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VIRUS-CELL INTERACTIONS

Fundamental Contribution and Host Range Determination of ANP32A and ANP32B in Influenza A Virus Polymerase Activity

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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 influence both polymerase activity and interspecies restriction. We report here that either human ANP32A or ANP32B is indispensable for human influenza A virus RNA replication. The contribution of huANP32B is equal to that of huANP32A, and together they play a fundamental role in the activity of human influenza A virus polymerase, while neither human ANP32A nor ANP32B supports the activity of avian viral polymerase. Interestingly, we found that avian ANP32B was naturally inactive, leaving avian ANP32A alone to support viral replication. Two amino acid mutations at sites 129 to 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 and ANP32B as key factors for both virus replication and adaptation.

IMPORTANCE The key host factors involved in the influenza A viral polymerase activity and RNA replication remain largely unknown. We provide evidence here 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 viral RNA replication. However, unlike avian ANP32A, the avian ANP32B is evolutionarily nonfunctional in supporting viral replication because of a mutation at sites 129 and 130. These sites play an important role in ANP32A/ANP32B and viral polymerase interaction and therefore determine viral replication, suggesting a novel interface as a potential target for the development of anti-influenza strategies.

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

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 replica-

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tion. 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 (9–28). Many host factors have been reported to interact with the vRNP complex to help viral replication (7, 29–40). Of these proteins, the acidic nuclear phosphoprotein 32 family, members A and B (ANP32A and ANP32B), have been found to regulate viral RNA synthesis (7). Interestingly, chicken ANP32A (chANP32A) has been reported to specifically promote avian influenza replication due to the 33-amino-acid 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 (41). Furthermore, ANP32A from birds has splicing variants without SUMO interaction motif also support viral replication and polymerase adaptation (42). These studies suggest that ANP32A plays an important role in viral replication.

The acidic (leucine-rich) nuclear phosphoprotein 32-kDa (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 (43). Although ANP32A is considered to be an important cofactor 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 huANP32A and huANP32B play fundamental roles in the facilitation of human influenza A viral RNA synthesis, and that both huANP32A and huANP32B 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.

(This article was submitted to an online preprint archive [44].)

RESULTS

Human ANP32A&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 (3, 4, 29). All of 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 virus-like luciferase RNA was expressed, together with the viral polymerases PB1, PB2, PA, and NP, to determine the polymerase activity in these cell lines. First, we tested the polymerase activity of 2009 pandemic H1N1 virus A/Sichuan/01/2009 ($H1N1_{SC09}$) (45) on these knockout 293T cells and found that individual knockout of NIBP, ZC3H15, or DDX17 results in a 2- to 4-fold decrease in viral polymerase activity, which is consistent with previous results (3, 29). 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 since ANP32A and ANP32B (ANP32A&B) were reported to be important host factors supporting viral polymerase activity (7, 8, 41, 42). Since ANP32A and ANP32B have high similarity in both structure and known functions, we predicted that in single-knockout cell lines of

