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**The host RNA polymerase II C-terminal domain is the anchor  
for replication of the influenza virus genome**

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## 26 **Summary**

27           The current model is that the influenza virus polymerase (FluPol) binds either to host RNA  
28 polymerase II (RNAP II) or to the acidic nuclear phosphoprotein 32 (ANP32), which drives its  
29 conformation and activity towards transcription or replication of the viral genome, respectively. Here,  
30 we provide evidence that the FluPol-RNAP II binding interface has a so far overlooked function for  
31 replication of the viral genome. Using a combination of cell-based and *in vitro* approaches, we show  
32 that the RNAP II C-terminal-domain, jointly with ANP32, enhances FluPol replication activity and we  
33 propose a model in which the host RNAP II is the anchor for transcription and replication of the viral  
34 genome. Our data open new perspectives on the spatial coupling of viral transcription and replication  
35 and the coordinated balance between these two activities.

36

37 **Keywords :** Influenza virus, RNA-dependent RNA polymerase, cellular RNA polymerase II, ANP32,  
38 transcription, replication

39

## 40 **Introduction**

41 Influenza viruses are major human pathogens which cause annual epidemics (type A, B and C viruses)  
42 (Krammer et al., 2018) and have zoonotic and pandemic potential (type A viruses) (Harrington et al.,  
43 2021). The viral RNA dependent RNA polymerase (FluPol) is a key determinant of viral host-range  
44 and pathogenicity, and a prime target for antiviral drugs (Mifsud et al., 2019). It is a ~260 kDa  
45 heterotrimer composed of the subunits PB1 (polymerase basic protein 1), PB2 (polymerase basic protein  
46 2) and PA (polymerase acidic protein) (Wandzik et al., 2021). In viral particles each of the eight  
47 negative-sense RNA genomic segments (vRNAs) is associated with one copy of the viral polymerase  
48 and encapsidated with multiple copies of the nucleoprotein (NP) to form viral ribonucleoproteins  
49 (vRNPs) (Eisfeld et al., 2015). Upon viral infection, incoming vRNPs are imported into the nucleus in  
50 which FluPol performs transcription and replication of the viral genome through distinct primed (Plotch  
51 et al., 1979) and unprimed (Deng et al., 2006) RNA synthesis mechanisms, respectively. Determination  
52 of high-resolution FluPol structures by X-ray crystallography and cryogenic electron microscopy (cryo-  
53 EM) have revealed that a remarkable conformational flexibility allows FluPol to perform mRNA  
54 transcription (Dias et al., 2009; Guilligay et al., 2008; Kouba et al., 2019; Pflug et al., 2018; Wandzik  
55 et al., 2020). Recently, first insights into the molecular process of FluPol genome replication were  
56 revealed, however the molecular details remain largely to be determined (Carrique et al., 2020; Keown  
57 et al., 2022; Wang et al., 2022).

58 Transcription of negative-sense vRNAs into mRNAs occurs via a process referred to as “cap-  
59 snatching”, whereby nascent 5'-capped host RNA polymerase II (RNAP II) transcripts are bound by  
60 the PB2 cap-binding domain (Guilligay et al., 2008), cleaved by the PA endonuclease domain (Dias et  
61 al., 2009), and used as capped primers to initiate transcription (Kouba et al., 2019). Polyadenylation is  
62 achieved by a noncanonical stuttering mechanism at a 5'-proximal oligo(U) polyadenylation signal  
63 present on each vRNA (Poon et al., 1999; Wandzik et al., 2020). Influenza mRNAs therefore harbour  
64 5'-cap and 3'-poly(A) structures characteristic of cellular mRNAs and are competent for translation by  
65 the cellular machinery. The accumulation of primary transcripts of incoming vRNPs leads to *de novo*

66 synthesis of viral proteins, including FluPol and NP, which are thought to trigger genome replication  
67 upon nuclear import (Zhu et al., 2023).

68 Replication generates exact, full-length complementary RNAs (cRNAs) which in turn serve as  
69 templates for the synthesis of vRNAs. Unlike transcription, replication occurs through a primer-  
70 independent, *de novo* initiation mechanism which occurs either at the first nucleotide of the 3'-vRNA  
71 template or at nucleotide 4 and 5 of the 3'-cRNA template followed by realignment prior to elongation  
72 (Deng et al., 2006; Oymans and Te Velthuis, 2018; Zhang et al., 2010). Nascent cRNAs and vRNAs  
73 are encapsidated by NP and FluPol to form cRNPs and vRNPs, respectively (York et al., 2013). Progeny  
74 vRNPs can then perform secondary transcription and replication or, late during the infection cycle, be  
75 exported from the nucleus to the cytoplasm to be incorporated into new virions.

76 FluPol associates dynamically with many cellular proteins, among which the interaction with  
77 the host RNAP II transcription machinery (Krischuns et al., 2021) and the nuclear acidic nuclear  
78 phosphoprotein 32 (ANP32) (Long et al., 2016) are essential for a productive viral infection. A direct  
79 interaction between FluPol and the disordered C-terminal domain (CTD) of the largest RNAP II subunit  
80 is essential for “cap-snatching” (Engelhardt et al., 2005). In mammals, the CTD consists of 52 repeats  
81 of the consensus sequence T<sub>1</sub>-S<sub>2</sub>-P<sub>3</sub>-T<sub>4</sub>-S<sub>5</sub>-P<sub>6</sub>-S<sub>7</sub> (Harlen and Churchman, 2017). To perform “cap-  
82 snatching”, FluPol binds specifically to the CTD when phosphorylated on serine 5 residues (pS5 CTD),  
83 which is a hallmark of RNAP II in a paused elongation state (Core and Adelman, 2019). Structural  
84 studies revealed bipartite FluPol CTD-binding sites for influenza type A, B and C viruses (Krischuns  
85 et al., 2022; Lukarska et al., 2017; Serna Martin et al., 2018). For type A viruses, both CTD-binding  
86 sites, denoted site 1A and site 2A, are on the PA-C-terminal domain (PA-Cter) and consist of highly  
87 conserved basic residues that directly interact with pS5 moieties of the CTD (Lukarska et al., 2017).

88 The host ANP32 proteins consist of a structured N-terminal leucine-rich repeat (LRR) domain  
89 and a disordered C-terminal low complexity acidic region (LCAR) and are essential for influenza virus  
90 genome replication (Nilsson-Payant et al., 2022; Swann et al., 2023). Differences in avian and  
91 mammalian ANP32 proteins represent a major driver of FluPol adaptation upon zoonotic infections of  
92 mammalian species with avian influenza viruses (Baker et al., 2018; Long et al., 2016; Swann et al.,  
93 2023). A type C virus FluPol (FlucPol) co-structure with ANP32A shows that the ANP32A LRR

94 domain binds to an asymmetrical influenza polymerase dimer, which is presumed to represent the  
95 FluPol replication complex (Carrique et al., 2020). The two FluPol conformations observed in this dimer  
96 would represent the RNA-bound replicase (FluPol<sub>(R)</sub>), which synthesises *de novo* genomic RNA and  
97 the encapsidase (FluPol<sub>(E)</sub>), which binds the outgoing replication product and nucleates formation of the  
98 progeny RNP (Zhu et al., 2023). In addition, the ANP32 LCAR domain was shown to interact with NP,  
99 leading to a model in which the ANP32 LCAR domain recruits NP to nascent exiting RNA, thus, in  
100 combination with the FluPol<sub>(E)</sub>, ensuring efficient co-replicative encapsidation of *de novo* synthesised  
101 genomic RNAs (Wang et al., 2022).

102         Structural, biochemical and functional studies to date have led to a model in which the FluPol  
103 binds either to the pS5 CTD or to ANP32, which drives RNPs towards transcription or replication,  
104 respectively (Zhu et al., 2023). Here, we provide genetic evidence that the FluPol CTD-binding  
105 interface has a so far overlooked function in that it is essential not only for transcription but also for  
106 replication of the viral genome. We show that the CTD, jointly with ANP32, enhances FluPol  
107 replication activity and we demonstrate in structural studies that CTD-binding to the FluPol is consistent  
108 with replication activity. We therefore propose a model in which transcription and replication of the  
109 influenza virus genome occur in association to the RNAP II CTD thereby allowing an efficient  
110 switching between the two activities.

## 111 Results

### 112 The FluPol CTD-binding interface is essential for replication of the viral genome

113 Previous studies demonstrated that the FluPol PA-Cter domain is crucial for FluPol  
114 transcription by mediating the interaction between the FluPol transcriptase (FluPol<sub>(T)</sub>) and the host  
115 RNAP II pS5 CTD for “cap-snatching” through direct protein-protein interaction (**Fig. 1A**, FluPol<sub>(T)</sub>)  
116 (Krischuns et al., 2022; Lukarska et al., 2017). Recent cryo-EM structures of Flu<sub>C</sub>Pol suggest that the  
117 PA-Cter domain is also involved in the formation of the FluPol replication complex (Carrique et al.,  
118 2020). Based on these Flu<sub>C</sub>Pol structures, we generated a model of the Flu<sub>A</sub>Pol replicase-encapsidase  
119 complex (**Fig. 1B** FluPol<sub>(R)</sub> and FluPol<sub>(E)</sub>). According to our model, the residues essential for FluPol  
120 binding to the CTD are surface exposed and potentially competent for CTD-binding in the FluPol<sub>(R)</sub> as  
121 well as the FluPol<sub>(E)</sub> conformation. Strikingly, FluPol<sub>(E)</sub> CTD-binding residues are in close proximity to  
122 the ANP32-binding interface (**Fig. 1B and S1A**). We therefore hypothesised that the FluPol CTD-  
123 binding interface may have a role beyond the described association with the pS5 CTD in the FluPol<sub>(T)</sub>  
124 conformation namely to facilitate assembly of the FluPol<sub>(R)</sub>-ANP32-FluPol<sub>(E)</sub> replication complex.

125 To assess this dual role hypothesis, we examined whether binding of the A/WSN/1933 (WSN)  
126 FluPol to ANP32A is altered by previously described FluPol CTD-binding mutations (PA K289A,  
127 R454A, K635A, or R638A) (Lukarska et al., 2017). For this purpose, we made use of cell-based  
128 *G. princeps* split-luciferase protein-protein complementation assays as described before (Mistry et al.,  
129 2020; Morel et al., 2022). As expected from our previous work (Lukarska et al., 2017), steady-state  
130 levels of the wild-type and mutant PA protein are similar or slightly reduced in the case of the  
131 PA K635A mutant (**Fig. S1B**) and each mutation reduces FluPol binding to the CTD (**Fig. 1C**, grey  
132 bars) as well as FluPol activity as measured in a vRNP reconstitution assay (**Fig. 1C**, hatched bars).  
133 Remarkably, they also reduce FluPol binding to ANP32A (**Fig. 1C**, blue bars). We then tested double  
134 mutations (PA K635A+R638A, K289+R454A) or mutations shown to disrupt CTD-binding site 2A  
135 more severely (PA S420E, Δ550-loop, highlighted in orange in **Fig S1A**) (Krischuns et al., 2022).  
136 Steady-state levels of the mutant PA proteins remain unchanged (**Fig. S1C**) while stronger reductions  
137 in ANP32A-binding and FluPol activity are observed (**Fig. S1D**, blue and hatched bars). CTD-binding

138 signals remain in the comparable range of 25-50% signal reduction as with the initial single mutations  
139 (**Fig. S1D**, grey bars) indicating that disruption of a single CTD-binding site (site 1A or site 2A) can  
140 largely preserve the CTD-FluPol interaction signal through binding to the remaining second FluPol  
141 CTD-binding site.

142 We then quantified the accumulation of viral RNA species (NA mRNA, cRNA, and vRNA) in  
143 the vRNP reconstitution assay by strand-specific RT-qPCR (Kawakami et al., 2011). The PA K289A,  
144 R454A, K635A, and R638A mutants show a ~80% and ~50% decrease of mRNA:vRNA and  
145 cRNA:vRNA ratios, respectively, indicating that both transcription and replication are affected  
146 (**Fig. 1D**). The absolute levels of cRNA and vRNA are decreased for the PA R454A and K635A  
147 mutants, as well as with the PA S420E and  $\Delta$ 550-loop mutants (**Fig. S1E**). Intriguingly, in the presence  
148 of the PA K289A and R638A mutants, cRNA and vRNA levels appear unchanged or even increased  
149 (**Fig. S1E**). As the vRNP reconstitution assay was shown to be skewed towards transcription (Robb et  
150 al., 2009), it is likely that although a replication defect exists, it is masked by the transcription defect as  
151 more FluPols become available for replication activity, especially when the defect in ANP32 binding is  
152 moderate as observed for the PA R638A mutant (**Fig. 1C**).

153 To further support the notion that the FluPol CTD-binding interface is essential for FluPol  
154 replication, we made use of transcription-defective (FluPol<sub>(T-)</sub>, PA D108A, (Dias et al., 2009) and  
155 replication-defective (FluPol<sub>(R-)</sub>, PA K664M, Drncova et al., unpublished) FluPol mutants that trans-  
156 complement each other in a vRNP reconstitution assay (**Fig. 1E**). FluPol CTD-binding mutants were  
157 either co-expressed with the FluPol<sub>(R-)</sub> mutant to reveal a defect in transcription (**Fig. S1F**), or with the  
158 FluPol<sub>(T)</sub> mutant to reveal a defect in replication (**Fig. S1G**). Upon co-expression with the FluPol<sub>(R-)</sub>,  
159 trans-complementation is observed for most of the investigated CTD-binding mutants. Moreover, the  
160 FluPol<sub>(R-)</sub>-mediated fold-change (FC) shows a strong positive correlation when plotted against the  
161 reduction in mRNA levels, confirming that the tested FluPol mutants are transcription-defective  
162 (**Fig. 1F**). Similarly, upon co-expression with the FluPol<sub>(T-)</sub>, trans-complementation is observed for  
163 most of the investigated mutants, with positive correlations being observed between the FluPol<sub>(T-)</sub>-  
164 mediated FC and the reduction of cRNA as well as vRNA levels (**Fig. 1G**) demonstrating that the  
165 investigated FluPol mutants are defective for replication.



166 Overall, our findings show that the FluPol CTD-binding interface, beyond its function for  
167 FluPol “cap-snatching”, is also involved in the FluPol-ANP32 interaction and is essential for genome  
168 replication. They are not limited to the WSN strain, as a dual-effect of the mutations PA K289A,  
169 R454A, K635A, or R638A on CTD- and ANP32A-binding is also observed with the FluPol of the  
170 human isolate A/Anhui/01/2013 (H7N9) (Anhui-H7N9), a virus of avian origin which harbours the  
171 human-adaptation signatures PB2 K627 and N701 (**Fig. S1H**). Sequence alignments of the PB1, PB2,  
172 PA and NP proteins from the influenza A viruses used in this study are shown in **Fig. S2**.

173

#### 174 **Serial passaging of FluPol PA K289A, R454A, K635A, R638A mutant viruses selects for adaptive** 175 **mutations which restore FluPol binding to the CTD and ANP32A**

176 We previously rescued by reverse genetic procedures recombinant WSN PA mutant IAVs  
177 (PA K289A, R454A, K635A, R638A mutants) that were highly attenuated, formed pinhead-sized  
178 plaques and had acquired multiple non-synonymous mutations (Lukarska et al., 2017). In order to  
179 investigate whether and how the recombinant viruses adapt to defective CTD and ANP32-binding  
180 (**Fig. 1**), we subjected them to eight serial cell culture passages in MDCK-II cells followed by titration  
181 and next generation sequencing (NGS) of the viral genome at passage 1 to 4 and 8 (**Fig. 2A**, p1-4 and  
182 p8). The plaque size of the passaged viruses progressively increased (**Fig. 2B** and **S3A**), which went  
183 along with distinct patterns of adaptive mutations (**Source data file 1**), suggesting that cell culture  
184 passaging increased the replicative capacity of the PA mutant viruses. True reversions that require at  
185 least two nucleotide substitutions did not emerge. The observed second-site mutations are primarily  
186 located on the FluPol subunits as well as the NP (**Fig. 2C**, **S3B** and **S4A**) and are mostly surface exposed  
187 (**Fig. 2D** and **S4B-D**). Several FluPol second-site mutations are located close to the FluPol CTD-binding  
188 sites (**Fig. 2D**, PA D306N, C489R, R490I, R496Q) while some PB2 second-site mutations are located  
189 in flexible hinge-subdomains (**Fig. 2D**, PB2 D253G, S286N, M535I). Overall, the FluPol second-site  
190 mutations show no local clustering or common function, indicating that diverse routes of adaptation  
191 occurred. NP second-site mutations are also surface exposed, but to our knowledge have not been  
192 associated to date with any specific function (**Fig. S4D**) (Tang et al., 2021).

193 We first focused on the reversion pathway of PA K289A mutant virus. Recombinant viruses  
194 with combinations of FluPol second site mutations observed along the PA K289A reversion pathway  
195 at p1, p4 and p8 recapitulate the progressive increase in plaque size observed during serial passaging  
196 (**Fig. 2E and S3C**), demonstrating that the FluPol second-site mutations are leading to the observed  
197 increase in viral replicative capacity. PB2 and PA proteins with second-site mutations which occurred  
198 during passaging of PA K289A mutant virus accumulate at similar levels compared to the WT proteins  
199 (**Fig. S3D**). However, the combinations of FluPol second site mutations result in sequential increases  
200 and decreases of CTD-binding, ANP32A-binding and/or FluPol activity (**Fig. 2F**). The highest levels  
201 of CTD-binding, ANP32A-binding and FluPol activity are observed for the combination of mutations  
202 observed at p8 (PA K289A+PB2 D253G+PA C489R), which goes along with the highest replicative  
203 capacity observed for the passaged virus at p8 (**Fig. 2B and 2E**).

204 The reversion pathways of the PA R454A, K635A and R638A mutant viruses were analysed  
205 in the same way (**Fig. S3E**). Across all tested combinations of primary FluPol mutations and second-  
206 site mutations that occurred during passaging, FluPol activity shows a strong positive correlation with  
207 ANP32A-binding (**Fig. 2G**, left panel) as well as a positive correlation with CTD-binding (**Fig. 2G**,  
208 middle panel) but not with FluPol-binding to other known cellular partners, such as DDX5 (**Fig. 2G**,  
209 right panel) or RED (**Fig. S3F**).

210 Overall, we show that serial cell culture passaging of PA K289A, R454A, K635A, and R638A  
211 mutant viruses selects for adaptive mutations that rescue viral polymerase activity by restoring FluPol  
212 binding to the CTD and/or ANP32A, which is in agreement with our initial observation that the  
213 investigated FluPol mutants are impaired for CTD-binding as well as ANP32A-binding (**Fig. 1**).

214

### 215 ***De novo* FluPol replication activity is enhanced *in vitro* in the presence of CTD and ANP32A**

216 To further document a potential interplay between CTD and ANP32-binding to FluPol, we  
217 tested the effect of mCherry-CTD (CTD-WT) overexpression on FluPol binding to ANP32A as well as  
218 FluPol dimerisation in cultured cells. A FluPol binding-deficient CTD in which all serine 5 residues  
219 were replaced with alanines was used as a specificity control (CTD-S5A) (Krischuns et al., 2022).  
220 mCherry-CTD-WT and -CTD-S5A accumulate to similar levels (**Fig. S5A**), however only mCherry-

221 CTD-WT leads to a dose-dependent increase of FluPol ANP32A-binding, suggesting that the CTD  
222 facilitates FluPol-binding to ANP32 (**Fig. 3A**). CTD overexpression also leads to a dose-dependent  
223 increase in WSN FluPol dimerisation (**Fig. 3B**), as measured in a split-luciferase complementation assay  
224 (Chen et al., 2019). A similar increase was also observed with the Anhui-H7N9 and  
225 B/Memphis/13/2003 FluPols (**Fig. S5B**). Various oligomeric FluPol species that have been described  
226 (Carrique et al., 2020; Chang et al., 2015; Fan et al., 2019). There is evidence that the split-luciferase-  
227 based dimerisation assay, when FluPol is over-expressed in the absence of any other overexpressed  
228 viral or cellular protein, detects mostly the symmetrical FluPol dimer species (Chen et al., 2019; Fan et  
229 al., 2019). Here however, we observed that the increase in FluPol dimerisation upon CTD-WT  
230 overexpression is less pronounced in cells depleted for ANP32A and ANP32B (ANP32AB KO)  
231 (**Fig. S5C-D**), which strongly suggests that i) the split-luciferase-based assay also detects the ANP32-  
232 dependent asymmetrical dimer species, and ii) CTD overexpression enhances the formation of  
233 ANP32AB-dependent dimers. The effect of ANP32A and ANP32B depletion is significant for Anhui-  
234 H7N9 and B/Memphis/13/2003 FluPols but not for WSN FluPol (**Fig. S5C**), suggesting that other  
235 proteins, e.g. ANP32E, can support CTD-dependent dimer-formation for WSN FluPol (Zhang et al.,  
236 2020).

237 To investigate by biochemical and structural approaches whether and how *in vitro* replication  
238 is dependent on ANP32A and pS5 CTD, we performed unprimed RNA synthesis assays using  
239 recombinant A/Zhejiang/DTID-ZJU01/2013(H7N9) FluPol (Kouba et al., 2023) bound to a 51-mer  
240 vRNA loop template (denoted v51\_mut\_S). This polymerase, referred to below as Zhejiang-H7N9,  
241 originates from a human isolate of an avian strain possessing the characteristic PB2 E627 and N701  
242 residues (**Fig. S2**) (Chen et al., 2013). The sequence of the v51\_mut\_S template was derived by joining  
243 of the first 30 and the last 21 nucleotides of the respective 5' and 3' vRNA ends of Zhejiang-H7N9  
244 segment 4, and was adapted so that a stalled elongation product of 34 nts could be produced by replacing  
245 UTP in the reaction by non-hydrolysable UpNHpp (**Fig. 3C**). This would produce a 34-mer cRNA  
246 product, estimated to be long enough that its 5' hook (1-10) could bind to the encapsidase according to  
247 the modelled Zhejiang-H7N9 replicase-encapsidase structure. The addition of all NTPs would produce  
248 a full-length 51-mer replication product without poly-adenylation (A17, only 4xU), assuming RNA

249 synthesis proceeded to the 5' end of the template, rather than terminating prior to reading through the 5'  
250 hook.

251 *De novo* replication assays were performed with WT Zhejiang-H7N9 FluPol, using three  
252 (ACG) or four (ACGU) nucleotides, (a) excess apo-polymerase, that could serve as encapsidase, (b)  
253 huANP32A and (c) 6-repeats pS5CTD peptide (**Fig. 3D**, pS5 CTD(6mer)). The results show that in  
254 reactions with ACG only (**Fig. 3D**, lane 1-4), the principal product is the stalled 34-mer cRNA, whose  
255 significant production is only visible in the presence of CTD and ANP32A (**Fig. 3D**, lane 4). When all  
256 four nucleotides are added (**Fig. 3D**, lane 5-8), the presence of CTD and ANP32A again strongly  
257 enhances RNA synthesis with the production of both stalled product (34-mer) and a significant amount  
258 of full-length replication product (**Fig. 3D**, lane 8).

259 We further investigated which domains of huANP32A are essential for this *de novo* replication  
260 activity increase in the presence, or not, of pS5 CTD(6mer) peptide. By comparing the full-length  
261 ANP32 (“FL” lane 1-6) with ANP32A subdomains corresponding to the LRR (“1-149” lane 7-10), the  
262 LRR and LCAR N-terminal region (“1-199” lane 11-14), or the LCAR and LRR C-terminal region  
263 (“144-249” lane 15-18) (**Fig. 3E**, left panel), we show that the 149-199 region of ANP32A is most  
264 critical to observe a joint CTD- and ANP32-dependent enhancement of replication product synthesis  
265 (**Fig. 3E**, right panel). These *in vitro* data are consistent with cell-based assays in which FluPol activity  
266 was investigated with the same C-terminal deletion mutants of ANP32A (**Fig. 3F and 3G**).

267 To confirm whether a 5' triphosphorylated full-length replication product was actually  
268 synthesised, we performed next-generation sequencing (NGS) of all RNAs present in the reaction mix  
269 (**Fig. 3D**, lane 8). Prior to NGS and to simplify the analysis of this heterogeneous RNA sample, we first  
270 degraded non-5' triphosphorylated RNAs with a 5'-phosphate-dependent exonuclease. This removes the  
271 v51\_mut\_S template, which carries a 5' monophosphate, and which is perfectly complementary to the  
272 expected product and could have formed unwanted double-stranded RNA (**Fig. S5E**). NGS analysis  
273 revealed that the majority of reads (*i.e.*, premature and near full-length products) start with 5'-  
274 pppGC... instead of the expected 5'-pppAGC... (**Fig. 3H**). Despite the presence of many premature  
275 termination products (**Fig. S5F**), around 80% of near full-length products, are 50-mers, lacking the  
276 expected 5' pppA, with few 51-mers (5'-pppAGC...) and 49-mers (5'-pppC...) (**Fig. 3I**). This

277 suggests that, *in vitro*, most RNA synthesis products are initiated *de novo* with a 5'-pppG at the second  
278 position of the v51\_mut\_S template (3'-UCG...).

