Research Article

# Equine ANP32 proteins support influenza A virus RNA polymerase activity

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ABSTRACT

Host ANP32 family proteins are crucial for maintaining the activity of influenza RNA polymerase and play an important role in the cross-species transmission of influenza viruses. To date, the molecular properties of equine ANP32 (eqANP32) protein are poorly understood, particularly the mechanisms that affect equine influenza virus (EIV) RNA polymerase activity. Here, we found that there are six alternative splicing variants of equine ANP32A (eqANP32A) with different levels of expression. Further studies showed that these six splicing variants of eqANP32A supported the activity of EIV RNA polymerase to varying degrees, with the variant eqANP32A_X2 having the highest expression abundance and exhibiting the highest support of polymerase activity. Sequence analysis demonstrated that the differences in the N-Cap regions of the six splicing variants significantly affected their N-terminal conformation, but did not affect their ability to bind RNA polymerase. We also demonstrated that there is only one transcript of eqANP32B, and that this transcript showed only very low support to the EIV RNA polymerase. This functional defect in eqANP32B is caused by the sequence of the 110–259 amino acids at its C-terminus. Our results indicated that it is the eqANP32A_X2 protein that mainly determines the efficiency of the EIV replication in horses. In conclusion, our study parsed the molecular properties of eqANP32 family proteins and revealed the sequence features of eqANP32A and eqANP32B, suggesting for the first time that the N-Cap region of ANP32A protein also plays an important role in supporting the activity of the influenza virus polymerase.

## 1. Introduction

Influenza A viruses (IAVs) belong to the *Orthomyxoviridae* family and are common zoonotic pathogens responsible for global and seasonal epidemics in humans and other animals. Due to their rapid mutation rate and diverse subtypes, IAVs pose a significant public health threat. Wild birds are generally considered to be the natural hosts of IAVs (Spackman, 2008). Avian influenza viruses (AIVs) have the potential to transmit to mammals and cause pandemics, posing a threat to animal and human health (Brody, 2019). Horses, along with humans and pigs, have long been known to be susceptible to IAVs (Fatima et al., 2019). Equine influenza (EI) is caused by the equine influenza virus (EIV), and is characterized by acute outbreaks of illness and rapid transmission (Chambers, 2022) and is listed as a notifiable animal disease (category B) by the World Organization for Animal Health (WOAH). Horses infected

with EIV develop typical respiratory disorders, with the most common clinical signs being fever, lethargy, anorexia, nasal discharge, and a nonproductive dry cough. EIVs can be spread through the air and via direct contact. With the rapid development of the global horse racing, rodeo, and equestrian sports industries, horse health has received increasing attention. Despite the availability of vaccines and the current strongly controlled international surveillance, EI outbreaks occur persistently worldwide, causing huge economic losses to the global equine industry and posing a serious threat to the stable development of global commerce and economy (Timoney, 1996; van Maanen and Cullinane, 2002; Yamanaka et al., 2008). The agents responsible for EIV are, to date, thought to be limited to the subtypes H7N7 (which has not been isolated since 1980 and seems to have disappeared from horse herds worldwide) (Webster, 1993; Madić et al., 1996) and H3N8 (which is found worldwide, except for Iceland and New Zealand) (Laabassi et al.,

2015; Rash et al., 2017; Brister et al., 2019; Dilai et al., 2021). An outbreak of H3N8 EIV was reported in Colorado, USA, on April 23, 2022, resulting in the deaths of 102 wild horses from the West Douglas region of Utah. About 40%–60% of the infected horses had a runny nose, cough, fever, and other clinical symptoms (Huang, 2022). However, the mechanism underlying EIV pathogenicity is still a seriously neglected research field, and therefore there is an urgent need to study these mechanisms in horses to provide a better strategy for the control of EI.

The influenza virus RNA-dependent RNA polymerase (RdRp) comprises three polypeptides [polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA)]. RdRp is able to co-opt host factors, which are essential to viral transcription and replication. Host acidic nuclear phosphoprotein 32 (ANP32) proteins have been found to be broadly associated with influenza virus infection (Shapira et al., 2009; Long et al., 2016; Yu et al., 2022). The ANP32 family includes three conserved family members: ANP32A, ANP32B, and ANP32E. A large number of studies have reported that ANP32A and ANP32B proteins interact with the influenza RNA polymerase complex and are able to support its activity (Bradel-Tretheway et al., 2011; Sugiyama et al., 2015). Research from our lab demonstrated that ANP32A and ANP32B are key host molecules for the function of IAV RNA polymerase in different species (Zhang H. et al., 2019). Previous studies have shown that the RNA polymerase activity of various subtypes of IAV decreases more than 10000-fold in ANP32 A/B double knockout 293T cells (DKO). ANP32E barely has any effect on IAV RNA polymerase activity, although it shows weak support for the influenza B virus (Zhang Z. et al., 2020). Moreover, avian ANP32 (avANP32) is known to specifically support AIV replication (Long et al., 2016). Further studies verified that an additional 33 amino acid insert in avANP32A has a potential SUMOylation modification that facilitates the binding of avANP32A to the viral RNA polymerase complex. This interaction further promotes avian RNA polymerase activity (Domingues and Hale, 2017; Carrique et al., 2020). Meanwhile, swine ANP32A (swANP32A) is a key host factor that specifically supports the replication of AIV in pigs (Peacock et al., 2020; Zhang H. et al., 2020).

ANP32 proteins play a significant role in supporting influenza virus replication and cross-transmission. However, the molecular characteristics of equine ANP32 (eqANP32) proteins and their effect on the activity of EIV RNA polymerase remain unknown. Here, we identified the molecular characteristics of eqANP32 and evaluated its ability to support the RNA polymerase activity of EIV. This research will contribute to a better understanding of EIV pathogenesis and its adaptation within host cells.

## 2. Materials and methods

### 2.1. Cells and tissues culture

Human embryonic kidney (HEK) 293T cells and human ANP32A and ANP32B double-knockout 293T (DKO cells, described previously) cell line (Zhang H. et al., 2019) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% fetal bovine serum, 1% penicillin, and streptomycin, and kept at 37 °C with 5% $CO_2$. The frozen tissue samples, including lung, liver, spleen, kidney, funicle, and placenta, obtained from aborted fetuses or adult horses, were all preserved in our laboratory (Lin et al., 2020; Wang et al., 2023).

### 2.2. Viruses

Plasmids carrying the polymerase from influenza A viruses from different species were used in this study, and included those from human influenza virus A/human/Anhui/01/2013 (H7N9$_{AH13}$, kindly provided by Dr. Hualan Chen); H1N1 swine influenza virus A/swine/North Carolina/3793/08 (H1N1$_{NC08}$, kindly provided by Dr Feng Li from South Dakota State University); H3N2 canine influenza virus A/canine/Guangdong/1/2011 (H3N2$_{GD12}$, kindly provided by Dr Shoujun Li); H9N2 avian influenza virus A/chicken/Zhejiang/B2013/2012

(H9N2$_{ZJ12}$, kindly provided by Dr Zejun Li); and H3N8 equine influenza virus A/equine/Xinjiang/1/2007 (H3N8$_{XJ07}$), which was preserved in our lab.

