

1 Swine ANP32A supports avian influenza 2 virus polymerase

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11 Abstract

12 Avian influenza viruses occasionally infect and adapt to mammals, including humans.
13 Swine are often described as ‘mixing vessels’, being susceptible to both avian and human
14 origin viruses, which allows the emergence of novel reassortants, such as the precursor to the
15 2009 H1N1 pandemic. ANP32 proteins are host factors that act as influenza virus polymerase
16 cofactors. In this study we describe how swine ANP32A, uniquely among the mammalian
17 ANP32 proteins tested, supports some, albeit limited, activity of avian origin influenza virus
18 polymerases. We further show that after the swine-origin influenza virus emerged in humans
19 and caused the 2009 pandemic it evolved polymerase gene mutations that enabled it to more
20 efficiently use human ANP32 proteins. We map the super pro-viral activity of swine ANP32A
21 to a pair of amino acids, 106 and 156, in the LRR and central domains and show these
22 mutations enhance binding to influenza virus trimeric polymerase. These findings help
23 elucidate the molecular basis for the ‘mixing vessel’ trait of swine and further our
24 understanding of the evolution and ecology of viruses in this host.

25 Introduction

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

32 One mammalian influenza host of significance are swine, which have been described
33 as susceptible to viruses of both human- and avian-origin (6). It has been hypothesised that
34 swine act as ‘mixing vessels’, allowing efficient gene transfer between avian- and mammalian-
35 adapted viruses. This leads to reassortants, which are able to replicate in humans, but to
36 which populations have no protective antibody responses, as best illustrated by the 2009
37 H1N1 pandemic (pH1N1) (7). The ability of pigs to act as ‘mixing vessels’ has generally been
38 attributed to the diversity of sialic acids, the receptors for influenza, found in pigs that would
39 enable co-infection of a single host by diverse influenza strains (8, 9). The husbandry of swine
40 has also been hypothesised to play a role in this ‘mixing vessel’ trait; swine are often exposed
41 to wild birds and it is likely their environments are often contaminated with wild bird
42 droppings containing avian influenza viruses.

43 For an avian-origin influenza virus to efficiently infect and transmit between mammals
44 several host barriers must be overcome. One major barrier is the weak activity of avian
45 influenza virus polymerases in the mammalian cell. The acidic (leucine-rich) nuclear
46 phosphoproteins of 32 kilodaltons (ANP32) proteins are key host factors responsible for the
47 restricted polymerase activity of avian influenza viruses in mammalian cells (10). ANP32
48 proteins possess an N-terminal domain composed of five leucine rich repeats (LRRs) and a C-
49 terminal low complexity acidic region (LCAR) separated by a short region termed the ‘central
50 domain’. In birds and most mammals three ANP32 paralogues are found: ANP32A, ANP32B
51 and ANP32E (11, 12). The roles of ANP32 proteins in cells are diverse and often redundant
52 between the family members but include histone chaperoning, transcriptional regulation,
53 regulation of nuclear export and apoptosis (12). In birds, such as chickens and ducks, an exon
54 duplication allows for the expression of an alternatively spliced, longer isoform of ANP32A
55 that effectively supports activity of polymerases of avian influenza viruses (10, 13). Mammals

56 only express the shorter forms of ANP32 proteins which do not efficiently support avian
57 polymerase unless the virus acquires adaptive mutations, particularly in the PB2 polymerase
58 subunit, such as E627K (10). A further difference between the ANP32 proteins of different
59 species is the level of redundancy in their ability to support influenza polymerase. In humans,
60 two paralogues – ANP32A and ANP32B – are essential but redundant influenza polymerase
61 cofactors (14, 15). In birds, only a single family member – ANP32A - supports influenza virus
62 polymerase activity, as avian ANP32B proteins are not orthologous to mammalian ANP32B
63 (11, 15, 16). In mice, only ANP32B can support influenza A polymerase activity (14, 15).
64 Neither avian nor mammalian ANP32E proteins have been shown to support influenza
65 polymerase activity (14-16).

66 In this study, we investigated the ability of a variety of mammalian ANP32 proteins to
67 support influenza virus polymerases derived from viruses isolated from a range of hosts. We
68 find differences in pro-viral efficiency that do not always coincide with the natural virus-host
69 relationship: for example, human ANP32B is better able to support bat influenza polymerases
70 than either bat ANP32 protein. Conversely, we describe evidence of human ANP32 adaptation
71 early during the emergence of the pH1N1 virus from pigs, and find that swine ANP32A is the
72 most potent pro-viral mammalian ANP32 protein tested, capable even of modestly
73 supporting avian virus polymerases. This can be attributed to amino acid differences in the
74 LRR4 and central domains that enhance the interaction between swine ANP32A and the
75 influenza polymerase complex, suggesting a mechanism for this super pro-viral activity and
76 giving support to the special status as potential ‘mixing vessels’ of swine in influenza
77 evolution.

78 Results

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

80 To investigate the ability of different mammalian ANP32A and ANP32B proteins to
81 support influenza virus polymerase activity, several mammalian-origin influenza virus
82 polymerase constellations were tested using an ANP32 reconstitution minigenome assay. A
83 previously described human cell line with both ANP32A and ANP32B ablated (eHAP dKO) was
84 transfected with ANP32A or ANP32B expressing plasmids from chicken, human, swine, horse,
85 dog, seal or bat, as well as the minimal set of influenza polymerase expression plasmids for
86 PB2, PB1, PA and nucleoprotein (NP), to drive amplification and expression of a firefly-
87 luciferase viral-like reporter RNA and a *Renilla*-luciferase expression plasmid as a transfection
88 control.

89 Initially, we tested a panel of polymerases derived from human, canine, equine and
90 bat influenza viruses. In contrast to chicken ANP32B, which does not support influenza virus
91 polymerase activity (11, 15, 16), chicken ANP32A and all mammalian ANP32A and ANP32B
92 proteins supported activity of the mammalian-origin viral polymerases to varying degrees
93 (Fig. 1a). Among the mammalian ANP32 proteins tested, for most polymerases, swine
94 ANP32A provided the strongest support of polymerase activity, whereas the ANP32B proteins
95 from dog, seal and bat displayed the least efficient pro-viral activity. These trends could not
96 be explained by differences in expression levels or nuclear localisation (Fig. 1b, c). The bat
97 influenza polymerases, along with (human) influenza B polymerase showed a different
98 pattern of ANP32 usage, being able to strongly utilise ANP32Bs from all mammalian species,
99 particularly human ANP32B (Fig. 1a). There was no evidence that influenza viruses adapted
100 to particular mammals had evolved to specifically use the corresponding ANP32 proteins. For

101 example, dog ANP32A or ANP32B were not the most efficient cofactors for canine influenza
102 virus polymerase and human ANP32B was better able to support the bat influenza
103 polymerase than either of the bat ANP32 proteins.

