

1 **The host factor ANP32A is required for influenza A virus vRNA and cRNA synthesis**

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3 Benjamin E. Nilsson-Payant^{1, #}, Benjamin R. tenOever¹, Aartjan J.W. te Velthuis^{2,3, #}

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5 ¹ Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, USA

6 ² Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK

7 ³ Lewis Thomas Laboratory, Department of Molecular Biology, Princeton University, Princeton, USA

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9 # Corresponding authors: benjamin.nilsson@mssm.edu; aj.te.velthuis@princeton.edu

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15 **ABSTRACT**

16 Influenza A viruses are negative-sense RNA viruses that rely on their own viral replication machinery to
17 replicate and transcribe their segmented single-stranded RNA genome. The viral ribonucleoprotein
18 complexes in which viral RNA is replicated consist of a nucleoprotein scaffold around which the RNA
19 genome is bound, and a heterotrimeric RNA-dependent RNA polymerase that catalyzes viral replication.
20 The RNA polymerase copies the viral RNA (vRNA) via a replicative intermediate, called the complementary
21 RNA (cRNA), and subsequently uses this cRNA to make more vRNA copies. To ensure that new cRNA and
22 vRNA molecules are associated with ribonucleoproteins in which they can be amplified, the active RNA
23 polymerase recruits a second polymerase to encapsidate the cRNA or vRNA. Host factor ANP32A has been
24 shown to be essential for viral replication and to facilitate the formation of a dimer between viral RNA
25 polymerases and differences between mammalian and avian ANP32A proteins are sufficient to restrict viral
26 replication. It has been proposed that ANP32A is only required for the synthesis of vRNA molecules from a
27 cRNA, but not vice versa. However, this view does not match recent molecular evidence. Here we use
28 minigenome assays, virus infections, and viral promoter mutations to demonstrate that ANP32A is essential
29 for both vRNA and cRNA synthesis. Moreover, we show that ANP32 is not only needed for the actively
30 replicating polymerase, but also for the polymerase that is encapsidating nascent viral RNA products.
31 Overall, these results provide new insights into influenza A virus replication and host adaptation.

32

33 **IMPORTANCE**

34 Zoonotic avian influenza A viruses pose a constant threat to global health and they have the potential to
35 cause highly pathogenic pandemic outbreaks. Species variations in host factor ANP32A play a key role in
36 supporting the activity of avian influenza A virus RNA polymerases in mammalian hosts. Here we show that
37 ANP32A acts at two stages in the influenza A virus replication cycle, supporting recent structural
38 experiments and in line with its essential role. Understanding how ANP32A supports viral RNA polymerase
39 activity and how it supports avian polymerase function in mammalian hosts is important for understanding
40 influenza A virus replication and the development of antiviral strategies against influenza A viruses.

41 **INTRODUCTION**

42 Influenza A viruses (IAV) are segmented negative-sense single-stranded RNA viruses that belong to the
43 *Orthomyxoviridae*. IAV viruses infect a wide range of host species including humans, with wild aquatic birds
44 being their natural reservoir (1). Humans are typically infected with seasonal, mammalian adapted IAV
45 strains and only occasionally infected with avian adapted IAV strains. This is in part because zoonotic avian
46 IAV strains are unable to efficiently transmit to and replicate in mammalian cells, unless multiple host species
47 barriers are overcome (2). The two most important adaptations are i) a change in receptor binding specificity
48 from α -2,3-linked sialic acids to α -2,6-linked sialic acids for effective viral entry into human cells (1), and ii)
49 restored binding of the viral RNA-dependent RNA polymerase (RdRp) of avian IAV strains to host factor
50 ANP32A for improved viral replication (3, 4).

51 The IAV RNA polymerase is composed of three subunits called polymerase basic 1 (PB1), PB2, and
52 polymerase acidic (PA). The RNA polymerase replicates and transcribes the IAV genome segments in the
53 context of viral ribonucleoprotein (vRNP) complexes (Fig 1A and 1B). In these vRNPs, the RNA polymerase
54 binds to the ends of a single-stranded negative-sense viral RNA genome segment, while the viral
55 nucleoprotein (NP) associates with the rest of the viral RNA (vRNA) segment in a double helical structure
56 (5). During viral genome replication, vRNA segments are replicated by the viral RdRp through a
57 complementary positive-sense RNA (cRNA) replicative intermediate, which requires at least one additional
58 RNA polymerase to encapsidate the cRNA into a cRNP (5) (Fig 1A). The RNA polymerase in the cRNP next
59 uses the cRNA as template to produce new vRNA genome segments, which is a process that requires at
60 least one additional RdRp complex acting in a *trans*-activating capacity (5), and an RNA polymerase that
61 encapsidates the vRNA into a new vRNP (6, 7) (Fig 1B).

62 One or more steps of avian-adapted IAV replication process are severely impaired in mammalian
63 cells (2, 8), but a single glutamic acid-to-lysine mutation in the PB2 subunit (PB2-E627K) is sufficient to
64 restore the activity of avian RNA polymerases in mammalian host cells (4). The molecular details underlying
65 the impaired activity of avian RNA polymerases in mammalian cells are not fully understood, but several
66 potential mechanisms have been proposed, including destabilized interactions of RNA polymerase and NP
67 during vRNP assembly (9–12), reduced viral promoter RNA binding (13, 14), differential interactions with
68 importin- α (15, 16), inhibitory or activating host factors (17, 18) and unstable cRNP structures of avian IAV
69 in mammalian cells (19). However, expression of acidic (leucine-rich) nuclear phosphoprotein 32 family
70 member A protein (ANP32A) from avian cells alone is sufficient to restore avian IAV RNA polymerase activity
71 in mammalian cells (20), suggesting that the PB2-E627K mutation arises to compensate for an impaired
72 interaction between mammalian ANP32A and the avian IAV RNA polymerase.

73 ANP32A consists of an N-terminal leucine-rich repeat (LRR) domain with four LRR motifs and a C-
74 terminal low-complexity acidic region (LCAR) (21–23). Although the avian and mammalian ANP32A
75 homologues have a high sequence identity, birds contain an additional exon duplication (24, 25). Alternative
76 splicing of the avian ANP32A gene leads to three ANP32A proteins: one containing an additional SUMO

77 interaction motif (SIM)-like and LCAR sequence encoded by an additional exon; one containing an
78 additional LCAR sequencing through partial splicing of the additional exon; and one lacking the extra exon
79 and being similar to the mammalian ANP32A protein (25–27). Restoring avian IAV RdRp activity in
80 mammalian host cells by avian ANP32A overexpression was shown to be dependent on the presence of the
81 avian-specific exon duplication (20). ANP32A's closely related family member ANP32B, which is required for
82 mammalian cell-adapted RdRp function (28, 29), does not appear to play a role in restoring replication of
83 avian-adapted IAV (30).

84 Interaction studies of ANP32A and the viral RNA polymerase have suggested that ANP32A only
85 interacts with the complete heterotrimeric RNA polymerase (27, 28, 31, 32). ANP32A proteins containing
86 the avian signature exon duplication were also shown to have a much stronger affinity for the viral RNA
87 polymerase (26, 27, 33). Presence of the avian glutamic acid residue 627 (627E) on the PB2 subunit
88 significantly weakens the interaction with ANP32A, necessitating the additional ANP32A exon or PB2
89 mutation E627K to stabilize the interaction (34). The determinant of the differential interaction was mapped
90 to the C-terminal LCAR domain of ANP32A, which directly interacts with the flexible PB2 627-domain on
91 the viral RdRp (27, 33).

