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

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12 Running Title: ANP32 proteins are indispensable for influenza virus

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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.

INTRODUCTION

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

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

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

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

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

RESULTS

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

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

influenza viral replication, we used a CRISPR/Cas9 system to establish a series of knockout 293T cell lines, and a model viral-like luciferase RNA was expressed together with viral polymerase PB1, PB2, PA, and NP, to determine the polymerase activity in these cell lines. Firstly, we tested the polymerase activity of 2009 pandemic H1N1 virus A/Sichuan/01/2009(H1N1_{sc09}) (38) on these knockout 293T cells, and found that individual knockout of NIBP, ZC3H15, or DDX17 results in a 2-4 fold decrease in viral polymerase activity, in which is consistent with previous results (23, 37). However, in none of the other single gene knockout cells was viral polymerase activity blocked (Fig. 1A). We did not observe any reduction of viral polymerase activity in ANP32A or ANP32B knockout cells, which was a surprising result as ANP32A and ANP32B were reported to be important host factors supporting viral polymerase activity (7, 8, 34, 35). Since ANP32A and ANP32B have high similarity in both structure and known functions, we predicted that in single knockout cell lines of ANP32A or ANP32B, the presence of either one of these two proteins could support viral replication in the absence of the other. We then developed an ANP32A and ANP32B double knockout cell line to confirm this hypothesis. In human ANP32A (huANP32A) knockout 293T cells (AKO cells), and human ANP32B (huANP32B) knockout 293T cells (BKO cells), the viral polymerases have similar activities to wild-type 293T cells, but when both ANP32A and ANP32B were knocked out (DKO) (Fig. S1 in the supplemental material), the polymerase

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

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

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

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

ANP32A&B are members of the evolutionarily conserved ANP32 family, which has various functions in the regulation of gene expression, intracellular transport, and cell death (36). We tried to knock out ANP32A&B in both chicken DF1 cells and human A549 cells, but the knockout caused cell death, indicating both ANP32A&B have crucial roles in cell proliferation in these cells. ANP32A&B exist in almost all eukaryotic cells, with the exception of early eukaryotic life (yeast and other fungi) (43). Molecular phylogenetic analysis of ANP32 proteins revealed an evolutionary relationship among different species (Fig. S3). We then investigated the support of ANP32A or ANP32B from different species for viral polymerase activity in DKO cells. ANP32A from human, chicken, duck, turkey, zebra finch, mouse, pig, and horse, and ANP32B from human and chicken, were expressed individually with minigenomes of either H1N1_{SC09}, human isolate H7N9_{AH13}, H3N2 Canine influenza virus 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

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

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

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

acid substitutions (Fig. 5A). Of these, a combined mutation N129I/D130N, but not others, on huANP32B impaired H1N1 polymerase activity (Fig. 5B), and the N129I showed stronger impairment than did the D130N mutation (Fig. 5C). We confirmed this phenotype on H7N9 polymerase activity (Fig. 5D). The 129-130 mutations also aborted the support of huANP32A of H1N1 and H7N9 polymerases (Fig. 5E and 5F). Furthermore, substitutions at 129-130 in chANP32A and chANP32B reversed the support of these proteins of the H7N9 virus human isolate (Fig. 5G). Interestingly, in the context of a chicken H7N9 viral minigenome, although mutation of 129-130 of chANP32A impaired the polymerase activity, the chANP32B reverse mutation did not restore its support to chicken-like H7N9 viral RNA replication (Fig. 5H), indicating that chicken virus replication may require both the 129-130 site and an extra 33-amino acid peptide as in chANP32A. The expression levels of mutant ANP32 proteins were assessed using western blotting (Fig. S6). Most terrestrial mammalian ANP32A&B proteins have a 129-ND-130 signature, but most of the avian ANP32Bs have 129-IN-130 residues (Table S1). Murine ANP32A (muANP32A) has 129-NA-130 and this may explain the impaired support of H1N1 polymerase by muANP32A (Fig. 4 and Fig. S7). To further confirm the function of the 129-130 site, we made inactivating mutations of huANP32A and huANP32B at the sites 129-130 (N129I, D130N) on the 293T genome using 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-immunoprecipitate 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

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

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

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

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

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

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

MATERIALS AND METHODS

Cells, Viruses, and Plasmids

Human embryonic kidney (293T, ATCC CRL- 3216) and Madin-Darby canine kidney (MDCK, CCL-34™) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) with 10% fetal bovine serum (FBS; Sigma), 1% penicillin and streptomycin (Gibco), and kept at 37°C with 5% CO₂. Certain

