Avian Influenza A Virus polymerase can utilise human ANP32 proteins to support cRNA but not vRNA synthesis

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Abstract

Host restriction limits the emergence of novel pandemic strains from the Influenza A Virus avian reservoir. For efficient replication in mammalian cells, the avian influenza RNA-dependent RNA polymerase must adapt to use human orthologues of the host factor ANP32, which lack a 33 amino acid insertion relative to avian ANP32A. Here we find that influenza polymerase requires ANP32 proteins to support both steps of replication: cRNA and vRNA synthesis. Nevertheless, avian strains are only restricted in vRNA synthesis in human cells. Therefore, avian polymerase can use human ANP32 orthologues to support cRNA synthesis, without acquiring mammalian adaptations. This implies a fundamental difference in the mechanism by which ANP32 proteins support cRNA vs vRNA synthesis.

26 Importance

27 In order to infect humans and cause a pandemic, avian influenza must first learn how
28 to use human versions of the proteins the virus hijacks for replication – instead of the
29 avian versions found in bird cells. One such protein is ANP32. Understanding the
30 details of how host proteins such as ANP32 support viral activity may allow the design
31 of new antiviral treatments that disrupt these interactions. In this work, we use cells
32 that lack ANP32 to unambiguously demonstrate ANP32 is needed for both steps
33 of influenza genome replication. Surprisingly however, we find that avian influenza can
34 use human ANP32 proteins for the first step of replication without any adaptation, but
35 only avian ANP32 for the second step of replication. This suggests ANP32 may have
36 an additional role in supporting the second step of replication, and it is this activity that
37 is specifically blocked when avian influenza infects human cells.

38 Introduction

39 Influenza A viruses (IAVs) pose a pandemic risk: whilst the natural hosts of IAV are
40 aquatic birds, the virus is associated with sporadic zoonotic jumps, which may trigger
41 widespread disease in an immunologically naïve population (1). The enormous
42 consequences of such events are illustrated by the historical 1918 'Spanish flu'
43 pandemic, and the ongoing coronavirus pandemic caused by SARS-CoV-2.

44

45 Prior to emerging as a pandemic strain, zoonotic IAV must adapt to overcome multiple
46 host range barriers. One important restriction is adaptation of the RNA-dependent
47 RNA polymerase (FluPol) for efficient activity within the mammalian cellular
48 environment (1, 2). FluPol is a heterotrimer consisting of three viral proteins: PB1, PB2
49 and PA. Each of the eight viral genomic RNAs (vRNAs) of the IAV genome are
50 encapsidated by nucleoproteins (NP) and associate with a FluPol protomer in a viral
51 ribonucleoprotein complex (vRNP). During infection, FluPol drives both transcription
52 and replication of the viral genome (3). Transcription occurs in a primer-dependent
53 manner to produce capped and poly-adenylated positive sense viral mRNAs. Genome
54 replication occurs in a two-step process: first, negative sense vRNA is copied into a
55 full-length positive-sense cRNA intermediate which is packaged into complementary
56 RNPs (cRNPs) by acquiring an encapsidating polymerase and NP. Nascent vRNPs
57 are then produced from cRNPs (3, 4). cRNA and vRNA synthesis differ in *de novo*
58 initiation strategy. Whilst cRNA synthesis uses terminal initiation, vRNA synthesis uses
59 internal initiation followed by primer realignment that is dependent on an additional
60 trans-activating FluPol (5–8).

61

62 Structural studies suggest the functional flexibility of FluPol is possible due to
63 rearrangements of peripheral domains outside the catalytic core (3). These depend on
64 the presence or absence and nature of the bound RNA promoter, or upon dimerization
65 with additional polymerase molecules (9–11). FluPol also co-opts host factors to co-
66 ordinate its activity, some of which may stabilise different functional states of the
67 polymerase (10, 12).
68
69 ANP32 proteins are a family of proviral host factors that are essential for influenza
70 replication (13, 14). Species differences between ANP32 proteins underly host
71 restriction of avian influenza polymerase in mammalian cells (15). Prototypical avian
72 influenza polymerase bearing a glutamic acid at position 627 of the PB2 protein
73 (hereafter referred to as FluPol 627E) cannot be supported by mammalian ANP32
74 proteins that lack the 33 amino acid insertion present in avian ANP32A orthologues,
75 and accordingly show restricted replication in human cells. In contrast, FluPol bearing
76 a single amino acid substitution at the PB2 627 position from glutamic acid to lysine
77 (the quintessential mammalian-adapting mutation, FluPol 627K), can utilise
78 mammalian ANP32 proteins that lack this insertion, and such viruses replicate to high
79 titres in human cells (2, 15). Interestingly, although chicken ANP32A (chANP32A)
80 shows enhanced binding to FluPol relative to human ANP32A (huANP32A), that is
81 dependent on the 33 amino acid insertion, this pattern occurs independent of FluPol
82 627 signature, i.e., species-specific functionality is not dictated by differences in
83 binding affinity (16–18).
84
85 Previous studies suggest that ANP32 proteins are specifically required to support
86 vRNA synthesis, and accordingly that host restriction occurs at this step of replication.

87 Sugiyama et al found that purified huANP32A stimulated RNA replication from a short
88 cRNA but not vRNA template in *in vitro* replication assays (19). Moreover, studies
89 comparing infection in mammalian cells with strains bearing either FluPol 627E or
90 627K found that 627E was specifically restricted in vRNA synthesis, but not cRNP
91 stabilisation (20, 21). Nevertheless, other reports implicate ANP32 in both steps of
92 replication (11, 22). The recent structures of huANP32A and chANP32A in complex
93 with FluPol from Influenza C virus demonstrate that the N terminal Leucine Rich
94 Repeat (LRR) domain of ANP32 bridges a novel asymmetric dimer of two FluPol
95 enzymes. The unstructured C terminal Low Complexity Acidic Region (LCAR) domain
96 of ANP32 remains largely unresolved, apart from additional density sandwiched in a
97 groove formed between the two 627 domains. This dimer was interpreted as a
98 'replication complex', with the RNA-bound FluPol ($FluPol_R$) adopting a replication-
99 competent structure, and the additional apo-enzyme ($FluPol_E$) adopting a novel
100 conformation: poised to encapsidate the nascent RNA into an RNP complex.
101 Interestingly, this structure was obtained with $FluPol_R$ bound to a 47nt short vRNA,
102 i.e., primed for cRNA synthesis, which was not previously implemented in requiring
103 support by ANP32. Moreover, mutations introduced to disrupt the FluPol asymmetric
104 dimer interface resulted in a significant reduction in cRNA encapsidation. This,
105 combined with the logic that both cRNA and vRNA require encapsidation into RNPs
106 during replication, suggests that ANP32 may play a role in cRNA synthesis as well as
107 vRNA synthesis (11).
108
109 Here we use ANP32 knockout cell lines to clarify the role of ANP32 proteins in cRNA
110 synthesis. We establish an RNA fluorescence in situ (FISH) assay for directly
111 visualising cRNA and demonstrate that ANP32 proteins are essential for primary

112 cRNA synthesis in authentic infection, as well as under experimental conditions in
113 which vRNA synthesis is inhibited. Nevertheless, we find that avian FluPol 627E does
114 not show restricted cRNA accumulation in mammalian cells. This observation is
115 consistent regardless of whether $FluPol_R$, $FluPol_E$ or both FluPol molecules in the
116 replication complex bear the avian-like 627E signature. Moreover, the cRNPs
117 produced by $FluPol_R$ 627E are functional for onward replication. To conclude, our
118 study suggests that huANP32 proteins are sufficient to support avian FluPol cRNA
119 synthesis, and that host restriction acts specifically at the level of vRNA synthesis. This
120 suggests a fundamental difference in the mechanism by which the host factor ANP32
121 supports vRNA and cRNA synthesis.