### 2.3. Plasmids

Plasmids encoding ANP32A isoforms of several species were preserved in our lab. These ANP32A isoforms included chicken ANP32A (chANP32A), swine ANP32A (swANP32A), human ANP32A (huANP32A), canine ANP32A (caANP32A), and human ANP32B (huANP32B). The other pCAGGS plasmids, which contained differential splicing variants of ANP32A and full length eqANP32B isoforms, were generated using PCR amplification according to the sequences deposited in GenBank, and fusing the FLAG-tag to the C-terminal by homologous recombination PCR, including equine ANP32A_X1 (eqANP32A_X1, XM_023654809.1, XP_023510577.1), equine ANP32A_X2 (eqANP32A_X2, XM_001495810.6, XP_001495860.2), equine ANP32A_X3 (eqANP32A_X3, XM_023654824.1, XP_023510592.1), equine ANP32A_X4 (eqANP32A_X4, XM_005602971.3, XP_005603028.1), equine ANP32A_X5 (eqANP32A_X5, XM_023654839.1, XP_023510607.1), equine ANP32A_X6 (eqANP32A_X6, XM_005602970.3, XP_005603027.1), equine ANP32B (eqANP32B, XM_023629723.1, XP_023485491.1) and truncated interchange mutants of eqANP32B and huANP32B (pcAGGS-eqANP32B110/huANP32B-Flag, pcAGGS-huANP32B110/eqANP32B-Flag) (Supplementary Table S1). All 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).

### 2.4. Polymerase activity assay

The eukaryotic expression plasmids of different species of influenza A virus RNA polymerase reporter system were constructed with pCAGGS vector as the skeleton. The firefly luciferase gene reporter plasmid pEZ-vLuc containing human PolI promoter was constructed and preserved in our laboratory. The pRL-TK plasmid (kindly provided by Dr. Jeremy Luban from the University of Massachusetts Medical School) containing the *Renilla* luciferase gene was used as the internal reference gene of the polymerase double fluorescence reporting system. Viral RNA polymerase, NP expression plasmids, pEZ-vLuc and pRL-TK were co-transfected to analyze the polymerase activity (Zhang H. et al., 2019; Zhang H. et al., 2020). To determine the effect of ANP32 proteins on viral RNA polymerase activity, DKO cells in 24-well plates were transfected with plasmids of different ANP32 proteins (20 ng), the PB1 (40 ng), PB2 (40 ng), PA (20 ng), and NP (80 ng), together with 40 ng minigenome reporter and 1 ng pRL-TK plasmids using polyethyleneimine (PEI) transfection reagent (a cationic polymer, prepared by our lab). Cells were lysed with 50 μL of passive lysis buffer (Promega) 20 h after transfection, and firefly and *Renilla* luciferase activities were measured using a Dual-luciferase kit (Promega) with a Centro XS LB 960 luminometer (Berthold Technologies). All the experiments were performed at least three times independently. Results represent the mean ± SEM of the replicates within one representative experiment. The expression levels of polymerase proteins on different cell lines were detected with western blotting, using mouse monoclonal antibodies specific for PB2 and NP proteins.

### 2.5. Immunoprecipitation

Cell lysates were prepared with lysis buffer (50 mmol/L Hepes-NaOH [pH 7.9], 100 mmol/L NaCl, 50 mmol/L KCl, 0.25% NP-40, and 1 mmol/L DTT) after transfection for 36 h, centrifuged at 12,000×$g$ and 4 °C for 10 min. After centrifugation, 1% of each cell lysate was saved as an input control, and the remaining supernatants were incubated with Anti-FLAG M2 Magnetic Beads (SIGMA-ALDRICH, M8823), at 4 °C for 12 h. Subsequently, the magnetic beads were collected using a magnetic bead

separator and were washed 5 times with phosphate-buffered saline (PBS), 80 μL of 3 × Flag peptide was added to each sample. The samples were then eluted at 4 °C for 30 min and then boiled in the 1 × sample loading buffer at 98 °C for 10 min. After obtaining the immunoprecipitation samples, western blotting analyses were performed using standard methods.

## 2.6. Western blotting

For determining protein expression levels, cell lines with different treatments were lysed using a RIPA Lysis Buffer (Solarbio, Beijing, China), and centrifuged at 13,000 rpm and 4 °C for 10 min. Samples were taken from each supernatant, separated on 4%–12% gels using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk powder in Tris-buffered saline (TBS) for 2 h. Incubation with the first antibody (Anti-Flag antibody from SIGMA (F1804), Anti-ANP32B from Abcam (ab200836), and with Anti-PB1, Anti-NP, Anti-PA, and Anti-PB2 (from our lab) were each performed for 12 h at 4 °C, followed by washing three times with TBST. The secondary antibody (Sigma, 1:20,000) was added, and membranes were incubated at room temperature for 1 h. Subsequently, membranes were washed three times for 10 min with TBST. Signals were detected using an LI-COR Odyssey Imaging System (LI-COR, Lincoln, NE, USA).

## 2.7. RNA isolation, reverse transcription, and quantification by RT-PCR

According to the manufacturer's instructions, total RNA from frozen horse tissues samples including lung, liver, spleen, kidney, funicle, and placenta was extracted using the RNeasy mini kit (Qiagen). A reverse transcription kit (Cat. RR047A, Takala, China) was used for reverse transcription. A 20 μL transcription reaction system was then set up with random primers and reverse transcriptase to synthesize cDNA, and 3 μg of RNA per sample was used for cDNA synthesis. The SYBR ® Premix Ex TaqTM II (Tli RNaseH Plus) (Cat: RR820A, TaKaRa, China) was used for real-time quantitative detection with the following specific primers: eqANP32A_X1 specific primers (5′-CTAAGATGCCAGCCTTTGAGC-3′ and 5′- TTTCTCGTGCCCATTTATT-3′), eqANP32A_X2 specific primers (5′-GGACGCCCTCTGATGTGAA-3′ and 5′-GTAAGTTTGCGACTGAGGTGAG-3′), eqANP32A_X3 specific primers (5′-AGGAGGGGGAAGGTGAGGGT-3′ and 5′-AGTTTGCGACTGAGGTGAG-3′), eqANP32A_X4 specific primers (5′-AGGAGGGGGAAGGTGAAAGA-3′ and 5′-CCCCTGA-GATTCTGTTATCG-3′), eqANP32A_X5 specific primers (5′-ACAG-CAAACTGAAATGCCC-3′ and 5′- TCAAATTCATCTGTGAGGC-3′), eqANP32A_X6 specific primers (5′-AGCCTTGATATCTGATATG-3′ and 5′-ATTCTGTTATCGCTTAGTTC-3′), eqANP32B specific primers (5′-TTTCCGTTTCAAATCTCCC-3′ and 5′-AAAGGTTCCAAGGTGCTGA-3′) and GAPDH specific primers (5′- GGTCACCAGGGCTGCTTTTAACTC-3′ and 5′-TCGCTCCTGGAAGATGGTGATGGC-3′). The comparative Ct method was used to determine the relative mRNA expression of genes normalized by the GAPDH.

