Research article

Host proteins interact with viral elements and affect the life cycle of highly pathogenic avian influenza A virus H7N9

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ABSTRACT

Host-virus interactions can significantly impact the viral life cycle and pathogenesis; however, our understanding of the specific host factors involved in highly pathogenic avian influenza A virus H7N9 (HPAI H7N9) infection is currently restricted. Herein, we designed and synthesized 65 small interfering RNAs targeting host genes potentially associated with various aspects of RNA virus life cycles. Afterward, HPAI H7N9 viruses were isolated and RNA interference was used to screen for host factors likely to be involved in the life cycle of HPAI H7N9. Moreover, the research entailed assessing the associations between host proteins and HPAI H7N9 proteins. Twelve key host proteins were identified: Annexin A (ANXA)2, ANXA5, adaptor related protein complex 2 subunit sigma 1 (AP2S1), adaptor related protein complex 3 subunit sigma 1 (AP3S1), ATP synthase F1 subunit alpha (ATP5A1), COPI coat complex subunit alpha (COP)A, COPG1, heat shock protein family A (Hsp70) member 1A (HSPA)1A, HSPA8, heat shock protein 90 alpha family class A member 1 (HSP90AA1), RAB11B, and RAB18. Co-immunoprecipitation revealed intricate interactions between viral proteins (hemagglutinin, matrix 1 protein, neuraminidase, nucleoprotein, polymerase basic 1, and polymerase basic 2) and these host proteins, presumably playing a crucial role in modulating the life cycle of HPAI H7N9. Notably, ANXA5, AP2S1, AP3S1, ATP5A1, HSP90A1, and RAB18, were identified as novel interactors with HPAI H7N9 proteins rather than other influenza A viruses (IAVs). These findings underscore the significance of host-viral protein interactions in shaping the dynamics of HPAI H7N9 infection, while highlighting subtle variations compared with other IAVs. Deeper understanding of these interactions holds promise to advance disease treatment and prevention strategies.

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1. Introduction

Avian influenza A virus (IAV) H7N9 was initially detected in China in February 2013 as a triple-reassortant comprising the hemagglutinin (HA) gene from H7N3, the neuraminidase (NA) gene from H7N9, and internal genes from H9N2 [1]. The poultry-to-human transmission of the H7N9 virus has resulted in more than 1600 cases of severe infections and a mortality rate exceeding 30% in humans [2–4], indicating its significant impact on public health. The respiratory droplets enabled H7N9, which was obtained from humans, to spread easily among ferrets indicating high transmissibility. However, there is no scientific evidence yet confirming the transmission of the virus between individuals [5]. IAV's genome typically includes eight separate segments of single-stranded RNA that encode at least twelve proteins. These proteins consist of hemagglutinin (HA), neuraminidase (NA), matrix 1 protein (M1), matrix 2 protein (M2), nucleoprotein (NP), polymerase acidic (PA) protein, polymerase basic 1 (PB1), polymerase basic 2 (PB2), non-structural protein-1(NS1), nuclear export protein (NEP/NS2), PB1F2, and PA-X [6,7]. Each of these viral proteins plays distinct and critical roles in the life cycle of IAV. HA plays a vital role in enabling attachment of the virus to host cells, PB2 enhances the efficiency of viral transcription, M1 stabilizes the viral ribonucleoprotein (vRNP) complex, whereas NS1, NS2, PBIF2, and PA-X significantly regulate the host's immune response [7–10]. The H7N9 virus has been found to exhibit the Q226I mutation in the receptor binding region of HA and the E627K mutation in PB2, enhancing its ability to spread from birds to humans [11]. Mutations found at positions G186 V/N/K in the HA protein likely modify receptor specificity, while the R292K mutation in the NA protein grants resistance against NA inhibitors [2–4].

In 2017, Southern China experienced a fifth epidemic caused by a novel and highly pathogenic avian influenza A virus H7N9 (HPAI H7N9). Studies indicate that about 39% of the HPAI H7N9 samples collected during this epidemic had mutations that could potentially impact the virus's biological behavior [12–14]. Research findings indicated that acquiring the PB2 627K or 701 N mutations has led to heightened virulence of the HPAI H7N9 virus in humans, emphasizing the importance of taking measures to avoid a potential global outbreak [13]. An experiment in mouse model showed that the A286V and T437 M mutations in NP eradicated virulence , which implies that the amino acid residues associated with virulence in NP could be potential targets for creating weakened influenza vaccines and developing antiviral drugs [15].

Nonetheless, the effect of genetic mutations or the molecular evolution of HPAI H7N9 on the interactions between viral and host factors that alter the effectiveness of viral infection is still unknown. A group of factors originating from the hosts has been documented to impact the life cycle of Influenza A Virus (IAV). The interplay between influenza A virus and host proteins has been demonstrated to impact the severity of viral illness. For example, Karlas et al. accurately identified and documented the presence of 287 specific host genes, including those encoding ATP synthase, COPI coat complex subunit alpha (COPA), heat shock proteins (HSPs), and RAB family-related proteins. The ATP synthase and COPA plays crucial roles in intracellular transport and maintaining organelle integrity within cells; HSPs and the RAB family could potentially impact the replication of influenza A virus; and the interaction between the host karyopherin subunit α 1 and IAV NP facilitates the nuclear import of NP [16,17]. Recently, a report has emerged indicating that the protein inhibitor of activated STAT 1 (PIAS1) exhibit interactions with IAV NP, PB1, and PB2 proteins, this interaction effectively impedes the activity of the viral RNP complex while significantly diminishing the stability of IAV PB2 [18], blocks the activation of the vRNP complex and hinders viral replication by a Bcl10-interacting protein isoform with CARD, known as BinCARD [19].

However, the precise information about the interactions between HPAI H7N9 virus and host proteins, as well as their impact on the virus's life cycle, is still undetermined. Although it has been absent for several years, the simultaneous transmission of SARS-CoV-2 and IAV has prompted worries regarding the possible resurgence of H7N9 [20].

