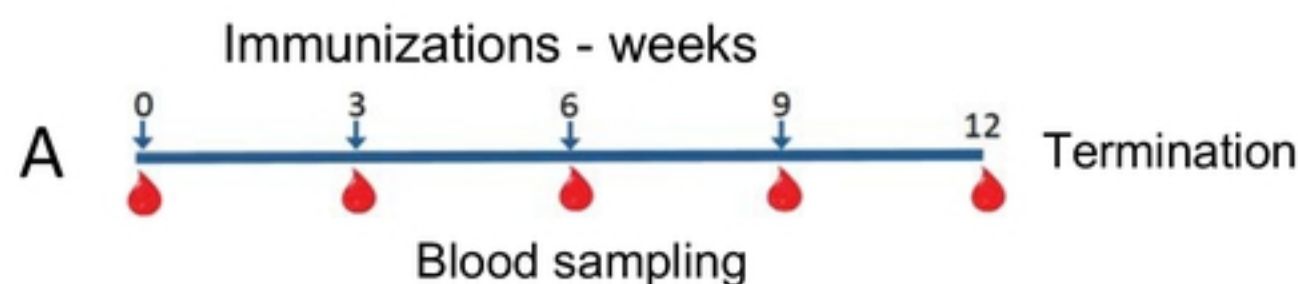


Supplementary Figure 10. Structural details of stabilizing mutations A316W and H66R present in ConS and ConM. (A) Surface representation of ConS structure, with ribbon representation of one protomer shown. The W316 and R66 side chains are shown as magenta spheres. (B) Zoomed in view of W316 and the base of the V3 loop in the ConS structure, highlighting a hydrophobic stacking interaction with Y318, a cation-pi interaction with R308, and a possible weak H-bond with S306. (C) Structure and cryo-EM density of two possible rotamers for the R66 side chain in ConS. R66 is modelled as rotamer #1 in both deposited ConS and ConM cryo-EM structures, but rotamer #2 is equally likely. In this conformation, R66 would probably form stabilizing contacts with T71. (D) Same as in (C), but for ConM cryo-EM structure.