## 2.8. Amino acid sequence alignment

The amino acid sequence alignment was performed using DNAMAN (DNAMAN Software, USA), and Geneious Prime (Geneious Prime Software, USA).

## 2.9. Structure prediction

The structures of differential splicing variants of equine ANP32A were predicted using the online Phyre2 Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), which predicted them with >90% confidence. Structures were subsequently analyzed using the Swiss-PBD Viewer 4.10 software (Swiss Institute of Bioinformatics, Basel, Switzerland).

## 2.10. Statistical analysis

Statistical analysis was performed in GraphPad Prism, version 7 (Graph Pad Software, USA). Statistical differences between groups were assessed using an unpaired Student's *t*-test, two-way analysis of variance (ANOVA) or 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. For Western blot assay, the relative band intensities were analyzed using the software ImageJ. NS, not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

## 3. Results

### 3.1. Gene expression patterns and tissue distribution of eqANP32A and eqANP32B in host tissues

The ANP32 family comprises ANP32A, ANP32B, ANP32C, ANP32D, and ANP32E. Among these, horses are likely to possess three conserved ANP32 family members, namely ANP32A, ANP32B, and ANP32E. These three equine ANP32s have been identified at different loci in the GenBank database. Previous studies have demonstrated that ANP32A and ANP32B proteins are crucial in supporting the polymerase activity of IAVs (Staller et al., 2019; Zhang H. et al., 2019), while ANP32E does not support the activity of IAV RNA polymerase (Zhang Z. et al., 2020). In this study, we analyzed the isoforms of eqANP32A and eqANP32B. According to the predicted gene sequences of eqANP32 family provided by the NCBI database, the eqANP32A gene is located on chromosome 1, contains seven exons, and has six predicted transcripts. The eqANP32B gene is located on chromosome 25, contains seven exons, and has one predicted transcript (Fig. 1A). In order to verify whether the predicted eqANP32 A/B genes can be transcribed to mRNA, the mRNAs of eqANP32A and eqANP32B were quantified in different equine tissues using qRT-PCR. The results demonstrated that the mRNA of the six splice variants of eqANP32A and eqANP32B were expressed similarly in different equine tissues, with the mRNA abundance of the *eqANP32A_X2* gene being the highest (Fig. 1B–G). The six splicing variants of eqANP32A and eqANP32B were then amplified from equine lung tissue and were inserted into the pCAGGS vector. The expression of these eqANP32 proteins was assessed with Western blotting (Fig. 1H and I). These results for the first time demonstrated that six splice variants of eqANP32A and one transcript of eqANP32B exist, but that the mRNA abundance of eqANP32 family genes varies in different tissues.

### 3.2. The eqANP32A splicing variants differentially impact EIV RNA polymerase activity

We previously constructed a 293T knockout cell line, DKO cells, in which the huANP32A and huANP32B were both knocked out. We demonstrated that the activities of almost all of polymerases from different subtypes of influenza A virus were abolished in DKO cells (Zhang H. et al., 2019). To assess the impact of eqANP32A splicing variants and eqANP32B protein on EIV RNA polymerase activity, the plasmids carrying *ANP32* genes were co-transfected with an H3N8 equine influenza virus A/equine/Xinjiang/1/2007(H3N8$_{XJ07}$) minigenome system into DKO cells. We found that the eqANP32 family proteins significantly (by approximately 100–1000 times) increased the activity of H3N8$_{XJ07}$ RNA polymerase (Fig. 2A). The six eqANP32A splice variants displayed obvious differences in their support of H3N8$_{XJ07}$ RNA polymerase activity. Of these variants, the eqANP32A_X2 variant showed the highest support to H3N8$_{XJ07}$ RNA polymerase activity. We further tested the ability of equine ANP32 proteins to support the activity of influenza virus RNA polymerases from different species. Our results showed that the six eqANP32A splice variants could significantly support the activities of RNA polymerase from several mammalian-derived
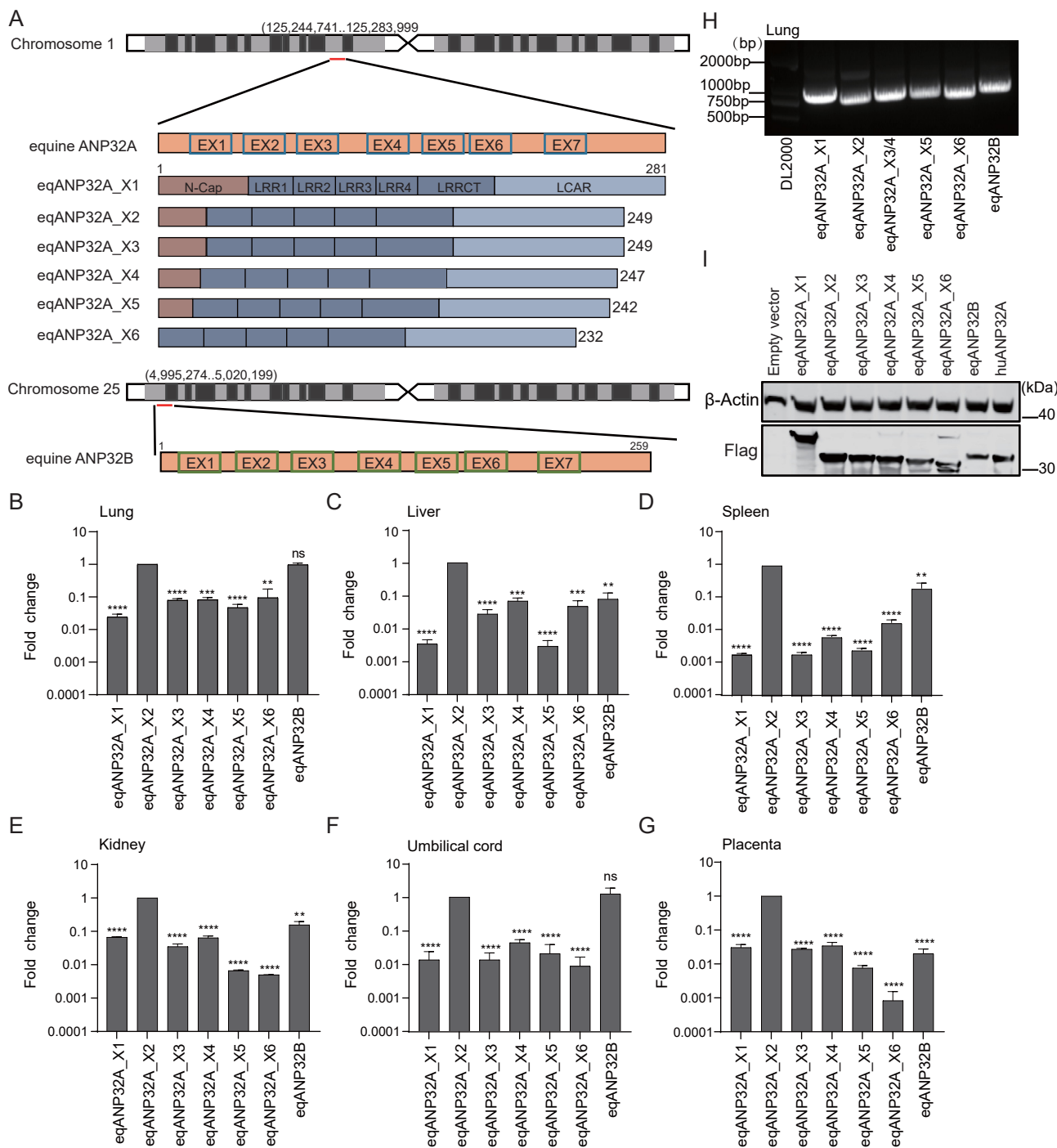
**Fig. 1.** Expression of eqANP32 family genes and their distribution in horse tissues. **A** Schematic diagram illustrating the genomic analysis of equine ANP32A and ANP32B within the chromosomes. **B**–**G** Expression of mRNA from eqANP32 family genes in different equine tissue samples, including lung (**B**), liver (**C**), spleen (**D**), kidney (**E**), funicle (**F**), and placenta (**G**). The comparative Ct method was used to determine the relative mRNA expression of genes normalized by the GAPDH. **H** Cloning of eqANP32 family genes in equine lung tissue. (**I**) Stable expression of 1 μg of each FLAG-tagged ANP32A in wild-type 293T cells. At 48 h post-transfection, cells were lysed in radio immunoprecipitation assay (RIPA) lysis buffer, and proteins were separated with 4%–12% SDS-PAGE, followed by immunoblotting with anti-β-actin antibody and anti-FLAG antibody to detect the FLAG-tagged ANP32 proteins. hu, human; eq, equine. Statistical differences between samples are indicated, according to a one-way ANOVA, followed by a Dunnett's test (ns, not significant; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Error bars represent the SEM within one representative experiment. The results represent at least three independent experiments.

