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Swine ANP32A supports avian influenza virus polymerase

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1 Swine ANP32A supports avian influenza
2 virus polymerase

3 Running title: Swine ANP32A and influenza virus polymerase

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17

18

19 Abstract (max 250 words)

20 Avian influenza viruses occasionally infect and adapt to mammals, including humans.
21 Swine are often described as ‘mixing vessels’, being susceptible to both avian and human
22 origin viruses, which allows the emergence of novel reassortants, such as the precursor to
23 the 2009 H1N1 pandemic. ANP32 proteins are host factors that act as influenza virus
24 polymerase cofactors. In this study we describe how swine ANP32A, uniquely among the
25 mammalian ANP32 proteins tested, supports activity of avian origin influenza virus
26 polymerases, and avian influenza virus replication. We further show that after the swine-
27 origin influenza virus emerged in humans and caused the 2009 pandemic it evolved
28 polymerase gene mutations that enabled it to more efficiently use human ANP32 proteins.
29 We map the enhanced pro-viral activity of swine ANP32A to a pair of amino acids, 106 and
30 156, in the leucine-rich repeat and central domains and show these mutations enhance
31 binding to influenza virus trimeric polymerase. These findings help elucidate the molecular
32 basis for the ‘mixing vessel’ trait of swine and further our understanding of the evolution
33 and ecology of viruses in this host.

34 Importance (max 150 words)

35 Avian influenza viruses can jump from wild birds and poultry into mammalian species
36 such as humans or swine, but only continue to transmit if they accumulate mammalian
37 adapting mutations. Pigs appear uniquely susceptible to both avian and human strains of
38 influenza and are often described as virus ‘mixing vessels’. In this study, we describe how a
39 host factor responsible for regulating virus replication, ANP32A, is different between swine
40 and humans. Swine ANP32A allows a greater range of influenza viruses, specifically those
41 from birds, to replicate. It does this through binding the virus polymerase more tightly than

42 the human version of the protein. This work helps to explain the unique properties of swine
43 as 'mixing vessels'.

44 Introduction

45 Influenza A viruses continuously circulate in their natural reservoir of wild aquatic
46 and sea birds. Occasionally, avian influenza viruses infect mammalian hosts, but these
47 zoonotic viruses have to adapt for efficient replication and further transmission. This limits
48 the emergence of novel endemic strains. Avian-origin, mammalian-adapted influenza
49 viruses have been isolated from a range of mammalian species including humans, swine,
50 horses, dogs, seals, and bats (1-6).

51 One mammalian influenza host of significance are swine, which have been described
52 as susceptible to viruses of both human- and avian-origin (6). It has been hypothesised that
53 swine act as 'mixing vessels', allowing efficient gene transfer between avian- and
54 mammalian-adapted viruses. This leads to reassortants, which are able to replicate in
55 humans, but to which populations have no protective antibody responses, as best illustrated
56 by the 2009 H1N1 pandemic (pH1N1) (7). The ability of pigs to act as 'mixing vessels' has
57 generally been attributed to the diversity of sialic acids, the receptors for influenza, found in
58 pigs that would enable co-infection of a single host by diverse influenza strains (8, 9). The
59 husbandry of swine has also been hypothesised to play a role in this 'mixing vessel' trait;
60 swine are often exposed to wild birds and it is likely their environments are often
61 contaminated with wild bird droppings containing avian influenza viruses (10, 11).

62 For an avian-origin influenza virus to efficiently infect and transmit between
63 mammals several host barriers must be overcome. One major barrier is the weak activity of
64 avian influenza virus polymerases in the mammalian cell (12, 13). The acidic, (leucine-rich)

65 nuclear phosphoproteins of 32 kilodaltons (ANP32) proteins are key host factors responsible
66 for the restricted polymerase activity of avian influenza viruses in mammalian cells (14).
67 ANP32 proteins possess an N-terminal domain composed of five leucine rich repeats (LRRs)
68 and a C-terminal low complexity acidic region (LCAR) separated by a short region termed the
69 'central domain'. In birds and most mammals three ANP32 paralogues are found: ANP32A,
70 ANP32B and ANP32E (15, 16). The roles of ANP32 proteins in cells are diverse and often
71 redundant between the family members but include histone chaperoning, transcriptional
72 regulation, regulation of nuclear export and apoptosis (16). In birds, such as chickens and
73 ducks, an exon duplication allows for the expression of an alternatively spliced, longer
74 isoform of ANP32A that effectively supports activity of polymerases of avian influenza
75 viruses (14, 17). Mammals only express the shorter forms of ANP32 proteins which do not
76 efficiently support avian polymerase unless the virus acquires adaptive mutations,
77 particularly in the PB2 polymerase subunit, such as E627K (14). A further difference
78 between the ANP32 proteins of different species is the level of redundancy in their ability to
79 support influenza polymerase. In humans, two paralogues – ANP32A and ANP32B – are
80 essential but redundant influenza polymerase cofactors (18, 19). In birds, only a single
81 family member – ANP32A - supports influenza virus polymerase activity, as avian ANP32B
82 proteins are not orthologous to mammalian ANP32B (15, 19, 20). In mice, only ANP32B can
83 support influenza A polymerase activity (18, 19). Neither avian nor mammalian ANP32E
84 proteins have been shown to support influenza polymerase activity (18-20).

85 In this study, we investigated the ability of a variety of mammalian ANP32 proteins
86 to support influenza virus polymerases derived from viruses isolated from a range of hosts.
87 We find differences in pro-viral efficiency that do not always coincide with the natural virus-
88 host relationship: for example, human ANP32B is better able to support bat influenza

89 polymerases than either bat ANP32 protein. Conversely, we describe evidence of human
90 ANP32 adaptation early during the emergence of the pH1N1 virus from pigs, and find that
91 swine ANP32A is the most potent pro-viral mammalian ANP32 protein tested, supporting
92 non-adapted avian virus polymerase activity and avian influenza virus replication
93 significantly better than human ANP32A. This can be attributed to amino acid differences in
94 the LRR4 and central domains that enhance the interaction between swine ANP32A and the
95 influenza polymerase complex, suggesting a mechanism for this enhanced pro-viral activity.
96 Our findings give support to the special status as potential 'mixing vessels' of swine in
97 influenza evolution.

98 Results

99 *Mammals naturally susceptible to influenza have two pro-viral ANP32 proteins.*

100 To investigate the ability of different mammalian ANP32A and ANP32B proteins to
101 support influenza virus polymerase activity, several mammalian-origin influenza virus
102 polymerase constellations were tested using an ANP32 reconstitution minigenome assay. A
103 previously described human cell line with both ANP32A and ANP32B ablated (eHAP dKO
104 (18)) was transfected with expression plasmids encoding ANP32A or ANP32B from chicken,
105 human, swine, horse, dog, seal or bat, as well as the minimal set of influenza polymerase
106 expression plasmids for PB2, PB1, PA and nucleoprotein (NP), to drive amplification and
107 expression of a firefly-luciferase viral-like reporter RNA and a *Renilla*-luciferase expression
108 plasmid as a transfection control.