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

XP_001495860.2), chicken ANP32B (chANP32B, NM_001030934.1, NP_001026105.1) and human ANP32B (huANP32B, NM_006401.2, NP_006392.1). Site-directed mutants of these sequences were generated using overlapping PCR and identified by DNA sequencing. Mutants of pcAGGS-huANP32B- Δ 216/190/165 and pcDNA3.1-PA-V5 were constructed according to the online In-Fusion [®]HD Cloning Kit User Manual (http://www.clontech.com/CN/Products/Cloning_and_Competent_Cells/Cloning_Kits/xxclt_searchResults.jsp). Briefly, the fragments of the pcAGGS/pcDNA3.1 vector and each target gene were amplified with a 15 bp homologous arm and were then fused using the In-Fusion HD Enzyme (Clontech, Felicia, CA, USA). To create the pcAGGS-huANP32B- Δ 216/190/165 plasmids, pcAGGS-huANP32B was used as the template to amplify the pcAGGS vector. This sequence was then fused with different truncated huANP32B fragments (huANP32B- Δ 216/190/165). To obtain pcDNA3.1-PA-V5 plasmid, pBD- H1N1_{SC09}-PA was used as the template to amplify the PA-V5 sequence, and then fused with pcDNA3.1 vector.

Knockout Cell Lines

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

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

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

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

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

Polymerase Assay

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

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

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

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

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

Quantitative ELISA for Determination of Virus Production

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

0.5 h, the plate was washed again and incubated with freshly prepared TMB peroxidase substrate (Galaxy Bio, Beijing, China) for 10 min at room temperature. The reaction was stopped by adding 2 M H₂SO₄, and the optical density at 450 nm wavelength (OD₄₅₀) was measured using the VersaMax Microplate Reader (BioTek, Winooski, USA). Dilution buffer was taken as a blank control and the purified IAV-NP protein was double diluted eight times in dilution buffer to obtain the standard curve. The virus production was calculated using the standard curve.

Influenza Virus Infection and Infectivity

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

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

Immunoprecipitation and Western Blotting

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

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

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

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REFERENCES

1. **Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ.** 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* **139**:1243-1254.
2. **Konig R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, Alamares JG, Tscherne DM, Ortigoza MB, Liang Y, Gao Q, Andrews SE, Bandyopadhyay S, De Jesus P, Tu BP, Pache L, Shih C, Orth A, Bonamy G, Miraglia L, Ideker T, Garcia-Sastre A, Young JA, Palese P, Shaw ML, Chanda SK.** 2010. Human host factors required for influenza virus replication. *Nature* **463**:813-817.
3. **Watanabe T, Kawakami E, Shoemaker JE, Lopes TJS, Matsuoka Y, Tomita Y, Kozuka-Hata H, Gorai T, Kuwahara T, Takeda E, Nagata A, Takano R, Kiso M, Yamashita M, Sakai-Tagawa Y, Katsura H, Nonaka N, Fujii H, Fujii K, Sugita Y, Noda T, Goto H, Fukuyama S, Watanabe S, Neumann G, Oyama M, Kitano H, Kawaoka Y.** 2014. Influenza Virus-Host Interactome Screen as a Platform for Antiviral Drug Development. *Cell Host & Microbe* **16**:795-805.
4. **Bradel-Tretheway BG, Mattiaccio JL, Krasnoselsky A, Stevenson C, Purdy D, Dewhurst S, Katze MG.** 2011. Comprehensive proteomic analysis of influenza virus polymerase complex reveals a novel association with mitochondrial proteins and RNA

564 polymerase accessory factors. J Virol **85**:8569-8581.

565 5. **Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, Gupta PB, Hao**

566 **T, Silver SJ, Root DE, Hill DE, Regev A, Hacohen N.** 2009. A physical and regulatory

567 map of host-influenza interactions reveals pathways in H1N1 infection. Cell **139**:1255-

568 1267.

569 6. **Karlas A, Machuy N, Shin Y, Pleissner K-P, Artarini A, Heuer D, Becker D, Khalil**

570 **H, Ogilvie LA, Hess S, Maurer AP, Muller E, Wolff T, Rudel T, Meyer TF.** 2010.

571 Genome-wide RNAi screen identifies human host factors crucial for influenza virus

572 replication. Nature **463**:818-822.