104 We next tested the ANP32 preference of a human 2009 (swine-origin) pH1N1 and two
105 polymerases from swine influenza isolates. Interestingly, these polymerases were robustly
106 supported by chicken and swine ANP32A, but not other mammalian ANP32 proteins, with the
107 Eurasian avian-like polymerase from A/swine/England/453/2006 (H1N1; sw/453) showing
108 the clearest effect (Fig. 2a). We went on to test a panel of avian-origin viral polymerases with
109 no known mammalian polymerase adaptations, including A/duck/Bavaria/77(H1N1), thought
110 to be an avian precursor of the Eurasian avian-like swine lineage (Fig. 2b). For all the avian
111 origin viral polymerases the stringent preference for avian ANP32A to support polymerase
112 activity was evident (co-expression of chicken ANP32A led to very strong polymerase activity).
113 However, amongst all the mammalian ANP32 proteins tested, only swine ANP32A was able
114 to significantly support avian influenza polymerase activity, though to a lesser degree than
115 chicken ANP32A (Fig. 2b). This unique pro-viral effect of swine ANP32A on swine and avian-
116 origin polymerases was maintained across a wide titration of plasmid doses (Fig. 2c).

117 *Swine influenza virus polymerases, adapting to humans, evolve to better use human*
118 *ANP32 proteins*

119 In 2009 the swine-origin pH1N1 influenza virus adapted from pigs for transmission
120 between humans causing an influenza pandemic (7). The pH1N1 polymerase genes were
121 derived from a triple reassortant constellation in which PB2 and PA originally derived from
122 avian influenza viruses in the mid-1990s (17). From 2009 to 2010 the virus continued to
123 circulate and adapt to humans in the second and third pandemic waves (18). We previously

124 showed that a single substitution in the PA subunit of the polymerase, N321K, contributed to
125 increased polymerase activity of third-wave pH1N1 viruses in human cells (18). We
126 hypothesised that this PA mutation might function by improving support for the emerging
127 virus polymerase by the human ANP32 proteins.

128 We performed minigenome assays with a first-wave pandemic virus,
129 A/England/195/2009(pH1N1; E195), and a third-wave pandemic virus
130 A/England/687/2010(pH1N1; E687), which differ in PA at position 321. As shown before, PA
131 321K enhances polymerase activity in general in both virus polymerase backgrounds in human
132 eHAP cells, as well as swine NPTr cells (Fig. 3a). However, the boost is far greater in the human
133 cells (~8-fold) than in the swine cells (~2-fold), implying this mutation may have arisen to
134 overcome the greater restriction seen upon the jump into humans (18).

135 We next tested the ability of human and swine ANP32 proteins to support the
136 different pH1N1 polymerases in eHAP dKO cells. Polymerases containing PA-321N are more
137 robustly enhanced by swine ANP32A (by around 3.5-fold compared to human ANP32A), as is
138 typical of swine-origin polymerases (Fig. 3b). Swine ANP32A, however, gives a much more
139 modest boost to polymerase activity compared to human ANP32A when 321K is present (<2-
140 fold). This suggests the PA N321K adaptation was selected in these viruses to adapt to the
141 more poorly supportive ANP32 proteins present in human cells.

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

144 We set out to identify the molecular basis for the unusually high activity of swine
145 ANP32A in comparison with the other mammalian ANP32 proteins. An alignment of ANP32A
146 primary sequences identified three amino acids outside the LCAR, that differed between

147 swine ANP32A and the other mammalian orthologues. Using reciprocal mutant ANP32A
148 proteins, the identity of amino acid position 156, naturally a serine in swine ANP32A but a
149 proline in most other mammalian ANP32A proteins, was shown to have a major, reciprocal
150 influence on activity (Fig. 4a). The amino acid at position 106 contributed to a lesser degree,
151 with swine-like valine enhancing pro-viral activity over human-like isoleucine. Position 228,
152 localised nearby the C-terminal nuclear localisation signal of ANP32A, had no appreciable
153 impact. In the background of human ANP32A, I106V generally gave between a 1.5- and 6-fold
154 increase in polymerase activity while P156S gave between a 3- and 16-fold boost, depending
155 on the polymerase constellation tested. The combined 106/156 mutant showed an additive
156 effect implying these two residues are, together, responsible for the enhanced pro-viral
157 activity of swine ANP32A (Fig. 4a). None of the mutations affected expression levels (Fig. 4b).
158 Positions 106 and 156 map to the LRR4 and central domains of ANP32 protein, respectively,
159 proximal to the previously characterised LRR5 residues, 129/130, that are responsible for the
160 lack of pro-viral activity of avian ANP32B proteins (Fig. 4c). This reinforces the concept that
161 the LRR4/LRR5/central region of ANP32 proteins is essential to their pro-viral function.
162 Indeed, we could show that introducing the mutation N129I into swine ANP32A abrogated its
163 ability to support influenza polymerase activity (Fig. 4a).

164 *An increase in binding to the polymerase accounts for the super pro-viral activity of swine*
165 *ANP32A*

166 Pro-viral ANP32 proteins from birds and mammals directly bind trimeric polymerase
167 in the cell nucleus (13, 19, 20). Moreover, the inability of avian ANP32B to support influenza
168 polymerase activity correlates with a lack of protein interaction conferred by amino acid
169 differences at residues 129 and 130 (11).

170 To assess the strength of interaction between swine ANP32A protein and influenza
171 polymerase, we used a split-luciferase assay, where the two halves of *Gaussia* luciferase are
172 fused onto PB1 and ANP32 protein (11, 20). Swine ANP32A interacted strongly with both
173 human-origin - E195 (pH1N1 2009) - and avian-origin - A/turkey/England/50-92/1992(H5N1)
174 - influenza polymerases, although not as strongly as chicken ANP32A (Fig. 5a). Furthermore,
175 the two residues identified as being responsible for strong pro-viral activity of swine ANP32A,
176 at positions 106 and 156, conferred this stronger polymerase binding, implying the mode of
177 action of these mutations is through enhancing ANP32-polymerase interactions (Fig. 5a). It
178 was also shown that N129I, the substitution naturally identified in chicken ANP32B and
179 previously shown to abolish binding and activity in chicken and human ANP32 proteins (11,
180 15), showed a similar phenotype in swine ANP32A, abolishing detectable binding and activity
181 (Fig. 5a,b). The ablations of the pro-viral activity of swine ANP32A and ANP32B by the
182 substitution N129I was not explained by reductions in expression of these mutant proteins
183 (Fig. 5b,c).

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

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

189 Very few mammals share the pro-viral marker, 156S, and the few that do mostly
190 constitute species not yet described as hosts for influenza viruses (Fig. 5d). A notable
191 exception is the pika which, in a similar manner to pigs, appears to be highly susceptible to

192 avian influenza viruses with minimal mammalian adaptation (21-23). Pigs are currently the
193 only known mammalian species that contain the secondary, minor pro-viral maker 106V.

