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Research Article

C1QTNF5 is a novel attachment factor that facilitates the entry of influenza A virus

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ABSTRACT

Influenza A virus (IAV) binds sialic acid receptors on the cell surface to enter the host cells, which is the key step in initiating infection, transmission and pathogenesis. Understanding the factors that contribute to the highly efficient entry of IAV into human cells will help elucidate the mechanism of viral entry and pathogenicity, and provide new targets for intervention. In the present study, we reported a novel membrane protein, C1QTNF5, which binds to the hemagglutinin protein of IAV and promotes IAV infection *in vitro* and *in vivo*. We found that the HA1 region of IAV hemagglutinin is critical for the interaction with C1QTNF5 protein, and C1QTNF5 interacts with hemagglutinin mainly through its N-terminus (1–103 aa). In addition, we further demonstrated that overexpression of C1QTNF5 promotes IAV entry, while blocking the interaction between C1QTNF5 and IAV hemagglutinin greatly inhibits viral entry. However, C1QTNF5 does not function as a receptor to mediate IAV infection in sialic acid-deficient CHO-Lec2 cells, but promotes IAV to attach to these cells, suggesting that C1QTNF5 is an important attachment factor for IAV. This work reveals C1QTNF5 as a novel IAV attachment factor and provides a new perspective for antiviral strategies.

1. Introduction

Influenza virus is an enveloped single stranded RNA virus, belonging to the *Orthomyxoviridae* family (Paules and Subbarao, 2017). Influenza virus has a wide host range and causes infection in humans and a variety of animals (Suzuki, 2005). Rapid and efficient transmission is a characteristic of influenza virus, and three transmission routes are widely accepted: aerosol, droplet and contact transmission (Killingley and Nguyen-Van-Tam, 2013; Paules and Subbarao, 2017). The clinical features of influenza in adults or adolescents mainly include fever, chills, headache, and cough (Cox and Subbarao, 1999; Nakagawa et al., 2017; Paules and Subbarao, 2017). According to the antigenic difference between the nucleoprotein (NP) and matrix 1 (M1) protein, influenza viruses are classified into A, B, C and D types (Bouvier and Palese, 2008; Hause et al., 2014). Among these types, influenza A virus (IAV) and influenza B virus (IBV) are more harmful and initiate seasonal influenza epidemics, resulting in 290,000 to 650,000 deaths each year (Javanian et al., 2021; Park and Ryu, 2018).

Viral tissue tropism is determined by the diversity of cell surface receptors and entry cofactors. The binding of viral proteins with cell surface receptors is the first step in viral infection (Sieczkarski and Whittaker, 2005; Smith and Helenius, 2004). To date, host cell receptors for many important viruses have been identified and confirmed, and different viruses utilize different cell surface receptors to enter cells (Grove and Marsh, 2011; Maginnis, 2018). For example, dipeptidyl peptidase 4 (DPP4) acts as a receptor for MERS-CoV to mediate entry (Raj et al., 2013); Both SARS-CoV and SARS-CoV-2 bind the receptor angiotensin-converting enzyme 2 (ACE2) to enter cells (Hoffmann et al., 2020; Li et al., 2003); Sodium taurocholate cotransporting polypeptide (NTCP) is an entry receptor for HBV (Yan et al., 2012). Influenza A virus binds and enters host cells through its viral receptor-binding protein hemagglutinin binding to glycoproteins or glycolipid-linked sialic acid on the cell surface (Gottschalk, 1959). In addition to known viral receptors, other cofactors such as co-receptors or attachment factors also play roles in viral entry and propagation. Many studies have reported that some proteins are co-receptors or attachment factors in viral infection and promote the entry of viruses (Eslami et al., 2022; Karakus