Based on the existing data and taking into account host factors linked to the IAV life cycle, the primary objective of this research was to pinpoint host proteins that may exert a substantial impact on the infection caused by HPAI H7N9 virus. Considering previous studies on genes related to membrane traffic machinery, endoplasmic reticulum-Golgi recycling, secretion, and lipid formation, the intricate development and production of 65 specific small interfering RNAs (siRNAs) were carried out to target genes known to be associated with different stages of the virus life cycle [16,20]. By utilizing RNA interference (RNAi), we proceeded to evaluate and scrutinize potential host factors linked to the life cycle of the H7N9 virus. Furthermore, we performed Co-IP experiments to assess potential connections among proteins derived from the host and the virus. This groundbreaking research stands as the initial thorough exploration of host elements that are likely to influence the life cycle of HPAI H7N9. Crucially, our results reveal meaningful connections between host proteins and crucial viral constituents including HA, M1, NA, NP, PA, and PB1 of HPAI H7N9.

2. Material and methods

2.1. Cell line and viruses

The A549 human lung epithelial cell line (ATCC, Rockville, MD, USA, Cat# CCL-185) was cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher, Waltham, MA, USA; Cat#10566016) containing 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA; Cat#10099141), 2 mM L-glutamine (Life Technologies, Waltham, MA, USA; Cat#25030081), and 1% penicillin-streptomycin (Life Technologies; Cat#10378016) at 37 °C with 5% CO_2. HPAI H7N9 was isolated from a diagnosed patient in Guangzhou, China in 2017. The sequence of the strain has been uploaded to the influenza gene bank, name A/Guangdong/HP001/2017 (abbreviated as HP001). HP001 studies were performed strictly according to the requirements of a biosafety level 3 laboratory (Registration No. CNAS BL0022).

2.2. 50% tissue culture infective dose (TCID50) and MOI of HP001

The virus stocks were quantified using the TCID50 method. HP001 was 10-fold serially diluted with DMEM from 10^{-1} to 10^{-10} concentrations. The dilutions of HP001 (100 µl) were added to wells in each row of a 96-well plate, and an A549 cell suspension (100 µl) was added to each well to a final cell density of 2×10^5 cells/ml. A549 cells without HP001 infection were included as controls. Cytopathic effects (CPEs) were observed using microscopy and recorded each day for 7 days. The obtained results were used to calculate TCID50 using the Reed-Muench method. The TCID50 values were transformed to multiplicity of infection (MOI) according to the equation MOI $= 0.7 \times$ TCID50/cell number (Supplementary Table 1).

2.3. SiRNA screening

65 siRNAs were fabricated and generated for the purpose of targeting precise genes, each siRNA sequence was matched to its specific target gene (Supplementary Table 2). The concentration gradient tests and transfection duration of the siRNAs were carefully examined (Supplementary Table 3). Additionally, the cytotoxic effects of the siRNAs were assessed by quantifying nuclei counts automatically. A hypotoxic classification was assigned if a minimum of 750 nuclei were observed within one well of a 384-well plate (Supplementary Table 4). Subsequently, the most effective RNAi sequence was determined using quantitative real-timer PCR (qPCR) analysis and selected for further experimentation (Supplementary Table 4). Briefly, 1.5 µl of siRNA, 4.5 µl of HiPerFect transfection reagent (Qiagen, Dusseldorf, Germany; Cat#301705), and 94 µl of opti-MEM medium (Invitrogen; Waltham, MA, USA, Cat#31985070) were added to each well of a 24-well plate and gently shaken for 1 min. After incubation at room temperature for 10 min, a cell suspension (400 µl) containing 1×10^5 A549 cells was added to give a final siRNA concentration of 75 nM. Cells were incubated at 37 °C and 5% CO_2 for 48 h. Following that, the A549 cells were washed with phosphate-buffered saline (PBS) and infected with HP001 (MOI = 0.1) diluted with IAV medium (consisting of 85% DMEM and 14% bovine serum albumin (BSA, the concentration of the stock solution was 7.5%, Solarbio, Beijing, China; Cat#H1130), 1% penicillin-streptomycin (Life Technologies, Cat#10378016) and 2 µg/ml Tosyl phenylalanyl chloromethyl ketone (TPCK)-Trypsin (Sigma, St. Louis, MO, USA; Cat#T1426)). During the infection process, plates would be shaken gently twice to distribute the virus evenly. After 2 h of infection, the cells were subjected to three washes with PBS, followed by incubation with IAV-medium at 37 °C with 5% CO2 for 48 h. A nontargeting control siRNA (Qiagen; Cat#1027310) was used for transfection.

2.4. RNA extraction and quantitative real-time reverse transcription PCR (qRT-PCR)

After being incubated for 48 h, the A549 cells were rinsed three times with PBS. Then, TRIzol (Invitrogen; Cat#15596018) was employed to extract the total cellular RNA. Subsequently, the extracted RNA underwent reverse transcription to generate complementary DNA (cDNA). The cDNA representing a viral NP gene fragment was subjected to qPCR using an H7N9 nucleic acid test kit (Zhijiang Bio-tech, Shanghai, China; Cat#RR-0226-02) on an ABI 7500 qPCR system (ABI, Foster City, CA, USA) comprising: 45 °C for 10 min and 95 °C for 15 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 30 s. All qRT-PCR tests were performed in triplicate and repeated independently three times.

2.5. Antibodies

Antibodies recognizing Annexin A (ANXA)2 (Cat# NB100-2724), heat shock protein family A (Hsp70) member 1A (HSPA)1A (Cat# NB120-2788), HSPA8 (Cat# NBP2-12880), influenza A virus PA (Cat# NBP2-42876), and influenza A virus PB1 (Cat# NBP2-42877) were purchased from Novus Biologicals (Littleton, CO, USA). Antibodies recognizing adaptor related protein complex 2 subunit sigma 1 (AP2S1) (Cat# sc-134261), adaptor related protein complex 3 subunit sigma 1 (AP3S1) (Cat# sc-1366388), COPA (Cat# sc-398099), COPG1 (Cat# sc-393977), RAB18 (Cat# sc-393168), and influenza A virus M1 (Cat# sc-69824) were bought from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies recognizing ANXA5 (Cat# ab54775), and influenza A virus NP (Cat# ab128193) were purchased from Abcam (Cambridge, UK). The antibody recognizing ATP synthase F1 subunit alpha (ATP5A1) (LS-C659451-100) was bought from LifeSpan BioSciences (Seattle, WA, USA). Antibodies recognizing heat shock protein 90 alpha family class A member 1 (HSP90AA1) (11445-T38), influenza A virus HA, and influenza A virus NA, were bought from Sino Biological Inc (Beijing, China). The antibody recognizing RAB11B (19742-1-AP) was bought from Proteintech Group Inc. (Rosemont, IL, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat# 2188), anti-mouse immunoglobulin G (IgG) (Cat# 7076S), and anti-rabbit IgG (Cat# 7074S) horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Normal mouse IgG (Cat# SC-2025) and normal rabbit IgG (Cat# SC-2027) obtained from Santa Cruz Biotechnology were used as isotype controls.