194 Discussion

195 In this study we describe the ability of different mammalian ANP32A and ANP32B
196 proteins to support activity of influenza virus polymerases isolated from a variety of hosts.
197 We found that swine ANP32A, uniquely among the ANP32 proteins, supports avian influenza
198 virus polymerase activity. Swine ANP32A does not harbour the avian-specific 33 amino acid
199 duplication that enables the strong interaction and efficient support of polymerase activity of
200 avian-origin viruses by avian ANP32A proteins. Thus, avian influenza viruses are restricted for
201 replication in swine as we have previously shown, and mammalian-adapting mutations
202 enhance their polymerase activity in pig cells (24). Nonetheless, this level of pro-viral activity
203 associated with swine ANP32A, albeit weak compared to avian ANP32As, may contribute to
204 the role of swine as mixing vessels: non-adapted avian influenza viruses that infect pigs could
205 replicate sufficiently to accumulate further mutations that allow for more efficient
206 mammalian adaptation and/or reassortment, enabling virus to either become endemic in
207 swine or to jump into other mammals, including humans.

208 We map this strongly pro-viral polymerase phenotype to a pair of mutations which
209 allow swine ANP32A to bind more strongly to influenza virus polymerase, potentially
210 explaining the mechanism behind its super pro-viral activity. These residues are only found in
211 a handful of other mammals including wild pigs and pika. It is conceivable these residues are
212 located at a binding interface between polymerase and ANP32, but resolution of the structure
213 of the host:virus complex will be required to confirm this hypothesis.

214 It has long been speculated that swine play a role as ‘mixing vessels’, by acting as host
215 to both human- and avian-origin influenza viruses. This trait may be partially attributed to
216 receptor patterns in swine allowing viruses that bind to both α 2,3 linked (i.e. avian-like
217 viruses) and α 2,6 linked sialic acid (i.e. human-like) to replicate alongside each other (8, 9).
218 However, replication of the avian-origin influenza virus genomes inside infected cells is also
219 required to enhance the opportunity for further adaptation or reassortment. We previously
220 developed a minigenome assay for assessing polymerase activity in swine cells and showed
221 that avian virus polymerases were restricted and that restriction could be overcome by typical
222 mutations known to adapt polymerase to human cells (24). Taken together the ability to enter
223 swine cells without receptor switching changes in the haemagglutinin gene, along with a
224 greater mutation landscape afforded in swine cells by the partially supportive pro-viral
225 function of swine ANP32A may have an additive effect to allow swine to act an intermediate
226 host for influenza viruses to adapt to mammals. Furthermore, our work implies other
227 mammals, such as the pika, could play a similar role which is of particular interest due to the
228 pika’s natural habitat often overlapping with that of wild birds and its (somewhat swine-like)
229 distribution of both α 2,3 and α 2,6-linked sialic acid receptors (25).

230 Upon crossing into humans from swine, it is likely that viruses would be under
231 selective pressure to adapt to human pro-viral factors, such as the ANP32 proteins. We use
232 the example of a pair of first- and third-wave pandemic H1N1 influenza viruses isolated from
233 clinical cases in 2009 and 2010 (18). The polymerase constellation of the 2009 pH1N1 virus
234 contains PB2 and PA gene segments donated from avian sources to a swine virus in a triple
235 reassortant constellation in the mid-1990s, then passed onto humans in 2009 (17). Although
236 the first-wave viruses, derived directly from swine, can clearly replicate and transmit between
237 humans, over time the PA substitution, N321K, was selected because it enabled more efficient

238 support of the viral polymerase by human ANP32 proteins – our data suggests this is a direct
239 adaptation to the less supportive human ANP32 proteins. This again illustrates how swine
240 have acted as a ‘halfway house’ for the step-wise adaptation of genes originating in avian
241 influenza viruses that have eventually become humanised.

242 Also of note, we show here that as for the human orthologues, the ANP32A and B
243 proteins of swine (as well as all other mammals tested here) are fairly redundant in their pro
244 influenza activity to support the viral polymerase. We further show that the substitution
245 N129I is able to partially or fully ablate the pro-viral activity of both swine ANP32A and
246 ANP32B. We suggest that the introduction of this substitution in both swine ANP32A and
247 ANP32B by genome editing would be a feasible basis generating influenza resistant, or
248 resilient, pigs, in a similar manner to that demonstrated for porcine respiratory and
249 reproductive syndrome virus resistant pigs, and proposed for influenza resistant, or resilient,
250 chickens (11, 26).

251 To conclude, we hypothesise that the superior pro-viral function of swine ANP32A for
252 supporting influenza replication may play a role in both the ability of swine to host avian
253 influenza viruses, but also upon the potential evolutionary ecology of swine influenza viruses.
254 This, in turn, may influence the ability of different swine influenza viruses to act as zoonotic
255 hosts or as potential pandemic viruses.

256 **Materials and methods**

257 *Cells*

258 Human engineered-Haploid cells (eHAP; Horizon Discovery) and eHAP cells with
259 ANP32A and ANP32B knocked out (dKO) by CRISPR-Cas9, as originally described in (14), were

260 maintained in Iscove's Modified Dulbecco's Medium (IMDM; ThermoFisher) supplemented
261 with 10% fetal bovine serum (FBS; Biosera), 1% non-essential amino acids (NEAA; Gibco) and
262 1% Penicillin-streptomycin (pen-strep; invitrogen). Human embryonic kidney (293Ts, ATCC)
263 and Newborn Pig Trachea cells (NPTr; ATCC) were maintained in Dulbecco's Modified Eagle
264 Medium (DMEM) supplemented with 10% FBS, 1% NEAA and 1% pen-strep. All cells were
265 maintained at 37°C, 5% CO₂.

266 *ANP32 plasmids constructs*

267 Animal ANP32 constructs were codon optimised and synthesised by GeneArt
268 (ThermoFisher). Sequences used were pig (*Sus scrofa*) ANP32B (XP_020922136.1), Horse
269 (*Equus caballus*) ANP32A (XP_001495860.2) and ANP32B (XP_023485491.1), Dog (*Canis lupus*
270 *familiaris*) ANP32A (NP_001003013.2), Dingo (*Canis lupus dingo*) ANP32B (XP_025328134.1),
271 Monk Seal (*Neomonachus schauinslandi*) ANP32A (XP_021549451.1) and ANP32B
272 (XP_021546921.1), and Common Vampire Bat (*Desmodus rotundus*) ANP32A
273 (XP_024423449.1) and ANP32B (XP_024415874.1). All isoforms were chosen due to their
274 equivalence to the known functional human isoforms. Species of origin were chosen due to
275 being flu hosts or the most-commonly related species to flu hosts (in the case of Monk Seal
276 which are closely related to Harbour Seal whereas common vampire bats belong to the same
277 family as little yellow-shouldered and flat-faced bats). Dingo ANP32B was substituted for dog
278 ANP32B as the equivalent isoform used for all other ANP32Bs is unannotated in the dog
279 genome due to a gap in the scaffold. All ANP32 expression constructs included a C-terminal
280 GSG-linker followed by a FLAG tag and a pair of stop codons. Overlap extension PCR was used
281 to introduce mutations into the ANP32 constructs which were then subcloned back into
282 pCAGGS and confirmed by sanger sequencing.

