Fundamental contribution and host range determination of ANP32 protein family in influenza A virus polymerase activity

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Running Title: ANP32 proteins are indispensable for influenza virus

ABSTRACT

The polymerase of the influenza virus is part of the key machinery necessary for viral replication. However, the avian influenza virus polymerase is restricted in mammalian cells. The cellular protein ANP32A has been recently found to interact with viral polymerase, and to both influence polymerase activity and interspecies restriction. Here we report that either ANP32A or ANP32B is indispensable for influenza A virus RNA replication. The contribution of ANP32B is equal to that of ANP32A, and together they play a fundamental role in the activity of mammalian influenza A virus polymerase, while neither human ANP32A nor ANP32B support the activity of avian viral polymerase. Interestingly, we found that avian ANP32B was naturally inactive, leaving ANP32A alone to support viral replication. Two amino acid mutations at sites 129-130 in chicken ANP32B lead to the loss of support of viral replication and weak interaction with the viral polymerase complex, and these amino acids are also crucial in the maintenance of viral polymerase activity in other ANP32 proteins. Our findings strongly support ANP32A&B as key factors for both virus replication and adaption.

KEYWORDS: ANP32A; ANP32B; influenza A virus; polymerase activity; RNA replication; interspecies transmission

IMPORTANCE

The key host factors involved in the influenza A viral the polymerase activity and RNA replication remain largely unknown. Here we provide evidence that ANP32A and ANP32B from different species are powerful factors in the maintenance of viral polymerase activity. Human ANP32A and ANP32B contribute equally to support human influenza virus RNA replication. However, unlike avian ANP32A, the avian ANP32B is evolutionarily non-functional in supporting viral replication because of a 129-130 site mutation. The 129-130 site plays an important role in ANP32A/B and viral polymerase interaction, therefore determine viral replication, suggesting a novel interface as a potential target for the development of anti-influenza strategies.

53 **INTRODUCTION**

54 Virus transmission from its natural host species to a different species
55 reflects mechanisms of molecular restriction, evolution, and adaptation. Birds
56 harbor most of the influenza A viruses, which are typically replication-restricted
57 in human hosts because of receptor incompatibility and limited viral polymerase
58 activity in human cells. Although many host factors have been reported to be
59 involved in viral replication (1-8), the key mechanisms that determine viral
60 polymerase activity and host range are poorly understood.

61 Influenza A viral ribonucleoprotein (vRNP), the viral minimum replicon,
62 comprises the viral genome, the heterotrimeric RNA polymerase PB1, PB2, PA,
63 and the nucleoprotein (NP), and carries out viral RNA transcription and
64 replication in infected cells. Avian influenza A polymerases have very limited
65 activity in mammalian cells, indicating an unknown host-specific restriction
66 mechanism that directly affects viral RNA replication. The adaptation of avian
67 viruses to mammals, such as occurred with the H5N1 and H7N9 avian viruses,
68 occurs along with substitutions on the viral polymerase, mainly on the PB2
69 subunit (E627K and other signatures), which enhance viral replication (9-22).
70 Many host factors have been reported to interact with the vRNP complex to
71 help viral replication (7, 23-33). Of these proteins, chicken acidic nuclear
72 phosphoprotein 32 family member A (chANP32A) has been reported to
73 specifically promote avian influenza replication due to the 33 amino acids insert

74 (8). This 33 amino acid insert includes a hydrophobic SUMO interaction motif,
75 which connects to host SUMOylation to partially contribute to the promotion of
76 avian viral polymerase activity (34). Furthermore, ANP32A from birds has
77 splicing variants without SUMO interaction motif also support viral replication
78 and polymerase adaption (35). Interestingly, a previous study showed that
79 ANP32A&B (acidic nuclear phosphoprotein 32 family, members A&B) have
80 been found to regulate viral RNA synthesis (7). These studies suggest that
81 ANP32A plays an important role in viral replication.

82 The acidic (leucine-rich) nuclear phosphoprotein 32kDa (ANP32) family
83 comprises several members, including ANP32A, ANP32B, and ANP32E, which
84 have various functions in the regulation of gene expression, intracellular
85 transportation, and cell death (36). Although ANP32A is considered to be an
86 important co-factor of the influenza virus polymerase and to influence the viral
87 host range, the roles of different ANP32 members in viral replication, and the
88 extent to which the proteins are involved in the activity of the polymerase
89 remain unclear. In this study, by using CRISPR/Cas9 knockout screening, we
90 found that ANP32A and ANP32B play fundamental roles in the facilitation of
91 influenza A viral RNA synthesis, and that both ANP32A and ANP32B contribute
92 equally. The mammalian ANP32 proteins give no or only limited support to the
93 avian virus polymerase. Human ANP32A&B do not support the replication of
94 the avian influenza virus, but this restriction can be overcome by E627K

95 substitution in the viral PB2 protein. Furthermore, we found that chicken
96 ANP32B has no effect on polymerase activity, which can be ascribed to
97 mutations in two key amino acid residues of ANP32A&B that determine their
98 activity in supporting viral RNA replication. Together, these data reveal
99 fundamental roles for ANP32A and ANP32B in supporting influenza virus A
100 polymerase activity as well as a site key for their function, and show that both
101 ANP32A and B determine viral polymerase adaptation and host range.

102

103 **RESULTS**

104 **HuANP32A&B Are Critical Host Factors that Determine Viral Polymerase**
105 **Activity and Virus Replication**

106 Previous studies have reported that several host factors, including BUB3,
107 CLTC, CYC1, NIBP, ZC3H15, C14orf173, CTNNB1, ANP32A, ANP32B,
108 SUPT5H, HTATSF1, and DDX17, interact with influenza viral polymerase and
109 that some of these factors have an effect on viral polymerase activity (4, 23,
110 37). All these observations were based on the gene transient knockdown
111 technique, meaning that the results may vary because of the different
112 knockdown efficiency of target genes. Using a CRISPR/Cas9 system, however,
113 allows rapid knockout of certain genes and accurate evaluation of target
114 proteins. To identify the critical roles of the above-mentioned host factors in

115 influenza viral replication, we used a CRISPR/Cas9 system to establish a series
116 of knockout 293T cell lines, and a model viral-like luciferase RNA was
117 expressed together with viral polymerase PB1, PB2, PA, and NP, to determine
118 the polymerase activity in these cell lines. Firstly, we tested the polymerase
119 activity of 2009 pandemic H1N1 virus A/Sichuan/01/2009($H1N1_{SC09}$) (38) on
120 these knockout 293T cells, and found that individual knockout of NIBP, ZC3H15,
121 or DDX17 results in a 2-4 fold decrease in viral polymerase activity, in which is
122 consistent with previous results (23, 37). However, in none of the other single
123 gene knockout cells was viral polymerase activity blocked (Fig. 1A). We did not
124 observe any reduction of viral polymerase activity in ANP32A or ANP32B
125 knockout cells, which was a surprising result as ANP32A and ANP32B were
126 reported to be important host factors supporting viral polymerase activity (7, 8,
127 34, 35). Since ANP32A and ANP32B have high similarity in both structure and
128 known functions, we predicted that in single knockout cell lines of ANP32A or
129 ANP32B, the presence of either one of these two proteins could support viral
130 replication in the absence of the other. We then developed an ANP32A and
131 ANP32B double knockout cell line to confirm this hypothesis. In human ANP32A
132 (huANP32A) knockout 293T cells (AKO cells), and human ANP32B
133 (huANP32B) knockout 293T cells (BKO cells), the viral polymerases have
134 similar activities to wild-type 293T cells, but when both ANP32A and ANP32B
135 were knocked out (DKO) (Fig. S1 in the supplemental material), the polymerase

136 activity was abolished (about 10000-fold reduction) (Fig 1B), although the
137 polymerase was expressed equally in the WT, AKO, BKO, and DKO cells (Fig.
138 1B). We then tested the polymerase activities of 2013 China H7N9 human
139 isolate A/Anhui/01/2013 ($H7N9_{AH13}$) (39), and H1N1 virus A/WSN/1933 (WSN)
140 on AKO, BKO and DKO cell lines, and found that the viral polymerase complex
141 lost all activity in the DKO cells, but not in the AKO or BKO cells (Fig. 1C and
142 1D). We further confirmed the effect of huANP32A&B on viral infectivity by
143 infecting 293T cells and DKO cells with WSN virus. In DKO cells, but not AKO
144 or BKO cells, the infectivity of WSN decreased about 100000-fold (Fig. 1E).
145 These results indicate a crucial role for both ANP32A&B in viral RNA replication.

146 The knockout of both ANP32A and ANP32B led to dramatic loss of viral
147 polymerase activity (about 10000 fold), which is distinct from the previous report,
148 which used a gene knockdown method and resulted in about 3-5 fold reduction
149 of viral polymerase activity (8), indicating that ANP32A and ANP32B are of
150 fundamental importance in viral replication. We found that reconstitution of
151 either ANP32A or ANP32B, or both of them, in the DKO cells restored the viral
152 polymerase activities of $H1N1_{SC09}$, $H7N9_{AH13}$, and WSN viruses (Fig. 2A to 2C).
153 Expression of huANP32A or huANP32B in DKO cells supported the $H1N1_{SC09}$
154 viral polymerase activity in a dose-dependent manner (Fig. 2D). Reconstitution
155 of huANP32A or huANP32B or both of them in DKO cells restored full viral
156 infectivity (Fig. 2E). We confirmed that the required level of expression of

huANP32A or B is very low, and overdose expression has a negative effect (Fig. S2), suggesting an explanation of the confusing phenotype previously observed, that in normal 293T cells overexpression of ANP32 protein decreased viral polymerase activity (8). These data indicate that ANP32A and ANP32B are key factors required for polymerase activity, that they have similar functions in the support of viral replication, and that they can function independently and contribute equally to influenza virus polymerase activity.

Influenza A virus replication starts from the transcription and replication of the negative single-stranded viral RNA (vRNA) by the vRNP complex. vRNA is transcribed into positive complementary RNA (cRNA) and mRNA, then the cRNA is used as a template to amplify into new vRNA (40, 41). We found that vRNA, cRNA, and mRNA synthesis were dramatically reduced in the DKO cells. However, when huANP32A was reconstituted in the DKO cells, vRNA, cRNA, and mRNA synthesis fully recovered (Fig. 3A to 3C). We observed similar results from reconstitution of ANP32B or both (Fig. 3A to 3C), indicating that huANP32A&B are key factors in triggering the replication of the viral genome.

We next used an eight-plasmid reverse genetics system in $H1N1_{SC09}$ and tested the virus production in the supernatant of the transfected cells using an antigen capture ELISA (42). The result showed that the DKO cells had very low NP production in the cell supernatant, compared with the 293T, AKO, or BKO cells (Fig. 3D). The NP production was recovered when the huANP32A and/or

178 huANP32B was expressed in the DKO cells (Fig. 3E). Taken together, these
179 results suggest that huANP32A and huANP32B determine viral RNA replication
180 efficiency and subsequent viral production in 293T cells.