influenza viruses, including H7N9$_{AH13}$, H3N2$_{GD11}$ and H1N1$_{NC08}$ (Fig. 2B–D), while none showed support for the RNA polymerase activity of AIV H9N2$_{ZJ12}$ (Fig. 2E). Moreover, the support activity of eqAN-P32A_X2 was significantly higher than other variants, and even slightly higher than huANP32A. The ability of eqANP32B to support influenza

virus RNA polymerases from different species was significantly lower than that of eqANP32A_X2. These data suggested that different variants of eqANP32A and eqANP32B have different levels of support for influenza virus RNA polymerase, with eqANP32A_X2 likely to contribute most to the EIV polymerase activity.
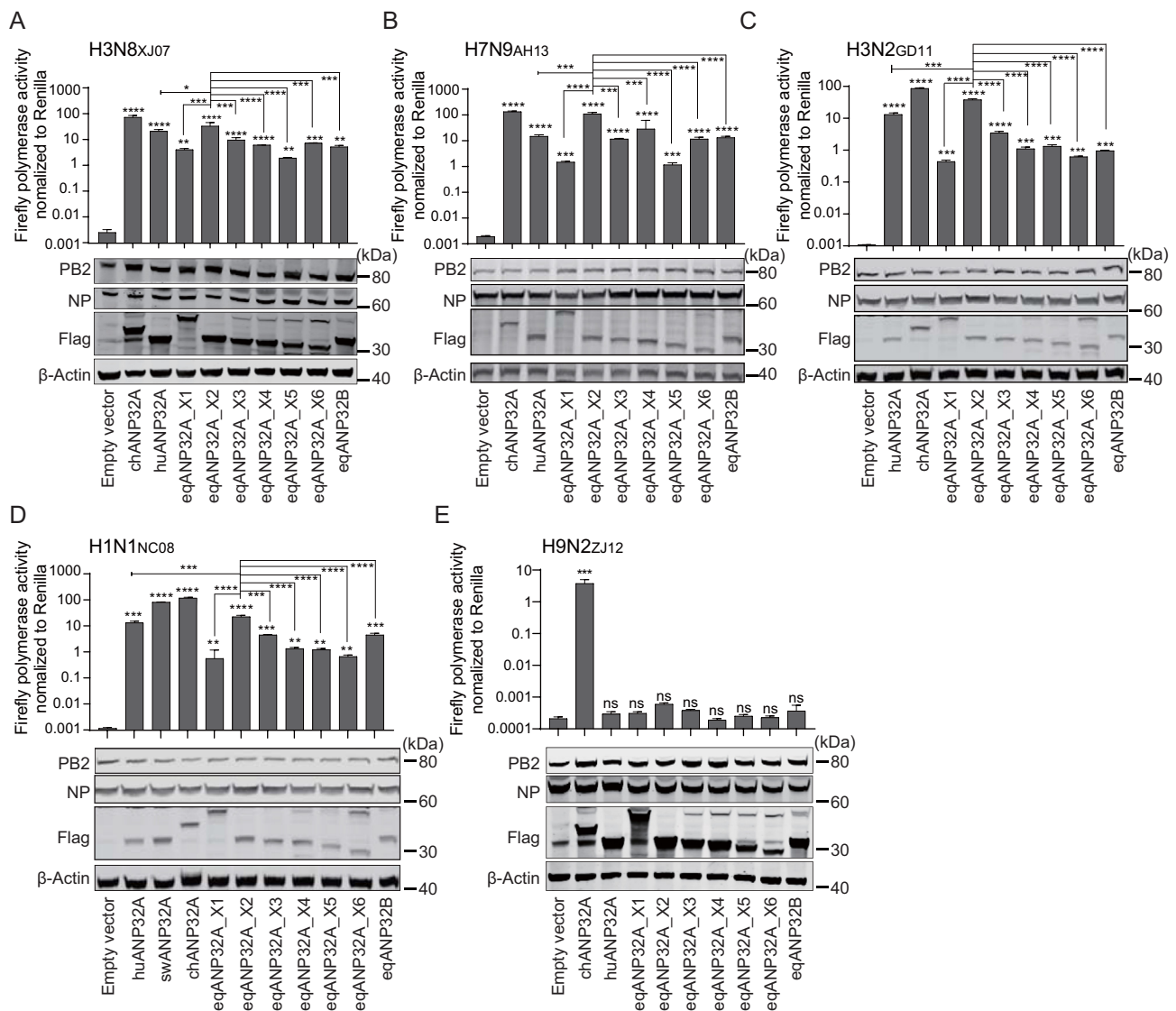
**Fig. 2.** Support of equine influenza viral replication by equine ANP32A or ANP32B, and species-dependent support of equine ANP32A or B for influenza A viral replication. Vectors carrying 20 ng of *ANP32A* or *ANP32B* genes, or empty vectors, were co-transfected into DKO cells (a 293T knockout cell line), together with a minigenome reporter, a *Renilla* expression control, and influenza virus polymerases from either equine influenza H3N8$_{XJ07}$ (**A**); human influenza H7N9$_{AH13}$ (**B**); canine influenza H3N2$_{GD11}$ (**C**); swine influenza H1N1$_{NC08}$ (**D**); avian influenza H9N2$_{ZJ12}$ (**E**). Luciferase activity was measured 20 h later, 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; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Error bars represent the SEM within one representative experiment. The results represent at least three independent experiments. Western blotting to detect the ANP32A and viral proteins used specific antibodies: PA antibody and PB2 antibody (from our lab), PB1 antibody (NBP2-42877, NOVUS), Anti-Flag antibody (F1804, SIGMA) and Anti-β-actin antibody (F1804, SIGMA). ch, chicken; sw, swine; hu, human; eq, equine.