122 Results

123 *cRNA synthesis is inhibited in human cells lacking ANP32A/B*

124 Human cells express three members of the ANP32 protein family: huANP32A,
125 huANP32B and huANP32E (23). Previous work has demonstrated that huANP32A/B
126 are functionally redundant for proviral activity, whilst huANP32E does not support
127 FluPol activity (13, 14). To investigate the role of ANP32 proteins in cRNA synthesis,
128 wild type human eHAP cells (eHAP WT) and human eHAP cells in which ANP32A/B
129 have been ablated (eHAP dKO) (13) were infected with A/Puerto Rico/8/1934(H1N1)
130 (PR8) and the accumulation of segment 4 (haemagglutinin (HA)) vRNA, cRNA and
131 mRNA quantified using a tagged RT-qPCR approach (24). Following infection, there
132 was no increase in either vRNA or cRNA levels over time in the absence of ANP32A/B,
133 confirming ANP32 proteins are essential for replication (Figure 1A,B). Significantly, no
134 increase in cRNA accumulation was observed over input, suggesting a direct role for
135 ANP32 proteins in the pioneering round of cRNA synthesis. In contrast, HA mRNA
136 transcripts increased 50-fold in dKO cells from 0 hours post infection (hpi) to 2hpi,
137 illustrating that primary transcription does not require ANP32 proteins (Figure 1C). In
138 a separate experiment, we analysed the accumulation of segment 6 (neuraminidase
139 (NA)) vRNA, cRNA and mRNA at an early and late timepoint in eHAP WT and dKO
140 cells, using an analogous tagged RT-qPCR approach. As with segment 4, at late
141 timepoints a significant reduction in all three RNA species was observed (Figure
142 1D,E,F). At 3hpi, a ~10 fold reduction in the accumulation of cRNA was already
143 apparent in the dKO cells, despite no difference in vRNA accumulation having yet
144 occurred. Again, this suggested a direct role for huANP32A/B in supporting cRNA
145 synthesis.

146

147 *Human ANP32 proteins play a direct role in primary cRNA synthesis*

148 To eliminate the possibility that ANP32 proteins are only required for vRNA synthesis,
149 and the lack of cRNA accumulation is an indirect effect, we made use of cRNP
150 stabilisation assays. cRNP stabilisation assays manipulate cellular conditions to allow
151 only the primary round of cRNA synthesis from incoming viral genomes (Vreede et al,
152 2004, Nilsson et al, 2017). vRNA synthesis, and consequently further secondary
153 rounds of cRNA synthesis, are inhibited. The assay involves pre-expressing FluPol
154 and NP, and then infecting cells with virus. Importantly, the FluPol that is exogenously
155 expressed is a catalytically dead mutant (PB1 D446Y) that will allow stabilisation of
156 nascent cRNA into catalytically inactive cRNPs. Cells are then drug treated with either
157 actinomycin D (ActD) or cycloheximide (CHX) during infection to inhibit the synthesis
158 of nascent viral proteins which would otherwise enable normal replication.

159

160 Initially, ActD-treated cRNP stabilisation assays were performed in eHAP WT and dKO
161 cells. ActD inhibits viral transcription, therefore in these assays only primary cRNA
162 synthesis occurs (Figure 2A). When analysed by RT-qPCR (Figure 2B,C,D) a highly
163 significant reduction in cRNA was observed in dKO cells as compared to WT cells. As
164 expected, no accumulation of NA vRNA or mRNA occurred over background levels
165 (controls lacking transfected PB1, dotted line), in either cell type. Equal transfection
166 efficiency was confirmed by western blot (Figure S1A).

167

168 We next established *in situ* assays for directly visualising IAV replication products to
169 allow single cell, spatial information to be collected alongside bulk assay readouts. To
170 achieve this, the RNA FISH assay RNAscope® was used (25). Probes were designed
171 to target either segment 6 negative sense RNA (NA vRNA probe) or positive sense

172 RNA (NA +RNA probe). The NA +RNA probe is unable to distinguish NA cRNA/mRNA
173 due to the minimal sequence differences between these two RNA species. To counter
174 this, simultaneous cRNP stabilisation and replication assays were performed with
175 ActD treatment, to inhibit viral transcription and allow +RNA staining to be attributed
176 to cRNA. Replication assays are performed as for cRNP stabilisation assays, however
177 active polymerase is pre-expressed, so that multiple rounds of cRNA and vRNA
178 synthesis take place. Validation of the approach is shown in Figure S1B-F. In an *in*
179 *situ* cRNP stabilisation assay, +RNA accumulation could be clearly detected 3hpi in
180 the nuclei of infected WT cells (Figure 2E). In contrast, whilst incoming vRNPs could
181 be observed in the nuclei of dKO cells, no +RNA staining was observed. This
182 corroborates the view that huANP32A/B are required for primary cRNA synthesis.
183
184 Our infection data (Figure 1) suggest that ANP32 proteins are not required for primary
185 transcription. Nevertheless, at later infection timepoints, reduced mRNA accumulation
186 is observed in dKO cells, compared to WT cells (Figure 1C,F). To confirm this is an
187 indirect effect due to reduced vRNP template, we chose to undertake a CHX-treated
188 cRNP stabilisation assay in eHAP wild type, dKO or in eHAP cells lacking expression
189 of all three huANP32 proteins: A, B and E (eHAP tKO). In this version of the assay,
190 both cRNA synthesis and primary transcription occur in the absence of vRNA
191 synthesis (Figure 2F). No accumulation of vRNA over input was observed in any of
192 the cell types over time (Figure 2G), as expected. In agreement with ActD cRNP
193 stabilisation assays (Figure 2C), a significant decrease in cRNA accumulation was
194 seen in both dKO and tKO cells compared to WT by 5hpi (Figure 2H). A small but
195 significant difference (p=0.0018) was observed between cRNA accumulation in the
196 dKO and tKO cells, implying huANP32E may support a low level of cRNP stabilisation.

197 In contrast, no significant difference was observed in mRNA accumulation in the
198 presence or absence of any ANP32 proteins from 0 to 5hpi (Figure 2I). This confirms
199 that primary transcription does not require ANP32 proteins. Comparable transfection
200 efficiency was confirmed via western blot (Figure S2G).
201
202 *Avian polymerase is not restricted in cRNA synthesis in mammalian cells*
203 Restriction of avian signature FluPol (FluPol 627E) in mammalian cells is attributed to
204 species differences in ANP32 proteins. Consequently, as we have confirmed that
205 ANP32 is required to support both cRNA and vRNA synthesis, we would expect FluPol
206 627E to be restricted in both steps of replication. Nevertheless, previous work maps
207 restriction to occur specifically at the level of vRNA synthesis (20, 21, 26). To
208 investigate this apparent contradiction, we used a pair of isogenic viruses based on
209 the avian strain A/turkey/England/50-92/1991 (H5N1) (5092) that differ only in the
210 residue at PB2 position 627: either the wild type PB2 627E (hereafter referred to as
211 5092E) or the humanising mutation PB2 E627K (5092K) (27).
212
213 First, we confirmed that 5092 polymerase is also dependent on ANP32 proteins for
214 cRNA synthesis both during authentic infection (Figure S2A,B,C) and in cRNP
215 stabilisation assays (Figure S2D,E,F). Next, we undertook simultaneous cRNP
216 stabilisation and replication assays in human cells, where only the shorter
217 huANP32A/B/E proteins, that are incompatible with FluPol 627E, are available. We
218 pre-expressed NP with either catalytically dead 5092 FluPol 627E or 627K (cRNP
219 stabilisation assay) or WT 5092 FluPol 627E or 627K (replication assay), followed by
220 infection with either 5092K or 5092E virus in the presence of ActD. Significantly, these
221 assays allow the effect of PB2 residue 627 in either $FluPol_R$ or $FluPol_E$ to be

222 differentiated. The resulting FluPol combinations for both cRNA and vRNA synthesis
223 are outlined in Figure 3A.
224
225 During the pioneering round of cRNA synthesis, measured in the cRNP stabilisation
226 assay, $FluPol_E$ is provided by the pre-expressed polymerase whilst $FluPol_R$ is brought
227 in on the vRNPs from the infecting virus. No vRNA accumulated in the cRNP
228 stabilisation assay, as expected (Figure 3B). Interestingly, neither the PB2 signature
229 of $FluPol_R$ nor $FluPol_E$ impacted cRNP stabilisation, with all four FluPol combinations
230 accumulating equivalent amounts of cRNA (Figure 3C). This demonstrates that avian
231 and human signature virus can undergo cRNA synthesis equally well in human cells,
232 i.e., avian FluPol is compatible with huANP32 proteins for cRNA synthesis and
233 stabilisation.
234
235 In the replication assay, samples with pre-expressed FluPol 627K accumulated
236 equivalent quantities of vRNA and cRNA, regardless of whether the incoming virus
237 was 5092K or 5092E (Figure 3Ai,ii,B,C). Similarly, samples with pre-expressed FluPol
238 627E were equally impaired in vRNA and cRNA synthesis, independent of infecting
239 virus signature (Figure 3Aiii,iv,B,C). It has previously been described that host
240 restricted FluPol is impaired in nuclear import, due to incompatibilities with the
241 importin-alpha isoforms present in human cells (28). However, this cannot explain the
242 data obtained here, as FluPol 627E was fully functional in supporting the pioneering
243 round of cRNA synthesis and stabilisation within the nucleus during cRNP stabilisation
244 assays. As replication assays allow multiple cycles of replication, the reduced cRNA
245 synthesis observed with pre-expressed FluPol 627E is likely due to the secondary

246 effect of reduced vRNP template. Exogenous expression of chANP32A was able to
247 rescue replication for all FluPol combinations (Figure 3D,E).