109 Initially, we tested a panel of polymerases derived from human, canine, equine and
110 bat influenza viruses. In contrast to chicken ANP32B, which does not support influenza virus
111 polymerase activity (15, 19, 20), chicken ANP32A and all mammalian ANP32A and ANP32B

112 proteins supported activity of the mammalian-origin viral polymerases to varying degrees
113 (Fig. 1a). Among the mammalian ANP32 proteins tested, for most polymerases, swine
114 ANP32A provided the strongest support of polymerase activity, whereas the ANP32B
115 proteins from dog, seal and bat displayed the least efficient pro-viral activity, lower than
116 those species' respective ANP32A proteins. These trends could not be explained by
117 differences in expression levels or nuclear localisation (Fig. 1b, c). The bat influenza
118 polymerases, along with (human) influenza B polymerase showed a different pattern of
119 ANP32 usage, being able to strongly utilise ANP32Bs from all mammalian species,
120 particularly human ANP32B (Fig. 1a). There was no evidence that influenza viruses adapted
121 to particular mammals had evolved to specifically use the corresponding ANP32 proteins.
122 For example, dog ANP32A or ANP32B were not the most efficient cofactors for canine
123 influenza virus polymerase and human ANP32B was better able to support the bat influenza
124 polymerase than either of the bat ANP32 proteins.

125 *Swine ANP32A, but not other mammalian ANP32 proteins, can support the polymerase*
126 *activity and virus replication of avian-origin influenza viruses*

127 We next tested the ANP32 preference of a human 2009 (swine-origin) pH1N1 and
128 two polymerases from swine influenza isolates. Interestingly, these polymerases were
129 robustly supported by chicken and swine ANP32A, but not other mammalian ANP32
130 proteins, with the Eurasian avian-like polymerase from A/swine/England/453/2006 (H1N1;
131 sw/453) showing the clearest effect (Fig. 2a). We went on to test a panel of avian-origin viral
132 polymerases with no known mammalian polymerase adaptations, including
133 A/duck/Bavaria/77(H1N1; Bav), thought to be an avian precursor of the Eurasian avian-like
134 swine lineage (Fig. 2b)(5). For all the avian origin viral polymerases the stringent preference

135 for avian ANP32A to support polymerase activity was evident (co-expression of chicken
136 ANP32A led to very strong polymerase activity). However, amongst all the mammalian
137 ANP32 proteins tested, only swine ANP32A was able to significantly support avian influenza
138 polymerase activity, though to a lesser degree than chicken ANP32A (Fig. 2b). This unique
139 pro-viral effect of swine ANP32A on swine and avian-origin polymerases was maintained
140 across a wide titration of plasmid doses (Fig. 2c).

141 Furthermore, we tested the relative ability of human and swine cells to support
142 replication of a non-adapted avian influenza virus. Isogenic recombinant
143 A/turkey/England/50-92/1991(H5N1; 50-92) virus containing either wild-type PB2 (E627) or
144 the mammalian adaptation PB2-E627K were used to infect wild type human eHAP and swine
145 NPT_r cells (Fig 3a). Although E627K significantly increased the virus replication in both cell
146 lines, the magnitude of difference was less in the swine cells than the human cells at earlier
147 time points (for example 17-fold vs 110x-fold at 12 hours post-infection). The less drastic
148 reduction in replication of the virus with non-adapted avian origin polymerase compared
149 with the adapted control in swine cells is consistent with the hypothesis that swine ANP32A
150 can support replication of avian influenza viruses.

151 To investigate whether this difference was indeed accounted for by differences in
152 ANP32A proteins, chicken, swine, or human ANP32A were pre-expressed in eHAP dKO cells
153 that were then infected with 50-92 wild type and E627K recombinant viruses (Fig. 3b). As
154 shown previously, when empty vector was expressed no virus replication took place (18).
155 For the mammalian adapted PB2-E627K virus it made little difference which ANP32A protein
156 was expressed although a trend was seen for chicken ANP32A supporting higher titres than
157 swine ANP32A, which, in turn, supported higher titres than human ANP32A. For the non-

158 adapted PB2-E627 virus, however, a greater and significant difference was seen – chicken
159 ANP32A clearly supported virus replication better than either mammalian ANP32A protein.
160 Swine ANP32A supported replication of the avian influenza virus to a higher level than
161 human ANP32A at all time points, and this difference was significant ($P < 0.05$) at 24 hours
162 post infection. Overall, this indicates that swine ANP32A is better able to both support avian
163 influenza virus polymerase activity, as well as virus replication, than human ANP32A.

164 *The pH1N1 swine influenza virus polymerase, adapting to humans, evolved to better use*
165 *human ANP32 proteins*

166 In 2009 the swine-origin pH1N1 influenza virus adapted from pigs for transmission
167 between humans causing an influenza pandemic (7). The pH1N1 polymerase genes were
168 derived from a swine triple reassortant constellation in which PB2 and PA originally derived
169 from avian influenza viruses in the mid-1990s (21). From 2009 to 2010 the virus continued
170 to circulate and adapt to humans in the second and third pandemic waves (22). pH1N1
171 viruses contain the PB2 polymerase adaptations T271A, G590S, and Q591R, which appear to
172 compensate for the lack of E627K in enabling replication in mammalian cells and these
173 amino acids did not change between the first and third waves of the pandemic (23). We
174 previously showed that a single substitution in the PA subunit of the polymerase, N321K,
175 contributed to increased polymerase activity of third-wave pH1N1 viruses in human cells
176 (22). We hypothesised that this PA mutation might function by improving support for the
177 emerging virus polymerase by the human ANP32 proteins.

178 We performed minigenome assays with a first-wave pandemic virus,
179 A/England/195/2009(pH1N1; E195), and a third-wave pandemic virus
180 A/England/687/2010(pH1N1; E687), which differ in PA at position 321. As shown before, PA

181 321K enhances polymerase activity in general in both virus polymerase backgrounds in
182 human eHAP cells, as well as swine NPTr cells (Fig. 4a). However, the boost is far greater in
183 the human cells (~7-fold) than in the swine cells (~2-fold), implying this mutation may have
184 arisen to overcome the greater restriction seen upon the jump into humans (22).