283 *Viral minigenome plasmid constructs*

284 Viruses and virus minigenome full strain names used through this study were
285 A/Victoria/1975(H3N2; Victoria), A/England/195/2009(pH1N1; E195),
286 A/England/687/2010(pH1N1; E687), A/Japan/WRAIR1059P/2008(H3N2; Japan),
287 B/Florida/4/2006 (B/Florida), A/Anhui/2013(H7N9; Anhui), A/duck/Bavaria/1/1977(H1N1,
288 Bavaria), A/turkey/England/50-92/1992(H5N1; 50-92), A/chicken/Pakistan/UDL-
289 01/2008(H9N2; UDL1/08), A/canine/New York/dog23/2009(H3N8; CIV H3N8),
290 A/canine/Illinois/41915/2015(H3N2; CIV H3N2), A/equine/Richmond/1/2007(H3N8;
291 Richmond), A/swine/England/453/2006(EAH1N1; sw/453), A/swine/Hubei/221/2016(H1N1;
292 Hubei), A/little yellow-shouldered bat/Guatemala/164/2009(H17N10; H17) and A/flat-faced
293 bat/Peru/033/2010(H18N11; H18). Viral minigenome expression plasmids (for PB2, PB1, PA
294 and NP) for H3N2 Victoria, H5N1 50-92, H1N1 E195, H1N1 E687 IBV Florida/06, H9N2 UDL1/08
295 and H1N1 Bavaria have been previously described (10, 18, 24, 27). Viral minigenome plasmids
296 for H1N1 swine/453, H3N2 Japan, H3N2 CIV, H3N8 CIV, Hubei and Richmond were subcloned
297 from reverse genetics plasmids or cDNA into pCAGGS expression vectors using virus segment
298 specific primers.

299 pCAGGs minigenome reporters for H17N10 and H18N11 bat influenza viruses were a
300 kind gift from Professor Martin Schwemmler, Universitätsklinikum Freiburg (28). pCAGGs
301 minigenome reporters for H7N9 were a kind gift from Professor Munir Iqbal, The Pirbright
302 Institute, UK. Reverse genetics plasmids for H3N8, Richmond were a kind gift from Adam Rash
303 of the Animal Health Trust, Newmarket, UK. Reverse genetics plasmids for H3N2 CIV and
304 H3N8 CIV were a kind gift from Dr. Colin Parrish of the Baker Institute for Animal Health,

305 Cornell University (29, 30). Viral RNA from sw/453 was kindly provided by Dr. Sharon Brookes,
306 Animal Plant and Health Agency, Weybridge, UK.

307 *Minigenome assay*

308 eHAP dKO cells were transfected in 24 well plates using lipofectamine[®] 3000 (thermo
309 fisher) with a mixture of plasmids; 100ng of pCAGGs ANP32/empty vector, 40ng of pCAGGs
310 PB2, 40ng of pCAGGs PB1, 20ng of pCAGGs PA, 80ng of pCAGGs NP, 40ng of pCAGGs *Renilla*
311 luciferase, 40ng of poll vRNA-Firefly luciferase. Transfections in wild-type eHap cells were
312 performed similarly but without ANP32. Transfections in NPTr cells were carried out in 12 well
313 plates using the same ratios above. 24 hours post-transfection cells were lysed with passive
314 lysis buffer (Promega) and luciferase bio-luminescent signals were read on a FLUOstar Omega
315 plate reader (BMG Labtech) using the Dual-Luciferase[®] Reporter Assay System (Promega).
316 Firefly signal was divided by *Renilla* signal to give relative luminescence units (RLU).

317 *Split Luciferase Assay*

318 Split luciferase assays were undertaken in 293Ts seeded in 24 well plates. 30ng each
319 of PB2, PA, and PB1, with the N-terminus of *Gaussia* Luciferase (Gluc1) tagged to its C-
320 terminus after a GGSGG linker, were co-transfected using lipofectamine 3000 along with
321 ANP32A, tagged with the C-terminus of *Gaussia* Luciferase (Gluc2) on its C-terminus (after a
322 GGSGG linker). 24 hours later cells were lysed in 100µl of *Renilla* lysis buffer (Promega) and
323 *Gaussia* activity was measured using a *Renilla* luciferase kit (Promega) on a FLUOstar Omega
324 plate reader (BMG Labtech). Normalised luminescence ratios (NLR) were calculated by
325 dividing the values of the tagged PB1 and ANP32 wells by the sum of the control wells which
326 contained 1) untagged PB1 and free Gluc1 and 2) untagged ANP32A and free Gluc2 as
327 described elsewhere (11, 31).

328 *Western Blotting*

329 To confirm equivalent protein expressing during mini-genome assays transfected cells
330 were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS,
331 50mM TRIS, pH 7.4) supplemented with an EDTA-free protease inhibitor cocktail tablet
332 (Roche).

333 Membranes were probed with mouse α -FLAG (F1804, Sigma), rabbit α -Vinculin
334 (AB129002, Abcam), rabbit α -PB2 (GTX125926, GeneTex) and mouse α -NP ([C43] ab128193,
335 Abcam). The following near infra-red (NIR) fluorescent secondary antibodies were used:
336 IRDye[®] 680RD Goat Anti-Rabbit (IgG) secondary antibody (Ab216777, Abcam) and IRDye[®]
337 800CW Goat Anti-Mouse (IgG) secondary antibody (Ab216772, Abcam). Western Blots were
338 visualised using an Odyssey Imaging System (LI-COR Biosciences).

339 *Immunofluorescence*

340 For investigating localisation of exogenously expressed ANP32 proteins, eHAP ANP32
341 dKO cells were cultured on 8 well chambered cover slips (Ibidi) and transfected with 125 ng
342 of the indicated FLAG-tagged ANP32 protein. Cells were fixed in PBS, 4% paraformaldehyde
343 24 hours post transfection, then permeabilised in PBS, 0.2% Triton X-100. Cells were blocked
344 in PBS, 2% bovine serum albumin and 0.1% tween. FLAG-tagged ANP32 proteins were
345 detected using mouse anti-FLAG M2 primary antibody (Sigma), followed by goat anti-mouse
346 Alexa Fluor 568 (Invitrogen). Nuclei were counterstained with DAPI. Images were obtained
347 using a Zeiss Cell Observer widefield microscope with ZEN Blue software, using a Plan-
348 Apochromat 63x 1.40-numerical aperture oil objective (Zeiss) and processed using FIJI
349 software (32).