248 Discussion

249 Here we have used cell lines that lack expression of ANP32 proteins to unambiguously
250 confirm huANP32 is required to support cRNA synthesis. Nevertheless, we did not
251 observe restriction of avian virus cRNA synthesis in human cells, confirming that host
252 range restriction imparted by species differences in ANP32 acts at the level of vRNA
253 synthesis.

254

255 In the structure of the ANP32 and FluPol complex described by Carrique et al, the
256 ANP32 LRR domain bridges the FluPol dimer. The flexible LCAR remains largely
257 unresolved, although there is additional density present in a groove formed between
258 the two FluPol 627 domains. As the groove is acidic in FluPol 627E, the acidic
259 huANP32 LCAR is likely incompatible with this interaction, whereas the mixture of
260 acidic and basic residues in the 33 amino acid insertion of avian chANP32A
261 overcomes this block (11). Our data suggests this mismatch between the huANP32
262 LCAR and FluPol 627E can be tolerated for cRNA synthesis. This conclusion aligns
263 with the observation that addition of chANP32A to human cells does not stimulate
264 FluPol 627E cRNA synthesis (26).

265

266 The ANP32-stabilised replication complex is thought to be required to load an
267 encapsidating FluPol onto the promoter of the extruded strand of nascent c/vRNA.
268 Moreover, it has been proposed that the unstructured ANP32 LCAR domain may act
269 as a molecular whip, which recruits NP for loading onto the nascent RNA (11, 29).
270 Previous studies have suggested that host restricted avian FluPol can generate cRNA
271 in human cells, but that the resulting cRNPs are non-functional for onwards replication
272 (20, 22, 26). However, here we show that cRNA produced from $FluPol_R$ 627E is fully

273 functional for vRNA synthesis, provided the encapsidating FluPol bears 627K for
274 onward vRNA synthesis. Therefore, functional cRNPs are produced in the absence of
275 a compatible 627-LCAR interaction, suggesting host restriction does not act at the
276 level of cRNA encapsidation. In combination, our data therefore suggests that the 627-
277 LCAR interaction is required for a further mechanistic role that is specific to vRNA
278 synthesis. For example, a compatible 627-LCAR interaction could be of critical
279 importance to recruit the trans-activating FluPol (York et al., 2013).
280
281 In summary, this work has established that huANP32 is required for cRNA synthesis,
282 as well as vRNA synthesis, in human cells. Nonetheless, host restriction of avian
283 FluPol 627E acts specifically at the level of vRNA synthesis, as huANP32 paralogues
284 are sufficient for supporting avian cRNA synthesis and encapsidation. This suggests
285 a fundamental difference in the mechanism by which ANP32 proteins support cRNA
286 vs vRNA synthesis. We have also established an RNA FISH assay which allows
287 visualisation of FluPol replication products. This allows single-cell, spatial information
288 to be collated, which in future could be used to improve our understanding of the
289 spatial elements of FluPol regulation.

290 Materials and methods

291 *Cells and cell culture*

292 Human-engineered Haploid cells (eHAP, Horizon Discovery), eHAP cells with both
293 huANP32A and huANP32B (eHAP dKO) ablated via CRISPR-Cas9, as previously
294 described (13), or eHAP cells with huANP32A, huANP32B and huANP32E ablated via
295 CRISPR-Cas9 (eHAP tKO) (gift from Ecco Staller and Ervin Fodor) were maintained
296 in Iscove's modified Dulbecco's medium (IMDM, Thermo Fisher) supplemented with
297 10% fetal bovine serum (FBS, Labtech), 1% penicillin-streptomycin (pen-strep, Gibco)
298 and 1% non-essential amino acids (NEAA, Gibco). Human embryonic kidney (293Ts,
299 ATCC) and Madin-Darby canine kidney (MDCK, ATCC) cells were maintained in
300 Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1% pen-
301 strep and 1% NEAA. When used for infection, 293T cells were cultured on poly-L-
302 lysine coated plates to aid adherence. All cells were maintained at 37°C and 5% CO_2.

303

304 *Plasmids*

305 pCAGGS expression plasmids encoding the polymerase subunits (PB2, PB1, PA) and
306 NP from PR8 were subcloned from pPolI plasmids. pCAGGS expression plasmids
307 encoding 50-92 PB2, PB1, PA and NP have previously been described (30). The
308 catalytic mutant PB1 D446Y has previously been described (31, 32). pCAGGS
309 expression plasmid encoding FLAG-tagged chicken ANP32 has been described
310 previously (15).

311

312 *Viral infections*

313 The full strain names of viruses used in this study are A/Puerto Rico/8/1934(H1N1)
314 (PR8) and A/turkey/England/50-92/1991(H5N1) (50-92). For experiments using 50-

315 92, all infections were performed with recombinant viruses containing the HA, NA and
316 M segments from PR8, the PB1, PA, NP and NS segments from 50-92 and either the
317 WT 50-92 PB2 segment containing a lysine at position 627 (5092E) or a modified PB2
318 with a glutamic acid at position 627 (5092K), as previously described (27). For
319 infections, virus was diluted in serum-free media to the correct multiplicity of infection
320 (described in figure legends). For comparative 50-92E/K experiments, viral inputs
321 were normalised based on genome copy number. To synchronise infection, viral
322 inoculation was performed at 4°C. In brief, cells were pre-incubated at 4°C for 15
323 minutes, before addition of viral inoculum and a further incubation at 4°C for 45
324 minutes. Viral inoculum was then replaced with pre-warmed full media and infected
325 plates incubated at 37°C, 5% CO_2. At the appropriate time point, cells were processed
326 for RT-qPCR analysis, imaging analysis or immunoblot as described below.
327
328 *Replication/cRNP stabilisation assays*
329 For replication/cRNP stabilisation assays, cells were transfected using Lipofectamine
330 3000 (Invitrogen) with pCAGGS expression plasmid mixtures encoding polymerase
331 components in the ratios 2:2:1:4, PB2:PB1:PA:NP, where 1=20ng, 40ng or 80ng (24
332 well plate, 12 well plate or 6 well plate respectively). For experiments including
333 chANP32A, pCAGGS expression plasmid encoding FLAG-tagged chANP32A was
334 included in the transfection mix at a ratio of 4. For replication assays WT PB1 plasmid
335 was pre-expressed, whilst for cRNP stabilisation assays catalytically dead polymerase
336 (PB1 D446Y) was pre-expressed. 20 hours post transfection, cells were infected as
337 described above, with the addition of actinomycin D (5 μg/mL), cycloheximide (100
338 μg/mL) or DMSO control as indicated. At the appropriate time point, cells were
339 processed for RT-qPCR analysis, imaging analysis or immunoblot as described below.