185 We next tested the ability of human and swine ANP32 proteins to support the
186 different pH1N1 polymerases in eHAP dKO cells. Polymerases containing PA-321N are more
187 robustly enhanced by swine ANP32A (by around 3.5-fold compared to human ANP32A), as is
188 typical of swine-origin polymerases (Fig. 4b). Swine ANP32A, however, gives a much more
189 modest boost to polymerase activity compared to human ANP32A when 321K is present
190 (<2-fold). This suggests the PA N321K adaptation was selected in these viruses to adapt to
191 the more poorly supportive ANP32 proteins present in human cells. We could further show
192 that endogenous swine ANP32A protein is predominantly localised in the nucleus in swine
193 NPTr cells, consistent with our previous over-expression data (Fig. 4c).

194 *Differences in swine and human ANP32A pro-viral activity can be mapped to the LRR4*
195 *and central region.*

196 We set out to identify the molecular basis for the unusually high activity of swine
197 ANP32A in comparison with the other mammalian ANP32 proteins. An alignment of ANP32A
198 primary sequences identified three amino acids outside the LCAR, that differed between
199 swine ANP32A and the other mammalian orthologues. Using reciprocal mutant ANP32A
200 proteins, the identity of amino acid position 156, naturally a serine in swine ANP32A but a
201 proline in most other mammalian and all avian ANP32A proteins, was shown to have a
202 major, reciprocal influence on activity (Fig. 5a). The amino acid at position 106 contributed
203 to a lesser degree, with swine-like valine enhancing pro-viral activity over human-like

204 isoleucine when complementing the swine influenza polymerase constellation, though
205 changes at this residue appeared to have more minor effects on proviral activity supporting
206 the 50-92 and Bav avian virus polymerases. Position 228, localised nearby the C-terminal
207 nuclear localisation signal of ANP32A, had no appreciable impact. In the background of
208 human ANP32A, I106V generally gave between a 1.5- and 6-fold increase in polymerase
209 activity while P156S gave between a 3- and 16-fold boost, depending on the polymerase
210 constellation tested. The combined 106/156 mutant showed an additive effect implying
211 these two residues are, together, responsible for the enhanced pro-viral activity of swine
212 ANP32A (Fig. 5a,c). None of the mutations affected expression levels (Fig. 5b). Positions 106
213 and 156 map to the LRR4 and central domains of ANP32 protein, respectively, proximal to
214 the previously characterised LRR5 residues, 129/130, that are responsible for the lack of
215 pro-viral activity of avian ANP32B proteins (Fig. 5c)(15, 19). This reinforces the concept that
216 the LRR4/LRR5/central region of ANP32 proteins is essential to their pro-viral function.
217 Indeed, we could show that introducing the mutation N129I into swine ANP32A abrogated
218 its ability to support influenza polymerase activity (Fig. 5a).

219 *An increase in binding to the polymerase accounts for the enhanced pro-viral activity of*
220 *swine ANP32A*

221 Pro-viral ANP32 proteins from birds and mammals directly bind trimeric polymerase
222 in the cell nucleus (17, 24, 25). Moreover, the inability of avian ANP32B to support influenza
223 polymerase activity correlates with a lack of protein interaction conferred by amino acid
224 differences at residues 129 and 130 (15).

225 To assess the strength of interaction between swine ANP32A protein and influenza
226 polymerase, we used a split-luciferase assay, where the two halves of *Gaussia* luciferase are

227 fused onto PB1 and ANP32 protein (15, 25). As seen previously (25), the interaction
228 between influenza virus polymerase and human ANP32A was weak but detectable above
229 background (huA, Fig. 6a). Swine ANP32A interacted more strongly with both human-origin -
230 E195 (pH1N1 2009) - and avian-origin - A/turkey/England/50-92/1991(H5N1) - influenza
231 polymerases, although not as strongly as chicken ANP32A (Fig. 6a). Furthermore, the two
232 residues identified as being responsible for strong pro-viral activity of swine ANP32A, at
233 positions 106 and 156, enhanced polymerase binding by human ANP32A and the reciprocal
234 mutations decrease the swine ANP32A interaction, implying the mode of action of these
235 mutations is through enhancing swine ANP32A-polymerase interactions (Fig. 6a). It was also
236 shown that N129I, the substitution naturally identified in chicken ANP32B and previously
237 shown to abolish binding and activity in chicken and human ANP32 proteins (15, 19),
238 showed a similar phenotype in swine ANP32A, abolishing detectable binding and activity
239 (Fig. 6a,b). The ablations of the pro-viral activity of swine ANP32A and ANP32B by the
240 substitution N129I was not explained by reductions in expression of these mutant proteins
241 (Fig. 6b,c).

242 *Estimating the pro-viral activity of ANP32A proteins from other mammalian species*

243 Based on the molecular markers described in this study it is possible to survey
244 ANP32A proteins from all mammals to predict which other species may have highly
245 influenza polymerase supportive proteins and therefore potential to act as mixing vessels
246 for reassortment between avian and mammalian-adapted influenza viruses.

247 Very few mammals share the pro-viral marker, 156S, and the few that do mostly
248 constitute species not yet described as hosts for influenza viruses (Fig. 6d). A notable
249 exception is the pika which, in a similar manner to pigs, are known to often become infected

250 with avian influenza viruses with minimal mammalian adaptation (26-28). Pigs are currently
251 the only known mammalian species with a publicly available ANP32A sequence that contain
252 the secondary, minor pro-viral maker 106V.

253 Discussion

254 In this study we describe the ability of different mammalian ANP32A and ANP32B
255 proteins to support activity of influenza virus polymerases isolated from a variety of hosts.
256 We found that swine ANP32A, uniquely among the ANP32 proteins, supports avian
257 influenza virus polymerase activity and virus replication. Swine ANP32A does not harbour
258 the avian-specific 33 amino acid duplication that enables the strong interaction and efficient
259 support of polymerase activity of avian-origin viruses by avian ANP32A proteins (14). Thus,
260 avian influenza viruses are restricted for replication in swine as we have previously shown,
261 and mammalian-adapting mutations enhance their polymerase activity in pig cells (11).
262 Nonetheless, this level of pro-viral activity associated with swine ANP32A, albeit weaker
263 than avian ANP32As, may contribute to the role of swine as mixing vessels: non-adapted
264 avian influenza viruses that infect pigs could replicate sufficiently to accumulate further
265 mutations that allow for more efficient mammalian adaptation and/or reassortment,
266 enabling virus to either become endemic in swine or to jump into other mammals, including
267 humans.

268 We map this strongly pro-viral polymerase phenotype to a pair of mutations which
269 allow swine ANP32A to bind more strongly to influenza virus polymerase, potentially
270 explaining the mechanism behind its enhanced pro-viral activity. These residues are only
271 found in a handful of other mammals including pika. It is conceivable these residues are

272 located at a binding interface between polymerase and ANP32, but resolution of the
273 structure of the host:virus complex will be required to confirm this hypothesis.