573 7. **Sugiyama K, Kawaguchi A, Okuwaki M, Nagata K.** 2015. pp32 and APRIL are host

574 cell-derived regulators of influenza virus RNA synthesis from cRNA. Elife **4**.

575 8. **Long JS, Giotis ES, Moncorge O, Frise R, Mistry B, James J, Morisson M, Iqbal**

576 **M, Vignal A, Skinner MA, Barclay WS.** 2016. Species difference in ANP32A underlies

577 influenza A virus polymerase host restriction. Nature **529**:101-104.

578 9. **Subbarao EK, London W, Murphy BR.** 1993. A single amino acid in the PB2 gene of

579 influenza A virus is a determinant of host range. J Virol **67**:1761-1764.

580 10. **Xiao C, Ma W, Sun N, Huang L, Li Y, Zeng Z, Wen Y, Zhang Z, Li H, Li Q, Yu Y,**

581 **Zheng Y, Liu S, Hu P, Zhang X, Ning Z, Qi W, Liao M.** 2016. PB2-588 V promotes the

582 mammalian adaptation of H10N8, H7N9 and H9N2 avian influenza viruses. Sci Rep

583 **6**:19474.

584 11. **Song W, Wang P, Mok BW, Lau SY, Huang X, Wu WL, Zheng M, Wen X, Yang S,**

- 585 **Chen Y, Li L, Yuen KY, Chen H.** 2014. The K526R substitution in viral protein PB2
586 enhances the effects of E627K on influenza virus replication. *Nat Commun* **5**:5509.
- 587 12. **Sediri H, Schwalm F, Gabriel G, Klenk HD.** 2015. Adaptive mutation PB2 D701N
588 promotes nuclear import of influenza vRNPs in mammalian cells. *Eur J Cell Biol*
589 **94**:368-374.
- 590 13. **Manz B, de Graaf M, Mogling R, Richard M, Bestebroer TM, Rimmelzwaan GF,**
591 **Fouchier RA.** 2016. Multiple Natural Substitutions in Avian Influenza A Virus PB2
592 Facilitate Efficient Replication in Human Cells. *J Virol* **90**:5928-5938.
- 593 14. **Liu Q, Qiao C, Marjuki H, Bawa B, Ma J, Guillosoy S, Webby RJ, Richt JA, Ma W.**
594 2012. Combination of PB2 271A and SR polymorphism at positions 590/591 is critical
595 for viral replication and virulence of swine influenza virus in cultured cells and in vivo.
596 *J Virol* **86**:1233-1237.
- 597 15. **Zhang H, Li X, Guo J, Li L, Chang C, Li Y, Bian C, Xu K, Chen H, Sun B.** 2014. The
598 PB2 E627K mutation contributes to the high polymerase activity and enhanced
599 replication of H7N9 influenza virus. *J Gen Virol* **95**:779-786.
- 600 16. **Mok CK, Lee HH, Lestra M, Nicholls JM, Chan MC, Sia SF, Zhu H, Poon LL, Guan**
601 **Y, Peiris JS.** 2014. Amino acid substitutions in polymerase basic protein 2 gene
602 contribute to the pathogenicity of the novel A/H7N9 influenza virus in mammalian hosts.
603 *J Virol* **88**:3568-3576.
- 604 17. **Hu M, Yuan S, Zhang K, Singh K, Ma Q, Zhou J, Chu H, Zheng BJ.** 2017. PB2
605 substitutions V598T/I increase the virulence of H7N9 influenza A virus in mammals.

- 606 Virology **501**:92-101.
- 607 18. **Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y,**
608 **Yu K.** 2005. Molecular basis of replication of duck H5N1 influenza viruses in a
609 mammalian mouse model. J Virol **79**:12058-12064.
- 610 19. **Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y, Li Z, Guan Y, Tian G, Li Y, Shi J, Liu**
611 **L, Zeng X, Bu Z, Xia X, Kawaoka Y, Chen H.** 2009. Identification of amino acids in HA
612 and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian
613 host. Plos Pathog **5**:e1000709.
- 614 20. **Steel J, Lowen AC, Mubareka S, Palese P.** 2009. Transmission of Influenza Virus in
615 a Mammalian Host Is Increased by PB2 Amino Acids 627K or 627E/701N. Plos Pathog
616 **5**(1):e1000252.
- 617 21. **Hatta M, Gao P, Halfmann P, Kawaoka Y.** 2001. Molecular basis for high virulence of
618 Hong Kong H5N1 influenza A viruses. Science **293**:1840-1842.
- 619 22. **Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T.** 2010. PB2 residue 271
620 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian
621 host cells. J Virol **84**:4395-4406.
- 622 23. **Bortz E, Westera L, Maamary J, Steel J, Albrecht RA, Manicassamy B, Chase G,**
623 **Martinez-Sobrido L, Schwemmle M, Garcia-Sastre A.** 2011. Host- and strain-specific
624 regulation of influenza virus polymerase activity by interacting cellular proteins. MBio
625 **2**:e00151-00111.
- 626 24. **Diot C, Fournier G, Santos MD, Magnus J, Komarova A, Werf SVD, Munier S,**