181

182 **Support of Influenza A Viral Replication by ANP32A or B from Different**
183 **Species**

184 ANP32A&B are members of the evolutionarily conserved ANP32 family,
185 which has various functions in the regulation of gene expression, intracellular
186 transportat, and cell death (36). We tried to knock out ANP32A&B in both
187 chicken DF1 cells and human A549 cells, but the knockout caused cell death,
188 indicating both ANP32A&B have crucial roles in cell proliferation in these cells.
189 ANP32A&B exist in almost all eukaryotic cells, with the exception of early
190 eukaryotic life (yeast and other fungi) (43). Molecular phylogenetic analysis of
191 ANP32 proteins revealed an evolutionary relationship among different species
192 (Fig. S3). We then investigated the support of ANP32A or ANP32B from
193 different species for viral polymerase activity in DKO cells. ANP32A from human,
194 chicken, duck, turkey, zebra finch, mouse, pig, and horse, and ANP32B from
195 human and chicken, were expressed individually with minigenomes of either
196 $H1N1_{SC09}$, human isolate $H7N9_{AH13}$, H3N2 Canine influenza virus
197 A/canine/Guangdong/1/2011($H3N2_{GD11}$), H3N8 equine influenza virus

198 A/equine/Xinjiang/1/2007(H3N8$_{XJ07}$), A/equine/Jilin/1/1989(H3N8$_{JL89}$), or H9N2
199 chicken virus A/chicken/Zhejiang/B2013/2012(H9N2$_{ZJ12}$), in DKO cells (Fig 4
200 and Fig. S4A). We found interestingly that chicken and other avian ANP32A
201 proteins, which contain an additional 33 amino acids compared to the ANP32
202 proteins of mammals (8), supported viral polymerase activities in all cases,
203 whereas the ANP32A proteins from human, pig, and horse, as well as human
204 ANP32B, supported mammalian influenza virus polymerase activities, but not
205 that of chicken H9N2$_{ZJ12}$, or the H3N8$_{JL89}$ (an avian-like virus). This result is
206 consistent with previous reports that avian ANP32A can promote avian viral
207 polymerase activity in human cells (8, 35). PB2 is the most important
208 polymerase subunit, and affects host range (25, 44). Almost all the avian
209 viruses had a glutamic acid (E) at PB2 residue 627, while it could be rapidly
210 selected as a lysine (K) when the virus adapted to mammals, accompanied with
211 increased pathogenicity and transmission abilities. The PB2 E627K mutation
212 has long been regarded as a key signature of the avian influenza virus in
213 overcoming the block to replicate in mammalian cells (9, 20, 21, 45-49). We
214 observed that the K627E mutation in H7N9$_{AH13}$ lost support from mammalian
215 ANP32 proteins (Fig. S4A and S4B), while E627K mutations in isolates of avian
216 origin (horse H3N8$_{JL89}$, or chicken H9N2$_{ZJ12}$) dramatically changed viral fitness
217 to mammalian ANP32 proteins (Fig. S4B and S4C). These results revealed that
218 ANP32A&B play important roles in influenza A viral replication across different

219 species, and that mammalian ANP32 proteins provide poor support for avian
220 influenza viral RNA replication.

221

222 **A Novel 129-130 Site Vital for Influenza Polymerase Activity in ANP32A&B**

223 Surprisingly, chicken ANP32B did not support any viral RNA replication, and
224 mouse ANP32A showed limited support to $H1N1_{SC09}$ and $H3N8_{XJ07}$, indicating
225 a functional loss in these molecules (Fig. 4). The ANP32 protein family has a
226 conserved structure containing an N-terminal leucine-rich repeat (LRR) and a
227 C-terminal low-complexity acidic region (LCAR) domain (36). ANP32A&B have
228 been reported to interact directly with the polymerase complex (7, 35). The key
229 functional domains of ANP32 proteins remain largely unknown. We noticed that
230 the chicken ANP32B (chANP32B) was functionally inactive and hypothesized
231 that certain amino acids may be responsible for this phenotype. Alignment of
232 huANP32B, pgANP32B, and chANP32B revealed scattered substitutions on
233 the proteins (Fig. S5A). Chimeric clones between chicken and human ANP32B
234 were constructed and evaluated (Fig. S5B). We found that the replacement of
235 amino acids 111-160 of huANP32B with those of chANP32B aborted the activity
236 of this protein, while chANP32B with human fragment 111-161 gained the ability
237 to boost viral polymerase activity (Fig. S5C and S5D). Further comparing of
238 ANP32B fragments 111-161 of chicken, human, and pig showed eight amino

239 acid substitutions (Fig. 5A). Of these, a combined mutation N129I/D130N, but
240 not others, on huANP32B impaired H1N1 polymerase activity (Fig. 5B), and the
241 N129I showed stronger impairment than did the D130N mutation (Fig. 5C). We
242 confirmed this phenotype on H7N9 polymerase activity (Fig. 5D). The 129-130
243 mutations also aborted the support of huANP32A of H1N1 and H7N9
244 polymerases (Fig. 5E and 5F). Furthermore, substitutions at 129-130 in
245 chANP32A and chANP32B reversed the support of these proteins of the H7N9
246 virus human isolate (Fig. 5G). Interestingly, in the content of a chicken H7N9
247 viral minigenome, although mutation of 129-130 of chANP32A impaired the
248 polymerase activity, the chANP32B reverse mutation did not restore its support
249 to chicken-like H7N9 viral RNA replication (Fig. 5H), indicating that chicken
250 virus replication may require both the 129-130 site and an extra 33-aminal acid
251 peptide as in chANP32A. The expression levels of mutant ANP32 proteins were
252 assessed using western blotting (Fig. S6). Most terrestrial mammalian
253 ANP32A&B proteins have a 129-ND-130 signature, but most of the avian
254 ANP32Bs have 129-IN-130 residues (Table S1). Murine ANP32A (muANP32A)
255 has 129-NA-130 and this may explain the impaired support of H1N1
256 polymerase by muANP32A (Fig. 4 and Fig. S7). To further confirm the function
257 of the 129-130 site, we made inactivating mutations of huANP32A and
258 huANP32B at the sites 129-130 (N129I, D130N) on the 293T genome using
259 CRISPR-Cas9 mediated recombination (Fig. 6A and Fig. S8); the double

mutated proteins expressed well and fully impaired the replication of both $H1N1_{SC09}$ and $H7N9_{AH13}$ (Fig. 6B and 6C). Interestingly, the single ANP32A or ANP32B mutations lead to slightly decreased polymerase activities compared with wildtype 293T cells, suggesting competition between the mutated proteins and the native proteins for viral polymerase binding.

The 129-130 Site in ANP32B Affects Its Interaction with Viral Polymerase

It has been reported that ANP32A variants interact with the polymerase subunit PB2 and that the avian ANP32A can enhance avian viral RNP assembly in human cells (35). ANP32A and ANP32B can interact with polymerase only when 3 subunits of the polymerase are present (7). ANP32B proteins from different species have a conserved structure that comprises a N-terminal leucine-rich repeat (LRR) region and a C terminal low complexity acidic region (LCAR) (Fig. 7A). To investigate the function of the 129-130 site in ANP32B in the interaction of this protein with viral polymerase, we used variants of huANP32B, chANP32B to co-transfect with WSN minireplicon into DKO cells. Co-immunoprecipitations detected strong interactions between huANP32A and the polymerase subunits PB1 and PA (Fig. 7B, Lane 1), but weak interactions when chANP32B was present (Fig. 7B, Lane 2). No interactions were detected when PB1 was absent (Fig. 7B, Lane 3), indicating this interaction occurred

between ANP32B and the viral trimeric polymerase complex, which is consistent with previous findings (7, 34). As controls, we also observed that the LCAR truncations of huANP32B were unable to interact with the viral polymerase (Fig. 7B, Lane 4-6), in agreement with previous observations (34). When we mutated the functional human ANP32B at the site 129-130 to the chicken ANP32B signature, the huANP32B lost the ability to interact with viral polymerase. Conversely, chANP32B gained the ability to co-immumoprecipitate with viral polymerase when its 129-130 sites were mutated to the human ANP32B signature (Fig. 7C). Together these results revealed a fundamental function of ANP32A and ANP32B in supporting influenza A viral polymerase activity and a novel 129-130 site of ANP32A&B in different species that may influence influenza virus replication.

DISCUSSION

Although the host factors that are involved in influenza A virus replication have been long investigated, and genome-wide screening has shown that many host proteins interact with viral polymerase, the key mechanisms that determine viral polymerase activity in different cells remain largely unclear. ANP32A and ANP32B have been previously identified binding with viral polymerase and

300 promoting human influenza viral vRNA synthesis (7). Furthermore, a recent
301 study showed for the first time that chicken ANP32A can rescue avian
302 polymerase activity in human cells because the chicken ANP32A harbors an
303 extra stretch of 33 amino acids which is absent in mammalian ANP32A (8).
304 However, the potencies of ANP32A and ANP32B (as well as other host factors)
305 in viral replication are not well investigated in different hosts. In our study, we
306 used CRISPR/Cas9 knockout cells to screen the candidate host proteins
307 involved in viral RNA replication. We identified that ANP32A and ANP32B are
308 key host co-factors that determine influenza A virus polymerase activity. Without
309 ANP32A&B, the viral polymerase activity decreased by 10000 fold and the viral
310 infectivity decreased by more than 100000 fold. In contrast, the DDX17
311 knockout cells showed about 3-fold decreased viral polymerase activity (Fig.
312 1A), which was in agreement with prior reports (23). We found that ANP32A or
313 ANP32B can independently restore viral polymerase activity, indicating that
314 both have a similar function in viral replication (7).

315 Influenza viruses and their hosts have a long co-evolution history. ANP32
316 family members are expressed in animal and plant cells, with both conserved
317 LRR and LCAR domains (43). Three conserved ANP32 members (ANP32A,
318 ANP32B, and ANP32E) in vertebrates were reported to have several functions
319 in cells. We found that knockout of both ANP32A and ANP32B abolished the
320 influenza viral replication, while the ANP32E may not be involved in viral

321 replication. Interestingly, we found that the chicken ANP32A keeps the
322 conserved function to support both avian and mammalian virus polymerase
323 activity, while human ANP32A or B did not support avian viral replication. Thus,
324 we demonstrate here that ANP32A and ANP32B are key host factors that play
325 a fundamental role in influenza A viral RNA replication.

326 A recent study revealed that avian ANP32A has a hydrophobic SUMO
327 interaction motif (SIM) in an extra 33 amino acid insert region which connects
328 to host SUMOylation to specifically promote avian viral polymerase activity (34).
329 However, this SIM-like domain is not present in all of the ANP32B proteins from
330 different hosts, and neither is it found in mammalian ANP32A. We showed that
331 most ANP32Bs from different species are functional as ANP32As in support of
332 viral replication, but chANP32B is naturally unable to support polymerase
333 activity (Fig. 4). We finally found that the amino acids 129I and 130N of
334 chANP32B are responsible for the loss of this function. Sequence analysis and
335 mutagenesis studies suggest that the 129-130 sites are important for
336 maintaining the function of avian ANP32A and human ANP32A/B in viral
337 replication (Fig. 5). The co-immunoprecipitation assay showed that mutations
338 on these 129-130 sites changed the interaction efficiency between the ANP32
339 proteins and viral polymerase complex (Fig. 7). Mutating the 129-130 sites in
340 chANP32B to the functional signature 129N and 130D enables chANP32B to
341 support polymerase activity in human viruses, but not chicken viruses with PB2

342 627E, indicating that the ANP32B may undergo selection in two areas during
343 co-evolution with the avian influenza virus: chANP32B does not harbor an extra
344 insert as chANP32A and the 129-130 mutations. This result also suggests that
345 avian ANP32A is the only protein from the avian ANP32 family to support avian
346 viral replication.