274 A recent study from Zhang and colleagues independently corroborated the superior
275 ability of swine ANP32A amongst mammalian ANP32 proteins to support avian influenza
276 virus polymerase activity (29). Moreover, they also correlated this phenotype with amino
277 acids at position 106 and 156 that increased the strength of interactions between the host
278 factor and the viral polymerase complex. In their studies the interaction between ANP32
279 proteins and viral polymerase was measured by co-immunoprecipitation, making it unlikely
280 that the similar differences we measured using our quantitative split luciferase assay were
281 due to re-orientation of the luciferase tags.

282 It has long been speculated that swine play a role as 'mixing vessels', by acting as
283 host to both human- and avian-origin influenza viruses (30). This trait may be partially
284 attributed to receptor patterns in swine allowing viruses that bind to both α 2,3 linked (i.e.
285 avian-like viruses) and α 2,6 linked sialic acid (i.e. human-like) to replicate alongside each
286 other (8, 9). However, replication of the avian-origin influenza virus genomes inside infected
287 cells is also required to enhance the opportunity for further adaptation or reassortment. We
288 previously developed a minigenome assay for assessing polymerase activity in swine cells
289 and showed that avian virus polymerases were restricted and that restriction could be
290 overcome by typical mutations known to adapt polymerase to human cells (11). Taken
291 together the ability to enter swine cells without receptor switching changes in the
292 haemagglutinin gene, along with a greater mutation landscape afforded in swine cells by the
293 partially supportive pro-viral function of swine ANP32A may have an additive effect to allow
294 swine to act an intermediate host for influenza viruses to adapt to mammals. Furthermore,

295 our work implies other mammals, such as the pika, could play a similar role which is of
296 particular interest due to the pika's natural habitat often overlapping with that of wild birds
297 and its (somewhat swine-like) distribution of both α 2,3 and α 2,6-linked sialic acid receptors
298 (31).

299 Upon crossing into humans from swine, it is likely that viruses would be under
300 selective pressure to adapt to human pro-viral factors, such as the ANP32 proteins. We use
301 the example of a pair of first- and third-wave pandemic H1N1 influenza viruses isolated from
302 clinical cases in 2009 and 2010 (22). The polymerase constellation of the 2009 pH1N1 virus
303 contains PB2 and PA gene segments donated from avian sources to a swine virus in a triple
304 reassortant constellation in the mid-1990s, then passed onto humans in 2009 (21). Although
305 the first-wave viruses, derived directly from swine, can clearly replicate and transmit
306 between humans, over time the PA substitution, N321K, was selected because it enabled
307 more efficient activity of the viral polymerase in human cells. Our data suggests this is a
308 direct adaptation to human ANP32 proteins. This again illustrates how swine have acted as a
309 'halfway house' for the step-wise adaptation of genes originating in avian influenza viruses
310 that have eventually become humanised.

311 Also of note, we show here that as for the human orthologues (18, 19), the ANP32A
312 and B proteins of swine (as well as all other mammals tested here) are redundant in their
313 ability to support the viral polymerase. We further show that the substitution N129I is able
314 to partially or fully ablate the pro-viral activity of swine ANP32A and ANP32B. We suggest
315 that the introduction of this substitution in both swine ANP32A and ANP32B by genome
316 editing would be a feasible basis for generating influenza resistant, or resilient, pigs, in a

317 similar manner to that demonstrated for porcine respiratory and reproductive syndrome
318 virus resistant pigs, and proposed for influenza resistant, or resilient, chickens (15, 32).

319 To conclude, we hypothesise that the superior pro-viral function of swine ANP32A
320 for supporting influenza replication may enable swine to act as intermediary hosts for avian
321 influenza viruses, and also affect the way the viruses evolve as they pass from birds, through
322 swine, and onto humans. This, in turn, may influence the ability of different swine influenza
323 viruses to act as zoonotic agents or as potential pandemic viruses.

324 Materials and methods

325 *Cells*

326 Human engineered-Haploid cells (eHAP; Horizon Discovery) and eHAP cells with
327 ANP32A and ANP32B knocked out (dKO) by CRISPR-Cas9, as originally described in (18),
328 were maintained in Iscove's Modified Dulbecco's Medium (IMDM; ThermoFisher)
329 supplemented with 10% fetal bovine serum (FBS; Biosera), 1% non-essential amino acids
330 (NEAA; Gibco) and 1% Penicillin-streptomycin (pen-strep; invitrogen). Human embryonic
331 kidney (293Ts, ATCC), Newborn Pig Trachea cells (NPTr; ATCC), and Madin-Darby Canine
332 Kidney cells (MDCK; ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM)
333 supplemented with 10% FBS, 1% NEAA and 1% pen-strep. All cells were maintained at 37°C,
334 5% CO₂.

335 *ANP32 plasmids constructs*

336 Animal ANP32 constructs were codon optimised and synthesised by GeneArt
337 (ThermoFisher). Sequences used were pig (*Sus scrofa*) ANP32B (XP_020922136.1), Horse
338 (*Equus caballus*) ANP32A (XP_001495860.2) and ANP32B (XP_023485491.1), Dog (*Canis*

339 *lupus familiaris*) ANP32A (NP_001003013.2), Dingo (*Canis lupus dingo*) ANP32B
340 (XP_025328134.1), Monk Seal (*Neomonachus schauinslandi*) ANP32A (XP_021549451.1) and
341 ANP32B (XP_021546921.1), and Common Vampire Bat (*Desmodus rotundus*) ANP32A
342 (XP_024423449.1) and ANP32B (XP_024415874.1). All isoforms were chosen based on their
343 orthology and synteny to the known functional human isoforms. Species of origin were
344 chosen due to being influenza hosts or the most-commonly related species to influenza
345 hosts (in the case of Monk Seal which are closely related to Harbour Seal whereas common
346 vampire bats belong to the same family as little yellow-shouldered and flat-faced bats).
347 Dingo ANP32B was substituted for dog ANP32B as the equivalent isoform used for all other
348 ANP32Bs is unannotated in the dog genome due to a gap in the scaffold. All ANP32
349 expression constructs included a C-terminal GSG-linker followed by a FLAG tag and a pair of
350 stop codons. Overlap extension PCR was used to introduce mutations into the ANP32
351 constructs which were then subcloned back into pCAGGS and confirmed by Sanger
352 sequencing.