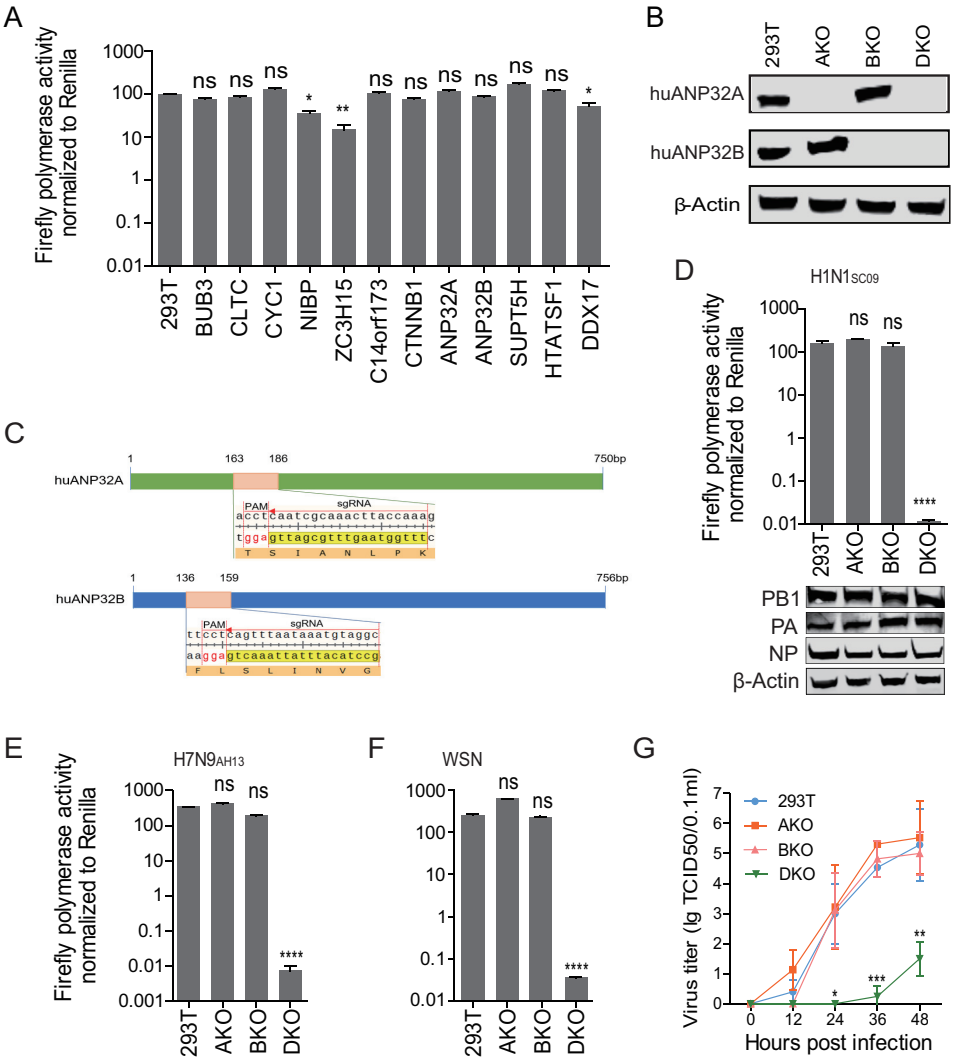

FIG 1 huANP32A&B are indispensable for influenza A virus polymerase activity and viral replication. (A) Wild-type 293T cells and single-gene-knockout 293T cell lines, including BUB3, CLTC, CYC1, NIBP, ZC3H15, C14orf173, CTNNB1, ANP32A, ANP32B, SUPT5H, HTATSF1, and DDX17, were transfected with firefly minigenome reporter, *Renilla* expression control, and the polymerase of $H1N1_{SC09}$. The luciferase activity was measured 24 h after transfection, and data indicate the firefly luciferase gene activity normalized to the *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) Wild-type 293T cells were transfected with pMJ920 vector (a plasmid expressing eGFP and Cas9) and gRNAs targeting huANP32A and huANP32B to generate huANP32A&B double-knockout cells (DKO). The endogenous proteins of different cells (293T cells, huANP32A knockout cells [AKO], huANP32B knockout cells [BKO], and DKO cells) were identified by Western blotting with antibodies against β-actin, huANP32A, and huANP32B as described in Materials and Methods. (C) Design scheme of sgRNAs for huANP32A and huANP32B. (D to F) Wild-type 293T cells, 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 (D), $H7N9_{AH13}$ polymerase (E), or WSN polymerase (F). The luciferase activity was measured at 24 h posttransfection. For panels D to F, the data are the firefly luciferase gene activity normalized to that of *Renilla* luciferase activity. 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 replicates within one representative experiment. The expression levels of $H1N1_{SC09}$ polymerase proteins were assessed by Western blotting in panel D. (G) Wild-type 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 postinfection, and the virus titers were determined by endpoint titration in MDCK cells. Error bars indicate the SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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. Human ANP32A (huANP32A) knockout 293T cells (AKO cells), human ANP32B (huANP32B) knockout 293T cells (BKO cells), and ANP32A and ANP32B double-knockout cells (DKO) were confirmed by Western blotting with ANP32A- and ANP32B-specific antibodies (Fig. 1B). The target sequences of sgRNAs for huANP32A and huANP32B are shown in Fig. 1C. In AKO and BKO cells, the viral polymerases have similar activities to wild-type (WT) 293T cells, but when both ANP32A and ANP32B were knocked out (DKO), the polymerase activity was abolished (~10,000-fold reduction) (Fig. 1D), although the polymerase was expressed equally in the 293T, AKO, BKO, and DKO cells (Fig. 1D). We then tested the polymerase activities of 2013 China H7N9 human isolate A/Anhui/01/2013 ($H7N9_{AH13}$) (46), 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. 1E and F). We further confirmed the effect of huANP32A&B on viral infectivity by infecting 293T cells and KO cells with WSN virus. In DKO cells, but not AKO or BKO cells, the infectivity of WSN decreased by >10,000-fold (Fig. 1G). 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 (~10,000-fold), which is distinct from a previous report that used a gene knockdown method and resulted in an approximately 3- to 5-fold reduction in viral polymerase activity (8). We found that reconstitution of either huANP32A or huANP32B, or both of them, in the DKO cells restored the viral polymerase activities of $H1N1_{SC09}$, $H7N9_{AH13}$, and WSN viruses (Fig. 2A to C). Expression of huANP32A or huANP32B in DKO cells supported the $H1N1_{SC09}$ viral polymerase activity in a dose-dependent manner (Fig. 2D). We confirmed that the required level of expression of huANP32A or B is very low, and overdose expression has a negative effect (Fig. 2E), suggesting an explanation of the confusing phenotype previously observed, that in normal 293T cells overexpression of ANP32 protein decreased viral polymerase activity (8). Reconstitution of huANP32A or huANP32B or both of them in DKO cells restored full viral infectivity (Fig. 2F), indicating that huANP32A and huANP32B are of fundamental importance in human influenza viral replication. These data proved that huANP32A and huANP32B 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 cRNA (cRNA) and mRNA; the cRNA is then used as a template to amplify into new vRNA (47, 48). We found that vRNA, cRNA, and mRNA synthesis was dramatically reduced in DKO cells. However, when huANP32A was reconstituted in the DKO cells, vRNA, cRNA, and mRNA synthesis was fully recovered (Fig. 3A to C). We observed similar results from the reconstitution of ANP32B or both ANP32A and ANP32B (Fig. 3A to C), indicating that huANP32A&B are key factors in triggering the replication of the human influenza 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 enzyme-linked immunosorbent assay (ELISA) (49). The result showed that the DKO cells had very low NP production in the cell supernatant compared to the 293T, AKO, or BKO cells (Fig. 3D). The NP production was recovered when the huANP32A and/or huANP32B was expressed in 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 ANP32B 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 (43). ANP32A&B exist in almost all eukaryotic cells, with the exception of early

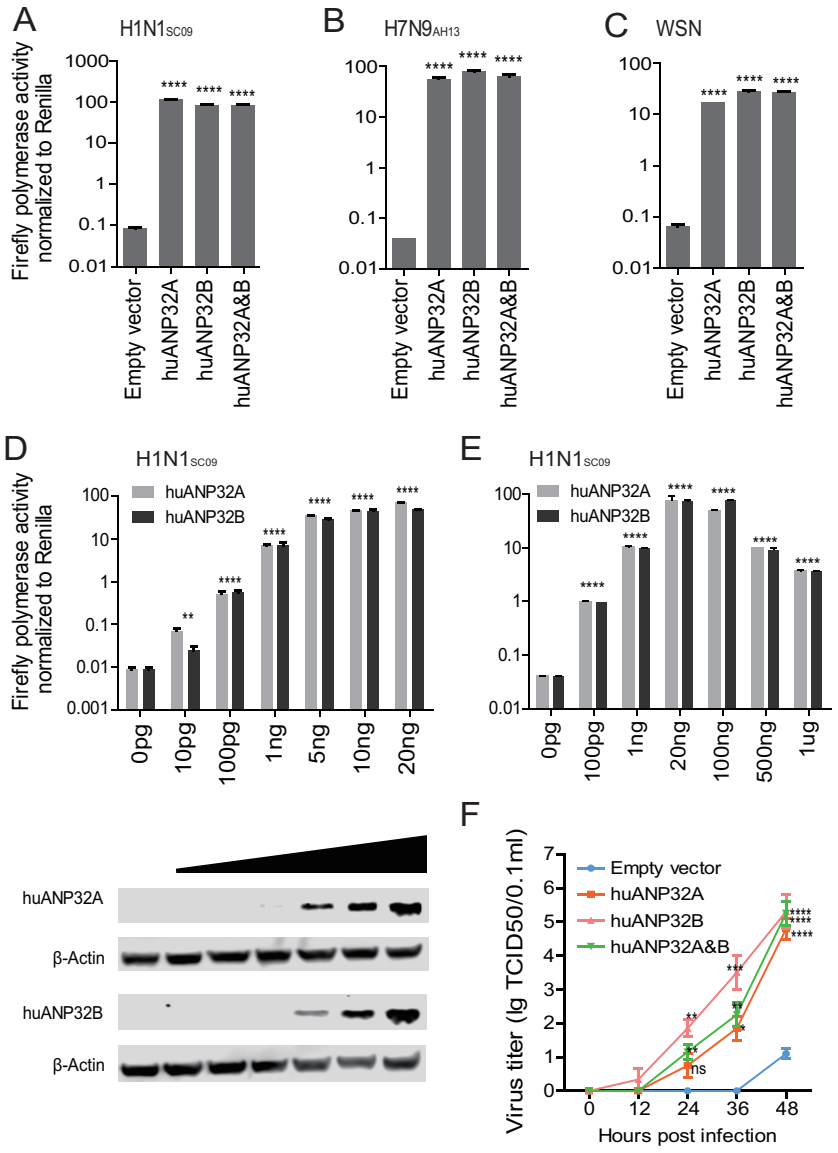

FIG 2 Reconstitutions of huANP32A&B rescue polymerase activity and replication of influenza A virus. We cotransfected 20 ng of huANP32A or huANP32B, 10 ng of huANP32A and 10 ng of huANP32B, or 20 ng of empty vector with either $H1N1_{SC09}$ polymerase (A), $H7N9_{AH13}$ polymerase (B), or WSN polymerase (C) into DKO cells and then assayed the luciferase activity at 24 h after transfection. (D and E) Increasing doses of huANP32A or huANP32B were cotransfected with $H1N1_{SC09}$ polymerase into DKO cells. The luciferase activity was measured at 24 h posttransfection. For panels A to E, the data are the firefly luciferase gene activities normalized to that of the *Renilla* luciferase activity. 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. (F) DKO cells were transfected with 1 μg of huANP32A and/or huANP32B, 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 postinfection, and the virus titers in these supernatants were determined as described above. Error bars indicate the SD from three independent experiments (NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

eukaryotic life (yeast and other fungi) (50). 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 sources and ANP32B from human and chicken sources 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 A/equine/Xinjiang/1/2007($H3N8_{XJ07}$), A/equine/Jilin/1/1989($H3N8_{JL89}$), or H9N2 chicken virus A/chick-