92 Interestingly, the interaction between ANP32A and the viral RNA polymerase is significantly
93 strengthened in the presence of viral RNA (26, 33), suggesting that ANP32A binds the RNA polymerase in
94 the context of an RNP. Recent cryo-electron microscopy structures of an influenza C virus RNA polymerase
95 dimer revealed that ANP32A formed a bridge between an RNA polymerase bound to RNA and an apo form
96 of the RNA polymerase (7), suggesting that ANP32A mediates the assembly of the viral replicase complex.
97 In line with this idea, ANP32A appears to be required for viral RNA genome replication, but not primary
98 transcription, in both avian and mammalian hosts (20, 28). Moreover, using recombinant purified protein,
99 human ANP32A has been shown to be an enhancer of vRNA synthesis by human-adapted IAV RNA
100 polymerases (28). Based on the latter observation, it has been proposed that ANP32A is only required for
101 the synthesis of vRNA molecules from a cRNA template, but not vice versa. However, both cRNA and vRNA
102 molecules are encapsidated and it is unlikely that IAV evolved separate encapsidation complexes for vRNA
103 and cRNA nascent strands.

104 To investigate at which stage in IAV replication ANP32A is critical, we used a combination of cell-
105 based minigenome assays, single-step promoter mutants, and recombinant IAV strains to systematically
106 characterize the role of ANP32A in IAV genome replication. We find that an IAV RNA polymerase with a
107 627E mutation is unable to efficiently synthesize both vRNA and cRNA, and that expression of avian ANP32A
108 is sufficient for the 627E RNA polymerase to restore both vRNA and cRNA synthesis. Overall, this study
109 provides new insights into the role of ANP32A in mediating IAV replicase function. Together these insights
110 lead to a better understanding of IAV genome replication and host adaptation.

111

112

113 **RESULTS**

114 **Avian ANP32A stimulates infection and replication of IAV containing PB2 627E.** Avian IAV RNA
115 polymerase activity is significantly restricted in mammalian host cells (14, 17, 35) and it has been
116 demonstrated that the expression of avian host protein ANP32A, but not ANP32B, is sufficient to restore
117 avian IAV RNA polymerase activity in mammalian cells (20, 30). However, the exact step at which ANP32A
118 is involved in RNA polymerase activity remains unclear. We set out to systematically analyze the role of
119 ANP32A using PB2 627E as tool and exchanged the lysine of the mammalian-adapted influenza strain
120 A/WSN/33 (H1N1) (abbreviated as WSN) with the avian-specific glutamic acid (WSN-K627E) using reverse
121 genetics. As has been reported previously (17), the avian-like WSN-K627E virus was significantly restricted
122 in both viral replication and transcription in human cells compared to WSN (Fig 2A). However, in avian cells,
123 no significant difference in viral replication and transcription was observed between both viruses. To confirm
124 that infection of human cells with the avian-like WSN-K627E virus would be restored by avian ANP32A, we
125 transiently expressed chicken ANP32A (chANP32A) in HEK-293T cells and infected the cells with either wild-
126 type WSN or avian-like WSN-K627E virus. While WSN-K627E was unable to grow in human cells in the
127 absence of avian ANP32A, expression of chANP32A was sufficient to support WSN-K627E growth similar to
128 the wild-type WSN virus (Fig 2B). Moreover, analysis of the accumulation of viral RNA in human cells showed
129 that transient expression of chANP32A, but not human ANP32A (huANP32A), was able to fully restore
130 restricted viral RNA synthesis by WSN-K627E, while exogenous expression of either chANP32A or
131 huANP32A had no discernible effect on the replication of the wild-type WSN (Fig 2C).

132

133 **Avian ANP32A stimulates replication, but not transcription by 627E-containing RNA polymerases.** In
134 order to confirm that the above observations are linked to the activity of the viral RNA polymerase and no
135 other viral factors, we transiently reconstituted the minimal viral components necessary for genome
136 transcription and replication in HEK-293T cells and analyzed the accumulation of the three viral RNA species
137 cRNA, vRNA and mRNA. In agreement with the above findings, we found that vRNPs containing the PB2-
138 627E subunit were significantly restricted in their activity, showing reduced production of all viral RNA
139 species, and that viral RNA accumulation was restored in the presence of chANP32A (Fig 2D).

140 Accumulation of viral mRNA products is dependent on viral replication, which can confound
141 analysis of the effect of ANP32A. As cellular protein translation is blocked by cycloheximide and protein
142 translation is necessary for viral genome replication, but not transcription, cycloheximide can be used to
143 obtain insight into primary transcription of incoming infecting vRNPs. To confirm that primary transcription
144 by the 627E IAV vRNPs was independent of ANP32A in mammalian cells, we infected human HEK-293T cells
145 at a high MOI with the WSN or WSN-K627E virus in the presence of cycloheximide. In agreement with
146 previous reports, we found that there was no difference in mRNA synthesis, suggesting that the previously
147 observed reduced mRNA levels were a consequence of impaired genome replication and consequently
148 reduced vRNA template levels (Fig 2E).

149 The above result indicates that avian ANP32A is only required for the genome replication of avian-
150 like IAVs and not for their transcription. However, the findings do not prove that mammalian ANP32A plays
151 no role in the transcription of mammalian-adapted IAV RNA polymerases. Therefore, we knocked down
152 both human ANP32A and ANP32B, which have been shown to be redundant in mammalian cells (29), in
153 human alveolar epithelial A549 cells, and infected these cells with WSN at a high MOI. We found that early
154 in the infection cycle, no difference in primary transcription was observed in control and knockdown cells,
155 while a strong reduction in viral genome replication could be seen (Fig 2F and 2G). Overall, our findings
156 suggest that ANP32A is only involved in IAV replication and they confirm previous reports that an IAV RNA
157 polymerase with a 627E residue requires the presence of the avian ANP32A host factor to perform efficient
158 genome replication, but not transcription.

159

160 **The N-terminal third of ANP32A LCAR domain is required for efficient IAV replication.** An exon
161 duplication in the avian ANP32A gene was linked to the stimulation of avian IAV RNA polymerase (20, 24,
162 25). More recently, the central domain of ANP32A immediately upstream of the avian exon duplication was
163 linked to avian IAV RNA polymerase stimulation as well (30). In order to gain a better understanding of the
164 role of the ANP32A domains in IAV replication, internal and C-terminal deletions were introduced into a
165 chANP32A expression construct (Fig 3A and 3B). The ability of these truncated chANP32A proteins to
166 enhance viral replication by an avian-like RNA polymerase was determined using minigenome assays (Fig
167 3C). Deletion of the exon duplication in the chANP32A protein led to a loss in 627E RNA polymerase activity
168 relative to transient expression of the full-length chANP32A. However, in the presence of the exon
169 duplication a C-terminal or an internal truncation of 31 amino acids in the LCAR domain was compatible
170 with 627E RNA polymerase activity, while longer deletions of the LCAR domain led to a loss in 627E RNA
171 polymerase activity. Together, these results suggest that the LCAR domain of ANP32A plays a pivotal role
172 in supporting IAV RNA polymerase activity.

173

174 **Promoter binding is not restricted in avian RNPs.** Some reports have suggested that an avian RdRp
175 forms inherently weaker interactions with the vRNA template than a mammalian adapted IAV RdRp (13, 14).
176 The 627E residue is located far away from the promoter binding site and our previous work has shown that
177 the 627-domain does not affect the basic functions of the RNA polymerase (36). In an effort to exclude that
178 a single point mutation in PB2-627 leads to a significant difference in vRNA promoter binding and possible
179 vRNP assembly, the IAV RNA polymerase, either carrying a PB2-627K (K) or PB2-627E (E) subunit, a
180 catalytically inactive PB1 subunit (D445A/D446A) and a TAP-tagged PA subunit, was transiently expressed
181 in human HEK-293T cells together with a 76 nucleotide-long vRNA based on segment 5. Following
182 immunoprecipitation of the RNA polymerase, bound RNA was extracted and the amount of co-
183 immunoprecipitated vRNA was determined (Fig 4A). We observed that vRNA binding of a PB2-627E carrying

184 RNA polymerase was not impaired compared to a PB2-627K RNA polymerase. In addition, expression of
185 chANP32A did not have any impact on vRNA binding.