279

## 280 **Structural analysis shows that both replication and transcription are consistent with CTD-** 281 **binding to the FluPol**

282 Using cryo-EM, we then sought to determine structures of actively replicating FluPol, in the  
283 presence of ANP32A, pS5 CTD(6mer) peptide mimic and excess apo-FluPol, by freezing grids with  
284 the *de novo* reaction stalled with UpNHpp (**Fig. 3D**, lane 4). However, instead of using the WT  
285 Zhejiang-H7N9 FluPol, which forms a robust symmetrical dimer *in vitro* (Krischuns et al., 2022) that  
286 could compete with the formation of an active asymmetric FluPol<sub>(R)</sub>-FluPol<sub>(E)</sub> dimer, we used a  
287 Zhejiang-H7N9 FluPol mutant that harbours four mutations (PA E349K, PA R490I, PB1 K577G, and  
288 PB2 G74R) and is referred to below as the 4M mutant. PA E349K, PB1 K577G, and PB2 G74R were  
289 shown to disrupt the symmetrical apo FluPol dimer interface (Chen et al., 2019), while PA R490I  
290 appeared during passaging of the PA R638A mutant virus and increases FluPol-ANP32A binding in a  
291 cell-based assay (**Fig. S3E**). Indeed, Zhejiang-H7N9 4M shows enriched monomeric particles while  
292 preserving a similar *in vitro* activity when compared to the Zhejiang-H7N9 WT FluPol (**Fig. S6A-B**).  
293 Accordingly, in cell-based assays Anhui-H7N9 4M shows decreased FluPol dimerisation, increased  
294 binding to ANP32A, while showing activity levels comparable to the WT Anhui-H7N9 FluPol as  
295 measured in a vRNP reconstitution assay (**Fig. S6C**).

296 Several high-resolution structures were obtained from a Krios data collection (**Table 1a, Fig. 4,**  
297 **Fig. S7**) including FluPol with PB2-C in a replicase-like configuration at 2.9 Å resolution (**Fig. 4A**, left  
298 panel). However, the PA endonuclease domain (PA-ENDO) is still in an unrotated transcriptase-like  
299 conformation, thus precluding the usual interaction with the PB2-NLS domain, as previously observed  
300 for H5N1 or Flu<sub>C</sub>Pol replicase conformations (**Fig. 4A**, middle and right panel) (Carrique et al., 2020;  
301 Fan et al., 2019). A second structure, obtained at a higher resolution (2.5 Å), is in a similar state, but  
302 PB2-C is not visible (**Fig. 4B**). For both structures, FluPol is in the pre-initiation state mode A, with the  
303 3' end of the v51\_mut\_S template (3'-UCG...) in the active site and the priming loop fully extended and  
304 well ordered (**Fig. 4C, D; Fig. S8A**). The template is positioned with C2 and G3 respectively at the -1

305 and +1 positions, whilst only the phosphate of U1 is visible (**Fig. 4C-D**). Finally, a stalled elongation  
306 state was obtained at 2.9 Å resolution in which FluPol encloses a template-product duplex, with  
307 incoming UpNHpp at the +1 active site position, and the 3' vRNA end bound to the secondary site  
308 (**Fig. 4E, Fig. S8B**). In all structures, the pS5 CTD is observed bound in sites 1A and 2A. Continuous  
309 density between the two sites, with 25 pS5 CTD residues coming from four repeats, is observed for the  
310 pre-initiation state and the elongation structures (**Fig. 4F**), whereas for the replicase-like structure, the  
311 connectivity is lost between both sites. Despite extensive cryo-EM data analysis, ANP32A was never  
312 visualised nor the putative replicase-encapsidase dimer.

313 Altogether, these results show that *in vitro*, *de novo* RNA synthesis assays result in a  
314 heterogeneous mix of FluPol conformations including replicase-like and transcriptase-like  
315 configurations, reflecting the flexibility of the peripheral PB2-C domains (**Fig. S7**). Importantly, a  
316 replicase-like initiation complex is observed for the first time, characterised by the radically different  
317 position of the cap-binding domain compared to the transcriptase conformation (**Fig. 4A**) (Thierry et  
318 al., 2016), which has never been observed during extensive studies of cap-dependent transcription  
319 (Wandzik et al., 2020). However, the observed stalled elongation state, in which the translocated 3' end  
320 of the template has reached the secondary binding site (**Fig. 4E**), is similar to the previously described  
321 pre-termination transcription state (Wandzik et al., 2020). Finally, all structures have the CTD peptide  
322 mimic bound in both sites 1A and 2A, suggesting that both replication and transcription are consistent  
323 with CTD binding (**Fig. 4A-B, E**).

324 The pre-initiation state structures show that at least *in vitro*, and using highly purified  
325 recombinant FluPol, the preferred position of the 3' end of the vRNA template is with C2, rather than  
326 U1, at the -1 position. This leads to formation of pppGpC at the beginning of the product, consistent  
327 with the NGS results, or alternatively, would allow efficient priming by pppApG, if this dinucleotide  
328 was already available from another source. Internal initiation at position 2 of vRNA has been previously  
329 described (Vreede and Brownlee, 2007; Zhang et al., 2010), but other authors report pppApG formation  
330 at position 1 (Deng et al., 2006). We note that initiation at position 2 of vRNA implies that the 5' end  
331 of the cRNA product probably forms a less stable hook structure since the A1:A10 non-canonical base  
332 pair would be disrupted. This could explain why we do not observe in cryo-EM the cRNA hook bound

333 in a putative encapsidase, even though there is excess apo-polymerase. This could further explain why  
334 the putative asymmetric FluPol<sub>(R)</sub>-FluPol<sub>(E)</sub> dimer is also not observed, since it seems likely that *in vitro*,  
335 the Flu<sub>A</sub>Pol FluPol<sub>(R)</sub>-FluPol<sub>(E)</sub> -ANP32 complex is not stable in itself, unlike the case of Flu<sub>C</sub>Pol, where  
336 the FluPol<sub>(R)</sub>-FluPol<sub>(E)</sub> complex is stable even in the absence of ANP32 (Carrique et al., 2020). These  
337 observations suggest that other cellular or viral factors and the RNP context might be required to  
338 recapitulate true terminal initiation of vRNA replication and further work is required to clarify this issue  
339 (Drncova et al., unpublished).

340

### 341 **Restoration of CTD-binding function enhances FluPol binding to ANP32A and FluPol replication** 342 **activity**

343 Second-site mutations PA C489R and PB2 D253G were selected during passaging of the  
344 recombinant WSN virus with a PA K289A mutation (**Fig. 2C**) and conferred elevated FluPol-binding  
345 to the CTD as well as to ANP32A (**Fig. 2F**, grey and blue bars, respectively). PA C489 is in close  
346 proximity to the phosphoserine binding site in CTD-binding site 2A (**Fig. 5A**). The mutation PA C489R  
347 could therefore plausibly compensate for the loss of the positive charge of PA K289A and rescue the  
348 interaction with the CTD. Indeed, structural analysis by cryo-EM of Zhejiang-H7N9 WT FluPol in the  
349 symmetric dimeric form (**Table 1b** and **Fig. S9**) and bearing the double mutation K289A+C489R  
350 confirms that C489R points towards the phosphoserine binding site, although at a slightly greater  
351 distance than K289 (**Fig. 5B-C**). Despite this, the structure did not reveal CTD binding to the Zhejiang-  
352 H7N9 PA K289A+C489R FluPol in this conformation. Therefore, we sought to analyse the functional  
353 impact of the single PA C489R reversion on the viral phenotype, and in cellular assays, on FluPol  
354 activity and FluPol-binding to the CTD and ANP32A (**Fig. 5D-I**).

355 A recombinant WSN virus with the PA K289A+C489R mutations shows larger plaques  
356 compared to the PA K289A mutant virus, demonstrating that the PA C489R second-site mutation  
357 provides a significant growth advantage to PA K289A mutant virus (**Fig. 5D** and **Fig. S10A**). Indeed,  
358 WSN FluPol activity as measured in a vRNP reconstitution assay increases significantly when the  
359 PA C489R mutation is combined with PA K289A (**Fig. 5E**). This increase correlates with a restored  
360 mRNA:vRNA ratio (**Fig. 5F** and **Fig. S10B**) as well as an increased FluPol-binding to the CTD

361 (Fig. 5G). Importantly, steady-state levels of the wild-type and mutant PA proteins are similar  
362 (Fig. S10C). These observations suggest that charge restoration in CTD-binding site 2A of the WSN  
363 FluPol in the transcriptase conformation rescues the interaction with the host RNAP II for “cap-  
364 snatching” in cell-based assays and during live-virus infection. In cell-based assays, the Anhui-H7N9  
365 FluPols with PA K289A and PA K289A+C489R show similar trends to those observed with their WSN  
366 FluPol counterparts, although with much less pronounced phenotypes (Fig. S10D). Therefore we  
367 speculate that the fact that CTD-binding is not restored *in vitro* for Zhejiang-H7N9 PA K289A+C489R  
368 could be due to strain-specificities (Fig. S2) and/or to the presence of cellular factors that favor binding  
369 of the CTD to the PA K289A+C489R and are missing in the *in vitro* assay.

370 Beyond the rescue of FluPol transcription, cRNA accumulation levels increase significantly  
371 when PA C489R is combined with PA K289A (Fig. S10B), which leads to a rescue of the imbalanced  
372 cRNA:vRNA ratio associated with the PA K289A mutant (Fig. 5H). This goes along with increased  
373 ANP32A-binding (Fig. 5J). Taken together our observations suggest that the second-site mutation  
374 PA C489R has another functional impact, namely restoring FluPol replication by enhancing ANP32A-  
375 binding. Similar results are obtained for the PA C453R revertant which has been shown to restore  
376 FluPol-binding to the CTD in site 1A by compensating the loss of positive charge of the PA R638A  
377 mutation (Fig. S10E) (Fodor et al., 2003; Lukarska et al., 2017). PA C453R rescues the attenuated viral  
378 plaque phenotype (Fig. S10F-G) as well as the reduced FluPol activity (Fig. S10H) associated with  
379 PA R638A, while it does not affect PA steady-state levels (Fig. S10J). Strikingly, PA C453R enhances  
380 ANP32A-binding when tested in combination with PA R638A (Fig. S10I), again mimicking at CTD-  
381 binding site 1A the effects of PA C489R at site 2A.

382 The positive impact of the PA C489R and PA C453R mutations on ANP32A-binding could  
383 possibly be mediated by an increased binding of the pS5 CTD in site 1A or 2A in either the FluPol<sub>(R)</sub>  
384 and/or FluPol<sub>(E)</sub> conformation. According to our model of the Flu<sub>A</sub>Pol replicase-encapsidase complex  
385 (Fig. 1B) the FluPol CTD-binding residues on the encapsidase do not mediate a direct interaction  
386 between the FluPol<sub>(E)</sub> and ANP32A. Within the space lined by ANP32A and FluPol<sub>(E)</sub>, it is possible to  
387 model CTD repeats, which make contacts with the FluPol<sub>(E)</sub> CTD-binding sites (1A and 2A) on one  
388 side and ANP32A on the other side (Fig. 5J). Moreover, Flu<sub>C</sub>Pol X-ray electron density maps revealed



389 an asymmetric FluPol dimer crystal packing which share a similar inter-polymerase interface compared  
390 to the ANP32A-bound FlucPol replication complex, with the exception of PB2 peripheral domains  
391 (**Fig. S10K-L**). The observed CTD bound to FlucPol<sub>(R2)</sub> in site 2C is mostly compatible with ANP32A  
392 binding and in a similar position as in our CTD-bound Flu<sub>A</sub>Pol replication complex model. Therefore,  
393 although we cannot rule out that the CTD-binding sites 1A and 2A are involved in direct protein-protein  
394 interaction between the FluPol<sub>(E)</sub> and ANP32A, it seems more likely that CTD repeats are bound in  
395 between the FluPol<sub>(E)</sub> and ANP32 to stabilise the FluPol replication complex thereby facilitating FluPol  
396 replication.

## 397 Discussion

### 398 A model for RNAP II CTD-anchored transcription and replication of the influenza virus genome

399 There is long-standing and extensive evidence that binding of FluPol to the pS5 CTD repeats  
400 of host RNAP II is essential for transcription of viral mRNAs (Chan et al., 2006; Engelhardt et al., 2005;  
401 Newcomb et al., 2009). Here, we challenge the prevailing concept that CTD binding exclusively  
402 stabilises the transcriptase conformation of the influenza virus polymerase (FluPol<sub>(T)</sub>) (Zhu et al., 2023).  
403 We show that CTD-binding also enhances replication of the viral genome, in conjunction with the host  
404 protein ANP32A, which was recently described to bridge two FluPol moieties in an asymmetric  
405 replicase-encapsidase dimer (FluPol<sub>(R)</sub>-FluPol<sub>(E)</sub>) (Carrique et al., 2020). Our findings open new  
406 perspectives on the spatial coupling of viral transcription and replication and the coordinated balance  
407 between these two activities.

408 Early in infection, incoming vRNPs serve as a template for both primary mRNA transcription  
409 and the first round of cRNA synthesis (Vreede et al., 2004). Based on our findings, we propose a model  
410 in which incoming vRNPs switch from primary transcription to cRNA synthesis while remaining bound  
411 to the RNAP II CTD (**Fig. 5K, a**). It has been long understood that primary transcription requires  
412 FluPol<sub>(T)</sub> association with the RNAP II CTD to enable “cap-snatching” for the priming of viral mRNA  
413 synthesis in the FluPol<sub>(T)</sub> conformation (**Fig. 5K, b-c**). Our structural and biochemical data reveal that  
414 a vRNA- and CTD-bound FluPol can adopt a FluPol<sub>(R)</sub> conformation and initiate vRNA to cRNA  
415 synthesis *de novo*, and that CTD binding to the FluPol enhances cRNA synthesis. Importantly, the  
416 switch from a transcribing FluPol<sub>(T)</sub> to a replicating FluPol<sub>(R)</sub> can occur only after *de novo* synthesised  
417 FluPol and NP are imported into the nucleus (**Fig. 5K, d**) and an asymmetric FluPol<sub>(R)</sub>-ANP32A-FluPol<sub>(E)</sub>  
418 complex is assembled, ensuring encapsidation of the nascent viral RNA by the FluPol<sub>(E)</sub> moiety. Our  
419 cell-based and *in vitro* assays show that the RNAP II CTD and ANP32 jointly enhance cRNA synthesis.  
420 The data support a model in which the CTD binds at the interface between FluPol<sub>(E)</sub> and ANP32, thereby  
421 forming a FluPol<sub>(R)</sub>-ANP32A-FluPol<sub>(E)</sub> complex in which both the FluPol<sub>(R)</sub> and FluPol<sub>(E)</sub> are bound to  
422 the CTD (**Fig. 5K, e-f**). The CTD is a low complexity disordered region and there is evidence that it  
423 drives phase separation and the formation of biomolecular condensates that concentrate RNAP II and

424 transcription regulatory factors (Cramer, 2019). In infected cells, the RNPA II condensates may in  
425 addition concentrate the FluPol and its viral and cellular partners, allowing CTD-bound FluPols to  
426 switch efficiently between transcription or replication depending on the relative availability of, e.g. 5'  
427 capped RNAs, NP and/or apo FluPol (**Fig. 5K, g**), and perform both activities in a coordinated manner.

428         Despite many attempts we were unable to provide structural evidence for a ANP32A-CTD-  
429 FluPol<sub>(E)</sub> complex. A possible explanation is that such complexes are stably assembled only when the  
430 FluPol<sub>(R)</sub> is in the context of a vRNP, or in the presence of additional viral (Newcomb et al., 2009)  
431 and/or cellular factor(s). Another, non-exclusive explanation, could relate to the fact the CTD-  
432 mimicking peptides used for *in vitro* experiments are six repeats in length whereas the full-length CTD  
433 consists of 52 repeats in mammals. Moreover, the observed FluPol *de novo* initiation at position 2 of  
434 the vRNA (Drncova et al., unpublished) may result in a hook structure at the 5' end of the nascent  
435 cRNA that is less stable and therefore less likely to be bound by a FluPol<sub>(E)</sub> moiety, which could explain  
436 why the asymmetric FluPol<sub>(R)</sub>-FluPol<sub>(E)</sub> dimer is not observed.

437         Although a structure for the asymmetric FluPol<sub>(R)</sub>-ANP32-FluPol<sub>(E)</sub> replicase-encapsidase  
438 dimer has so far been obtained only for vRNA-bound FluPol<sub>(R)</sub>, it is generally thought that the same  
439 type of asymmetric dimer is formed by a cRNA-bound FluPol<sub>(R)</sub> (Zhu et al., 2023). Therefore, the model  
440 proposed for incoming vRNPs (**Fig. 5K**) can be extended to neo-synthesised, replicating cRNPs and  
441 vRNPs, consistent with vRNA synthesis being detected mostly in the chromatin-associated fractions  
442 (López-Turiso et al., 1990; Takizawa et al., 2006). In our model, the FluPol<sub>(R)</sub>-associated RNPs could  
443 remain anchored to the same CTD repeats and undergo several rounds of cRNA->vRNA or vRNA-  
444 >cRNA synthesis, while the FluPol<sub>(E)</sub>-associated RNPs could form novel CTD-associated FluPol<sub>(R)</sub> or  
445 FluPol<sub>(T)</sub> after RNA synthesis is complete. Our model that the RNAP II CTD provides a recruitment  
446 platform for the different types of monomeric and multimeric polymerases raises many questions for  
447 future studies. What is the sequence of events leading to CTD-anchored replicase complexes? Are the  
448 CTD and/or host factors recruited by the CTD controlling the balance between transcription and  
449 replication? Are transcribing and replicating FluPol complexes localised to the same RNAP II  
450 condensates? Could the viral-induced degradation of RNAP II observed at late time-points during  
451 influenza virus infection (Rodriguez et al., 2007; Vreede et al., 2010) favour the release of neo-

452 synthesised vRNPs from the chromatin and their nuclear export ? Fully addressing these questions will  
453 require to overcome technical challenges such as structural analyses on the FluPol in the context of  
454 vRNPs and cRNPs, or high-resolution imaging in live-infected cells to visualise the subnuclear  
455 localisation of FluPol transcription and replication.

## 456 **Material and Methods**

### 457 **Cells**

458 HEK-293T cells (ATCC CRL-3216) were grown in complete Dulbecco's modified Eagle's medium  
459 (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin  
460 (Gibco). MDCK cells (provided by the National Influenza Center, Paris, France) were grown in  
461 Modified Eagle's medium (MEM, Gibco) supplemented with 5% FBS and 1% penicillin-streptomycin.  
462

### 463 **Plasmids**

464 The A/WSN/33 (WSN) pcDNA3.1-PB2, -PB1, -PA and pCI-NP plasmids were described previously  
465 (Krischuns et al., 2022; Lukarska et al., 2017). The A/Anhui/1/2013 (Anhui-H7N9) pCAGGS-PB2, -  
466 PB1, -PA and NP plasmids were described previously (Mänz et al., 2016) and were kindly provided by  
467 R. Fouchier (Erasmus Medical Center, Netherlands). The B/Memphis/13/2003 pcDNA3.1-PB2, PA  
468 plasmids were described previously (Guilligay et al., 2008). The WSN reverse genetics plasmids were  
469 kindly provided by G. Brownlee (Sir William Dunn School of Pathology, Oxford, UK) (Fodor et al.,  
470 1999). For vRNP reconstitution assays, a pPolI-Firefly plasmid encoding the Firefly luciferase sequence  
471 in negative polarity flanked by the 5' and 3' non-coding regions of the IAV NS segment was used and  
472 the pTK-Renilla plasmid (Promega) was used as an internal control. For RNA quantifications in vRNP  
473 reconstitution assays by strand-specific qRT-PCR a pPR7-WSN-NA reverse genetic plasmid was used  
474 (Vieira Machado et al., 2006). The WSN and Anhui-H7N9 pCI-PB1-G1 and pCI-PB1-G2 plasmids  
475 used for *G. princeps* split-luciferase-based protein-protein complementation assays were constructed as  
476 described previously (Cassonnet et al., 2011). The Anhui-H7N9 PA open-reading frame (ORF) was  
477 subcloned into the pCI vector and the PA-X ORF was deleted when indicated by introducing silent  
478 mutations at the site of ribosomal frameshifting (Jagger et al., 2012). For CTD-binding assays, a C-  
479 terminal stretch of RPB1 (108 amino acids) in conjunction with 14 CTD repeats were tagged C-  
480 terminally with the *G. princeps* fragments as described previously (Morel et al., 2022). For ANP32A-  
481 binding assays, ANP32A was tagged C-terminally with the *G. princeps* fragments as described  
482 previously (Long et al., 2019). The pCI-mCherry-CTD-WT and mCherry-CTD-S5A plasmids were

483 generated by replacing the G2 sequence in previously described pCI-G2-CTD constructs (Krischuns et  
484 al., 2022). ANP32A encoding the amino acids 1-249 (full-length), 1-199 and 1-149 were subcloned into  
485 the pCI vector with a C-terminal V5-tag and SV40 nuclear localisation signals (NLS). pCI-G2-DDX5  
486 and -G2-RED were described previously (Fournier et al., 2014; Munier et al., 2013). All mutations were  
487 introduced by an adapted QuikChange site-directed mutagenesis (Agilent Technologies) protocol  
488 (Zheng et al., 2004). All primer and plasmid sequences are available upon request.

489

#### 490 **Generation of HEK-293T ANP32A and ANP32B knockout cells**

491 CRISPR-Cas9-mediated HEK-293T ANP32A and ANP32B knockout cells (HEK-293T ANP32A  
492 KO) and a control cell line (HEK-293T CTRL) were generated by lentiviral transduction. Guide RNAs  
493 (gRNAs) targeting ANP32A (5'-ACCGCTTTGGTAAGTTTGCGATTG-3' and 5'-  
494 AAACCAATCGCAAACCTTACCAAAG-3'), ANP32B (5'-ACCGGGAGCTGAGGAACCGGACCC-  
495 3' and 5'-AAACGGGTCCGGTTCCTCAGCTCC-3') and a non-targeting control (5'-  
496 ACCGGTATTACTGATATTGGTGGG-3' and 5'-AAACCCACCAATATCAGTAATAC-3') were  
497 annealed and cloned into the BsmBI site of pSicoR-CRISPR-PuroR (a kind gift from Robert Jan  
498 Lebbink, (van Diemen et al., 2016)). Replication-incompetent lentiviral particles were generated by  
499 transient transfection of HEK-293T cells with the pSicoR-CRISPR-PuroR plasmid harbouring gRNAs  
500 targeting either ANP32A, ANP32B or a non-targeting control gRNA as well as with the packaging  
501 plasmids pMD2.G and psPAX2 (gifts from Didier Trono, Addgene plasmids # 12259 and # 12260,  
502 respectively). Supernatants were harvested 72 hpt, centrifuged to remove cellular debris and passed  
503 through 0.45 µm sterile filters. Fresh HEK-293T cells were plated in 6 well plates precoated with poly-  
504 D-lysine and were infected with 3 ml of lentiviral supernatant by constant centrifugation at 2.000 rpm  
505 for 40 min in the presence of 10 µg/ml Polybrene (Sigma-Aldrich). At 72 hpi, the medium was  
506 exchanged to selection medium containing 1 µg/ml puromycin (PAA Laboratories) for one week.  
507 Surviving cells were then cloned by limiting dilution in 96-well plates. Individual cell clones were  
508 grown and ANP32A and ANP32B protein levels in cellular lysates of individual clones was assessed  
509 by immunoblotting. To generate HEK293T cells with a double KO for ANP32A and ANP32B, two  
510 rounds of targeting were performed.

511

## 512 **Protein complementation assays**

513 HEK-293T cells were seeded in 96-well white plates (Greiner Bio-One) the day before transfection  
514 using polyethylenimine (PEI-max, #24765-1 Polysciences Inc). Cells were lysed 20-24 hpt in *Renilla*  
515 lysis buffer (Promega) for 45 min at room temperature (RT) under steady shaking. *G. princeps*  
516 luciferase enzymatic activity due to luciferase reconstitution was measured on a Centro XS LB960  
517 microplate luminometer (Berthold Technologies) using a reading time of 10 s after injection of 50 µl  
518 *Renilla* luciferase reagent (Promega). Mean relative light units (RLUs) of technical triplicates of  
519 *G. princeps* split-luciferase-based protein-protein complementation assays are represented. In the  
520 graphs each dot represents an independently performed biological replicate while at least three  
521 experiments were performed in each case. For FluPol-binding assays cells were always co-transfected  
522 with plasmids encoding the viral polymerase subunits (PB2, PB1, PA) tagged to one part of the  
523 *G. princeps* luciferase and the target protein tagged to the other part. The following combinations of  
524 *G. princeps* tags were used while the naming order indicates C- or N-terminal tagging: PB1-G2 and  
525 CTD-G1, PB1-G1 and CTD-G2, PB1-G2 and ANP32A-G1, PB1-G1 and ANP32A-G2, PB2-G1 and  
526 G2-RED, PB2-G1 and G2-DDX5. For FluPol dimerisation PB2, PA, PB1-G1 and PB1-G2 were co-  
527 transfected as described previously (Chen et al., 2019) and when indicated an expression plasmid for  
528 mCherry, mCherry-CTD-WT or mCherry-CTD-S5A was added while the total amount of transfected  
529 plasmid was adjusted using an empty control plasmid.