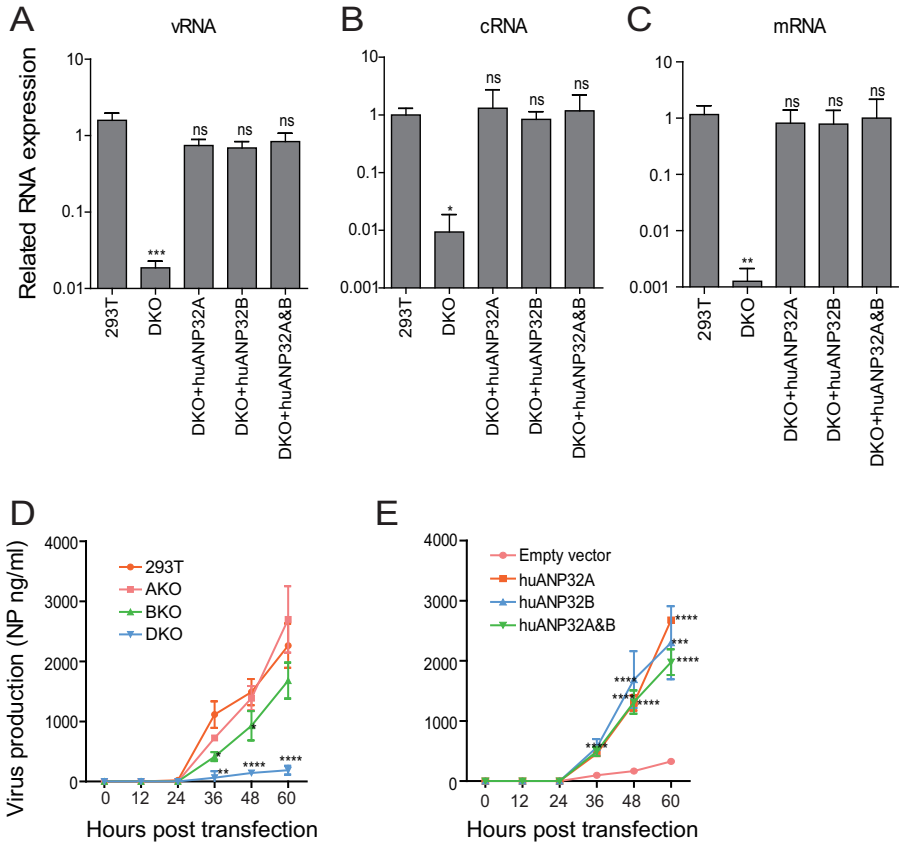

FIG 3 huANP32A&B determine viral RNA replication efficiency and viral production. (A to C) Wild-type 293T or DKO cells were transfected with the H1N1$_{SC09}$ minigenome reporting system, together with 20 ng of empty vector, huANP32A, huANP32B, or huANP32A plus huANP32B. The cells were incubated at 37°C for 24 h before reverse transcription, followed by quantitative PCR (qRT-PCR) for vRNA, mRNA, and cRNA of the luciferase gene. Error bars represent the SD from three independent experiments (NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (D and E) Replication kinetics of H1N1$_{SC09}$ in ANP32 knockout cells. 293T, AKO, BKO, and DKO cells in 6-well plates were transfected with 0.5 μg each of the eight plasmids of H1N1$_{SC09}$ (D), or, in DKO cells, 40 ng of huANP32A, 40 ng of huANP32B, 20 ng each of huANP32A and huANP32B, or 40 ng of empty vector was cotransfected with the eight plasmids of H1N1$_{SC09}$ (E). The cells were cultured at 37°C, and supernatants were collected at 0, 12, 24, 48, and 60 h posttransfection and subjected to virus production assay by ELISA as described in Materials and Methods. Error bars indicate the SD from three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

en/Zhejiang/B2013/2012(H9N2$_{ZJ12}$) in DKO cells (Fig. 4A to G). Interestingly, we found that chicken and other avian ANP32A proteins, which contain an additional 33 amino acids compared to the ANP32 proteins of mammals (8), supported viral polymerase activities in all cases, whereas the ANP32A proteins from humans, pigs, and horses, as well as human ANP32B, supported mammalian influenza virus polymerase activities, but not that of chicken H9N2$_{ZJ12}$ or the H3N8$_{JL89}$ (an avian-like virus). This result is consistent with previous reports that avian ANP32A can promote avian viral polymerase activity in human cells (8, 42). PB2 is the most important polymerase subunit and affects host range (31, 51, 52). Almost all the avian viruses had a glutamic acid (E) at PB2 residue 627, while it could be rapidly selected as a lysine (K) when the virus adapted to mammals, accompanied with increased pathogenicity and transmission abilities. The PB2 E627K mutation has long been regarded as a key signature of the avian influenza virus in overcoming the block to replicate in mammalian cells (9, 20, 22, 27, 28, 45, 53, 54). The H7N9 virus strain A/Anhui/01/2013 is an avian original virus with E627K (human viral signature) on the PB2 subunit; however, other key residues of PB2, including 588A, 591Q, 598 V, and 701D, are all avian viral signatures. We observed that the K627E mutation in H7N9$_{AH13}$ lost support from mammalian ANP32 proteins (Fig. 4H), while