340

341 *Tagged RT-qPCR against vRNA, cRNA and mRNA*

342 For RT-qPCR analysis, 293T or eHAP cells were cultured in 24 well plates, with each
343 condition in triplicate. Following infection/transfection, cells were lysed using buffer
344 RLT or RLT plus (Qiagen), frozen at -80, then total RNA was extracted using either
345 the RNeasy RNA extraction kit (Qiagen) with 30 minute on column DNAseI digest, or
346 the QIAsymphony RNA kit (Qiagen). Quantification for segment 4 and segment 6
347 vRNA, cRNA and mRNA was based on the tagged primer approach developed by
348 Kawakami et al (24). For each sample, four reverse transcription reactions were set
349 up using 200ng RNA/reaction, RevertAid H Minus Reverse Transcriptase (Thermo
350 Scientific) as per the manufacturer's instructions, plus a tagged primer targeting either
351 vRNA or cRNA, a tagged polydT (for viral mRNA) or an untagged polydT (for GAPDH
352 internal control). For NA vRNA, cRNA and mRNA, primers used were
353 GGCCGTCATGGTGGCGAATGAAACCATAAAAAGTTGGAGGAAG,
354 GCTAGCTTCAGCTAGGCATCAGTAGAAACAAGGAGTTT and
355 CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTT respectively, tags underlined))
356 whilst primers used for HA vRNA, cRNA and mRNA were
357 GGCCGTCATGGTGGCGAATGGAGAGTGCCCAAAATACGT,
358 GCTAGCTTCAGCTAGGCATCAGTAGAAACAAGGGTGTT and
359 CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTT respectively, tags underlined).
360 Tagged cDNA was then diluted 1 in 10 and quantified using real-time quantitative PCR
361 using Fast SYBR green master mix (Thermo Scientific). Primer pairs used were:
362 CCTTCCCCTTTTCGATCTTG/ GGCCGTCATGGTGGCGAAT (NA vRNA),
363 CTTTTTGTGGCGTGAATAGTG/ GCTAGCTTCAGCTAGGCATC (NA cRNA),
364 CTTTTTGTGGCGTGAATAGTG/ CCAGATCGTTCGAGTCGT (NA mRNA),

365 CATACCATCCATCTATCATTCC/ GGCCGTCATGGTGGCGAAT (HA vRNA),
366 GGGGGCAATCAGTTTCTG/ GCTAGCTTCAGCTAGGCATC (HA cRNA),
367 GATTCTGGCGATCTACTCAACTGTC/ CCAGATCGTTCGAGTCG (HA mRNA) and
368 AATCCCATCACCATCTTCCA/ TGGACTCCACGACGTACTCA (GAPDH). qPCR
369 analysis was carried out in duplicate or triplicate on a Viia 7 real-time PCR system
370 (Thermo Fisher). Fold changes in gene expression relative to either input (0hpi) or
371 mock infected controls (as indicated in figure legends) were calculated using the 2^{-}
372 $^{\Delta\Delta CT}$ method with GAPDH expression as internal control.
373
374 *RNAscope/immunofluorescence co-staining*
375 For imaging analysis, cells were cultured on glass cover slips coated in poly-L-lysine
376 in 12 well plates. At the appropriate timepoint, infected cells were washed in phosphate
377 buffered saline (PBS, Gibco) and fixed in 4% paraformaldehyde for 30 minutes, prior
378 to further washes in PBS and dehydration in an ethanol gradient (50% EtOH, 5 mins,
379 70% EtOH, 5 mins, 100% EtOH, 5 mins, fresh 100% EtOH, 10 mins). Cover slips were
380 stored in 100% ethanol at -20°C until further processing. For
381 RNAscope/immunofluorescence co-staining, RNA was stained first using RNAscope
382 probes (ACDBio). Probes were designed to target PR8 NA vRNA (channel 1) and PR8
383 NA cRNA/mRNA (+RNA) (channel 2). Cover slips were rehydrated in an ethanol
384 gradient (70% EtOH, 2 mins, 50% EtOH, 2 mins, PBS, 10 mins), treated with protease
385 III diluted 1 in 15 in PBS and staining undertaken using the fluorescent multiplex kit
386 v1, following the manufacturer's instructions up until and including incubation in the
387 final fluorophore mixture (Fl-Amp4). At this point, cover slips were blocked in PBS with
388 2% bovine serum albumin (BSA) and 0.1% tween for 30 minutes, incubated in rabbit
389 α-PB2 (catalog no. GTX125926, Genetex) antibody for 1 hour at room temperature

390 (RT), followed by goat α-rabbit AF647 (Invitrogen) plus DAPI for 1 hour, RT. Images
391 were obtained using a Leica SP5 inverted confocal microscope and processing
392 undertaken using FIJI software (33, 34).
393
394 *Immunoblot analysis*
395 To confirm equivalent protein expression during replication/cRNP stabilisation assays,
396 cells transfected in parallel were lysed with homemade radioimmunoprecipitation
397 assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1%
398 SDS, 50 mM Tris, pH 7.4) supplemented with an EDTA-free protease inhibitor cocktail
399 tablet (Roche). Lysates were clarified, then mixed with 4x Laemmli sample buffer (Bio-
400 Rad) with 10% β-mercaptoethanol. Membranes were probed with rabbit α-vinculin
401 (EPR8185, Abcam) or mouse α-tubulin (ab7291, Abcam) and mouse α-NP (C43,
402 Abcam), followed by near-infrared secondary antibodies (IRDye 680RD goat anti-
403 rabbit (IgG) secondary antibody (Abcam) and IRDye 800CW goat anti-mouse (IgG)
404 secondary antibody (Abcam)). Western blots were visualised using an Odyssey
405 imaging system (Li-Cor Biosciences).
406
407 *Statistical analysis*
408 Statistics throughout this study were performed using one-way analysis of variance
409 (ANOVA) or student's T-test as described in figure legends.

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418
419 O.C.S., A.M.B., C.M.S and W.S.B. conceived and planned experiments; O.C.S. and
420 A.M.B. performed experiments and analysed data, O.C.S., T.P.P., C.M.S. and W.S.B.
421 provided supervision, O.C.S. and W.S.B. wrote the original manuscript, O.C.S.,
422 A.M.B., T.P.P., C.M.S. and W.S.B. reviewed and edited the manuscript.
423
424 The authors declare no competing interests.

425 References

426 1. Long JS, Mistry B, Haslam SM, Barclay WS. 2019. Host and viral determinants
427 of influenza A virus species specificity. Nature Reviews Microbiology. Nature
428 Publishing Group https://doi.org/10.1038/s41579-018-0115-z.

429 2. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2
430 gene of influenza A virus is a determinant of host range. J Virol 67:1761–4.

431 3. Pflug A, Lukarska M, Resa-Infante P, Reich S, Cusack S. 2017. Structural
432 insights into RNA synthesis by the influenza virus transcription-replication
433 machine. Virus Research. Elsevier B.V.
434 https://doi.org/10.1016/j.virusres.2017.01.013.

435 4. Fodor E, Velthuis AJWT. 2020. Structure and function of the influenza virus
436 transcription and replication machinery. Cold Spring Harbor Perspectives in
437 Medicine 10:1–14.

438 5. Fan H, Walker AP, Carrique L, Keown JR, Serna Martin I, Karia D, Sharps J,
439 Hengrung N, Pardon E, Steyaert J, Grimes JM, Fodor E. 2019. Structures of
440 influenza A virus RNA polymerase offer insight into viral genome replication.
441 Nature 573:287–290.

442 6. York A, Hengrung N, Vreede FT, Huiskonen JT, Fodor E. 2013. Isolation and
443 characterization of the positive-sense replicative intermediate of a negative-
444 strand RNA virus. Proc Natl Acad Sci U S A 110.

445 7. Deng T, Vreede FT, Brownlee GG. 2006. Different De Novo Initiation
446 Strategies Are Used by Influenza Virus RNA Polymerase on Its cRNA and
447 Viral RNA Promoters during Viral RNA Replication. Journal of Virology
448 80:2337–2348.