530

## 531 **vRNP reconstitution assays**

532 HEK-293T cells were seeded in 96-well white plates (Greiner Bio-One) the day before transfection.  
533 Cells were co-transfected with plasmids encoding the vRNP protein components (PB2, PB1, PA, NP),  
534 a pPoli-Firefly plasmid encoding a negative-sense viral-like RNA expressing the Firefly luciferase and  
535 the pTK-Renilla plasmid (Promega) as an internal control. For FluPol trans-complementation assays,  
536 equal amounts of expression plasmids expressing the trans-complementing PA mutants were co-  
537 transfected. The fold-change shown for FluPol trans-complementation assays represents the increase  
538 above the integrated background of the respective FluPol mutants alone. For FluPol activity rescue

539 experiments in HEK-293T ANP32AB KO cells, expression plasmids encoding FL or truncated versions  
540 of ANP32A were co-transfected. Firefly luciferase activity due to viral polymerase activity and Renilla  
541 luciferase activity due to RNAP II activity were measured using the Dual-Glo Luciferase Assay system  
542 (Promega) according to the manufacturer's instructions. The graphs represent viral polymerase activity  
543 normalised to RNAP II activity. Luciferase activities were measured in technical duplicates at 20-24  
544 hpt or at 48 hpt when indicated. In the graphs each dot represents an independently performed biological  
545 replicate while at least three experiments were performed in each case. For the quantification of mRNA,  
546 cRNA and vRNA levels, HEK-293T cells were seeded in 12-well plates and transfected with plasmids  
547 encoding the vRNP protein components (PB2, PB1, PA, NP) and a low amount of WSN-NA RNA  
548 expressing plasmid (5 ng/well). Total RNA was isolated at 24 hpt with RNeasy Mini columns according  
549 to the manufacturer's instructions (RNeasy Kits, Qiagen) and strand-specific RT-qPCRs were  
550 performed as described previously (Kawakami et al., 2011). Briefly, total RNA was reverse transcribed  
551 using primers specific for NA mRNA, cRNA, vRNA and, when indicated, the cellular glyceraldehyde  
552 3-phosphate dehydrogenase (GADPH) with SuperScript<sup>TM</sup> III Reverse Transcriptase (Invitrogen) and  
553 quantified using SYBR-Green (Roche) with the LightCycler 480 system® (Roche). RNA levels were  
554 normalised to GAPDH when indicated and analysed using the  $2^{-\Delta\Delta CT}$  as described before (Livak and  
555 Schmittgen, 2001).

556

### 557 **Antibodies and immunoblots**

558 Total cell lysates were prepared in RIPA cell lysis buffer as described before (Krischuns et al., 2018).  
559 Proteins were separated by SDS-PAGE using NuPAGE<sup>TM</sup> 4-12% Bis-Tris gels (Invitrogen) and  
560 transferred to nitrocellulose membranes which were incubated with primary antibodies directed against  
561 pS5 CTD (clone 3E8), mCherry (26765-1-AP), V5 (SV5-Pk1), Tubulin (B-5-1-2), PA (Da Costa et al.,  
562 2015), PB2 (GTX125925, GeneTex), ANP32A (AV40203, Sigma), ANP32B (EPR14588) and  
563 subsequently with HRP-tagged secondary antibodies (Jackson Immunoresearch). Membranes were  
564 revealed with the ECL2 substrate according to the manufacturer's instructions (Pierce) and  
565 chemiluminescence signals were acquired using the ChemiDoc imaging system (Bio-Rad) and analysed  
566 with ImageLab (Bio-Rad).



567

## 568 **Production and characterisation of recombinant viruses**

569 The recombinant PA mutant influenza viruses (PA K289A, R454A, K635A, R638A) used for serial cell  
570 culture passaging were generated by reverse genetics followed by plaque purification under agarose  
571 overlay and one round of viral amplification (P1) as described previously (Lukarska et al., 2017). For  
572 the serial passaging MDCK cell were infected at a multiplicity of infection (MOI) of 0.0001 and  
573 incubated for 3 days at 37°C in DMEM containing TPCK-Trypsin (Sigma) at a final concentration of  
574 1 µg/mL. Between each passage, viral supernatants were titrated on MDCK cells in a plaque assay as  
575 described before (Matrosovich et al., 2006). Viral RNA was extracted from 140 µL of viral stocks using  
576 the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. For next-  
577 generation sequencing of the full viral genome reverse transcription and amplification of the eight  
578 genomic segments were performed using the RT-qPCR protocol adapted by the National Influenza  
579 Center (Institut Pasteur) (Watson et al., 2013) as described previously (Chen et al., 2019). Next-  
580 generation sequencing was performed by the P2M facility at Institut Pasteur using the Nextera XT DNA  
581 Library Preparation kit (Illumina), the NextSeq 500 sequencing systems (Illumina) and the CLC  
582 Genomics Workbench 9 software (Qiagen) for analysis. Recombinant viruses with a selected subset of  
583 observed second-site mutations were generated by reverse genetics as described previously sja(Sjaastad  
584 et al., 2018). In brief, HEK-293T cells were seeded in 6-well plates the day before transfection with the  
585 WSN reverse genetics plasmids in Opti-MEM (Gibco) using FuGene®6 (Promega) according to the  
586 manufacturer's instructions (Fodor et al., 1999). The next day, MDCK were added to the wells in  
587 500 µL Opti-MEM containing 0.5 µg/mL TPCK trypsin (Sigma). The following days, 500 µL Opti-  
588 MEM containing 1 and 2 µg/mL TPCK trypsin, respectively, were added. One day later, the  
589 supernatants were harvested, centrifuged and stored at -80°C. The reverse genetics supernatants were  
590 titrated on MDCK cells and plaque diameters were measured upon staining with crystal violet using  
591 Fiji (Schindelin et al., 2012).

592

593

594

595 **Influenza virus polymerase Zhejiang-H7N9 (WT, 4M, PA K289A+C489R)**

596 The previously described and cloned pFastBac Dual vector encoding for the A/Zhejiang/DTID-  
597 ZJU01/2013 (H7N9) polymerase heterotrimer subunits, PA (Uniprot: M9TI86), PB1  
598 (Uniprot: M9TLW3), and PB2 (Uniprot: X5F427) was used as a starting point (Zhejiang-H7N9 WT)  
599 (Kouba et al., 2023). The mutations PA E349K, PA R490I, PB1 K577G and PB2 G74R were  
600 introduced by a combination of PCRs and Gibson assembly (Zhejiang-H7N9 4M). A similar approach  
601 has been applied to clone the FluPol Zhejiang-H7N9 PA K289A+C489R mutant. Plasmid sequences  
602 were confirmed by sanger sequencing for each polymerase subunit. All primer and plasmid sequences  
603 are available upon request.

604 FluPols Zhejiang-H7N9 (WT / 4M / PA K289A+C489R) were produced using the baculovirus  
605 expression system in *Trichoplusia ni* High 5 cells. For large-scale expression, cells at 0.8-1E06 cells/mL  
606 concentration were infected by adding 1% of virus. Expression was stopped 72 to 96 h after the day of  
607 proliferation arrest and cells were harvested by centrifugation (1.000g, 20 min at 4°C).

608 Cells were disrupted by sonication for 4 min (5 s ON, 20 s OFF, 40% amplitude) on ice in lysis buffer  
609 (50 mM HEPES pH 8, 500 mM NaCl, 0.5 mM TCEP, 10% glycerol) with cOmplete EDTA-free  
610 Protease Inhibitor Cocktail (Roche). After lysate centrifugation at 48.384g during 45 min at 4°C,  
611 ammonium sulfate was added to the supernatant at 0.5 g/mL final concentration. The recombinant  
612 protein was then collected by centrifugation (45 min, 4°C at 70.000g), re-suspended in the lysis buffer,  
613 and the procedure was repeated another time. FluPol Zhejiang-H7N9 was then purified using His60  
614 NiNTA Superflow resin (Takara Bio) from the soluble fraction. Bound proteins were subjected to two  
615 sequential washes steps using (i) the lysis buffer supplemented by 1 M NaCl and (ii) the lysis buffer  
616 supplemented by 50 mM imidazole. Remaining bound proteins were eluted using the lysis buffer  
617 supplemented by 500 mM imidazole. Fractions with FluPol Zhejiang-H7N9 were pooled and directly  
618 subjected to a strep-tactin affinity purification (IBA, Superflow). Bound proteins were eluted using the  
619 lysis buffer supplemented by 2.5 mM d-desthiobiotin and protein-containing fractions were pooled and  
620 diluted with an equal volume of buffer (50 mM HEPES pH 8, 0.5 mM TCEP, 10% glycerol) before  
621 loading on a third affinity column HiTrap Heparin HP 5 mL (Cytiva). A continuous gradient of lysis  
622 buffer supplemented with 1 M NaCl was applied over 15 CV, and FluPol Zhejiang-H7N9 was eluted

623 as single species at ~800 mM NaCl. Pure and acid nucleic free FluPol Zhejiang-H7N9 was dialysed  
624 overnight in a final buffer (50 mM HEPES pH 8, 500 mM NaCl, 2 mM TCEP, 5% glycerol),  
625 concentrated with Amicon Ultra-15 (50 kDa cutoff), flash-frozen and stored at -80°C for further use.

626

#### 627 **Influenza virus nucleoprotein Anhui-H7N9 monomeric mutant (R416A)**

628 The influenza virus nucleoprotein (NP) Anhui-H7N9 gene was amplified from a pCAGGS plasmid (gift  
629 from Ron Fouchier) (Mänz et al., 2016) and introduced by Gibson assembly in a pLIB vector with an  
630 N-terminal double strep-tag followed by a human rhinovirus (HRV) 3C protease cleavage site. The  
631 NP R416A mutation was introduced by a combination of PCRs and Gibson assembly. Introduction of  
632 the desired mutation was confirmed by sanger sequencing. Monomeric influenza virus Anhui-H7N9  
633 NP R416A was produced using the baculovirus expression system in *Trichoplusia ni* High 5 cells. For  
634 large-scale expression, cells at 0.8-1E06 cells/mL concentration were infected by adding 1% of virus.  
635 Expression was stopped 72 to 96 h after the day of proliferation arrest and cells were harvested by  
636 centrifugation (1.000g, 20 min at 4°C).

637 Cells were disrupted by sonication for 4 min (5 s ON, 15 s OFF, 40% amplitude) on ice in lysis buffer  
638 (50 mM HEPES pH 8, 500 mM NaCl, 2 mM TCEP, 5% glycerol) with cComplete EDTA-free Protease  
639 Inhibitor Cocktail (Roche). After lysate centrifugation at 48.384g for 45 min at 4°C, the soluble fraction  
640 was loaded on a StrepTrap HP 5 mL (Cytiva). Bound proteins were eluted using the lysis buffer  
641 supplemented by 2.5 mM d-desthiobiotin. Protein-containing fractions were pooled and diluted with an  
642 equal volume of buffer (50 mM HEPES pH 8, 2 mM TCEP, 5% glycerol) before loading on an affinity  
643 column HiTrap Heparin HP 5 mL (Cytiva). A continuous gradient of lysis buffer supplemented with  
644 1 M NaCl was applied over 15 CV, and Anhui-H7N9 NP R416A was eluted as single species at ~500  
645 mM NaCl without nucleic acids ( $A_{260/280}$ : 0.6). Pure and acid nucleic free Anhui-H7N9 NP R416A was  
646 dialysed overnight in a final buffer (50 mM HEPES pH 8, 300 mM NaCl, 1 mM TCEP, 5% glycerol)  
647 together with N-terminal his-tagged HRV 3C protease (ratio 1:5 w/w). Tag-cleaved Anhui-H7N9  
648 NP R416A was subjected to a last Ni-sepharose affinity to remove the HRV 3C protease, further  
649 concentrated with Amicon Ultra-15 (10 kDa cutoff), flash-frozen and stored at -80°C for later use.

650

## 651 **Acidic Nuclear Phosphoprotein 32A**

652 Human and chicken ANP32A genes (GeneScript) were introduced in a pETM11 vector with an N-  
653 terminal 6xHis-tag followed by a Tobacco Etch Virus (TEV) protease cleavage site. ANP32A  
654 constructs were expressed in BL21(DE3) *E.coli* cells. Expression was induced when absorbance  
655 reached 0.6, with 1 mM IPTG, incubated for 4 h at 37°C. Cells were harvested by centrifugation  
656 (1.000g, 20 min at 4°C).

657 Cells were disrupted by sonication for 5 min (5 s ON, 15 s OFF, 50% amplitude) on ice in lysis buffer  
658 (50 mM HEPES pH 8, 150 mM NaCl, 5 mM beta-mercaptoethanol (BME)) with cComplete EDTA-free  
659 Protease Inhibitor Cocktail (Roche). After lysate centrifugation at 48.384g for 45 min at 4°C, the soluble  
660 fraction was loaded on a HisTrap HP 5 mL column (Cytiva). Bound proteins were subjected to a wash  
661 step using the lysis buffer supplemented by 50 mM imidazole. Remaining bound proteins were eluted  
662 using the lysis buffer supplemented by 500 mM imidazole. Fractions containing ANP32A were dialysed  
663 overnight in the lysis buffer (50 mM HEPES pH 8, 150 mM NaCl, 5 mM BME) together with N-  
664 terminal his-tagged TEV protease (ratio 1:5 w/w). Tag-cleaved ANP32A protein was subjected to a Ni-  
665 sepharose affinity column to remove the TEV protease, further concentrated with Amicon Ultra-15  
666 (3 kDa cutoff) and subjected to a Size-Exclusion Chromatography using a Superdex 200 Increase  
667 10/300 GL column (Cytiva) in a final buffer containing 50 mM HEPES pH 8, 150 mM NaCl, 2 mM  
668 TCEP. Fractions containing exclusively ANP32A were concentrated with Amicon Ultra-15 (3 kDa  
669 cutoff), flash-frozen and stored at -80°C for later use.

670 Human ANP32A truncation constructs (1-199 and 144-249) were generated, expressed and protein  
671 purified as previously described (Camacho-Zarco et al., 2020). The human ANP32A 1-149 construct  
672 was a gift from Cynthia Wolberger (Addgene plasmid # 67241, (Huyton and Wolberger, 2007)) and  
673 was expressed and protein purified as previously described (Camacho-Zarco et al., 2020).

674

## 675 ***De novo* FluPol replication activity**

676 Synthetic vRNA loop (“v51\_mut\_S”) (5’-pAGU AGA AAC AAG GGU GUA UUU UCC CCU CUU  
677 UUU GUU UCC CCU GCU UUU GCU -3’) (IDT) was used for all *in vitro* replication activity assays.  
678 For all *de novo* replication activity assays, 2.4 µM FluPol Zhejiang-H7N9 (WT or 4M) were mixed with

679 (i) 0.8  $\mu\text{M}$  v51\_mut\_S and/or (ii) 8  $\mu\text{M}$  ANP32A and/or (iii) 16  $\mu\text{M}$  pS5 CTD(6mer) (respective molar  
680 ratio: 3 FluPols: 1 template: 10 ANP32: 20 pS5 CTD). Reactions were launched at 30°C for 4 h by  
681 adding ATP/GTP/CTP/UTP (AGCU) or only ATP/GTP/CTP (AGC) at 100  $\mu\text{M}$ /NTP, 0.75  $\mu\text{Ci/ml}$   $\alpha$ -  
682 32P ATP and  $\text{MgCl}_2$  in a final assay buffer containing 50 mM HEPES pH 8, 150 mM NaCl, 2 mM  
683 TCEP, 100  $\mu\text{M}$ /NTP, 1 mM  $\text{MgCl}_2$ . Reactions were stopped by adding 2X RNA loading dye, heating  
684 5 min at 95°C and immediately loaded on a 20% TBE-7M urea-polyacrylamide gel. Each gel was  
685 exposed on a storage phosphor screen and read with an Amersham Typhoon scanner (Cytiva). For each  
686 gel the decade markers system (Ambion) was used.

687

### 688 **Next Generation Sequencing of *in vitro* FluPol replication products**

689 To confirm the identity of the replication products (full-length cRNA product and stalled cRNA  
690 product) and address the discrepancy between expected size and urea-PAGE migration, sequencing of  
691 the total reaction product was performed from a *de novo* FluPol replication assay. 2.4  $\mu\text{M}$  FluPol  
692 Zhejiang-H7N9 4M were mixed with (i) 0.8  $\mu\text{M}$  v51\_mut\_S, (ii) 8  $\mu\text{M}$  ANP32A and (iii) 16  $\mu\text{M}$   
693 pS5 CTD(6mer) (respective molar ratio: 3 FluPols: 1 template: 10 ANP32A: 20 pS5 CTD). Reaction  
694 was launched at 30°C for 4 h by adding ATP/GTP/CTP/UTP at 100  $\mu\text{M}$ /NTP and  $\text{MgCl}_2$  in a final assay  
695 buffer containing 50 mM HEPES pH 8, 150 mM NaCl, 2 mM TCEP, 100  $\mu\text{M}$ /NTP, 1 mM  $\text{MgCl}_2$ . After  
696 completion of the reaction, proteins were removed using the Monarch RNA Cleanup Kit (NEB) and 5'-  
697 mono-phosphorylated v51\_mut\_S templates were specifically digested with a terminator 5'-phosphate-  
698 dependent exonuclease (Biosearch technologies) and subjected again to the Monarch RNA Cleanup Kit  
699 (NEB). Total remaining RNAs were used for next-generation sequencing.

700 Sample concentration and fragment size distribution were checked with the RNA Pico assay  
701 (Bioanalyzer, Agilent). 6 ng of sample was treated with 2 units T4 Polynucleotide Kinase (NEB) and  
702 incubated at 37°C for 30 min, followed by heat inactivation at 65°C for 20 min. The treated samples  
703 were then taken into library preparation following the Takara SMARTer small RNA sequencing kit  
704 according to the manufacturer's instructions with 13 cycles of PCR. The final library fragment size  
705 distribution was checked with the High Sensitivity Bioanalyzer assay. 8 pM of library was sequenced  
706 on a MiSeq to generate 76 bp single-end reads.

707 Resulting total reads (13.073.371) were trimmed using Cutadapt v2.3 (Kechin et al., 2017) with settings  
708 recommended by the library preparation kit (-u 3 -a 'AAAAAAAAAA') omitting the filter for only  
709 reads longer than 20 bases (10.991.864). Trimmed reads were subsequently used as an input for pattern  
710 lookup performed using Python scripts (**Source data file 1**). All reads exactly starting by these motifs  
711 have been kept for further analysis:  
712 "AGCAAAGCA"/"GCAAAGCA"/"CAAAGCA"/"AAAAGCA" (7.776.959 reads). Each read  
713 starting exactly by these motifs "5'-AGCAAAGCAGGGG" / "5'-GCAAAGCAGGGG" / "5'-  
714 CAAAGCAGGGG" / "5'-AAAAGCAGGGG" were counted and plotted (**Fig. 3.H**). Each read  
715 matching the exact sequence of a full-length replication product starting by "5'-AGC AAA...ACU-3'"  
716 (51-mer), "5'-\_GC AAA... ACU-3'" (50-mer), "5'-\_\_C AAA... ACU-3'" (49-mer) or "5'-\_\_\_ AAA...  
717 ACU-3'" (48-mer) were counted and plotted as percentage (**Figure 3.I**).

718

### 719 **Electron microscopy**

720 *De novo* replication states were trapped by mixing 2.4  $\mu\text{M}$  FluPol Zhejiang-H7N9 4M with (i) 0.8  $\mu\text{M}$   
721 v51\_mut\_S, (ii) 8  $\mu\text{M}$  chANP32A, (iii) 16  $\mu\text{M}$  pS5 CTD(6mer) and (iv) 4  $\mu\text{M}$  Anhui-H7N9 NP R416A  
722 in a final buffer containing 50 mM HEPES pH 8, 150 mM NaCl, 2 mM TCEP, 1 mM  $\text{MgCl}_2$ ,  
723 ATP/GTP/CTP at 100  $\mu\text{M}$ /NTP, and 100  $\mu\text{M}$  of non-hydrolysable UpNHpp (Jena Bioscience). The  
724 *de novo* reaction mix was incubated for 4 h at 30°C. Before proceeding to grids freezing, the sample  
725 was centrifuged for 5 min at 11.000g and stored at 4°C before proceeding to grids freezing. For grids  
726 preparation, 1.5  $\mu\text{l}$  of sample was applied on each sides of plasma cleaned (Fischione 1070 Plasma  
727 Cleaner: 1 min 10 s, 90% oxygen, 10% argon) grids (Ultrafoil 1.2/1.3, Au 300). Excess solution was  
728 blotted for 3 to 5 s, blot force 0, 100% humidity, at 4°C, with a Vitrobot Mark IV (ThermoFisher) before  
729 plunge-freezing in liquid ethane. Automated data collection was performed on a TEM Titan Krios G3  
730 (Thermo Fisher) operated at 300 kV equipped with a K3 (Gatan) direct electron detector camera and a  
731 BioQuantum energy filter, using EPU. Coma and astigmatism correction were performed on a carbon  
732 grid. Micrographs were recorded in counting mode at a  $\times 105,000$  magnification giving a pixel size of  
733 0.84 Å with defocus ranging from -0.8 to -2.0  $\mu\text{m}$ . Gain-normalised movies of 40 frames were  
734 collected with a total exposure of  $\sim 40 \text{ e}^-/\text{\AA}^2$ .

735 For Zhejiang-H7N9 PA K289A+C489R structures, 0.8  $\mu\text{M}$  FluPol Zhejiang-H7N9 PA K289A+C489R  
736 was mixed with (i) 0.8  $\mu\text{M}$  v51\_mut\_S and 16  $\mu\text{M}$  pS5 CTD(6mer) in a final buffer containing 50 mM  
737 HEPES pH 8, 150 mM NaCl, 2 mM TCEP. After 1 h incubation on ice, the sample was centrifuged for  
738 5 min, 11,000g and stored at 4°C before proceeding to grids freezing. For grid preparation, 1.5  $\mu\text{l}$  of  
739 sample was applied on each sides of plasma cleaned (Fischione 1070 Plasma Cleaner: 1 min 10 s, 90%  
740 oxygen, 10% argon) grids (UltrAufoil 1.2/1.3, Au 300). Excess solution was blotted for 3 to 5 s, blot  
741 force 0, 100% humidity, at 4°C, with a Vitrobot Mark IV (ThermoFisher) before plunge-freezing in  
742 liquid ethane. Automated data collection was performed on a TEM Glacios (ThermoFisher) operated at  
743 200 kV equipped with a F4i (ThermoFisher) direct electron detector camera and a SelectrisX energy  
744 filter, using EPU. Coma and astigmatism correction were performed on a carbon grid. Micrographs  
745 were recorded in counting mode at a  $\times 130,000$  magnification giving a pixel size of 0.907  $\text{\AA}$  with defocus  
746 ranging from  $-0.8$  to  $-2.0$   $\mu\text{m}$ . EER movies were collected with a total exposure of  $\sim 40 \text{ e}^-/\text{\AA}^2$ .

747

#### 748 **Image processing**

749 For the TEM Titan Krios dataset, movie drift correction was performed using Relion's Motioncor  
750 implementation, with 7x5 patch, using all movie frames (Zheng et al., 2017). All additional initial image  
751 processing steps were performed in cryoSPARC v3.3 (Punjani et al., 2017). CTF parameters were  
752 determined using "Patch CTF estimation", realigned micrographs were then manually inspected and  
753 low-quality images were manually discarded. To obtain an initial 3D reconstruction of FluPol Zhejiang-  
754 H7N9 4M, particles were automatically picked on few hundreds micrographs using a circular blob with  
755 a diameter ranging from 100 to 140  $\text{\AA}$ . Particles were extracted using a box size of 360 x 360 pixels<sup>2</sup>,  
756 2D classified and subjected to an "ab-initio reconstruction" job. The best initial model was further used  
757 to prepare 2D templates. Template picking was then performed using a particle diameter of 120  $\text{\AA}$  and  
758 particles extracted from dose-weighted micrographs. Successive 2D classifications were used to  
759 eliminate particles displaying poor structural features. All remaining particles were then transferred to  
760 Relion 4.0. Particles were divided in subset of 300k to 500k particles and subjected to multiple 3D  
761 classification with coarse image-alignment sampling using a circular mask of 180  $\text{\AA}$ . For each similar  
762 FluPol conformation, particles were grouped and subjected to 3D masked refinement followed by

763 multiple 3D classification without alignment or using local angular searches. Once particles properly  
764 classified, bayesian polishing was performed and re-extracted “shiny” particles were subjected to a last  
765 3D masked refinement. Post-processing was performed in Relion using an automatically or manually  
766 determined B-factor. For each final map, reported global resolution is based on the FSC 0.143 cut-off  
767 criteria. Local resolution variations were estimated in Relion. Detailed image processing information is  
768 shown in Fig. S7.