353 *Viral minigenome plasmid constructs*

354 Viruses and virus minigenome full strain names used through this study were
355 A/Victoria/1975(H3N2; Victoria), A/England/195/2009(pH1N1; E195),
356 A/England/687/2010(pH1N1; E687), A/Japan/WRAIR1059P/2008(H3N2; Japan),
357 B/Florida/4/2006 (B/Florida), A/Anhui/2013(H7N9; Anhui), A/duck/Bavaria/1/1977(H1N1,
358 Bavaria), A/turkey/England/50-92/1991(H5N1; 50-92), A/chicken/Pakistan/UDL-
359 01/2008(H9N2; UDL1/08), A/canine/New York/dog23/2009(H3N8; CIV H3N8),
360 A/canine/Illinois/41915/2015(H3N2; CIV H3N2), A/equine/Richmond/1/2007(H3N8;
361 Richmond), A/swine/England/453/2006(EAH1N1; sw/453), A/swine/Hubei/221/2016(H1N1;

362 Hubei), A/little yellow-shouldered bat/Guatemala/164/2009(H17N10; H17) and A/flat-faced
363 bat/Peru/033/2010(H18N11; H18). Viral minigenome expression plasmids (for PB2, PB1, PA
364 and NP) for H3N2 Victoria, H5N1 50-92, H1N1 E195, H1N1 E687 IBV Florida/06, H9N2
365 UDL1/08 and H1N1 Bavaria have been previously described (11, 14, 22, 33). Viral
366 minigenome plasmids for H1N1 swine/453, H3N2 Japan, H3N2 CIV, H3N8 CIV, Hubei and
367 Richmond were subcloned from reverse genetics plasmids or cDNA into pCAGGS expression
368 vectors using virus segment specific primers.

369 pCAGGS minigenome reporters for H17N10 and H18N11 bat influenza viruses were a
370 kind gift from Professor Martin Schwemmler, Universitätsklinikum Freiburg (34). pCAGGS
371 minigenome reporters for H7N9 were a kind gift from Professor Munir Iqbal, The Pirbright
372 Institute, UK. Reverse genetics plasmids for H3N8, Richmond were a kind gift from Adam
373 Rash of the Animal Health Trust, Newmarket, UK. Reverse genetics plasmids for H3N2 CIV
374 and H3N8 CIV were a kind gift from Dr. Colin Parrish of the Baker Institute for Animal Health,
375 Cornell University (35, 36). Viral RNA from sw/453 was kindly provided by Dr. Sharon
376 Brookes, Animal Plant and Health Agency, Weybridge, UK.

377 *Minigenome assay*

378 eHAP dKO cells were transfected in 24 well plates using lipofectamine® 3000
379 (thermo fisher) with a mixture of plasmids; 100ng of pCAGGS ANP32/pCAGGS empty, 40ng
380 of pCAGGS PB2, 40ng of pCAGGS PB1, 20ng of pCAGGS PA, 80ng of pCAGGS NP, 40ng of
381 pCAGGS *Renilla* luciferase, 40ng of poll vRNA-Firefly luciferase. Transfections in wild-type
382 eHap cells were performed similarly but without ANP32. Transfections in NPTr cells were
383 carried out in 12 well plates using the same ratios above. 24 hours post-transfection cells
384 were lysed with passive lysis buffer (Promega) and luciferase bio-luminescent signals were

385 read on a FLUOstar Omega plate reader (BMG Labtech) using the Dual-Luciferase® Reporter
386 Assay System (Promega). Firefly signal was divided by *Renilla* signal to give relative
387 luminescence units (RLU). All assays were performed with 2 or 3 separate repeats on
388 different days, representative experiments are shown.

389 *Viruses replication assays*

390 All virus replication assays were performed with recombinant viruses containing the
391 HA, NA and M genes of A/Puerto Rico/8/1934(H1N1; PR8) and the remaining genes from
392 the avian influenza virus 50-92 containing PB2 627 E (wild type) or K, as has been described
393 previously (11). eHAP dKO cells pre-transfected 24 hours prior with 400ng of pCAGGs-
394 ANP32A (chicken, swine or human) or pCAGGs-empty, or wild type eHAP or NPTr cells were
395 infected at a multiplicity of infection of 0.001 in 6 well plates. Virus growth media, either
396 IMDM or DMEM (for eHAP cells and NPTr cells, respectively) was made from serum-free
397 media containing 1 µg/ml of N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin
398 (Worthington-Biochemical). Virus containing supernatants were collected at 12, 24, 48, 72
399 hours post-inoculation and stored at -80°C. Titres were assessed by infectious plaques on
400 MDCKs. All time points were taken in triplicate and all virus growth curves were performed
401 at least twice with a representative repeat shown.

402 *Split Luciferase Assay*

403 Split luciferase assays were undertaken in 293Ts seeded in 24 well plates. 30ng each
404 of PB2, PA, and PB1, with the N-terminus of *Gaussia* Luciferase (Gluc1) tagged to its C-
405 terminus after a GGSGG linker, were co-transfected using lipofectamine 3000 along with
406 ANP32A, tagged with the C-terminus of *Gaussia* Luciferase (Gluc2) on its C-terminus (after a
407 GGSGG linker). 24 hours later cells were lysed in 100µl of *Renilla* lysis buffer (Promega) and

408 *Gussia* activity was measured using a *Renilla* luciferase kit (Promega) on a FLUOstar Omega
409 plate reader (BMG Labtech). Normalised luminescence ratios (NLR) were calculated by
410 dividing the values of the tagged PB1 and ANP32 wells by the sum of the control wells which
411 contained 1) untagged PB1 and free Gluc1 and 2) untagged ANP32A and free Gluc2 as
412 described elsewhere (15, 37).

413 *Western Blotting*

414 To confirm equivalent protein expressing during mini-genome assays transfected
415 cells were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1%
416 SDS, 50mM TRIS, pH 7.4) supplemented with an EDTA-free protease inhibitor cocktail tablet
417 (Roche).

418 Membranes were probed with mouse α -FLAG (F1804, Sigma), rabbit α -Vinculin
419 (AB129002, Abcam), rabbit α -PB2 (GTX125926, GeneTex) and mouse α -NP ([C43] ab128193,
420 Abcam). The following near infra-red (NIR) fluorescent secondary antibodies were used:
421 IRDye[®] 680RD Goat Anti-Rabbit (IgG) secondary antibody (Ab216777, Abcam) and IRDye[®]
422 800CW Goat Anti-Mouse (IgG) secondary antibody (Ab216772, Abcam). Western Blots were
423 visualised using an Odyssey Imaging System (LI-COR Biosciences).

424 *Immunofluorescence*

425 For investigating localisation of exogenously expressed ANP32 proteins, eHAP ANP32
426 dKO cells were cultured on 8 well chambered cover slips (Ibidi) and transfected with 125 ng
427 of the indicated FLAG-tagged ANP32 protein. Cells were fixed in PBS, 4% paraformaldehyde
428 24 hours post transfection, then permeabilised in PBS, 0.2% Triton X-100. Cells were blocked
429 in PBS, 2% bovine serum albumin and 0.1% tween. FLAG-tagged ANP32 proteins were
430 detected using mouse anti-FLAG M2 primary antibody (Sigma), followed by goat anti-mouse

431 Alexa Fluor 568 (Invitrogen). Nuclei were counterstained with DAPI. Images were obtained
432 using a Zeiss Cell Observer widefield microscope with ZEN Blue software, using a Plan-
433 Apochromat 63x 1.40-numerical aperture oil objective (Zeiss) and processed using FIJI
434 software (38).