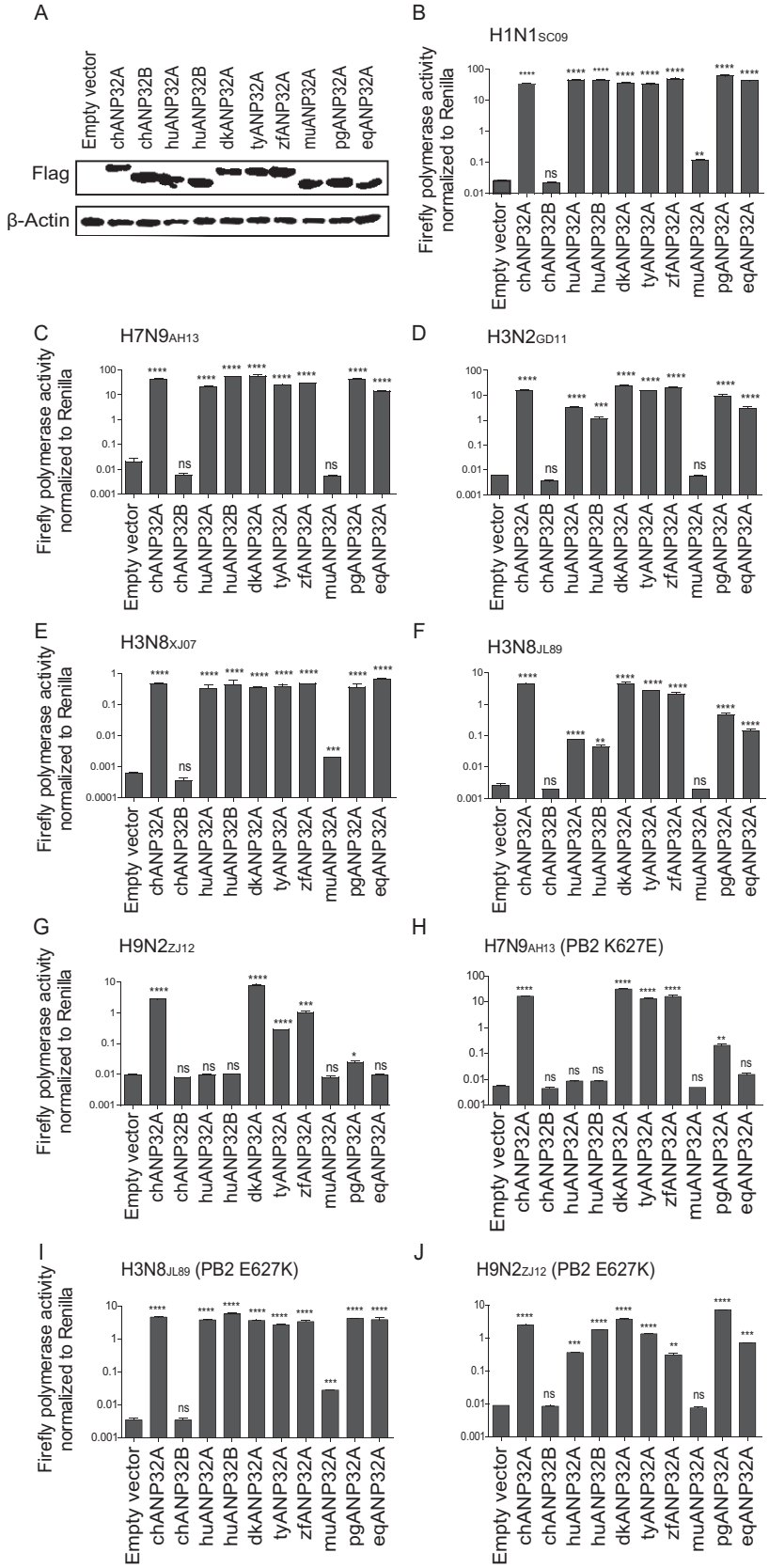

FIG 4 Support of influenza A viral replication by ANP32A or ANP32B from different species. (A) One microgram of each ANP32 plasmid was transfected into 293T cells using Lipofectamine 2000. At 48 h posttransfection, the cell lysates were analyzed using SDS-PAGE and Western blotting with antibodies

(Continued on next page)

E627K mutations in isolates of avian origin (horse $H3N8_{JL89}$ or chicken $H9N2_{ZJ12}$) dramatically changed viral fitness to mammalian ANP32 proteins (Fig. 4I and J). These results revealed that 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, which may be the major determinant for the adaptation of influenza A virus to different host.

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 (43). ANP32A&B have been reported to interact directly with the polymerase complex (7, 42). 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. 5A). Chimeric clones between chicken and human ANP32B were constructed and evaluated (Fig. 5B). We found that the replacement of amino acids 111 to 160 of huANP32B with those of chANP32B aborted the activity of this protein, while chANP32B with the human fragment from amino acids 111 to 161 (fragment 111-161) gained the ability to boost viral polymerase activity (Fig. 5C and D). Further comparing of ANP32B fragments 111-161 for chickens, humans, and pigs showed eight amino acid substitutions (Fig. 6A). Of these, a combined mutation N129I/D130N, but not others, on huANP32B impaired H1N1 polymerase activity (Fig. 6B), and the N129I showed stronger impairment than did the D130N mutation (Fig. 6C). We confirmed this phenotype on H7N9 polymerase activity (Fig. 6D). The 129-130 mutations also aborted the support of huANP32A of H1N1 and H7N9 polymerases (Fig. 6E and F). Furthermore, substitutions at amino acids 129 and 130 in chANP32A and chANP32B reversed the support of these proteins of the H7N9 virus human isolate (Fig. 6G). Interestingly, in the context of a chicken H7N9 viral minigenome, although the mutation of amino acids 129 and 130 of chANP32A impaired the polymerase activity, the chANP32B reverse mutation did not restore its support to chicken-like H7N9 viral RNA replication (Fig. 6H), indicating that chicken virus replication may require both the 129-130 site and an extra 33-amino-acid peptide as in chANP32A. Most terrestrial mammalian ANP32A&B proteins have a 129-ND-130 signature, but most of the avian ANP32Bs have 129-IN-130 residues (Table 1). Murine ANP32A (muANP32A) has 129-NA-130, and this may explain the impaired support of H1N1 polymerase by muANP32A (Fig. 4 and 6I). To further confirm the function of the 129-130 site, we made inactivating mutations of huANP32A and huANP32B at the sites 129 and 130 (N129I, D130N) on the 293T genome using CRISPR-Cas9 mediated recombination (Fig. 7A to D); the double mutated proteins expressed well and fully impaired the replication of both $H1N1_{SC09}$ and $H7N9_{AH13}$ (Fig. 7E and F). Interestingly, the single ANP32A or ANP32B mutations led to slightly decreased polymerase activities compared to wild-type 293T cells, suggesting competition between the mutated proteins and the native proteins for viral polymerase binding.

FIG 4 Legend (Continued)
against Flag peptide and β-actin. (B to J) Twenty nanograms of either empty vector or ANP32A or ANP32B from one of several different species was cotransfected with a minigenome reporter, *Renilla* expression control, and human influenza virus polymerase from $H1N1_{SC09}$ (B), $H7N9_{AH13}$ (C), canine influenza virus $H3N2_{GD11}$ (D), equine influenza virus $H3N8_{XJ07}$ (E) and an avian origin equine influenza virus $H3N8_{JL89}$ (F), avian influenza virus $H9N2_{ZJ12}$ (G), $H7N9_{AH13}$ with PB2 K627E (H), $H3N8_{XJ07}$ with PB2 E627K (I), or $H9N2_{ZJ12}$ with PB2 E627K (J) into DKO cells. The luciferase activity was measured 24 h later. Data indicate the firefly luciferase gene activity normalized to that of *Renilla* luciferase activity. 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.