627 **Naffakh N.** 2016. Influenza A Virus Polymerase Recruits the RNA Helicase DDX19 to
628 Promote the Nuclear Export of Viral mRNAs. *Scientific Reports* **6**:33763.

629 25. **Eisfeld AJ, Neumann G, Kawaoka Y.** 2015. At the centre: influenza A virus
630 ribonucleoproteins. *Nat Rev Microbiol* **13**:28-41.

631 26. **Gabriel G, Klingel K, Otte A, Thiele S, Hudjetz B, Arman-Kalcek G, Sauter M,**
632 **Shmidt T, Rother F, Baumgarte S, Keiner B, Hartmann E, Bader M, Brownlee GG,**
633 **Fodor E, Klenk HD.** 2011. Differential use of importin-alpha isoforms governs cell
634 tropism and host adaptation of influenza virus. *Nature Communications* **2**(2):156.

635 27. **Landeras-Bueno S, Jorba N, Pérez-Cidoncha M, Ortín J.** 2011. The Splicing Factor
636 Proline-Glutamine Rich (SFPQ/PSF) Is Involved in Influenza Virus Transcription. *Plos*
637 *Pathog* **7**:e1002397.

638 28. **Marcosvillar L, Pazo A, Nieto A.** 2016. Influenza Virus and Chromatin: Role of the
639 CHD1 Chromatin Remodeler in the Virus Life Cycle. *Journal of Virology* **90**:3694.

640 29. **Momose F, Naito T, Yano K, Sugimoto S, Morikawa Y, Nagata K.** 2002. Identification
641 of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J Biol*
642 *Chem* **277**:45306-45314.

643 30. **Shaw ML, Stertz S.** 2017. Role of Host Genes in Influenza Virus Replication. In: Tripp
644 R., Tompkins S. (eds) *Roles of Host Gene and Non-coding RNA Expression in Virus*
645 *Infection. Current Topics in Microbiology and Immunology*, vol 419. Springer, Cham.

646 31. **Su WC, Hsu SF, Lee YY, Jeng KS, Lai MM.** 2015. A Nucleolar Protein, Ribosomal
647 RNA Processing 1 Homolog B (RRP1B), Enhances the Recruitment of Cellular mRNA

648 in Influenza Virus Transcription. Journal of Virology **89**:11245.

649 32. **Ver LS, Marcos-Villar L, Landeras-Bueno S, Nieto A, Ortín J.** 2015. The Cellular

650 Factor NXP2/MORC3 Is a Positive Regulator of Influenza Virus Multiplication. Journal

651 of Virology **89**:10023-10030.

652 33. **Zhang J, Li G, Ye X.** 2010. Cyclin T1/CDK9 Interacts with Influenza A Virus Polymerase

653 and Facilitates Its Association with Cellular RNA Polymerase II. Journal of Virology

654 **84**:12619-12627.

655 34. **Domingues P, Hale BG.** 2017. Functional Insights into ANP32A-Dependent Influenza

656 A Virus Polymerase Host Restriction. Cell reports **20**:2538-2546.

657 35. **Baker SF, Ledwith MP, Mehle A.** 2018. Differential Splicing of ANP32A in Birds Alters

658 Its Ability to Stimulate RNA Synthesis by Restricted Influenza Polymerase. Cell reports

659 **24**:2581-2588 e2584.

660 36. **Reilly PT, Yu Y, Hamiche A, Wang L.** 2014. Cracking the ANP32 whips: important

661 functions, unequal requirement, and hints at disease implications. Bioessays **36**:1062-

662 1071.