2.6. Co-IP and immunoblot analysis

Protein extracts from A549 cells infected with HP001 (MOI = 0.1) for 48 h were prepared, followed by incubation overnight with appropriate antibodies recognizing host proteins plus Protein G beads (GE healthcare, Uppsala, Sweden; Cat#28-9440-08).

The immunoprecipitates were subjected to three washes using a low-salt lysis buffer, followed by elution with $2 \times$ sodium dodecyl sulfate (SDS) loading buffer and resolved through SDS polyacrylamide gel electrophoresis (PAGE). Subsequently, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes and individually incubated with anti-HA, M1, NA, NP, PA, or PB1

antibodies. Following this step, secondary antibodies were applied and the immunoreactive proteins were detected using the Super Signal West Pico chemiluminescent substrate (Thermo Fisher; Cat#34580) and the imaging system (Biorad, ChemiDoc™XRS; Cat#1708265). Immunoblot analysis of target host proteins after siRNA transfection was performed to confirm siRNA interference, and host proteins and HP001 proteins from the above extracts were tested before and after Co-IP, separately, to confirm the specificity of the antibodies. Normal mouse or rabbit IgG served as isotype controls, GAPDH was used as an internal control. In parallel, control groups were established by combining antibodies, protein G beads, and cell extracts in the absence of HP001 infection. Additionally, control groups consisting of protein G beads and HP001-treated cell extracts without antibodies were also included.

2.7. GeneMANIA methodology

To integrate multiple functional interaction networks of the identified host factors and make predictions about the functions of
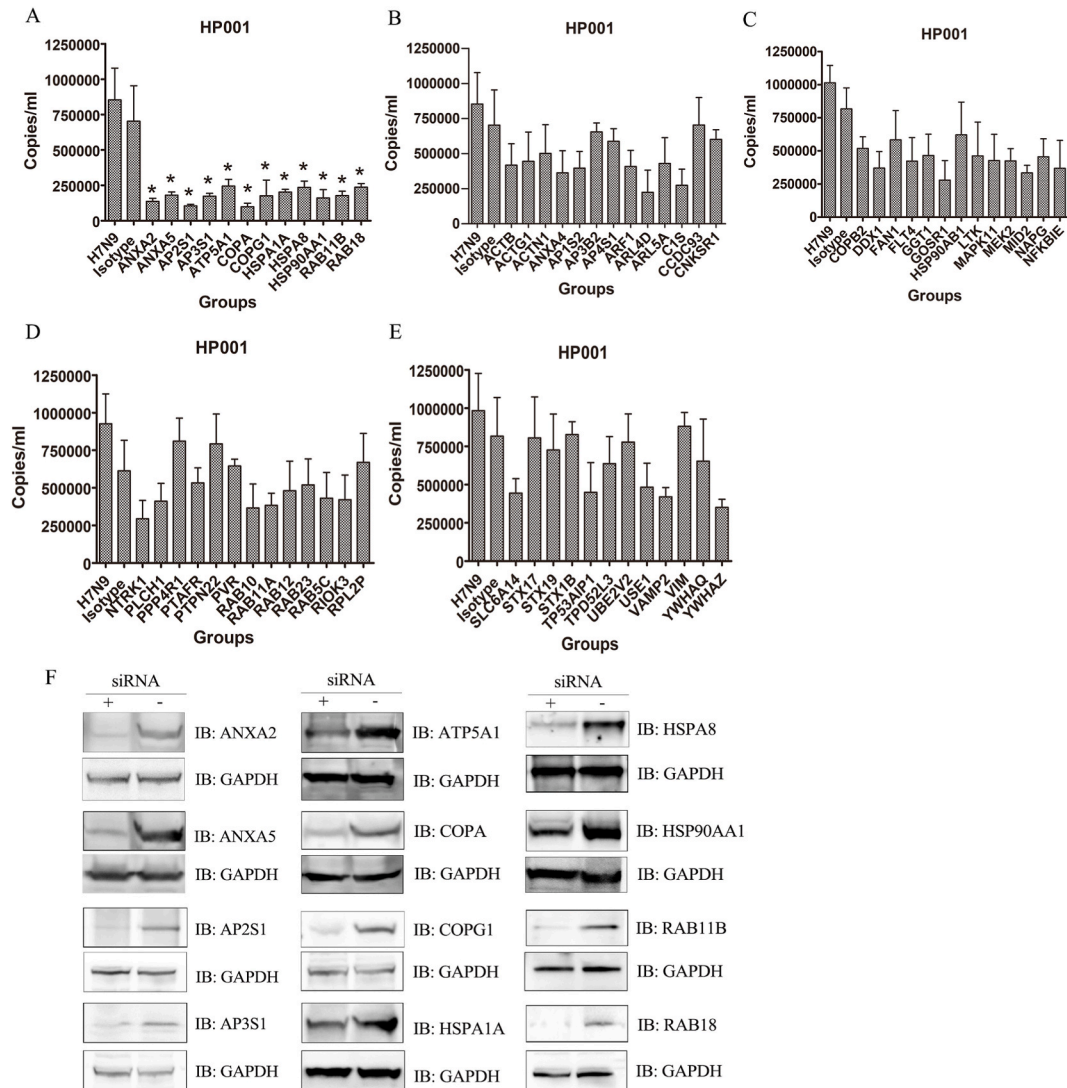