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et al., 2020; Moore, 1997; Sasaki et al., 2018; Wang et al., 2017). Neuropilin-1 (NRP1) significantly promotes the cell entry and infectivity of SARS-CoV-2 (Cantuti-Castelvetri et al., 2020); Fibronectin promotes the entry of EV71 by interacting with the viral protein VP1 (He et al., 2018); Vimentin is an attachment factor that facilitates SARS-CoV-2 entry into human endothelial cells (Amraei et al., 2022); A disintegrin and metalloproteinase-17 (ADAM17) is an essential attachment factor for classical swine fever virus (Yuan et al., 2021); Heat shock protein member 8 (HSPA8) is an attachment factor for infectious bronchitis virus (Zhu et al., 2020). All of these cofactors can promote the viral entry. In addition, cofactors promoting viral entry have also been found in influenza virus infection. Wang et al. found that the membrane surface protein HSP90AA1 promotes IAV entry into A549 cells by interacting with the viral glycoprotein hemagglutinin (Wang et al., 2020). However, whether there are other cofactors that promote IAV entry into cells and enhance viral pathogenicity has not been fully explored or confirmed.

The C1q-tumor necrosis factor 5 (C1QTNF5) protein belongs to the C1q and tumor necrosis factor-related superfamily (C1q/TNF), and is a membrane-associated protein (Mandal et al., 2006; Schaffler and Buechler, 2012). Human C1QTNF5 comprises 243 amino acids and three domains: a signal peptide (residues 1–15), a collagen domain (residues 30–98) and a gC1q domain (residues 103–243) (Tu and Palczewski, 2012). It is expressed in almost all human tissues, but is the most highly expressed in the retinal pigment epithelium and ciliary epithelium (Hayward et al., 2003; Wong et al., 2008). Interestingly, a human C1QTNF5 expression profile showed that C1QTNF5 is also highly expressed in human lung tissues in addition to the highest expression in the retina (Lei et al., 2016). The biological functions of C1QTNF5 have not been fully defined. Some studies have confirmed that C1QTNF5 affects disease occurrence and metabolism. A missense mutation (Ser 163 to Arg) changes the conserved amino acids in the gC1q domain of C1QTNF5, resulting in late-onset retinal degeneration (L-ORD) (Ayyagari et al., 2005; Hayward et al., 2003; Mandal et al., 2006). In terms of metabolism, C1QTNF5 induces AMPK phosphorylation to promote glucose uptake and mediates the phosphorylation of acetyl-CoA carboxylase (ACC) to facilitate fatty acid oxidation (Park et al., 2009; Schaffler and Buechler, 2012). Loss of the C1QTNF5 protein improves insulin action and reduces hepatic steatosis in C1QTNF5-deficient mice (Lei et al., 2016). However, the function of C1QTNF5 in viral infection and pathogenesis has not been reported. Due to its relatively high expression in lung tissue, the lung is the main infection target of influenza viruses, it implies that C1QTNF5 protein may play a role in influenza virus infection. In this study, we showed that C1QTNF5 binds to the glycoprotein hemagglutinin of IAV and acts as an attachment factor to promote IAV entry and enhances viral infection. Moreover, blocking the interaction between C1QTNF5 and hemagglutinin by knocking down C1QTNF5, treating with anti-C1QTNF5 antibody and recombinant C1QTNF5 protein greatly inhibits viral entry. C1QTNF5 does not act as a receptor to mediate IAV infection in sialic acid-deficient CHO-Lec2 cells, but can promote IAV binding to these cells, suggesting that C1QTNF5 is an important attachment factor for IAV.