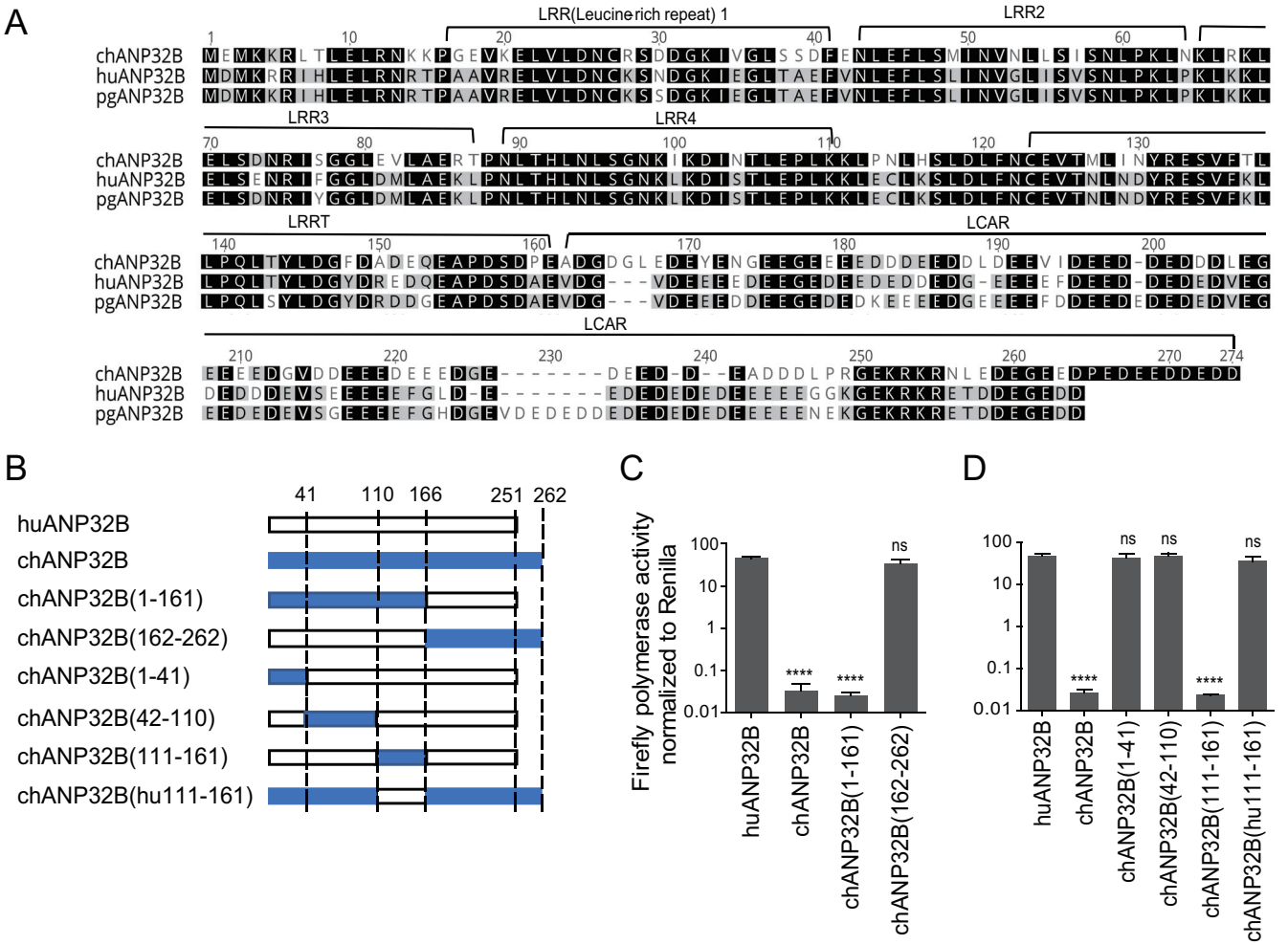

FIG 5 Mapping of crucial sites in ANP32 proteins that influence viral polymerase activity. (A) ANP32B of chicken, human, and pig sequences were aligned using Geneious R6 software. The notches are marked with dashes. The similarity of amino acid identity is highlighted in different colors. (B) Schematic diagram of chimeric clones between chicken and human ANP32B, constructed according to the known domains (separated by dotted lines: 1 to 41 amino acids, LRR1 region; 42 to 110 amino acids, LRR2,3&4 region; 111 to 161 amino acids, LRRCT region; 162 to 251 amino acids, LCAR region [as showed in panel A]). The bars indicate the origins of the genes by color as follows: white, huANP32B; and blue, chANP32B. (C and D) Chimeric clones were cotransfected with minigenome reporter, *Renilla* expression control, and $H1N1_{SC09}$ polymerase into DKO cells. The luciferase activity was measured 24 h later. Data indicate the polymerase activity normalized to that of *Renilla*. The statistical differences between samples are indicated, following one-way ANOVA and subsequent Dunnett's test (NS, not significant; ****, $P < 0.0001$). Error bars represent the SEM of replicates within a representative experiment.

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 (42). ANP32A and ANP32B can interact with polymerase only when three subunits of the polymerase are present (7). ANP32B proteins from different species have a conserved structure that comprises a N-terminal LRR region and a C-terminal LCAR (Fig. 8A). To investigate the function of the 129-130 site in ANP32B in the interaction of this protein with viral polymerase, we used ANP32B and its variants to cotransfect with the WSN minireplicon into DKO cells. Coimmunoprecipitations detected strong interactions between huANP32A and the polymerase subunits PB1 and PA (Fig. 8B, lane 1) but weak interactions when chANP32B was present (Fig. 8B, lane 2). No interactions were detected when PB1 was absent (Fig. 8B, lane 3), indicating that this interaction occurred between ANP32B and the viral trimeric polymerase complex, which is consistent with previous findings (7, 41). As controls, we also observed that the LCAR truncations of huANP32B were unable to interact with the viral polymerase (Fig. 8B, lanes 4 to 6), in agreement with previous observations (41). When we mutated the functional human

