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dépendante de l'ARN trimérique des virus influenza :
son impact sur notre compréhension des interactions de
la polymérase avec les facteurs viraux et cellulaires**

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Structure resolution of the trimeric RNA-dependent RNA polymerase of influenza viruses: impact on our understanding of polymerase interactions with host and viral factors.

Elise Biquand and Caroline Demeret

Unité de Génétique Moléculaire des Virus à ARN, CNRS, UMR 3569, Paris, France; Université Paris Diderot, Sorbonne Paris Cité, Institut Pasteur, 28, Rue du Dr. Roux, 75724 Paris cedex 15, France

1 **SUMMARY**

2 Influenza viruses are segmented negative-sense RNA viruses encoding their own RNA-
3 dependent RNA polymerase (RdRp), whose multiple activities are central for the viral
4 life cycle. The RdRp is composed of three subunits, PB1, PB2 and PA. It binds to the
5 extremities of each vRNA segments encapsidated with multiple copies of the
6 Nucleoprotein (NP), altogether constituting the viral RiboNucleoprotein (vRNP). The
7 RdRp performs both vRNA transcription and replication in the context of vRNP in the
8 nuclei of infected cells. The temporal regulation of RdRp-associated activities is essential
9 for the successful completion of the virus life cycle, but its understanding has been
10 limited by the lack of structural information about the polymerase complex. The atomic-
11 resolution of polymerase complexes from influenza virus type A, type B and type C came
12 out in the past two years. We compile here the data provided by the near-concomitant
13 resolution of several influenza polymerase crystal structures. We will highlight how
14 structural information can contribute to our understanding of the interactions between
15 the RdRp and viral or host factors.

16
17

18 Influenza viruses are segmented negative-stranded RNA viruses of long-term human
19 health concerns, which still remains largely unresolved despite the therapeutic advances
20 provided by vaccines and anti-viral therapeutic drugs. A specific difficulty to durably
21 combat influenza infection resides in the high variability of influenza viruses, which
22 leads to the continuous apparition of variants that resist antiviral drugs or escape
23 vaccine-induced immunity. The low fidelity of the viral RNA-dependent RNA polymerase
24 (RdRp) accounts for such variability. The RdRp is a heterotrimer composed of the
25 polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase
26 acidic protein A (PA) proteins. The complex is bound to conserved sequences of the
27 Non-Coding Regions (NCR) at the extremities of each viral RNA segment. The vRNA
28 wraps around oligomers of the NP protein, thereby forming encapsidated structures
29 designed viral ribonucleoproteins (vRNP). The RdRp catalyzes both the transcription
30 and the replication of viral RNA segments in the nucleus of infected cells, and is thus
31 able to perform distinct activities on the vRNA templates. The RdRp operates only in the
32 context of vRNP, which are the functional units for RdRp transcription and replication
33 activities (for reviews see [1-3]).

34 Upon vRNP entry into the nucleus a first round of transcription occurs, where the
35 polymerase transcribes vRNA into viral messenger RNAs (mRNA) [1]. The vRNA 5'NCR
36 and 3' NCR constitutes the promoter for transcription of each viral segment, which relies
37 on the primed synthesis of the mRNA using host capped oligonucleotides. To initiate
38 transcription, the viral polymerase first captures a capped noncoding or pre-messenger
39 host RNA associated with the host transcribing RNA-polymerase II, via the Cap-binding
40 domain of PB2. Then PA cleaves it 8-14 nucleotides from the cap via its endonuclease
41 domain. The resulting short-capped oligonucleotides serve as primers for the synthesis

42 of viral mRNAs, which are last poly-adenylated by the stuttering of the polymerase on a
43 poly-U sequence located near the 5' end of the RNA template.

44 The viral replication is taking place in two steps. The RNA segment of the vRNPs
45 are first copied in complementary cRNA strands of positive polarity, which form cRNPs
46 after the loading of NP oligomers and the association of a polymerase complex. cRNP
47 serve as templates for the synthesis of vRNA segments, giving rise to progeny vRNPs
48 (reviewed in [4, 5]. Newly synthesized vRNPs are either transcribed to provide high
49 levels of viral protein expression (secondary transcription), or are exported from the
50 nucleus to be incorporated into novel virions. For vRNA replication, the RdRp operates
51 an un-primed RNA polymerisation, and this process is dependent upon newly
52 synthesized PB1, PB2, PA and NP proteins. The PB1 sub-unit performs the RNA
53 polymerization, and harbors functional motifs common of all RNA-dependent RNA
54 polymerases [6]. The influenza polymerase is devoid of proof reading activity (or 3'-5'
55 exonuclease activity), accounting for the high mutation rate in the replicated viral
56 segments. Other RNA replication independent activities of the polymerase complex are
57 likely to be involved in the viral cycle, such as a role in the splicing of the NS1 viral
58 mRNA [7], or in the trafficking of vRNP through the cytoplasm (reviewed in [3]).

59 Influenza virus RdRp therefore appears as a multifaceted protein complex,
60 performing different activities directed toward the vRNAs, whose temporal coordination
61 ensures the proper execution of the viral cycle. The processes driving these activities
62 and their regulation are little understood at the molecular level so far. Intrinsic
63 characteristics of the polymerase complex, as well as interaction with different sets of
64 viral or host factors, are thought to determine the capacity of influenza virus polymerase
65 to proceed to replication or transcription. This notion is nevertheless supported by few
66 experimentally derived pieces of evidence, hampered in particular by the lack of
67 structural knowledge regarding the polymerase complex. Until recently, information
68 was available for the structure of the PA endonuclease N-terminal domain (PA-ENDO),
69 and for PA C-terminal domain in complex with the sixteen N-terminal residues of PB1 [8,
70 9]. For PB2, the structure of the Cap binding domain (PB2-CAP, [10], and of two C-
71 terminal domains, termed the 627K and NLS domains [11, 12] had been resolved. In
72 addition, the extreme N-terminus of PB2 had been co-crystallized in complex with the C-
73 terminus of PB1 [13]. Nothing was known about the structure of the PB1 sub-unit except
74 its extreme N and C termini associated with the PA and PB2 sub-unit respectively. The
75 atomic resolution of the crystal structures of the RdRp of influenza viruses type A, type B
76 and type C came out in a remarkably short period of time, concluding long-lasting
77 intensive attempts that confronted challenges of producing sufficient quantities of
78 purified polymerase sub-units to be crystallized [14-17].

79 Two papers published in the Nature issue of the 18th december, 2014, described the
80 structure of the influenza virus polymerases co-crystallized with a synthetic v3'+v5' RNA,
81 mimicking the viral promoter. One was on the RdRp from a bat influenza A virus [16]
82 the other on a human influenza B virus RdRp [15]. Later on came out the structure of the
83 RdRp from the influenza virus C polymerase without any bound viral RNA (apo fluC)
84 [14]. Then a study published in the early 2016 still incremented the picture, in particular
85 by providing the structure of RdRp from influenza virus B bound to a 5'cRNA [17]. These
86 studies first provided some insights into the mechanistic aspects of the cap-snatching,
87 the primed and un-primed RNA polymerization, which are reviewed in the broader
88 context of negative-strand viral RNA-dependent RNA polymerases in [18]. They also
89 enabled the immediate appreciation that influenza virus polymerase complex can adopt
90 different conformations (figure 1-a). We will describe here the main characteristics of

91 influenza polymerase structures that have been brought out, and focus on polymerase
92 interaction with viral or host factors.

93

94 **The RdRp Core : a central invariant fold**

95 The RdRp organizes around an invariant central polymerase body made of the
96 PB1 protein, the N-terminal part of PB2 (PB2-N) and the C-terminal part of PA (PA-C)
97 (figure 1-b). The PB1 sub-unit adopts in its central region a canonical RNA polymerase
98 fold, conserved among RNA-dependent RNA polymerases [19]. It consists in a palm sub-
99 domain, exhibiting the functional motifs for RNA polymerization, as well as fingers and
100 thumbs set on both sides of the palm. A large internal cavity provides the catalytic
101 chamber for RNA binding and polymerization. A priming loop emerges within the
102 central cavity from the thumb of PB1 (figure 2-a) and is involved in the terminal
103 initiation of unprimed RNA synthesis [20]. This fold is flanked on both sides by PB2-N
104 and PA-C (figure 2-b), involving an intricate intertwining of the polymerase subunits.
105 The buried surface of the central RdRp core is far more extended than envisioned from
106 former crystallographic studies with sub-unit domains [9, 13, 21]. This results in a
107 somewhat compact structure, from which a long β ribbon of PB1 is extruding, which
108 contains the bipartite NLS of the influenza A and B PB1 proteins (figure 2-b)

109

110 **Binding of the vRNA promoter to the core polymerase**

111 The bat influenza virus A (fluA) and the human influenza virus B (fluB) polymerases
112 were both crystallized with bound vRNA 3' and 5' extremities mimicking the viral
113 promoter, thereby providing a picture of viral promoter binding to the influenza virus
114 polymerase. The distal region of the vRNA promoter is base-paired and not associated
115 with the polymerase, while 3' and 5' extremities are separated (figure 3-a), and bind to
116 different interfaces of the polymerase core.

117 The binding of 3' vRNA involves all three sub-units, and numerous base-specific RNA-
118 protein interactions (figure 3-b). The bound 3' vRNA adopts an arc shape, its 3'
119 extremity lies close to the distal double-stranded portion of the promoter (figure 3-c). It
120 is predictable that the 3' end of RNA template changes orientation to enter into the
121 catalytic site, which probably involves modifications of polymerase core conformation at
122 the vicinity of RNA binding interfaces. The 5' promoter end forms a stem loop structure
123 through internal base-pairing (figure 3-a) essentially as predicted [22]. It binds to a
124 pocket formed by the PB1 and PA sub-units through multiple amino acids-base
125 interactions (figure 3-d). The binding characteristics of 5'vRNA were found similar when
126 only a 5'cRNA or 5'vRNA are bound to the polymerase [17]. In all, the binding of viral
127 RNA to the core turns out to be full part of the polymerase structures.

128

129 **Distinctively arranged domains of RdRp**

130 The PB2 two-third C-terminal (PB2-C) and PA N-terminal endonuclease domain
131 (PA-ENDO) turn out to be variously arranged according to the polymerase structures,
132 highlighting a noteworthy plasticity relative to the central core, and resulting in notably
133 differing overall shapes of the polymerase complexes (figure 1-a). These domains are
134 thus likely to be key players of the versatility of the polymerase. The individual
135 structural modules that can be identified within PB2-C, consisting in mid, cap-binding,
136 cap-627 linker, 627 and NLS domains [17], remain individually unchanged, but differ in
137 their respective position. Indeed, the cap-binding and 627 domains are differently
138 rotated around a rigid element made of the mid and cap-627 linker (figure 4). The PB2
139 sub-unit therefore adopts distinct conformations according to the polymerase

140 configurations, and the same conformation as the one found in the c5'RNA bound/Apo
141 polymerase conformation was also recovered upon the expression of isolated PB2-C
142 (pdb 5FMM and pdb 5FML) [17], except that in the latter the NLS domain of the protein
143 could not be resolved.

144

145 **Transcription-competent polymerase conformation**

146 The polymerases bound to the v5'+v3' vRNA mimicking viral promoter (Bat fluA
147 pdb 4WSB and human fluB pdb 4WSA)[15, 16], or to v5'only-bound polymerase (pdb
148 5FMZ,[17]), adopt a similar U-shape fold (figure 1).