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462 Figure legends

463 **Figure 1 – Most mammalian species have two ANP32 proteins capable of supporting**
464 **influenza polymerase.** a) Minigenome assays performed in human eHAP dKO with ANP32
465 proteins from different avian or mammalian species co-transfected. Green bars indicate
466 species the influenza virus polymerase was isolated from, orange bars indicate recent species
467 the virus has jumped from. b) Western blot assay showing protein expression levels of FLAG-
468 tagged ANP32 proteins during a minigenome assay. c) Immunofluorescence images showing
469 nuclear localisation of all FLAG-tagged ANP32 proteins tested. Abbreviations: ch – chicken, hu
470 – human, sw – swine, eq – equine. Statistical significance was determined by one-way ANOVA
471 with multiple comparisons against empty vector. *, $0.05 \geq P > 0.01$; **, $0.01 \geq P > 0.001$; ***,
472 $0.001 \geq P > 0.0001$; ****, $P \leq 0.0001$.

473

474 **Figure 2. swANP32A can support the activity of minimally mammalian-adapted or**
475 **completely unadapted polymerases.** Minigenome assays of swine (a) and avian (b)
476 polymerases performed in human eHAP dKO cells with ANP32 proteins from different avian
477 or mammalian species co-transfected. Green bars indicate species the influenza virus
478 polymerase was isolated from, orange bars indicate recent species the virus has jumped from.
479 c) ANP32 protein titrations with several different virus polymerases. ANP32 proteins were
480 diluted in a series of 3x dilutions starting with 100ng. Statistical significance was determined
481 by one-way ANOVA with multiple comparisons against empty vector. **, $0.01 \geq P > 0.001$; ***,
482 $0.001 \geq P > 0.0001$; ****, $P \leq 0.0001$.

483

484 **Figure 3. Third-wave pandemic H1N1 viruses adapt to human ANP32 proteins through the**
485 **PA mutation N321K.** a) Minigenome assays of first- and third-wave pH1N1 viruses (E195 and
486 E687, respectively) performed in wild-type human eHAP cells and swine NPTr cells. b)
487 Minigenome assays performed in human eHAP cells with ANP32A and ANP32B knocked out
488 with ANP32 proteins from human or swine co-transfected in. Statistical significance was
489 determined by one-way ANOVA with multiple comparisons. ****, $P \leq 0.0001$.

490

491 **Figure 4. The pro-avian activity of swine ANP32A can be mapped to amino acids in LRR4 and**
492 **the central domain.** a) Minigenome assays performed in human eHAP dKO cells with
493 human/swine ANP32A reciprocal mutants expressed. b) Western blot analysis showing
494 expression levels of human/swine ANP32A from minigenome assays. c) Crystal structure of
495 ANP32 (PDBID: 2JE1) with residues found to affect pro-viral activity mapped (33). The

496 unresolved, unstructured LCAR shown as a yellow line. Schematic made using PyMol (34).
497 Statistical significance was determined by one-way ANOVA with multiple comparisons. *, 0.05
498 $\geq P > 0.01$; ***, $0.001 \geq P > 0.0001$; ****, $P \leq 0.0001$.

499

500 **Figure 5. Amino acid residues responsible for the pro-avian polymerase activity of swine**
501 **ANP32A are also responsible for it binding more strongly to influenza trimeric polymerase.**

502 a) Split luciferase assays showing the relative binding of different ANP32 proteins to trimeric
503 polymerase from different influenza virus strains. PB1 was tagged with the N-terminal part of
504 *Gaussia* luciferase while ANP32 proteins were tagged with the C-terminal part. NLR,
505 normalised luminescence ratio, calculated from the ratio between tagged and untagged
506 ANP32/PB1 pairs. Assay performed in 293T cells. Statistical significance was determined by
507 one-way ANOVA with multiple comparisons between the swA and huA wild-types and
508 mutants. ***, $0.001 \geq P > 0.0001$; ****, $P \leq 0.0001$. b) Minigenome assays performed in
509 human eHAP cells with ANP32A and ANP32B knocked out with different ANP32 mutants
510 expressed. The N129I naturally occurs in chicken ANP32B. c) Western blot assay showing
511 protein expression levels of FLAG-tagged ANP32 proteins during a minigenome assay. d)
512 phylogenetic tree of mammalian ANP32A proteins, species which contain the highly pro-viral
513 156S shown in green, species with 156P shown in black. Phylogenetic trees made using the
514 neighbour-joining method based on amino acid sequence. Statistical significance was
515 determined by one-way ANOVA with multiple comparisons against empty vector. ****, $P \leq$
516 0.0001.