769 For the TEM Glacios dataset, EER fractionation was set to 24, giving  $\sim 1e^{-7}\text{\AA}^2$  per resulting fraction.  
770 Movie drift correction was performed using Relion’s Motioncor implementation, with 5x5 patch (Zheng  
771 et al., 2017). All additional initial image processing steps were performed in cryoSPARC v4.1 (Punjani  
772 et al., 2017). CTF parameters were determined using “Patch CTF estimation”, realigned micrographs  
773 were then manually inspected and low-quality images were manually discarded. To obtain an initial 3D  
774 reconstruction of FluPol Zhejiang-H7N9 PA K289A+C489R, particles were automatically picked on  
775 few hundreds micrographs using a circular blob with a diameter ranging from 100 to 140 Å. Particles  
776 were extracted using a box size of 340 x 340 pixels<sup>2</sup>, 2D classified and subjected to an “ab-initio  
777 reconstruction” job. The best initial model was further used to prepare 2D templates. Template picking  
778 was then performed using a particle diameter of 120 Å and particles extracted from dose-weighted  
779 micrographs. Successive 2D classifications were used to eliminate particles displaying poor structural  
780 features. All remaining particles were subjected to an “Heterogenous refinement” job, with 3  
781 symmetrical dimers and 3 monomers as initial 3D models. Particles assigned to the monomeric 3D  
782 classes were subjected to a final non-uniform refinement. Post-processing was performed in Relion  
783 using an automatically determined B-factor. Reported global resolution is based on the FSC 0.143 cut-  
784 off criteria. Local resolution variations were estimated in Relion. Detailed image processing information  
785 is shown in Fig. S9.

786

### 787 **Model building and refinement**

788 Using the FluPol Zhejiang-H7N9 elongation complex as starting point (PDB: 7QTL) (Kouba et al.,  
789 2023), atomic models were constructed by iterative rounds of manual model building with COOT and  
790 real-space refinement using Phenix (Afonine et al., 2018). Validation was performed using Phenix.



791 Model resolution according to the cryo-EM map was estimated at the 0.5 FSC cutoff. Figures were  
792 generated using ChimeraX (Goddard et al., 2018).  
793

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807

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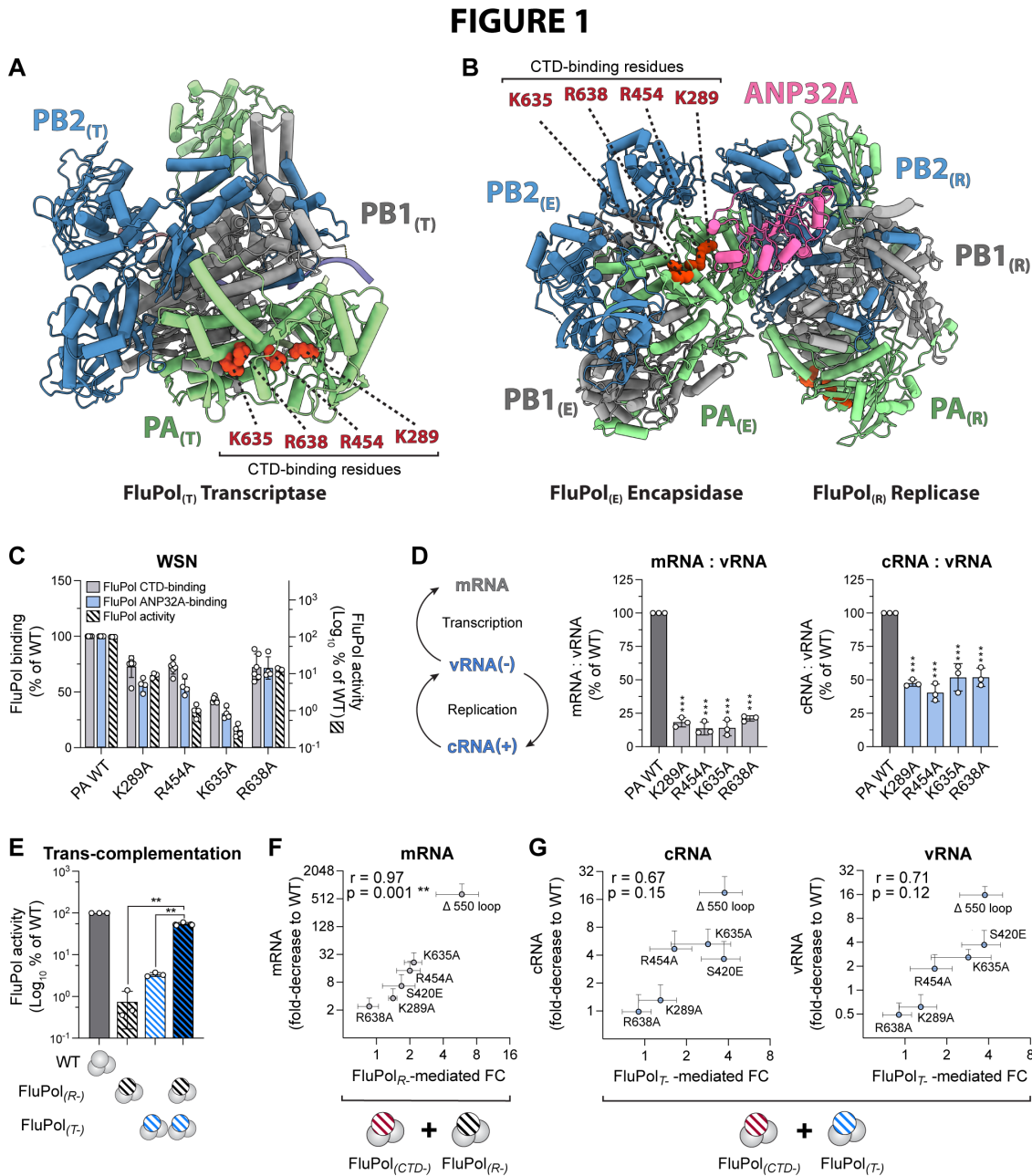
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1062 Main Figures (5) and Table (1):



1063

1064 **Figure 1: The FluPol CTD-binding interface is essential for replication of the viral genome**

1065 (A) Representation of the Cryo-EM structure of the Flu<sub>A</sub>Pol heterotrimer in the FluPol<sub>(T)</sub> conformation,  
 1066 bound to a 3'5' vRNA promoter and a short capped RNA primer (A/NT/60/1968, PDB: 6RR7, (Fan et  
 1067 al., 2019)). Ribbon diagram representation with PA in green, PB1 in grey, and PB2 in blue. Key FluPol  
 1068 CTD-binding residues PA K289, R454, K635 and R638 are highlighted in red.

1069 (B) Model of the actively replicating Zhejiang-H7N9 Flu<sub>A</sub>Pol replicase-ANP32A-encapsidase (FluPol<sub>(R)</sub>,  
 1070 FluPol<sub>(E)</sub> and ANP32A) The colour code is as in (A) with ANP32A in pink. The model was constructed  
 1071 by superposing equivalent Zhejiang-H7N9 Flu<sub>A</sub>Pol domains (elongating H7N9 Flu<sub>A</sub>Pol, including



1072 product-template duplex, PDB: 7QTL, (Kouba et al., 2023)) for the FluPol<sub>(R)</sub> and 5' hook bound apo-  
1073 dimer Zhejiang-H7N9 Flu<sub>A</sub>Pol (PDB: 7ZPL, (Kouba et al., 2023)) for the FluPol<sub>(E)</sub>, on those of Flu<sub>C</sub>Pol  
1074 replication complex (PDB: 6XZQ, (Carrique et al., 2020)). The ANP32A was left unchanged. Key  
1075 FluPol CTD-binding residues PA K289, R454, K635 and R638 are highlighted in red on the FluPol<sub>(R)</sub>  
1076 as well as the FluPol<sub>(E)</sub>.

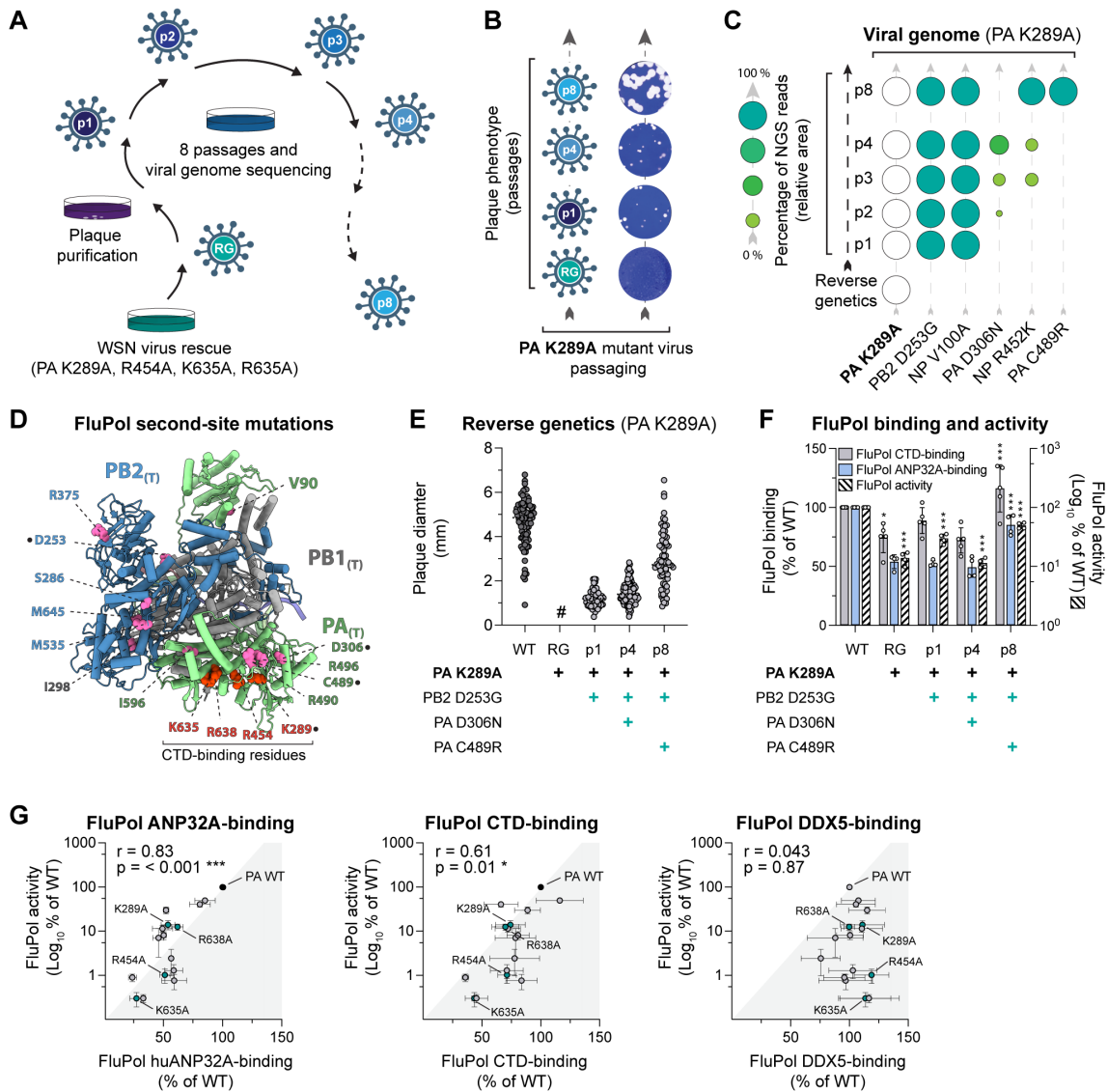
1077 **(C)** Cell-based WSN FluPol binding and activity assays. Left Y-axis (linear scale): FluPol binding to  
1078 the CTD (grey bars) and ANP32A (blue bars) was assessed using split-luciferase-based  
1079 complementation assays. HEK-293T cells were co-transfected with expression plasmids for the CTD  
1080 or ANP32 tagged with one fragment of the *G. princeps* luciferase and FluPol tagged with the other  
1081 fragment either with PA WT or with the indicated PA CTD-binding mutants. Luminescence due to  
1082 luciferase reconstitution was measured and the signals are represented as a percentage of PA WT. Right  
1083 Y-axis (logarithmic scale): FluPol PA CTD-binding mutant activity (hatched bars) was assessed by  
1084 vRNP reconstitution in HEK-293T cells upon transient transfection, using a model vRNA encoding the  
1085 Firefly luciferase. Luminescence was measured, normalised to a transfection control and is represented  
1086 as a percentage of PA WT. \* $p < 0.033$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$  (one-way ANOVA; Dunnett's  
1087 multiple comparisons test).

1088 **(D)** WSN vRNPs with the indicated PA mutations were reconstituted in HEK-293T cells by transient  
1089 transfection using the NA vRNA segment. Steady-state levels of NA mRNA, cRNA and vRNA were  
1090 quantified by strand-specific RT-qPCR (Kawakami et al., 2011) and are presented as ratios of mRNA  
1091 to vRNA (grey bars) or cRNA to vRNA (blue bars) levels relative to PA WT. The corresponding  
1092 mRNA, vRNA and cRNA levels are presented in Fig. S1E. \*\*\* $p < 0.001$  (one-way ANOVA; Dunnett's  
1093 multiple comparisons test).

1094 **(E)** FluPol trans-complementation of a transcription-defective (FluPol<sub>(T-)</sub>, PA D108A, (Dias et al.,  
1095 2009)) and a replication-defective (FluPol<sub>(R-)</sub>, PA K664M, Drncova et al., unpublished) FluPol was  
1096 measured by vRNP reconstitution in HEK-293T cells upon transient transfection, using a model vRNA  
1097 encoding the Firefly luciferase. Luminescence was measured at 48 hpt and normalised to a transfection  
1098 control. The data are represented as percentage of PA WT. \*\* $p < 0.002$  (one-way repeated measure  
1099 ANOVA; Dunnett's multiple comparisons test).

1100 **(F-G)** WSN vRNPs with the indicated PA CTD-binding mutations were trans-complemented with (F)  
1101 a replication-defective (FluPol<sub>(R-)</sub>, PA K664M) or (G) a transcription-defective (FluPol<sub>(T-)</sub>, PA D108A,  
1102 (Dias et al., 2009)) FluPol. The FluPol<sub>(R-)</sub> and FluPol<sub>(T-)</sub>-mediated fold-change (FC) relative to the  
1103 background (x-axis, represented separately in Fig. S1F-G) is plotted against the fold-decrease of viral  
1104 m-, c- and vRNA levels relative to PA WT as measured in a vRNP reconstitution assay (y-axis,  
1105 represented separately in Fig. S1E).

## FIGURE 2



1106

1107 **Figure 2: Serial passaging of mutant viruses with mutations at the FluPol-CTD interface selects**  
 1108 **for adaptive mutations which restore FluPol binding to the CTD and ANP32A**

1109 (A) Schematic representation of the cell culture passaging experiment. Recombinant WSN mutant  
 1110 viruses (PA K289A, R454A, K635A and R638A) were plaque purified on MDCK-II cells after reverse  
 1111 genetics (p1) and were subjected to seven serial passages in MDCK-II cells (p2 to p8) followed by  
 1112 titration and short-read next generation sequencing (NGS) of the viral genome.

1113 (B) Plaque phenotype of passaged PA K289A mutant viruses. Representative images of crystal violet  
 1114 stained plaque assays of the supernatants collected after reverse genetics (RG), p1, p4 and p8 are shown.

1115 (C) The viral genome of passaged PA K289A mutant viruses was sequenced by NGS after p1 - p4 and  
 1116 p8 (from bottom to top). The initial FluPol CTD-binding mutation (PA K289A, white circles) and the  
 1117 second site mutations on PB1, PB2, PA and NP (green circles) are indicated. Only second-site mutations  
 1118 found in  $\geq 10\%$  of reads in at least one passage are shown. The fraction of reads showing a given

1119 mutation is indicated by the area of the circle, and by different shades of green that represent <25, 25-  
1120 50, 50-75 and >75 % of reads (schematic on the left).

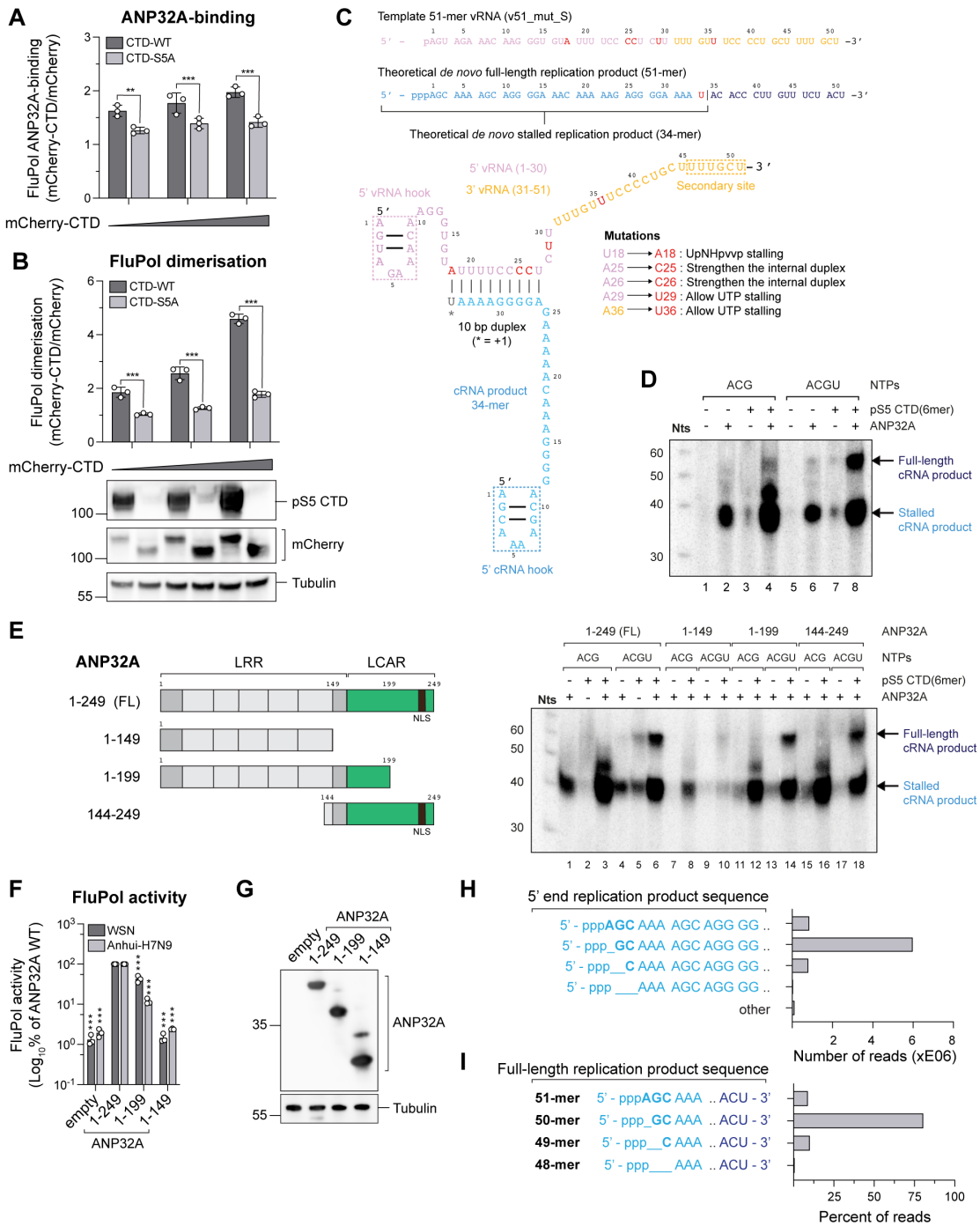
1121 **(D)** Representation of the Cryo-EM structure of the FluPol heterotrimer in the FluPol<sub>(T)</sub> conformation,  
1122 bound to a 3'5' vRNA promoter and a short capped RNA primer (A/NT/60/1968, PDB: 6RR7, (Fan et  
1123 al., 2019)). Ribbon diagram representation with PA in green, PB1 in grey, and PB2 in blue. Initially  
1124 mutated FluPol PA residues (PA K289A, R454A, K635A and R638A) are highlighted in red and  
1125 residues corresponding to FluPol second-site mutations in pink. Second-site mutations which appeared  
1126 during passaging of the WSN PA K289A mutant virus are highlighted with a dot.

1127 **(E)** Characterisation of recombinant WSN mutant viruses. Recombinant viruses with the primary  
1128 FluPol mutation PA K289A and the indicated combinations of FluPol second-site mutations (PB2  
1129 D253G, PA D306N, PA C489R) were generated by reverse genetics. The supernatants of two  
1130 independently performed RG experiments were titrated on MDCK cells and stained by crystal violet.  
1131 Plaque diameters (mm) were measured and each dot represents one viral plaque. Representative plaque  
1132 assays are shown in Fig. S3C. (#) not measurable pinhead-sized plaques.

1133 **(F)** The indicated combinations of WSN FluPol second-site mutations which occurred during passaging  
1134 of the PA K289A mutant virus were characterised in cell-based FluPol binding and activity assays. Left  
1135 Y-axis (linear scale): FluPol binding to the CTD (grey bars) and ANP32A (blue bars) was assessed  
1136 using split-luciferase-based complementation assays as in Fig. 1C. Luminescence signals due to  
1137 luciferase reconstitution are represented as a percentage of FluPol WT. Right Y-axis (logarithmic scale):  
1138 FluPol activity (hatched bars) was measured by vRNP reconstitution in HEK-293T cells as in Fig. 1C.  
1139 Normalised luminescence signals are represented as a percentage of FluPol WT. Stars indicate statistical  
1140 significance compared to the previous passage. \* $p < 0.033$ , \*\*\* $p < 0.001$  (one-way ANOVA; Tukey's  
1141 multiple comparisons test).

1142 **(G)** For each initial FluPol mutant (PA K289A, R454A, K635A, R638A, green dots) and FluPol  
1143 genotypes observed upon serial passaging (grey dots), FluPol binding to ANP32A, CTD and DDX5 (x-  
1144 axis in the left, middle and right panel, respectively) were plotted against the FluPol activity as measured  
1145 in a vRNP reconstitution assay (y-axis). The FluPol binding and FluPol activity data are represented  
1146 separately in Fig. 2F for PA K289A and in Fig. S3E for PA R454A, K635A, R638A. \* $p < 0.033$ , \*\*\* $p$   
1147  $< 0.001$  (Pearson Correlation Coefficient ( $r$ )).

## FIGURE 3



1148

1149 **Figure 3: FluPol replication activity is enhanced in the presence of CTD and ANP32A**

1150 (A-B) Cell-based assays for (A) WSN FluPol-binding to ANP32A and (B) WSN FluPol dimerisation  
1151 in the presence of increasing amounts of pS5 CTD. HEK-293T cells were co-transfected with  
1152 expression plasmids for ANP32A tagged with one fragment of the *G. princeps* luciferase and FluPol  
1153 tagged with the other fragment. FluPol dimerisation was assessed using split-luciferase-based  
1154 complementation assays as described previously (Chen et al., 2019). Plasmids encoding untagged

1155 mCherry or mCherry tagged to CTD-WT or CTD-S5A in which all serine 5 residues were replaced with  
1156 alanines were co-transfected in increasing amounts. Luminescence due to luciferase reconstitution was  
1157 measured and the signals are represented as fold-changes compared to untagged mCherry co-  
1158 expression. In parallel, cell lysates of the FluPol dimerisation assay were analysed by western blot using  
1159 antibodies specific for the pS5 CTD, mCherry and tubulin. \*\*\* $p < 0.001$  (two-way ANOVA; Sidak's  
1160 multiple comparisons test).

1161 **(C)** Zhejiang-H7N9 FluPol vRNA loop template design strategy. The 51-mer vRNA template  
1162 (v51\_mut\_S) used in *in vitro* replication activity assays and expected theoretical RNA replication  
1163 products sequences are displayed. The v51\_mut\_S sequence is derived from segment 4 of  
1164 A/Zhejiang/DUID-ZJU01/2013(H7N9)/KJ633805. The 5' vRNA end (1-30) is coloured in pink. The  
1165 3' vRNA end (31-51) is coloured in gold. v51\_mut\_S introduced mutations are in bold, coloured in red  
1166 and their functions are summarised. The theoretical *de novo* full-length replication product sequence is  
1167 coloured in light blue (1-34) and dark blue (35-51). The expected stalled *de novo* replication product  
1168 sequence using non-hydrolysable UpNHpp is annotated with U34 in red. A schematic representation of  
1169 the expected stalled elongation state is displayed and coloured accordingly. The 5' end vRNA hook, the  
1170 5' end cRNA hook of the replication product and the secondary site are highlighted by a dotted  
1171 rectangle.