449 8. Jorba N, Coloma R, Ortín J. 2009. Genetic trans-complementation establishes
450 a new model for influenza virus RNA transcription and replication. PLoS
451 Pathogens 5.
452 9. Hengrung N, el Omari K, Serna Martin I, Vreede FT, Cusack S, Rambo RP,
453 Vonrhein C, Bricogne G, Stuart DI, Grimes JM, Fodor E. 2015. Crystal
454 structure of the RNA-dependent RNA polymerase from influenza C virus.
455 Nature 527:114–117.
456 10. Thierry E, Guilligay D, Kosinski J, Bock T, Gaudon S, Round A, Pflug A,
457 Hengrung N, el Omari K, Baudin F, Hart DJ, Beck M, Cusack S. 2016.
458 Influenza Polymerase Can Adopt an Alternative Configuration Involving a
459 Radical Repacking of PB2 Domains. Molecular Cell 61:125–137.
460 11. Carrique L, Fan H, Walker AP, Keown JR, Sharps J, Staller E, Barclay WS,
461 Fodor E, Grimes JM. 2020. Host ANP32A mediates the assembly of the
462 influenza virus replicase. Nature 587:638–643.
463 12. Peacock TP, Sheppard CM, Staller E, Barclay WS. 2019. Host Determinants
464 of Influenza RNA Synthesis. Annual Reviews in Virology 6:215–233.
465 13. Staller E, Sheppard CM, Neasham PJ, Mistry B, Peacock TP, Goldhill DH,
466 Long JS, Barclay WS. 2019. ANP32 Proteins Are Essential for Influenza Virus
467 Replication in Human Cells. Journal of Virology 93.
468 14. Zhang H, Zhang Z, Wang Y, Wang M, Wang X, Zhang X, Ji S, Du C, Chen H,
469 Wang X. 2019. Fundamental Contribution and Host Range Determination of
470 ANP32A and ANP32B in Influenza A Virus Polymerase Activity. Journal of
471 Virology 93.
472 15. Long JS, Giotis ES, Moncorgé O, Frise R, Mistry B, James J, Morisson M,
473 Iqbal M, Vignal A, Skinner MA, Barclay WS. 2016. Species difference in

474 ANP32A underlies influenza A virus polymerase host restriction. Nature
475 529:101–104.
476 16. Baker SF, Ledwith MP, Mehle A. 2018. Differential Splicing of ANP32A in Birds
477 Alters Its Ability to Stimulate RNA Synthesis by Restricted Influenza
478 Polymerase. Cell Reports 24:2581-2588.e4.
479 17. Domingues P, Hale BG. 2017. Functional Insights into ANP32A-Dependent
480 Influenza A Virus Polymerase Host Restriction. Cell Reports 20:2538–2546.
481 18. Mistry B, Long JS, Schreyer J, Staller E, Yusef Sanchez-David R, Barclay WS.
482 2020. Elucidating the Interactions between Influenza Virus Polymerase and
483 Host Factor ANP32A. Journal of Virology 94.
484 19. Sugiyama K, Kawaguchi A, Okuwaki M, Nagata K. 2015. PP32 and APRIL are
485 host cell-derived regulators of influenza virus RNA synthesis from cRNA. Elife
486 4:1–19.
487 20. Mänz B, Brunotte L, Reuther P, Schwemmle M. 2012. Adaptive mutations in
488 NEP compensate for defective H5N1 RNA replication in cultured human cells.
489 Nature Communications 3.
490 21. Nilsson BE, te Velthuis AJW, Fodor E. 2017. Role of the PB2 627 Domain in
491 Influenza A Virus Polymerase Function. Journal of Virology 91.
492 22. Nilsson-Payant BE, tenOever BR, te Velthuis AJW. 2021. The host factor
493 ANP32A is required for influenza A virus vRNA and cRNA synthesis. Journal
494 of Virology https://doi.org/10.1128/jvi.02092-21.
495 23. Reilly PT, Yu Y, Hamiche A, Wang L. 2014. Cracking the ANP32 whips:
496 Important functions, unequal requirement, and hints at disease implications.
497 BioEssays 36:1062–1071.

498 24. Kawakami E, Watanabe T, Fujii K, Goto H, Watanabe S, Noda T, Kawaoka Y.
499 2011. Strand-specific real-time RT-PCR for distinguishing influenza vRNA,
500 cRNA, and mRNA. Journal of Virological Methods 173:1–6.
501 25. Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ,
502 Luo Y. 2012. RNAscope: A novel in situ RNA analysis platform for formalin-
503 fixed, paraffin-embedded tissues. Journal of Molecular Diagnostics 14:22–29.
504 26. Bi Z, Ye H, Wang X, Fang A, Yu T, Yan L, Zhou J. 2019. Insights into species-
505 specific regulation of ANP32A on the mammalian-restricted influenza virus
506 polymerase activity. Emerging Microbes and Infections 8:1465–1478.
507 27. Long JS, Howard WA, Núñez A, Moncorgé O, Lycett S, Banks J, Barclay WS.
508 2013. The Effect of the PB2 Mutation 627K on Highly Pathogenic H5N1 Avian
509 Influenza Virus Is Dependent on the Virus Lineage. Journal of Virology
510 87:9983–9996.
511 28. Gabriel G, Herwig A, Klenk HD. 2008. Interaction of polymerase subunit PB2
512 and NP with importin α1 is a determinant of host range of influenza A virus.
513 PLoS Pathogens 4.
514 29. Wang F, Sheppard CM, Mistry B, Staller E, Barclay WS, Grimes JM, Fodor E,
515 Fan H. 2022. The C-terminal LCAR of host ANP32 proteins interacts with the
516 influenza A virus nucleoprotein to promote the replication of the viral RNA
517 genome. Nucleic Acids Research https://doi.org/10.1093/nar/gkac410.
518 30. Moncorgé O, Mura M, Barclay WS. 2010. Evidence for Avian and Human Host
519 Cell Factors That Affect the Activity of Influenza Virus Polymerase. Journal of
520 Virology 84:9978–9986.

521 31. Cauldwell A v., Moncorge O, Barclay WS. 2013. Unstable Polymerase-
522 Nucleoprotein Interaction Is Not Responsible for Avian Influenza Virus
523 Polymerase Restriction in Human Cells. Journal of Virology 87:1278–1284.
524 32. Biswas SK, Nayak DP. 1994. Mutational Analysis of the Conserved Motifs of
525 Influenza A Virus Polymerase Basic Protein 1. Journal of Virology 68:1819–
526 1826.
527 33. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T,
528 Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ,
529 Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source
530 platform for biological-image analysis. Nat Methods 9:676–82.
531 34. Schneider C a, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25
532 years of image analysis. Nature Methods 9:671–675.
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535 *Figure 1. cRNA synthesis is inhibited in human cells lacking ANP32A/B.* (A-C)
536 Segment 4 (A) vRNA (B) cRNA or (C) mRNA accumulation over time in eHAP WT vs
537 dKO cells following infection with PR8 (MOI=3). Fold change was calculated over input
538 (0hpi). n=3 biological replicates, plotted as mean ± s.d.. Significance was assessed at
539 2hpi using an unpaired t-test following log transformation. (D-F) Segment 6 (D) vRNA
540 (E) cRNA or (F) mRNA accumulation over time in eHAP WT vs dKO cells following
541 infection with PR8 (MOI=3). Fold change was calculated over mock infected cells. n=3
542 biological replicates, plotted as mean ± s.d.. Significance was assessed using multiple
543 unpaired t-tests following log transformation, corrected for multiple comparisons using
544 false discovery rate. hpi=hours post infection. ns=not significant; **=p<0.01;
545 ***=p<0.001; ****=p<0.0001.
546
547 *Figure 2. Human ANP32 proteins play a direct role in primary cRNA synthesis.* (A)
548 Schematic illustrating influenza polymerase activity under the conditions of a cRNP
549 stabilisation assay with ActD. (B-E) cRNP stabilisation assay with ActD in eHAP WT
550 vs dKO cells, following infection with PR8 (MOI=3), 3hpi. Segment 6 (B) vRNA (C)
551 cRNA or (D) mRNA accumulation. Dotted line indicates background RNA present in
552 control samples transfected with a plasmid mix lacking PB1. Fold change was
553 calculated over mock infected cells. n=3 biological replicates, plotted as mean ± s.d..
554 Significance was assessed using an unpaired t-test following log transformation. (E)
555 Accumulation of segment 6 vRNA and +RNA in eHAP WT and dKO cells, analysed
556 using RNAscope. Magenta arrowheads highlight a subset of NA vRNA-stained puncta.
557 Images are representative maximum intensity projections. (F) Schematic illustrating
558 influenza polymerase activity under the conditions of a cRNP stabilisation assay with
559 CHX. (G-I) cRNP stabilisation assay with CHX in eHAP WT, dKO and tKO cells.