### 3.3. The N-Cap region is a key domain for the ability of eqANP32A to support RNA polymerase activity

To illustrate the molecular mechanism by which eqANP32A variants can exhibit varying support activity for the influenza virus RNA polymerase, the sequences of the eqANP32A variants were aligned (Fig. 3A). The leucine-rich repeat domain (LRR) and C-terminal low-complexity acidic region (LCAR) of the six variants of eqANP32A were highly conserved and showed 100% homology between the variants. However, the N-Cap regions of the eqANP32A variants had differences in sequence length and amino acid residue sequence (Fig. 3A). The sequence of the N-Cap domain of eqANP32A_X1 was the longest, containing 49 amino acids. The N-Cap domain sequences of eqANP32A_X2 and eqANP32A_X3 were the same length, both containing 17 amino acids, while these two splicing variants showed different levels of support for RNA polymerase

activity. The differences in amino acid sequences between eqANP32A_X2 and eqANP32A_X3 may be the reason that eqANP32A_X2 has significantly higher support for influenza A virus RNA polymerase activity than does eqANP32A_X3. This conjecture is worth further exploration. The amino acid sequences of the N-Cap domains of eqANP32A_X3 and eqANP32A_X4 were exactly the same from aa 1 to aa 15. However, eqANP32A_X4 lacks two amino acids at the end of the sequence, resulting in the slightly weaker support of eqANP32A_X4 for RNA polymerase activity of influenza A virus. The N-Cap region of eqANP32A_X5 lacks 7 amino acids, and eqANP32A_X6 was the shortest, lacking a full N-Cap region. Both of these showed weaker support for the RNA polymerase activity of H3N8$_{XJ07}$ than did eqANP32A_X2 (Fig. 2A). Of these splicing variants, eqANP32A_X2 demonstrated the highest support for the RNA polymerase activity of H3N8$_{XJ07}$ and RNA polymerases of influenza viruses from other species (Fig. 2A–D).
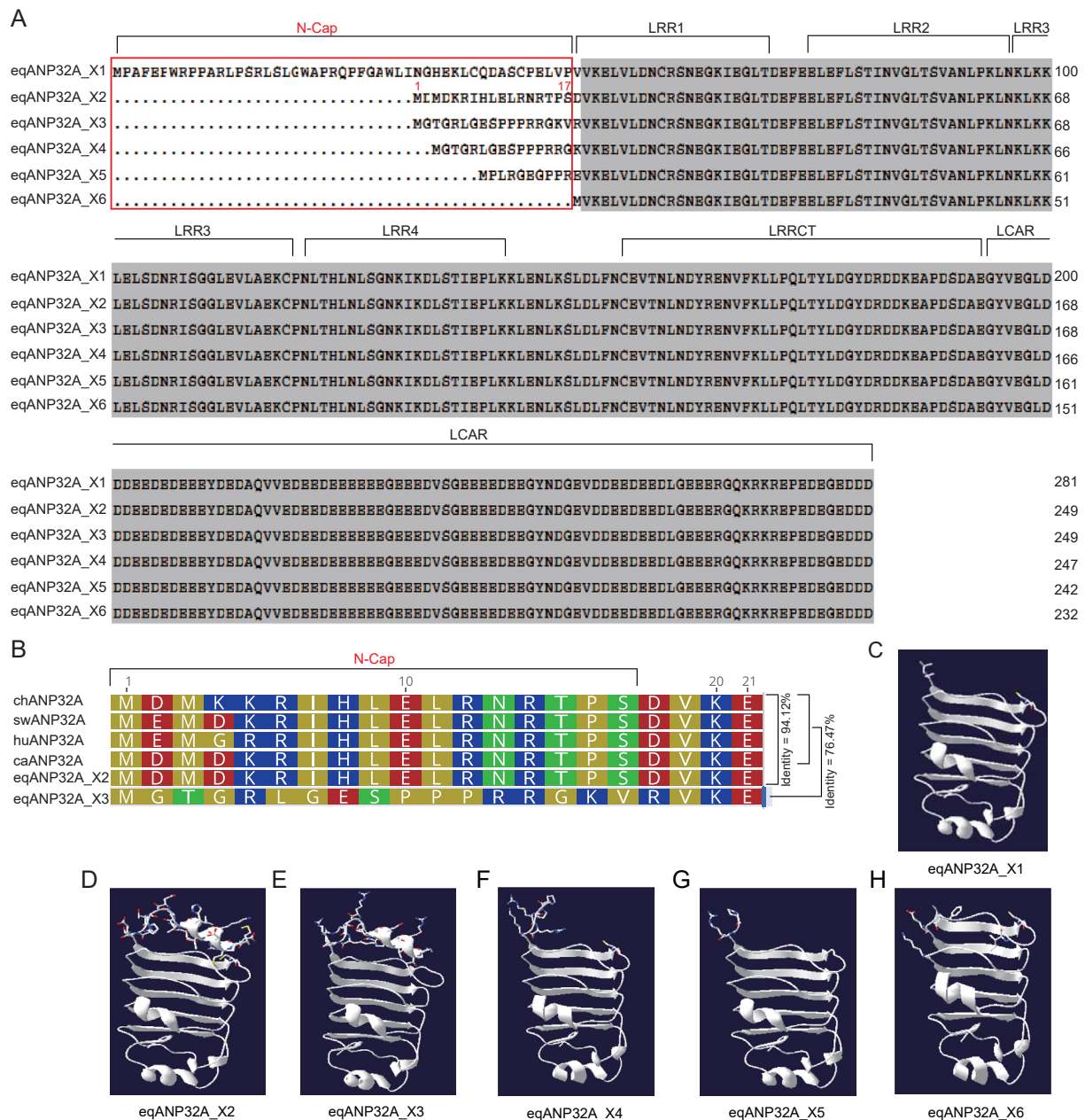
**Fig. 3.** Differences in the N-Cap region of eqANP32A lead to changes in the N-terminal conformation of eqANP32A proteins. **A** Amino acid sequences from differential splicing variants of equine ANP32A were aligned using the DNAMAN software. Gaps are marked with dashes. **B** The amino acid sequences of the N-Cap domain of eqANP32A and ANP32A proteins from other species were aligned in the Geneious Prime software. **C–H** Structure prediction of differential splicing of equine ANP32A (eqANP32A_X1, eqANP32A_X2, eqANP32A_X3, eqANP32A_X4, eqANP32A_X5 and eqANP32A_X6) using Phyre2. The analysis was performed using the Swiss-PBD Viewer software. ch, chicken; sw, swine; hu, human; eq, equine; ca, canine.