1172 **(D)** The *de novo* replication activity of Zhejiang-H7N9 FluPol using v51\_mut\_S is enhanced in the  
1173 presence of both ANP32A and pS5 CTD. The assays are done in the presence of either 3 NTPs (AUG)  
1174 or 4 NTPs (AUGC), with or without pS5 CTD and ANP32A. Full-length and stalled replication products  
1175 are indicated on the right side of the gel and coloured as in C. The decade molecular weight marker  
1176 (Nts) is shown on the left side of the gel. Uncropped gels are provided as a source data file.

1177 **(E)** (left) Schematic representation of the full-length (FL, 1-249) ANP32A and the investigated deletion  
1178 mutants: ANP32A 1-149, 1-199, 144-249. LRR: leucine-rich repeat; LCAR: low-complexity acidic  
1179 region; NLS: nuclear localization signal. (right) The *de novo* replication activity of Zhejiang-H7N9  
1180 FluPol using v51\_mut\_S is dependent on both the LRR and LCAR domains of ANP32A. The assays  
1181 are done in the presence of either 3 NTPs (AUG) or 4 NTPs (AUGC), with or without pS5 CTD and  
1182 with either FL ANP32A or the deletion mutants represented in (E). *De novo* replication products are  
1183 annotated on the right side of the gel and coloured as in C. The decade molecular weight marker (Nts)  
1184 is shown on the left side of the gel. Uncropped gels are provided as a source data file.

1185 **(F)** WSN (dark grey bars) and Anhui-H7N9 (light grey bars) vRNPs were reconstituted in HEK-293T  
1186 ANP32AB KO cells with a model vRNA encoding the Firefly luciferase, and co-expressed with either  
1187 the FL ANP32 or the indicated deletion mutants, tagged at their C-terminus to a V5 tag and an SV40  
1188 nuclear localisation sequence. Luminescence was measured and normalised to a transfection control.  
1189 The data are represented as a percentage of ANP32A FL. \*\*\* $p < 0.001$  (two-way ANOVA; Sidak's  
1190 multiple comparisons test).

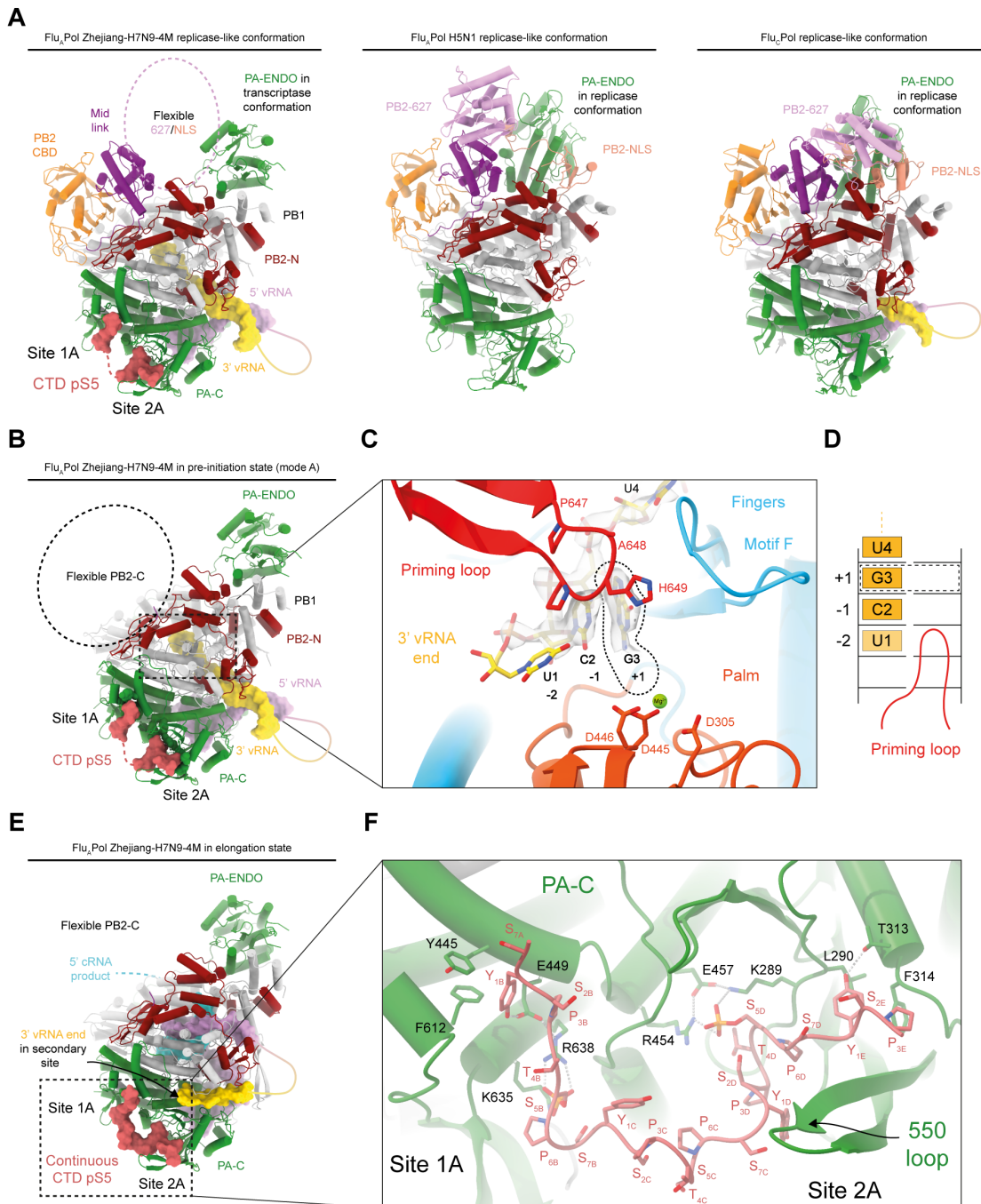
1191 **(G)** HEK-293T cells were transfected with the indicated ANP32A expression plasmids. Cells lysates

1192 were analysed by western blot using antibodies specific for the V5-tag and tubulin. Uncropped gels are  
1193 provided as a source data file.

1194 **(H)** RNA-sequencing analysis of Zhejiang-H7N9-4M FluPol *de novo* replication products in the  
1195 presence of all NTPs, the pS5 CTD and ANP32A. The number of reads is plotted according to the  
1196 recurrence of the exact 5' cRNA replication product motifs indicated on the left. Other reads that do not  
1197 encompass these motifs are plotted as "Other". NGS data are provided as a source data file.

1198 **(I)** RNA-sequencing analysis of FluPol Zhejiang-H7N9-4M *de novo* replication products in the  
1199 presence of pS5 CTD and ANP32A. The percentage of full-length replication products are plotted  
1200 according to the 5' terminal nucleotide which is indicated on the left. NGS data are provided as a source  
1201 data file.

## FIGURE 4



1202

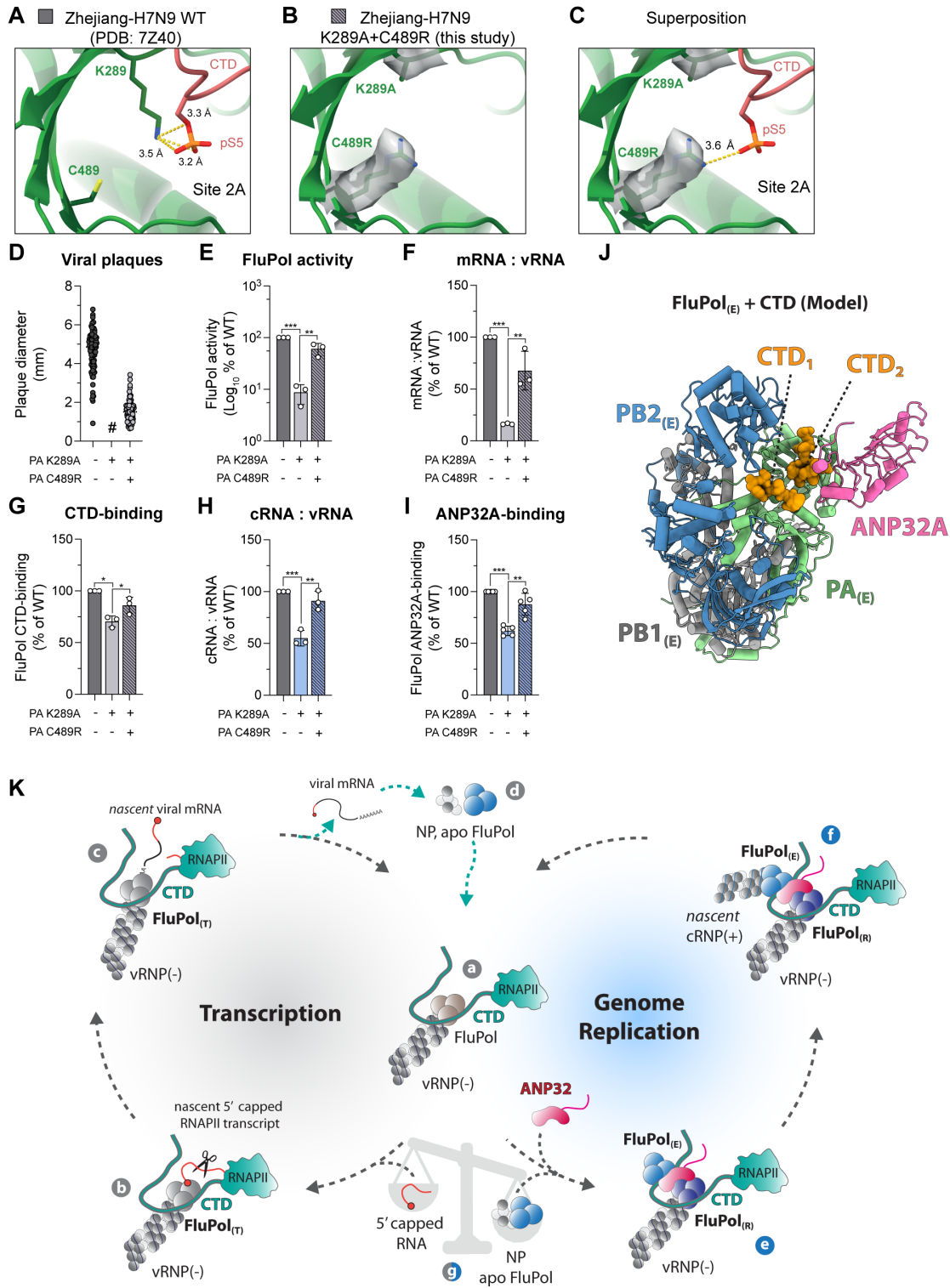
1203 **Figure 4: FluPol replication is consistent with CTD-binding to the FluPol**

1204 (A) Cartoon representations of Flu<sub>A</sub>Pol Zhejiang-H7N9-4M in a replicase-like conformation bound to  
 1205 CTD mimicking-peptides (obtained in this study) and as a reference, previously published Flu<sub>A</sub>Pol  
 1206 replicase-like (A/duck/Fujian/01/2002(H5N1), PDB: 6QPF (Fan et al., 2019)) and the Flu<sub>C</sub>Pol  
 1207 replicase-like conformation extracted from the Flu<sub>C</sub>Pol<sub>R</sub>-Flu<sub>C</sub>Pol<sub>E</sub>-ANP32A asymmetric dimer  
 1208 (C/Johannesburg/1/1966, PDB: 6XZG, (Carrique et al., 2020)). FluPol subunits and domains are  
 1209 coloured as follow: PA subunit in green, PB1 subunit in light grey, PB2-N in dark red, PB2 cap-binding

1210 domain (PB2-CBD) in orange, PB2 mid-link in pink, PB2 627 in light violet and PB2 NLS in salmon.  
1211 The pS5 CTD bound to FluPol Zhejiang-H7N9-4M in site 1A and 2A is coloured in red and displayed  
1212 as surface. The pS5 CTD discontinuity between both sites is shown as a dotted line. The flexibility of  
1213 Flu<sub>A</sub>Pol Zhejiang-H7N9-4M PB2-627/NLS domains is highlighted as a dotted circle. The three  
1214 displayed FluPols are in the same orientation, aligned on the PB1 subunit. In the Flu<sub>A</sub>Pol Zhejiang-  
1215 H7N9-4M structure the PA-Endonuclease domain (PA-ENDO) remains in a transcriptase  
1216 conformation, while it adopts a replicase conformation, interacting with the PB2-NLS domain, in the  
1217 FluPol Fujian-H5N1 and Flu<sub>C</sub>Pol structures. RNAs are displayed as surfaces. The 5' vRNA end is  
1218 coloured in pink and the 3' vRNA end in yellow. Flexible nucleotides are represented as solid line.  
1219 **(B)** Cartoon representation of FluPol Zhejiang-H7N9-4M structure in the pre-initiation state (mode A),  
1220 obtained at 2.5 Å resolution by cryo-EM. The colour code is the same as in A. PB2 C-terminal domains  
1221 (PB2-C) are flexible and highlighted as a dotted circle.  
1222 **(C)** Close-up view on the 3' vRNA end template (v51\_mut\_S) position in FluPol Zhejiang-H7N9-4M  
1223 active site. The Coulomb potential map of the template is shown. The 3' vRNA end is coloured in  
1224 yellow, by heteroatom. The 3' vRNA terminal U1 is displayed but remains unseen in the cryo-EM map.  
1225 The 3' vRNA G3 is in the +1 active site position, highlighted by a dotted line. The priming loop is  
1226 coloured in red and tip residues are shown (P647, A648, H649, P650). The palm domain is coloured in  
1227 orange and the catalytic aspartic acids (D445, D446 and D305) are displayed, coordinating a Mg<sup>2+</sup> ion,  
1228 coloured in green. The finger domain is coloured in blue and the beta-hairpin motif F is annotated.  
1229 **(D)** Schematic representation of the 3' vRNA end terminal nucleotides according to their active site  
1230 position, as seen in C. 3'-U1 is in position "-2", C2 in position "-1", G3 in position "+1". The +1 active  
1231 site position is highlighted by a dotted rectangle. The priming loop position is indicated.  
1232 **(E)** Cartoon representation of FluPol Zhejiang-H7N9-4M in a stalled elongation state. The colour code  
1233 is the same as in A. PB2-C domains are flexible. The visible part of the cRNA *de novo* replication  
1234 product is displayed as surface, while the flexible part is shown as a dotted line. The 3' vRNA end is  
1235 bound to the secondary site. The CTD pS5 is continuous between sites 1A and 2A.  
1236 **(F)** Close-up view on the pS5 CTD interaction with FluPol Zhejiang-H7N9-4M PA-C domain. FluPol  
1237 residues interacting with the pS5 CTD are shown for sites 1A and 2A. Each CTD pS5 repeat is indicated  
1238 (S<sub>7A</sub> | Y<sub>1B</sub>S<sub>2B</sub>P<sub>3B</sub>T<sub>4B</sub>S<sub>5B</sub>P<sub>6B</sub>S<sub>7B</sub> | Y<sub>1C</sub>S<sub>2C</sub>P<sub>3C</sub>T<sub>4C</sub>S<sub>5C</sub>P<sub>6C</sub>S<sub>7C</sub> | Y<sub>1D</sub>S<sub>2D</sub>P<sub>3D</sub>T<sub>4D</sub>S<sub>5D</sub>P<sub>6D</sub>S<sub>7D</sub> | Y<sub>1E</sub>S<sub>2E</sub>P<sub>3E</sub>). The PA-  
1239 550 loop is annotated.



## FIGURE 5



1240

1241 **Figure 5: Restoration of CTD-binding enhances FluPol binding to ANP32A and FluPol**  
 1242 **replication activity - a model for RNAP II CTD-anchored transcription and replication of the**  
 1243 **influenza virus genome**

1244 **(A)** Cartoon representation of the pS5 CTD bound to FluPol Zhejiang-H7N9 in site 2A (PDB: 7Z40,

1245 (Krischuns et al., 2022)). The PA subunit is coloured in green and the pS5 CTD is coloured in red.  
1246 PA K289 and PA C489 residues are displayed. Putative hydrogen bonds between PA K289 and pS5 are  
1247 drawn as yellow dashed lines and the corresponding distances are indicated.

1248 **(B)** Cartoon representation of FluPol Zhejiang-H7N9 PA K289A+C489R (obtained in this study). The  
1249 PA subunit is coloured in green. The Coulomb potential map of PA K289A and C489R is shown and  
1250 the corresponding residues are displayed. The pS5 CTD is not present.

1251 **(C)** Superposition of the pS5 CTD, extracted from the structure shown in A, with the FluPol Zhejiang-  
1252 H7N9 PA K289A+C489R structure shown in B. The putative hydrogen bond between PA C489R and  
1253 pS5 is shown and the corresponding distance is indicated.

1254 **(D-I)** Phenotypes associated with the WSN FluPol PA K289A mutation and the second-site mutation  
1255 PA C489R were characterised. (D) Plaque phenotype of recombinant WSN mutant viruses produced  
1256 by reverse genetics. The supernatants of two independently performed RG experiments were titrated on  
1257 MDCK cells and stained by crystal violet. Plaque diameters (mm) were measured and each dot  
1258 represents one viral plaque. Representative plaque assays are shown in Fig. S10A (#) not measurable  
1259 pinhead-sized plaques. (E) WSN FluPol activity was measured by vRNP reconstitution in HEK-293T  
1260 cells, using a model vRNA encoding the Firefly luciferase. Luminescence was measured and normalised  
1261 to a transfection control. The data are represented as a percentage of PA WT. (F) WSN vRNPs were  
1262 reconstituted in HEK-293T cells by transient transfection using the NA vRNA segment. The steady-  
1263 state levels of NA mRNA and vRNA were quantified by strand-specific RT-qPCR (Kawakami et al.,  
1264 2011), normalised to GAPDH by the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001) and are presented as  
1265 ratios of mRNA to vRNA levels relative to PA WT. The corresponding RNA levels are presented in  
1266 Fig. S10B. (G) WSN FluPol binding to the CTD was assessed using a split-luciferase-based  
1267 complementation assay. HEK-293T cells were co-transfected with expression plasmids for the CTD  
1268 tagged with one fragment of the *G. princeps* luciferase and FluPol tagged with the other fragment.  
1269 Luminescence due to luciferase reconstitution was measured and the data are represented as a  
1270 percentage of PA WT. (H) Accumulation levels of cRNA and vRNA in a vRNP reconstitution assay  
1271 were determined by strand-specific RT-qPCR (Kawakami et al., 2011) as in F. The corresponding RNA  
1272 levels are presented in Fig. S10B. (I) ANP32A was tagged with one fragment of the *G. princeps*  
1273 luciferase and binding to WSN FluPol was determined as in G. \* $p < 0.033$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$   
1274 (one-way ANOVA; Dunnett's multiple comparisons test).

1275 **(J)** Model of the encapsidating Zhejiang-H7N9 FluPol<sub>(E)</sub>-ANP32 interface. The model was constructed  
1276 by superposing equivalent Zhejiang-H7N9 Flu<sub>A</sub>Pol domains of 5' hook bound apo-dimer Zhejiang-  
1277 H7N9 Flu<sub>A</sub>Pol (PDB: 7ZPL, (Kouba et al., 2023)) for the FluPol<sub>(E)</sub> on those of Flu<sub>C</sub>Pol replication  
1278 complex (PDB: 6XZQ, (Carrique et al., 2020)). The ANP32A was left unchanged. The pS5 CTD  
1279 peptide mimic was added to the model by superposing the H17N10 Flu<sub>A</sub>Pol structure with bound  
1280 pS5 CTD peptide (PDB:5M3H, (Lukarska et al., 2017)) on the FluPol<sub>(E)</sub>.

1281 **(K)** A model for RNAP II CTD-anchored transcription and replication of the influenza virus genome.

1282 Upon influenza virus infection, incoming vRNPs are imported into the nucleus and bind to the host  
1283 RNA polymerase II (RNAP II) C-terminal domain (CTD) through bipartite interaction sites on the  
1284 influenza virus polymerase (FluPol) **(a)**. This intimate association allows FluPol, in the transcriptase  
1285 conformation (FluPol<sub>(T)</sub>), to cleave short capped oligomers derived from nascent RNAP II transcripts  
1286 in a process referred to as “cap-snatching” to initiate primary transcription of viral mRNAs **(b)**.  
1287 Polyadenylation is achieved by a non-canonical mechanism involving stuttering of the viral polymerase  
1288 at a polyadenylation signal **(c)**. The 5' and 3' vRNA extremities always remain bound to the polymerase  
1289 which allows efficient recycling from the termination to the initiation state **(c-a)**. Upon translation of  
1290 viral mRNAs, de novo synthesised FluPols in an apo state (not viral RNA-bound) and NPs are imported  
1291 into the nucleus **(d)**. The apo FluPol, in conjunction with the host factor ANP32, associates with the  
1292 parental CTD-associated FluPol<sub>(T)</sub> and triggers its conformational transition into a replicating FluPol<sub>(R)</sub>,  
1293 to form an asymmetric FluPol<sub>(R)</sub>- FluPol<sub>(E)</sub> dimer **(e)** where FluPol<sub>(E)</sub> is encapsidating the newly  
1294 synthesized cRNA in conjunction with NP **(f)**. The FluPol<sub>(R)</sub>- FluPol<sub>(E)</sub>-ANP32 replication complex  
1295 remains associated to the RNAP II through direct binding of the CTD to FluPol<sub>(R)</sub>, FluPol<sub>(E)</sub> as well as  
1296 ANP32 throughout synthesis of the progeny cRNP. Anchoring of the parental vRNP to the CTD allows  
1297 it to engage into successive cycles of either viral genome replication or mRNA transcription, depending  
1298 on the availability of NP, apo FluPol and/or nascent capped oligomers derived from actively  
1299 transcribing RNAP II **(g)**. Such switching between both activities allows efficient adaption to waving  
1300 levels of de novo synthesised vRNP components in the nucleus of an infected cell and is key to ensure  
1301 a correct balance between genome replication and mRNA transcription.