Fig. 1. RNAi screening of host factors related to highly pathogenic H7N9 avian influenza A virus (HPAI H7N9) HP001. **(A)** RNAi analysis of host factors required for the HP001 life cycle. After incubation with siRNA for 48 h, cells were infected with HP001 for another 48 h. Total RNA was extracted and the absolute quantity of HP001 RNA was measured by using a H7N9 nucleic acid test kit. All qRT-PCR experiments were performed in triplicate and repeated three times independently. Cells without siRNA interference but infected with HP001 served as a positive control (H7N9 group); cells transfected with isotype siRNA and infected with HP001 served as a negative control (isotype group). SiRNAs that reduced the number of viral copies significantly compared with the H7N9 group and isotype group were identified as possibly related to the HP001 life cycle (*p < 0.05). **(B–E)** RNAi silencing analysis of host factors that failed to inhibit HP001 efficiently. **(F)** Western blotting analysis of the targeted host proteins to verify siRNA knockdown efficiency. Normal 293T cells and cells transfected with non-targeting siRNAs served as controls. RNAi, RNA interference; qRT-PCR, quantitative real-time reverse transcription PCR; siRNA, small interfering RNA.

those genes or proteins, we employed GeneMANIA to calculate a network relevance for gene prediction [2]. The gene names were imputed into the online software GeneMANIA (http://genemania.org/) and their network relevance was calculated according to its algorithm. The combined network was later created by integrating information from various sources, including pathways, gene expression patterns, protein localization, physical interactions, common protein domains, and genetic interactions. From this combined network, the researchers were able to identify the top seven predicted functions. The top seven predicted functions were then identified from this combined network.

2.8. Statistical analysis

The qRT-PCR data, obtained from three replicates in each test and three independently repeated culture experiments, are presented as the mean ± standard deviation following a normal distribution. Student's t-test comparisons were performed between the host-target siRNA with the non-target control siRNA. An independent sample *t*-test was used for variables with a normal distribution and homogeneous variance. A paired *t*-test was used to compare the overall median differences between groups. All analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Differences with $p < 0.05$ between group means were considered statistically significant.
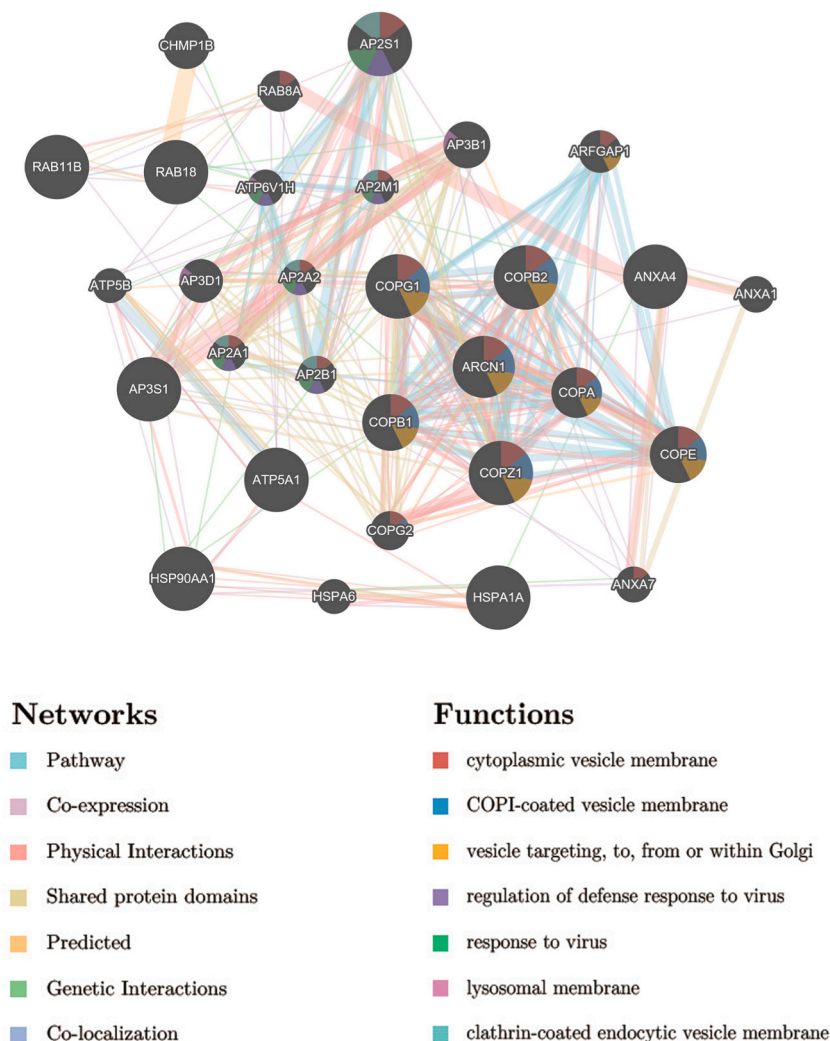