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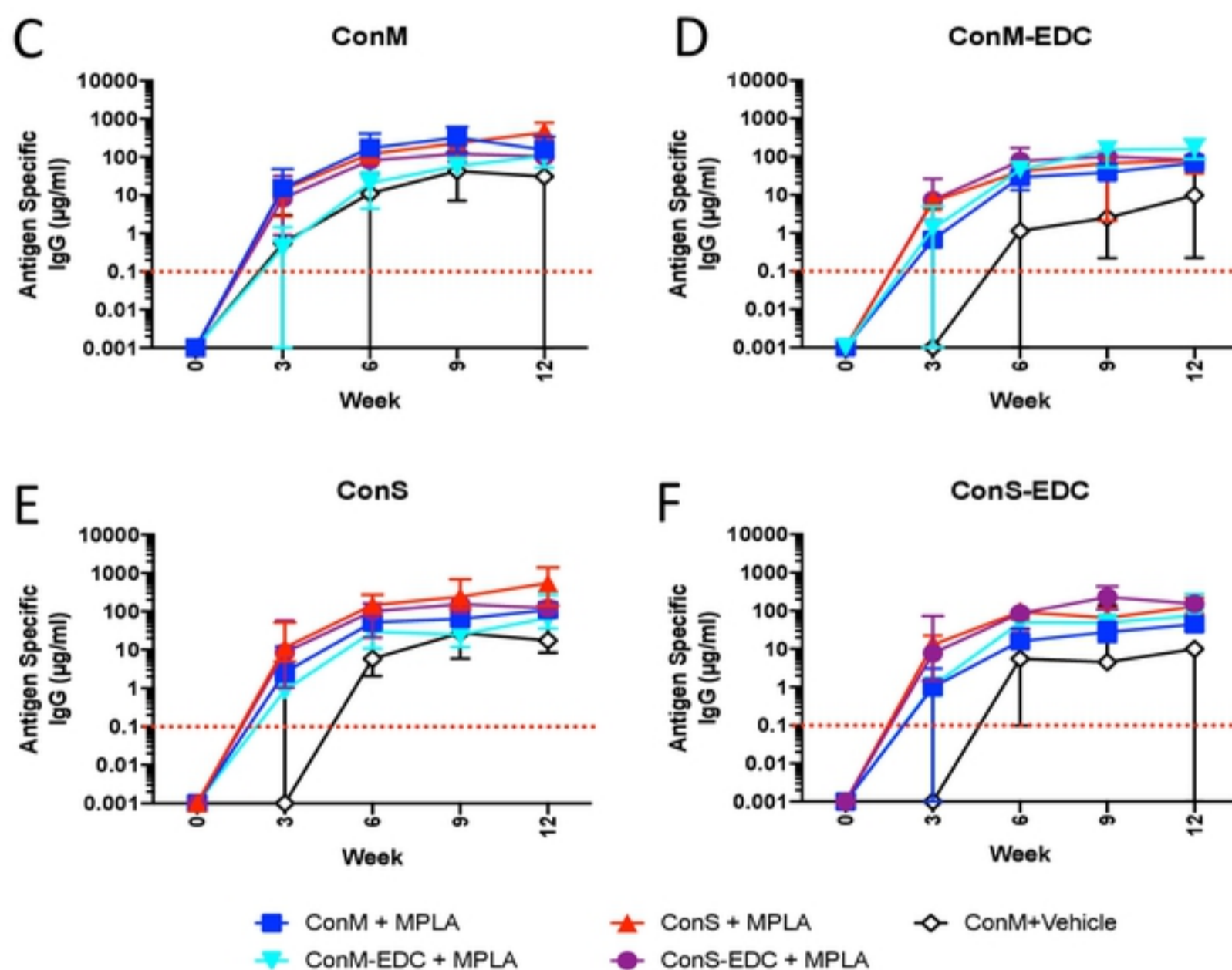
Supplementary Figure 11. Structures of select bnAb epitopes and exposure of glutamate, aspartate, and lysine side chains. (A) BG505-PGT145 cryo-EM structure (PDB 5V8L). PGT145 binding is to a large extent driven by glycan contacts. **(B)** ConS-PGT122 cryo-EM structure (this paper), showing, as with PGT145, a glycan-dependent epitope minimally by EDC. **(C)** ConS cryo-EM structure with VRC01 docked in, based on PDB 6V8X. Though not directly observed in the cryo-EM structures, a fraction of exposed Glu and Asp side chains could have been modified by EDC crosslinking, accounting for reduced binding to EDC modified ConS and ConM. Alternatively, the limited conformational flexibility in crosslinked SOSIPs could prevent rearrangements required to mediate high affinity CD4 binding site bnAb binding. **(D)** ConS cryo-EM structure with docked 35022, based on PDB 5W6D. Although 35022 binding to EDC-crosslinked ConS and ConM is largely conserved (as with VRC01) numerous exposed Glu and Asp side chains could potentially be modified and impact bnAb binding at this site.



B

Group Number	Test Item	Concentration	Dose Volume	Dose Volume/Site (mL)	No. of Rabbits
1	Con M + MPLA	0.2 mg/mL (Con M); 1 mg/mL (MPLA)	500 μ L (Con M) + 500 μ L (MPLA)	0.5	6
2	Con M-edc + MPLA	0.1 mg/mL (Con M-edc); 1 mg/mL (MPLA)	1000 μ L (Con M-edc) + 500 μ L (MPLA)	0.75	6
3	Con S + MPLA	0.2 mg/mL (Con M); 1 mg/mL (MPLA)	500 μ L (Con S) + 500 μ L (MPLA)	0.5	6
4	Con S-edc + MPLA	0.1 mg/mL (Con M-edc); 1 mg/mL (MPLA)	1000 μ L (Con S-edc) + 500 μ L (MPLA)	0.75	6
5	Con M + Vehicle	0.2 mg/mL (Con M); 0.9 % Sodium Chloride (Vehicle)	500 μ L (Con M) + 500 μ L (0.9% Sodium Chloride)	0.5	6

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Supplementary Figure 12. Rabbit IgG responses to antigen in MPLA immunization for toxicity study. **A)** immunization and sampling regimen where arrows represent immunizations and red drops represent bleeds. The protocol was terminated at week 12. **B)** Schedule of dosing and volumes, animals were injected at 2 sites per administration. Volumes differ between unmodified and EDC cross-linked administrations due to differing starting concentrations of GMP product. **C-F)** IgG purified from sera from rabbits immunized as in **A)** were titrated onto plates coated with: **C)** ConM, **D)** ConM-EDC, **E)** ConS, **F)** ConS-EDC, and results reported as binding curves in μ g/mL IgG.