149 In this configuration, both PB2-CAP and PA-ENDO form protruding arms facing
150 each other across a solvent-exposed channel, and the channel entry joins the catalytic
151 center of the core polymerase (figure 5-a). Two alternative positions of the PB2-CAP can
152 be distinguished. In the bat fluA structure, the cap-binding site is pointing to the
153 endonuclease active site across the separating channel (Figure 5-a). In such orientation,
154 upon binding of the host pre mRNA cap, the 10th to -15th nt would face the endonuclease
155 catalytic site of PA, and cleavage could occur at these positions. This is in agreement
156 with the observed lengths of capped oligonucleotides released by PA-mediated cleavage.
157 The cap-binding domain of PB2 in the promoter-bound fluB polymerase structure is
158 rotated by 70 Å compared to its position in the fluA polymerase, so that the cap binding
159 site of PB2 is directed towards the polymerase RNA catalytic cavity, and away from the
160 endonuclease domain of PA (figure 5-a and 5-b). In this configuration, the 3' end of the
161 capped oligonucleotide would be channeled into the polymerase catalytic center, so that
162 this polymerase configuration is assumed to be competent for the initiation of primed
163 viral mRNA synthesis [15]. The differences in both promoter-bound structures suggest
164 that a conformational switch must occur for viral mRNA synthesis during transcription
165 [15].

166 Based on modelling with the template-product elongation complex from the poliovirus
167 polymerase [23], the template exit would be blocked by an α helical bundle of PB2-N
168 named helical lid [15]. This lid has been suggested to participate to product-template
169 strands separation, which in turn should exit the core polymerase through distinct
170 tunnels [23]. The transcript strand has been proposed to leave the polymerase core
171 between the PB2-CAP, and 627-domains of PB2 [15] (figure 5-c). When elongation
172 proceeds close to the 5' end of RNA template, template strand progression would be
173 opposed by the tight binding of the 5' extremity to the core, thereby provoking the
174 stuttering of the polymerase on the oligo-U sequence 17-22 nucleotides upstream the 5'
175 extremity, which has been shown to generate addition of poly-A to the viral mRNA [24].

176

177 **5'cRNA and apo fluc C conformations**

178 In the polymerase bound to a 5'cRNA end [17], there is an entirely different
179 arrangement of the PB2 sub-unit. In particular the PB2-627 and PB2-NLS domains are
180 displaced relative to the transcription-competent polymerase (figure 6-a).

181 The PB2-CAP contacts both the PB1 sub-unit and PB2 cap-627 linker. The cap-
182 binding site is packed against the PB2 cap-627 linker and consequently it is not
183 accessible (figure 6-b). A similar organization is observed in the apo-flu C polymerase
184 structure (figure 6-a) [14]. The obstruction of the Cap binding site of PB2 suggests a
185 debilitated cap-snatching activity, which has been confirmed experimentally [14, 17],
186 and argues that the primed synthesis of viral mRNA is not possible in such configuration.
187 In contrast, the 5'cRNA polymerase configuration might be operational for the cRNA to
188 vRNA step of replication [17]. The apo-Flu conformation has been proposed to be a

189 closed conformation [14]. It might nevertheless perform replication upon stabilization
190 of the priming loop, which is disordered in the resolved structure [14].

191 In the 5' cRNA-bound fluB polymerase, the NLS domain of PB2 is tightly apposed
192 to PA-ENDO through a large interaction interface, and this domain packing also involves
193 a rotation of PA-ENDO relative to the promoter bound structure. In this configuration,
194 the PB2 NLS domain lies apart from PB2 627 domain. In the apo-fluC, similar interaction
195 interfaces exist between PB2-NLS and PA-ENDO (P3 in fluC), but in such orientation that
196 the PB2-NLS is lying close to PB2-627 (figure 6-a).

197
198

199 **The remarkable flexibility of influenza virus polymerase**

200 The various crystal structures resolved first outline divergent structural organization of
201 the promoter-bound and 5'cRNA-bound or apo- polymerases. Analysis of the behavior in
202 solution of different polymerase complexes highlights various degrees of compaction
203 [17]. Indeed, the apo-polymerase demonstrates an extended conformation, which was
204 also detected for a polymerase complex bound to only a 3' vRNA end, and seems to be
205 flexible owing to the conformational heterogeneity observed in solution. Other
206 polymerase forms, consistently including a bound 5'RNA, are more compact in solution.
207 The hypothesis is that the high-affinity binding of a 5'vRNA (in the range of 2 nM [25])
208 or 5'cRNA extremity to the core rigidifies the polymerase in a compacted conformation
209 and somehow shapes different polymerase conformations according to the type of 5'
210 RNA end (5'vRNA or 5'cRNA) binding [17]. A wider range of alternative polymerase
211 conformation have been detected in cross-linking experiments than in the crystal
212 structures. The structural modules of PB2-C (*ie* mid, cap binding, cap-627-linker, NLS)
213 seem to adopt a large variety of relative dispositions, both at the level of intra-protein
214 subdomains packing and relative to other sub-units PB1 and PA [17].

215
216

216 **Potential higher ordered polymerase forms**

217 An additional level of complexity arises from the possible involvement of higher
218 oligomerisation states of the polymerase trimeric complex. Indeed, cryo-electron
219 microscopy studies with the polymerase complex of an influenza A H5N1virus devoid of
220 the PB2 C-terminal domain (PB2 N 1-130) showed that this incomplete polymerase,
221 termed subcomplex I, assembled into dimers in solution, and further formed tetramers
222 upon binding of 5'v or cRNA strands [26]. These data led to the proposal that binding of
223 the vRNA promoter may regulate higher degree of polymerase oligomerisation. The
224 level of resolution reached in these studies allowed to specify the oligomerisation
225 interfaces, but did not provide all the precise locations of the involved residues. It
226 nevertheless indicated that the dimer interface involves α helices from the PA-C (aa
227 292/291 to 355/352, coordinates for the H5N1 human Flu A and human Flu B
228 respectively) and PB2-N domains (attributed to aa 86/88-130/132 for H5N1 Flu A and
229 human Flu B respectively [26]), which are lying adjacent in the cryo-EM derived
230 structure (figure 7-a) [26]. A weakest interaction mainly involving PB1-N α helical
231 fingers would mediate tetramer formation, which however could not be precisely
232 characterized except for the participation of PB1 aa 180-205 and 355-375.

233 Oligomerization of polymerase is compatible with the trans-replication model, which
234 proposes the recruitment of a soluble polymerase to the cRNA through interactions with
235 the resident RNP-bound polymerase [27]. In contrast, the synthesis of viral mRNA would
236 be carried out by the RNP-bound polymerase (*cis* conformation) [4, 27]. Polymerase
237 oligomerization state transitions are proposed to take part to the regulation between

238 the transcription and replication activities, and the polymerase conformation
239 compatible with dimer and tetramer formation to correspond to the replication active
240 state [26].

241 In the c5'RNA-bound/apo- polymerases however, the participating PB2 and PA α helices
242 are not contiguous, and the PB2 α helices are partly embedded, thus not fully accessible
243 to the polymerase surface (figure 7-b). Overall, the crystal structures obtained so far,
244 including the promoter-bound polymerase conformation, are not compatible with the
245 cryo-EM model for dimer formation. Nevertheless, it still can be proposed that
246 polymerase conformations that are competent for dimer (and possibly tetramer)
247 formation have not yet been captured by crystallographic studies, owing to the
248 polymerase flexibility.

249

250 **Polymerase in the context of the RNP**

251 The functional transcription/replication unit of influenza viruses is the RNP. Assembly
252 of viral RNA segments into RNPs is necessary for the viral replication process, which
253 requires the binding of NP to nascent vRNA or cRNA products. In contrast, the viral
254 mRNAs are not encapsidated by NP upon synthesis during the transcription process. For
255 both replication and transcription, it is thought that the template RNA strand locally
256 disassemble from NP to enter the catalytic core of the polymerase, and binds back to NP
257 upon exit. The NP protein is also proposed to be involved in the regulation of
258 transcription-replication switch of the polymerase activities [28].

259 The cryo-EM based three dimensional reconstruction of a mini-RNP, made with shorten
260 vRNA, indicated protein contacts between the promoter-proximal NP monomers and the
261 polymerase complex, without precise identification of the molecular interfaces [29, 30].
262 The structure nevertheless suggested specific interactions between the PB1 and PB2
263 polymerase subunits and the two molecules of NP adjacent to the polymerase, in line
264 with previous studies showing interaction of NP with PB1 and PB2, but not with PA [31,
265 32]. By fitting the polymerase structure into the mini-RNP pseudo-atomic model, the
266 interaction interface between PB1 and NP has been located to the flexible β ribbon of
267 extruding from the core polymerase, which contains the PB1 NLS at least for influenza A
268 and B, and also takes part to the binding interface of the polymerase with the 3' vRNA
269 end [15]. This PB1 β ribbon projects away from the polymerase core in the promoter-
270 bound polymerases (figure 8-a), where it is predicted to lie in close proximity to the
271 promoter-proximal NP protein. In the absence of 3'vRNA, this β ribbon is packed against
272 the polymerase core [15, 17].

273 The PB2 sub-unit lacked electron density in the pseudo atomic structure of mini-RNP, so
274 that only the first α helices (aa 1-24) of PB2 could be fitted in the mini RNP structure,
275 lying close to NP [15]. These extreme N-terminal PB2 α helices are consistently at the
276 surface of the polymerase core, close to the PB1 β -ribbon binding the other NP
277 monomer (figure 8-a). They also correspond to the PB2-N/PB1-C interaction interface
278 resolved from isolated domain [13], which may explain why a strong overlap had been
279 detected between the interaction domains with PB1 and with NP in the PB2 protein
280 [33]. The PB2 N-terminal α helices are lying close to the exit of the template strand
281 (figure 8-b), suggesting that they constitute NP binding interfaces relevant for the
282 loading of outgoing RNA template with NP (figure 8-b). Additional PB2/NP binding
283 interfaces have been observed in the mini-RNP structure but could not be mapped [29,
284 30].

285 On the opposite side of the polymerase, the PB2 627K domain has been shown to
286 interact with NP [32, 33]. The host adaptive 627 (generally a K in human polymerases

287 and a E in avian polymerases) and the 630 residues have been shown to modulate PB2
288 binding with NP and to affect RNP activities [32], although it has been challenged [34].
289 In the c5'RNA-bound configuration, the 627K domain is well accessible, protruding from
290 the rest of the polymerase, with both 627 and 630 residues exposed. Considering the
291 possibility that this configuration can perform the c to v RNA replication, the binding of
292 NP to PB2-627K domain would be involved in the loading of NP on nascent RNA
293 replicates, which would imply that the exit region of replicated RNA strand lies in the
294 vicinity of PB2 627K domain. In such context, it can be postulated that the exit region of
295 the outgoing replicated strand follows the tunnel delimited on one side by the packed
296 PB2-NLS/PA-ENDO, and on the other side by the mid/cap-627K linker sub domains of
297 PB2-C (figure 8-b). The 627K domain is more closely packed against the polymerase
298 core in other polymerase conformations, suggesting a reduced flexibility and a limited
299 surface availability for interaction with NP (figure 8-b).

300

301 **Amino acids involved in host adaptation**

302 Mammalian adaptation of influenza A viruses is a field of extended research, and several
303 residues have been found implicated in the host adaptation process. Compiling residues
304 identified in a series of studies as being under adaptation pressure pointed to 24
305 residues in PB2, 6 on PB1, PB2 and 3 on PA proteins [35, 36]. These residues, designed
306 thereafter as adaptative residues, are thought to optimize interactions with factors of the
307 novel host. The problematic of host specificity is more relevant for the influenza A
308 viruses, but the position of the adaptative mutations will be shown in fluB polymerase
309 structures, because there are the only ones enabling a comparison between
310 transcription-competent and 5'cRNA-bound polymerases. Given the high similarity
311 between the structures of fluA and fluB polymerases, it can be assumed that the
312 positions of adaptive residues are transferable to the Flu A polymerase.