186 To get a more quantitative insight into vRNA and cRNA promoter binding activity of the mammalian
187 or avian-adapted IAV RNA polymerase, we performed single-molecule Förster resonance energy transfer
188 (sm-FRET) assays using recombinant catalytically inactive but RNA-binding competent RNA polymerases
189 and short fluorescently labelled viral RNA promoters. In an unbound state, both vRNA and cRNA promoters
190 form largely double-stranded RNA molecules (37) . However, if bound by the IAV RNA polymerase, the 5'
191 and 3' promoter ends are separated in space, which increases the distance between donor and acceptor
192 dyes (Fig 4B and 4C). We observed that the vRNA-bound FRET populations were similar, irrespective of
193 whether the RNA polymerase added to the vRNA carried a lysine or glutamic acid at position 627 or in fact
194 lacked the entire 627-domain (Fig 4D). Moreover, similar FRET populations, with a clear shift from the
195 unbound state, were observed for the cRNA promoter after addition of the different RNA polymerases (Fig
196 4E). Together, these data demonstrate that the 627-domain of the PB2 subunit does not play a role in vRNA
197 or cRNA promoter binding and that the nature of position PB2-627 has no impact on RNA polymerase-
198 RNA binding.

199

200 **ANP32A function in RNA polymerase activity is NP-independent.** The interaction between PB2 and NP
201 has been implicated several times as the cause for avian RNA polymerase restriction in mammalian cells (9–
202 12). Previously, it was reported that avian RNA polymerase activity was less diminished or even not
203 diminished on short viral RNA templates that do not require NP for viral replication (14). In order to
204 determine whether ANP32A's function is NP or vRNA template length dependent, we therefore transiently
205 expressed the IAV RNA polymerase in the presence or absence of NP and chANP32A together with a short
206 segment 5-based 76 nucleotide-long vRNA and analysed the accumulation of viral RNA in this NP-
207 independent minigenome assay (Fig 5A). We find that even in this NP-independent replication assay, the
208 PB2-K627E mutation led to a significant loss in RNA polymerase activity, which was restored in the presence
209 of chANP32A. The absence or presence of NP did not affect these results, suggesting that NP does not play
210 any major role in ANP32A function. Next, we tested whether using shorter vRNA templates affects ANP32A
211 function. Using both a 47 nucleotide-long (Fig 5B) and a 30 nucleotide-long (Fig 5C) segment 6-based vRNA
212 template we confirmed our observation that avian RNA polymerase restriction and the role of ANP32A in
213 IAV RNA replication is independent of both template length and NP.

214

215 **ANP32A is required for both cRNA and vRNA synthesis in mini genome assays.** Several studies have
216 suggested that a pair of mutations in the 3' vRNA promoter (G3A and C8U, Fig 6A) can improve avian RNA
217 polymerase activity in mammalian cells (13, 14). We wondered if we could use the RNA polymerase
218 promoter to uncouple vRNA synthesis and cRNA synthesis, and thus study at what step of viral replication
219 ANP32A is essential. This can only be addressed using minigenome assays, since both cRNA and vRNA

220 synthesis must occur during a viral infection. As a first step towards this aim, we performed minigenome
221 assays using either a construct expressing wild-type segment 6 vRNA or a segment 6 vRNA containing these
222 promoter mutations (3A8U), and found that viral replication was greatly enhanced by these promoter
223 mutations compared to the wild-type vRNA template (Fig 6B). However, even at elevated levels, replication
224 was still significantly, albeit less markedly, restricted by the 627E RNA polymerase. Additional expression of
225 chANP32A fully restored the activity of the 627E RNA polymerase, in line with our observations above.

226 Next, we introduced an additional mutation in the conserved 5' vRNA stem-loop (G5U, Fig 6C) to
227 address whether the 3A8U mutation affects cRNA synthesis. The G5U mutation in the 5' vRNA promoter
228 prevents internal initiation on the resulting cRNA product and therefore only allows primary mRNA as well
229 as primary cRNA synthesis but block subsequent vRNA synthesis and secondary transcription. This becomes
230 especially apparent when combining the G5U and 3A8U mutations (Fig 6D). Interestingly, minigenome
231 assays with vRNA templates carrying the G5U 5' vRNA promoter mutation clearly demonstrated that the
232 627E RNA polymerase mutation prevented efficient primary cRNA synthesis. Activity of the 627E RNA
233 polymerase was restored by the additional expression of chANP32A (Fig 6E). These observations were
234 evident when using both the wild-type or the 3A8U 3' vRNA promoter, lending further proof to our finding
235 that the 3A8U promoter mutation alone is not sufficient to overcome the restriction of the avian-like 627E
236 RNA polymerase.

237 In order to determine whether only cRNA synthesis or also vRNA synthesis is restricted in a 627E
238 RNA polymerase and whether ANP32A is required for efficient vRNA synthesis, we introduced a pair of
239 mutations (2C9G) in the 5' cRNA promoter that would prevent 3' terminal initiation on the resulting vRNA
240 product, thereby abolishing further cRNA synthesis, but not transcription (Fig 6F). Indeed, in a minigenome
241 assay no vRNA synthesis could be observed in the presence of the 2C9G cRNA template and the 627E RNA
242 polymerase (Fig 6G). Additional expression of chANP32A again was able to significantly increase RNA
243 polymerase activity.

244

245 **ANP32A is required for both cRNA and vRNA synthesis during infection.** In order to test whether these
246 results could be recapitulated in the context of an infection and whether avian RNA polymerase restriction
247 occurs at the level of the actively replicating RNA polymerase or at the level of a secondary RdRp necessary
248 to form nascent RNP, we assessed the ability of an incoming vRNP to synthesize cRNA, and of the resulting
249 cRNP to perform further genome replication. Specifically, we pre-expressed either catalytically inactive
250 (PB1a) or active (wt) RNA polymerase, NP and chANP32A as indicated, before infecting cells with WSN in
251 the presence of the cellular transcription inhibitor Actinomycin D (Fig 6H). Therefore, viral replication is
252 entirely reliant on the presence of pre-expressed viral components. In the absence of IAV RNA polymerase,
253 no cRNA stabilization could be observed. Both PB2-627K or PB2-627E RNA polymerases were able to
254 stabilize nascent cRNA equally well when active replication by pre-expressed RNA polymerases was
255 prevented by the D445A/D446A mutation in the PB1 subunit. However, when further replication was

256 enabled by pre-expression of a catalytically active RNA polymerase, reduced levels of vRNA synthesis were
257 observed by the now actively replicating PB2-627E RNA polymerase, which was overcome by the addition
258 of chANP32A.

259 Overall, these results suggest that IAV RNA polymerases containing a PB2-K627E mutation are impaired at
260 the level of the actively replicating RNA polymerase and not the encapsidating RNA polymerase that is
261 required for nascent RNA binding. Furthermore, it also demonstrates that avian ANP32A is only required
262 for the actively replicating and not the encapsidating 627E RNA polymerase.

263

264

265 **DISCUSSION**

266 The RNA polymerase activity of avian IAV is significantly impaired in mammalian host cells and it has been
267 proposed that host factor ANP32A may restrict one step of the viral replication process. Here, we report
268 that ANP32A is required for both efficient vRNA as well as cRNA synthesis, but not viral transcription. This
269 is in agreement with previous studies (20, 28). In addition, we found that the N-terminal part of the LCAR
270 of ANP32A is critical for successful IAV RNA polymerase stimulation, in line with previous reports (27, 38).
271 Moreover, we show that the reduced activity of an IAV RNA polymerase containing PB2-627E in mammalian
272 cells is independent of vRNA or cRNA promoter binding, in contrast with previous reports that implicated
273 weaker promoter binding capabilities of avian-adapted RNA polymerases (13, 14). These findings clearly
274 confirm that ANP32A plays a more fundamental role in IAV genome. In addition, while a previous study
275 found that avian RdRp restriction was diminished or altogether abolished when using shorter vRNA
276 templates, we found that an inability to replicate vRNA templates remained, even when using templates of
277 30 nucleotides in length (14).