Figure 1

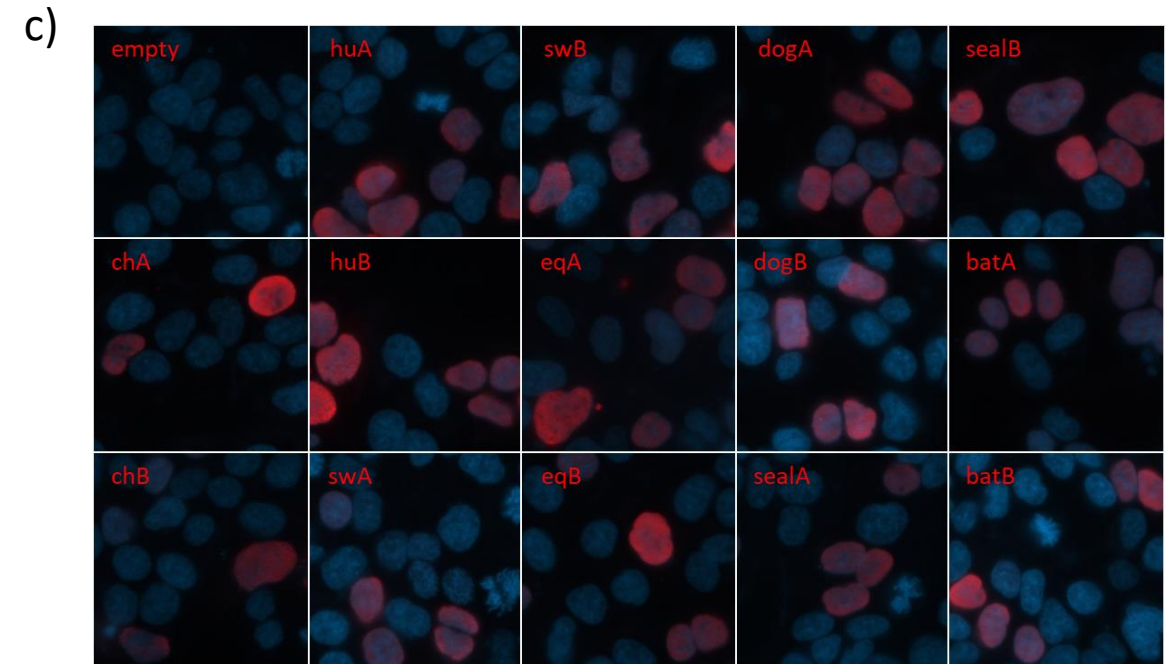
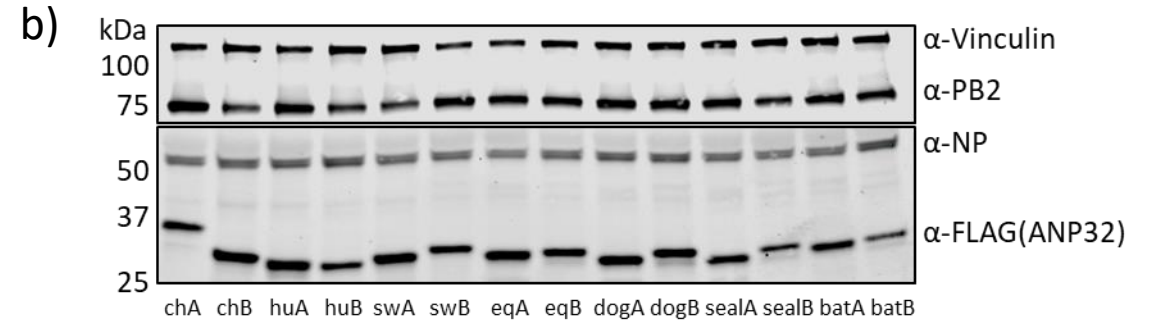
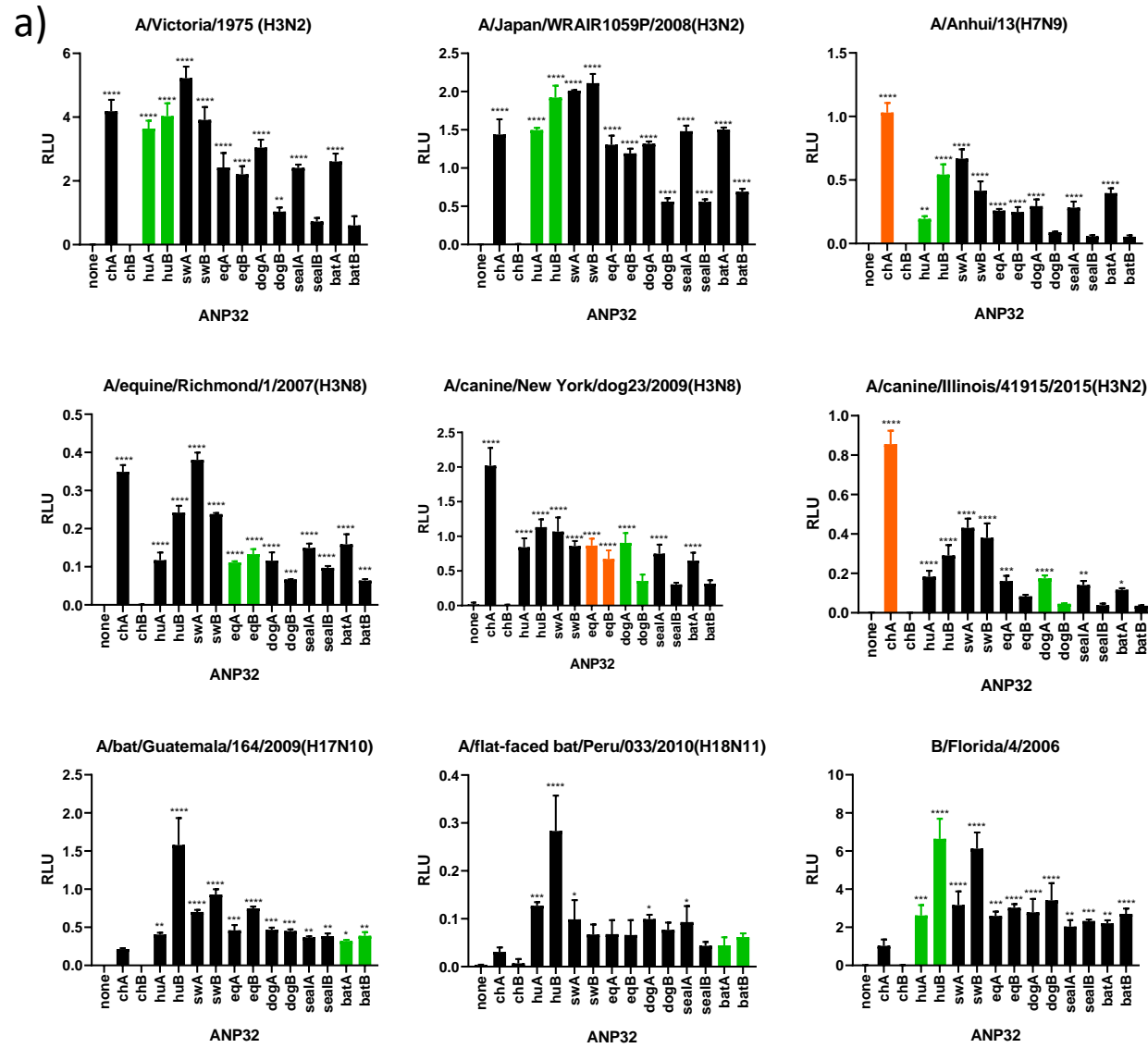