Fig. 2. Characterization of the targeted host proteins involved in the regulation of the HP001 life cycle. Networks, interactions, and functions of the 12 host proteins were evaluated using the GeneMANIA database. The functions were connected to COPI-coated vesicle function, vesicle-mediated transport related to Golgi and ER, COP9 signalosome, and defense response to virus and lysosomal membrane. COPI, coat protein; ER, endoplasmic reticulum; COP9, constitutive photomorphogenesis 9.
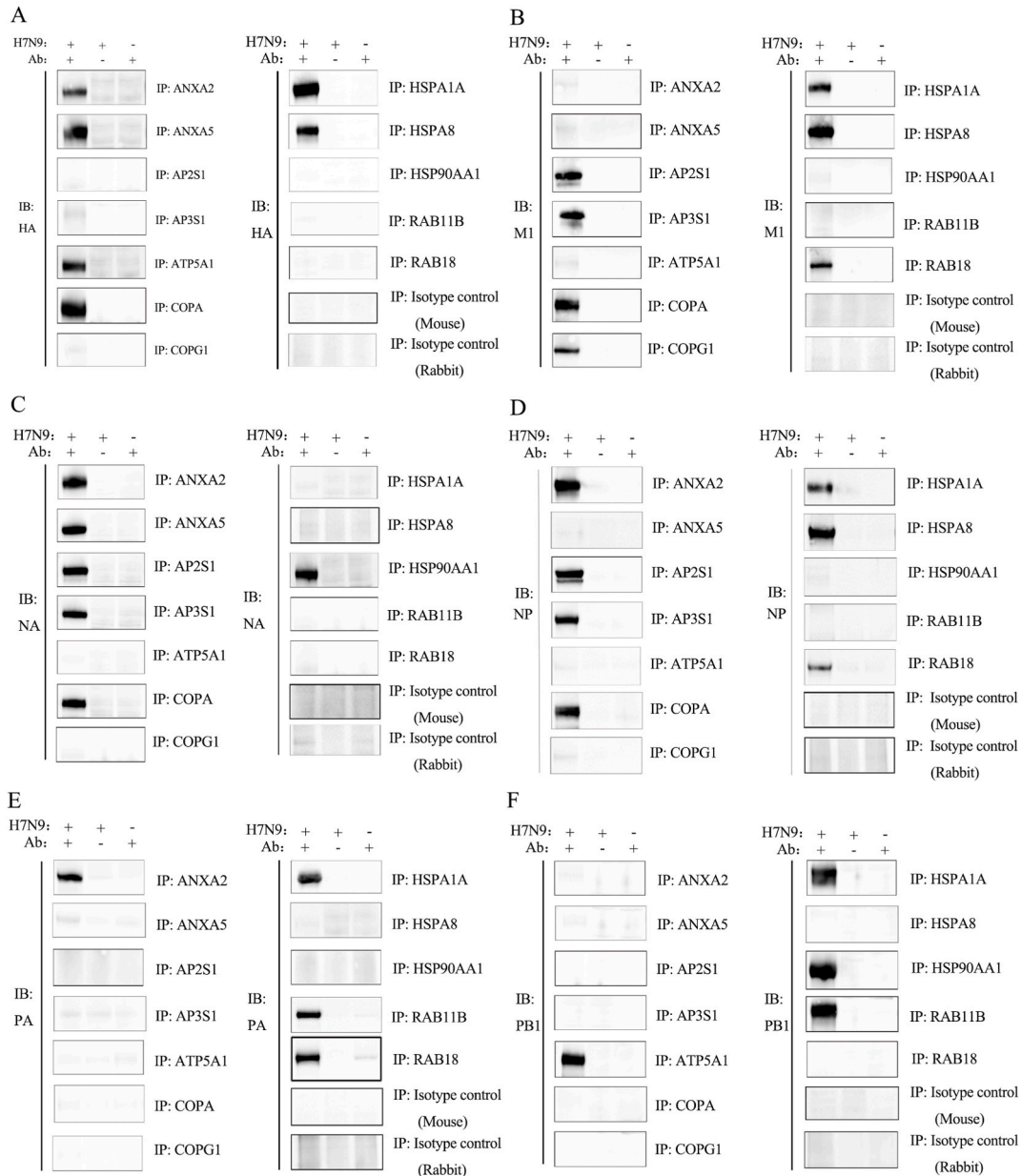
Fig. 3. Co-immunoprecipitation (Co-IP) and immunoblotting analysis of the interactions between targeted host proteins and HP001 proteins. Extracts from A549 cells infected with HP001 were incubated with antibodies recognizing targeted host proteins plus Protein G beads; proteins pulled down were detected using western blotting using anti-HA, M1, NA, NP, PA, and PB1 antibodies. Extracts containing HP001 incubated with Protein G beads without any antibody, and extracts from normal cells without HP001 mixed with Protein G beads and antibodies served as controls. Isotype antibodies served as negative controls. (A) Six host proteins (ANXA2, ANXA5, ATP5A1, COPA, HSPA1A, and HSPA8) interacted with HA. (B) Seven host proteins (AP2S1, AP3S1, COPA, COPG1, HSPA1A, HSPA8 and RAB18) interacted with M1. (C) Six host proteins (ANXA2, ANXA5, AP2S1, AP3S1, COPA and HSP90AA1) interacted with NA. (D) Seven host proteins (ANXA2, AP2S1, AP3S1, COPA, HSPA1A, HSPA8, and RAB18) interacted with NP. (E) Four host proteins (ANXA2, HSPA1A, RAB11B and RAB18) interacted with PA. (F) Four host proteins (ATP5A1, HSPA1A, HSP90AA1 and RAB11B) interacted with PB1. HA, hemagglutinin; M1, matrix 1 protein; NA, neuraminidase; NP; nucleoprotein; PA, polymerase basic 1; PB1, polymerase basic 2; ANXA2, annexin A2; ANXA5 annexin A5; ATP5A1, ATP synthase F1 subunit alpha; COPA, COPI coat complex subunit alpha; HSPA1A, heat shock protein family A (Hsp70) member 1A; HSPA8, heat shock protein family A (Hsp70) member 8; AP2S1, AP3S1, COPG1, COPI coat complex subunit G1; HSP90AA1, heat shock protein 90 alpha family class A member 1.

3. Results

3.1. SiRNA knockdown of host factors decreases HP001 replication

To determine the host factors involved in the life cycle of HP001, we performed tests on siRNAs that target 65 host genes in A549 cells infected with HP001 (MOI = 0.1). SiRNAs that knocked down ANXA2, ANXA5, AP2S1, AP3S1, ATP5A1, COPA, COPG1, HSPA1A, HSPA8, HSP90AA1, RAB11B, and RAB18 efficiently inhibited HP001 replication ($p < 0.05$; Fig. 1A). The results indicated that the proteins encoded by these host genes likely participate in the life cycle of HPAI H7N9. Meanwhile, siRNA knockdown of the other host genes did not interfere with HP001 replication (Fig. 1B–E). To validate the efficiency of siRNA interference, western blotting analysis was employed to quantify the levels of the target proteins, which confirmed a substantial reduction in the level of each protein (Fig. 1F).

3.2. Bioinformatic analysis of identified host gene hits

Bioinformatic analysis of the identified hits revealed the biological functions associated with the proteins encoded by these genes. Among the selected candidate host factors, ANXA2 and ANXA5 belong to the Annexin group, and AP2S1 and AP3S1 are involved in clathrin-dependent endocytosis, antigen processing, regulation of defense response to viruses, as well as the Golgi network, and cargo transportation. Additionally, ATP5A1 serves as a proton-transporting ATP synthase and a transmembrane transporter. COPA and COPG1 are responsible for ER-to-Golgi vesicle-mediated transport, intracellular protein transport, and retrograde vesicle-mediated transport (Golgi-to-ER). HSPA1A, HSPA8, and HSP90AA1 play crucial roles in various cellular processes, including the constitutive photomorphogenesis 9 (COP9) signalosome that functions in the ubiquitin–proteasome pathway. RAB11B and RAB18 are small GTPases that act as pivotal regulators of intracellular membrane trafficking. Further bioinformatic analysis conducted using Gene-MANIA revealed strong connections between these genes and signaling pathways associated with COPI-coated vesicle function, vesicle-mediated transport related to Golgi and ER compartments, COP9 signalosome activity, defense response against viruses, as well as lysosomal membrane functions (Fig. 2).