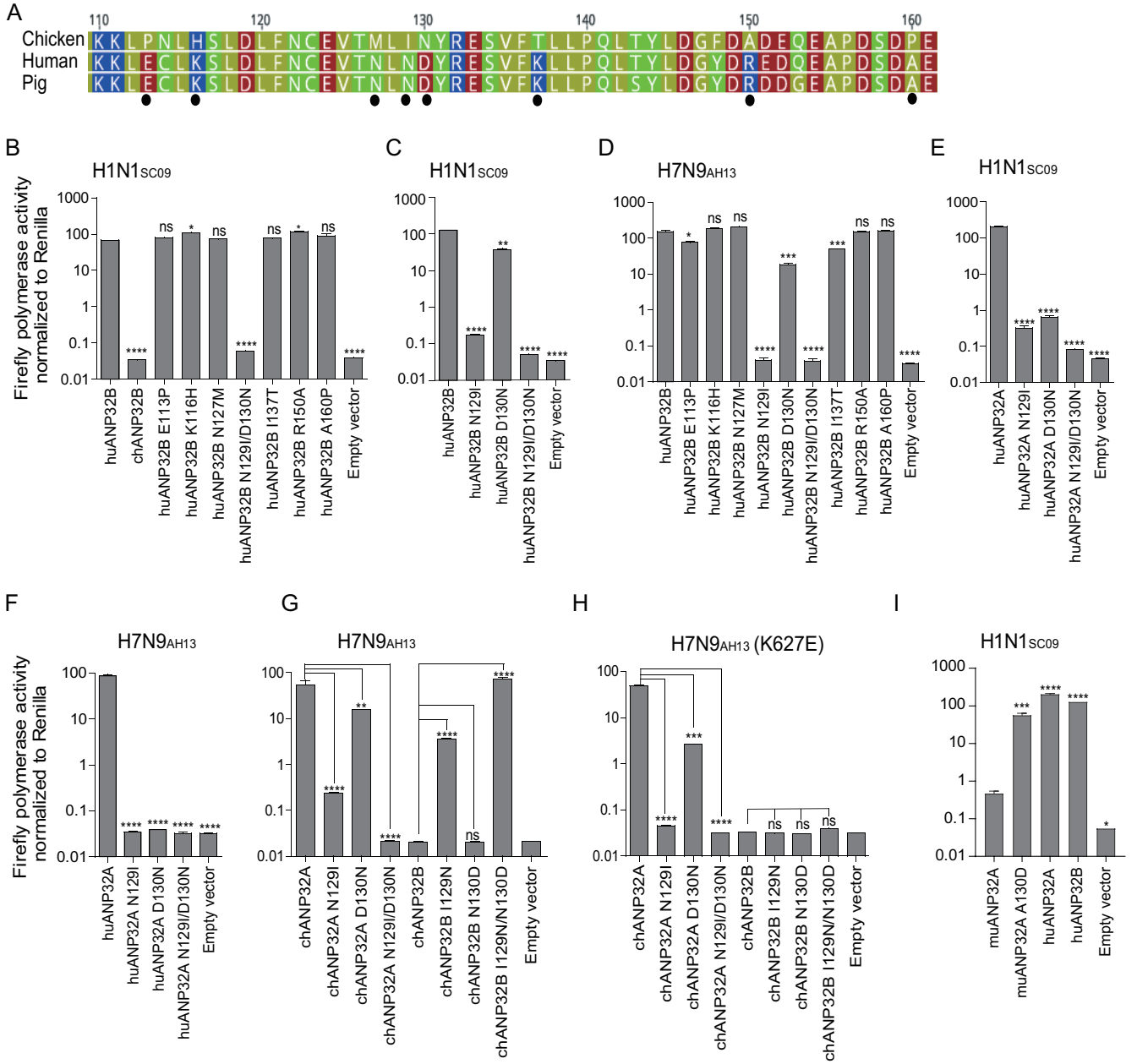

FIG 6 Key amino acids in ANP32A&B determine the activity of influenza viral polymerases. (A) Amino acid sequence comparison for chicken, human, and pig ANP32B proteins (amino acids 110 to 161). The amino acid sequences are available in GenBank. The black dots indicate the different amino acid within these proteins. (B to D) huANP32B mutants were cotransfected with polymerase plasmids from $H1N1_{SC09}$ (B and C) or $H7N9_{AH13}$ (D), plus a minigenome reporter and a *Renilla* luciferase control, into DKO cells. The luciferase activity was then assayed 24 h after transfection. (E and F) huANP32A mutants were cotransfected with polymerase plasmids from $H1N1_{SC09}$ (E) or $H7N9_{AH13}$ (F), plus a minigenome reporter and a *Renilla* luciferase control, into DKO cells. (G and H) chANP32A or chANP32B mutants were cotransfected with polymerase plasmids from $H7N9_{AH13}$ with either PB2 627K (G) or 627E (H), respectively. (I) Mutated muANP32A or human ANP32 proteins were cotransfected with minigenome reporter, *Renilla* expression control, and $H1N1_{SC09}$ polymerase into DKO cells. Luciferase activity was analyzed as mentioned above. The data indicate the firefly luciferase gene activity normalized to that of the *Renilla* luciferase activity. Statistical differences between cells are indicated, 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 a representative experiment.

ANP32B at the 129-130 site to the chicken ANP32B signature, the huANP32B lost the ability to interact with viral polymerase. Conversely, chANP32B gained the ability to coimmunoprecipitate with viral polymerase when its 129-130 sites were mutated to the human ANP32B signature (Fig. 8C). Together, these results revealed a fundamental function of ANP32A and ANP32B in supporting influenza A viral polymerase activity and

TABLE 1 Summary of amino acids at positions 129 and 130 in ANP32 proteins

	Residue at amino acid position 129 or 130[a]			
	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

[a]The sequences of different species ANP32 proteins were retrieved from the National Center for Biotechnology Information.

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 that 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 cofactors that determine influenza A virus polymerase activity. Without ANP32A&B, the viral polymerase activity decreased by 10,000-fold, and the viral infectivity decreased by >10,000-fold. In contrast, the DDX17 knockout cells showed ~3-fold-decreased viral polymerase activity (Fig. 1A), which was in agreement with prior reports (29). 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 coevolution history. ANP32 family members are expressed in animal and plant cells, with both conserved LRR and LCAR domains (50). 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 influenza viral replication, whereas 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 ANP32B 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 (41). However, this SIM-like domain is not present in all of the ANP32B proteins from different hosts, nor 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 chANP32B are responsible for the loss of this function. Sequence analysis and