Figure 2

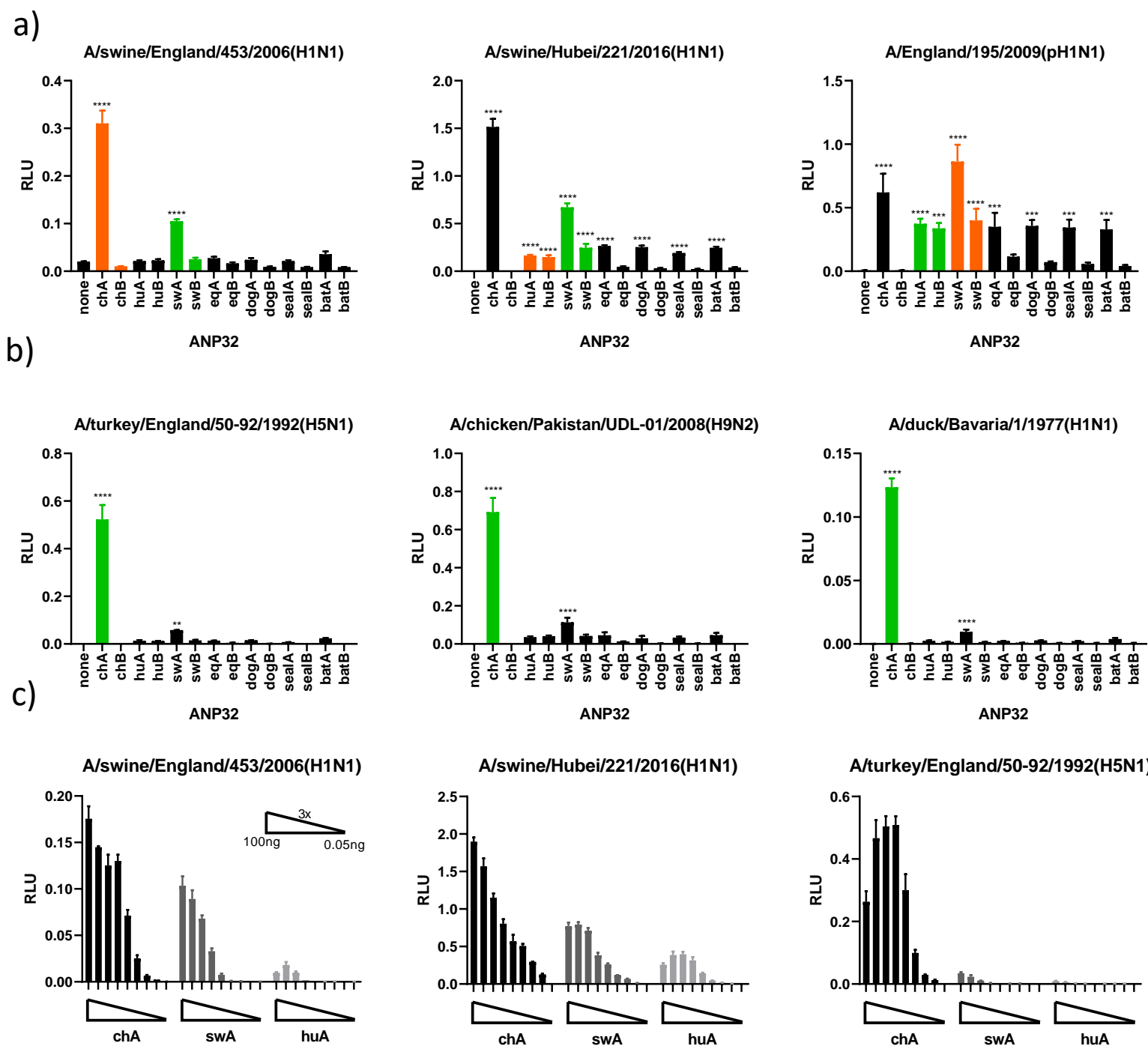


Figure 3

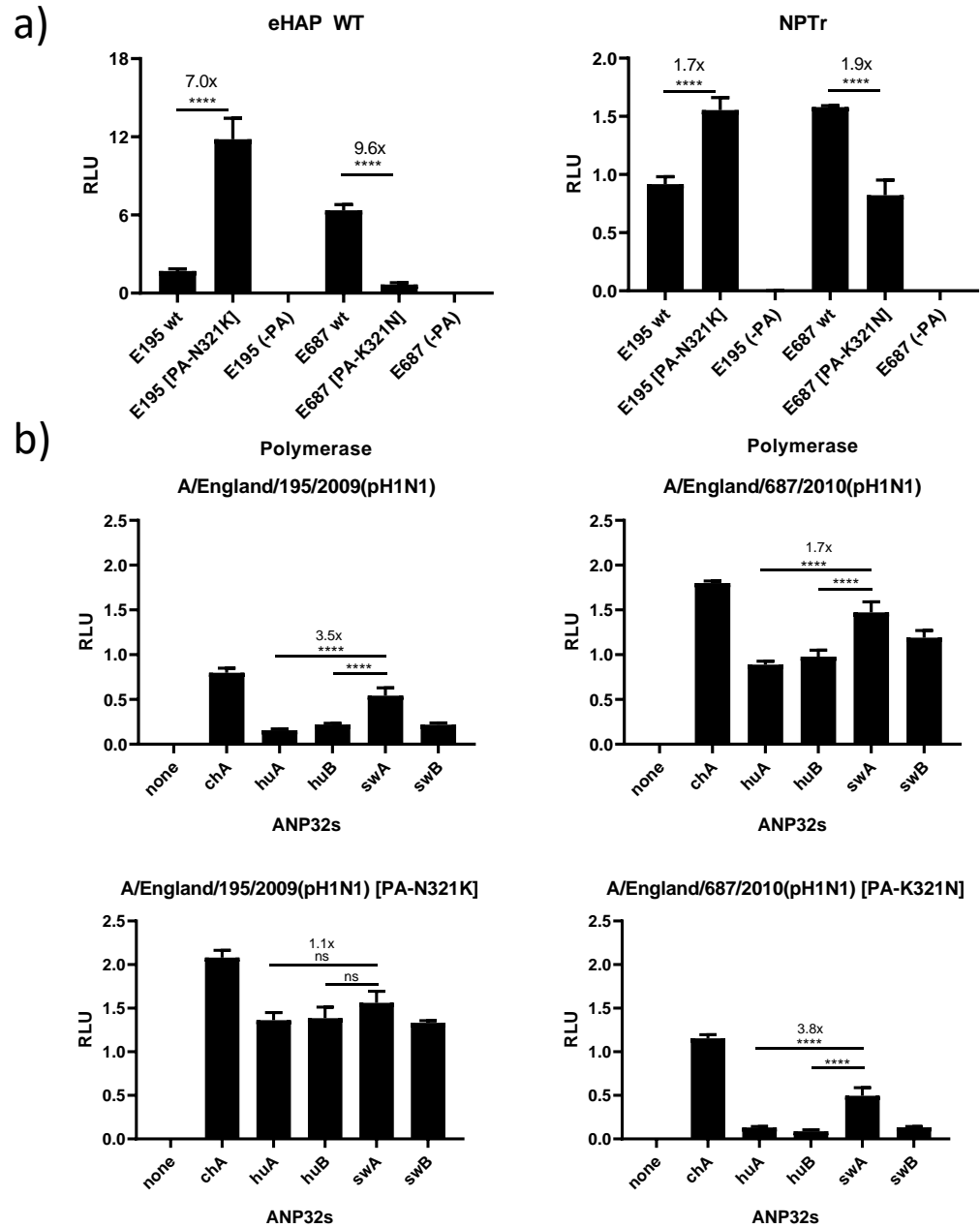


Figure 4