347 Together, out data give new insights into the functions of ANP32A and
348 ANP32B. The 129-130 substitution could be used as a novel target to modify
349 the genome of animals to develop influenza-A-resistant transgenic chickens, or
350 other animals, which will benefit the husbandry industry, as well as animal and
351 human health. Further investigation into the molecular mechanisms that
352 determine how ANP32 proteins work with the viral polymerase complex, for
353 example, the structure of the ANP32 and vRNP complex, and the fitness of
354 different virus subtypes would contribute to the understanding viral
355 pathogenesis and host defense.

356 **MATERIALS AND METHODS**

357 **Cells, Viruses, and Plasmids**

358 Human embryonic kidney (293T, ATCC CRL- 3216) and Madin-Darby
359 canine kidney (MDCK, CCL-34™) cells were maintained in Dulbecco's modified
360 Eagle's medium (DMEM, Hyclone) with 10% fetal bovine serum (FBS; Sigma),
361 1% penicillin and streptomycin (Gibco), and kept at 37°C with 5% CO_2. Certain

362 reagents were kindly provided by the following persons: polymerase plasmids
363 of H1N1 human influenza A virus A/Sichuan/01/2009 ($H1N1_{SC09}$), and H7N9
364 A/Anhui/01/2013 ($H7N9_{AH13}$) by Dr. Hualan Chen; H1N1 human influenza virus
365 A/WSN/1933 (WSN) by Dr Kawoaka; H3N2 canine influenza virus
366 A/canine/Guangdong/1/2011 ($H3N2_{GD12}$) by Dr Shoujun Li from China Southern
367 Agriculture University; and H9N2 avian influenza virus
368 A/chicken/Zhejiang/B2013/2012 ($H9N2_{ZJ12}$) by Dr Zejun Li from Shanghai
369 Veterinary Research Institute of CAAS. H3N8 equine influenza viruses
370 A/equine/Jilin/1/1989 ($H3N8_{JL89}$) and A/equine/Xinjiang/1/2007 ($H3N8_{XJ07}$)
371 were preserved in our lab. The reverse genetics system based on the pBD
372 vector for the $H1N1_{SC09}$ virus was established in Dr Hualan Chen's lab. The
373 pCAGGS plasmids of containing full length ANP32A isoforms of several species
374 were generated by gene synthesis (Synbio technologies, China) according to
375 the sequences deposited in GenBank, including chicken ANP32A (chANP32A,
376 XM_413932.5, XP_413932.3), human ANP32A(huANP32A, NM_006305.3,
377 NP_006296.1), zebra finch ANP32A (zfANP32A, XM_012568610.1,
378 XP_012424064.1), duck ANP32A (dkANP32A, XM_005022967.1,
379 XP_005023024.1), turkey ANP32A (tyANP32A, XM_010717616.1,
380 XP_010715918.1), pig ANP32A (pgANP32A, XM_003121759.6,
381 XP_003121807.3), mouse ANP32A (muANP32A, NM_009672.3,
382 NP_033802.2), equine ANP32A (eqANP32A, XM_001495810.5,

383 XP_001495860.2), chicken ANP32B (chANP32B, NM_001030934.1,
384 NP_001026105.1) and human ANP32B (huANP32B, NM_006401.2,
385 NP_006392.1). Site-directed mutants of these sequences were generated
386 using overlapping PCR and identified by DNA sequencing. Mutants of pcAGGS-
387 huANP32B-Δ216/190/165 and pcDNA3.1-PA-V5 were constructed according
388 to the online In-Fusion ®HD Cloning Kit User Manual
389 (http://www.clontech.com/CN/Products/Cloning_and_Competent_Cells/Clonin
390 g_Kits/xxclt_searchResults.jsp). Briefly, the fragments of the
391 pcAGGS/pcDNA3.1 vector and each target gene were amplified with a 15 bp
392 homologous arm and were then fused using the In-Fusion HD Enzyme
393 (Clontech, Felicia, CA, USA). To create the pcAGGS-huANP32B-Δ216/190/165
394 plasmids, pcAGGS-huANP32B was used as the template to amplify the
395 pcAGGS vector. This sequence was then fused with different truncated
396 huANP32B fragments (huANP32B-Δ216/190/165). To obtain pcDNA3.1-PA-V5
397 plasmid, pBD- $H1N1_{SC09}$-PA was used as the template to amplify the PA-V5
398 sequence, and then fused with pcDNA3.1 vector.

399

400 **Knockout Cell Lines**

401 To generate knockout cell lines for host proteins BUB3(AF081496.1),
402 CLTC(NM_004859.3), CYC1 (CR541674.1), NIBP (BC006206.2), ZC3H15

403 (NM_018471.2), C14orf173 (DQ395340.1), CTNNB1 (NM_001904.3), ANP32A
404 (NM_006305.3), ANP32B (NM_006401.2), SUPT5H (U56402.1), HTATSF1
405 (NM_014500), and DDX17 (NM_006386, NM_001098504) (4, 23, 37), the
406 gRNAs design tool (http://crispr.mit.edu/) was used for gRNAs design and off-
407 target prediction (50). DNA fragments that contained the U6 promoter, gRNAs
408 specific for host factors, a guide RNA scaffold, and U6 termination signal
409 sequence were synthesized and subcloned into the pMD18-T backbone vector.
410 The Cas9-eGFP expression plasmid (pMJ920) was a gift from Jennifer Doudna
411 (Addgene plasmid # 42234) (51). Briefly, 293T cells in 6-well plates were
412 transfected with 1.0 μg pMJ920 plasmids and 1.0 μg gRNA expression
413 plasmids by Lipofectamine® 2000 Transfection Reagent (Invitrogen,
414 Cat.11668-027) using the recommended protocols. GFP-positive cells were
415 sorting by fluorescence-activated cell sorting (FACS) at 48 h post-transfection,
416 then monoclonal knockout cell lines were screened by western blotting and/or
417 DNA sequencing.

418

419 **Generation of Site-directed Amino-acid-substituted 293T Cell Line**

420 High efficiency guide sequences for ANP32A and ANP32B which bind
421 upstream and downstream with close proximity to the target (129/130 ND) were
422 chosen. The gRNA expression plasmids were constructed as described above.

423 An 80nt oligo with the desired mutations at the target site was used as the donor
424 DNA. 293T cells were transfected with 1 μg pMJ920 plasmids, 1 μg gRNA
425 expression plasmids, and 50 pmol donor DNA. 48 h later, GFP positive cells
426 were isolated by FACS, and site-directed mutagenesis clones were identified
427 after screening by sequencing and western blotting using Anti-PHAP1 antibody
428 (ab51013) and Anti-PHAPI2 / APRIL antibody EPR14588 (ab200836). Cell lines
429 with double gene mutations were generated by second round transfection and
430 selection.

431

432 **Polymerase Assay**

433 A minigenome reporter, which contains the firefly luciferase gene flanked
434 by the non-coding regions of the influenza HA gene segment with a human polI
435 promoter and a mouse terminator sequence (52), was transfected with viral
436 polymerase and NP expression plasmids to analyze the polymerase activity.
437 Mutants of PB2 genes were generated using overlapping PCR and identified
438 by DNA sequencing. To determine the effect of ANP32 proteins on viral
439 polymerase activity, 293T or KO cells in 12-well plates were transfected with
440 plasmids of the PB1 (80 ng), PB2 (80 ng), PA (40 ng) and NP (160 ng), together
441 with 80 ng minigenome reporter and 10 ng *Renilla* luciferase expression
442 plasmids (pRL-TK, kindly provided by Dr Luban), using Lipofectamine 2000

443 transfection reagent (Invitrogen) according to the manufacturers' instructions.
444 Cells were incubated at 37°C. Cells were lysed with 100ul of passive lysis buffer
445 (Promega) at 24 h after transfection, and firefly and *Renilla* luciferase activities
446 was measured using a Dual-luciferase kit (Promega) with Centro XS LB 960
447 luminometer (Berthold technologies). The function of ANP32 was examined
448 using a polymerase assay by co-transfection of DKO cells with different ANP32
449 proteins for 24 h. All the experiments were performed independently at least
450 three times. Results represent the mean ± SEM of the replicates within one
451 representative experiment. The expression levels of polymerase proteins on
452 different cell lines were detected by western blotting, using specific mouse
453 monoclonal antibodies for NP and PB1 proteins, anti-V5 tag antibody (ab27671)
454 for PA-V5 protein.

455

456 **RNA isolation, Reverse Transcription, and Quantification by RT- PCR**

457 Total RNA from 293T cells was extracted using an RNeasy mini kit (Qiagen),
458 following the manufacturer's instructions. For the synthesis of first strand cDNA
459 derived from firefly luciferase RNAs driven by influenza polymerase, equal
460 concentrations of RNA were subject to cDNA synthesis using a Reverse
461 Transcription Kit (PrimeScript^{TM} RT reagent Kit with a gDNA Eraser (Perfect
462 Real Time), Cat.RR047A). Primers used in the RT reaction were as follows: 5'-

463 CATTTCGCAGCCTACCGTGGTGTT-3' for the firefly luciferase vRNA; 5'-
464 AGTAGAAACAAGGGTG-3' for the firefly luciferase cRNA; oligo-dT20 for the
465 firefly luciferase mRNA (53). The cDNA samples were subjected to
466 quantification by real-time PCR with specific primers F (5'-
467 GATTACCAGGGATTTCAGTCG-3') and R (5'-GACACCTTTAGGCAGACCAG-
468 3') using SYBR ® Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa Cat:
469 RR820A). Fold change in RNA was calculated by double-standard curve
470 methods and β-actin as an internal control.

471

472 **Quantitative ELISA for Determination of Virus Production**

473 The ELISA assay has been previously described(42). Briefly, a 96-well
474 microtiter plate (Costar, Bodenheim, Germany) was coated with 1μg/well of the
475 mouse monoclonal anti-IAV NP protein antibody in phosphate-buffered saline
476 (PBS), incubated overnight at 4 °C or 37 °C for 2 h. The plate was washed three
477 times with washing buffer (PBS containing 0.1% Tween-20, PBST) and blocked
478 with 200 μl 5% calf serum at 37 °C for 2 h. After washing three times with PBST
479 buffer, 100 μl of virus/VLPS-containing samples were added in dilution buffer
480 (PBS containing 10% calf serum and 0.1% Triton X-100) and incubated at 37 °C
481 for 1 h. The plate was then washed, and 100 μl of a 1:2000 dilution of HRP-
482 conjugated anti-IAV NP protein mAb was added. After incubation at 37 °C for

483 0.5 h, the plate was washed again and incubated with freshly prepared TMB
484 peroxidase substrate (Galaxy Bio, Beijing, China) for 10 min at room
485 temperature. The reaction was stopped by adding 2 M H_2SO_4, and the optical
486 density at 450 nm wavelength (OD450) was measured using the VersaMax
487 Microplate Reader (BioTek, Winooski, USA). Dilution buffer was taken as a
488 blank control and the purified IAV-NP protein was double diluted eight times in
489 dilution buffer to obtain the standard curve. The virus production was calculated
490 using the standard curve.