278 Our finding that avian-adapted RNA polymerases are able to stabilize nascent cRNA products
279 equally well in human host cells as mammalian-adapted RNA polymerases is in agreement with previous
280 studies (19, 36). However, here we additionally demonstrate that avian-adapted cRNPs are unable to
281 produce vRNA efficiently, suggesting that vRNA synthesis is impaired in the absence of the species-specific
282 ANP32A host protein. Furthermore, we also illustrate that not only vRNA synthesis is restricted, but that in
283 the context of minigenome assays, where only single-round replication cycles are permitted, both vRNA
284 and cRNA synthesis are inhibited by the PB2-K627E mutation and subsequently restored by the expression
285 of chANP32A. These data are consistent with recent structural findings where ANP32A is found to mediate
286 the assembly of the IAV replicase complex consisting of an actively replicating vRNP bound by ANP32A
287 which recruits an encapsidating RdRp (7).

288 In summary, these findings confirm that IAVs hijack ANP32A to facilitate viral genome replication
289 by mediating assembly of the replicase complex. It is tempting to speculate that IAVs evolved this
290 dependency in avian species. Because the mammalian ANP32A is shorter than its avian homologue, it
291 cannot efficiently support assembly of the IAV replicase complex at both stages of viral replication. Zoonotic

292 IAV strains must therefore acquire adaptive mutations to restore the ANP32A-RNA polymerase interaction.
293 These findings strengthen previous hypotheses about IAV host range restriction and shed further light on
294 the intricate details of IAV genome replication.

295

296

297 **MATERIALS AND METHODS**

298

299 **Cell Culture.** Human alveolar basal epithelial carcinoma cells (A549, ATCC, CCL-185), human embryonic
300 kidney cells (HEK-293T, ATCC, CRL-3216), chicken embryonic fibroblasts (DF-1, ATCC, CRL-12203) and
301 Madin-Darby Canine kidney epithelial cells (MDCK, ATCC, CCL-34) were commercially obtained. All cells
302 were maintained at 37°C and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with
303 10% Fetal Bovine Serum (FBS) and penicillin and streptomycin.

304

305 **Plasmids.** Plasmids pcDNA-NP, pcDNA-PA, pcDNA-PB1, pcDNA-PB2, pcDNA-3a (45), pcDNA-PB1a (46),
306 pcDNA-PB2-K627E (10), pcDNA-PB2Δ535-667 (36), pcDNA-PA-TAP (47), pPOLI-NA (48), pPOLI-cNA (49),
307 pPOLI-NA47 (14), pPOLI-NP76 (50), as well as the pHW2000 constructs expressing the eight influenza
308 A/WSN/33 (H1N1) virus segments (51) have been described previously.

309 Plasmids pCMV-3xFLAG-GFP, pCMV-3xFLAG-huANP32A and pCMV-3xFLAG-chANP32A were generated by
310 InFusion cloning of coding sequences for GFP, huANP32A and chANP32A (synthesized by Integrated DNA
311 Technologies) into an empty pCMV-3Tag-1A vector (Agilent Technologies).

312 Plasmids pHW2000-PB2-K627E, pPOLI-NA-3A8U, pPOLI-NA-G5U, pPOLI-NA-3A8U/G5U and pPOLI-cNA-
313 2C9G were generated by site-directed PCR mutagenesis using the primers detailed in Table 1. Plasmids
314 pPOLI-NA30, pCMV-3xFLAG-chANP32AΔ180-208, pCMV-3xFLAG-chANP32AΔ209-281, pCMV-3xFLAG-
315 chANP32AΔ220-281, pCMV-3xFLAG-chANP32AΔ251-281 and pCMV-3xFLAG-chANP32AΔ220-250 were
316 generated by deletion PCR mutagenesis using primers detailed in Table 1.

317

318 **Virus infections.** Recombinant influenza A/WSN/33 (H1N1) virus was generated using the pHW2000
319 eight-plasmid reverse genetics system as previously described (51). To generate a recombinant virus with
320 the avian signature lysine-to-glutamic acid point mutation at residue 627 of the PB2 segment, virus rescue
321 was performed with either the wild-type pHW2000-PB2 (WSN) or the mutated pHW2000-PB2-K627E (WSN-
322 K627E) segment.

323 For viral infections, approximately 1 × 10⁶ human HEK-293T or chicken DF-1 cells were infected with WSN
324 or WSN-K627E at an MOI of 1 in DMEM supplemented with 0.5% FBS.

325 For analysis of primary viral transcription, infections were performed in the presence of 200 µg/ml
326 cycloheximide at an MOI of 10 in DMEM supplemented with 0.5% FBS.

327 For viral infections of transiently transfected cells, approximately 1×10^6 human HEK-293T cells were
328 transiently transfected with 1 μ g of pCMV-3xFLAG-chANP32A, pCMV-3xFLAG-huANP32A or pCMV-
329 3xFLAG-GFP using Lipofectamine 2000 and Opti-MEM according to the manufacturer's instructions. 24 h
330 post-transfection, cells were infected at an MOI of 1 in DMEM supplemented with 0.5% FBS.

331 Total RNA was extracted from all infected samples at 6 h post-infection using TRIzol (Invitrogen) and
332 reconstituted in 20 μ l nuclease-free water.

333

334 **Viral growth curves.** Approximately 1×10^6 HEK-293T cells were transiently transfected with 1 μ g of
335 pCMV-3xFLAG-chANP32A or pCMV-3xFLAG-GFP using Lipofectamine 2000 and Opti-MEM according to
336 the manufacturer's instructions. 24 h post-transfection, cells were infected with influenza A/WSN/33 (H1N1)
337 virus bearing either the wild-type PB2-627K or the avian signature PB2-627E amino acid residue at an MOI
338 of 0.001 in DMEM supplemented with 0.5% FBS. Cell culture media containing viral particles was collected
339 at 6, 24 and 48 h post-infection. The concentration of infectious viral particles (plaque-forming units/ml)
340 was determined by plaque assay on MDCK cells.

341

342 **RNP reconstitutions.** In order to reconstitute vRNPs in a minigenome assay, approximately 1×10^6 HEK-
343 293T cells were transiently transfected with 1 μ g of pcDNA-PA, pcDNA-PB1/pcDNA-PB1a, pcDNA-
344 PB2/pcDNA-PB2-K627E, pcDNA-NP, pCMV-3xFLAG-chANP32A/pCMV-3xFLAG-huANP32A/pCMV-
345 3xFLAG-GFP and a plasmid expressing a vRNA or cRNA segment (pPOLI-NA, pPOLI-cNA, pPOLI-NA-3A8U,
346 pPOLI-G5U, pPOLI-3A8U-G5U, pPOLI-cNA-2C9G) using Lipofectamine 2000 and Opti-MEM according to
347 the manufacturer's instructions. When using plasmids encoding short truncated vRNA templates (pPOLI-
348 NP76, pPOLI-NA47, pPOLI-NA30), transfections were performed in the absence of pcDNA-NP unless
349 otherwise stated. To ensure that equal amounts of DNA was transfected in all conditions, an empty pcDNA-
350 3a vector was used to balance the transfections. Total RNA was extracted 48 h post-transfection using TRIzol
351 (Invitrogen) and reconstituted in 20 μ l of nuclease-free water.

352

353 **Primer extension analysis.** The accumulation of viral mRNA, cRNA and vRNA was analyzed by primer
354 extension using 32 P-labelled primers specific for negative- or positive-sense segment 6 RNA as well as 5S
355 rRNA as an internal loading control as described previously (52). Primer sequences are detailed in Table 1.
356 Primer extension products were analyzed by 6% 7 M urea PAGE for full-length RNA templates or 12% 7 M
357 urea PAGE for truncated RNA templates and detected by autoradiography. ImageJ was used to analyze and
358 quantify 32 P-derived detected signal (53).