313 Two residues of PA that play a role in adaptation to host (M352 and G548 in fluB, R356
314 and T552 in fluA) are located at the surface of the core polymerase, one is exposed at the
315 surface of the endonuclease domain (fluB E98, fluA T97) (figure 9). For PB1, six residues
316 have been described, two of them are exposed at the surface of the polymerase (fluB
317 52K, 583K; fluA 52K, 584K) whereas others are embedded into polymerase (105A and
318 516V fluB, 105N and 517V in fluA). Of note, one residue, 13P (fluB and flu A) is hidden in
319 the transcription configuration but well exposed in the 5'cRNA-bound configuration,
320 while the reverse applies to D677 (fluB)/S678 (fluA) (figure 9-a). These residues may be
321 accessible for interactions with host factors contributing respectively to the replication
322 and to the transcription of vRNA segments in human cells.

323 Most of the adaptative mutations concern the PB2 sub-unit. In the N-terminal part of
324 PB2 within the polymerase core, several adaptative mutations are lining the interaction
325 interface with PB1 (figure 9-b). This suggests that these residues could be involved in
326 interactions of the isolated PB2 sub-unit with host factors. They may alternatively be
327 necessary for an efficient polymerase complex formation through optimized interaction
328 with a mammalian PB1 sub-unit. However, equivalent mutations lining inter sub-unit
329 interfaces are not found in the PB1 and PA sub-units. The PB2 adaptative mutations
330 located in the C-terminal 627K and NLS domains *ie* E570/T569, M589/T588,
331 Q592/R591, K627/K627, Q664/M661, R686/G682, D705/D701, P706/K702 (fluB/fluA
332 coordinates) are mostly lying at the surface. Their exposure may nevertheless vary
333 according to the polymerase configuration. In the transcription competent form (figure
334 9-c, left), only E570 is readily accessible since turned toward the exterior of the
335 polymerase structure, while M589/Q592/K627 form a cluster somewhat embedded in

336 exit mRNA tunnel, and R686 lies close to the PA subunit (figure 9-c). The D705 (fluB)
337 D701 (fluA) residue is well exposed on the PB2 NLS domain only in the transcription
338 form of the polymerase, where it forms an exposed cluster with the P706 (fluB) in the
339 vicinity of the PA sub-unit (figure 9-c). One residue under selection pressure, Leu 476,
340 lies within the cap binding of domain of PB2, suggesting that some host-specific
341 characteristics may be involved in the recognition of capped mRNA.

342 The overall accessibility of the adaptive residues is more obvious in the replication
343 competent form of the polymerase, owing to the outward position of PB2-627K. Indeed,
344 the M589/Q592/K627 cluster is facing the exterior, as Q664, E570, and 718E (figure 9-
345 c). Two adaptive residues in the extreme C-terminal NLS containing peptide, E718 and
346 V762, are lying on the exposed face of the NLS packed against the PA endonuclease
347 domain in the replication competent polymerase form. The corresponding peptide is not
348 resolved in the transcription competent polymerase, which probably reflects an
349 extended conformation (figure 9-c). The particularly well accessible positions of PB2-
350 627K adaptive residues in the replication form favor the hypothesis that they engage
351 interactions mostly involved in the replication process.

352

353 **Amino acids important for host factor binding**

354 It is hypothesized that the influenza virus polymerase exploits host cell factors to
355 perform the transcription and replication of vRNA segments in a regulated manner. A
356 series of host factors have been identified as being involved in viral life cycle, through
357 high throughput studies of targeted depletion strategies using si-RNA or sh-RNA [37-
358 40]. On another side, interaction mapping led to the identification of potential host
359 factors interacting with the polymerase [41-44]. However, the overlaps between
360 functional and interaction mapping are limited, and only few of the polymerase/host
361 factors interactions have been mapped precisely. In most cases, it is even not known
362 whether interaction involves the PB1/PA/PB2 trimer or individual sub-units. The
363 available structures now provide some insight on the interplay between polymerase and
364 host factors.

365

366 Interaction with importins

367 Upon synthesis in the cytoplasm, the PB2 sub-unit from one side, and the PB1-PA dimer
368 on another side are directed to the nucleus [11, 45]. Nuclear import of the PB1-PA dimer
369 relies on its binding of the PB1 sub-unit to RanBP5, involving a protein interface which
370 contain the residues of its bipartite NLS located in the flexible β ribbon extruding from
371 the polymerase complex [46]. In the vRNP, the same β -ribbon seems to be engaged in an
372 interaction with one promoter-proximal NP monomer, it is therefore likely involved in
373 RanBP5-mediated nuclear import only in the context of the PB1-PA dimer.

374 Five amino acids have been described as being involved in the interaction of the PB2 C-
375 terminal peptide with α -importins [47]: 701D 737R, 738K, 752K and 755R (fluA) or
376 705D, 740R, 741K, 756K and 759R (fluB). It has been suggested that the binding of PB2
377 to importin α is involved in the efficiency of the polymerase activity of 627K-human
378 adapted polymerase, independently of its role in nuclear import of PB2 [48]. In the
379 replication-competent form, the PB2 NLS-containing peptide folds as a long α helix
380 packing on the endonuclease domain of PA, and the residues interacting with α
381 importins are all accessible except 705D (figure 10-a). However, it has been show that
382 unfolding of the C-terminal PB2 NLS peptide is required for its efficient binding to α -
383 importin [11], and consequently would not be possible in the 5'-cRNA bound
384 polymerase. By contrast, the binding to importin α could probably occur in the

385 transcription-competent polymerase configuration, where the extreme C-terminal NLS
386 peptide of PB2 could be in a flexible extended state as deduced from its lack of electron
387 density, suggesting that importins would impact on the transcription process mediated
388 by the human adapted 627K polymerase rather than on the replication process. The
389 PB2/importin interaction in the promoter-bound polymerase configuration could also
390 take part for the nuclear import of the incoming vRNP, which however has been shown
391 to be mainly mediated by that the NP protein [3]. Interaction of PB2 with importin has
392 been suggested to be part of the host adaptation mechanism, as importin isoforms from
393 human or avian hosts are differentially bound to PB2 from avian or human viruses [49].
394 This most probably impacts on the efficiency of nuclear translocation of the isolated
395 PB2.

396

397 Interaction with the cellular RNA polymerase II and transcription modulator hCLE

398 The viral influenza polymerase complex has been shown to interact with the C-terminal
399 domain of transcriptionally-active RNA polymerase II (polII) [50]. In the context of
400 vRNP, this is thought to enable the binding to host capped-mRNA required for cap-
401 snatching. A direct interaction with RNA polII has been recently detected, while its
402 binding interface on the influenza polymerase complex is not elucidated [50]. The
403 association of polymerase complex with RNA polII had been proposed earlier to be
404 mediated by the human transcription modulator hCLE [51]. In the influenza virus
405 polymerase complex, two regions of PA have been identified as interaction domains
406 with hCLE [52] and are lying in a pocket close to the vRNA binding site, and are
407 therefore not accessible (figure 10-b). This does not support the proposed role in
408 recruiting RNA polymerase II to facilitate CAP-snatching. The location of PA interface
409 with hCLE suggests an involvement in processes involved in both the replication and the
410 transcription.

411 The degradation of RNA polymerase II during influenza viral infection was identified as
412 a determinant of pathogenicity, with residues 504 in PB2 and 550 in PA (flu A
413 coordinates) found to be involved in the ability of the virus to induce such degradation
414 [53]. The PA 550 is near the published hCLE interaction interface, in a well-exposed
415 position (figure 10-b), suggesting a functional link between hCLE binding and induction
416 of RNA pol II degradation. The PB2 504 residue is positioned on the opposite side of the
417 polymerase, in the cap-627 linker proximal to the cap-binding site (figure 10-b). Given
418 the distance between these two residues, an additional factor could be recruited by the
419 viral polymerase to induce the degradation of RNA polymerase II. Such degradation is
420 associated with an increased ubiquitination of RNA polII, suggesting that a factor of the
421 host Ubiquitin-Proteasome System could be involved [54]. The PB2 504 seems more
422 accessible in the transcription competent polymerase form of fluB polymerase than in
423 5'-c-RNA bound form, where it is lying in a pocket of PB2, whereas it is buried in the apo-
424 conformation.

425

426 Host factors interaction from interactomics studies.

427 A number of other host factors have been identified as acting on the viral life
428 cycle via an interaction with the polymerase in low-throughput studies [3]. More
429 recently, high-throughput interaction mappings led to the identification of numerous
430 host factors interacting either with the RNP in an infectious context [41, 43], or with
431 isolated polymerase sub-units [38, 42, 44]. Despite limited overlap in the polymerase
432 host partners identified, several cell functions emerged as being targeted by the viral
433 polymerase, among which protein chaperoning, RNA metabolism, and nucleo-

434 cytoplasmic transport. The functional links between these interactions and the activities
435 of the polymerase or of its isolated sub-units are however almost not deciphered.

436 From the currently known structures, one can deduce which binding interfaces
437 are differently exposed according to the polymerase configuration. In the 5'+3' RNA-
438 bound polymerase, exposed surfaces are provided by one face of the Cap binding
439 domain and amino acids patches of PB2-mid (figure 11). These surfaces are not
440 accessible because they are packed against the polymerase complex in the 5'cRNA-
441 bound or apo-flu polymerase configurations (figure 11). They consequently could
442 mediate interactions engaged by the polymerase bound to 5' and 3' vRNA ends. One
443 such interaction could be with the rab11 protein, which has been shown to bind the
444 vRNP through PB2 [55]. This interaction is involved in the cytoplasmic transport of the
445 produced vRNP unit, and thus should engage with newly synthesized polymerases
446 bound to 5'+3' vRNA. Similarly, the α helix 6 of PA endonuclease domain provides a well-
447 exposed surface in the polymerase bound to 5'+3' vRNA ends, while being buried in the
448 5'cRNA-bound configuration owing to its packing against the NLS domain of PB2 (figure-
449 11).

450 The PB2 627 domain is well separated and highly accessible from the rest of the
451 polymerase only in the 5'cRNA-bound polymerase, while in the transcription-competent
452 forms it is packed by contacts with the PB1 and PA sub-unit in a somewhat buried
453 position along the mRNA exit pathway (figure 11). The PB2-627 domain could be an
454 important driver of interactions with host factors instrumental for the replication
455 activity of the polymerase. For example, the ANP32 protein has recently been shown to
456 be involved in the replication competence of influenza polymerase in human cells,
457 depending upon the 627 residue of PB2 [56]. The role of ANP32 could thus be mediated
458 by an interaction with the PB2 627K domain in the replication-competent polymerase.
459 The fourth helix α on the PA subunit is exposed only in the 5'cRNA-bound polymerase,
460 lying under the NLS C-terminal peptide of PB2, and may represent an interaction
461 interface for factors involved in the replication activity (figure 12). One such interaction
462 has been described involving PA and several sub-units of the human MCM
463 (minichromosome maintenance element) [57].

464 In addition, a number of residues are exposed in all forms of polymerase resolved
465 so far, but harbor different locations as a result of the distinctive PB2-C and PA-ENDO
466 arrangements. One can therefore propose that a number of polymerase activity-specific
467 interactions could be mediated through specific patterns of exposed residues patches. In
468 contrast, the exposed surfaces of the polymerase core essentially remain unchanged,
469 and possibly mediate interactions with host factors required for all polymerase
470 activities.

471

472

473 Concluding remarks

474 The recently characterized influenza virus polymerase structures highlight a
475 number of key points regarding influenza RNA polymerase biology. From a general
476 point of view, they showed that the influenza polymerase encompasses a central
477 catalytic fold which is shared by other RNA-dependent RNA polymerases, thus
478 contributing to an emerging picture of the catalytic fold of negative strand RNA-
479 dependent RNA polymerases [18].