491

492 **Influenza Virus Infection and Infectivity**

493 Human influenza virus WSN was rescued from a 12-plasmid rescue system
494 (54). Briefly, 293T culture in a 6-well plate was transfected with 0.1μgof each
495 pPolI plasmids and 1ug each of pCAGGS-NP, pCAGGS-PA, pCAGGS-PB1,
496 and pCAGGS-PB2 using Lipofectamine 2000 (Invitrogen) in Opti-MEM. Eight
497 hours post-transfection, medium was changed to DMEM plus 10% FBS. The
498 supernatant was harvested after 48 h and injected into 9-day-old specific
499 pathogen-free embryonated eggs for virus propagation. Eggs were incubated
500 at 35 °C for 72 h, the allantoic fluid from eggs was detected by
501 haemagglutination assay and the titers of virus on 293T cells were determined
502 using the method of Reed and Muench (55). Then 293T cells, huANP32A

503 knockout 293T cells (AKO cells), huANP32B knockout 293T cells (BKO cells),
504 and huANP32A &B double knocked out 293T cells (DKO) were infected at a
505 multiplicity of infection (MOI) of 0.01 for 1 h, washed twice with PBS, and then
506 cultured at 37°C in Opti-MEM containing 0.5% FBS and tosylsulfonyl
507 phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma) at 1mg/ml. At the
508 indicated time points, the culture supernatant was harvested. Viral titers in
509 MDCK cells were determined as described above.

510 **Immunoprecipitation and Western Blotting**

511 For immunoprecipitation and western blotting, transfected cells were lysed
512 using an ice-cold lysis buffer (50 mM Hepes-NaOH [pH 7.9], 100 mM NaCl, 50
513 mM KCl, 0.25% NP-40, and 1 mM DTT), and centrifuged at 13,000× g and 4 °C
514 for 10 min. After centrifugation, the crude lysates were incubated with Anti-
515 FLAG M2 Magnetic Beads (SIGMA-ALDRICH, M8823) or Anti-NP Magnetic
516 Beads (MCE Protein A/G Magnetic Beads and NP antibody from our lab) at 4°C
517 for 2 hr. After incubation, the resins were collected using a magnetic separator
518 and washed three times with PBS. The resin-bound materials were eluted using
519 a 3X Flag peptide or by boiling in the SDS-PAGE loading buffer, subjected to
520 SDS-PAGE and then transferred onto nitrocellulose membranes. Membranes
521 were blocked with 5% milk powder in Tris-buffered saline (TBS) for 2 h.
522 Incubation with the first anti-mouse antibody (Anti-Flag antibody from SIGMA
523 (F1804), Anti-V5 antibody from Abcam (ab27671), Anti-NP and PB1 antibody

524 from our lab) was performed for 2 h at room temperature (RT), followed by
525 washing three times with TBST. The secondary antibody (Sigma, 1:10,000) was
526 then applied and samples were incubated at RT for 1 h. Subsequently,
527 membranes were washed three times for 10 min with TBST. Signals were
528 detected using a LI-COR Odyssey Imaging System (LI-COR, Lincoln, NE, USA).

529 **Statistics.** Statistical analysis was performed in GraphPad Prism, version 5
530 (Graph Pad Software, USA). Statistical differences between groups were
531 assessed by One-way ANOVA followed by a Dunnett's post-test. All the
532 experiments were performed independently at least three times. Error bars
533 represent the standard deviation (SD) or the standard error of the mean (SEM)
534 in each group, as indicated in figure legends. NS, not significant ($p>0.05$),
535 *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

536

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538

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541

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725

726 **FIG 1. HuANP32A&B are indispensable for influenza A virus polymerase activity**
727 **and viral replication. (A)** Wild type 293T cells and single gene knockout 293T cell
728 lines, including BUB3, CLTC, CYC1, NIBP, ZC3H15, C14orf173, CTNNB1, ANP32A,
729 ANP32B, SUPT5H, HTATSF1, and DDX17, were transfected with firefly minigenome
730 reporter, *Renilla* expression control, and the polymerase of $H1N1_{SC09}$. Luciferase
731 activity was measured 24 h after transfection and data are firefly Luciferase gene
732 activity normalized to *Renilla* Luciferase gene activity. (Statistical differences between
733 samples are indicated, according to a one-way ANOVA followed by a Dunnett's test;
734 NS= not significant, *P < 0.05, **P < 0.01, ****P < 0.0001. Error bars represent the SEM
735 within one representative experiment.). (**B to D)** 293T, huANP32A knockout cells
736 (AKO), huANP32B knockout cells (BKO), and huANP32A&B double knockout cells
737 (DKO), were transfected with firefly minigenome reporter, *Renilla* expression control,
738 and either $H1N1_{SC09}$ polymerase (**B**), $H7N9_{AH13}$ polymerase (**C**), or WSN polymerase
739 (**D**). Luciferase activity was measured at 24 h post transfection. (**B** to **D,** data are firefly
740 Luciferase gene activity normalized to that of *Renilla*, Statistical differences between
741 cells are indicated, following a one-way ANOVA and subsequent Dunnett's test; NS =
742 not significant, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent the SEM
743 of the replicates within one representative experiment.). The expression levels of
744 $H1N1_{SC09}$ polymerase proteins were assessed by western blotting **(B)**. (**E)** 293T, AKO,
745 BKO and DKO cells were infected with WSN virus at an MOI of 0.01. The supernatants
746 were sampled at 0, 12, 24, 36, and 48 h post infection and the virus titers were

747 determined by means of endpoint titration in MDCK cells. Error bars indicate SD from
748 three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

749

750 **FIG 2. Reconstitutions of huANP32A&B rescue polymerase activity and**
751 **replication of influenza A virus.** 20 ng huANP32A, 20 ng huANP32B,10 ng
752 huANP32A and 10 ng huANP32B, or 20 ng empty vector were co-transfected with
753 either $H1N1_{SC09}$ polymerase (**A**), $H7N9_{AH13}$ polymerase (**B**) or WSN polymerase (**C**)
754 into DKO cells, luciferase activity was assayed at 24 h after transfection. (**D)** Increasing
755 doses of huANP32A or huANP32B were co-transfected with $H1N1_{SC09}$ polymerase into
756 DKO cells. Luciferase activity was measured at 24 h post transfection. The expression
757 of ANP32 proteins was assessed by western blotting. (**A** to **D,** data are firefly Luciferase
758 gene activity normalized to that of *Renilla*, Statistical differences between cells are
759 indicated, following a one-way ANOVA and subsequent Dunnett's test; NS = not
760 significant, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent the SEM of
761 the replicates within one representative experiment). (**E**) DKO cells were transfected
762 with 1ug huANP32A or/and B, or empty vector. After 24 h, the cells were infected with
763 WSN virus at an MOI of 0.01. The supernatants were sampled at 0, 12, 24, 36, and 48
764 h post infection and the virus titers in these supernatants were determined as above.
765 Error bars indicate SD from three independent experiments. NS = not significant, *P <
766 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

767

768 **FIG 3. HuANP32A&B determine viral RNA replication efficiency and viral**
769 **production. (A-C)** 293T, or DKO cells, were transfected with the $H1N1_{SC09}$
770 minigenome reporting system together with either 20 ng empty vector or huANP32A.
771 The cells were incubated at 37 °C for 24 h before reverse transcription followed by
772 quantitative PCR (qRT–PCR) for vRNA, mRNA and cRNA of the Luciferase gene. Error
773 bars represent the SD from three independent experiments. NS = not significant,
774 ****P < 0.0001. **(D** and **E)** Replication kinetics of $H1N1_{SC09}$ in ANP32 knockout cells.
775 293T, AKO, BKO, and DKO cells in 6-well plates were transfected with 0.5 µg each of
776 the eight plasmids of $H1N1_{SC09}$ (**D**), or, in DKO cells, 40 ng huANP32A, 40ng
777 huANP32B, 20 ng each of huANP32A and huANP32B, or 40 ng empty vector were co-
778 transfected with the eight-plasmids of $H1N1_{SC09}$ (**E**). The cells were cultured at 37 °C
779 and supernatants collected at 0, 12, 24, 48 and 60 h post-transfection and subjected
780 to virus production assay by ELISA, as described in 'Methods'. Error bars represent
781 the SD from three independent experiments. Error bars indicate SD from three
782 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

783

784 **FIG 4. Support of influenza A viral replication by ANP32A or B from different**
785 **species.** Twenty nanograms of either empty vector or ANP32A or B from one of several
786 different species was co-transfected with a minigenome reporter, *Renilla* expression

787 control, and human influenza virus polymerase from $H1N1_{SC09}$ (**A**), $H7N9_{AH13}$ (**B**),
788 canine influenza virus $H3N2_{GD11}$ (**C**), equine influenza virus $H3N8_{XJ07}$ (**D**) and $H3N8_{JL89}$
789 (**E**), or avian influenza virus $H9N2_{ZJ12}$ (**F**) into DKO cells. Luciferase activity was
790 measured 24 h later. (Data are firefly Luciferase gene activity normalized to that of
791 *Renilla*, Statistical differences between cells are indicated, following a one-way ANOVA
792 and subsequent Dunnett's test; NS = not significant, *P < 0.05, **P < 0.01, ***P < 0.001,
793 ****P < 0.0001. Error bars represent the SEM of the replicates within one
794 representative experiment.) dk, duck; ty, turkey; zf, zebra finch; mu, mouse; pg, pig;
795 eq, equine.

796

797 **FIG 5. Key amino acids in ANP32A&B determine the activity of influenza viral**
798 **polymerases. (A)** Amino acid sequence comparison for chicken, human and pig
799 ANP32B proteins (amino acids 110–161). Amino acid sequences are available in
800 GenBank. The black dots indicate the different amino acid within these proteins. **(B** to
801 **D)** huANP32B mutants were co-transfected with polymerase plasmids from $H1N1_{SC09}$
802 (**B** and **C**), or $H7N9_{AH13}$ (**D**), plus minigenome reporter and *Renilla* luciferase control
803 into DKO cells. Luciferase activity was then assayed 24 h after transfection. **(E** and **F)**
804 huANP32A mutants were co-transfected with polymerase plasmids from $H1N1_{SC09}$ (**E**)
805 or $H7N9_{AH13}$ (**F**), plus a minigenome reporter and *Renilla* luciferase control, into DKO
806 cells. **(G** and **H)** chANP32A or B mutants were co-transfected with polymerase
807 plasmids from $H7N9_{AH13}$ with either PB2 627K (**G**), or 627E (**H**) respectively. Luciferase

activity was analyzed as mentioned above. (Data are firefly Luciferase gene activity normalized to that of *Renilla*, Statistical differences between cells are given, following one-way ANOVA and subsequent Dunnett's test; NS = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent the SEM of the replicates within one representative experiment.)

FIG 6. Amino-acids N129I/D130N substitute into ANP32A&B impaired influenza polymerase activity in 293T cells. (A) The endogenous protein expressions of different cell lines were identified by Western blotting using antibodies against β-actin, huANP32A and huANP32B. (B and C) Selected 293T cell lines were transfected with firefly minigenome reporter, *Renilla* expression control, and polymerase from $H1N1_{SC09}$ (B) or $H7N9_{AH13}$ (C). Cells were assayed for Luciferase activity. WT, wild-type 293T cells; A5#, ANP32A mutant cells; B32#, ANP32B mutant cells; AB93#, ANP32A/ANP32B double mutant cells. (Data are firefly Luciferase gene activity normalized to that of *Renilla*, Statistical differences between cells are given, following a one-way ANOVA and subsequent Dunnett's test; NS = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent the SEM of the replicates within one representative experiment.)