560 Segment 4 (G) vRNA (H) cRNA or (I) mRNA accumulation following infection with PR8
561 (MOI=3). Fold change was calculated over input (0hpi). n=3 biological replicates,
562 plotted as mean ± s.d.. Significance was assessed using multiple unpaired t-tests
563 following log transformation, corrected for multiple comparisons using false discovery
564 rate. hpi=hours post infection. ns=not significant; **=p<0.01; ***=p<0.001;
565 ****=p<0.0001.
566
567 *Figure 3. Avian polymerase is not restricted in cRNA synthesis in mammalian cells.*
568 (A) Schematic outlining assay set-up and expected polymerase combinations during
569 replication. As indicated on the left, in the cRNP stabilisation assay, only the first layer
570 of activity (primary cRNA synthesis) will occur. Both layers of activity can occur in
571 replication assays. Unknown polymerase activity is indicated by a dashed black arrow.
572 (B,C) Simultaneous cRNP stabilisation and replication assays with ActD, 6 hpi.
573 Segment 6 (B) vRNA and (C) cRNA accumulation following infection with either 5092E
574 or 5092K as indicated (MOI=0.1). Dotted line indicates levels of vRNA/cRNA present
575 in a control lacking PB2 in the transfection mix. Fold change was calculated over input
576 (0hpi). n=3 biological replicates, plotted as mean ± s.d.. Significance was assessed
577 using one way ANOVA with Dunnett's multiple comparison test, following log
578 transformation. (D,E) Replication assays with ActD and chANP32A, 6 hpi. Segment 6
579 (D) vRNA and (E) cRNA accumulation following infection with either 5092E or 5092K
580 as indicated (MOI=0.1). Dotted line indicates levels of vRNA/cRNA present in a control
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582 biological replicates, plotted as mean ± s.d.. Significance was assessed using one way
583 ANOVA with Dunnett's multiple comparison test, following log transformation. Pre-
584 expressed polymerase mixes: Stabilisation K=PB2 627K/PB1 D446Y/PA/NP;

585 Stabilisation E=PB2 627E/PB1 D446Y/PA/NP; Replication K=PB2 627K/PB1/PA/NP;
586 Replication E=PB2 627E/PB1/PA/NP. hpi=hours post infection. ns=not significant; *=
587 p<0.05; ****=p<0.0001.
588
589 *Figure S1. Supporting data for figure 2.* (A) Matched western blot for Fig. 2B,C,D. (B-
590 F) Validation of replication and stabilisation assays with ActD analysed by RNA FISH.
591 Assays were performed in HEK 293T cells, following infection with PR8 (MOI=3). (B)
592 Accumulation of NA vRNA and +RNA, 3hpi, analysed by RNAscope with indirect
593 immunofluorescence against PB2. Pre-transfected PR8 polymerase mixes are
594 indicated on the left-hand side: FluPol WT=PB2/PB1/PA/NP; FluPol D446Y=PB2/PB1
595 D446Y/PA/NP; -PB1=PB2/PA/NP. Images are representative maximum intensity
596 projections. (C) Matched western blot confirming equal transfection of different
597 polymerase mixtures. (D,E,F) Matched RT-qPCR analysis of segment 6 (D) vRNA (E)
598 cRNA and (F) mRNA accumulation, 6hpi. Pre-transfected polymerase mixes are
599 indicated on the x-axis. Dotted line indicates input levels of RNA (0hpi). Fold change
600 was calculated over mock infected cells. n=3 biological replicates, plotted as mean ±
601 s.d.. Significance compared to –PB1 was assessed using one-way ANOVA with
602 Dunnett's multiple comparison test, following log transformation. (G) Matched western
603 blot for Fig. 2G,H,I. Untransf= Untransfected; hpi=hours post infection. ns=not
604 significant; ****=p<0.0001.
605
606 *Figure S2. Human ANP32 proteins play a direct role in primary cRNA synthesis of*
607 *H5N1 5092.* (A-C) Segment 6 (A) vRNA (B) cRNA or (C) mRNA accumulation over
608 time in eHAP WT vs tKO cells following infection with 5092K (MOI=3). Fold change
609 was calculated over input (0hpi). n=3 biological replicates, plotted as mean ± s.d..

610 Significance at 2hpi was assessed using an unpaired t-test following log
611 transformation. (D,E,F) cRNP stabilisation assay with CHX in eHAP WT and tKO cells.
612 Segment 4 (D) vRNA (E) cRNA or (F) mRNA accumulation following infection with
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616 Significance was assessed using multiple unpaired t-tests following log
617 transformation, corrected for multiple comparisons using false discovery rate.
618 hpi=hours post infection. ns=not significant; *= $p<0.05$; **=$p<0.01$; ***=$p<0.001$;
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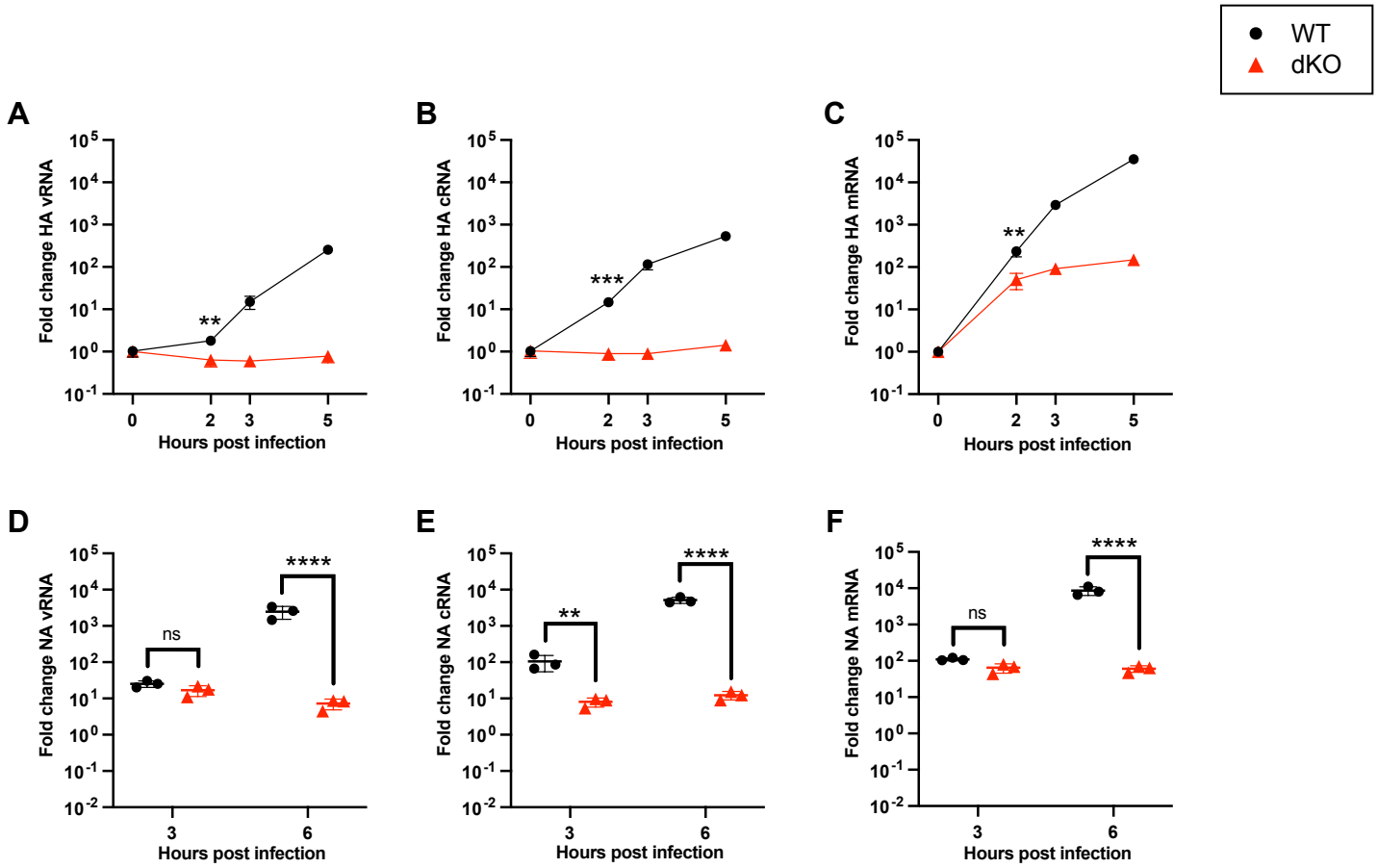
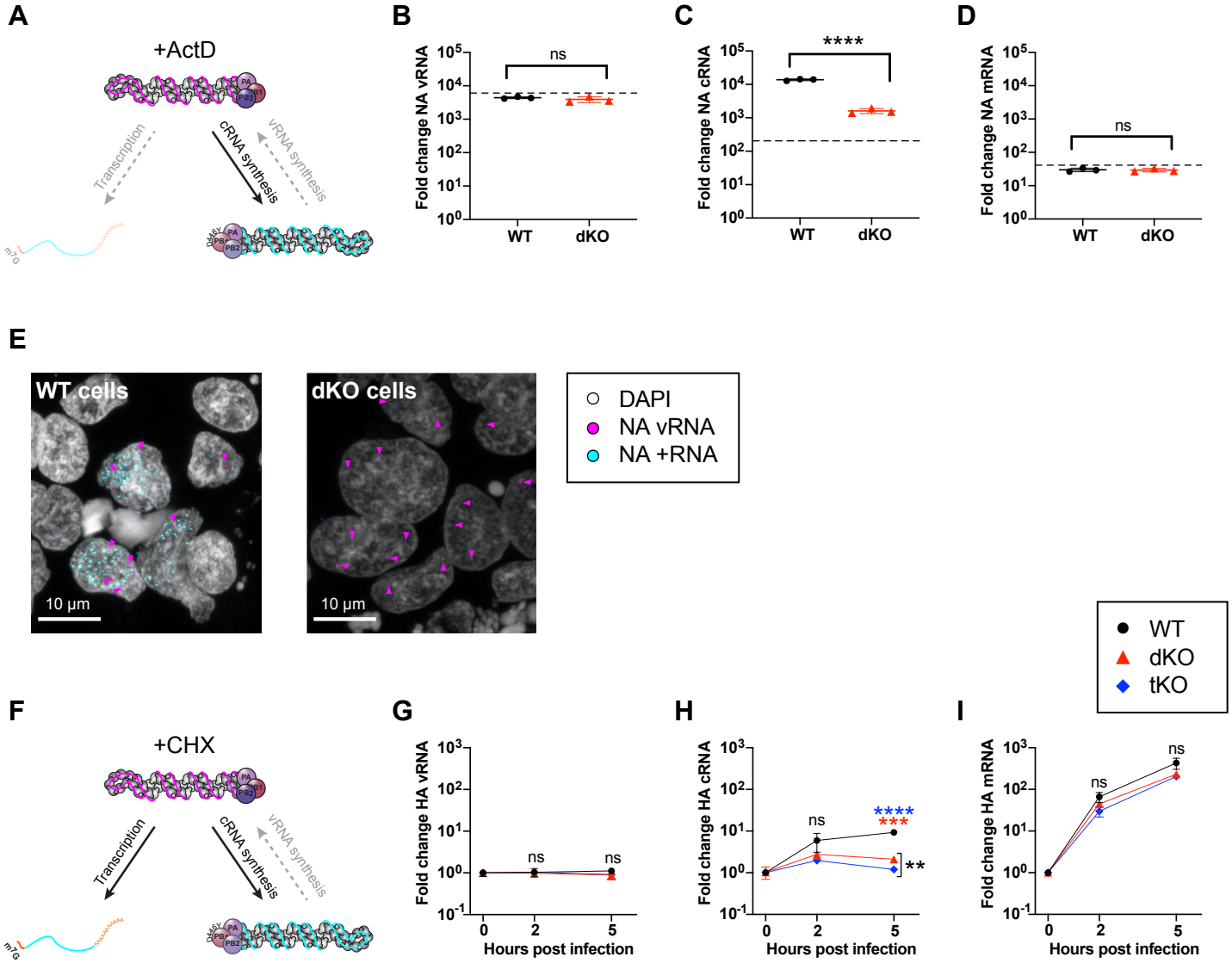