359

360 **siRNA-mediated knockdown of ANP32A/ANP32B.** Approximately 1×10^6 A549 cells were transfected
361 with 20 nM ON-TARGETplus Non-Targeting Control Pool (Dharmacon, D-001810-10-20) or 20 nM
362 siGENOME Human ANP32A siRNA SMARTpool (Dharmacon, M-016060-00-0005) and siGENOME Human

363 ANP32B siRNA SMARTpool (Dharmacon, M-020148-01-0005) using Lipofectamine RNAiMax and Opti-
364 MEM according to the manufacturer's instructions. 48 h post-transfection, cells were either lysed for
365 Western blot analysis or infected with influenza A virus/WSN/33 (H1N1) virus.

366 For infections, cells were washed with PBS prior to infection, followed by infection with influenza A/WSN/33
367 (H1N1) virus at an MOI of 1 in DMEM supplemented with 0.3% BSA for 15 h. Total RNA was extracted using
368 TRIzol (Invitrogen) and reconstituted in 20 μ l of nuclease-free water.

369

370 **Western blot analysis.** For Western blot analysis, cells were lysed in NP-40 lysis buffer containing 1 \times
371 cComplete Protease Inhibitor Cocktail (Roche) and 1 \times Phenylmethylsulfonyl fluoride (Sigma Aldrich) and
372 cleared from the insoluble fraction by centrifugation at 17,000 \times g for 5 min at 4°C. Samples were analyzed
373 by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with mouse
374 monoclonal anti-Actin (Thermo Scientific, MS-1295), mouse monoclonal anti-FLAG M2 (Sigma-Aldrich,
375 F1804), rabbit polyclonal anti-PHAP1 (Abcam, ab51013) and rabbit monoclonal anti-PHAPI2 (Abcam,
376 ab200836) antibodies. Primary antibodies were detected using HRP-conjugated secondary anti-mouse (GE
377 Healthcare, NA931V) and anti-rabbit (GE Healthcare, NA934V) antibodies and visualized using Immobilin
378 Western Chemiluminescent HRP substrate kit (Millipore) according to the manufacturer's instructions.

379

380

381 **RNA-binding assay.** Approximately 1 \times 10⁶ HEK-293T cells were transiently transfected with 1 μ g of
382 pcDNA-PA/pcDNA-PA-TAP, pcDNA-PB1a, pcDNA-PB2/pcDNA-PB2-K627E, pPOLI-NA47 and pCMV-
383 3xFLAG-chANP32A/pCMV-3xFLAG-GFP using Lipofectamine 2000 and Opti-MEM according to the
384 manufacturer's instructions. 48 h post-transfection, cells were lysed for 1 h at 4°C in 100 μ l lysis buffer (50
385 mM Tris-HCl [pH=8.0], 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM Phenylmethylsulfonyl fluoride, 1 mM
386 Na₃VO₄, 30 mM NaF, 10% glycerol, 1 \times cComplete Protease Inhibitor Cocktail). After clarification of lysates
387 from cellular debris by centrifugation (17,000 \times g, 5 min, 4°C), lysates were incubated for 24 h with
388 equilibrated IgG Sepharose 6 Fast Flow beads at 4°C. Beads were washed three times for 10 min at 4°C with
389 500 μ l wash buffer (10 mM Tris-HCl [pH=8.0], 150 mM NaCl, 0.1% NP-40, 10% glycerol). Beads were
390 collected by centrifugation (17,000 \times g, 5 min, 4°C) and supernatants were discarded. RNA was extracted
391 from whole cell lysates and IgG Sepharose beads using TRIzol and 20 μ g GlycoBlue Coprecipitant
392 (Invitrogen) according to the manufacturer's instructions. RNA was analysed by primer extension and 12%
393 7M urea PAGE as described above.

394

395 **Recombinant RdRp purification and smFRET.** Recombinant influenza A/WSN/33 (H1N1) polymerase
396 subunits containing a PB2 627K, 627E or delta 627 domain and a protein-A tag on the C-terminus of the PA
397 subunit were expressed in HEK-293T cells and purified using IgG-sepharose as described previously (54,

398 55). RNA and RNA polymerase were prepared and interactions between the molecules measured on a
399 custom-build confocal microscope as described previously. (54, 55)

400

401 **cRNA/vRNA stabilization assay.** The ability of the viral RdRp to either stabilize nascent cRNA products
402 during primary viral replication or to facilitate replication of both vRNA and cRNA, was tested by transiently
403 transfected approximately 1×10^6 HEK-293T cells with 1 μ g of pcDNA-PA, pcDNA-PB1/pcDNA-PB1a,
404 pcDNA-PB2/pcDNA-PB2-K627E, pcDNA-NP and pCMV-3xFLAG-chANP32A/pCMV-3xFLAG-GFP as
405 indicated using Lipofectamine 2000 and Opti-MEM according to the manufacturer's instructions. 48 h post-
406 transfection, cells were infected with influenza A/WSN/33 (H1N1) virus in the presence of 5 μ g/ml
407 Actinomycin D at an MOI of 5 in DMEM supplemented with 0.5% FBS. Total RNA was extracted 6 h post-
408 infection using TRIzol (Invitrogen) and reconstituted in 20 μ l of nuclease-free water. The accumulation of
409 viral mRNA, cRNA and vRNA was analyzed by primer extension using 32 P-labelled primers specific for
410 negative- or positive-sense segment 6 RNA as well as 5S rRNA as an internal loading control as described
411 previously (52). Primer extension products were analyzed by 6% 7 M urea PAGE in TBE buffer and detected
412 by autoradiography. ImageJ was used to analyze and quantify 32 P-derived detected signal (53).

413

414

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558

559

560 **FIGURE LEGENDS**

561

562 **FIG 1** Influenza A virus genome replication. **(A)** Schematic of vRNA-to-cRNA replication by the resident
563 RNA polymerase in vRNPs. Nascent cRNA is encapsidated by a newly synthesized RNA-free polymerase.
564 **(B)** Schematic of cRNA-to-vRNA replication by the resident RNA polymerase aided by a second regulatory
565 polymerase. Nascent vRNA is being encapsidated by a newly synthesized RNA-free polymerase.

566

567 **FIG 2** ANP32A is required for IAV genome replication. **(A)** Human HEK-293T cells or chicken DF-1 cells
568 were infected with influenza A/WSN/33 (H1N1) virus carrying either a lysine (K) or glutamic acid (E) on
569 residue 627 of the PB2 subunit for 6 h at an MOI of 1. The accumulation of viral RNA was analyzed by primer
570 extension and 6% PAGE. The graph shows the relative mean intensity of viral RNA normalized to 5S rRNA
571 from three independent biological replicates. **(B)** HEK-293T cells were transiently transfected with plasmids
572 expressing GFP or chANP32A. 24 h post-transfection, cells were infected with mammalian- (K) or avian- (E)
573 adapted influenza A/WSN/33 (H1N) at an MOI of 0.01. Infectious viral titers were determined by plaque
574 assay at the indicated timepoints post-infection from three independent biological replicates. **(C)** HEK-
575 293T cells were transfected with plasmids expressing huANP32A or chANP32A or GFP as a negative control.
576 24h post-transfection, cells were infected with wild-type influenza A/WSN/33 (H1N1) virus (WSN) or virus
577 with a PB2-K627E mutation (WSN-K627E) at an MOI of 1 for 6 h. The accumulation of viral RNA was analyzed
578 by primer extension and 6% PAGE. The graph shows the relative mean intensity of viral RNA normalized to
579 5S rRNA from three independent biological replicates. **(D)** HEK-293T cells were transfected with plasmids
580 expressing NP, PA, PB1, PB2 (627K or 627E), segment 6 vRNA and huANP32A, chANP32A or GFP. 48h post-
581 transfection, the accumulation of viral RNA was analyzed by primer extension and 6% PAGE. The graph
582 shows the relative mean intensity of viral RNA normalized to 5S rRNA from three independent biological
583 replicates. **(E)** HEK-293T cells were infected with wild-type influenza A/WSN/33 (H1N1) virus (WSN) or virus
584 with a PB2-K627E mutation (WSN-K627E) at an MOI of 10 for 6 h in the presence of cycloheximide. The
585 accumulation of viral RNA was analyzed by primer extension and 6% PAGE. The graph shows the relative
586 mean intensity of viral RNA normalized to 5S rRNA from three independent biological replicates. **(F-G)**
587 A549 cells were transfected with siRNA targeting human ANP32A and ANP32B. 48 h post-transfection cells
588 were either **(F)** lysed for Western blot analysis for ANP32A and ANP32B expression or **(G)** infected with
589 influenza A/WSN/33 (H1N1) virus (WSN) at an MOI of 1 for 12 h. The accumulation of viral RNA was analyzed
590 by primer extension and 6% PAGE. Error bars represent the standard deviation of the means and asterisks
591 represent a significant difference from the control group (two-tailed one-sample t test) as follows: ns, $P >$
592 0.05 ; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