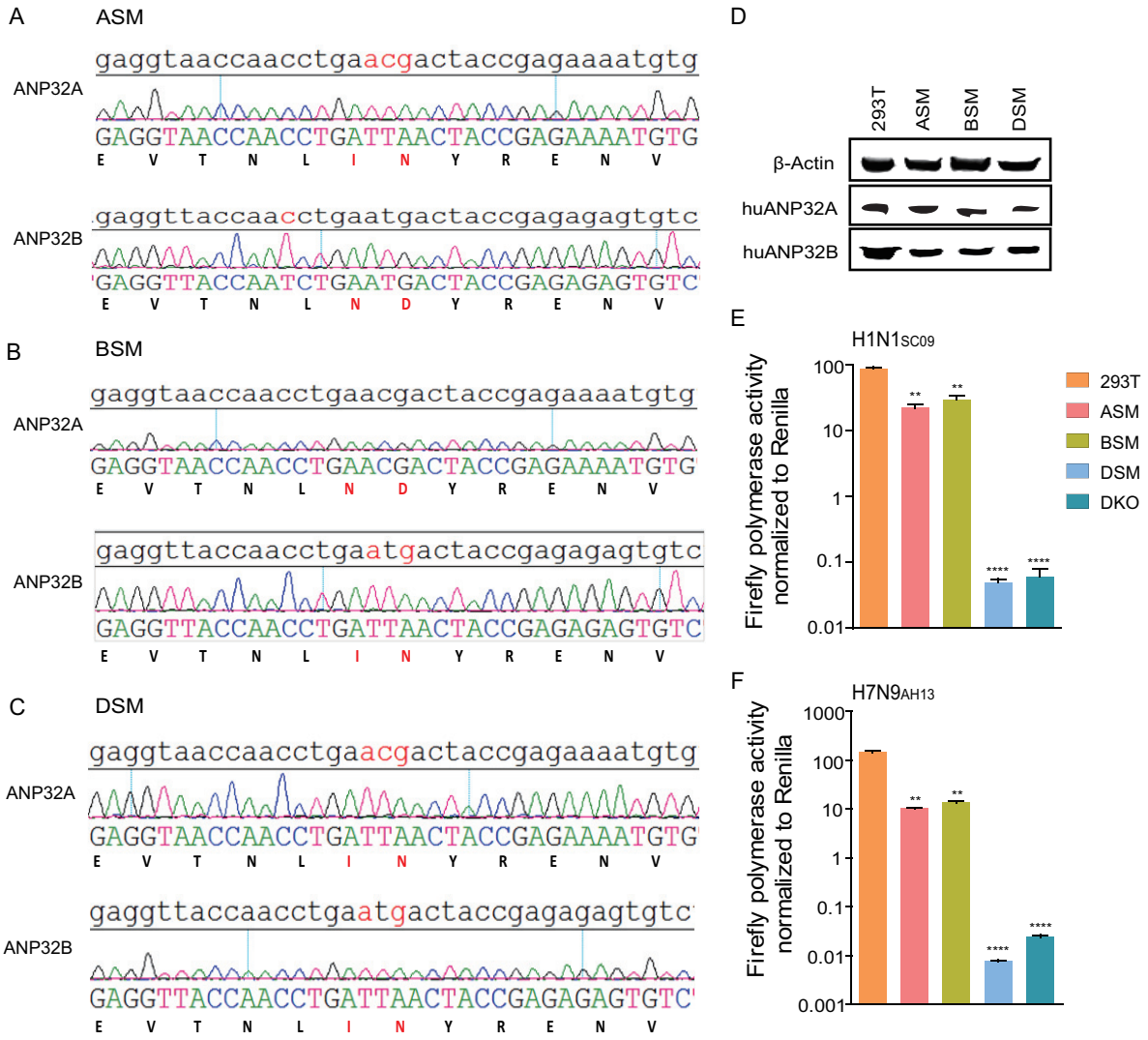

FIG 7 Amino acid N129I/D130N substitutions into ANP32A&B impaired influenza polymerase activity in 293T cells. N129I/D130N substitutions of huANP32A and ANP32B on 293T chromosome were generated by the CRISPR/Cas9 system. Positive colonies were identified by sequencing and Western blotting. (A) ANP32A stable mutated cells (ASM) were identified carrying the N129I/D130N substitutions on ANP32A but not on ANP32B. (B) ANP32B stable mutated cells (BSM) were identified harboring the N129I/D130N substitutions on ANP32B but not on ANP32A. (C) Double stable mutated cells (DSM) had N129I/D130N substitutions on both ANP32A&B. (D) The endogenous protein expressions of different cell lines were identified by Western blotting with antibodies against β-actin, huANP32A, and huANP32B. (E and F) Selected 293T cell lines were transfected with firefly minigenome reporter, *Renilla* expression control, and polymerase from $H1N1_{SC09}$ (E) or $H7N9_{AH13}$ (F). Cells were assayed for luciferase activity. The data indicate the firefly luciferase gene activity normalized to that of the *Renilla* luciferase activity. Statistical differences between cells are given, following a one-way ANOVA and subsequent Dunnett's test (NS, not significant; **, $P < 0.01$; ****, $P < 0.0001$). Error bars represent the SEM of the replicates within a representative experiment.

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. 6). The coimmunoprecipitation assay showed that mutations on these 129-130 sites changed the interaction efficiency between the ANP32 proteins and viral polymerase complex (Fig. 8). 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 coevolution 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. Currently, the vaccine is the best way to control avian influenza virus, such

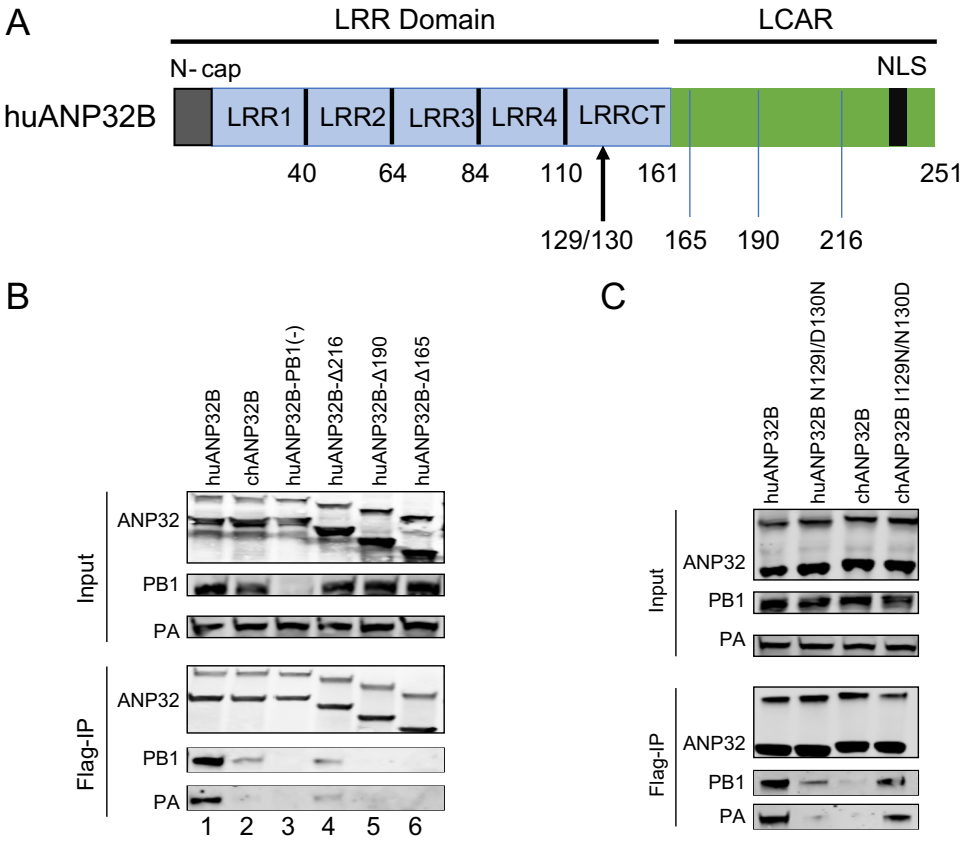

FIG 8 The 129-130 site in ANP32B contributes to the interaction with influenza polymerase. (A) Schematic showing the structure of ANP32B. LRR, leucine-rich repeat, including four LRR repeats and one C-terminal LRR domain (LRRCT); LCAR, low-complexity acidic region; NLS, nuclear localization signal. (B and C) 293T cells were transfected with different ANP32-Flag constructs, together with the viral polymerase subunits PA, PB1, and PB2. The coimmunoprecipitation of the anti-Flag antibodies and the proteins was assessed by Western blotting.

as H7N9 virus (55, 56). However, the 129-130 substitution of chANP32A could be used in the future as a novel target to develop transgenic chickens that may be totally resistant to influenza virus infection.