826 **FIG 7. The 129-130 site in ANP32B contributes to the interaction with influenza**
827 **polymerase.** (A) A schematic describing the structure of ANP32B. LRR, leucine-rich repeat,
828 including 4 LRR repeats and one C-terminal LRR domain (LRRCT); LCAR, low-complexity
829 acidic region; NLS, nuclear localization signal. (B) and (C) 293T cells were transfected with
830 different ANP32-Flag constructs, together with viral polymerase subunits PA, PB1, and
831 PB2. Coimmunoprecipitation of the anti-Flag antibodies and the proteins was assessed
832 using western blotting.
833

834 **SUPPORTING INFORMATION**

835 **FIG S1. The identification of huANP32A and huANP32B knockout cells.** 293T cells
836 were transfected with pMJ920 vector (plasmid expressing eGFP and Cas9) and
837 gRNAs targeting huANP32A or huANP32B to generate huANP32A knockout cells
838 (AKO), huANP32B knockout cells (BKO), and huANP32A&B double knockout cells
839 (DKO). The mRNA levels of huANP32A and B in different cell lines were determined
840 using qRT-PCR using primers specific for huANP32A (**A**) and huANP32B (**B**). The
841 primer sequences are given in 'Materials and Methods'. Fold change in RNA was
842 calculated by double-standard curve methods. β-actin was used as an internal control.
843 Statistical differences between samples are given, following one-way ANOVA and
844 subsequent Dunnett's test; NS = not significant, ****$P < 0.0001$. Error bars indicate SD
845 from three independent experiments. (**C**) The endogenous proteins were identified by
846 western blotting using antibodies against β-actin, huANP32A and huANP32B,

described as “Materials and Methods”.

FIG S2. Overdose expression of huANP32A&B impaired viral polymerase activity. Different doses of huANP32A or huANP32B were co-transfected with $H1N1_{SC09}$ polymerase (**A**) and $H7N9_{AH13}$ polymerase (**B**) into DKO cells. Luciferase activity was measured 24 h after transfection and data are firefly Luciferase gene activity normalized to that of *Renilla*. (Statistical differences between samples are given, following one-way ANOVA and subsequent Dunnett’s test; NS = not significant, *P < 0.05, ***P < 0.001, ****P < 0.0001. Error bars represent the SEM of the replicates within one representative experiment.). The expression levels of the huANP32A and huANP32B proteins were confirmed by western blotting.

FIG S3. Phylogenetic tree of amino acid sequences of ANP32A (A) and ANP32B (B). Trees were generated by the neighbor-joining method and evaluated by 1000 bootstrap pseudo replicates. The bar scale represents the genetic distance. The credibility value for each node is shown.

FIG S4. Expression of flag-tagged ANP32A or B proteins and PB2 E627K mutation overcomes human ANP32A&B restriction. (A) 1µg of each ANP32 plasmid was transfected into 293T cells using Lipofectamine® 2000. 48 h post

transfection, the cell lysates were analyzed using SDS-PAGE and Western Blotting using antibodies against Flag peptide and β-actin. (B-D) DKO cells were transfected with 20ng either empty vector or ANP32A or B from one of several different species, together with minigenome reporter, *Renilla* expression control, and different viral polymerase including: human $H7N9_{AH13}$ with PB2 K627E (**B**), an avian origin equine influenza virus $H3N8_{XJ07}$ with PB2 E627K (**C**), and avian influenza virus $H9N2_{ZJ12}$ with PB2 E627K (**D**). Luciferase activity was measured 24 h post transfection. (Data are polymerase activity normalized to *Renilla*; Statistical differences between cells are given, following one-way ANOVA and subsequent Dunnett's test; NS = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent the SEM of the replicates within one representative experiment.) dk, duck; ty, turkey; zf, zebra finch; mu, mouse; pg, pig; eq, equine.

FIG S5. Mapping of crucial sites in ANP32 proteins that influence viral polymerase activity. (A) ANP32B sequences from chicken, human, and pig were aligned using the Geneious R6 software. The notches are marked with dashes. The similarity of amino acid identity is highlighted in different colors. (B) Schematic diagram of chimeric clones between chicken and human ANP32B, constructed according to the known domains (Separated by dotted lines.1-41aa, LRR1 region; 42-110aa, LRR2,3 &4 region; 111-161aa, LRRCT region; 162-251aa, LCAR region, as showed in a). The bars show the origin of the genes as follows: white, huANP32B; blue, chANP32B. (56)

Chimeric clones were co-transfected with minigenome reporter, *Renilla* expression control, and $H1N1_{SC09}$ polymerase into DKO cells. Luciferase activity measured 24 h later. (Data are polymerase activity normalized to *Renilla*; Statistical difference between samples are given labeled, following one-way ANOVA and subsequent Dunnett's test; NS = not significant, ****$P < 0.0001$. Error bars represent the SEM of the replicates within one representative experiment.)

FIG S6. Expression of flag-tagged ANP32A or B mutants.

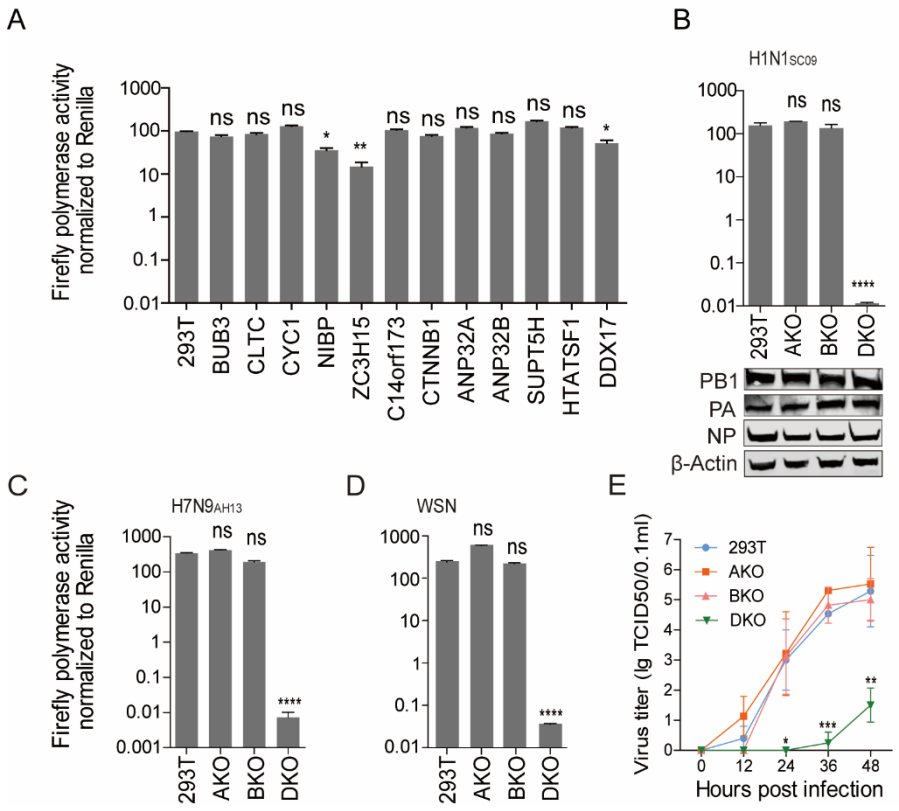
1μg of each mutant ANP32 and wildtype ANP32 plasmids were transfected in 293T cells by Lipofectamine® 2000. 48 h post transfection, the cells lysates were analyzed by SDS-PAGE and Western Blotting using antibodies against Flag peptide and β-actin.

FIG S7. Mouse ANP32A 130A impaired its support of viral replication. Mutated muANP32A or human ANP32 proteins were co-transfected with minigenome reporter, *Renilla* expression control, and $H1N1_{SC09}$ polymerase into DKO cells. Luciferase activity was assayed. (Data are polymerase activity normalized to *Renilla*; Statistical differences between cells are given, following one-way ANOVA and subsequent Dunnett's test; NS = not significant, *$P < 0.05$, ****$P < 0.0001$. Error bars represent the SEM of the replicates within one representative experiment.)

FIG S8. Identification of ANP32A&B site-mutation 293T cell lines. N129I/D130N substitutions in huANP32A and ANP32B on the 293T chromosome were generated

using a CRISPR/Cas9 system. Positive 293T colonies of N129I/D130N substitutions on huANP32A and ANP32B were identified by sequencing. (A) Cloned cell A5# was identified carrying the N129I/D130N on ANP32A but not ANP32B. (B) Cell B32# was identified harboring N129I/D130N on ANP32B but not ANP32A. (C) Cell AB93# has N129I/D130N on both ANP32A&B.

Zhang_Fig 1



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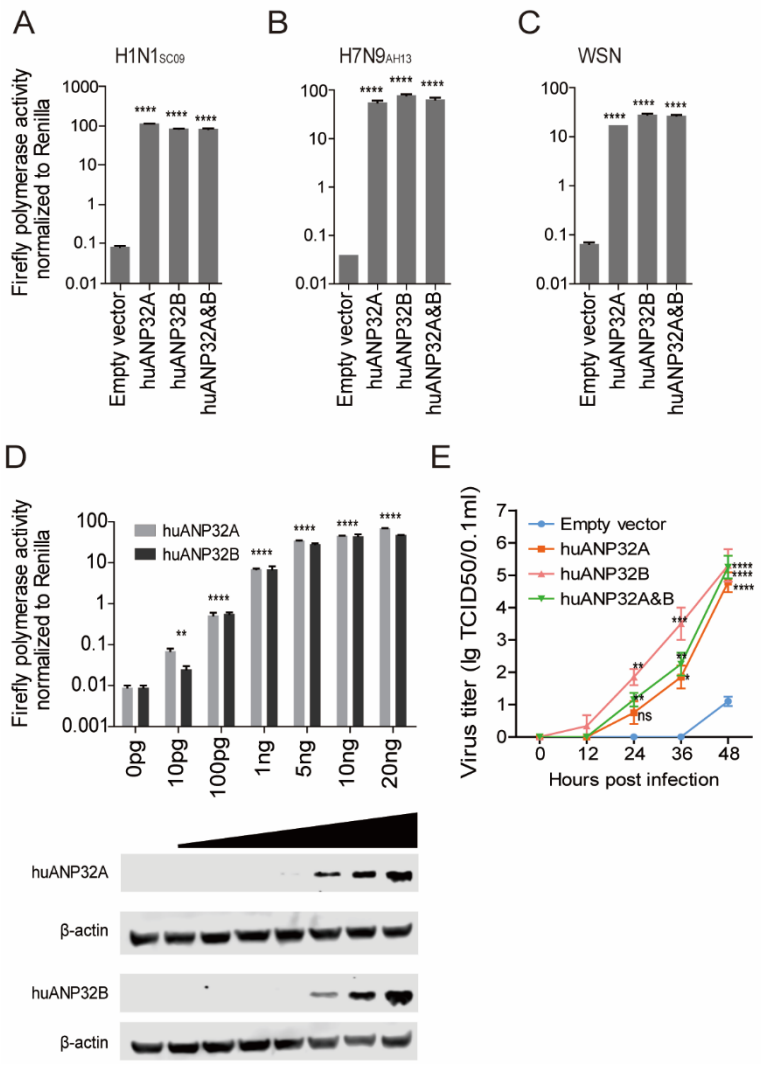