To further analyze the differences in the N-Cap domains of different eqANP32A variants, the N-Cap sequences of eqANP32A_X2, eqAN-P32A_X3, and ANP32A from other species were aligned. The N-Cap domain of eqANP32A_X2 shared 94.12% homology with ANP32A from other species, while eqANP32A X3 shared 76.47% homology (Fig. 3B). The N-Cap region of ANP32 protein helps to maintain the stability of its structure (Dao et al., 2015), but to date there has been no report regarding its impact on the RNA polymerase support function of ANP32. Based on their amino acid sequences, the structures of the six eqANP32A splicing variants were predicted using the online Phyre2 protein folding identification tool, and these were compared to the structures predicted by the Swiss-PBD Viewer software. As expected, the differences in the N-Cap region resulted in changes in the number and orientation of the N-terminal α-helices of the six splicing variants of

eqANP32A, which in turn led to changes in the N-terminal conformation (Fig. 3C–H).

Several studies have demonstrated that ANP32 proteins interact with the influenza RNA polymerase complex of PA/PB1/PB2 but not a certain protein subunit to regulate the activity of influenza RNA polymerase (Baker et al., 2018; Domingues et al., 2019; Zhang H. et al., 2019; Peacock et al., 2020; Zhang H. et al., 2020). To investigate the potential interactions between the eqANP32A splicing variants and the EIV RNA polymerase complex, the six splicing variants of eqANP32A and PB2, PB1, and PA of the H3N8$_{XJ07}$ RNA polymerase complex were co-transfected in DKO cells. Co-immunoprecipitation results showed that the six splicing variants of eqANP32A were able to specifically bind to the PB2, PB1 and PA subunits of the H3N8$_{XJ07}$ RNA polymerase (Fig. 4A) with no significant differences in their binding efficiency (Fig. 4B–C),
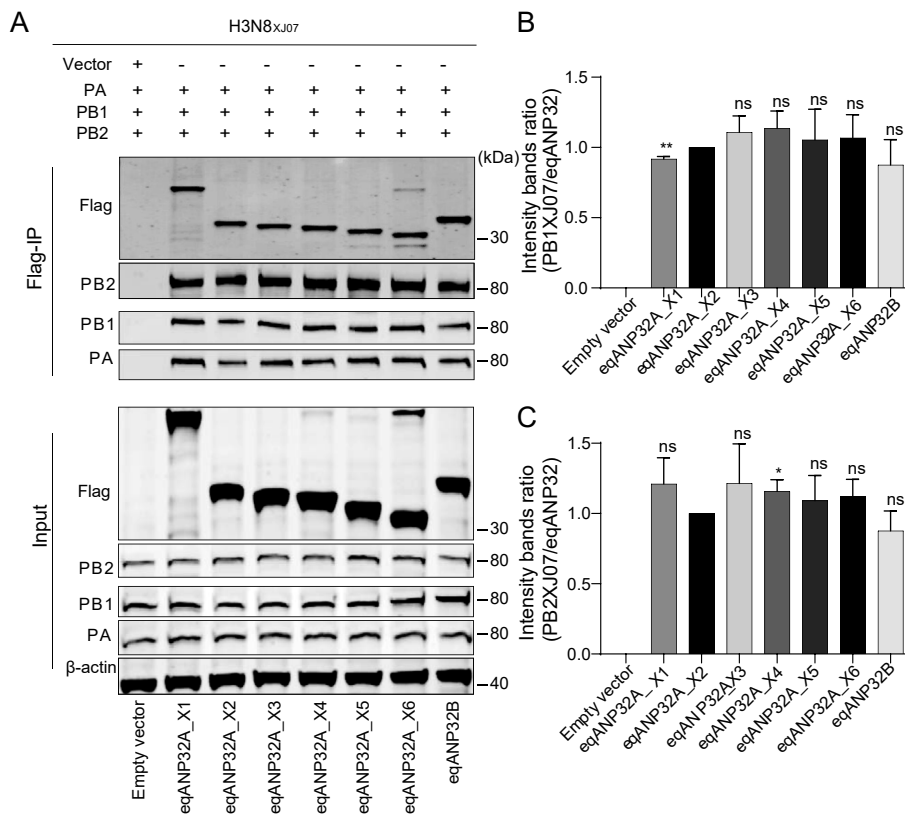
**Fig. 4.** The N-Cap domain does not affect the interaction of eqANP32A with the trimeric equine influenza virus polymerase complexes. **A** DKO cells (a 293T knockout cell line) were transfected with different ANP32A (0.6 μg) and polymerase plasmids (0.6 μg PA, 1 μg PB1, and 1 μg PB2) from equine influenza virus H3N8$_{XJ07}$. The cells were lysed at 36 h post-transfection. Co-IP was performed using anti-FLAG M2 magnetic beads, followed by Western blotting to detect the ANP32A and viral proteins using specific antibodies: PA antibody and PB2 antibody (from our lab), PB1 antibody (NBP2-42877, NOVUS), Anti-Flag antibody (F1804, SIGMA) and Anti-β-actin antibody (F1804, SIGMA). To compare the binding ability of eqANP32 with each of the polymerase protein, the intensity of each protein band was measured using the ImageJ software. The intensity bands ratio was the value of the intensity of each polymerase protein divided by that of eqANP32 (**B** and **C**). $P$ values were determined using one-way ANOVA followed by a Dunnett's multiple comparisons test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$. eq, equine.

indicating that the N-Cap region of the eqANP32A proteins did not affect their interactions with the polymerase. All these results suggested that the N-Cap domain of eqANP32A variants influences the support of these proteins for H3N8$_{XJ07}$ RNA polymerase activity, but does not influence their ability to bind RNA polymerase.

### 3.4. The C-terminal 111-259 aa of eqANP32B are responsible for its functional deficiency

As another important member of the conserved eqANP32 family, the influence of ANP32B on the activity of EIV RNA polymerase is also worth investigating. The support of eqANP32B protein for RNA polymerase of H3N8$_{XJ07}$ (Fig. 2A) or influenza viruses from other species was significantly lower than that of the eqANP32A_X2 variant (Fig. 2B–E). In order to further determine the function of eqANP32B, the activity of eqANP32B in support of polymerases from the equine influenza virus, the human influenza virus and the canine influenza virus was compared with that of ANP32B from other species. The results showed that eqANP32B exhibited significantly lower activity in supporting the activity of H3N8$_{XJ07}$ (Fig. 5A) and H3N2$_{GD11}$ (Fig. 5B) RNA polymerase compared to huANP32B or swANP32B. These results suggest that eqANP32B has lost its function to support EIV RNA polymerase activity to a certain extent.