435 For investigating endogenous levels of ANP32A in swine cells, NPTr cells were
436 cultured in Nunc™ 24 well tissue culture plates on cover slips (VWR) preincubated with 10%
437 (v/v) collagen (Rat's tail, Sigma-Aldrich) in PBS. Cells were fixed with PBS, 4%
438 paraformaldehyde for 20 minutes at room temperature. Cells were permeabilized with PBS,
439 1% Triton X-100 for 10 minutes, followed by 3 washes with PBS 0.1% Triton X-100 and
440 blocking with PBS, 5% (w/v) skim milk powder for 1 hour at room temperature. ANP32A was
441 detected using ab189110 (Abcam) incubated in PBS, 5% (w/v) skim milk powder overnight at
442 4°C, followed by incubation with anti-rabbit AlexaFluor488 (ab150077, Abcam). Phalloidin
443 was detected using an AlexaFluor647 conjugated antibody (ab176759, Abcam), incubated
444 during the secondary antibody application step at 1:10,000. Nuclei were counterstained
445 with DAPI (1:15,000, Thermo Fisher). Images were captured with a Leica DMLB fluorescence
446 microscope using Micro-Manager software at 40x or 20x for DAPI and Phalloidin
447 respectively. Images were processed using FIJI software.

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458 References

- 459 1. Li S, Shi Z, Jiao P, Zhang G, Zhong Z, Tian W, Long LP, Cai Z, Zhu X, Liao M, Wan XF. 2010.
460 Avian-origin H3N2 canine influenza A viruses in Southern China. *Infect Genet Evol* 10:1286-8.
- 461 2. Kandeil A, Gomaa MR, Shehata MM, El Taweel AN, Mahmoud SH, Bagato O, Moatasim Y,
462 Kutkat O, Kayed AS, Dawson P, Qiu X, Bahl J, Webby RJ, Karesh WB, Kayali G, Ali MA. 2019.
463 Isolation and Characterization of a Distinct Influenza A Virus from Egyptian Bats. *J Virol* 93.
- 464 3. Geraci JR, St Aubin DJ, Barker IK, Webster RG, Hinshaw VS, Bean WJ, Ruhnke HL, Prescott JH,
465 Early G, Baker AS, Madoff S, Schooley RT. 1982. Mass mortality of harbor seals: pneumonia
466 associated with influenza A virus. *Science* 215:1129-31.
- 467 4. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. 2005. Characterization of
468 the 1918 influenza virus polymerase genes. *Nature* 437:889-93.
- 469 5. Pensaert M, Ottis K, Vandeputte J, Kaplan MM, Bachmann PA. 1981. Evidence for the natural
470 transmission of influenza A virus from wild ducts to swine and its potential importance for
471 man. *Bull World Health Organ* 59:75-8.
- 472 6. Long JS, Mistry B, Haslam SM, Barclay WS. 2019. Host and viral determinants of influenza A
473 virus species specificity. *Nat Rev Microbiol* 17:67-81.
- 474 7. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL,
475 Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. 2009. Origins and evolutionary genomics
476 of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 459:1122-5.
- 477 8. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, Chang KC. 2010. Comparative
478 distribution of human and avian type sialic acid influenza receptors in the pig. *BMC Vet Res*
479 6:4.
- 480 9. Byrd-Leotis L, Liu R, Bradley KC, Lasanajak Y, Cummings SF, Song X, Heimburg-Molinario J,
481 Galloway SE, Culhane MR, Smith DF, Steinhauer DA, Cummings RD. 2014. Shotgun glycomics
482 of pig lung identifies natural endogenous receptors for influenza viruses. *Proc Natl Acad Sci*
483 *U S A* 111:E2241-50.
- 484 10. Van Poucke SG, Nicholls JM, Nauwynck HJ, Van Reeth K. 2010. Replication of avian, human
485 and swine influenza viruses in porcine respiratory explants and association with sialic acid
486 distribution. *Virol J* 7:38.
- 487 11. Moncorge O, Long JS, Cauldwell AV, Zhou H, Lycett SJ, Barclay WS. 2013. Investigation of
488 influenza virus polymerase activity in pig cells. *J Virol* 87:384-94.
- 489 12. Clements ML, Subbarao EK, Fries LF, Karron RA, London WT, Murphy BR. 1992. Use of single-
490 gene reassortant viruses to study the role of avian influenza A virus genes in attenuation of
491 wild-type human influenza A virus for squirrel monkeys and adult human volunteers. *J Clin*
492 *Microbiol* 30:655-62.
- 493 13. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza
494 A virus is a determinant of host range. *J Virol* 67:1761-4.
- 495 14. Long JS, Giotis ES, Moncorge O, Frise R, Mistry B, James J, Morisson M, Iqbal M, Vignal A,
496 Skinner MA, Barclay WS. 2016. Species difference in ANP32A underlies influenza A virus
497 polymerase host restriction. *Nature* 529:101-4.