663 37. **Watanabe T, Kawakami E, Shoemaker JE, Lopes TJ, Matsuoka Y, Tomita Y,**

664 **Kozuka-Hata H, Gorai T, Kuwahara T, Takeda E, Nagata A, Takano R, Kiso M,**

665 **Yamashita M, Sakai-Tagawa Y, Katsura H, Nonaka N, Fujii H, Fujii K, Sugita Y,**

666 **Noda T, Goto H, Fukuyama S, Watanabe S, Neumann G, Oyama M, Kitano H,**

667 **Kawaoka Y.** 2014. Influenza virus-host interactome screen as a platform for antiviral

668 drug development. Cell Host Microbe **16**:795-805.

- 669 38. **Zhang Y, Zhang Q, Kong H, Jiang Y, Gao Y, Deng G, Shi J, Tian G, Liu L, Liu J,**
670 **Guan Y, Bu Z, Chen H.** 2013. H5N1 hybrid viruses bearing 2009/H1N1 virus genes
671 transmit in guinea pigs by respiratory droplet. *Science* **340**:1459-1463.
- 672 39. **Zhang Q, Shi J, Deng G, Guo J, Zeng X, He X, Kong H, Gu C, Li X, Liu J, Wang G,**
673 **Chen Y, Liu L, Liang L, Li Y, Fan J, Wang J, Li W, Guan L, Li Q, Yang H, Chen P,**
674 **Jiang L, Guan Y, Xin X, Jiang Y, Tian G, Wang X, Qiao C, Li C, Bu Z, Chen H.** 2013.
675 H7N9 influenza viruses are transmissible in ferrets by respiratory droplet. *Science*
676 **341**:410-414.
- 677 40. **Hay AJ, Skehel JJ, McCauley J.** 1982. Characterization of influenza virus RNA
678 complete transcripts. *Virology* **116**:517-522.
- 679 41. **Young RJ, Content J.** 1971. 5'-terminus of influenza virus RNA. *Nat New Biol* **230**:140-
680 142.
- 681 42. **Wang M, Zhang Z, Wang X.** 2018. Strain-Specific Antagonism of the Human H1N1
682 Influenza A Virus against Equine Tetherin. *Viruses* **10**(5):264.
- 683 43. **Matilla A, Radrizzani M.** 2005. The Anp32 family of proteins containing leucine-rich
684 repeats. *Cerebellum* **4**:7-18.
- 685 44. **Almond JW.** 1977. A single gene determines the host range of influenza virus. *Nature*
686 **270**:617-618.
- 687 45. **Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu**
688 **H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L,**
689 **Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang**

690 **S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu**
691 **Y.** 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. *N Engl J*
692 *Med* **368**:1888-1897.

693 46. **Chen H, Bright RA, Subbarao K, Smith C, Cox NJ, Katz JM, Matsuoka Y.** 2007.
694 Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1
695 influenza viruses in mice. *Virus Res* **128**:159-163.

696 47. **Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y.** 2004. PB2 amino acid at
697 position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1
698 influenza A viruses in mice. *Virology* **320**:258-266.

699 48. **Mehle A, Doudna JA.** 2008. An inhibitory activity in human cells restricts the function
700 of an avian-like influenza virus polymerase. *Cell Host Microbe* **4**:111-122.

701 49. **Shi J, Deng G, Kong H, Gu C, Ma S, Yin X, Zeng X, Cui P, Chen Y, Yang H, Wan X,**
702 **Wang X, Liu L, Chen P, Jiang Y, Liu J, Guan Y, Suzuki Y, Li M, Qu Z, Guan L, Zang**
703 **J, Gu W, Han S, Song Y, Hu Y, Wang Z, Gu L, Yang W, Liang L, Bao H, Tian G, Li**
704 **Y, Qiao C, Jiang L, Li C, Bu Z, Chen H.** 2017. H7N9 virulent mutants detected in
705 chickens in China pose an increased threat to humans. *Cell Res* **27**:1409-1421.

706 50. **Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li YQ, Fine**
707 **EJ, Wu XB, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F.** 2013. DNA
708 targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**:827-+.

709 51. **Jinek M, East A, Cheng A, Lin S, Ma EB, Doudna J.** 2013. RNA-programmed
710 genome editing in human cells. *Elife* **2**: e00471.