3.3. Candidate host proteins interact with H7N9 elements

To determine the precise role of candidate host factors and assess their interactions with H7N9 viral proteins, Co-IP assays and immunoblotting analysis were conducted to validate the interactions between candidate host proteins and HP001 HA, M1, NA, NP, PA or PB1 proteins. Initially, we examined the interactions between host proteins and the HP001 HA protein. The data revealed that six host proteins (ANXA2, ANXA5, ATP5A1, COPA, HSPA1A, and HSPA8) interacted with HA; however, AP2S1, AP3S1, COPG1, HSP90AA1, RAB11B, and RAB18 did not interact with HA (with normal mouse or rabbit IgG serving as isotype controls; Fig. 3A). Likewise, the interactions of the 12 candidate host proteins with M1, NA, NP, PA, and PB1 proteins were assessed separately. Seven host proteins (AP2S1, AP3S1, COPA, COPG1, HSPA1A, HSPA8, and RAB18) exhibited interactions with M1; whereas ANXA2, ANXA5, ATP5A1, HSP90AA1, and RAB11B did not(Fig. 3B). Six host proteins (ANXA2, ANXA5, AP2S1, AP3S1, COPA and HSP90AA1) interacted with NA; whereas ATP5A1, COPA, HSPA1A, HSPA8, RAB11B, and RAB18 did not (Fig. 3C). Seven host proteins (ANXA2,
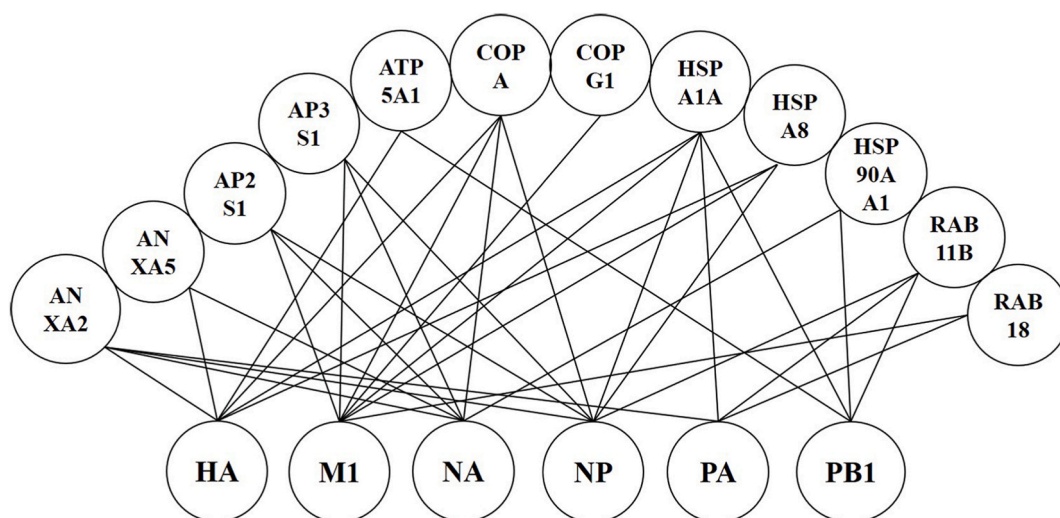

Fig. 4. Interactive network of 12 host proteins and six HP001 proteins. To summarize the interactions, an interactive network was constructed to allow an intuitive understanding of the interactions among the targeted host proteins and HP001 proteins. The connecting lines indicate interactions confirmed by co-immunoprecipitation (Co-IP).

AP2S1, AP3S1, COPA, HSPA1A, HSPA8. and RAB18) interacted with NP; whereas ANXA5, ATP5A1, HSP90AA1, COPG1 and RAB11B did not (Fig. 3D). Four host proteins, namely ANXA2, HSPA1A, RAB11B and RAB18, were found to interact with PA; whereas no interaction between ANXA5, AP2S1, AP3S1, ATP5A1, COPA, COPG1, HSPA8, and HSP90AA1 and PA was observed (Fig. 3E). Four host proteins (ATP5A1, HSPA1A, HSP90AA1 and RAB11B) interacted with PB1; whereas the remaining proteins showed no interactions
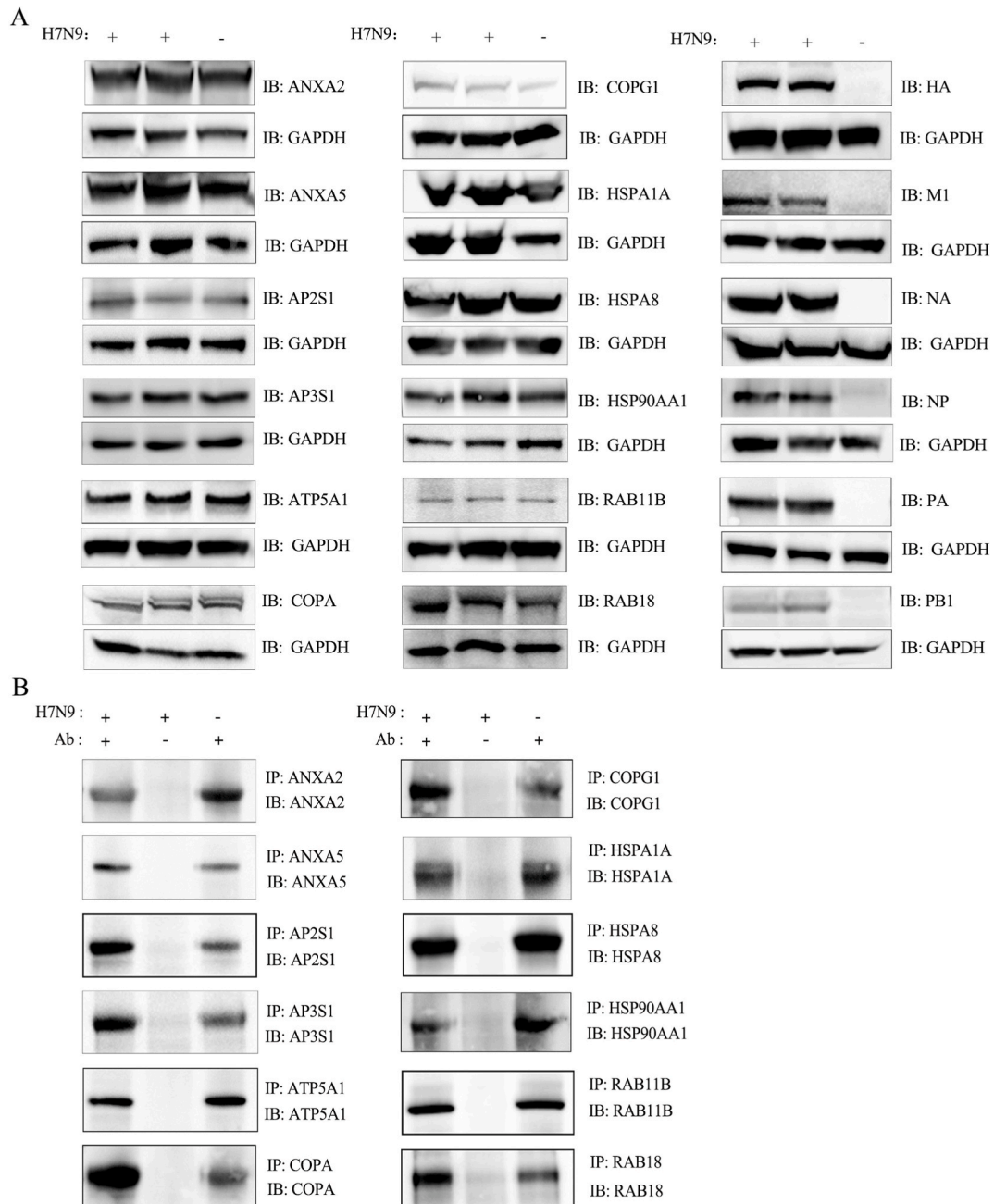