- 498 15. Long JS, Idoko-Akoh A, Mistry B, Goldhill D, Staller E, Schreyer J, Ross C, Goodbourn S,
499 Shelton H, Skinner MA, Sang H, McGrew MJ, Barclay W. 2019. Species specific differences in
500 use of ANP32 proteins by influenza A virus. *Elife* 8.
- 501 16. Reilly PT, Yu Y, Hamiche A, Wang L. 2014. Cracking the ANP32 whips: important functions,
502 unequal requirement, and hints at disease implications. *Bioessays* 36:1062-71.
- 503 17. Baker SF, Ledwith MP, Mehle A. 2018. Differential Splicing of ANP32A in Birds Alters Its
504 Ability to Stimulate RNA Synthesis by Restricted Influenza Polymerase. *Cell Rep* 24:2581-
505 2588 e4.
- 506 18. Staller E, Sheppard CM, Neasham PJ, Mistry B, Peacock TP, Goldhill DH, Long JS, Barclay WS.
507 2019. ANP32 proteins are essential for influenza virus replication in human cells. *J Virol*
508 doi:10.1128/JVI.00217-19.
- 509 19. Zhang H, Zhang Z, Wang Y, Wang M, Wang X, Zhang X, Ji S, Du C, Chen H, Wang X. 2019.
510 Fundamental Contribution and Host Range Determination of ANP32A and ANP32B in
511 Influenza A Virus Polymerase Activity. *J Virol* 93.
- 512 20. Park YH, Chungu K, Lee SB, Woo SJ, Cho HY, Lee HJ, Rengaraj D, Lee JH, Song CS, Lim JM, Han
513 JY. 2020. Host-Specific Restriction of Avian Influenza Virus Caused by Differential Dynamics
514 of ANP32 Family Members. *J Infect Dis* 221:71-80.
- 515 21. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E,
516 Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ,
517 Rivailier P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM,
518 Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr.,
519 Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P,
520 Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA,
521 Clark PA, Beatrice ST, Donis R, et al. 2009. Antigenic and genetic characteristics of swine-
522 origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 325:197-201.
- 523 22. Elderfield RA, Watson SJ, Godlee A, Adamson WE, Thompson CI, Dunning J, Fernandez-
524 Alonso M, Blumenkrantz D, Hussell T, Investigators M, Zambon M, Openshaw P, Kellam P,
525 Barclay WS. 2014. Accumulation of human-adapting mutations during circulation of
526 A(H1N1)pdm09 influenza virus in humans in the United Kingdom. *J Virol* 88:13269-83.
- 527 23. Liu Q, Qiao C, Marjuki H, Bawa B, Ma J, Guillosoy S, Webby RJ, Richt JA, Ma W. 2012.
528 Combination of PB2 271A and SR polymorphism at positions 590/591 is critical for viral
529 replication and virulence of swine influenza virus in cultured cells and in vivo. *J Virol*
530 86:1233-7.
- 531 24. Domingues P, Hale BG. 2017. Functional Insights into ANP32A-Dependent Influenza A Virus
532 Polymerase Host Restriction. *Cell Rep* 20:2538-2546.
- 533 25. Mistry B, Long JS, Schreyer J, Staller E, Sanchez-David RY, Barclay WS. 2019. Elucidating the
534 interactions between influenza virus polymerase and host factor ANP32A. *J Virol*
535 doi:10.1128/JVI.01353-19.
- 536 26. Yu Z, Cheng K, Sun W, Xin Y, Cai J, Ma R, Zhao Q, Li L, Huang J, Sang X, Li X, Zhang K, Wang T,
537 Qin C, Qian J, Gao Y, Xia X. 2014. Lowly pathogenic avian influenza (H9N2) infection in
538 Plateau pika (*Ochotona curzoniae*), Qinghai Lake, China. *Vet Microbiol* 173:132-5.
- 539 27. Su S, Xing G, Wang J, Li Z, Gu J, Yan L, Lei J, Ji S, Hu B, Gray GC, Yan Y, Zhou J. 2016.
540 Characterization of H7N2 Avian Influenza Virus in Wild Birds and Pikas in Qinghai-Tibet
541 Plateau Area. *Sci Rep* 6:30974.
- 542 28. Zhou J, Sun W, Wang J, Guo J, Yin W, Wu N, Li L, Yan Y, Liao M, Huang Y, Luo K, Jiang X, Chen
543 H. 2009. Characterization of the H5N1 highly pathogenic avian influenza virus derived from
544 wild pikas in China. *J Virol* 83:8957-64.
- 545 29. Zhang H, Li H, Wang W, Wang Y, Han GZ, Chen H, Wang X. 2020. A unique feature of swine
546 ANP32A provides susceptibility to avian influenza virus infection in pigs. *PLoS Pathog*
547 16:e1008330.

- 548 30. Ma W, Kahn RE, Richt JA. 2008. The pig as a mixing vessel for influenza viruses: Human and
549 veterinary implications. *J Mol Genet Med* 3:158-66.
- 550 31. Li Y, Xiao H, Huang C, Sun H, Li L, Su J, Ma J, Liu D, Wang H, Liu W, Gao GF, Li X, Yan J. 2015.
551 Distribution of sialic acid receptors and experimental infections with different subtypes of
552 influenza A viruses in Qinghai-Tibet plateau wild pika. *Virology* 12:63.
- 553 32. Burkard C, Opriessnig T, Mileham AJ, Stadejek T, Ait-Ali T, Lillico SG, Whitelaw CBA, Archibald
554 AL. 2018. Pigs Lacking the Scavenger Receptor Cysteine-Rich Domain 5 of CD163 Are
555 Resistant to Porcine Reproductive and Respiratory Syndrome Virus 1 Infection. *J Virol* 92.
- 556 33. Long JS, Howard WA, Nunez A, Moncorge O, Lycett S, Banks J, Barclay WS. 2013. The effect
557 of the PB2 mutation 627K on highly pathogenic H5N1 avian influenza virus is dependent on
558 the virus lineage. *J Virol* 87:9983-96.
- 559 34. Juozapaitis M, Aguiar Moreira E, Mena I, Giese S, Riegger D, Pohlmann A, Hoper D, Zimmer
560 G, Beer M, Garcia-Sastre A, Schwemmle M. 2014. An infectious bat-derived chimeric
561 influenza virus harbouring the entry machinery of an influenza A virus. *Nat Commun* 5:4448.
- 562 35. Feng KH, Gonzalez G, Deng L, Yu H, Tse VL, Huang L, Huang K, Wasik BR, Zhou B, Wentworth
563 DE, Holmes EC, Chen X, Varki A, Murcia PR, Parrish CR. 2015. Equine and Canine Influenza
564 H3N8 Viruses Show Minimal Biological Differences Despite Phylogenetic Divergence. *J Virol*
565 89:6860-73.
- 566 36. Rodriguez L, Nogales A, Reilly EC, Topham DJ, Murcia PR, Parrish CR, Martinez Sobrido L.
567 2017. A live-attenuated influenza vaccine for H3N2 canine influenza virus. *Virology* 504:96-
568 106.
- 569 37. Cassonnet P, Rolloy C, Neveu G, Vidalain PO, Chantier T, Pellet J, Jones L, Muller M, Demeret
570 C, Gaud G, Vuillier F, Lotteau V, Tangy F, Favre M, Jacob Y. 2011. Benchmarking a luciferase
571 complementation assay for detecting protein complexes. *Nat Methods* 8:990-2.
- 572 38. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden
573 C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona
574 A. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676-82.
- 575 39. Huyton T, Wolberger C. 2007. The crystal structure of the tumor suppressor protein pp32
576 (Anp32a): structural insights into Anp32 family of proteins. *Protein Sci* 16:1308-15.
- 577 40. Schrodinger, LLC. 2010. The PyMOL Molecular Graphics System, Version 1.3r1,
578 <https://www.pymol.org/>.