593

594 **FIG 3** The chANP32A LCAR domain is essential for avian RdRp stimulation. **(A)** Schematic of the wild-
595 type chANP32A protein and the internal and terminal truncations introduced in this study. **(B)** HEK-293T

596 cells were transfected with plasmids expressing NP, PA, PB1, PB2 or PB2-K627E, segment 6 vRNA and either
597 wild-type or truncated chANP32A. 48h post-transfection, the accumulation of viral RNA was analyzed by
598 primer extension and 6% PAGE. The graph shows the relative mean intensity of viral RNA normalized to 5S
599 rRNA from three independent biological replicates. Error bars represent the standard deviation of the means
600 and asterisks represent a significant difference from the control group (two-tailed one-sample t test) as
601 follows: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

602

603 **FIG 4** The PB2 627-domain does not affect viral RNA binding. **(A)** HEK-293T cells were transfected with
604 plasmids expressing PA, PB2 (627K or 627E), catalytically inactive PB1, a 47 nucleotide-long internally
605 truncated segment 6 and where indicated chANP32A. 48 h post-transfection, cells were lysed and the viral
606 RdRp was immunoprecipitated via a TAP-tag on PA. Co-precipitated vRNA was analyzed by primer
607 extension and 12% PAGE. **(B)** Schematic of the influenza A virus vRNA promoter before and after binding
608 with the viral RdRp. The distance between the Atto647N dye (red) and the Cy3 dye (orange) is indicated.
609 **(C)** Schematic of the influenza A virus cRNA promoter before and after binding with the viral RdRp. The
610 distance between the Atto647N dye (red) and the Cy3 dye (orange) is indicated. **(D)** vRNA promoter
611 binding by the influenza A virus RdRp as analyzed by smFRET. Recombinant viral RdRp, either with a PB2
612 subunit with a lysing (627K) or glutamic acid (627E) at position 627 or with a subunit lacking the entire 627-
613 domain (Δ 535-667) was used. The FRET populations were fit with a single Gaussian curve. The RNA only
614 (red) is shown as comparison. The means of the bound (black dotted line) and unbound (red dotted line)
615 signal is indicated in each graph. **(E)** cRNA promoter binding by the influenza A virus RdRp as analyzed by
616 smFRET. Recombinant viral RdRp, either with a PB2 subunit with a lysing (627K) or glutamic acid (627E) at
617 position 627 or with a subunit lacking the entire 627-domain (Δ 535-667) was used. The FRET populations
618 were fit with a double Gaussian curve representing the two conformations that the cRNA promoter adopts
619 in a pre-initiation state. The unbound RNA only (red line) is shown as comparison.

620

621 **FIG 5** Avian RdRp restriction in mammalian cells is independent of NP. **(A)** HEK-293T cells were
622 transfected with plasmids expressing a 76 nucleotide-long internally truncated segment 5 vRNA, PA, PB1,
623 PB2 and where indicated NP and chANP32A. 48 h post-transfection, the accumulation of viral RNA was
624 analyzed by primer extension and 12% PAGE. **(B)** HEK-293T cells were transfected with plasmids expressing
625 a 47 nucleotide-long internally truncated segment 6 vRNA, PA, PB1, PB2 and where indicated chANP32A.
626 48 h post-transfection, the accumulation of viral RNA was analyzed by primer extension and 12% PAGE.
627 The graph shows the relative mean intensity of viral RNA normalized to 5S rRNA from three independent
628 biological replicates. **(C)** HEK-293T cells were transfected with plasmids expressing a 30 nucleotide-long
629 internally truncated segment 6 vRNA, PA, PB1, PB2 and where indicated chANP32A. 48 h post-transfection,
630 the accumulation of viral RNA was analyzed by primer extension and 12% PAGE. The graph shows the
631 relative mean intensity of viral RNA normalized to 5S rRNA from three independent biological replicates.

632 Error bars represent the standard deviation of the means and asterisks represent a significant difference
633 from the control group (two-tailed one-sample t test) as follows: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.
634

635

636 **FIG 6** ANP32A is required for both vRNA and cRNA synthesis. **(A)** Schematic of the wild-type vRNA
637 promoter or a vRNA promoter carrying a G3A and C8U mutation in the 3' vRNA promoter (red). **(B)** HEK-
638 293T cells were transfected with plasmids expressing NP, PA, PB1, PB2 (627K or 627E), segment 6 vRNA and
639 chANP32A where indicated. 48h post-transfection, the accumulation of viral RNA was analyzed by primer
640 extension and 6% PAGE. The graph shows the relative mean intensity of viral RNA produced from the
641 mutant 3A8U vRNA template normalized to 5S rRNA from three independent biological replicates. **(C)**
642 Schematic of the vRNA promoter carrying a G5U mutation in the 5' vRNA promoter with and without the
643 3A8U 3' vRNA promoter mutation. **(D)** HEK-293T cells were transfected with plasmids expressing NP, PA,
644 PB1, PB2 (627K or 627E), segment 6 vRNA and chANP32A where indicated. 48h post-transfection, the
645 accumulation of viral RNA was analyzed by primer extension and 6% PAGE. The graph shows the relative
646 mean intensity of cRNA normalized to 5S rRNA from three independent biological replicates. **(E)** Schematic
647 of the wild-type cRNA promoter or a cRNA promoter carrying a G2C and C9G mutation in the 5' cRNA
648 promoter. **(F)** HEK-293T cells were transfected with plasmids expressing NP, PA, PB1, PB2 (627K or 627E),
649 segment 6 cRNA and chANP32A where indicated. 48h post-transfection, the accumulation of viral RNA was
650 analyzed by primer extension and 6% PAGE. The graph shows the relative mean intensity of vRNA produced
651 from the mutant 2C9G cRNA template normalized to 5S rRNA from three independent biological replicates.
652 **(G)** HEK-293T cells were transfected with NP, PA, PB1 or catalytically inactive PB1a, PB2 (627K or 627E) and
653 chANP32A where indicated. 24 h post-transfection, cells were infected with influenza A/WSN/33 (H1N1)
654 virus at an MOI of 5 for 6 h in the presence of Actinomycin D. The accumulation of viral RNA was analyzed
655 by primer extension and 6% PAGE. The graph shows the relative mean intensity of viral RNA normalized to
656 5S rRNA from four independent biological replicates. Error bars represent the standard deviation of the
657 means and asterisks represent a significant difference from the control group (two-tailed one-sample t test)
658 or between two non-control groups (two-tailed two-sample t test) as follows: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

660

661

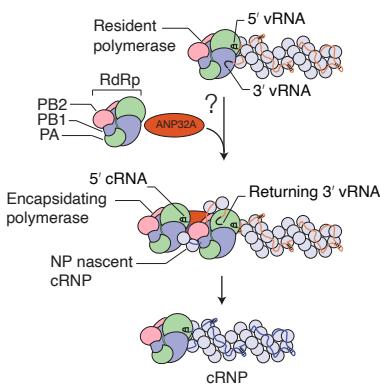
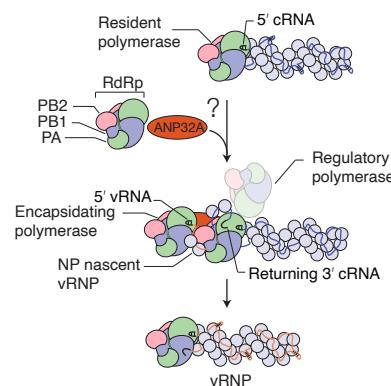
FIGURE 1**A****B**