480 One remarkable trait of the influenza virus polymerase lies in that it can adopt,
481 depending upon viral RNA ends binding, stable but distinctive packing of the PB2-C and
482 the PA-ENDO domains relative to a common polymerase core. The polymerase

483 conformations that appeared from the various crystallographic studies most likely
484 reflect different functional states of the polymerase. The polymerase complex seems to
485 exhibit a remarkable flexibility, with the PB2-C and PA-ENDO as key players of
486 conformational dynamics. Structural studies suggest that conformational switches are
487 underlying the multifunctional competency of the influenza virus polymerase. The rules
488 governing such switches still remain to be deciphered. The binding of RNA segments
489 extremities to the polymerase core seems to take an active part in determining
490 polymerase conformations, possibly through structural changes which somehow
491 transmit to the distal PB2-C and PA-ENDO flexibly linked domains.

492 One may speculate the association of host factors would assist polymerase
493 conformation remodeling and contribute to stabilize alternative polymerase
494 conformations. This is in line with the different patches of interaction motifs that are
495 exposed according to the type of RNA bound to the polymerase. A simultaneous
496 contribution of viral RNA binding and host-protein interactions is in fact likely to
497 underlie the multiple conformations, hence to the multiple activities, of the polymerase
498 complex. Studies of the polymerase-host interplays revealed a diversified panel of
499 cellular factors associating with influenza virus polymerase, but only few host factors
500 have been implicated in a specific activity of the polymerase. Further studies will be
501 required to identify sets of interacting host factors impacting on specific polymerase
502 activities. The advances in the structure knowledge of influenza virus polymerase
503 represent a powerful framework for such studies.

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510

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673
674

675 **FIGURE LEGENDS**

676 **Figure 1.** a. Overview of the different polymerase structures. The overall shape of the
677 polymerase complexes in four resolved structures are shown with a fixed position of the
678 central core. The PB1 sub-unit is colored beige, PB2 blue, PA green. The pdb are as
679 follows: bat FluA bound to 5'+3'-vRNA pdb 4WSB (Pflug et al., 2014); human fluB bound
680 to 5'+3'vRNA pdb4WSA (Reich et al., 2014); human fluB bound to 5'cRNA pdb 5EPI
681 (Thierry et al., 2016), human apo-fluC pdb5d9 (Hengrung et al., 2015). b. Schematics of
682 the PA /P3, PB1 and PB2 domain organization. Numbering is given for human bat FluA,
683 human flu B and human flu C as indicated. Hatched domains are those constituting the
684 central core polymerase.

685 **Figure 2.** The catalytic core of influenza polymerase. **a.** Ribbon diagram of the PB1
686 subunit, with the modules typical of an RNA dependent RNA polymerase fold indicated.
687 The conserved functional motifs of RNA polymerase lying in the palm domain are
688 colored in purple, the priming loop in red and the PB1 bipartite NLS in rust. **b** Diagram
689 representing the polymerase fold of fluA PB1 flanked by the PA-C on one side and by the
690 PB2-N on the other side of the thumb. PB1, PB2-N and PA-C are colored beige, blue and
691 green respectively the NLS of PB1 is represented in rust spheres. Example of the
692 polymerase fold of the flu A structure is given (pbd 4WRT) which is the only containing
693 a resolved structure of the priming loop.

694
695 **Figure 3.** RNA promoter binding to the polymerase. All images are generated from the
696 FluB structure pdb 4WRT (Reich et al., 2014) which is the only crystal containing full-
697 length 5' and 3' vRNA strands used as surrogate of viral promoter. **a.** Representation of
698 the 5' and 3' vRNA promoter, highlighting the base pairing of the distal region and
699 configuration of single-strand extremities. **b.** Surface representation of the polymerase
700 promoter-binding interface, showing the RNA-protein binding interface with the 3'
701 vRNA extremity. The 5' and 3' ends of vRNA strands are indicated. **c.** Surface
702 representation of the vRNA 3'end at the entry of the putative template canal modeled
703 from the superimposition with the primer-template structure of the Norwalk virus
704 polymerase (Reich et al., 2014). The 3' end of the 3' vRNA strand is indicated.

705 **d** Surface representation of the polymerase promoter-binding interface highlighting the
706 binding 5'vRNA hook in a pocket formed by PB1 and PA. The 5' end of the 5' vRNA
707 strand is indicated.

708 **Figure 4** Cartoon representation of the folding of PB2 in promoter-bound (shown for
709 fluB pdb 4WSA) and in the 5'cRNA bound (fluB pdb 5EPI) polymerase configurations.
710 The step-wise representation of the PB2 structure starts with the rigid axis constituted
711 by the cap-627 linker (purple) and mid domain (pink), then with the CAP (light blue),
712 627 (cyan) and NLS (violet) sub-domains, last in full PB2 protein (PB2-N in sky blue). N-
713 and C-terminus extremities of the corresponding polypeptides are indicated.

714
715 **Figure 5.** The transcription-competent polymerases. a. Structures of v5'+v3' RNA bound
716 polymerases bat flu A (pdb 4WSB) and human flu B (pdb 4WSA), shown with the PB2-C
717 and PA-ENDO domains in ribbon diagram and the rest of the polymerase in a surface
718 view. A cap bound to the PB2 cap-binding domain is shown in red, and the catalytic site
719 of PA endonuclease domain is colored purple. Color code for PB1, PB2 and PA subunits
720 are as in figure 1. b. Surface view of the human-fluB polymerase conformation (pdb
721 4WSA) highlighting the orientation of a cap bound to the PB2 cap binding site facing the
722 catalytic center. The view position is across the PB2 cap binding domain as indicated in
723 the panel a. c. Surface representation of the transcription-competent polymerase (from

724 pdb 4WSA) highlighting the position of template exit indicated by the location of the
725 obstructing PB2 helical lid (colored brown), and of the putative mRNA exit tunnel.
726

727 **Figure 6.** The different arrangements of the PB2 sub-unit in polymerase configurations.
728 **a.** Representation of the structures of the Flu B polymerase when bound to the 5'+3'
729 vRNA viral promoter (pdb 4WSA) or to the 5'cRNA only (pdb 5EPI), and of the apo-fluC
730 polymerase (pdb 5d9). The polymerase core is represented in surface grey, the PB2-C is
731 colored in a ribbon representation for CAP domain (light blue), cap-627 linker (purple)
732 and mid domain (pink). PB2-627 (cyan) and NLS (dark blue) domains are in surface
733 representation. The PA-ENDO is shown in ribbon and colored green. Polymerase
734 structures are represented with similar orientation of the polymerase core to highlight
735 the rearrangements of the PB2-C domains in the polymerase complex. **b.** Surface
736 representation of PB2-CAP bound to a cap represented in red spheres in the 5'cRNA
737 polymerase, highlighting the obstruction of the cap-binding site through apposition to
738 the PB2 cap-627 linker.
739

740 **Figure 7.** Polymerase dimer formation **a.** Dimer observed for fluA H5N1 subcomplex 1
741 (pdb3j9). Top : Ribbon representation of the dimer, PB1 is colored beige, PA is colored
742 green, PB2 α -helices (attributed to aa 86 to 130) are shown in cyan (monomer 1) or
743 blue (monomer 2). The PA α -helices involved in the dimer interface are shown in light
744 pink (monomer 1) and dark pink (monomer2), PB2 helices involved in dimer interface
745 are colored shown in cyan (monomer 1) and dark blue (monomer 2). Amino acids of
746 PB1 proposed to be involved in tetramer formation are in colored yellow. Bottom: the
747 surface of the dimer interface is shown to highlight the contacts between each monomer.
748 **b** structural elements proposed for dimer interface are shown in surface representation
749 colored as in one monomer of fluA H5N1 subcomplex 1, in the 5'cRNA bound form of
750 fluB polymerase (pdb 5 EPI) and in the apo-fluC polymerase (pdb5d9). They are shown
751 with the rest of the polymerase in ribbon (left) and in surface (right) representation to
752 highlight both the contiguity and the accessibility of the intervening α -helices
753

754 **Figure 8.** Interaction with NP. **a.** Interaction interface with NP. The PB1 β ribbon
755 interacting with NP is colored beige, 5' v or cRNA in yellow, 3'vRNA in orange, PB2 N-
756 terminal α helices involved in NP binding colored blue, the PB1-C-terminal α helices
757 involved in the PB2-N/PB1-C interaction interface observed in isolated domains colored
758 beige. The position of the PB1 NLS in the PB1 β ribbon is labelled red for the FluB
759 5'+3'vRNA-bound and 5'c-RNA bound polymerases, but not in the FluC apo polymerase
760 structure where it is not conserved. Both PB1 β ribbon and RNA strands are shown in
761 ribbon relative to the rest of the polymerase in white surface. Images are built from pdb
762 4WSA for the fluB promoter-bound polymerase, pdb 5EPI for 5'cRNA fluB bound
763 polymerase, pdb 5j9 for apo flu C polymerase. **b.** Location of the interaction interfaces
764 with NP in the promoter-bound fluB (left, pdb 4WSA), the 5'cRNA-bound fluB (right,
765 pdb 5EPI) and the apo-FluC C(pdb5d9) polymerases relative to the putative exit tunnels
766 for products (dark blue arrow for mRNA product, red arrow for replicate product) and
767 to the putative position of template exit, indicated by the location of the obstructing PB2
768 helical lid (colored brown). Note that the putative template exit is indicated on the face
769 of the polymerase where the template is expected to emerge. The PB2-627K domain
770 colored is cyan, residues involved in interaction with NP are in red. The PB2 627K,
771 PB1C/PB2N helices bundle and PB1 β ribbon are in ribbon representation.
772

773 **Figure 9.** Adaptative Residues in the polymerase **a.** Adaptative residues of influenza
774 polymerase. Residues are shown at the surface of the transcription-competent (pdb
775 5WSA, left) and 5'cRNA-bound (pdb 5EPI, right) forms of the Flu B polymerase.
776 Adaptative residues are colored red in PB2, purple in PA and brown in PB1, positions
777 are given for the PA and PB1 sub-units. **b.** Adaptative residues of the PB2 subunit
778 colored in red are shown in a surface representation of the 5'cRNA-bound fluB
779 polymerase, with transparency to highlight residues lying along the interaction interface
780 between PB2 and PB1 or PA (indicated by arrows). **c.** Adaptative residues in the 627/NLS
781 domains of PB2 are colored distinctly, their accessibility is shown in the transcription
782 competent (left), and 5'-cRNA bound (right) forms of the fluB polymerase.

783
784

785 **Figure 10** Polymerase interactions with host factors. **a.** Interaction with α importins.
786 Top (Left) Ribbon representation of the NLS domain of 5'cRNA-bound flu B polymerase
787 (pdb 5EPI) colored dark blue, with the rest of the polymerase shown in a surface
788 representation. The residues involved in interaction with α importins are colored red.
789 (right) surface representation of the PB2 NLS in the same polymerase configuration.
790 Bottom-the NLS domain in the promoter-bound FluB conformation (pdb 4WSA), only
791 partly resolved, is shown in ribbon representation, colored dark blue. The extreme C-
792 terminus peptide containing the NLS lacks electron density reflecting, a flexible non-
793 folded state and is represented with a dashed line. **b.** Interaction with hCLE. The
794 interaction interface of PA with the human factor hCLE is shown in surface
795 representation and colored in red in the FluB polymerase associated to 5'+3'vRNA
796 promoter (pdb 4WSA). Residues PB2 504 and PA 550 involved in RNA polII degradation
797 are represented in purple spheres.

798

799 **Figure 11** Potential distinctive binding interfaces . Surface representation of the FluB
800 polymerase bound 5'+3' vRNA (pdb4WSA, right) or to 5'cRNA bound (pdb 5EPI right).
801 Surfaces specifically exposed in the 5'+3'vRNA polymerase are colored brown (PB2) and
802 purple (PA), surfaces more exposed in the 5'cRNA-bound form are colored salmon (PB2)
803 and pink (PA). The PB2 residue 627K is colored yellow.