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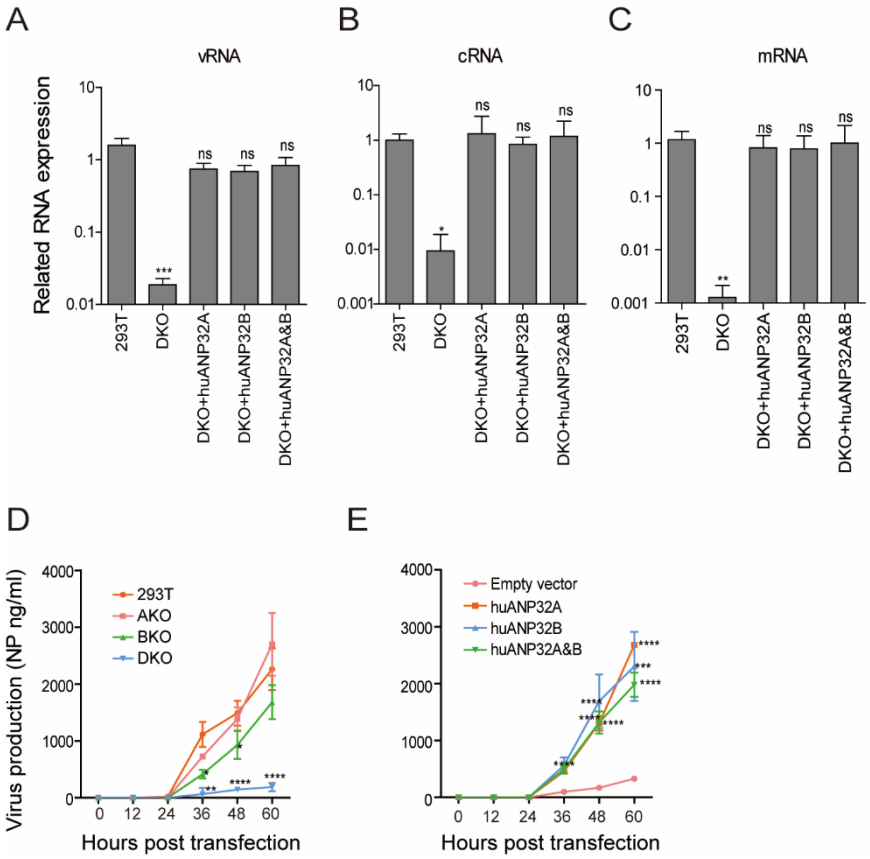

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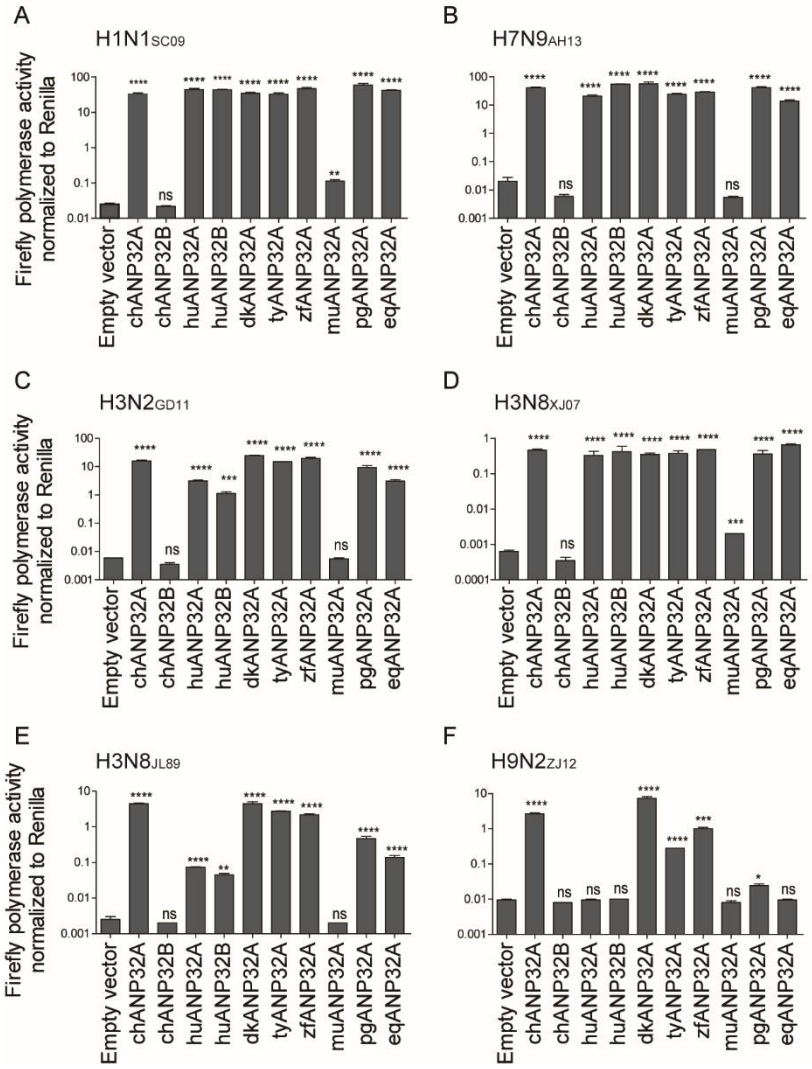

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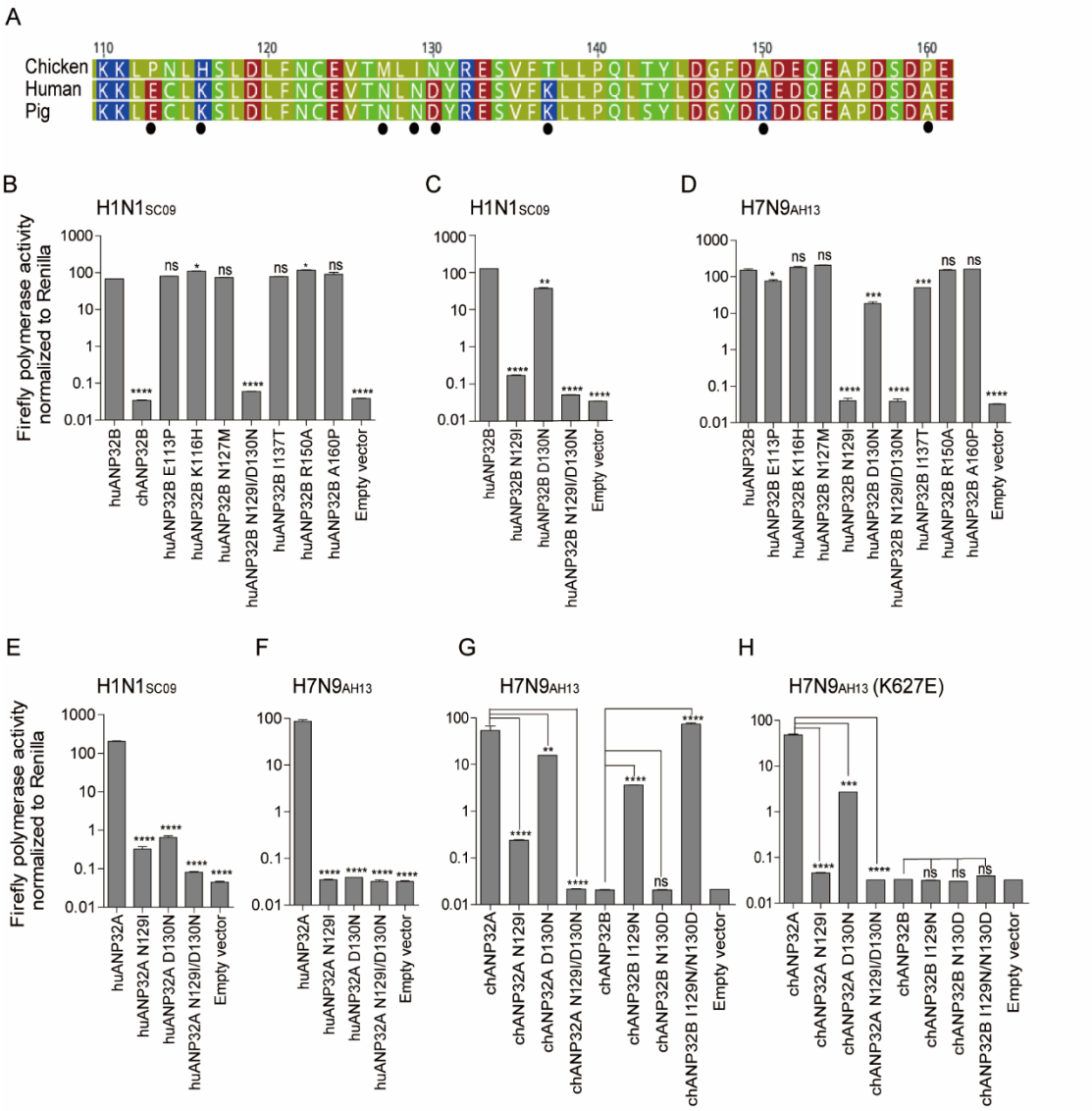

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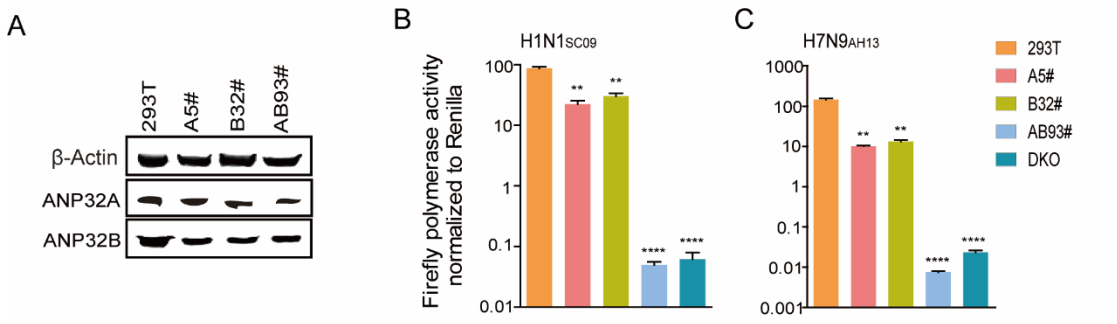