FIGURE 2

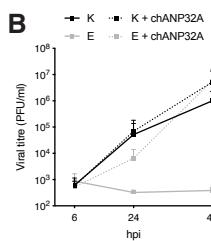
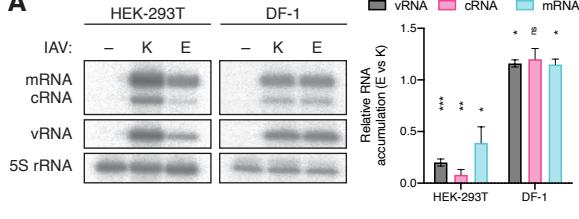
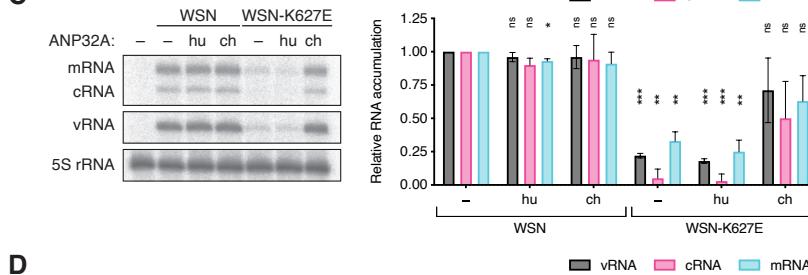
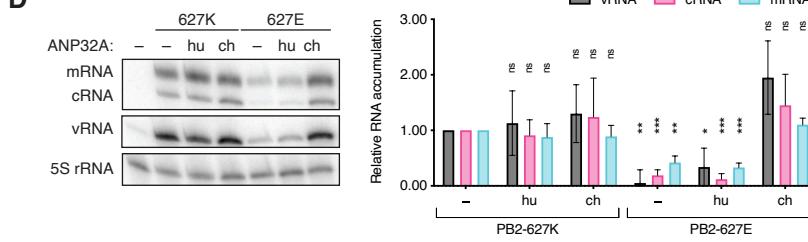
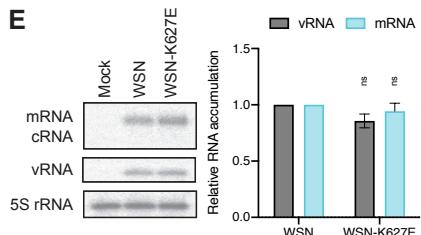
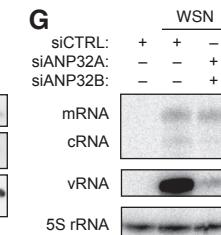
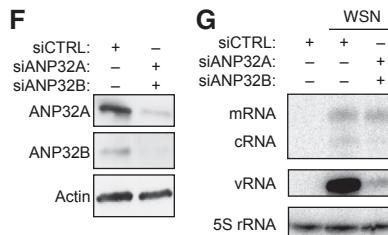
A**C****D****E****F**

FIGURE 3

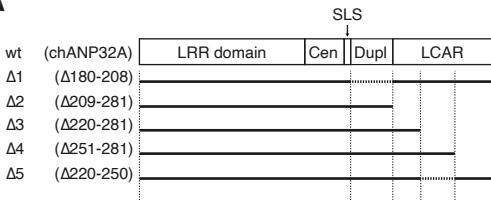
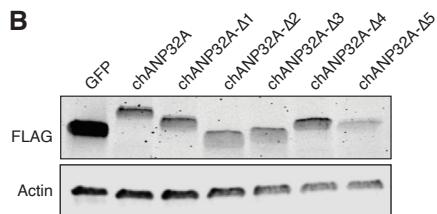
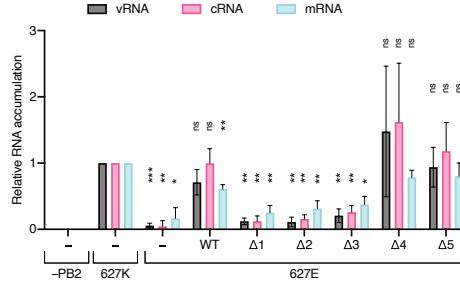
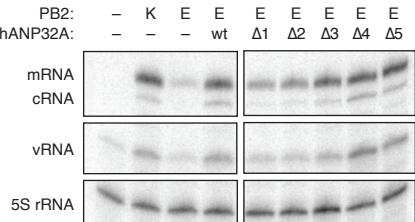
A**B****C**

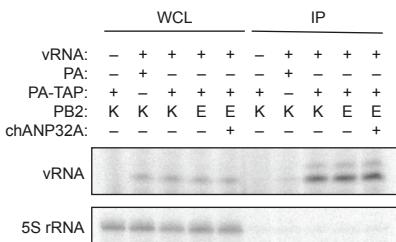
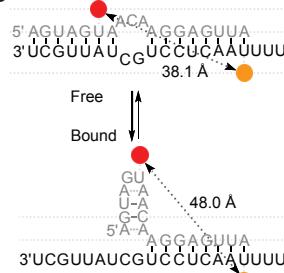
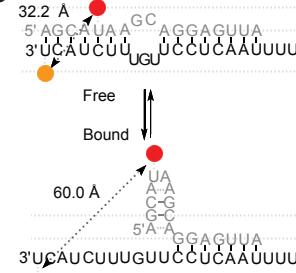
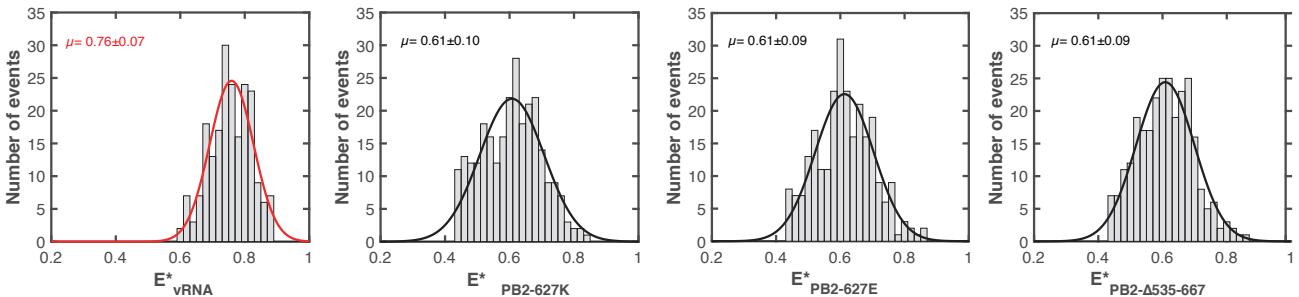
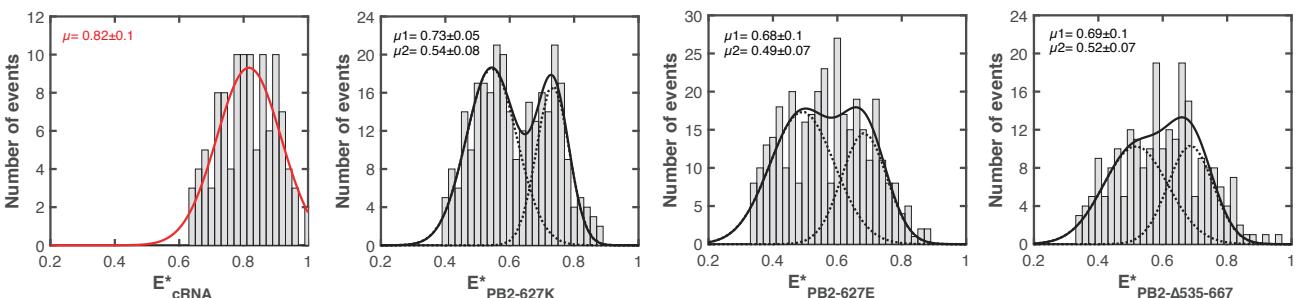
FIGURE 4**A****B****C****D****E**