804
805

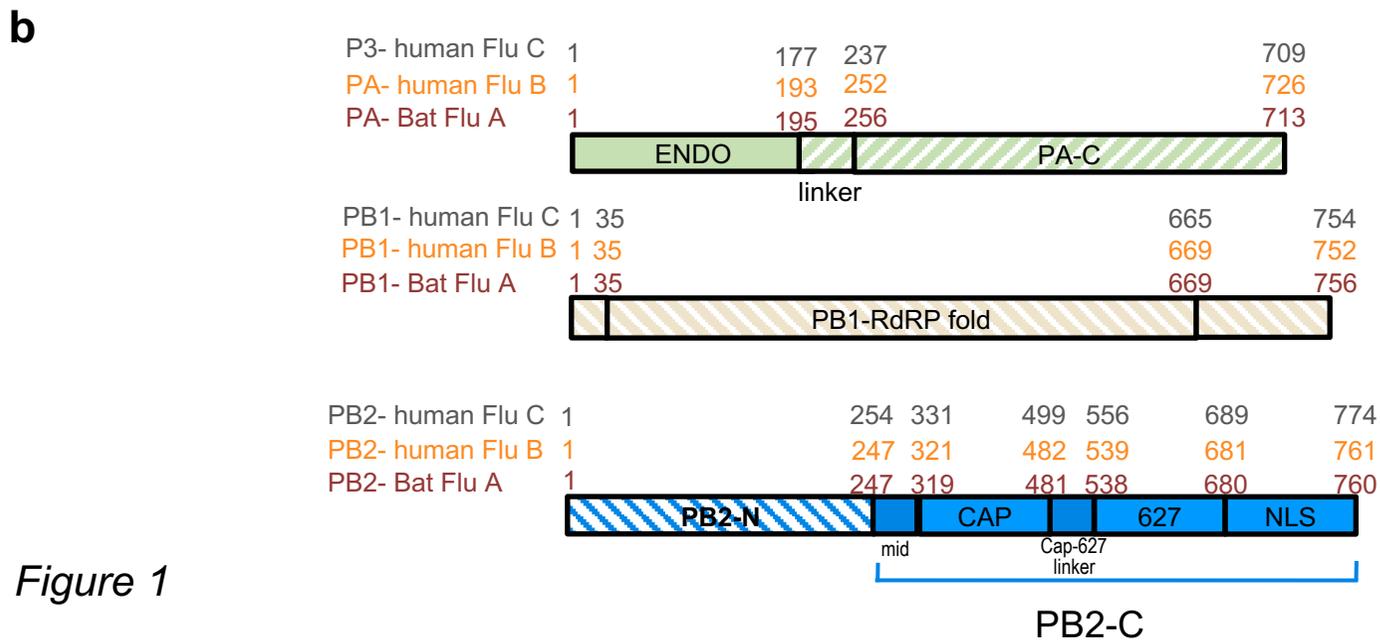
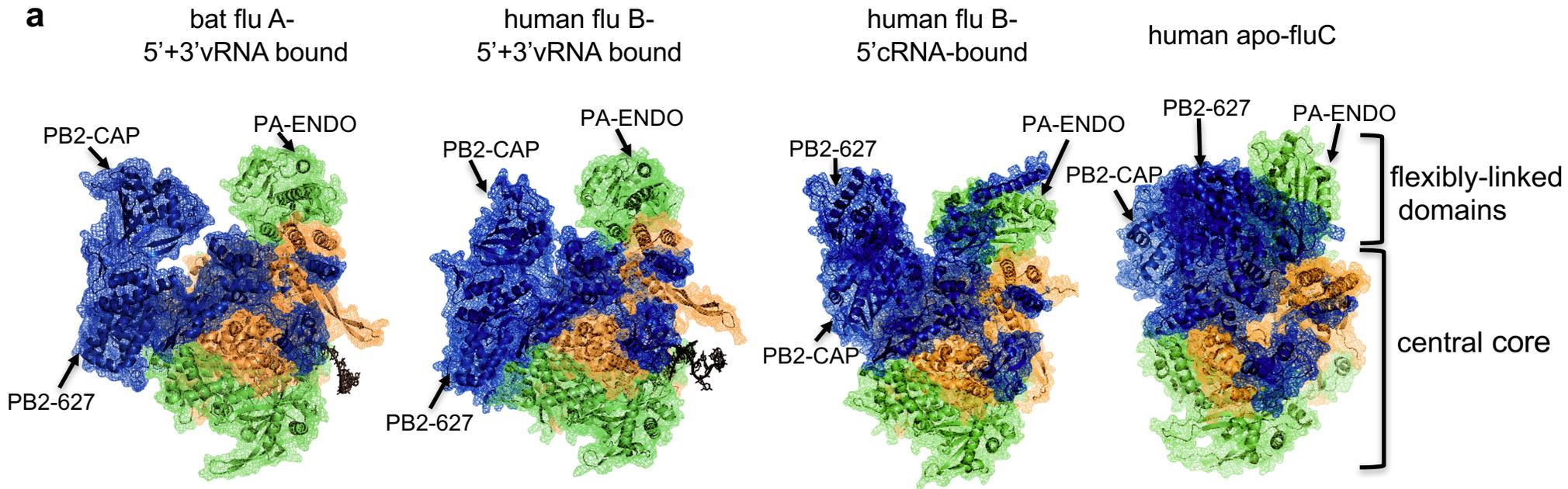


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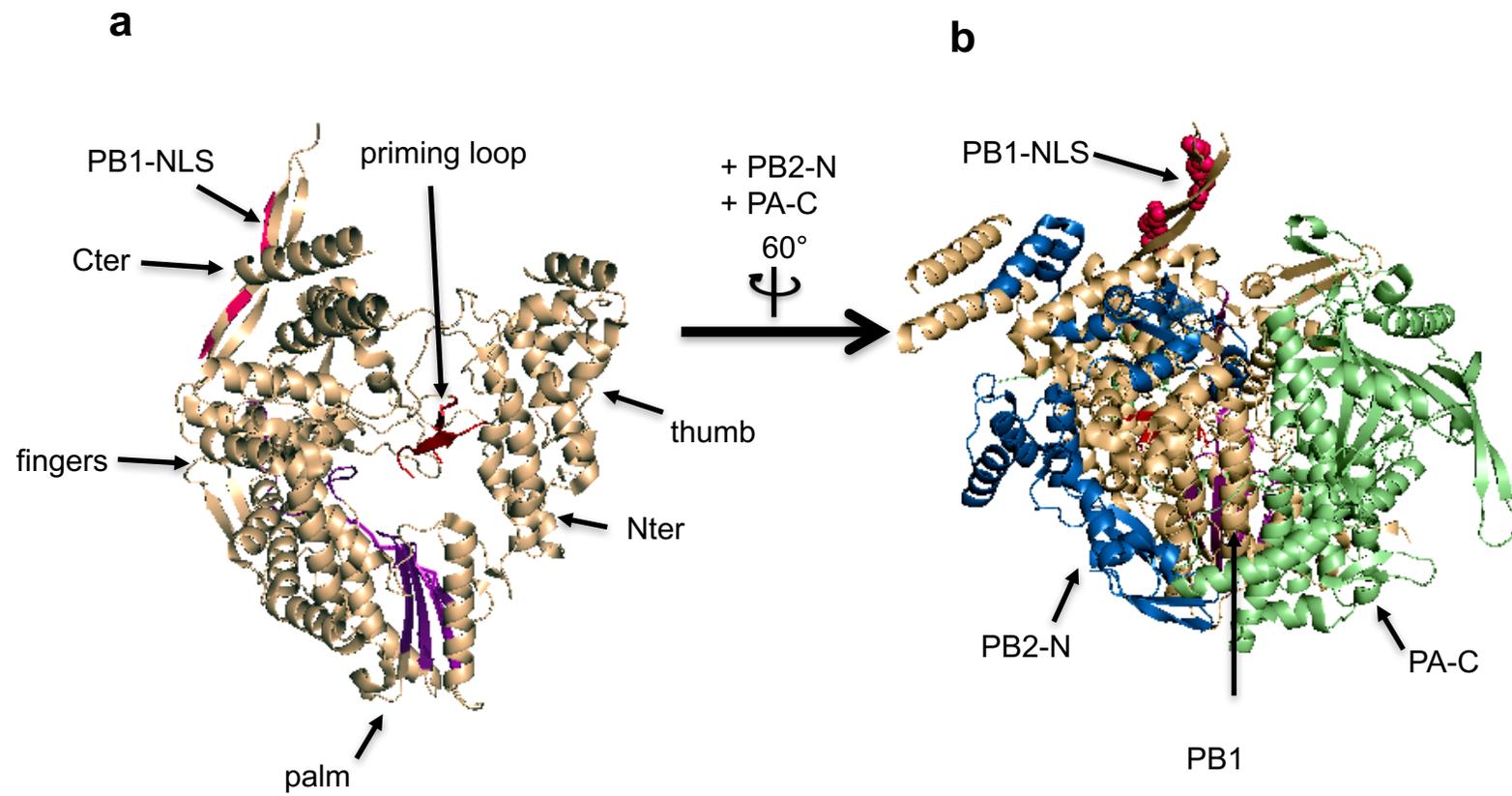


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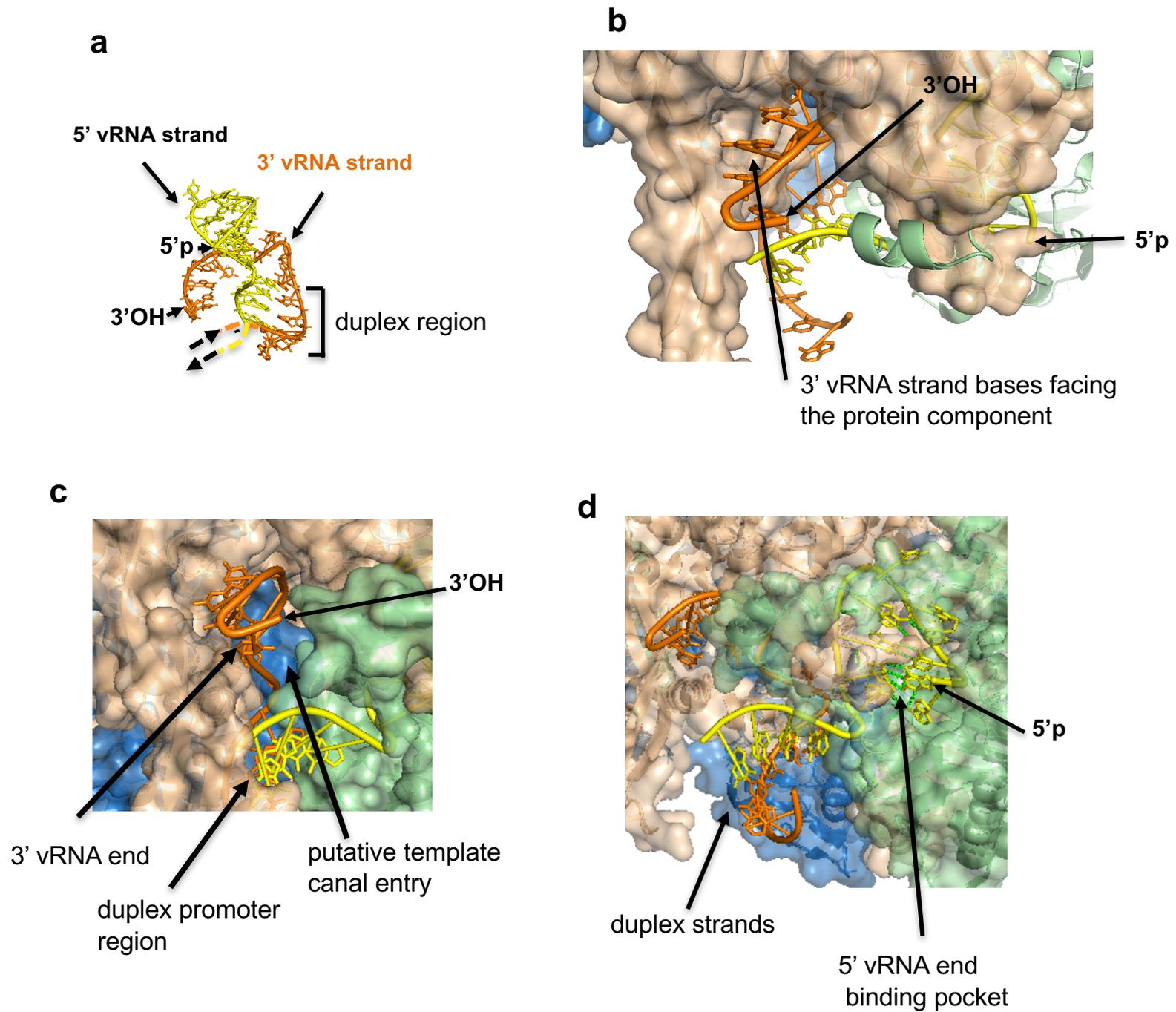