Figure 1. cRNA synthesis is inhibited in human cells lacking ANP32A/B. (A-C) Segment 4 (A) vRNA (B) cRNA or (C) mRNA accumulation over time in eHAP WT vs dKO cells following infection with PR8 (MOI=3). Fold change was calculated over input (0hpi). n=3 biological replicates, plotted as mean ± s.d.. Significance was assessed at 2hpi using an unpaired t-test following log transformation. (D-F) Segment 6 (D) vRNA (E) cRNA or (F) mRNA accumulation over time in eHAP WT vs dKO cells following infection with PR8 (MOI=3). Fold change was calculated over mock infected cells. n=3 biological replicates, plotted as mean ± s.d.. Significance was assessed using multiple unpaired t-tests following log transformation, corrected for multiple comparisons using false discovery rate. hpi=hours post infection. ns=not significant; **=p<0.01; ***=p<0.001; ****=p<0.0001.



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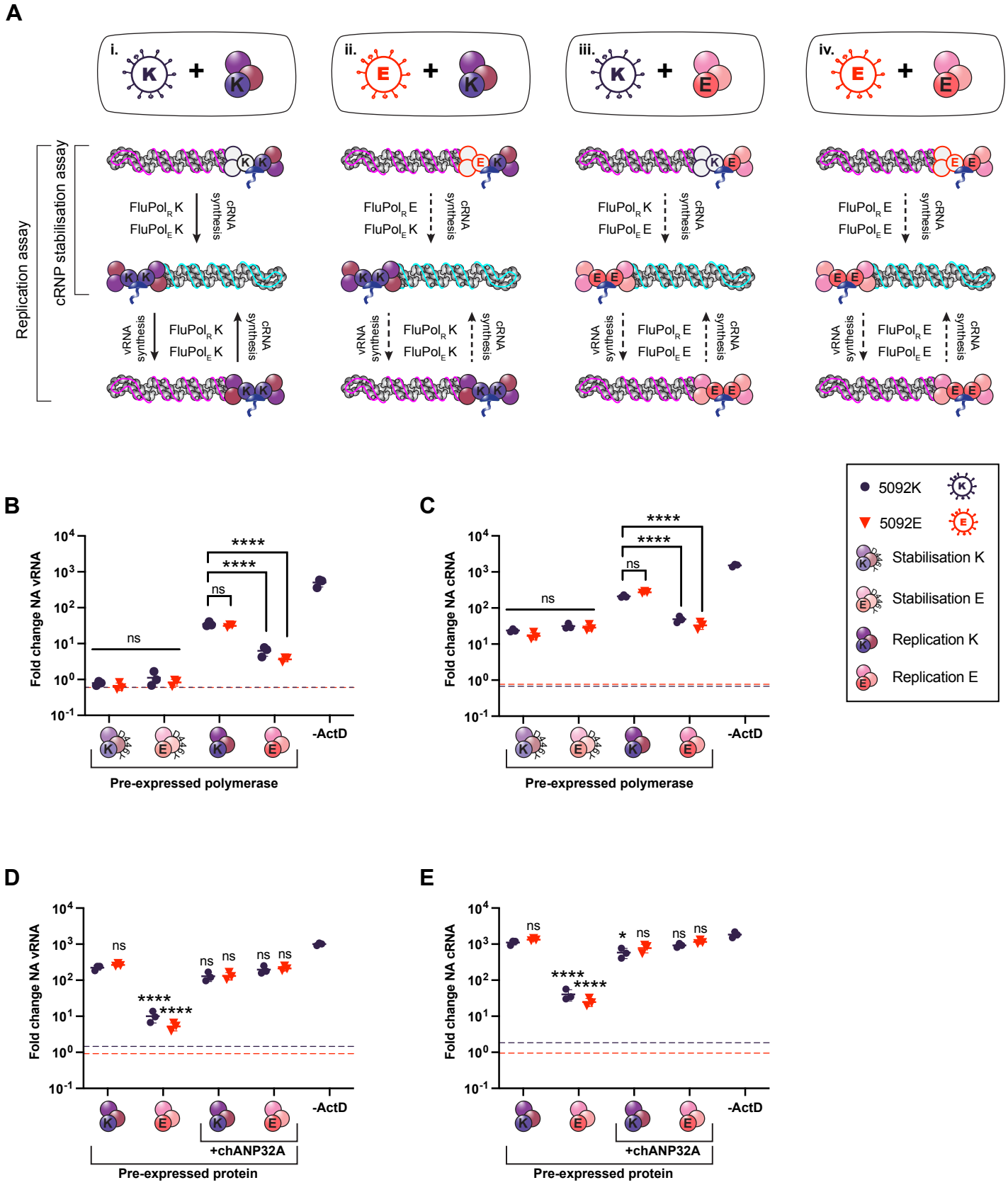
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ii.
iii.
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Replication assay
cRNP stabilisation assay
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FluPol$_E$ K
FluPol$_R$ E
FluPol$_E$ E
cRNA synthesis
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B
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Fold change NA vRNA
Fold change NA cRNA
ns
*
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-ActD
Pre-expressed polymerase
Pre-expressed protein
+chANP32A
5092K
5092E
Stabilisation K
Stabilisation E
Replication K
Replication E