FIGURE 5

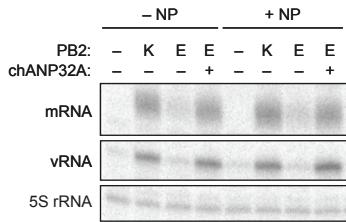
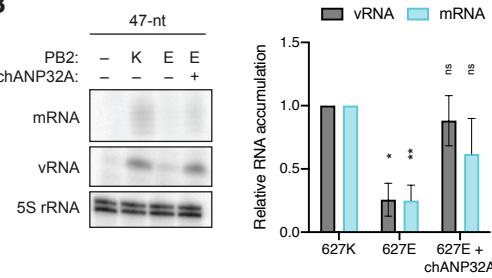
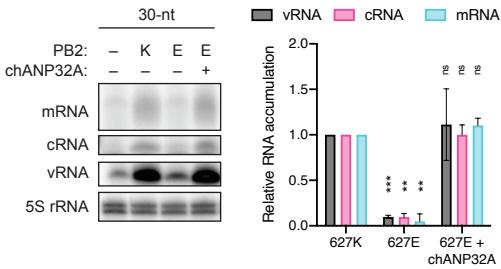
A**B****C**

FIGURE 6

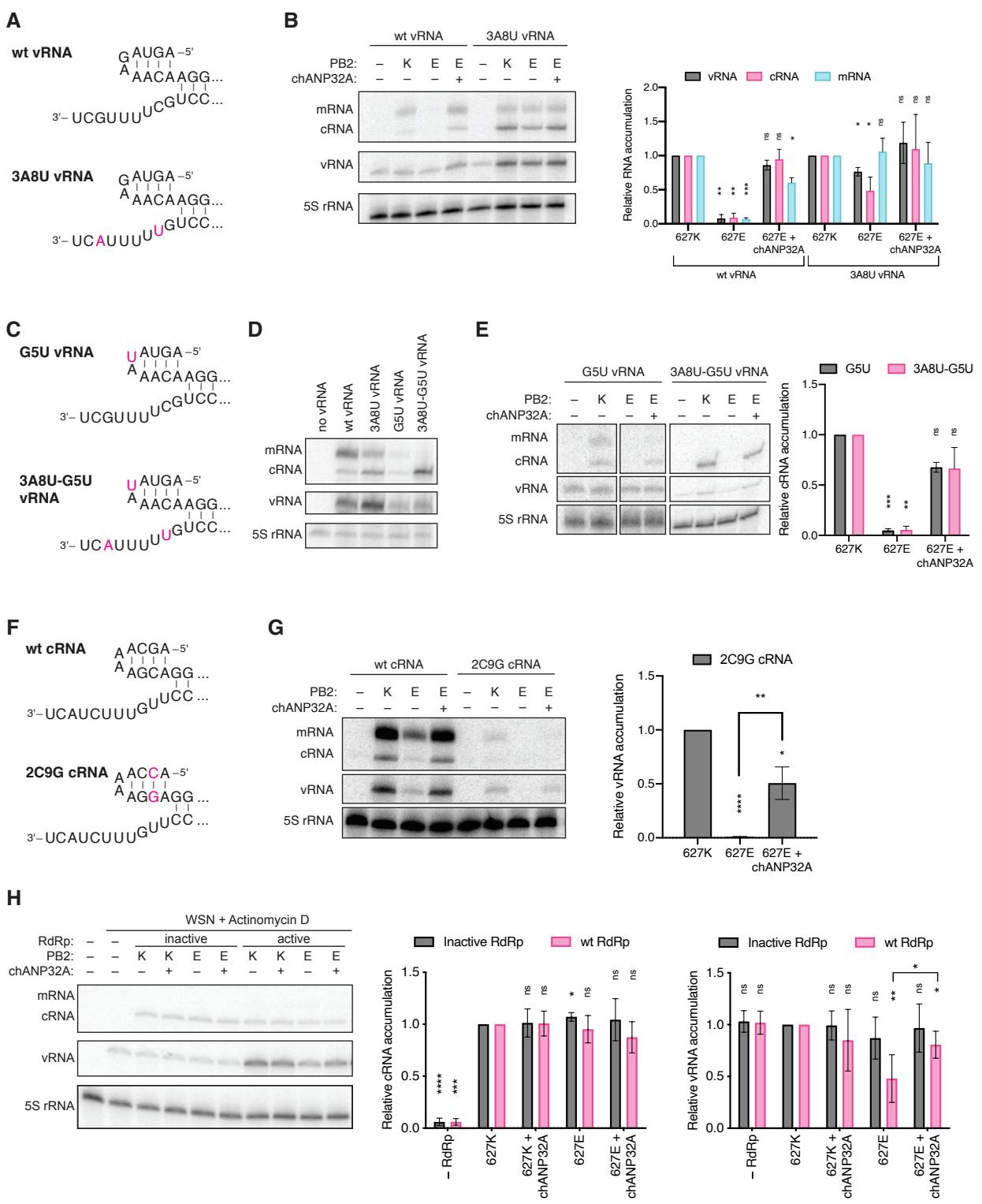


Table 1**Oligonucleotide sequences**

Mutagenesis primers	Sequence (5' -> 3')
PB2-K627E_F	cgcagccgctccaccagagcaaagtggaa
PB2-K627E_R	tccacttgcgtggagcggctgcgtcg
NA-3A8U_F	gggaccatgcggccagtaaaaacaggagttaaatgaatc
NA-3A8U_R	gattcattaaactcctgtttactggccggcatggtccc
NA-G5U_F	tgttcaaaaaactcctgtttatactaataacccggcggcc
NA-G5U_R	ggccgcgggttattagtataaaacaaggagttttgaaca
cNA-2C9G_F	ggcatttggccgggttattaccaaaggaggagttaaatga
cNA-2C9G_R	tcatttaactcctccttgtaataacccggcggccaaatgcc
NA30_F	ggccagcaaaagcaggagtctcctgtttactaata
NA30_R	tattagttagaaacaaggagactcctgctttgctggcc
chANP32AΔ180-208_F	gaggatgtgttggccttgaagaggagtacgatgac
chANP32AΔ180-208_R	gtcatcgtaactccttcaaggctcaacacatcctc
chANP32AΔ209-281_F	cgaagaagaagacgaagattaatgataaggatcccccg
chANP32AΔ209-281_R	cggggatccttatcattatacgacacctgggcatcg
chANP32AΔ220-281_F	acgatgccaggtcgataatgataaggatcccc
chANP32AΔ220-281_R	ggggatccttatcattatacgacacctgggcatcg
chANP32AΔ251-281_F	gaggggtataatgacggttaatgataaggatcccc
chANP32AΔ251-281_R	ggggatccttatcattaaaccgtcattataccctc
chANP32AΔ220-250_F	cgtatgccaggtcgtagatgtcgatgacgatg
chANP32AΔ220-250_F	catcgatcgacatctacgacactgggcatcg

Primer extension primers	Sequence (5' -> 3')
5S rRNA	TCCCAGGCGGTCTCCCATCC
NA (-)	TGGACTAGTGGGAGCATCAT
NA (+)	TCCAGTATGGTTTGATTCCG
NP76 (-)	CAGGGTAGATAATCACTCACAGAG
NP76 (+)	TGATTCGATGTCACTCTGTGAG
NA47 (-)	GTTAAAAAACTCCTTGTTC
NA47 (+)	GAGTTTTGAACCATTAAACTCCTG
NA30 (-)	AGCAAAAGCAGGAGTCTCCTG
NA30 (+)	GTAGAAACAAGGAGACTCCTGC