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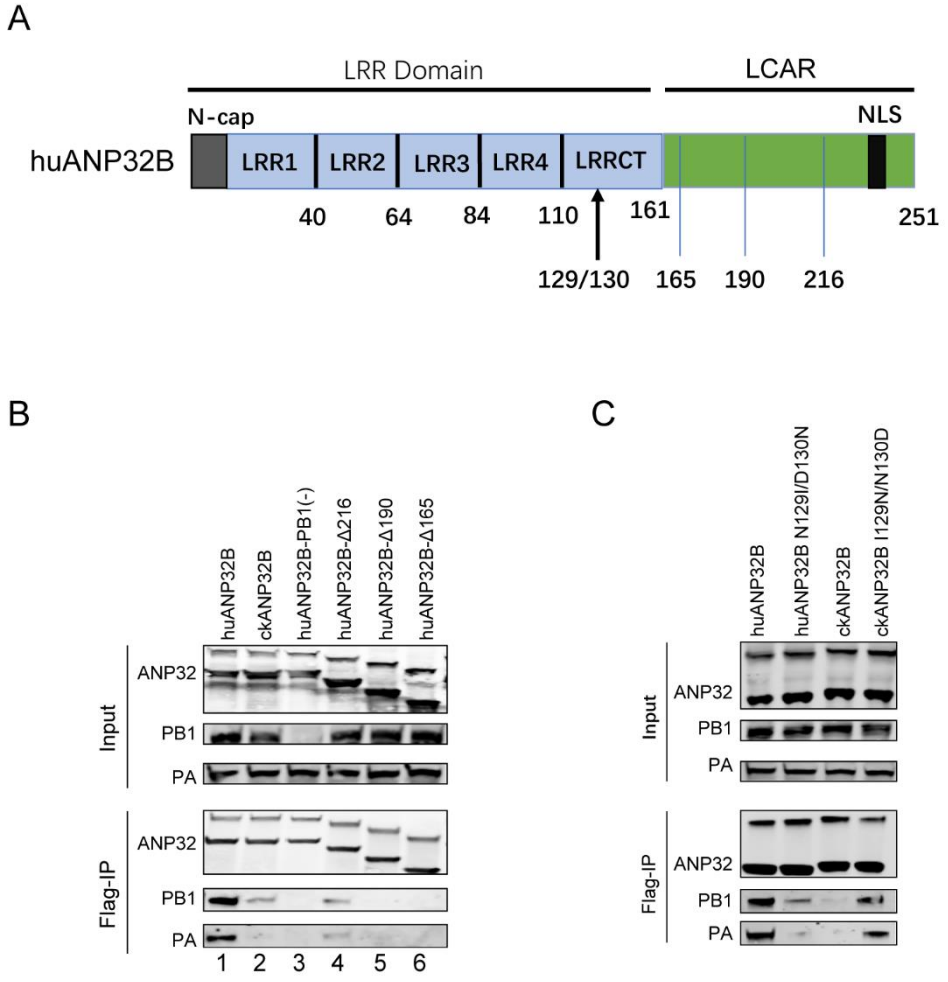
A
LRR Domain
LCAR
N-cap
NLS
huANP32B
LRR1
LRR2
LRR3
LRR4
LRRCT
40
64
84
110
161
251
129/130
165
190
216
B
huANP32B
ckANP32B
huANP32B-PB1(-)
huANP32B-Δ216
huANP32B-Δ190
huANP32B-Δ165
Input
Flag-IP
ANP32
PB1
PA
1 2 3 4 5 6
C
huANP32B
huANP32B N129I/D130N
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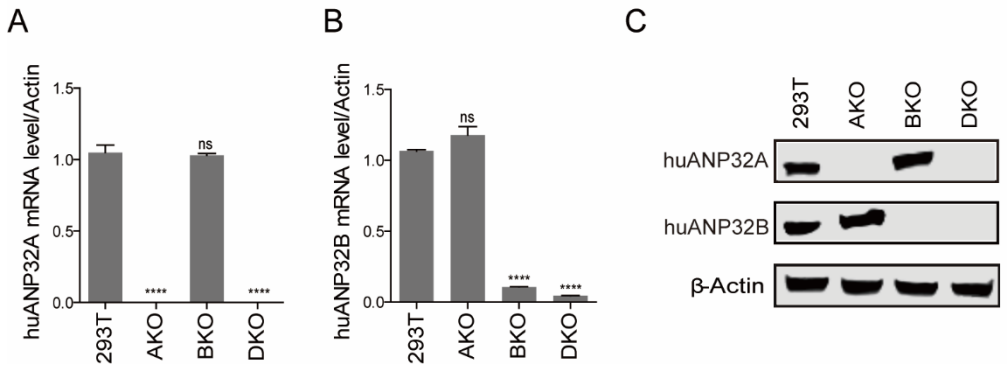
Zhang_Fig S1



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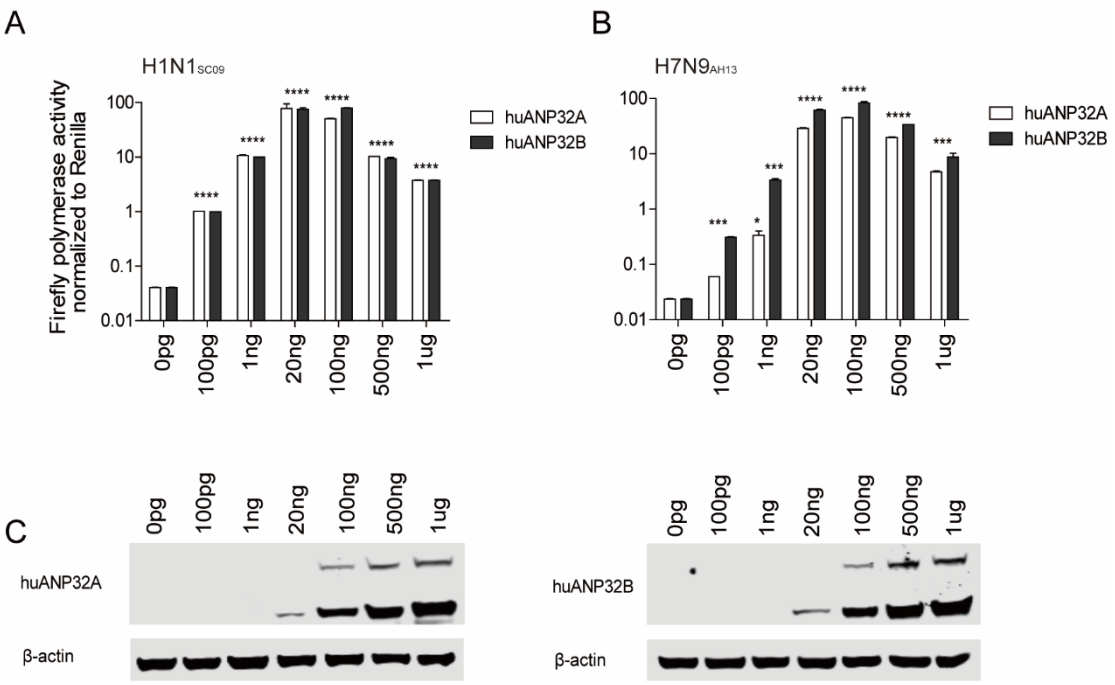

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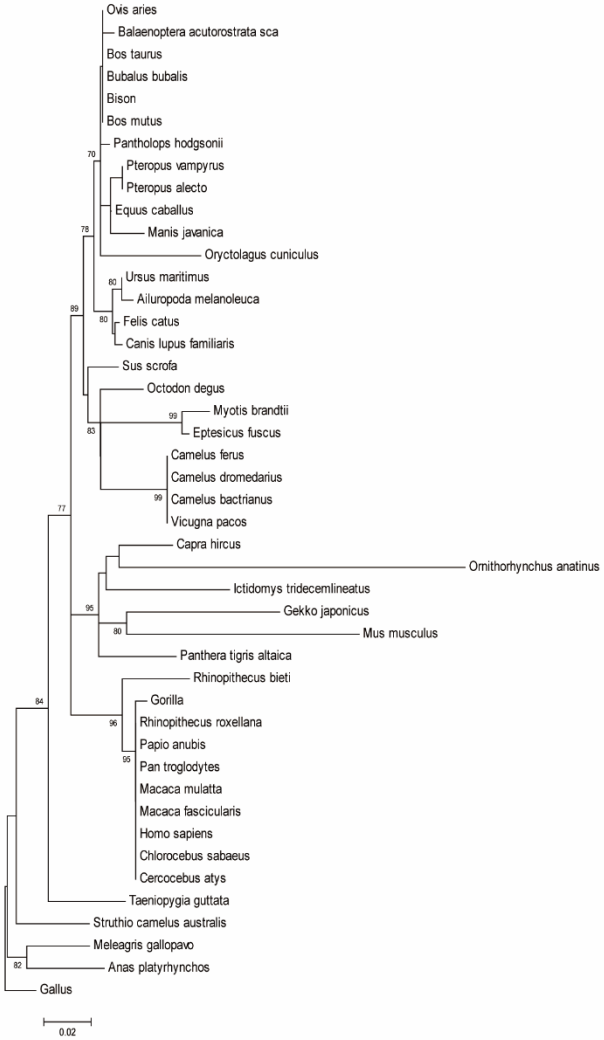
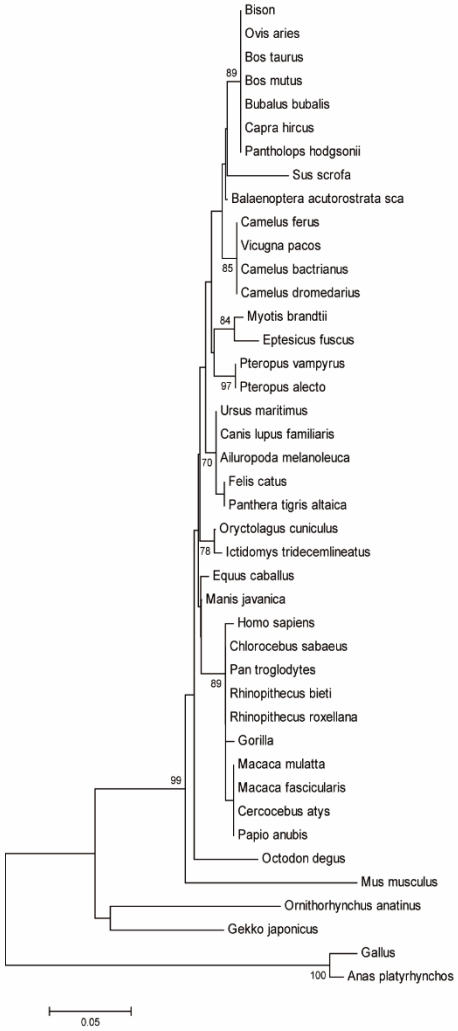
A