1302 **Table 1a. Cryo-EM structures data collection, refinement and validation statistics.**  
1303

<b>Name of structure</b>	<b>Influenza A/H7N9 polymerase in replicase-like conformation in pre-initiation state with Pol II pS5 CTD peptide mimic bound in site 1A/2A</b>	<b>Influenza A/H7N9 polymerase in pre-initiation state with continuous Pol II pS5 CTD peptide mimic bound in site 1A/2A</b>	<b>Influenza A/H7N9 polymerase in elongation state with continuous Pol II pS5 CTD peptide mimic bound in site 1A/2A</b>
PDB ID	PDB ID 8PM0	PDB ID 8PNP	PDB ID 8PNQ
EMDB ID	EMD-17755	EMD-17782	EMD-17783
<b>Data collection and processing</b>			
Microscope	ThermoFisher Krios TEM		
Voltage (kV)	300		
Camera	Gatan K3 direct electron detector mounted on a Gatan Bioquantum LS/967 energy filter		
Magnification	105000		
Nominal defocus range ( $\mu\text{m}$ )	-0.8 / -2.0		
Electron exposure ( $\text{e}^-/\text{\AA}^2$ )	40		
Number of frames collected (no.)	40		
Number of frames processed (no.)	40		
Pixel size ( $\text{\AA}$ )	0.84		
Initial micrographs (no.)	6000		
Final micrographs (no.)	5883		
<b>Refinement</b>			
Particles per class (no.)	33395	260565	14878
Map resolution ( $\text{\AA}$ ), 0.143 FSC	2.9	2.5	2.9
Model resolution ( $\text{\AA}$ ), 0.5 FSC	3.0	2.5	2.9
Map sharpening $B$ factor ( $\text{\AA}^2$ )	-20	-30	-20
Map versus model cross-correlation (CCmask)	0.8428	0.8901	0.8826
<b>Model composition</b>			
Non-hydrogen atoms	16673	14464	14917
Protein residues	1997	1716	1727
Nucleotide residues	31	29	45
Water	-	43	-

Ligands	1 Mg	-	2 Mg, 1 UpNHpp
<b>B factors (Å<sup>2</sup>)</b>			
Protein	74.00	67.24	70.02
Nucleotide	72.82	60.96	83.39
Ligand	48.38	-	68.99
Water	-	33.97	-
<b>R.M.S. deviations</b>			
Bond lengths (Å)	0.003	0.003	0.005
Bond angles (°)	0.475	0.499	0.465
<b>Validation</b>			
MolProbity score	1.53	1.35	1.40
All-atom clash score	4.79	4.32	3.86
Poor rotamers (%)	2.03	1.24	2.01
<b>Ramachandran plot</b>			
Favored (%)	97.77	97.70	98.24
Allowed (%)	2.23	2.24	1.76
Outliers (%)	0.00	0.06	0.00

1304

1305 **Table 1b. Cryo-EM structures data collection, refinement and validation statistics.**  
 1306 **K289A/C489R revertant.**  
 1307

<b>Name of structure</b>	<b>A/H7N9 K289A/C489R Apo-dimer with promoter</b>
PDB ID	PDB ID 8POH
EMDB ID	EMD-17792
<b>Data collection and processing</b>	
Microscope	ThermoFisher Glacios
Voltage (kV)	200
Camera	Falcon IV/Selectris X
Magnification	130k
Nominal defocus range (µm)	-0.8 / -2.0
Electron exposure (e <sup>-</sup> /Å <sup>2</sup> )	40
Number of fractions processed (no.)	24
Pixel size (Å)	0.907
Micrographs (no.)	5092
<b>Refinement</b>	
Particles per class (no.)	278761
Map resolution (Å), 0.143 FSC	3.26
Model resolution (Å), 0.5 FSC	3.38
Map sharpening <i>B</i> factor (Å <sup>2</sup> )	-140
Map versus model cross-correlation (CCmask)	0.7456
<b>Model composition</b>	
Non-hydrogen atoms	20729
Protein residues	2479
Nucleotide residues	38
Water	-
Ligands	1 Mg
<b><i>B</i> factors (Å<sup>2</sup>)</b>	
Protein	105.84
Nucleotide	123.86
Ligand	33.48
Water	-
<b>R.M.S. deviations</b>	
Bond lengths (Å)	0.003
Bond angles (°)	0.562
<b>Validation</b>	
MolProbity score	1.84
All-atom clash score	6.79

Poor rotamers (%)	2.37
<b>Ramachandran plot</b>	
Favored (%)	96.91
Allowed (%)	3.09
Outliers (%)	0.0

1308

1309

1310

## SUPPLEMENTARY FIGURES

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1314

### **The host RNA polymerase II C-terminal domain is the anchor**

1315

### **for replication of the influenza virus genome**

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1317

1318

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1321

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1326

1327 # Equal contributions

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1329

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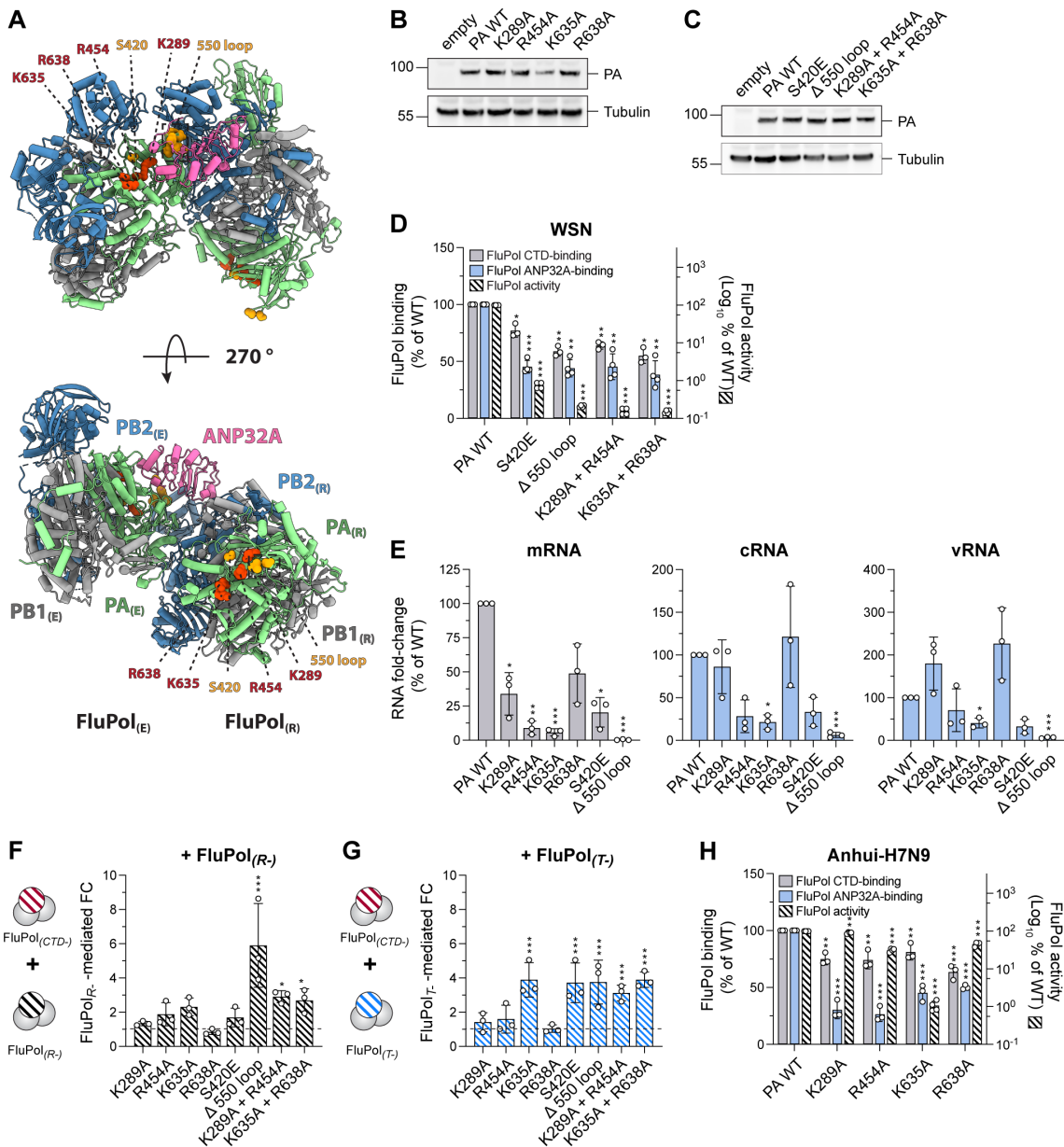
1333

1334

1335



## FIGURE S1



1336

### 1337 Figure S1: FluPol CTD-binding interface is essential for replication of the viral genome

1338 (A) Model of the Flu<sub>A</sub>Pol replication complex (FluPol<sub>(R)</sub>, FluPol<sub>(E)</sub> and ANP32A) based on a Flu<sub>C</sub>Pol  
 1339 Cryo-EM structure (C/Johannesburg/1/1966 structure, PDB: 6XZR, (Carrique et al., 2020) Ribbon  
 1340 diagram representation with PA in green, PB1 in grey, PB2 in blue and ANP32A in pink. Key FluPol  
 1341 CTD-binding residues PA K289, R454, K635 and R638 are highlighted in red and PA S420 and the PA  
 1342 550 loop in orange in either the FluPol<sub>(E)</sub> (top) or FluPol<sub>(R)</sub> (bottom) conformation.

1343 (B-C) HEK-293T cells were co-transfected with expression plasmids for WSN PB1, PB2 and PA with  
 1344 the indicated mutation. Cell lysates were analysed by western blot using antibodies specific for PA and  
 1345 tubulin. Uncropped gels are provided as a source data file.

1346 **(D)** Cell-based WSN FluPol binding and activity assays. Left Y-axis (linear scale): FluPol binding to  
1347 the CTD (grey bars) and ANP32A (blue bars) was assessed using split-luciferase-based  
1348 complementation assays as in Fig. 1C. Luminescence signals due to luciferase reconstitution are  
1349 represented as a percentage of PA WT. Right Y-axis (logarithmic scale): FluPol PA CTD-binding  
1350 mutant activity (hatched bars) was measured by vRNP reconstitution in HEK-293T cells as in Fig. 1C.  
1351 Luminescence signals are represented as a percentage of PA WT. \* $p < 0.033$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$   
1352 (one-way ANOVA; Dunnett's multiple comparisons test).

1353 **(E)** WSN vRNPs with the indicated PA mutations were reconstituted in HEK-293T cells by transient  
1354 transfection using a plasmid encoding the NA vRNA segment. Steady-state levels of NA mRNA, cRNA  
1355 and vRNA were quantified by strand-specific RT-qPCR (Kawakami et al., 2011) and are represented  
1356 as a percentage of PA WT. \* $p < 0.033$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$  (one-way repeated measure ANOVA;  
1357 Dunnett's multiple comparisons test).

1358 **(F-G):** FluPol trans-complementation assay. WSN vRNPs with the indicated PA CTD-binding  
1359 mutations were reconstituted in HEK-293T cells using a model vRNA encoding the Firefly luciferase,  
1360 and were co-expressed with (F) a replication-defective (FluPol<sub>(R-)</sub>, PA K664M) or (G) a transcription-  
1361 defective (FluPol<sub>(T-)</sub>, PA D108A, (Dias et al., 2009)) FluPol mutant. Luminescence was measured at 48  
1362 hpt and signals were normalised to a transfection control, and are represented as the fold-change (FC)  
1363 relative to the background. \* $p < 0.033$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$  (two-way ANOVA; Sidak's multiple  
1364 comparisons test).

1365 **(H)** Cell-based Anhui-H7N9 FluPol binding and activity assays using a plasmid encoding PA in which  
1366 the PA-X (PA-ΔX) ORF frame was deleted as described previously (Jagger et al., 2012). Left Y-axis  
1367 (linear scale): FluPol binding to the CTD (grey bars) and ANP32A (blue bars) was assessed using split-  
1368 luciferase-based complementation assays as in Fig. 1C. Luminescence signals due to luciferase  
1369 reconstitution are represented as a percentage of PA WT. Right Y-axis (logarithmic scale): FluPol PA  
1370 CTD-binding mutant activity (hatched bars) was measured by vRNP reconstitution in HEK-293T cells  
1371 as in Fig. 1C. Luminescence signals are represented as a percentage of PA WT. \*\* $p < 0.002$ , \*\*\* $p <$   
1372  $0.001$  (one-way ANOVA; Dunnett's multiple comparisons test).

## FIGURE S2

### PB2 Alignment

```
1 10 20 30 40 50 60 70 80
PB2-Anhui-H7N9 MERRIKELRDLMSQSRTREILTKTTVDHMAIIKKYTSGRQENKPNALRKMKMMAMKYPITADKRLMEMIERNRQOCTLWSK
PB2-Zhejiang-H7N9 MERRIKELRDLMSQSRTREILTKTTVDHMAIIKKYTSGRQENKPNALRKMKMMAMKYPITADKRLMEMIERNRQOCTLWSK
PB2-WSN MERRIKELRDLMSQSRTREILTKTTVDHMAIIKKYTSGRQENKPNALRKMKMMAMKYPITADKRLMEMIERNRQOCTLWSK

90 100 110 120 130 140 150 160
PB2-Anhui-H7N9 TNDAGSDRVMVSPPLAVTWNNRNGPTTSTVHYPKVYKTYFEKVERLKHGTFGPFVHFRNQVIRRRVDINPFGHADLSAKEAQ
PB2-Zhejiang-H7N9 TNDAGSDRVMVSPPLAVTWNNRNGPTTSTVHYPKVYKTYFEKVERLKHGTFGPFVHFRNQVIRRRVDINPFGHADLSAKEAQ
PB2-WSN TNDAGSDRVMVSPPLAVTWNNRNGPTTSTVHYPKVYKTYFEKVERLKHGTFGPFVHFRNQVIRRRVDINPFGHADLSAKEAQ

170 180 190 200 210 220 230 240
PB2-Anhui-H7N9 DVIMEVVPNEVGARILTSSESQLTITKEKKKELQDCKTAPLMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTCTW
PB2-Zhejiang-H7N9 DVIMEVVPNEVGARILTSSESQLTITKEKKKELQDCKTAPLMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTCTW
PB2-WSN DVIMEVVPNEVGARILTSSESQLTITKEKKKELQDCKTAPLMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTCTW

250 260 270 280 290 300 310 320
PB2-Anhui-H7N9 EQMYTPGGEVRRNDVDVQSLIIAARNIVRRATVSADPLASLLEMCHSTQIGGVRRMVDILRQNPTEEQAVDICKAAMGLRIS
PB2-Zhejiang-H7N9 EQMYTPGGEVRRNDVDVQSLIIAARNIVRRATVSADPLASLLEMCHSTQIGGVRRMVDILRQNPTEEQAVDICKAAMGLRIS
PB2-WSN EQMYTPGGEVRRNDVDVQSLIIAARNIVRRATVSADPLASLLEMCHSTQIGGVRRMVDILRQNPTEEQAVDICKAAMGLRIS

330 340 350 360 370 380 390 400
PB2-Anhui-H7N9 SSFSFGGFTFKRTSGSSVKREEEVLGNLQTLKIRVHEGYEFTMVGRRAATAIRKATRRLIQLIVSGKDEQSIAEAIIV
PB2-Zhejiang-H7N9 SSFSFGGFTFKRTSGSSVKREEEVLGNLQTLKIRVHEGYEFTMVGRRAATAIRKATRRLIQLIVSGKDEQSIAEAIIV
PB2-WSN SSFSFGGFTFKRTSGSSVKREEEVLGNLQTLKIRVHEGYEFTMVGRRAATAIRKATRRLIQLIVSGKDEQSIAEAIIV

410 420 430 440 450 460 470 480
PB2-Anhui-H7N9 AMVPSQEDCMIKAVRGDLNFRANRQRLNPMHQLLRHFQKDAKALPQNGIEPIDNNVMGIGILPDMPPSEMSLRGVRV
PB2-Zhejiang-H7N9 AMVPSQEDCMIKAVRGDLNFRANRQRLNPMHQLLRHFQKDAKALPQNGIEPIDNNVMGIGILPDMPPSEMSLRGVRV
PB2-WSN AMVPSQEDCMIKAVRGDLNFRANRQRLNPMHQLLRHFQKDAKALPQNGIEPIDNNVMGIGILPDMPPSEMSLRGVRV

490 500 510 520 530 540 550 560
PB2-Anhui-H7N9 SKMGVDBIESRTRVUVVSDRFLRVRDQRGNVLLSPEEVSETQGERLITTYSSSMWEINGPESVLVNTYQWIRRNHW
PB2-Zhejiang-H7N9 SKMGVDBIESRTRVUVVSDRFLRVRDQRGNVLLSPEEVSETQGERLITTYSSSMWEINGPESVLVNTYQWIRRNHW
PB2-WSN SKMGVDBIESRTRVUVVSDRFLRVRDQRGNVLLSPEEVSETQGERLITTYSSSMWEINGPESVLVNTYQWIRRNHW

570 580 590 600 610 620 630 640
PB2-Anhui-H7N9 RIQWSQDPTMLYNKMEFPPQSLVPKAARGQISGFVRVLFQOMRDVLDGTFDVIQIKLLPFAAAPKQSRMOPSSSLVNV
PB2-Zhejiang-H7N9 RIQWSQDPTMLYNKMEFPPQSLVPKAARGQISGFVRVLFQOMRDVLDGTFDVIQIKLLPFAAAPKQSRMOPSSSLVNV
PB2-WSN RIQWSQDPTMLYNKMEFPPQSLVPKAARGQISGFVRVLFQOMRDVLDGTFDVIQIKLLPFAAAPKQSRMOPSSSLVNV

650 660 670 680 690 700 710 720
PB2-Anhui-H7N9 RGSQMRILVVRGNSPFFNYNRAKTRRLTVLKGDAAGTMDPDEGTAGVESAVLRGFLILGKEDRKYGPALSINELSNLAKGE
PB2-Zhejiang-H7N9 RGSQMRILVVRGNSPFFNYNRAKTRRLTVLKGDAAGTMDPDEGTAGVESAVLRGFLILGKEDRKYGPALSINELSNLAKGE
PB2-WSN RGSQMRILVVRGNSPFFNYNRAKTRRLTVLKGDAAGTMDPDEGTAGVESAVLRGFLILGKEDRKYGPALSINELSNLAKGE

730 740 750
PB2-Anhui-H7N9 KANVLIGQGDVVLVMMKRRRDSILLTDSQATKRRIRMAIN
PB2-Zhejiang-H7N9 KANVLIGQGDVVLVMMKRRRDSILLTDSQATKRRIRMAIN
PB2-WSN KANVLIGQGDVVLVMMKRRRDSILLTDSQATKRRIRMAIN
```

### PB1 Alignment

```
1 10 20 30 40 50 60 70 80
PB1-Anhui-H7N9 MDVNPFLILKVPVQNAISTTFYTGDPYSHGTGTGTYMDTVNRTHYSEKGRMTNTTETGAPQNLNIDGFLPEDNEPS
PB1-Zhejiang-H7N9 MDVNPFLILKVPVQNAISTTFYTGDPYSHGTGTGTYMDTVNRTHYSEKGRMTNTTETGAPQNLNIDGFLPEDNEPS
PB1-WSN MDVNPFLILKVPVQNAISTTFYTGDPYSHGTGTGTYMDTVNRTHYSEKGRMTNTTETGAPQNLNIDGFLPEDNEPS

90 100 110 120 130 140 150 160
PB1-Anhui-H7N9 GYAQTDCVLEAMAFLEESHGPIFENSCLETMEIVQOTRVDKLTQGRQTYDWTLLNRNQPAAATALANIEVFRSNGLTANES
PB1-Zhejiang-H7N9 GYAQTDCVLEAMAFLEESHGPIFENSCLETMEIVQOTRVDKLTQGRQTYDWTLLNRNQPAAATALANIEVFRSNGLTANES
PB1-WSN GYAQTDCVLEAMAFLEESHGPIFENSCLETMEIVQOTRVDKLTQGRQTYDWTLLNRNQPAAATALANIEVFRSNGLTANES

170 180 190 200 210 220 230 240
PB1-Anhui-H7N9 GRLLDFLKDVMDSMKKEEMEITTHFORRRVRDNNMTKMMVTQRTIGKKQRNLNRSYLIRALTLNMTKDAERGLKRRRA
PB1-Zhejiang-H7N9 GRLLDFLKDVMDSMKKEEMEITTHFORRRVRDNNMTKMMVTQRTIGKKQRNLNRSYLIRALTLNMTKDAERGLKRRRA
PB1-WSN GRLLDFLKDVMDSMKKEEMEITTHFORRRVRDNNMTKMMVTQRTIGKKQRNLNRSYLIRALTLNMTKDAERGLKRRRA

250 260 270 280 290 300 310 320
PB1-Anhui-H7N9 IATPGMQIRGFVYFVEALARSICEKLEQSGPLVGGNEKKAKLANVVRKMMTNSQDTEISFTITGDNTKWNENQNPFRMFLA
PB1-Zhejiang-H7N9 IATPGMQIRGFVYFVEALARSICEKLEQSGPLVGGNEKKAKLANVVRKMMTNSQDTEISFTITGDNTKWNENQNPFRMFLA
PB1-WSN IATPGMQIRGFVYFVEALARSICEKLEQSGPLVGGNEKKAKLANVVRKMMTNSQDTEISFTITGDNTKWNENQNPFRMFLA

330 340 350 360 370 380 390 400
PB1-Anhui-H7N9 MITYTRNOPEWFRNVLISIAPIFMSNKMARLKGVMFESKSMKLRTOVPAEMLANIDLKYFNKSTRBKIEKIRPLLDIGT
PB1-Zhejiang-H7N9 MITYTRNOPEWFRNVLISIAPIFMSNKMARLKGVMFESKSMKLRTOVPAEMLANIDLKYFNKSTRBKIEKIRPLLDIGT
PB1-WSN MITYTRNOPEWFRNVLISIAPIFMSNKMARLKGVMFESKSMKLRTOVPAEMLASIDLKYFNKSTRBKIEKIRPLLDIGT

410 420 430 440 450 460 470 480
PB1-Anhui-H7N9 ASLSPGMMGMFNMLSTVLGVSILNLGQRKFKTTYWWDGLQSSDDFALIVNAPNHEGIAQVDRFYRTRCKLVGINNSKK
PB1-Zhejiang-H7N9 ASLSPGMMGMFNMLSTVLGVSILNLGQRKFKTTYWWDGLQSSDDFALIVNAPNHEGIAQVDRFYRTRCKLVGINNSKK
PB1-WSN ASLSPGMMGMFNMLSTVLGVSILNLGQRKFKTTYWWDGLQSSDDFALIVNAPNHEGIAQVDRFYRTRCKLVGINNSKK

490 500 510 520 530 540 550 560
PB1-Anhui-H7N9 KSYINRTGTFEFTSFFRYRQGVANFMSLELSPGVSGINEADMSVGVTVIKNNMINNDLGPATAQMALQLFKDYRYTYR
PB1-Zhejiang-H7N9 KSYINRTGTFEFTSFFRYRQGVANFMSLELSPGVSGINEADMSVGVTVIKNNMINNDLGPATAQMALQLFKDYRYTYR
PB1-WSN KSYINRTGTFEFTSFFRYRQGVANFMSLELSPGVSGINEADMSVGVTVIKNNMINNDLGPATAQMALQLFKDYRYTYR

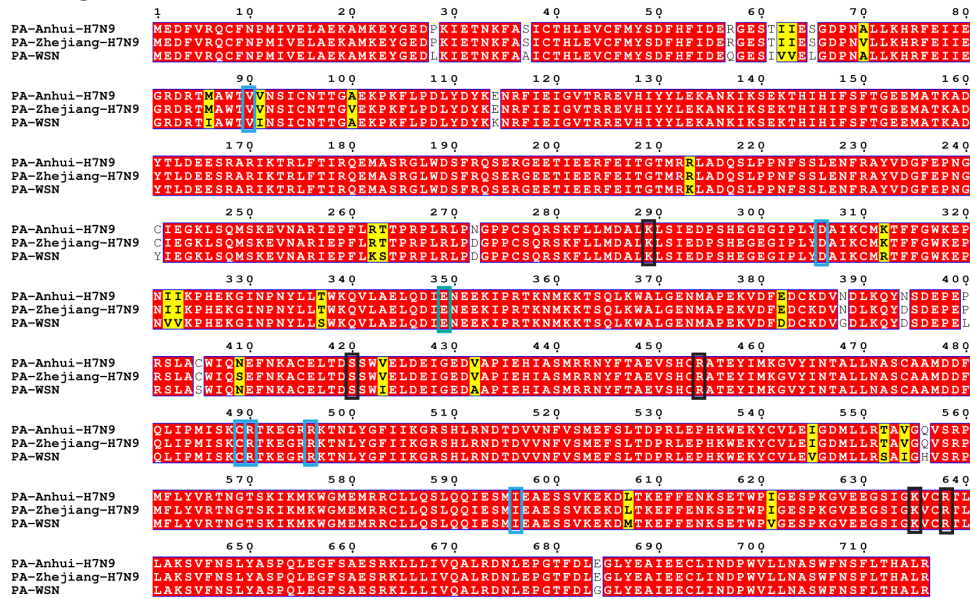
570 580 590 600 610 620 630 640
PB1-Anhui-H7N9 CHRGDQIQTRRAPELKLWEOQRKSKAGLLVSDGGPNLYIRNLHIFEVCLKWELMDEYQGRCLNPNPFFVSHKEDISV
PB1-Zhejiang-H7N9 CHRGDQIQTRRAPELKLWEOQRKSKAGLLVSDGGPNLYIRNLHIFEVCLKWELMDEYQGRCLNPNPFFVSHKEDISV
PB1-WSN CHRGDQIQTRRAPELKLWEOQRKSKAGLLVSDGGPNLYIRNLHIFEVCLKWELMDEYQGRCLNPNPFFVSHKEDISV

650 660 670 680 690 700 710 720
PB1-Anhui-H7N9 NNAVMPAHGPAKSMEDYDAVATTHSWIPKRRNSILNTSQRGILEDEQMYQKCCNLFKFFPSSSYRRPVGISSMVEAMVS
PB1-Zhejiang-H7N9 NNAVMPAHGPAKSMEDYDAVATTHSWIPKRRNSILNTSQRGILEDEQMYQKCCNLFKFFPSSSYRRPVGISSMVEAMVS
PB1-WSN NNAVMPAHGPAKSMEDYDAVATTHSWIPKRRNSILNTSQRGILEDEQMYQKCCNLFKFFPSSSYRRPVGISSMVEAMVS

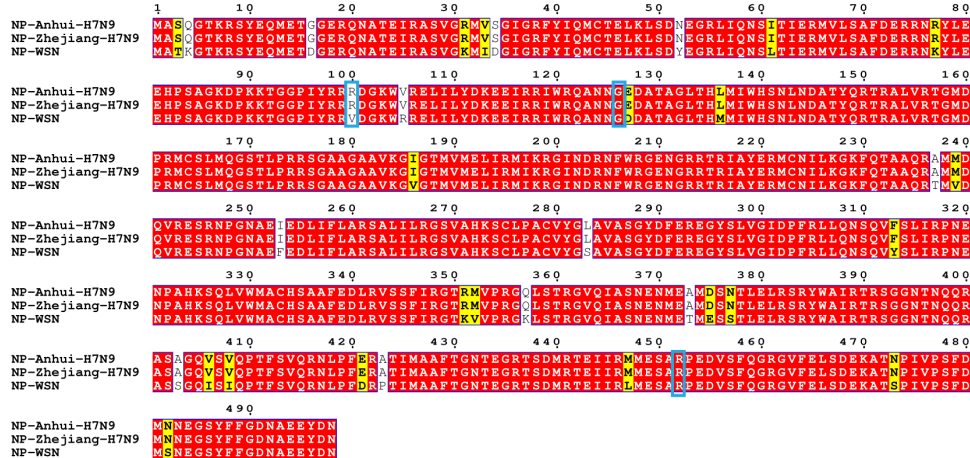
730 740 750
PB1-Anhui-H7N9 RARIDARIDFESGRIKKEEFAEIMKICSTIEELRRQK
PB1-Zhejiang-H7N9 RARIDARIDFESGRIKKEEFAEIMKICSTIEELRRQK
PB1-WSN RARIDARIDFESGRIKKEEFAEIMKICSTIEELRRQK
```