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 genomes 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, the structure of the ANP32 and vRNP complex, and the fitness of different virus subtypes, for example, would contribute to our understanding of 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 modified Eagle medium (DMEM; HyClone) with 10% fetal bovine serum (FBS; Sigma), and 1% penicillin and streptomycin (Gibco) and kept at 37°C in 5% CO_2. Certain reagents were kindly provided by the indicated individuals: polymerase plasmids of H1N1 human influenza A virus A/Sichuan/01/2009 ($H1N1_{SC09}$) and H7N9 A/Anhui/01/2013 ($H7N9_{AH13}$) were provided by Hualan Chen; H1N1 human influenza virus A/WSN/1933 (WSN) was provided by Yoshihiro Kawoaka; H3N2 canine influenza virus A/canine/Guangdong/1/2011 ($H3N2_{GD12}$) was provided by Shoujun Li from China Southern Agriculture University; and H9N2 avian influenza virus A/chicken/Zhejiang/B2013/2012 ($H9N2_{ZJ12}$) was provided by 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 Hualan Chen's lab. The pCAGGS plasmids 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-Δ216/190/165 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 then fused using In-Fusion HD enzyme (Clontech, Felicia, CA). To create the pcAGGS-huANP32B-Δ216/190/165 plasmids, pcAGGS-huANP32B was used as a template to amplify the pcAGGS vector. This sequence was then fused with different truncated huANP32B fragments (huANP32B-Δ216/190/165). 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) (3, 4, 29), the gRNA design tool (http://crispr.mit.edu/) was used for gRNAs design and off-target prediction (57). 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) (58). Briefly, 293T cells in 6-well plates were transfected with 1.0 μg of pMJ920 plasmids and 1.0 μg of gRNA expression plasmids by Lipofectamine 2000 transfection reagent (Invitrogen, catalog no. 11668-027) using the recommended protocols. Green fluorescent protein (GFP)-positive cells were sorting by fluorescence-activated cell sorting (FACS) at 48 h posttransfection, and then monoclonal knockout cell lines were screened by Western blotting and/or DNA sequencing.

Generation of a site-directed, amino-acid-substituted 293T cell line. High-efficiency guide sequences for ANP32A and ANP32B that 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 80-nucleotide oligonucleotide with the desired mutations at the target site was used as the donor DNA. 293T cells were transfected with 1 μg of pMJ920 plasmids, 1 μg of gRNA expression plasmids, and 50 pmol of donor DNA. After 48 h, GFP-positive cells were isolated by FACS, and site-directed mutagenesis clones were identified after screening by sequencing and Western blotting with 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 noncoding regions of the influenza hemagglutinin gene segment with a human polymerase I promoter and a mouse terminator sequence (59) 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 host proteins on viral polymerase activity, 293T or different host protein knockout 293T 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 of minigenome reporter and 10 ng of *Renilla* luciferase expression plasmids (pRL-TK, kindly provided by J. Luban), using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated at 37°C. The cells were lysed with 100 μl of passive lysis buffer (Promega) at 24 h after transfection, and the firefly and *Renilla* luciferase activities were measured using a dual-luciferase kit (Promega) with a Centro XS LB 960 luminometer (Berthold Technologies). The function of ANP32 was examined using a polymerase assay by cotransfection of DKO cells with different ANP32 proteins for 24 h. All of the experiments were performed independently at least three times. Results represent means ± the standard errors of the mean (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 the NP and PB1 proteins and anti-V5 tag antibody (ab27671) for the PA-V5 protein.

RNA isolation, reverse transcription, and quantification by RT-PCR. Total RNA from 293T cells was extracted using an RNeasy minikit (Qiagen) according to 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 subjected to cDNA synthesis using a reverse transcription (RT) kit (PrimeScript RT reagent kit with a gDNA Eraser [Perfect Real Time], catalog no. RR047A). Primers used in the RT reaction were as follows: 5′-CATTTCGCAGCCTACCGTGGTGTT-3′ for the firefly luciferase vRNA, 5′-AGTA GAAACAAGGGTG-3′ for the firefly luciferase cRNA, and oligo-dT20 for the firefly luciferase mRNA (60). The cDNA samples were subjected to quantification by real-time PCR with the specific primers F (5′-GATTACCAGGGATTTCAGTCG-3′) and R (5′-GACACCTTTAGGCAGACCAG-3′) using SYBR Premix Ex Taq II (Tli RNase H Plus; TaKaRa catalog no. RR820A). The fold change in RNA was calculated by double-standard curve methods, and β-actin served as an internal control.

Quantitative ELISA for determination of virus production. The ELISA has been previously described (49). Briefly, a 96-well microtiter plate (Costar, Bodenheim, Germany) was coated with 1 μg/well

of the mouse monoclonal anti-IAV NP protein antibody in phosphate-buffered saline (PBS), incubated overnight at 4 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 μl of 5% calf serum at 37°C for 2 h. After three washes with PBST buffer, 100-μl portions of virus or virus-like particle (VPL) samples were added in dilution buffer (PBS containing 10% calf serum and 0.1% Triton X-100), followed by incubation at 37°C for 1 h. The plate was then washed, and 100 μl of a 1:2,000 dilution of horseradish peroxidase-conjugated anti-IAV NP protein monoclonal antibody was added. After incubation at 37°C for 0.5 h, the plate was washed again, followed by incubation with freshly prepared TMB peroxidase substrate (Galaxy Bio, Beijing, China) for 10 min at room temperature. The reaction was stopped by adding 2 M H_2SO_4, and the optical density at 450 nm was measured using a VersaMax microplate reader (BioTek, Winooski, VT). 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 (61). Briefly, a 293T culture in a 6-well plate was transfected with 0.1 μg each of pPolI plasmids and 1 μg each of pCAGGS-NP, pCAGGS-PA, pCAGGS-PB1, and pCAGGS-PB2 using Lipofectamine 2000 (Invitrogen) in Opti-MEM. At 8 h posttransfection, the 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 hemagglutination assay, and the titers of virus on 293T cells were determined using the method of Reed and Muench (62). The 293T cells, huANP32A knockout 293T cells (AKO cells), huANP32B knockout 293T cells (BKO cells), and huANP32A&B double-knockout 293T cells (DKO) were infected at a multiplicity of infection (MOI) of 0.01 for 1 h, washed twice with PBS, and cultured at 37°C in Opti-MEM containing 0.5% FBS and tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma) at 1 mg/ml. At the indicated time points, the culture supernatant was harvested, and the virus 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, 1 mM dithiothreitol) and centrifuged at 13,000 $\times$ *g* and 4°C for 10 min. After centrifugation, the crude lysates were incubated with anti-FLAG M2 magnetic beads (Sigma-Aldrich, catalog no. M8823) or anti-NP magnetic beads (MCE protein A/G magnetic beads and NP antibody from our lab) at 4°C for 2 h. After incubation, the resins were collected using a magnetic separator and washed three times with PBS. The resin-bound materials were eluted using a 3$\times$ 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 [catalog no. F1804], anti-V5 antibody from Abcam [catalog no. ab27671], and anti-NP and PB1 antibody from our lab) was performed for 2 h at room temperature, followed by three washes with TBST. The secondary antibody (Sigma, 1:10,000) was then applied, and the samples were incubated at room temperature for 1 h. Subsequently, the membranes were washed three times for 10 min with TBST. Signals were detected using an Odyssey imaging system (LI-COR, Lincoln, NE).

Statistics. Statistical analysis was performed in GraphPad Prism (v5; GraphPad Software). Statistical differences between groups were assessed by one-way analysis of variance (ANOVA), followed by a Dunnett's post test. All the experiments were performed independently at least three times. Error bars represent standard deviations (SD) or standard errors of the mean (SEM) in each group, as indicated in the figure legends (NS, not significant; $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

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