Supplemental Table 1 – Cryo-EM data collection parameters & model statistics

	ConM_PGT122 PDB: 7LXM EMDB: EMD-23571	ConM-EDC_PGT122 PDB: 7LXN EMDB: EMD-23572	ConS_PGT122 PDB: 7LX2 EMDB: EMD-23564	ConS-EDC_PGT122 PDB: 7LX3 EMDB: EMD-23565
Data Collection				
Microscope	Titan Krios	Titan Krios	Titan Krios	Titan Krios
Detector	Gatan K2 Summit	Gatan K2 Summit	Gatan K2 Summit	Gatan K2 Summit
Voltage (kV)	300	300	300	300
Pixel size (Å)	1.026	1.026	1.026	1.026
Defocus range (µm)	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2
Total electron dose (e ⁻ /Å ²)	50	50	50	50
Dose rate (e ⁻ /Å ² /sec)	5.2	5.2	4.76	4.72
Frames per exposure	48	48	42	53
Data Processing				
Total micrograph movies	2758	2785	3504	5083
Particle images in map	144,741	91,987	161,340	132,156
Symmetry imposed	C3	C3	C3	C3
Map resolution (FSC=0.143; Å)	3.41	3.85	3.12	3.45
Map sharpening B-factor (Å ²)	-90.5	-141.6	-105.2	-134.1
Model Refinement				
No. of atoms in deposited model				
gp120	10,350	10,350	10,083	10,083
gp41	2850	2850	2598	2598
PGT122	5592	5592	5592	5592
Glycans	2028	2028	1881	1881
RMS deviations				
Bond lengths (Å)	0.019	0.02	0.02	0.02
Bond angles (°)	1.717	1.707	1.633	1.65
Validation				
MolProbity Score	0.89	0.94	0.88	0.84
Clashscore	1.49	1.81	1.43	1.21
EMRinger Score	2.38	2.59	2.98	2.78
Poor rotamers (%)	0.52	1.19	0.15	0.1
Ramachandran Plot				
Favored (%)	98.54	98.58	98.8	99.12
Allowed (%)	1.2	1.29	1.19	0.88
Outliers (%)	0.26	0.13	0	0
Average B-factor	59.1	91.6	35.9	59

Supplementary table 1. Cryo-EM data collection parameters and model-building statistics

Potential Crosslinks

<u>ConS</u>				<u>ConM</u>			
<u>Intra-protomer</u>		<u>Inter-protomer</u>		<u>Intra-protomer</u>		<u>Inter-protomer</u>	
D47	K46	D167.A	K169.C	D47	K46	D167.B	K169.E
D47	K487			D47	K487	E654.B	K601.D
D83	K227			E64	K207	E659.B.	K601.D
E91	K487			E83	K227		
D107	K574			E91	K487		
D113	K117			D107	K574		
D167	K168			D113	K117		
D180	K421			D133	K155		
D230	K232			E153	K178		
D230	K232			D167	K168		
E267	K231			D180	K421		
E268	K231			D230	K232		
E268	K232			D230	K240		
E269	K232			E267	K231		
E269	K348			E268	K231		
E275	K282			E268	K232		
E275	K97			E269	K232		
E351	K347			E269	K348		
E351	K348			E275	K282		
E370	K421			E275	K97		
E466	K356			E290	K340		
E492	K46			E351	K347		
E492	K490			E351	K348		
E621	K617			E370	K421		
E630	K633			E466	K356		
D632	K46			E492	K46		
E634	K617			E492	K490		
				E584	K588		
				E621	K617		
				E630	K633		
				D632	K46		
				E634	K617		

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Observed Crosslinks (all intra-protomer)

<u>ConS</u>		<u>ConM</u>	
E268	K232	E267	K231
E269	K348	E268	K232
E293	K337	E290	K340
E351	K348	E485	K231
E632	K46		
E634	K617		

Supplementary Table 2. Summary of potential and observed crosslinks in ConS and ConM. A separation distance of $\leq 6\text{\AA}$ between Glu/Asp and Lys side chains was used to define potential crosslinks, in addition to geometric constraints as observed by the structure. See Methods for determination of observed crosslinks.