Fig. 5. Immunoblot analysis of the samples before and after co-immunoprecipitation (Co-IP). (A) Immunoblot analyses of the samples before Co-IP proves the specificity of the antibodies. To verify the specificity of the antibodies against the target host proteins and HP001 proteins, extracts from A549 cells infected with or without HP001 were checked by immunoblotting analyses with antibodies recognizing the targeted host proteins or HP001 proteins before Co-IP. The results indicated a high degree of antibody specificity. (B) Immunoblotting analysis of the samples after Co-IP further demonstrated the specificity of the antibodies. Extracts from A549 cells infected with HP001 were incubated with antibodies recognizing the targeted host proteins plus Protein G beads; extracts containing HP001 incubated with Protein G beads without any antibody, and extracts from normal cells without HP001 mixed with Protein G beads and antibodies served as controls. After Co-IP incubation, samples were check by immunoblotting analyses with antibodies recognizing the targeted host proteins.

with PB1 (Fig. 3F). To provide a comprehensive overview of these interactions, we constructed a schematic diagram illustrating the intricate interplay between host proteins and H7N9 proteins (Fig. 4). Additionally, via immunoblotting analyses, we assessed the levels of the 12 candidate host proteins and six HP001 proteins before IP (Fig. 5A), followed by evaluating the specificity of antibodies for the 12 candidate host proteins using post-IP immunoblotting analyses (Fig. 5B). These results not only confirmed the existence of robust interactions between candidate host proteins and viral counterparts, but also underscored the remarkable specificity of the antibodies.

4. Discussion

Since late February 2017, the HPAI H7N9 viruses have swiftly spread from their initial case in Guangdong Province, China. After the H7N9 vaccination, further examination unveiled a rapid development into separate subtypes and genotypes [21,22]. However, the specific stages of the life cycle of highly pathogenic avian influenza (HPAI) H7N9 and how it interacts with cells in its host are still not fully understood. By elucidating the virus-host protein interactions, our understanding of IAVs has significantly progressed, providing valuable insights into the pivotal role played by host proteins in the life cycle of HPAI H7N9.

Using an RNAi screen, we discovered 12 host proteins (ANXA2, ANXA5, AP2S1, AP3S1, ATP5A1, COPA, COPG1, HSPA1A, HSPA8, HSP90AA1, RAB11B, and RAB18) linked to the HPAI H7N9 life cycle in our research. Bioinformatic analysis indicated these candidate host proteins predominantly belong to the following families: (1) Annexin families; (2) Adaptor protein complexes; (3) heat shock protein families; and (4) small GTPases families. Subsequent functional analysis further substantiated the strong association of these target host proteins with COPI-coated vesicle function, lysosomal membrane, vesicle-mediated transport related to Golgi and ER, the COP9 signalosome, and the defense response against viral infections. In the Co-IP and immunoblotting analyses, we assessed the interactions between 12 candidate host proteins and six H7N9 viral proteins. Ultimately, we constructed an intricate interaction network that reveals complex relationships between host and virus factors in the H7N9 life cycle (Fig. 4). For instance, ANXA2 has been found to interact with various viral proteins, including HA, NA, NP, and PA, indicating its role in viral entry, membrane fusion, and replication processes. Additionally, the interaction between HSPA1A and viral proteins HA, M1, NA, NP, PA, and PB1 suggests its probable involvement in multiple stages of the HAPI H7N9 life cycle.

The Annexin family of proteins play a crucial role in the regulation and coordination of processes such as vesicle transport, exocytosis, and the synthesis of calcium-regulated proteins [23,24]. Influenza A virus has been found to be linked with annexins. For instance, the protein ANXA2 facilitates the replication of the H5N1 virus by interacting with its NS1 protein; the formyl peptide receptor 2 (a receptor for Annexin A1) regulates endosomal export of influenza virus [23,25]. Despite the absence of any documented proof, no links have been established between the Annexin family members and HAPI H7N9.