579 Figure legends

580 **Figure 1 – Most common mammalian influenza hosts have two ANP32 proteins capable of**
581 **supporting influenza polymerase.** a) Minigenome assays performed in human eHAP dKO
582 with ANP32 proteins from different avian or mammalian species co-transfected. Green bars
583 indicate species the influenza virus polymerase was isolated from, orange bars indicate
584 recent species the virus has jumped from. Data indicates triplicate repeats plotted as mean
585 with standard deviation. Data for each polymerase normalised to chicken ANP32A. b)
586 Western blot assay showing protein expression levels of FLAG-tagged ANP32 proteins, NP
587 and PB2 during a minigenome assay. c) Immunofluorescence images showing nuclear

588 localisation of all FLAG-tagged ANP32 proteins (red) tested. Nuclei are stained with DAPI
589 (blue). Abbreviations: ch – chicken, hu – human, sw – swine, eq – equine. Statistical
590 significance was determined by one-way ANOVA with multiple comparisons against empty
591 vector or between ANP32 proteins from the same host. *, $0.05 \geq P > 0.01$; **, $0.01 \geq P >$
592 0.001 ; ***, $0.001 \geq P > 0.0001$; ****, $P \leq 0.0001$.

593

594 **Figure 2. swANP32A can support the activity of minimally mammalian-adapted or**
595 **completely non-adapted polymerases.** Minigenome assays of swine (a) and avian (b)
596 polymerases performed in human eHAP dKO cells with ANP32 proteins from different avian
597 or mammalian species co-transfected. Green bars indicate species the influenza virus
598 polymerase was isolated from, orange bars indicate recent species the virus has jumped
599 from. Data indicates triplicate repeats plotted as mean with standard deviation. Data for
600 each polymerase normalised to chicken ANP32A. c) ANP32 protein titrations with three
601 different virus polymerase constellations. ANP32 expression plasmids were diluted in a
602 series of 3x dilutions starting with 100ng. Data indicates triplicate repeats plotted as mean
603 with standard deviation. Statistical significance was determined by one-way ANOVA with
604 multiple comparisons against empty vector. **, $0.01 \geq P > 0.001$; ***, $0.001 \geq P > 0.0001$;
605 ****, $P \leq 0.0001$.

606

607 **Figure 3. Swine ANP32A can support avian influenza virus replication better than human**
608 **ANP32A.** Comparative growth kinetics of isogenic, recombinant avian influenza viruses
609 (A/turkey/England/50-92/1991(H5N1)) PB2 627E (wild type) vs E627K in (a) wild-type
610 human eHAP cells and swine NPT_r cells and (b) eHAP dKO cells pre-expressing empty vector,
611 chicken, swine or human ANP32A. Cells were infected at a multiplicity of infection (MOI) of

612 0.001. All time points taken in triplicate and mean viral titres determined by plaque assay in
613 MDCK cells with standard deviation shown. Graph is representative data of at least two
614 independent repeats showing the same trends. Statistical significance determined by
615 multiple Student's t-tests in panel (a) and one-way ANOVA with multiple comparisons in
616 panel (b). Value shown on graph in panel (a) indicate fold-change in mean titres. Dotted lines
617 on graphs indicate limits of detection. *, $0.05 \geq P > 0.01$; **, $0.01 \geq P > 0.001$; ***, $0.001 \geq P$
618 > 0.0001 ; ****, $P \leq 0.0001$.

619

620 **Figure 4. Third-wave pandemic H1N1 viruses adapt to human ANP32 proteins through the**
621 **PA mutation N321K.** a) Minigenome assays of polymerases derived from first- and third-
622 wave pH1N1 viruses (E195 and E687, respectively) performed in wild-type human eHAP cells
623 and swine NPTr cells. Data indicates triplicate repeats plotted as mean with standard
624 deviation. Data normalised to E195 wild type. b) Minigenome assays performed in human
625 eHAP cells with ANP32A and ANP32B knocked out and complemented with ANP32 proteins
626 from human or swine following co-transfection of expression plasmids. Data indicates
627 triplicate repeats plotted as mean with standard deviation. Data normalised to E195 wt with
628 chicken ANP32A. All experiments in parts a) and b) performed on two separate occasions
629 with a representative repeat shown. c) Indirect immunofluorescence images showing
630 endogenous nuclear localisation of swine ANP32A in swine NPTr cells. Statistical significance
631 was determined by one-way ANOVA with multiple comparisons. ****, $P \leq 0.0001$.

632

633 **Figure 5. The enhanced pro-viral activity of swine ANP32A maps to amino acids in LRR4**
634 **and the central domain.** a) Minigenome assays with polymerase constellations from a swine

635 or an avian influenza virus performed in human eHAP dKO cells with human/swine ANP32A
636 reciprocal mutants expressed. Data indicates triplicate repeats plotted as mean with
637 standard deviation repeated on two separate occasions with a representative repeat shown.
638 Data normalised to each polymerase with swine ANP32A wild type. b) Western blot analysis
639 showing expression levels of human/swine ANP32A from minigenome assays. c) Crystal
640 structure of ANP32A (PDBID: 2JE1) with residues found to affect pro-viral activity mapped
641 (39). The unresolved, unstructured LCAR shown as a yellow line. Schematic made using
642 PyMol (40). Statistical significance was determined by one-way ANOVA with multiple
643 comparisons. *, $0.05 \geq P > 0.01$; ***, $0.001 \geq P > 0.0001$; ****, $P \leq 0.0001$.

644

645 **Figure 6. Amino acid residues responsible for the enhanced support of polymerase activity**
646 **of swine ANP32A also mediate increased binding to influenza trimeric polymerase.** a) Split
647 luciferase assays showing the relative binding of different ANP32 proteins to trimeric
648 polymerase from human pH1N1 or avian H5N1 viruses. PB1 was tagged with the N-terminal
649 part of *Gaussia* luciferase while ANP32 proteins were tagged with the C-terminal part. NLR,
650 normalised luminescence ratio, calculated from the ratio between tagged and untagged
651 ANP32/PB1 pairs. Assay performed in 293T cells. Data indicates triplicate repeats plotted as
652 mean with standard deviation, repeated across two separate experiments with
653 representative data shown. Statistical significance was determined by one-way ANOVA with
654 multiple comparisons between the swA and huA wild-types and mutants. ***, $0.001 \geq P >$
655 0.0001 ; ****, $P \leq 0.0001$. b) Minigenome assays with reconstituted polymerases from 3
656 different influenza viruses, performed in human eHAP cells with ANP32A and ANP32B
657 knocked out and complemented with wild type swine ANP32A or B or N129I mutants
658 thereof. Data indicates triplicate repeats plotted as mean with standard deviation, repeated

659 across two separate experiments with representative data shown. Data normalised to each
660 polymerase with wild type swine ANP32A. c) Western blot assay showing protein expression
661 levels of FLAG-tagged swine ANP32 wild type or N129I proteins during a minigenome assay.
662 d) Phylogenetic tree of mammalian ANP32A proteins. Species which contain the highly pro-
663 viral 156S shown in red, species with 156P shown in black. Phylogenetic trees made using
664 the neighbour-joining method based on amino acid sequence. Statistical significance was
665 determined by one-way ANOVA with multiple comparisons against empty vector. ****, $P \leq$
666 0.0001.