## FIGURE S2 (continued)

### PA Alignment



### NP Alignment



■ CTD-binding residues

■ second-site mutations

■ 4M mutations

1374

1375

1376 **Figure S2: PB2, PB1, PA and NP protein sequence alignments of the influenza A virus strains**  
 1377 **used in this study**

1378 Protein sequences from A/WSN1933(H1N1), A/Anhui/01/2013 (H7N9) and A/Zhejiang/DTID-

1379 ZJU01/2013(H7N9) were obtained from UniProt (<https://www.uniprot.org/>), aligned with CLC Main

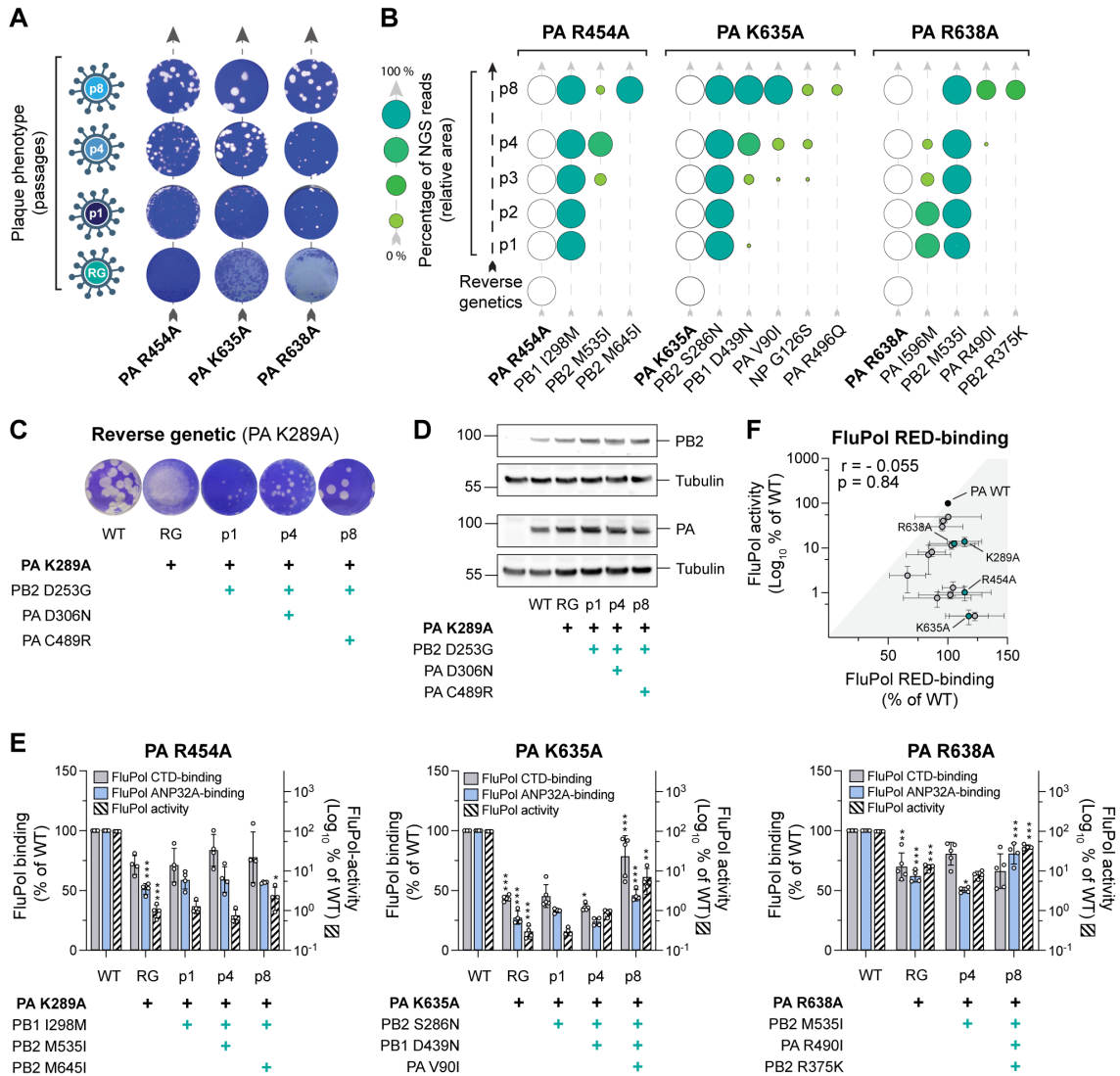
1380 Workbench 21 and visualised by Esprict 3.0 [55]. Identical and similar residues are indicated in red or

1381 yellow, respectively. FluPol CTD binding subjected to mutagenesis are indicated by grey boxes, FluPol

1382 second-site mutations which appeared upon passaging of FluPol CTD-binding mutant viruses by blue

1383 boxes and dimer interrupting FluPol 4M residues (PB2 G74, PA E349, PB1 K577) by green boxes.

## FIGURE S3



1384

1385 **Figure S3: Serial passaging of mutant viruses with mutations at the FluPol-CTD interface selects**  
 1386 **for adaptive mutations which restore FluPol binding to the CTD and ANP32A**

1387 **(A)** Plaque phenotypes of passaged WSN PA R454A, K635A, R638A mutant viruses. Representative  
 1388 images of crystal violet stained plaque assays of the supernatants collected after reverse genetics (RG),  
 1389 p1, p4 and p8 are shown.

1390 **(B)** The viral genomes of passaged WSN PA R454A, K635A, R638A mutant viruses were sequenced  
 1391 by short-read NGS after p1 - p4 and p8 (from bottom to top). Initial FluPol CTD-binding mutation  
 1392 (white circles) and second site mutations on PB1, PB2, PA and NP (green circles) are indicated. Only  
 1393 second-site mutations found in  $\geq 10\%$  of reads in at least one passage are shown. The fraction of reads  
 1394 showing a given mutation is indicated by the area of the circle, and by different shades of green that  
 1395 represent  $<25$ , 25-50, 50-75 and  $>75$  % of reads (schematic on the left).

1396 **(C)** Characterisation of recombinant WSN mutant viruses. Recombinant viruses with the primary

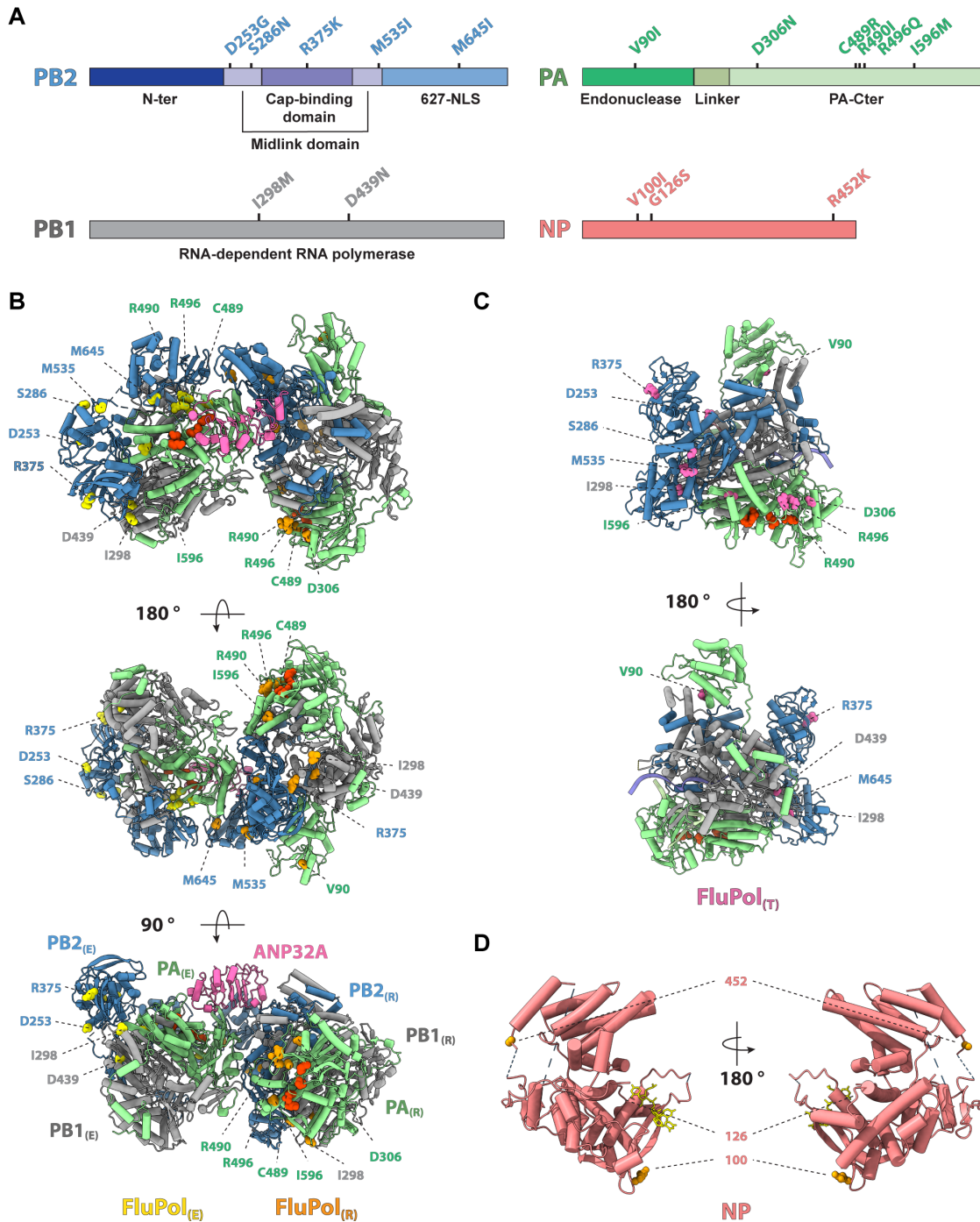
1397 FluPol mutation PA K289A and the indicated combinations of FluPol second-site mutations (PB2  
1398 D253G, PA D306N, PA C489R) were generated by reverse genetics and subjected to plaque assay.  
1399 Representative images of plaque assays stained by crystal violet are shown.

1400 **(D)** HEK-293T cells were co-transfected with expression plasmids for WSN PB1, PB2 and PA with the  
1401 indicated mutation. Cells lysates were analysed by western blot using antibodies specific for PB2, PA  
1402 and tubulin. Uncropped gels are provided as a source data file.

1403 **(E)** The indicated combinations of WSN FluPol second-site mutations which occurred during passaging  
1404 of PA R454A, K635A and R638A mutant viruses were characterised in cell-based FluPol binding and  
1405 activity assays. Left Y-axis (linear scale): FluPol binding to the CTD (grey bars) and ANP32A (blue  
1406 bars) was assessed using split-luciferase-based complementation assays as in Fig. 1C. Luminescence  
1407 signals due to luciferase reconstitution are represented as a percentage of FluPol WT. Right Y-axis  
1408 (logarithmic scale): FluPol activity (hatched bars) was measured by vRNP reconstitution in HEK-293T  
1409 cells as in Fig. 1C. Luminescence signals are represented as a percentage of FluPol WT. Stars indicate  
1410 statistical significance compared to the previous passage. \* $p < 0.033$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$  (one-  
1411 way ANOVA; Tukey's multiple comparisons test).

1412 **(F)** For each initial FluPol mutant (PA K289A, R454A, K635A, R638A, green dots) and FluPol  
1413 genotype observed during serial passaging (grey dots), FluPol binding to RED (x-axis) was plotted  
1414 against the FluPol activity as measured in a vRNP reconstitution assay (y-axis). (Pearson Correlation  
1415 Coefficient ( $r$ ))

## FIGURE S4



1416

1417 **Figure S4: FluPol and NP second-site mutations which were selected during serial cell culture**  
 1418 **passaging of PA mutant viruses**

1419 (A) Second-site mutations which were selected on FluPol and NP during serial passaging of  
 1420 recombinant plaque-purified PA mutant (K289A, R454A, K635A, R638A) viruses were mapped on a  
 1421 linear domain representation of PB2, PB1, PA and NP. Only second-site mutations found in  $\geq 10\%$  of  
 1422 reads in at least one passage are shown.

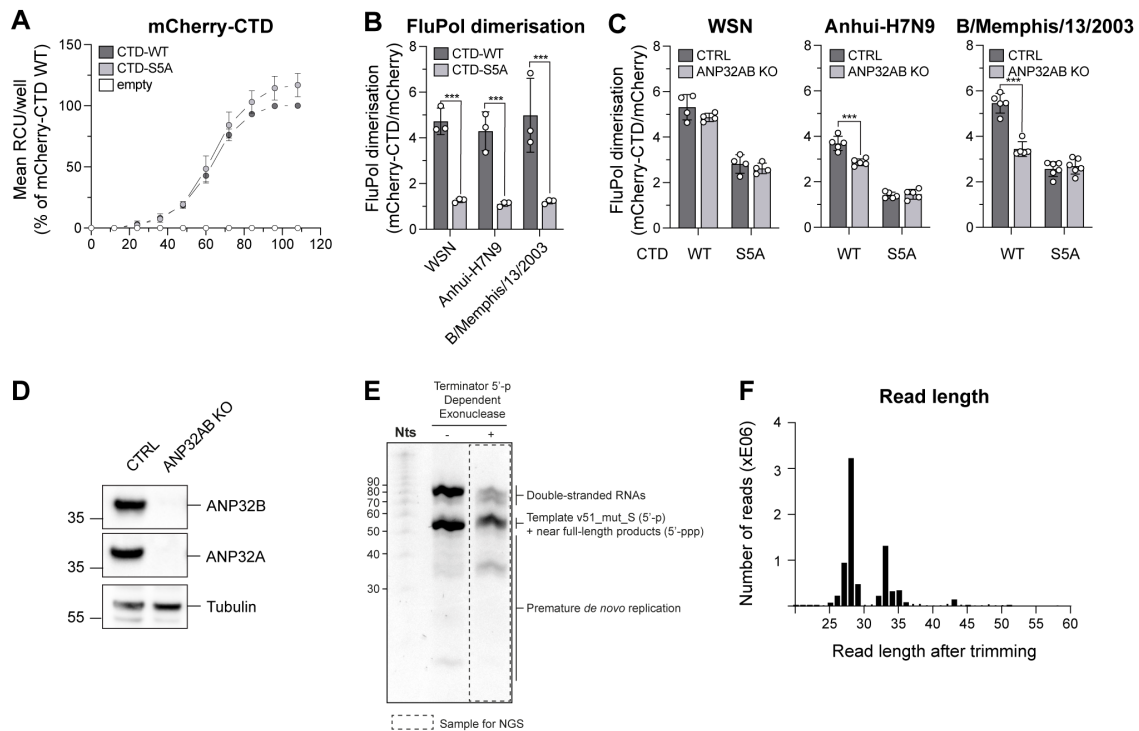
1423 **(B)** Model of the Flu<sub>A</sub>Pol replication complex (FluPol<sub>(R)</sub>, FluPol<sub>(E)</sub> and ANP32A) based on a Flu<sub>C</sub>Pol  
1424 Cryo-EM structure (C/Johannesburg/1/1966 structure, PDB: 6XZR, (Carrique et al., 2020)). Ribbon  
1425 diagram representation with PA in green, PB1 in grey, PB2 in blue and ANP32A in pink. Initially  
1426 mutated FluPol PA residues (PA K289A, R454A, K635A and R638A) are highlighted in red and  
1427 residues corresponding to FluPol second-site mutations were mapped on the FluPol<sub>(R)</sub> in orange and  
1428 FluPol<sub>(E)</sub> in yellow, respectively.

1429 **(C)** Representation of the Cryo-EM structure of the FluPol heterotrimer in the FluPol<sub>(T)</sub> conformation,  
1430 bound to a 3'5' vRNA promoter and a short capped RNA primer (A/NT/60/1968, PDB: 6RR7, (Fan et  
1431 al., 2019)). Ribbon diagram representation with PA in green, PB1 in grey, and PB2 in blue. Initially  
1432 mutated FluPol PA residues (PA K289A, R454A, K635A and R638A) are highlighted in red and  
1433 residues corresponding to FluPol second-site mutations in pink.

1434 **(D)** Residues corresponding to NP second-site mutations were mapped on the RNA-bound crystal  
1435 structure of a monomeric NP mutant (PDB: 7DXP, (Tang et al., 2021)). Ribbon diagram representation  
1436 with NP in salmon, RNA in yellow and residues corresponding to NP second-site mutations in orange.



## FIGURE S5



1437

1438 **Figure S5: FluPol replication activity is enhanced in the presence of CTD and ANP32A**

1439 **(A)** HEK-293T cells were transiently transfected with expression plasmids encoding for mCherry-CTD-  
1440 WT, mCherry-CTD-S5A or an empty plasmid control. Real-time monitoring of fluorescence was  
1441 performed by the acquisition of 5 images with a 10X objective per well every 12 h up to 108 hpt. Mean  
1442 fluorescence per well (RCU: Red Calibrated Units) is expressed as percentages of mCherry-CTD-WT  
1443 at 108 hpt.

1444 **(B)** WSN, Anhui-H7N9, B/Memphis/13/2003 FluPol dimerisation was assessed in a split-luciferase-  
1445 based complementation assay as described previously (Chen et al., 2019). Untagged mCherry or  
1446 mCherry tagged to CTD-WT or CTD-S5A in which all serine 5 residues were replaced with alanines  
1447 were co-expressed. Luminescence due to luciferase reconstitution was measured and the signals are  
1448 represented as a percentage of untagged mCherry co-expression. \*\*\* $p < 0.001$  (two-way ANOVA;  
1449 Sidak's multiple comparisons test).

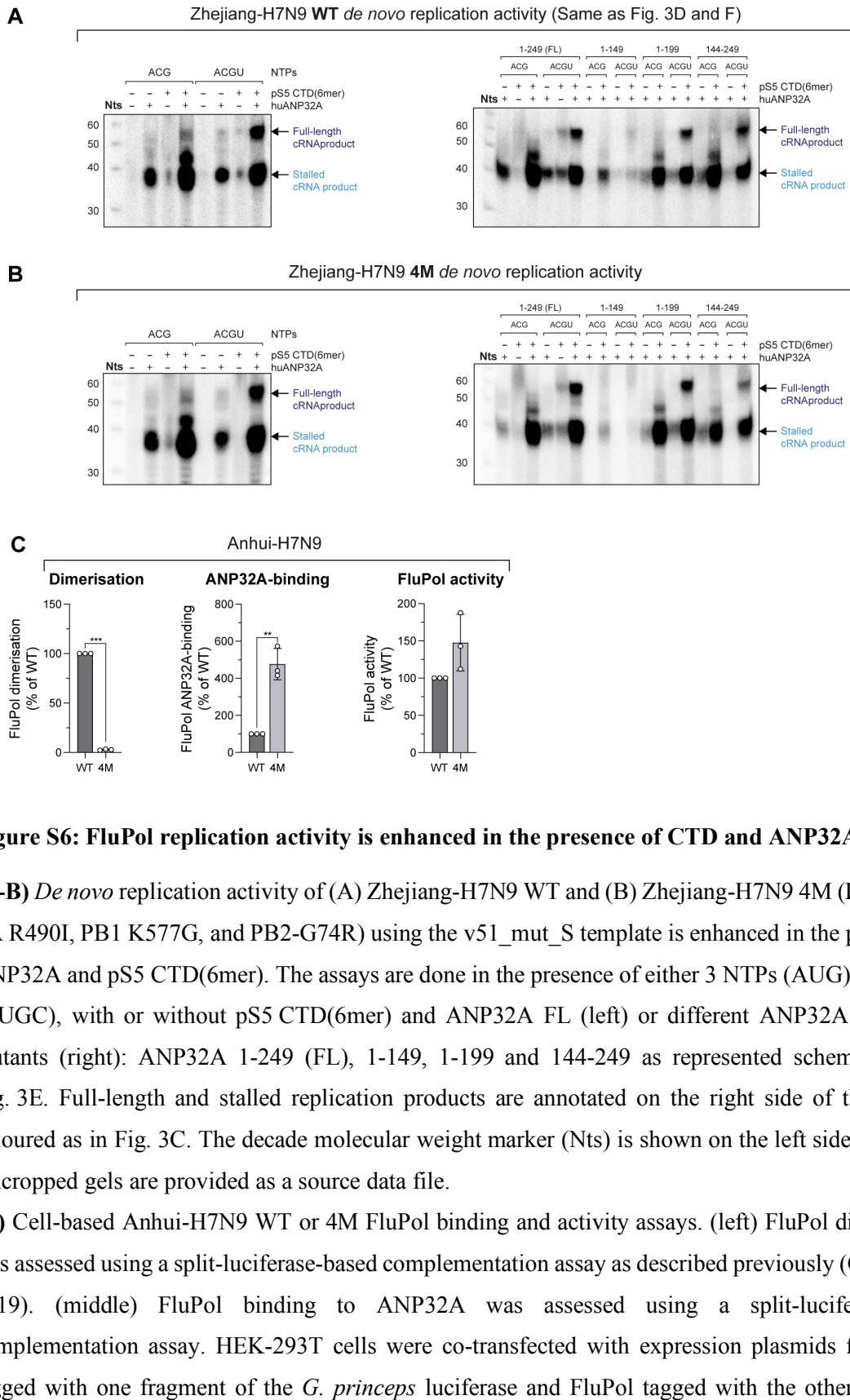
1450 **(C)** FluPol dimerisation levels of WSN, Anhui-H7N9 and B/Memphis/13/2003 were assessed as  
1451 described in (B) either in HEK-293T control (CTRL) or in HEK-293T cells in which ANP32A and  
1452 ANP32B were knocked out by CRISPR-Cas9 (ANP32AB KO). \*\*\* $p < 0.001$  (two-way ANOVA;  
1453 Sidak's multiple comparisons test).

1454 **(D)** HEK-293T CTRL and ANP32AB KO cell lysates were analysed by western blot using antibodies  
1455 specific for ANP32A, ANP32B and tubulin. Uncropped gels are provided as a source data file.