Adaptor protein complexes play a crucial role in cargo selection and vesicle formation and have the potential to interact with IAV 19 [26]. The viral NS1 protein binds to adaptor proteins CRK and CRKL, leading to the suppression of the antiviral-acting JUN N-terminal kinase (JNK)-activating transcription factor 2 (ATF2) pathway. Furthermore, the intrinsic cellular antiviral immune response and the spread of influenza A virus are controlled by the action of four and a half LIM domains 2 (FHL2) [27,28]. However, there is currently no available information regarding the relevance of adaptor molecules in HAPI H7N9 infection. Heat shock protein (HSP) families play critical roles in diverse cellular functions, including pathogen response, protein degradation, and safeguarding the proteome against external pressures [29,30]. HSPs exert significant influences on influenza A virus. For instance, HSP70 serves as a molecular chaperone for the polymerase of the influenza virus, playing a probable role in regulating its functionality. HSP40 plays a crucial role in the initial phase of infection by enabling the nuclear entry of IAV RNPs while enhancing the replication of the virus. Moreover, HSP90 interacts with the IAV NA protein, enhancing its stability and augmenting virus production in infected mammalian cells [31,32]. The results strongly endorse the critical function of HSPs in facilitating different stages of the IAV lifespan.

Small GTPase families play a crucial role in regulating intracellular membrane trafficking, encompassing vesicle formation, transport, tethering, and fusion processes [33,34]. Additionally, GTPases exhibit associations with the influenza A virus. For instance, IAV RNPs engage with RAB11 via viral RNA polymerase and exploit the RAB11-dependent recycling endosome machinery to facilitate viral apical plasma membrane trafficking [34]. However, additional research is necessary to achieve a complete understanding of the roles played by GTPases in the life cycle of the HAPI H7N9 virus.

Taken collectively, host proteins belonging to the Annexins, ATP, Adaptor protein complexes, COPI complexes, HSPs and small GTPases families interact with IAV proteins to significantly impact the viral life cycle. Notably, interactions involving ANXA2, COPA, COPG1, HSPA1A, HSPA8, and RAB11B have been substantiated in our study. Furthermore, we have discovered additional individuals within these family groups who also take part in the life cycle of HAPI H7N9. These include ANXA5, ATP5A1, HSP90AA1, RAB18, AP2S1, and AP3S1, which have not been previously reported in the literature pertaining to other strains of IAVs. The observation implies that IAVs, including HAPI H7N9, follow comparable mechanisms and engage in similar interactions with their host throughout their lifecycle; however, there are subtle differences in the specific details. Considering the complex and detailed character of host-virus interactions during viral infection, it is imperative to unveil the distinctive mechanisms and impacts of these interactions specific to the HPAI H7N9 life cycle compared to other Influenza A viruses. Protein interactions can occur through various methods, such as direct or indirect means, as well as larger molecular complexes involving mechanisms, including molecular recognition, subunit assembly, and cross-polymerization. Therefore, further evaluation is warranted to determine the modes and detailed mechanisms underlying the interactions between host proteins and HPAI H7N9 proteins.

In our research, we encountered specific constraints that affected our findings. Initially, all laboratory experiments were carried out utilizing A549 cells, therefore the connections between host proteins and HPAI H7N9 might not precisely mirror the biological circumstances in living organisms. Furthermore, a lack of understanding persists regarding the precise forms of engagement and intricate processes involved. Lastly, it is probable that the HAPI H7N9 virus invades various cell types using unique methods, resulting in

potential differences in the virus life cycle among different cells. Hence, forthcoming investigations will strive to ascertain the exact patterns of these interactions and establish the host-viral protein targeting's specificity, while also pinpointing distinct binding locations.

5. Conclusion

In this study, we successfully discovered 12 host proteins (ANXA2, ANXA5, AP2S1, AP3S1, ATP5A1, COPA, COPG1, HSPA1A, HSPA8, HSP90AA1, RAB11B, and RAB18) involved in the life cycle of HPAI H7N9 in A549 cells by implementing RNAi silencing. Our findings uncovered that six host proteins (ANXA2, ANXA5, ATP5A1, COPA, HSPA1A, and HSPA8) interact with the viral HA protein; seven (AP2S1, AP3S1, COPA, COPG1, HSPA1A, HSPA8, and RAB18) interact with the viral M1 protein; six (ANXA2, ANXA5, AP2S1, AP3S1, COPA, and HSP90AA1) interact with the viral NA protein; seven (ANXA2, AP2S1, AP3S1, COPA, HSPA1A, HSPA8, and RAB18) interact with the viral NP protein; four (ANXA2, HSPA1A, RAB11B, and RAB18) interact with the viral PA protein; and four (ATP5A1, HSPA1A, HSP90AA1 and RAB11B) interact with the viral PB1 protein. The study presents a compelling rationale for further investigation of host proteins that interact with HPAI H7N9 viral proteins and influence the virus life cycle. The discoveries we made provide a variety of viewpoints for researching the origin, spread, and harmfulness of the virus, ultimately aiding in the progress of developing therapeutic drugs.

Data availability

The data supporting the findings of this study are available from the corresponding author.

CRediT authorship contribution statement

Dong-Shan Yu: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Xiao-Xin Wu:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Tian-Hao Weng:** Writing – original draft, Methodology, Formal analysis, Data curation. **Lin-Fang Cheng:** Software, Project administration, Data curation. **Fu-Min Liu:** Validation, Software, Methodology. **Hai-Bo Wu:** Visualization, Validation, Supervision. **Xiang-Yun Lu:** Software, Methodology, Investigation. **Nan-Ping Wu:** Project administration, Funding acquisition, Conceptualization. **Shui-Lin Sun:** Writing – review & editing, Funding acquisition, Conceptualization. **Hang-Ping Yao:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

Co-IP	Co-immunoprecipitation
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal calf serum
HA	Hemagglutinin
HPAI H7N9	Highly pathogenic H7N9 avian influenza A virus
IAV	Influenza A virus
M1	Matrix 1 protein
M2	Matrix 2 protein
NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleoprotein
NS1	Non-structural protein 1
PA	Polymerase acidic protein
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PAGE	polyacrylamide gel electrophoresis

PVDF   Polyvinylidene fluoride
QRT-PCR   Quantitative Real-time Reverse Transcription PCR
RNAi   RNA interferences
RNP   Ribonucleoprotein
SDS   Sodium dodecyl sulfate

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28218.

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