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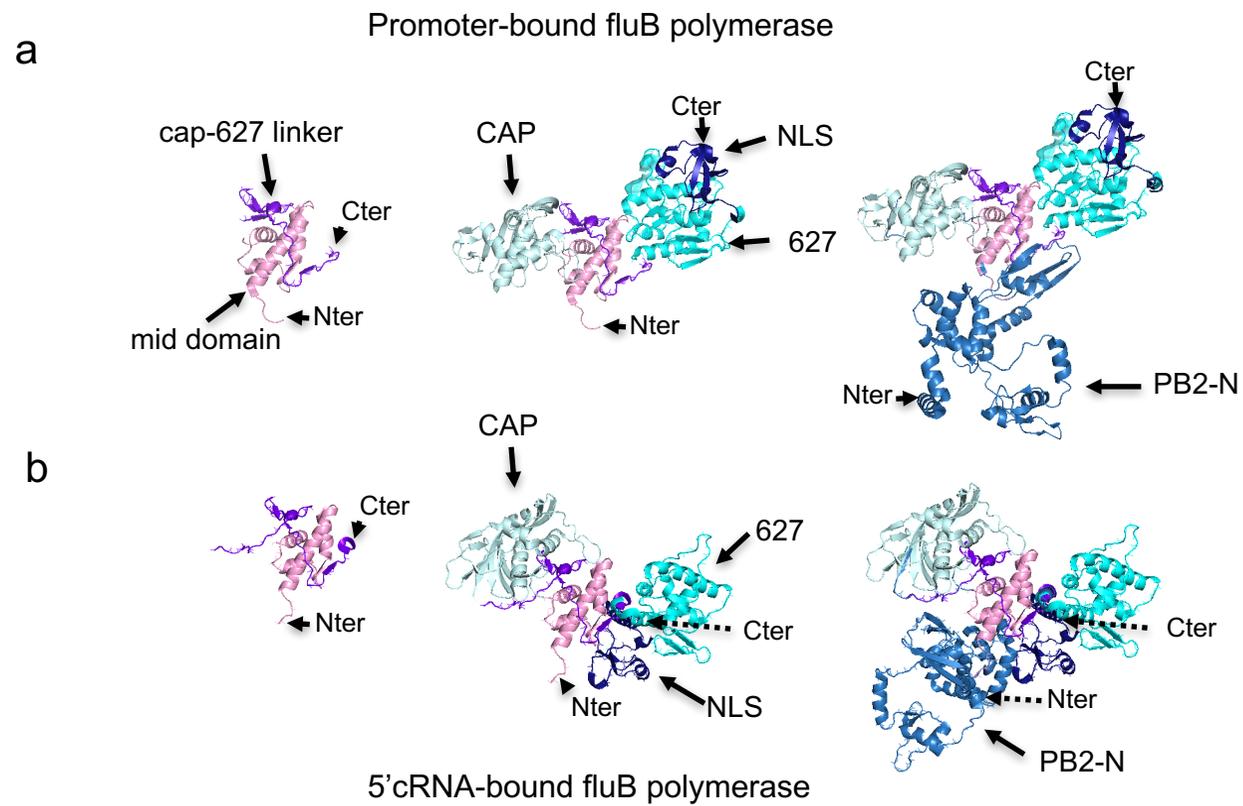


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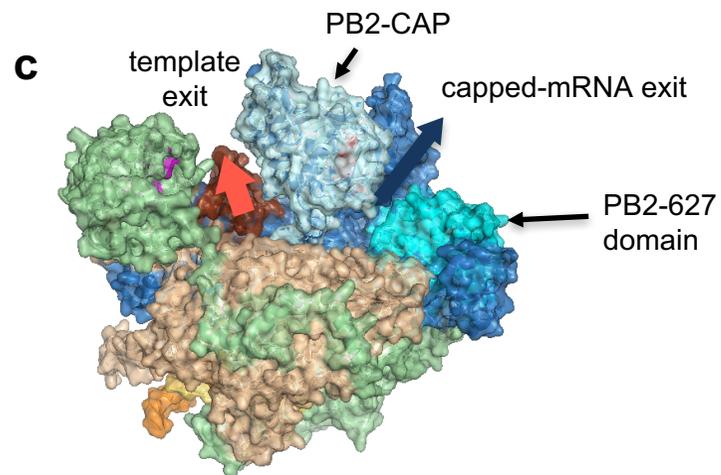
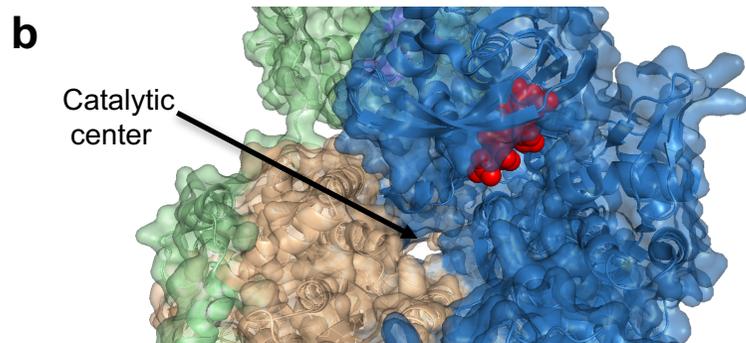
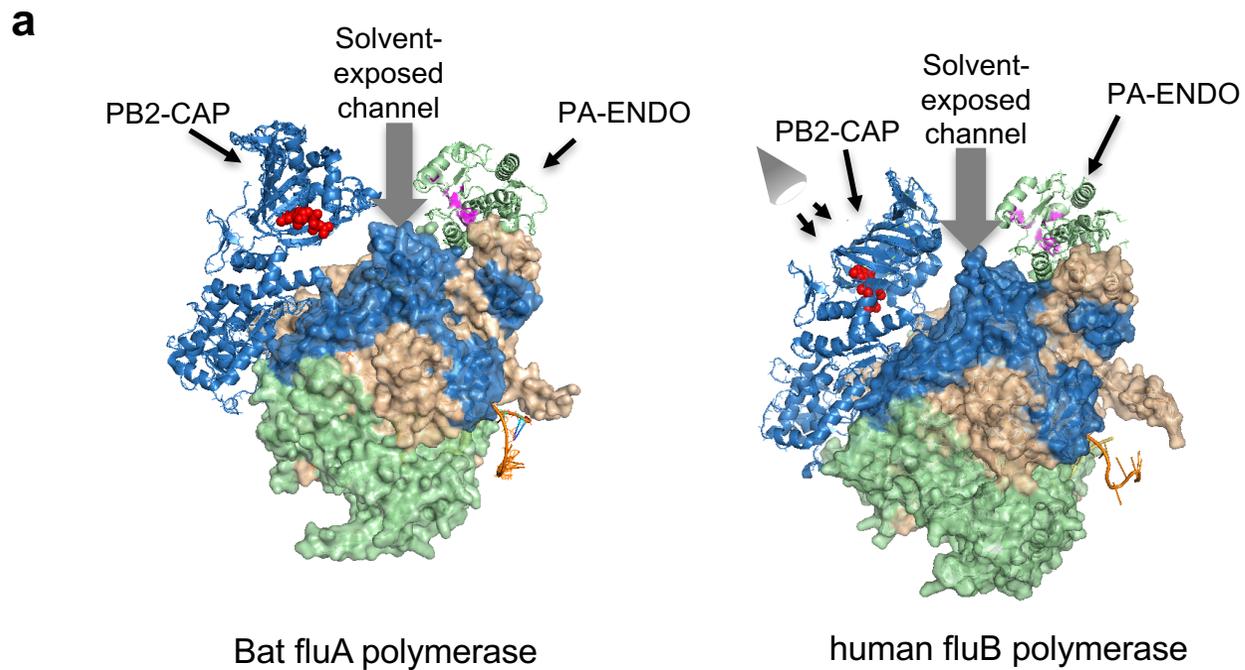


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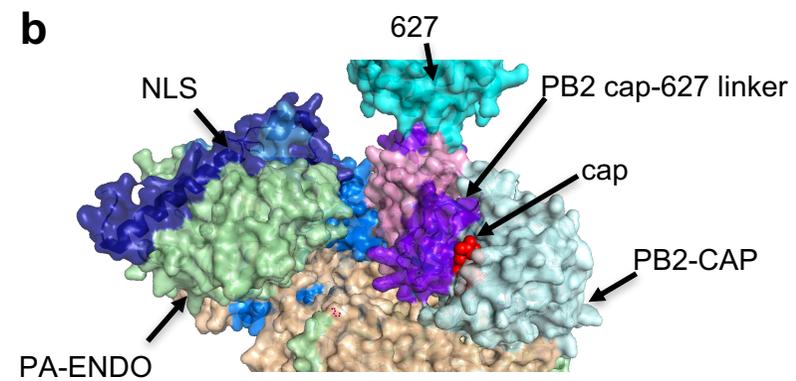
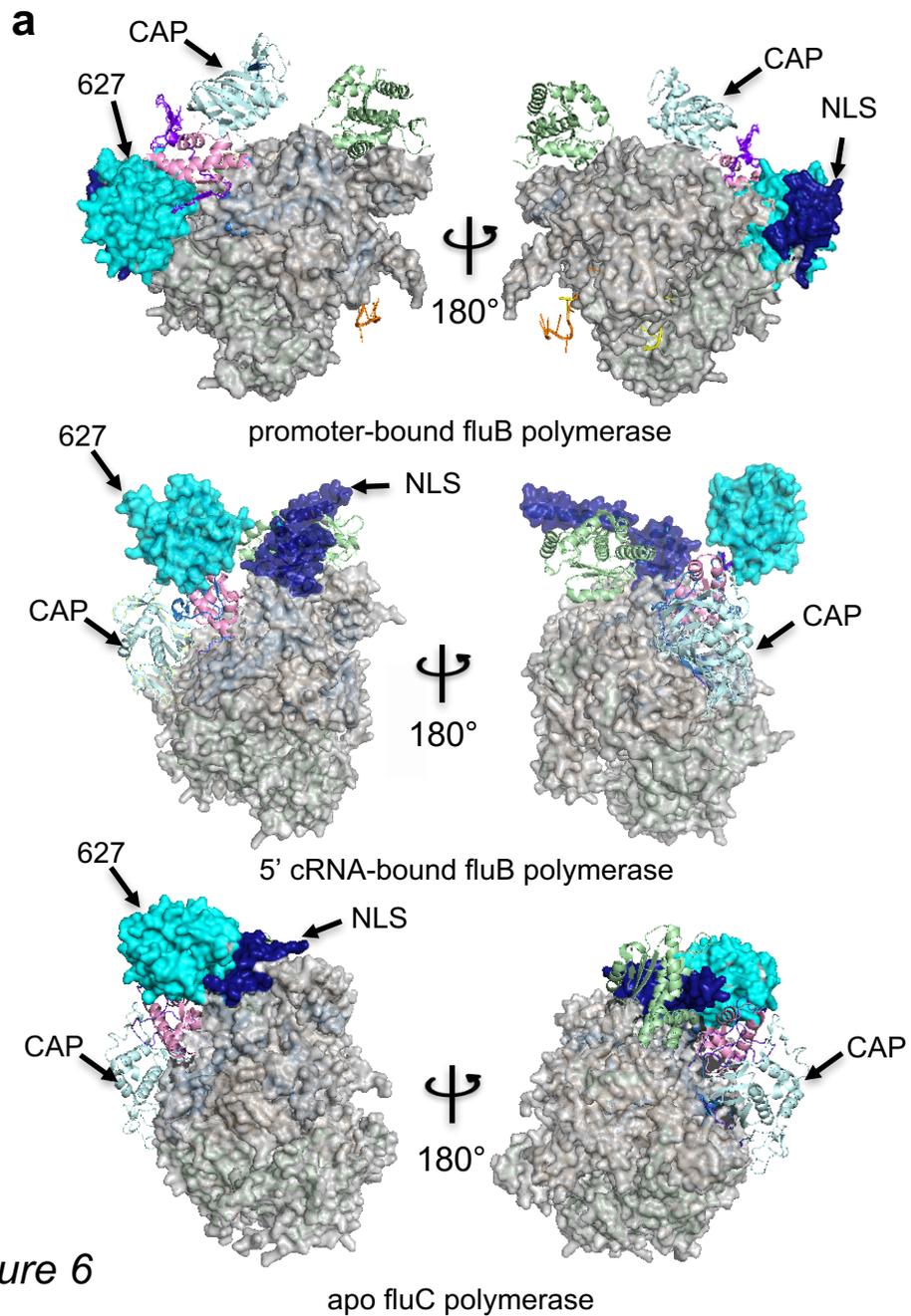