Amino acid sequences alignment of eqANP32B and huANP32B revealed highly homology in the LRR and LRRCT regions, while the sequence differences between the two were mainly concentrated in the LCAR region (Fig. 6A). To determine the key domains responsible for the functional deficiencies of eqANP32B, chimeric clones between equine
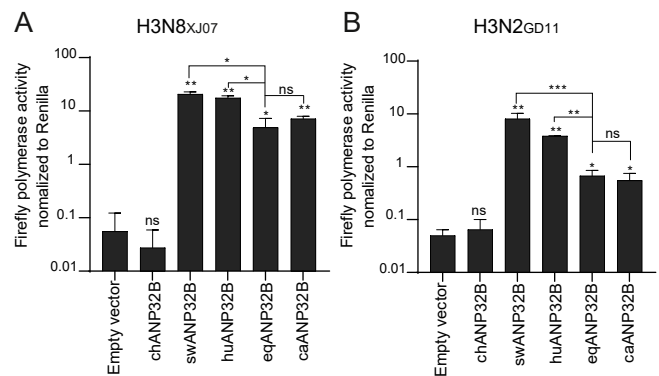


**Fig. 5.** Differences in ANP32B support the activity of viral polymerases from influenza viruses infecting different species. DKO cells were co-transfected with expression plasmids carrying PB1 (40 ng), PB2 (40 ng), PA (20 ng) and NP (80 ng) from equine influenza H3N8$_{XJ07}$ (**A**); canine influenza H3N2$_{GD11}$ (**B**), together with 40 ng minigenome reporter and 1 ng *Renilla* luciferase expression plasmids (pRL-TK, as an internal control) in the presence of chANP32B, swANP32B, eqANP32B, huANP32B or caANP32B protein or empty vector. Cells were then lysed using passive lysis buffer, and luciferase activity was measured at 20 h post transfection. Statistical differences between samples are indicated, according to a one-way ANOVA, followed by a Dunnett's test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Error bars represent the SEM within one representative experiment. The results represent at least three independent experiments. ch, chicken; sw, swine; hu, human; ca, canine.

and human ANP32B were constructed and evaluated (Fig. 6B). We then systematically validated the expression of the above mutants in vitro (Fig. 6C) and performed polymerase activity assays. We found that the replacement of amino acids 111 to 259 of eqANP32B with those of huANP32B aborted the activity of this huANP32B mutant, and in terms of polymerase activity, the activity of this chimera was similar to that of eqANP32B. Conversely, eqANP32B with amino acids 111 to 251 (fragment 111–251) from the human protein gained the ability to boost viral polymerase activity (Fig. 6D). The above data indicate that the key area responsible for the functional defect in eqANP32B is the 111-259 aa fragment.

## 4. Discussion

The influenza virus hijacks many host proteins to help its self-replication in host cells. Among these host factors, the ANP32 family proteins have proven to be the most important host factors in the replication and cross-transmission of influenza A and B viruses. Their functions have been found to depend on their interactions with the influenza virus RNA polymerase (Long et al., 2016; Domingues and Hale, 2017; Domingues et al., 2019; Long et al., 2019; Zhang H. et al., 2019; Zhang Z. et al., 2020). The study of the function of eqANP32 family proteins will help to elucidate the pathogenic mechanism of EIV and will provide potential new targets for anti-viral drugs. Here, we analyzed the sequence characteristics of the eqANP32 family proteins and assessed the expression of the six splicing variants of eqANP32A and eqANP32B (Fig. 1). Crucially, we analyzed the roles of the six splicing variants of eqANP32A and eqANP32B in supporting the RNA polymerase activity of influenza viruses from different species (Fig. 2). Of these, the splicing variant

eqANP32A_X2 has the highest specific supporting effect on the RNA polymerase activity of classic EIVs (Fig. 2).

Numerous studies have demonstrated that the molecular structural basis of ANP32 proteins in supporting influenza virus replication is strongly associated with the key functional domains of these proteins. chANP32A was the first host factor found to be related to the species-specificity of avian influenza virus polymerase (Long et al., 2016). Further research revealed that chANP32A contains 33 amino acid-specific insertion sequences between the LRR and LCAR regions, while mammalian ANP32A lacks this insertion. The 33 amino acid-specific insertion sequences are key functional domains and allow chANP32A to specifically support the activity of the AIV polymerase (Long et al., 2019). Researchers have also found a SUMO interaction base sequence (VLSLV) within this 33-aa insertion, which is necessary for the binding of chANP32A to PB2-627E (avian-origin IAV) and PB2–627K (mammalian-originated IAV) polymerase. Deletion or mutation of VLSLV significantly reduced the support activity of chANP32A towards avian influenza virus RNA polymerase (Baker et al., 2018). Subsequently, the 129/130 site was found to be a key motif in ANP32 proteins of all species to support viral polymerase activity. Both the 33 aa insertions and the 129 N/130D site are indispensable for the support activity of chANP32A towards AIV polymerase (Zhang H. et al., 2019). Researchers subsequently confirmed that Asp149 (D149) and Asp152 (D152) were key to the interaction between huANP32A and viral RNA polymerase (Park et al., 2021). Moreover, the tight binding of LCAR to the PB2-627 domain is essential to stabilize the interaction between ANP32A and influenza virus RNA polymerase (Carrique et al., 2020). Recently, LCAR has been found to be able to interact directly with NP and recruit NP to nascent RNAs during influenza virus genome replication (Wang et al., 2022).
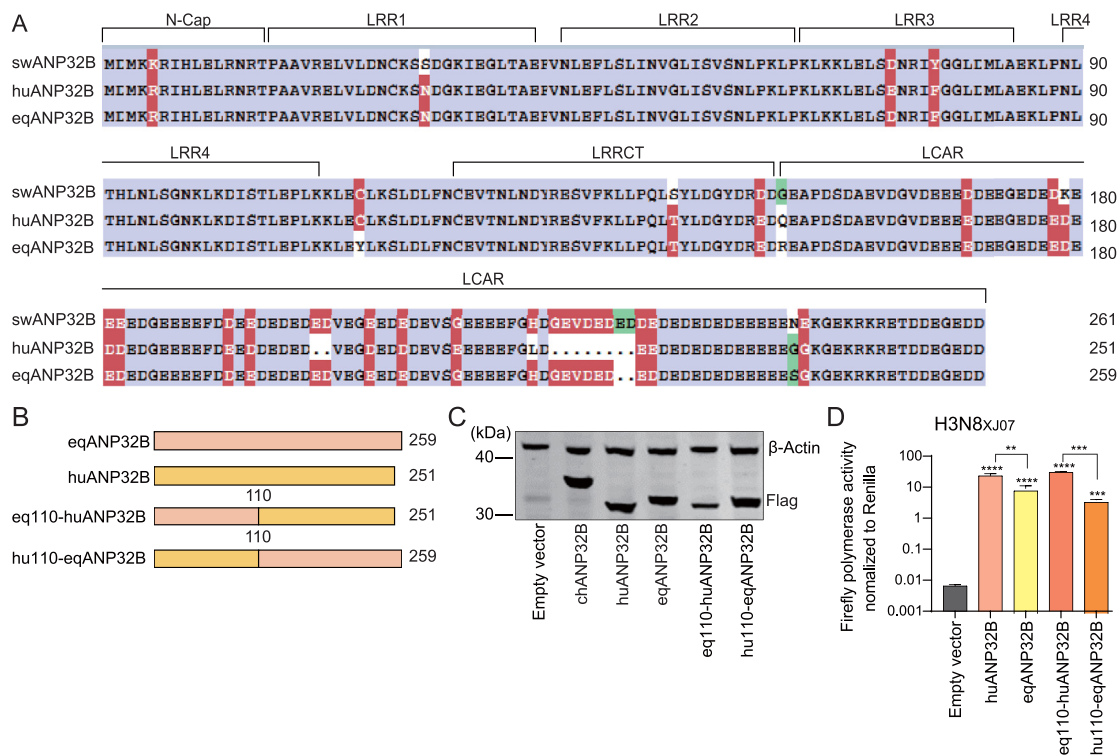