ANP32A

B



ANP32B

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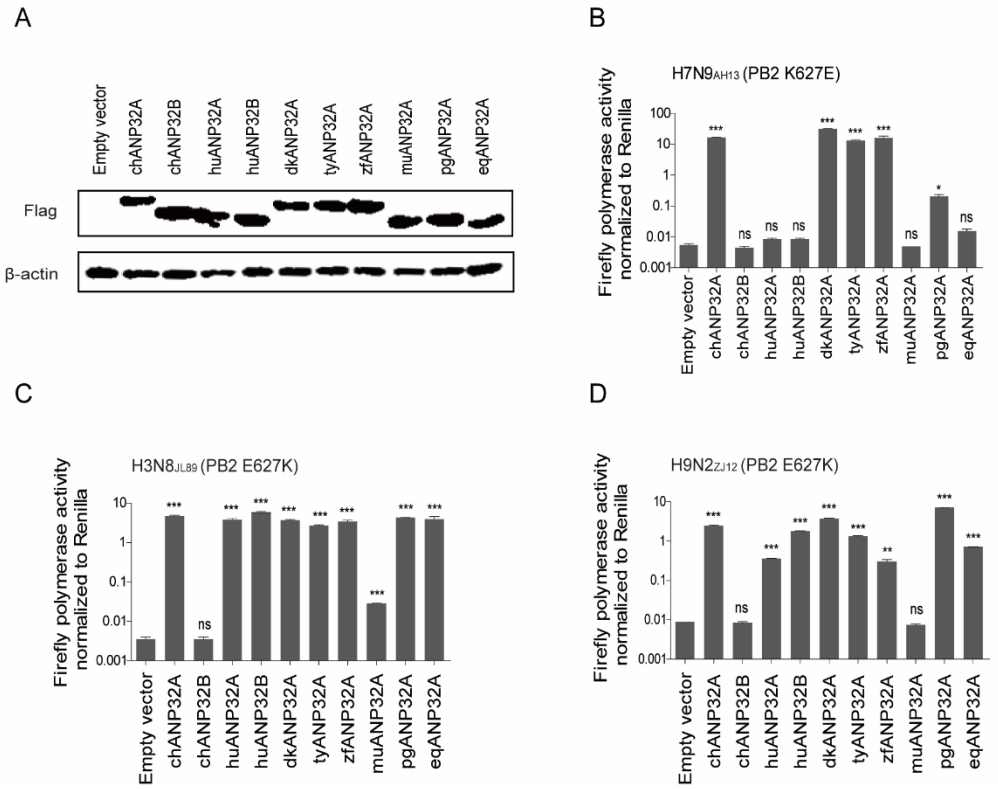

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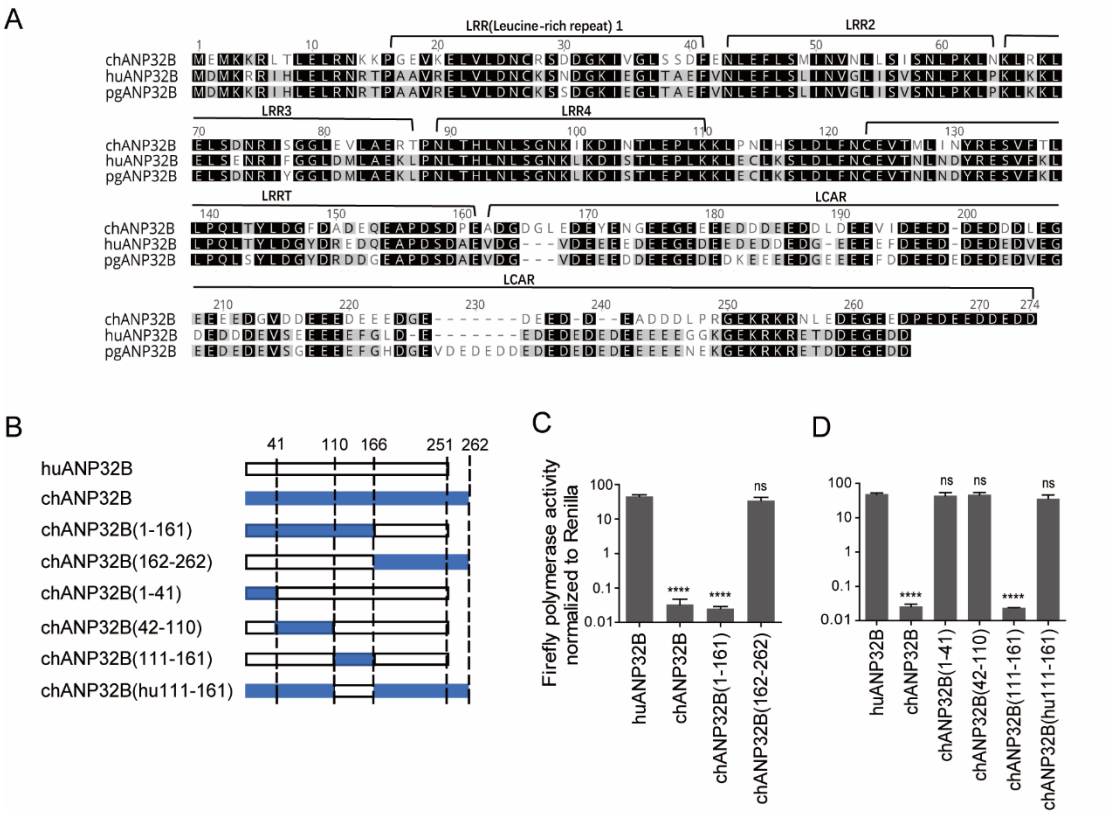

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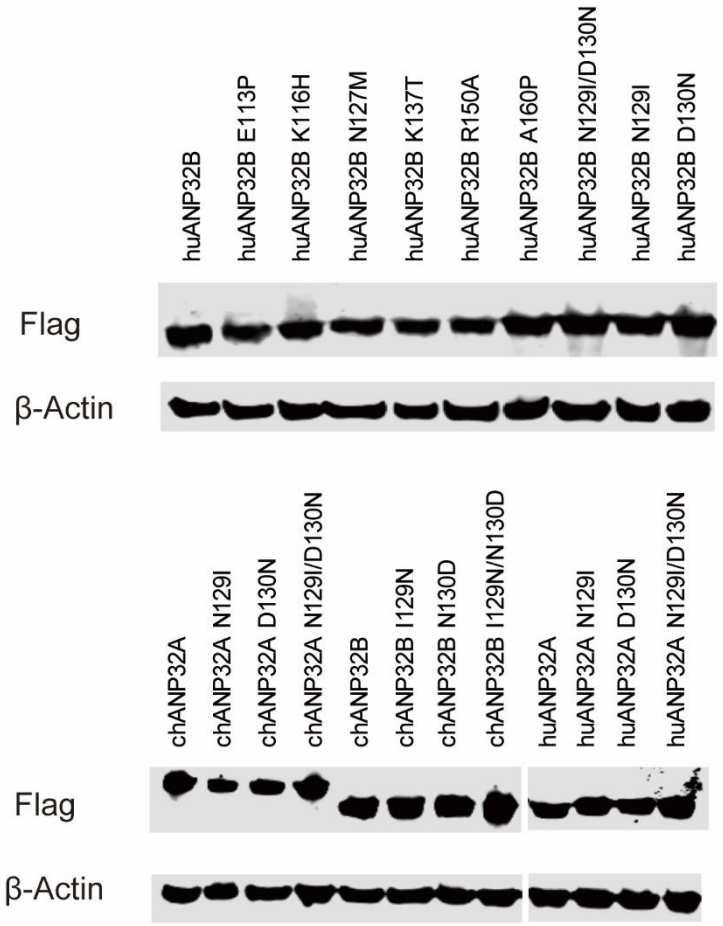

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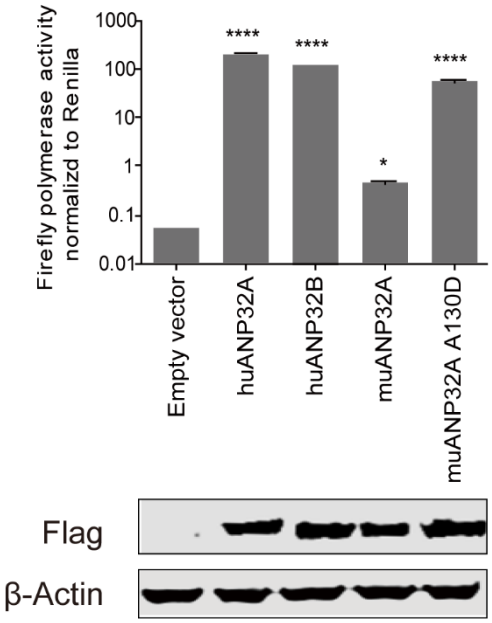

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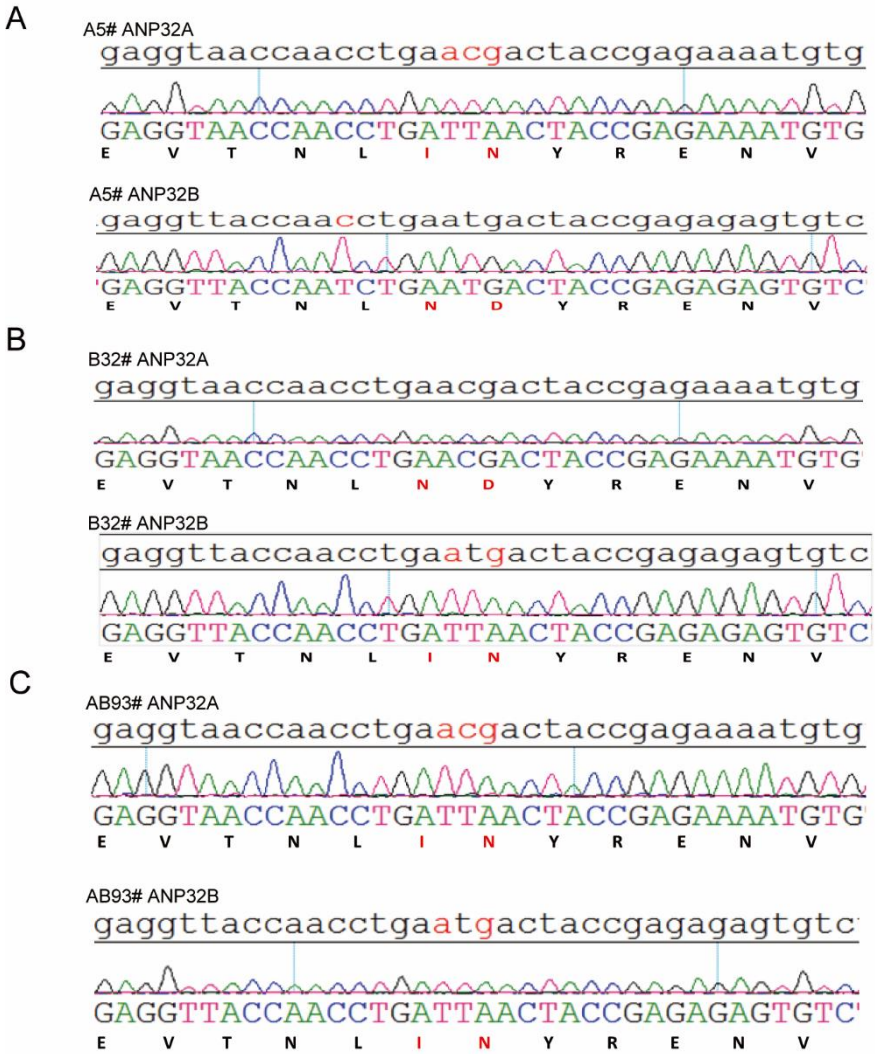

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Table S1. Summary of amino acids at 129-130 of ANP32 proteins

	Residue at amino acid position*			
	ANP32A		ANP32B	
Host	129	130	129	130
Gallus	N	D	I	N
Anas platyrhynchos	N	D	I	N
Homo sapiens	N	D	N	D
Gorilla gorilla	N	D	N	D
Felis catus	N	D	N	D
Canis lupus familiaris	N	D	N	D
Bos taurus	N	D	N	D
Sus scrofa	N	D	N	D
Mus musculus	N	A	S	D
Equus caballus	N	D	N	D

*The sequences of different species ANP32 proteins were retrieved from NCBI