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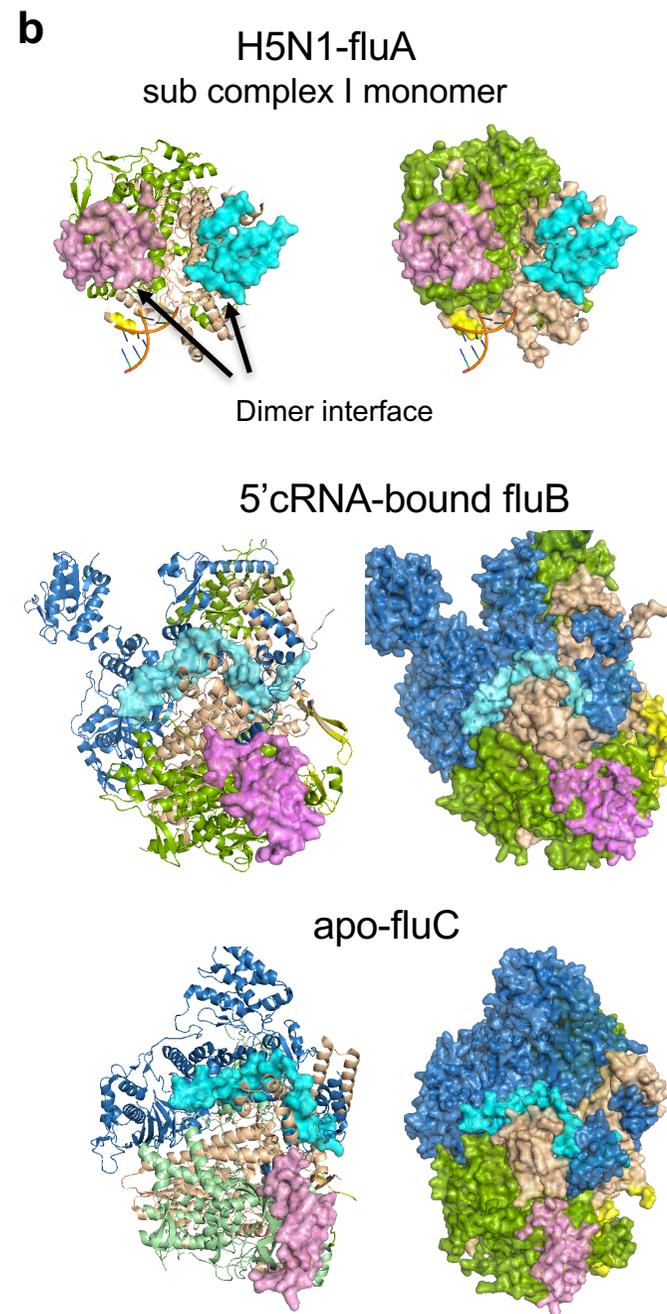
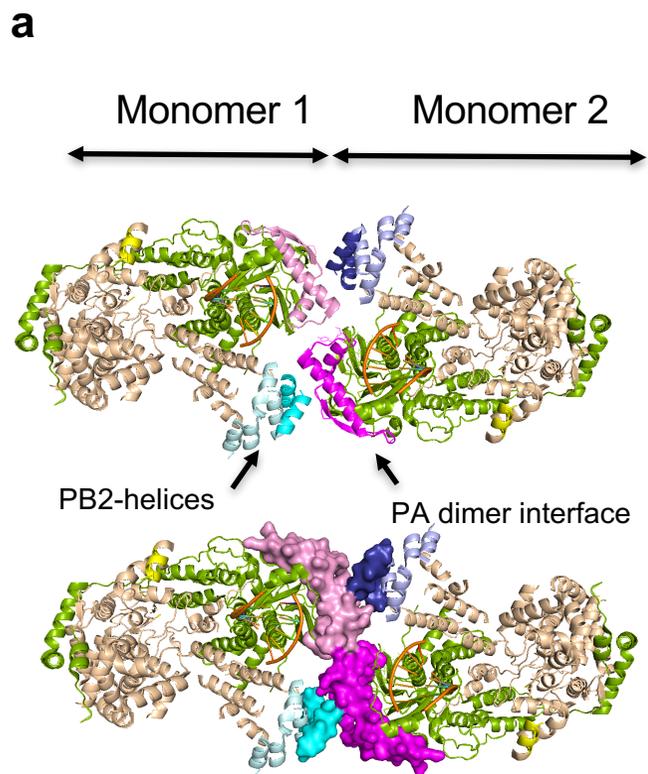


Figure 7

a

5'+3' vRNA-bound polymerase

5' cRNA-bound polymerase

apo polymerase

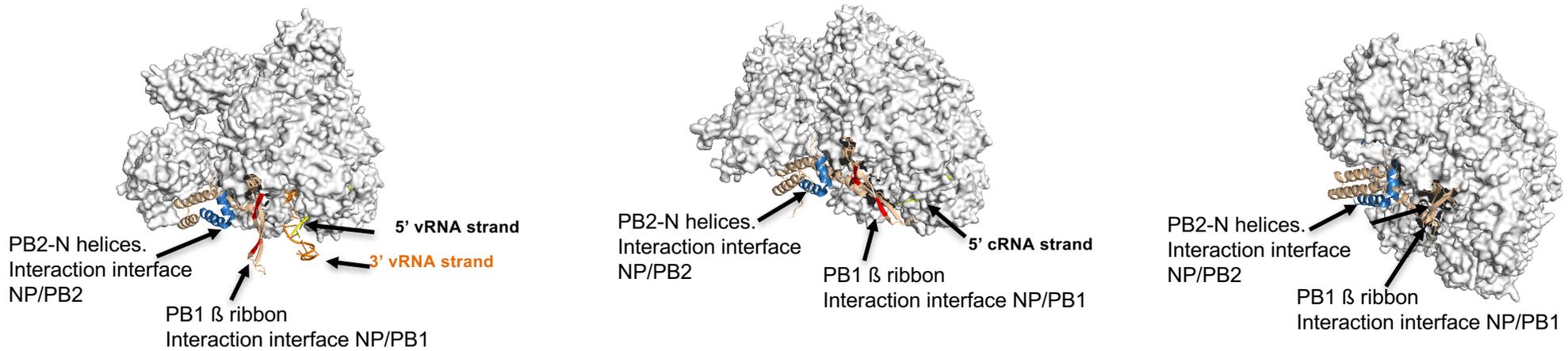
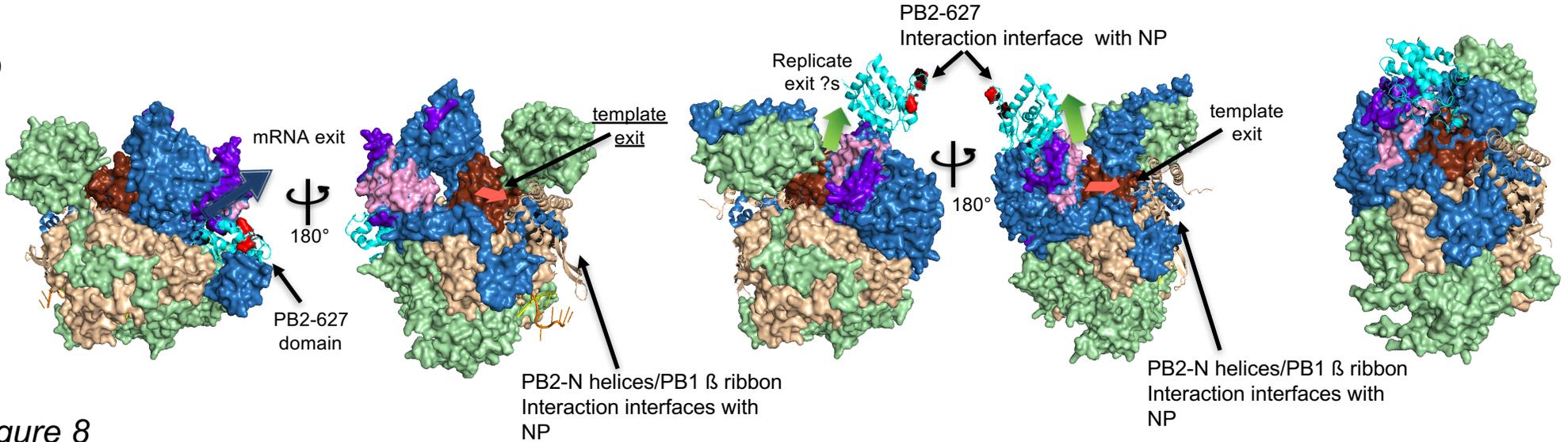
**b**