**Fig. 6.** The key region responsible for the functional deficiency of eqANP32B is 111–259aa. **(A)** Amino acid sequences from swine, eqANP32B and huANP32B were aligned using the DNAMAN software. Gaps are marked with dashes. **(B)** Schematic diagram of chimeric clones between equine and human ANP32B, constructed according to the known domains. The bars indicate the origins of the genes by color as follows: pink, eqANP32B; and orange, huANP32B. **(C)** Stable expression of 1 μg of FLAG-tagged eq110-huANP32B and hu110-eqANP32B in wild-type 293T cells. At 48 h post-transfection, cells were lysed and analyzed by Western blotting. **(D)** Plasmids (20 ng) carrying ANP32B or mutants, or empty vectors were co-transfected with plasmids carrying polymerase from H3N8$_{XJ07}$. Luciferase activity was assayed at 20 h after transfection. (Data are firefly activity normalized to *Renilla*. Statistical differences between cells are labeled according to a one-way ANOVA followed by a Dunnett's test; **, $P < 0.01$; ***$P < 0.001$, ****$P < 0.0001$). sw, swine; hu, human; eq, equine.

Preliminary research in our laboratory also found that the 106/156 site of swANP32A, which is located in the LRR domain and LCAR domain, is a key amino acid in the support of cross-species replication of AIV (Zhang H. et al., 2020). However, functional domain studies into the impact of ANP32 protein on influenza virus replication have mainly focused on the LRR or LCAR region of ANP32A, and little is known of the impact of the N-Cap region on influenza viruses. Here, we showed that the N-Cap region of eqANP32A also plays a critical role in supporting influenza viral RNA polymerase activity (Figs. 2 and 3). We found that the six splicing variants of eqANP32A have differences in sequence length and amino acid residues in their N-Cap regions. The eqANP32A with the longest N-Cap region did not have the highest support for EIV RNA polymerase activity. Conversely, the support for the EIV RNA polymerase activity of the eqANP32A lacking an N-Cap region was not the lowest. Of these splicing variants, eqANP32A_X2 had the highest ability to support the RNA polymerase activity, because its N-Cap domain shared the highest homology with ANP32A from other species. Our data demonstrate that the length and sequence of the amino acid residues in the N-Cap region of eqAN32A are key factors that affect its ability to support EIV RNA polymerase activity.

The typical structure of ANP32 proteins is divided into a LRR and a LCAR domain (Dao et al., 2015). The LRR domain of ANP32A protein consists of five β-sheet-containing repeats with highly similar structures. The N-Cap domain of ANP32 proteins is critical for maintaining the stability of the LRR structure (Dao et al., 2014). So far, only the 3D structure of the first 161 amino acids has been elucidated. Loïc Carrique and colleagues revealed the biological basis of host factor ANP32A-mediated influenza virus replication by analyzing the structure of the ANP32A protein and influenza virus polymerase complex with electron microscopy (Carrique et al., 2020). In our study, by predicting the structure of the eqANP32A protein, we found that although the N-Cap region of eqANP32A is located at the N-terminus of the LRR, the differences in the N-Cap region of the six splice variants of eqANP32A also significantly affect the spatial conformation of eqANP32A (Fig. 3C–H). However, further co-immunoprecipitation results found that all the eqANP32A variants were able to bind to the H3N8$_{XJ07}$ RNA polymerase complex, with no significant differences in their binding efficiency (Fig. 4), indicating that the N-Cap region of eqANP32A proteins regulated their abilities to support polymerase via a mechanism independent of binding. Although our team and other researchers have found that ANP32 proteins promote the RNA polymerase activity by interacting with the polymerase complex, this study and another study (Baker et al., 2018) demonstrated that ANP32 proteins are also able to enhance the polymerase activity via a protein: protein interaction-independent mechanism. Further study is needed to elucidate the mechanism underlying this phenomenon.

It has been reported that chANP32A is differentially spliced to produce three ANP32A splice variants: chANP32A$_{33}$, chANP32A$_{29}$ and chANP32A$_{\triangle}$. The genetic polymorphism and sequence differences in chANP32A determine its ability to support avian influenza virus RNA polymerase in mammalian cells (Domingues and Hale, 2017). It also has been discovered that different bird species express the three splice variants of chANP32A at different levels. Some species, such as swans, ostriches and others, mainly express human-like ANP32A (chANP32A$_{\triangle}$). These differences can potentially be used to predict the adaptability of polymerases from AIV- and mammalian-derived viruses, and suggest that the differential expression of ANP32 splice variants in hosts can affect the evolution of influenza virus (Domingues et al., 2019). Here, we found that eqANP32 also has multiple splice variants. Differential splicing of eqANP32A yields six splicing variants, while there is one transcript of eqANP32B. We found that the levels of mRNA expression for each of the six eqANP32A splice variants was different in different tissues (Fig. 1B–G). Levels of eqANP32A_X2 mRNA expression were the highest (Fig. 1B–G), and the support of influenza A virus RNA polymerase activity was also the highest for this variant (Fig. 2A–D), suggesting that eqANP32A_X2 plays a dominant role in supporting EIV RNA polymerase

activity. Whether the differential splicing of eqANP32A also plays an important role in the adaptation and evolution of equine influenza virus RNA polymerase remains unknown and is worth exploring further.

## 5. Conclusions

In summary, we investigated polymorphism and sequence differences in the host factor eqANP32 family genes. We demonstrated that the *eqANP32A* gene has multiple splicing variants with significant differences in the N-Cap region of the eqANP32A sequence. Our data highlight that the host factor eqANP32A_X2 is a key host factor supporting efficient EIV RNA polymerase replication in horses. In addition, our research has demonstrated for the first time that the N-Cap region of eqANP32A is a key domain that supports influenza virus RNA polymerase activity and thus provides a new target for the development of antiviral drugs, which is of great significance for preventing future epidemics of influenza.

## Data availability

All of the data generated during the current study are included in the manuscript.

## Ethics statement

This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The horses used in this study were approved by the Harbin Veterinary Research Institute (HVRI), the Chinese Academy of Agricultural Sciences (CAAS). The Animal Ethics Committee approval number is Heilongjiang-SYXK (Hei) 2017–009. All physical procedures associated with this work were done under anesthesia to minimize pain and distress in accordance with the recommendations of the Ethics Committees of the HVRI.

## Author contributions

Yuan Zhang: conceptualization, methodology, investigation, formal analysis, data curation, software, writing-original draft preparation. Xing Guo: methodology, formal analysis, data curation, software. Mengmeng Yu: methodology, formal analysis. Liuke Sun: methodology, formal analysis. Yuxing Qu: methodology, validation. Kui Guo: methodology. Zhe Hu: methodology, formal analysis. Diqiu Liu: methodology. Haili Zhang: conceptualization, methodology, investigation, formal analysis, data curation, funding acquisition, resources, supervision, writing-review and editing. Xiaojun Wang: conceptualization, data curation, formal analysis, funding acquisition, investigation, project administration, resources, software, supervision, writing-review and editing.

## Declaration of competing interest

The authors declare no conflicts of interest, financial or otherwise.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.10.009.

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