1456 **(E)** NGS sample preparation of FluPol Zheijiang-H7N9 4M *de novo* replication products. Urea-PAGE

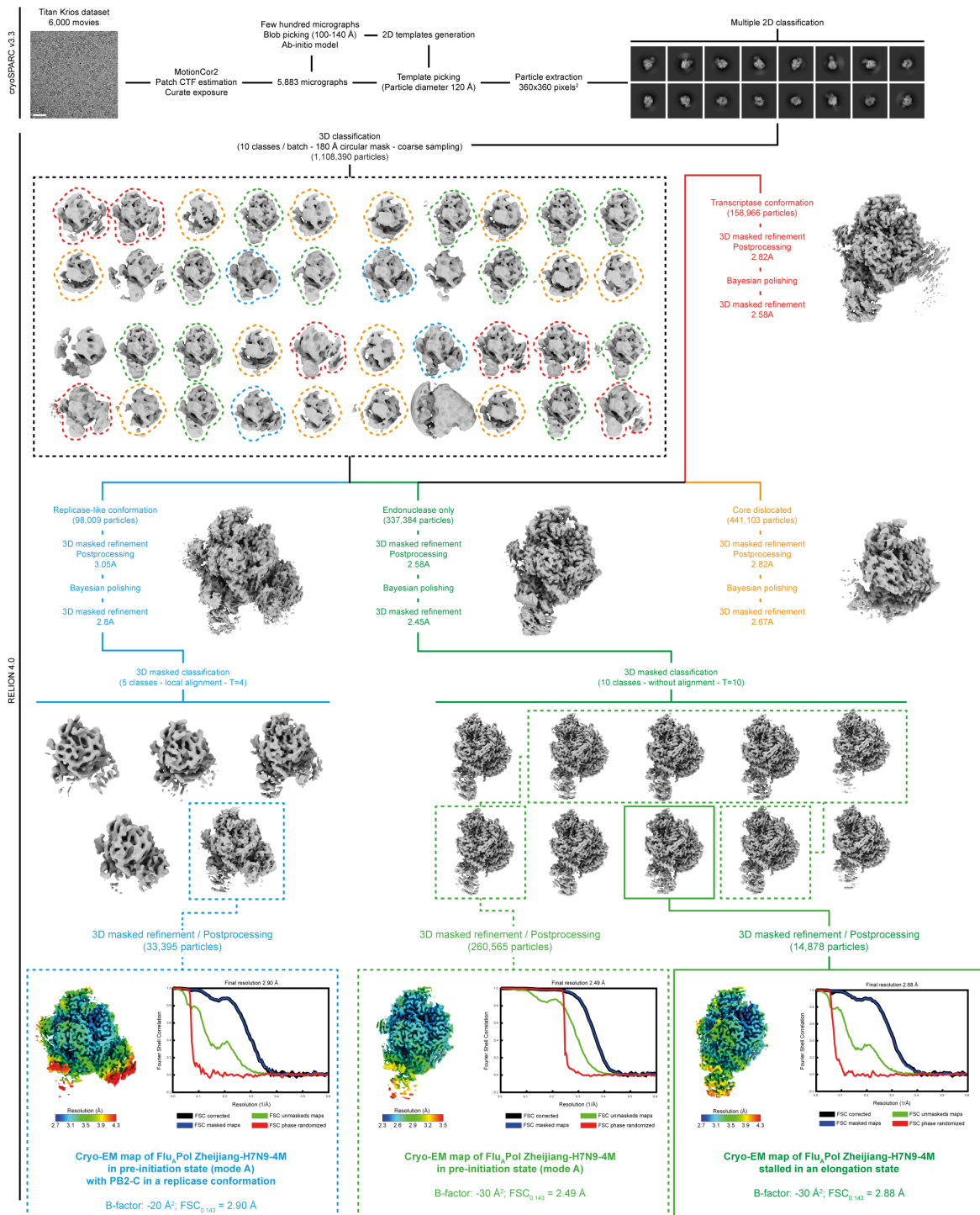
1457 gel stained with SYBR-Gold of the *de novo* reaction (Fig. 3 lane 8) before (-) and after (+) 5'-  
1458 monophosphate (5'-p) RNA digestion using a terminator 5'-p-dependent exonuclease. The dotted  
1459 rectangle corresponds to the sample sent for NGS. The decade molecular weight marker (Nts) is shown  
1460 on the left side of the gel. The uncropped gel is provided as a source data file.  
1461 **(F)** RNA-sequencing analysis of FluPol Zhejiang-H7N9 4M *de novo* replication products in the  
1462 presence of pS5 CTD(6mer) and ANP32A. After trimming, the number of reads are plotted according  
1463 to their lengths. NGS data are provided in source data table 1.

## FIGURE S6



1479 Luminescence due to luciferase reconstitution was measured and the signals are represented as a  
1480 percentage of PA WT. (right) FluPol activity was measured by vRNP reconstitution in HEK-293T cells,  
1481 using a model vRNA encoding the Firefly luciferase. Luminescence was measured and normalised to a  
1482 transfection control. The data are represented as a percentage of PA WT. \*\*p < 0.002, \*\*\*p < 0.001  
1483 (one-way ANOVA; Dunnett's multiple comparisons test).

## FIGURE S7



1484

1485 **Figure S7: Image processing FluPol Zhejiang-H7N9-4M *de novo* replication reaction (Krios)**

1486 Schematics of the image processing strategy used to obtain (i) the Cryo-EM map of FluPol Zhejiang-

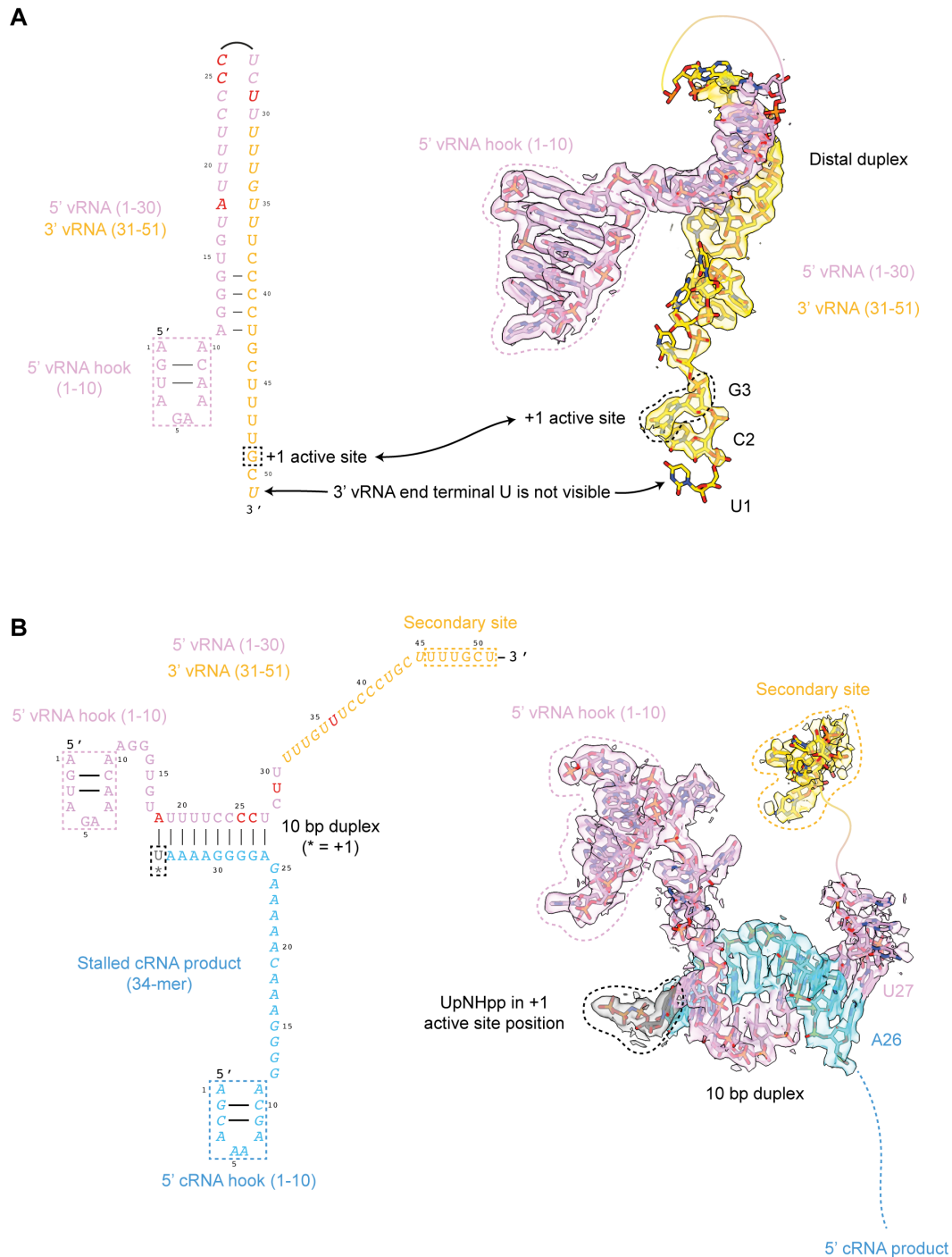
1487 H7N9-4M in a pre-initiation state (mode A), with PB2-C in a replicase conformation, (ii) the cryo-EM

1488 map of FluPol Zhejiang-H7N9-4M in pre-initiation state (mode A) without PB2-C, and (iii) the cryo-

1489 EM map of FluPol Zhejiang-H7N9-4M stalled in an elongation state using UpNHpp. Further processing

1490 steps for the transcriptase conformation and dislocated FluPols are not shown. Representative cropped  
1491 micrograph from the TEM Titan Krios dataset, 2D class averages, 3D class averages and intermediate  
1492 structures are displayed. CTF stands for “Contrast Transfer Function”. The final number of particles,  
1493 filtered EM maps according to the local resolution and corresponding Fourier Shell Correlation curves  
1494 (FSC) are shown. Scale bar = 200 Å.

## FIGURE S8



1495

1496 **Figure S8: RNA conformation schematics, densities and models of the different states obtained**

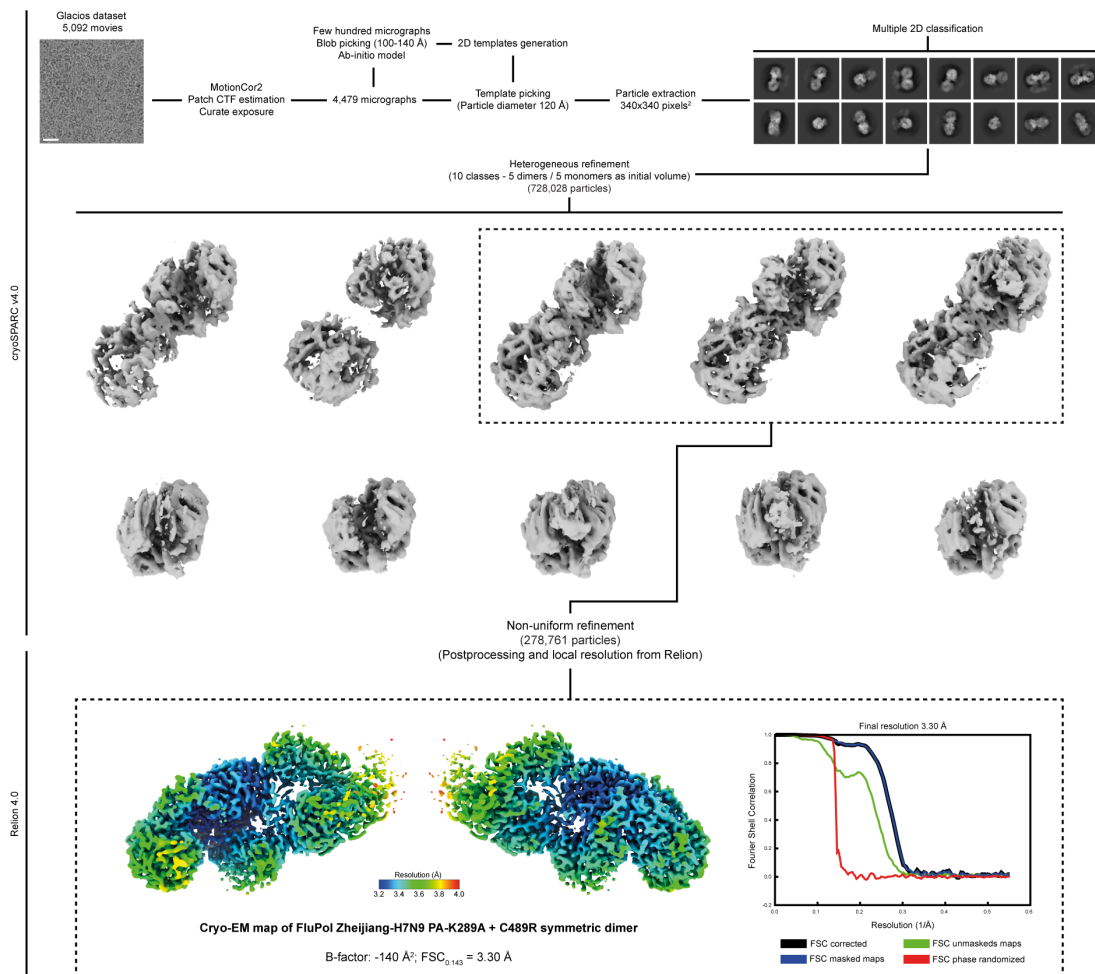
1497 **(A)** (Left) Schematics of the v51\_mut\_S template conformation present in both (i) the cryo-EM map of  
 1498 FluPol Zhejiang-H7N9-4M in pre-initiation state (mode A) without PB2-C or (ii) with PB2-C in  
 1499 replicase conformation. The 5' vRNA end (1-30) is coloured in pink. The 3' vRNA end (31-51) is  
 1500 coloured in gold. Introduced mutations are coloured in red. Unseen nucleotides are in italics. The +1

1501 active site position is indicated by a dotted rectangle. (Right) Coulomb potential of the v51\_mut\_S  
1502 isolated from the cryo-EM map of FluPol Zhejiang-H7N9 4M in pre-initiation state (mode A) without  
1503 PB2-C, at an overall resolution of 2.5 Å. 3'-U1 is displayed but not visible in the cryo-EM map. 3'-G3  
1504 is in the +1 active site position.

1505 **(B)** (Left) Schematics of the v51\_mut\_S template and the *de novo* cRNA replication product (34-mer)  
1506 conformations present in the cryo-EM map of FluPol Zhejiang-H7N9-4M stalled in an elongation state  
1507 using UpNHpp. The template is coloured as in A. The *de novo* cRNA product is coloured in blue.  
1508 Unseen nucleotides are in italics. The +1 active site position is indicated. (Right) Coulomb potential of  
1509 the v51\_mut\_S template and the *de novo* cRNA replication product (34-mer) isolated from the cryo-  
1510 EM map of FluPol Zhejiang-H7N9-4M stalled in an elongation state using UpNHpp, at an overall  
1511 resolution of 2.9 Å. The non-hydrolysable UpNHpp is in the +1 active site position, coloured in dark  
1512 grey and circled by a dotted line. Flexible nucleotides are shown as dotted lines.



## FIGURE S9

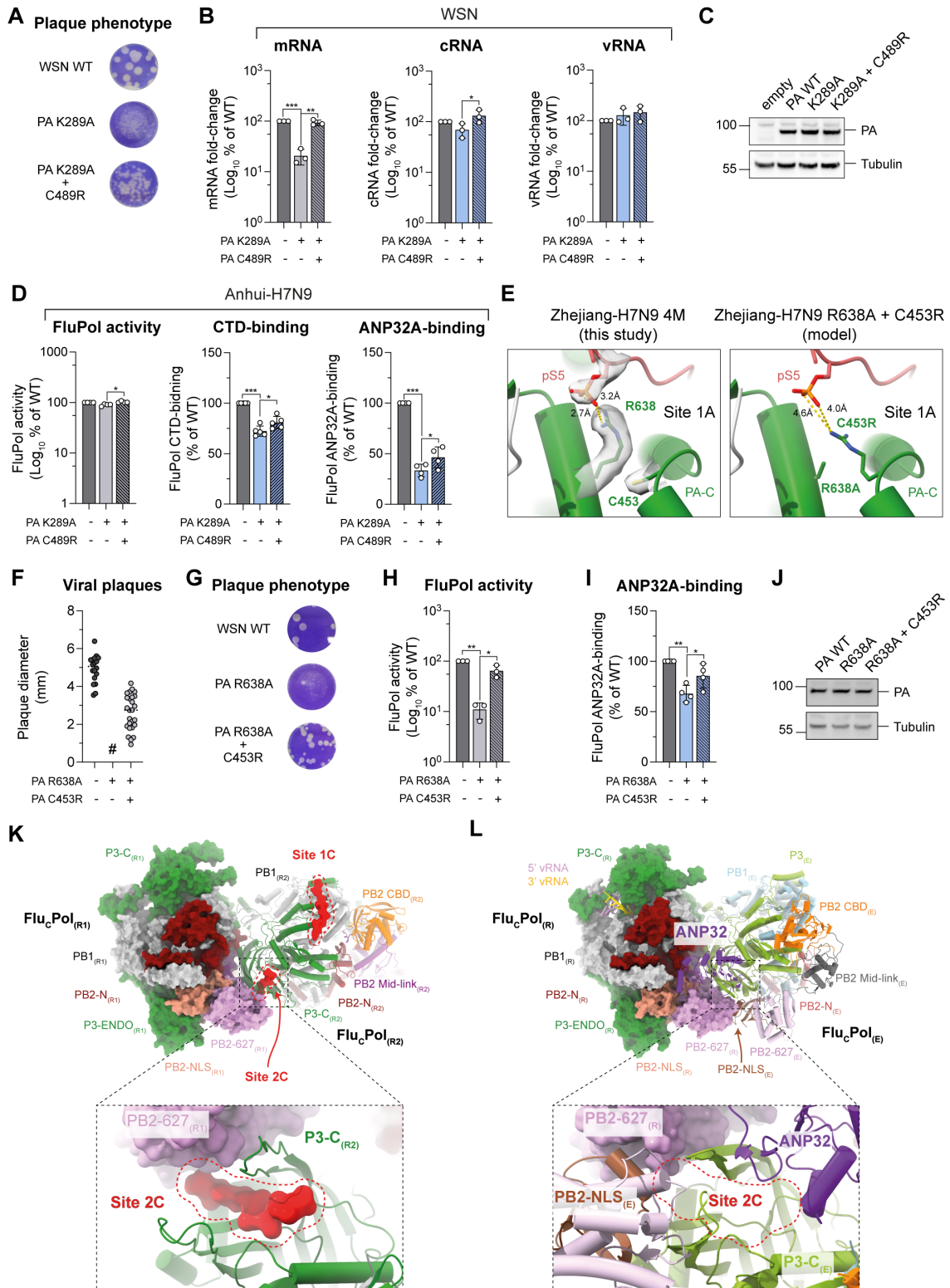


1513

### 1514 **Figure S9: Image processing FluPol Zhejiang-H7N9 PA K289A+C489R (Glacios)**

1515 Schematics of the image processing strategy used to obtain the cryo-EM map of FluPol Zhejiang-H7N9  
 1516 PA K289A+C489R. Representative cropped micrograph from the TEM Glacios dataset, 2D class  
 1517 averages, 3D class averages and intermediate structures are displayed. CTF stands for “Contrast  
 1518 Transfer Function”. The final number of particles, filtered EM maps according to the local resolution  
 1519 and corresponding Fourier Shell Correlation curves (FSC) are shown. Scale bar = 200 Å.

## FIGURE S10



1520

1521 **Figure S10: Restoration of CTD-binding enhances FluPol binding to ANP32A and FluPol**  
1522 **replication activity**

1523 **(A)** Recombinant WSN mutant viruses were produced by reverse genetics. Representative plaque  
1524 assays as quantified in Fig. 5D of mutant viruses harbouring PA K289A and the second-site mutation

1525 PA C489R are shown. The supernatants of two independently performed RG experiments were titrated  
1526 on MDCK cells and stained by crystal violet.

1527 **(B)** WSN vRNPs with the PA K289A mutation or the double mutation PA K289A+C489R were  
1528 reconstituted in HEK-293T cells by transient transfection using a plasmid encoding the NA vRNA  
1529 segment. Steady-state levels of NA mRNA, cRNA and vRNA were quantified by strand-specific RT-  
1530 qPCR (Kawakami et al., 2011) normalised to GAPDH by the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen,  
1531 2001) and are represented as a percentage of PA WT. \* $p < 0.033$ , \*\* $p < 0.002$ , \*\*\* $p < 0.001$  (one-way  
1532 ANOVA; Dunnett's multiple comparisons test).

1533 **(C)** HEK-293T cells were co-transfected with expression plasmids for WSN PB1, PB2 and PA with the  
1534 indicated mutation. Cell lysates were analysed by western blot using antibodies specific for PA and  
1535 tubulin. Uncropped gels are provided as a source data file.

1536 **(D)** Anhui-H7N9 Flu<sub>A</sub>Pol with the PA K289A mutation and the second-site mutation PA C489R were  
1537 characterised. (left) Anhui-H7N9 Flu<sub>A</sub>Pol activity was measured by vRNP reconstitution (PB2, PB1,  
1538 PA-ΔX, NP) in HEK-293T cells, using a model vRNA encoding the Firefly luciferase. Luminescence  
1539 was measured and normalised to a transfection control. The data are represented as a percentage of  
1540 PA WT. (middle) Anhui-H7N9 Flu<sub>A</sub>Pol binding to the CTD was assessed using a split-luciferase-based  
1541 complementation assay. HEK-293T cells were co-transfected with expression plasmids for the CTD  
1542 tagged with one fragment of the *G. princeps* luciferase and Flu<sub>A</sub>Pol tagged with the other fragment.  
1543 Luminescence due to luciferase reconstitution was measured and the data are represented as a  
1544 percentage of PA WT. (right) ANP32A was tagged with one fragment of the *G. princeps* luciferase and  
1545 binding to WSN Flu<sub>A</sub>Pol was determined as for Flu<sub>A</sub>Pol I-binding to the CTD. \* $p < 0.033$ , \*\*\* $p < 0.001$   
1546 (one-way ANOVA; Dunnett's multiple comparisons test).

1547 **(E)** (left) Cartoon representation of the pS5 CTD bound to Flu<sub>A</sub>Pol Zhejiang-H7N9 z4M (replicase-like  
1548 conformation) in CTD-binding site 1A. The PA subunit is coloured in green and the pS5 CTD is  
1549 coloured in red. PA C453 and PA R638 residues are displayed. Putative hydrogen bonds between  
1550 PA R638 and pS5 are drawn as yellow dashed lines and corresponding distances are indicated. The  
1551 Coulomb potential map of PA C453, PA R638, and pS5 is shown. (right) Model derived from Flu<sub>A</sub>Pol  
1552 Zhejiang-H7N9 4M structure bearing PA C453R and PA R638A mutations. The PA subunit is coloured  
1553 in green and the pS5 CTD is coloured in red. PA C453R and PA R638A residues are displayed.  
1554 Hypothetical distances between PA C453R and pS5 are shown.

1555 **(F-I)** Phenotypes associated with the WSN Flu<sub>A</sub>Pol PA R638A mutation and the second-site mutation  
1556 PA C453R were characterised. (F) Plaque phenotype of recombinant WSN mutant viruses produced by  
1557 reverse genetics. RG supernatants were titrated on MDCK cells and stained by crystal violet. Plaque  
1558 diameters (mm) were measured and each dot represents one viral plaque. Representative plaque assays  
1559 are shown in (G) (#) not measurable pinhead-sized plaques. (H) WSN Flu<sub>A</sub>Pol activity was measured  
1560 by vRNP reconstitution in HEK-293T cells, using a model vRNA encoding the Firefly luciferase.  
1561 Luminescence was measured and normalised to a transfection control. The data are represented as a

1562 percentage of PA WT. **(I)** WSN Flu<sub>A</sub>Pol binding to the ANP32A was assessed using a split-luciferase-  
1563 based complementation assay. HEK-293T cells were co-transfected with expression plasmids for the  
1564 CTD tagged with one fragment of the *G. princeps* luciferase and Flu<sub>A</sub>Pol tagged with the other fragment.  
1565 Luminescence due to luciferase reconstitution was measured and the data are represented as a  
1566 percentage of PA WT. \* $p < 0.033$ , \*\* $p < 0.002$  (one-way ANOVA; Dunnett's multiple comparisons  
1567 test).

1568 **(J)** HEK-293T cells were co-transfected with expression plasmids for WSN PB1, PB2 and PA with the  
1569 indicated mutation. Cell lysates were analysed by western blot using antibodies specific for PA and  
1570 tubulin. v

1571 **(K)** Flu<sub>C</sub>Pol asymmetric dimer bound to pS5 CTD (PDB: 6F5P, (Serna Martin et al., 2018)). Both  
1572 Flu<sub>C</sub>Pols are in replicase conformation. The first Flu<sub>C</sub>Pol<sub>R1</sub> (Replicase(1)) is displayed as surface. The  
1573 second Flu<sub>C</sub>Pol<sub>R2</sub> (Replicase(2)) is displayed as cartoon. P3 subunit is coloured in green, PB1 subunit  
1574 in light grey, PB2-N in dark red, PB2 Mid-link in purple, PB2 CBD in orange, PB2 627 in pink,  
1575 PB2 NLS in beige. Both pS5 CTD binding sites (1C, 2C) are circled in red. Each bound pS5 CTD  
1576 peptides are displayed as surface and coloured in red. Close-up view on pS5 CTD bound to the site 2C  
1577 is shown below.

1578 **(L)** Flu<sub>C</sub>Pol asymmetric dimer bound to ANP32A (PDB: 6XZG, (Carrique et al., 2020)). The first  
1579 Flu<sub>C</sub>Pol is in a replicase conformation (Replicase<sub>(R)</sub>), displayed and coloured as in K. The 5' vRNA end  
1580 is coloured in pink and the 3' vRNA end is coloured in gold. The second Flu<sub>C</sub>Pol is in an encapsidase  
1581 conformation (Encapsidase<sub>(E)</sub>), displayed as cartoon. P3<sub>E</sub> subunit is colored in light green, PB1<sub>E</sub> subunit  
1582 in light blue, PB2-N<sub>(E)</sub> in salmon, PB2 Mid-link<sub>(E)</sub> in dark grey, PB2 CBD<sub>(E)</sub> in orange, PB2 627<sub>(E)</sub> in  
1583 light pink, PB2 NLS<sub>(E)</sub> in brown. ANP32A is displayed as cartoon and coloured in purple. Close-up  
1584 view on the pS5 CTD in binding site 2C is shown.