Figure 8

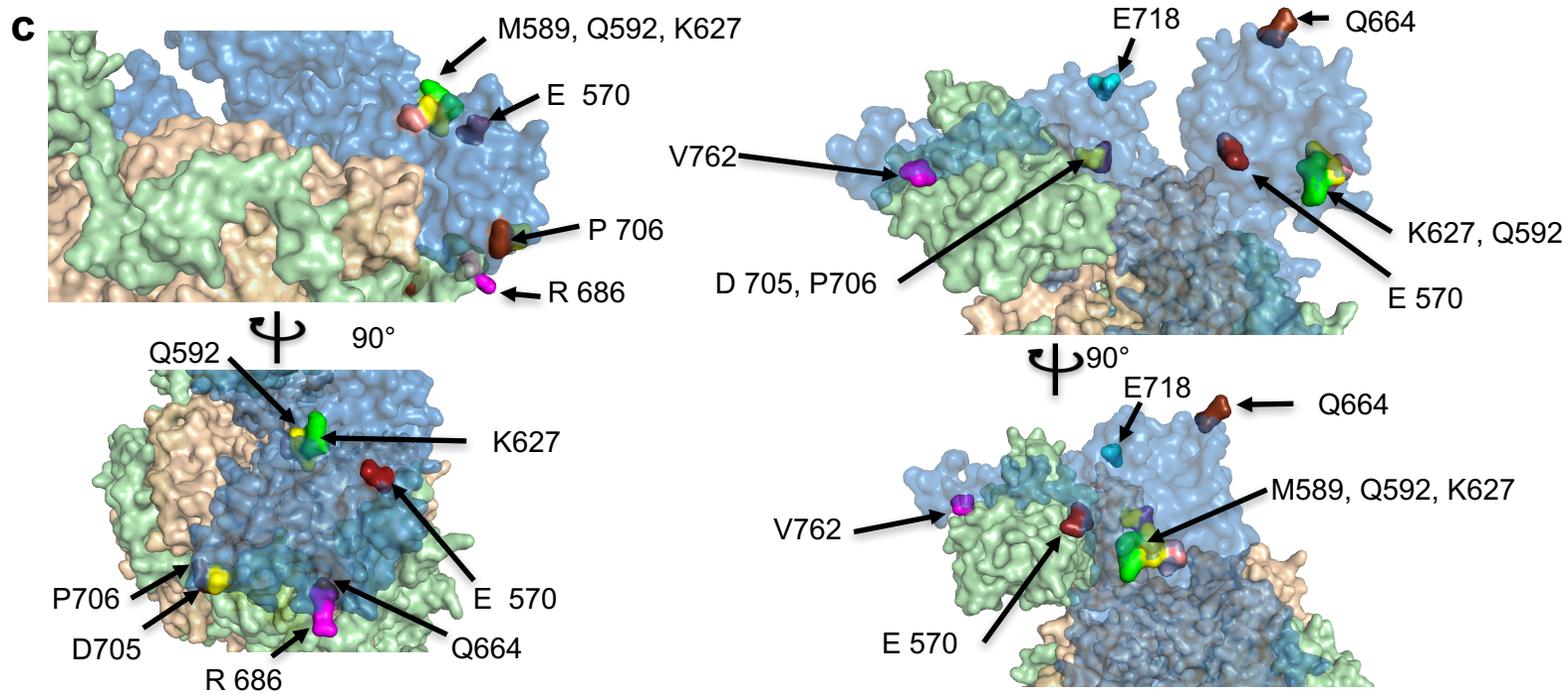
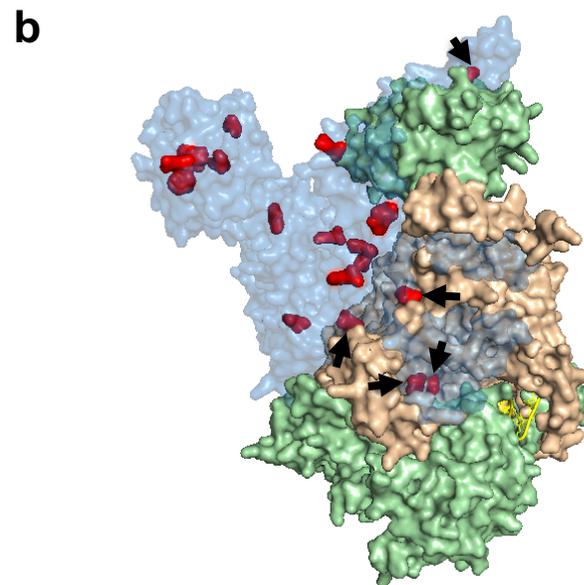
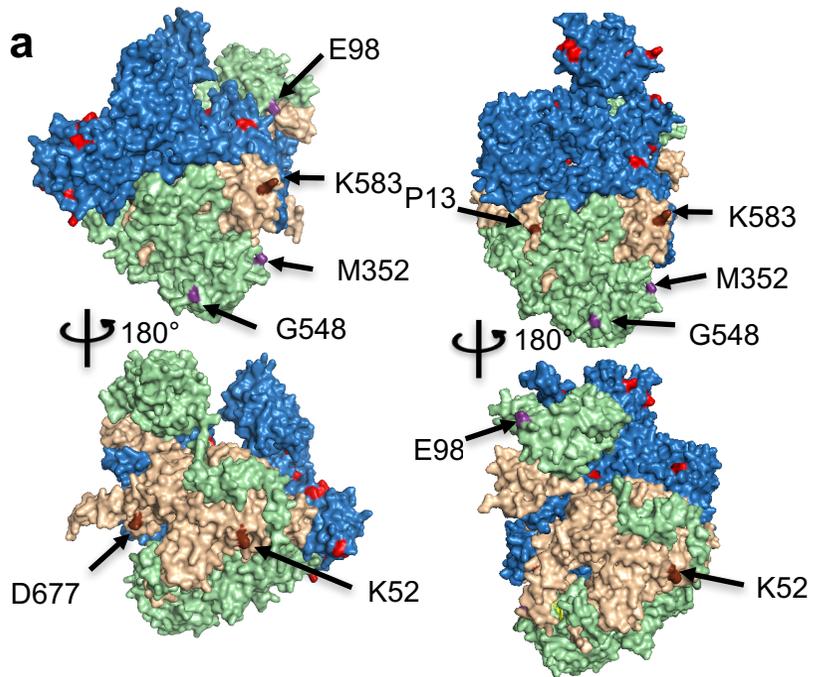
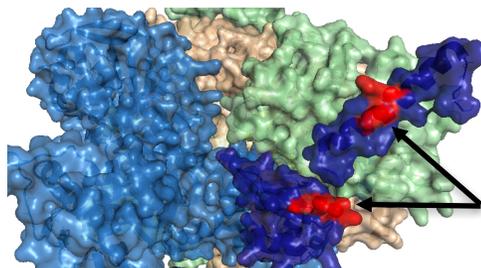
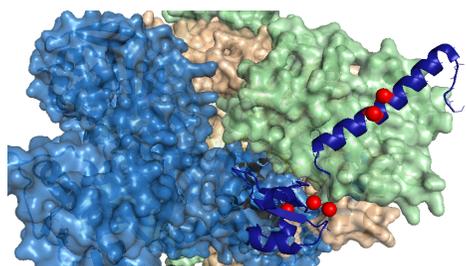


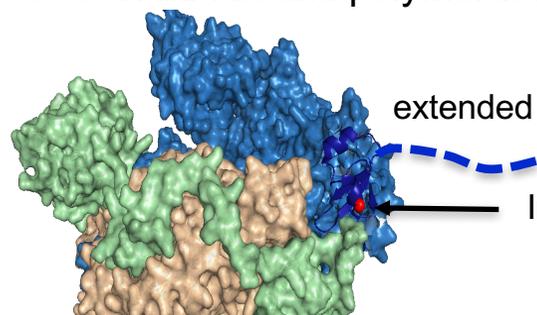
Figure 9

a 5' cRNA-bound polymerase



Interaction with α importins

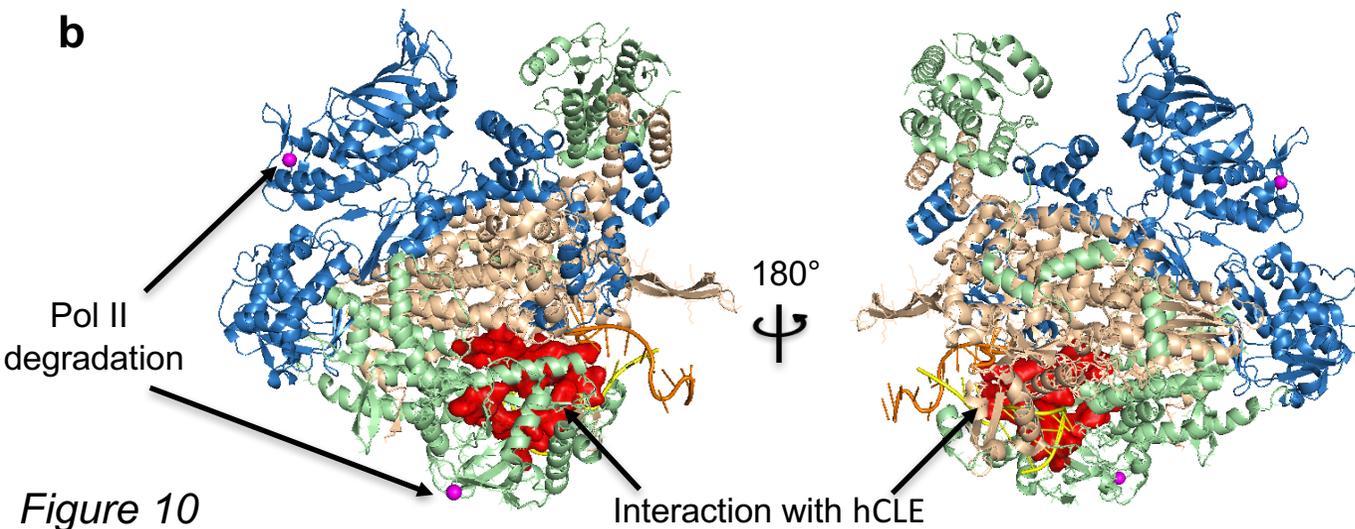
5'+3' vRNA-bound polymerase



extended C-terminal NLS peptide

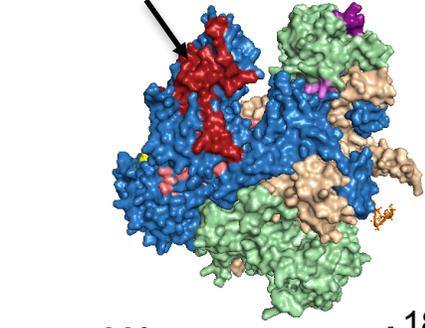
Interaction with α importins

b



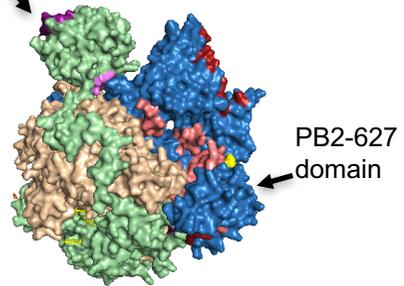
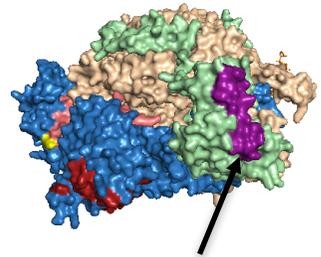
5'+3' vRNA-bound polymerase

PB2-CAP+mid
exposed surface



90°

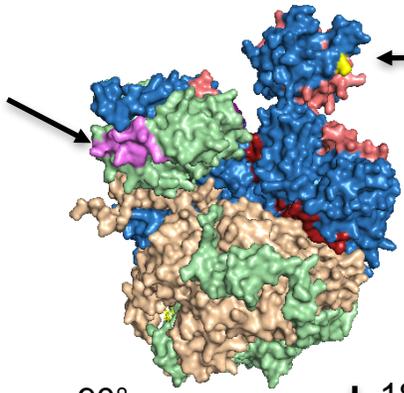
180°



Exposed PA
 α 6 helix

5'cRNA -bound polymerase

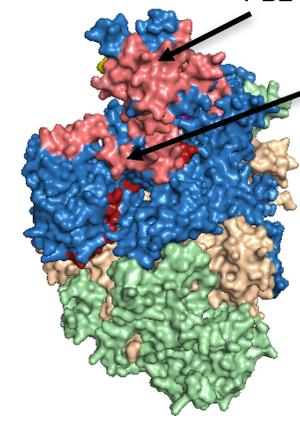
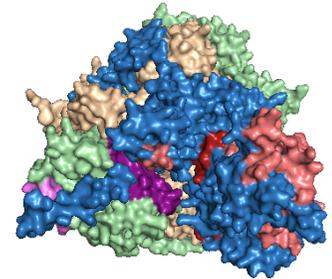
Exposed PA
 α 4 helix



PB2-627 domain

90°

180°



PB2-627 exposed surface

PB2-CAP-cap 627 linker
exposed surface

Figure 11