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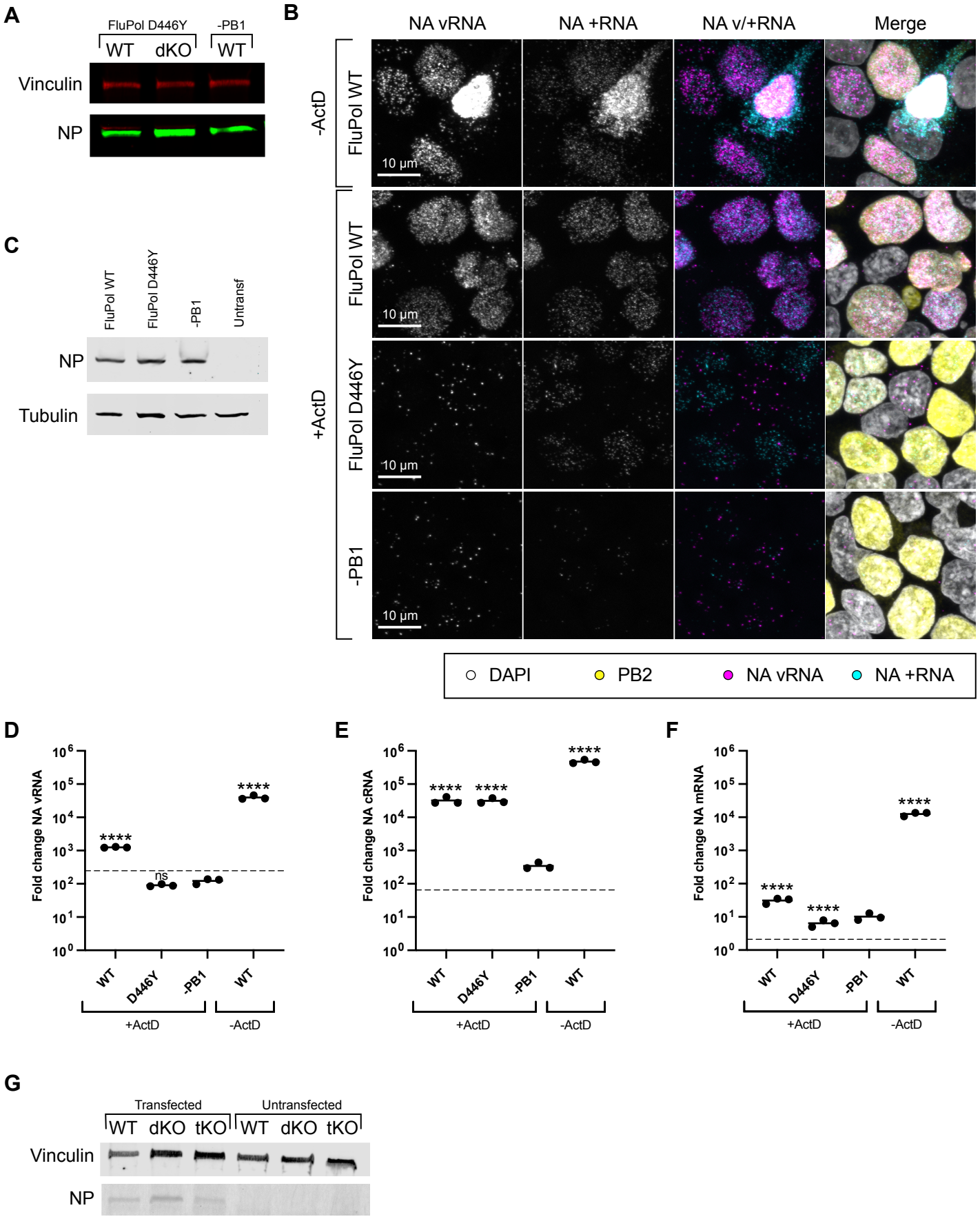
A
FluPol D446Y
-PB1
WT
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Vinculin
NP
B
NA vRNA
NA +RNA
NA v/+RNA
Merge
-ActD
FluPol WT
+ActD
FluPol WT
FluPol D446Y
-PB1
10 µm
DAPI
PB2
NA vRNA
NA +RNA
C
FluPol WT
FluPol D446Y
-PB1
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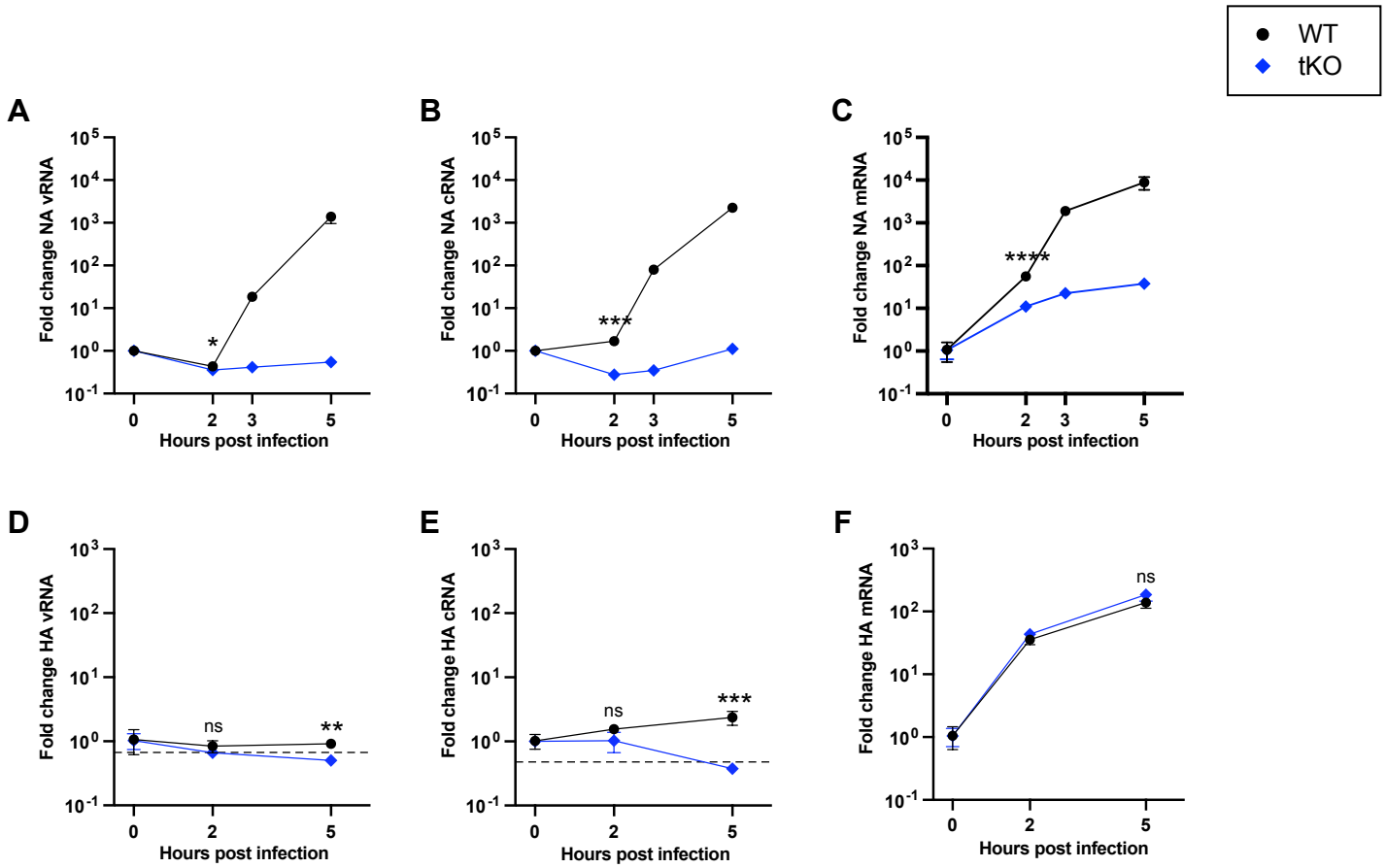

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