- 711 52. **Pleschka S, Jaskunas R, Engelhardt OG, Zurcher T, Palese P, Garcia-Sastre A.**
712 1996. A plasmid-based reverse genetics system for influenza A virus. *J Virol* **70**:4188-
713 4192.
- 714 53. **Kawakami E, Watanabe T, Fujii K, Goto H, Watanabe S, Noda T, Kawaoka Y.** 2011.
715 Strand-specific real-time RT-PCR for distinguishing influenza vRNA, cRNA, and mRNA.
716 *J Virol Methods* **173**:1-6.
- 717 54. **Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR,**
718 **Donis R, Hoffmann E, Hobom G, Kawaoka Y.** 1999. Generation of influenza A viruses
719 entirely from cloned cDNAs. *Proc Natl Acad Sci U S A* **96**:9345-9350.
- 720 55. **Muench H, Reed L.** 1938. A simple method of estimating fifty percent endpoints. *27* (3):
721 493-497.
- 722 56. **Zhu W, Zhu Y, Qin K, Yu Z, Gao R, Yu H, Zhou J, Shu Y.** 2012. Mutations in
723 polymerase genes enhanced the virulence of 2009 pandemic H1N1 influenza virus in
724 mice. *PLoS One* **7**:e33383.

725

FIG 1. HuANP32A&B are indispensable for influenza A virus polymerase activity

and viral replication. (A) Wild type 293T cells and single gene knockout 293T cell

lines, including BUB3, CLTC, CYC1, NIBP, ZC3H15, C14orf173, CTNNB1, ANP32A,

ANP32B, SUPT5H, HTATSF1, and DDX17, were transfected with firefly minigenome

reporter, *Renilla* expression control, and the polymerase of H1N1_{SC09}. Luciferase

activity was measured 24 h after transfection and data are firefly Luciferase gene

activity normalized to *Renilla* Luciferase gene activity. (Statistical differences between

samples are indicated, according to a one-way ANOVA followed by a Dunnett's test;

NS= not significant, *P < 0.05, **P < 0.01, ****P < 0.0001. Error bars represent the SEM

within one representative experiment.). **(B to D)** 293T, huANP32A knockout cells

(AKO), huANP32B knockout cells (BKO), and huANP32A&B double knockout cells

(DKO), were transfected with firefly minigenome reporter, *Renilla* expression control,

and either H1N1_{SC09} polymerase **(B)**, H7N9_{AH13} polymerase **(C)**, or WSN polymerase

(D). Luciferase activity was measured at 24 h post transfection. **(B to D)**, data are firefly

Luciferase gene activity normalized to that of *Renilla*, Statistical differences between

cells are indicated, following a one-way ANOVA and subsequent Dunnett's test; NS =

not significant, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent the SEM

of the replicates within one representative experiment.). The expression levels of

H1N1_{SC09} polymerase proteins were assessed by western blotting **(B)**. **(E)** 293T, AKO,

BKO and DKO cells were infected with WSN virus at an MOI of 0.01. The supernatants

were sampled at 0, 12, 24, 36, and 48 h post infection and the virus titers were

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

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

control, and human influenza virus polymerase from H1N1_{SC09} (**A**), H7N9_{AH13} (**B**), canine influenza virus H3N2_{GD11} (**C**), equine influenza virus H3N8_{XJ07} (**D**) and H3N8_{JL89} (**E**), or avian influenza virus H9N2_{ZJ12} (**F**) into DKO cells. Luciferase activity was measured 24 h later. (Data are firefly Luciferase gene activity normalized to that of *Renilla*, Statistical differences between cells are indicated, 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.) dk, duck; ty, turkey; zf, zebra finch; mu, mouse; pg, pig; eq, equine.

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

FIG 7. The 129-130 site in ANP32B contributes to the interaction with influenza

polymerase. (A) A schematic describing the structure of ANP32B. LRR, leucine-rich repeat, including 4 LRR repeats and one C-terminal LRR domain (LRRCT); LCAR, low-complexity acidic region; NLS, nuclear localization signal. (B) and (C) 293T cells were transfected with different ANP32-Flag constructs, together with viral polymerase subunits PA, PB1, and PB2. Coimmunoprecipitation of the anti-Flag antibodies and the proteins was assessed using western blotting.

SUPPORTING INFORMATION

FIG S1. The identification of huANP32A and huANP32B knockout cells.

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

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

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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
<i>Gallus</i>	N	D	I	N
<i>Anas platyrhynchos</i>	N	D	I	N
<i>Homo sapiens</i>	N	D	N	D
<i>Gorilla gorilla</i>	N	D	N	D
<i>Felis catus</i>	N	D	N	D
<i>Canis lupus familiaris</i>	N	D	N	D
<i>Bos taurus</i>	N	D	N	D
<i>Sus scrofa</i>	N	D	N	D
<i>Mus musculus</i>	N	A	S	D
<i>Equus caballus</i>	N	D	N	D

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