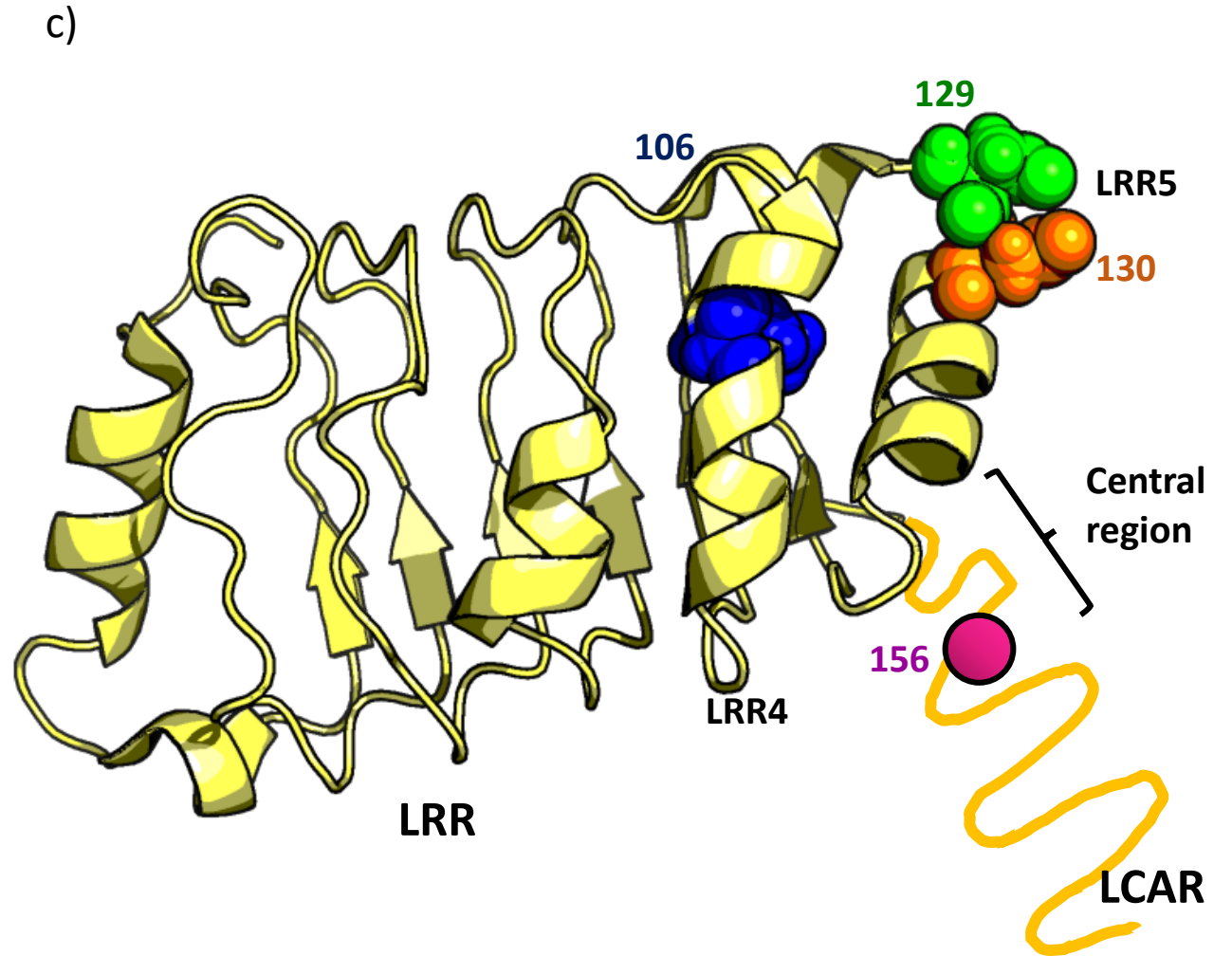
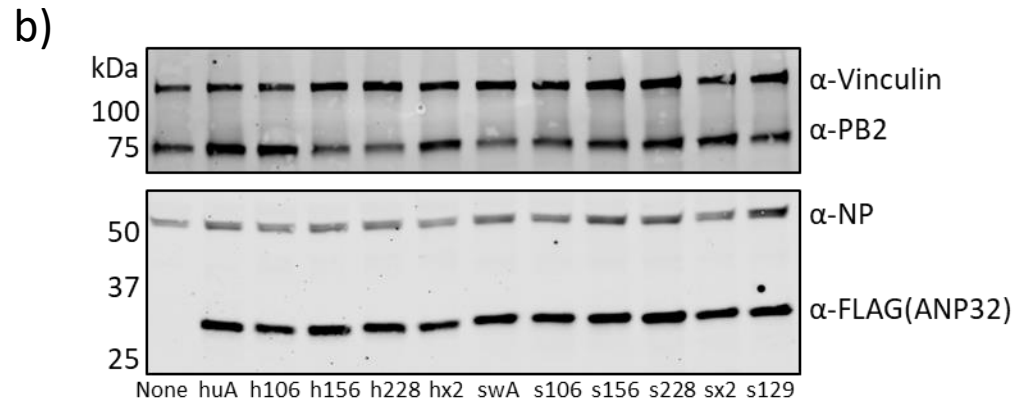
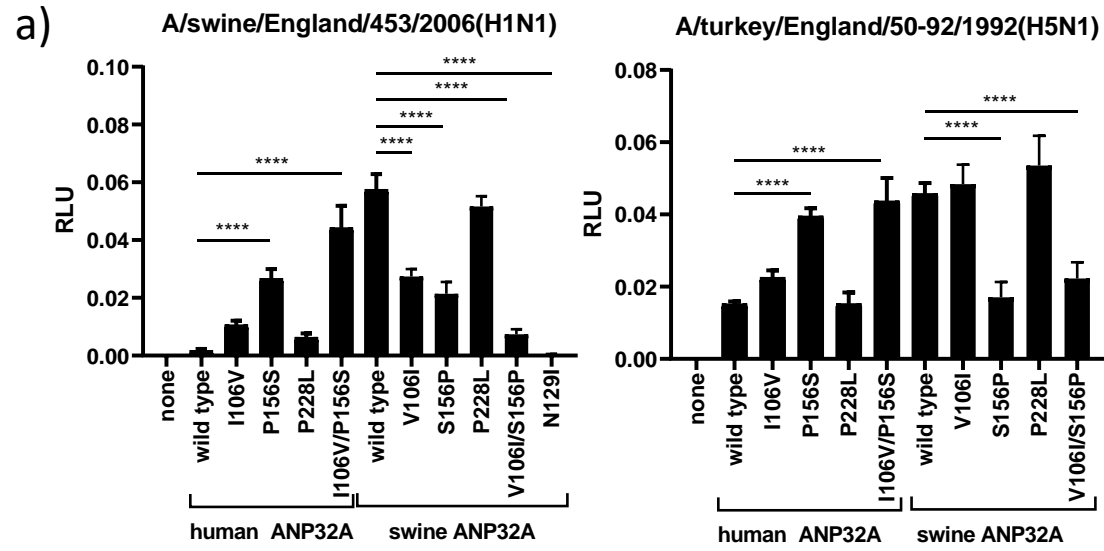


Figure 5