2. Materials and methods

2.1. Cells and viruses

The human embryonic kidney (HEK) 293T cells, human lung carcinoma A549 cells, and Madin-Darby canine kidney (MDCK) cells were sourced from our laboratory and cell lines were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Biological Industries) supplemented with 10% fetal bovine serum (FBS, Biological Industries), 1% antibiotics (penicillin and streptomycin, Gibco). The stable cell lines HEK293T-C1QTNF5, A549-C1QTNF5 were established by lentiviruses infection, blasticidin (sigma) with final concentration of 10 μg/mL was added to the culture medium to maintain the screening during cultivation. The CHO-K1 and CHO-Lec2 cell lines were kind gifts from Dr. Yunsen Li (Soochow University), and were cultured in α-minimal essential medium (αMEM, Biological Industries) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics. All cell lines were cultured at 37 °C and 5% CO_2. The influenza virus PR8 (A/Puerto Rico/8/34), influenza PR8-Renilla luciferase reporter virus (PR8-Rluc), influenza H3N2 strain, mouse-adapted influenza PR8 (A/Puerto Rico/8/34) strain were provided by the State Key Laboratory of Virology (Wuhan University). These viruses were propagated in MDCK cells or 10 day embryonated chicken eggs, and virus titers were measured by plaque assays in MDCK cells.

2.2. Antibodies and reagents

The antibodies used in this study were as follows: anti-C1QTNF5, anti-Lamin B1, anti-Na^+/K^+-ATPase, anti-Myc, anti-β-Actin, anti-GAPDH, anti-GST and anti-His (A3021, A1910, A11683, AE070, AC026, AC002, AE001 and AE003, respectively; ABclonal, China); anti-α-Tubulin, anti-Flag and anti-HA (T6199, F1804 and H6908, respectively; Sigma); anti-NP (ab128193; Abcam); anti-H1N1 HA (11684-MM03; Sino Biological); goat anti-mouse or anti-rabbit IgG (115-035-174 and 111-005-144; Jackson Immuno Research Laboratories); goat anti-mouse antibodies conjugated with Alexa Fluor 488 (A-11001; Invitrogen). The main reagents used in this study were as follows: anti-Flag M2 beads, TPCK, sialidase and blasticidin (A2220, T1426, N7885 and 203350, respectively; Sigma); Lipofectamine 2000 (11668019; Thermo Fisher Scientific); Dual luciferase reporter assay kit (E1980; Promega); Neofect™ DNA transfection reagent (TF20121201; Neofect); Minute™ plasma membrane protein and cell component separation kit (SM-005; Invent Biotechnologies).

2.3. Plasmids and transfection

The human C1QTNF5 (NM_015645) was cloned into the mammalian expression vectors pCMV-HA and pCMV-Myc. C1QTNF5 truncation mutants were constructed on pCMV-HA vector. GST-C1QTNF5 and His-HA were constructed on the prokaryotic expression vectors pGEX-4T and PET-30A, respectively. Flag tagged PR8-HA (Hemagglutinin) protein expression plasmid and PR8 HA1 truncated plasmid were kind gifts from Dr. Boli Hu (Zhejiang University, China), PR8 HA2 truncated plasmid were subcloned by standard molecular biology techniques use PR8-HA plasmid as template. Expression plasmids pCAGGS-PB1, -PB2, -PA and -NP, luciferase reporter pPolI-NP-Luc, and pTK-RL were sourced from our laboratory. Plasmids and siRNAs transfection were performed using lipofectamine 2000 or polyethylenimine according to the instructions. All cloning primers and siRNAs sequences are shown in Supplementary Table S1.

2.4. Establishment of stable C1QTNF5-expressing or knockdown cell lines

To establish the stable C1QTNF5-overexpressing cell lines, 293T cells were cotransfected with C1QTNF5-overexpressing lentiviral plamid (pCDH-CMV-Flag-IRES-Blast-C1QTNF5) or empty vector together with Δ8.9 packaging plasmid and pVSV-G plasmid. After 48 h, the lentiviruses were harvested to infect 293T and A549 cells. At 48 h post-infection, the cells were screened with blasticidin (25 μg/mL), and immunoblotting assay was performed to detect the expression of C1QTNF5. To establish C1QTNF5 knockdown cell lines, sgRNA sequences for C1QTNF5 were cloned into the lenti-CRISPR-V2 vector, which was cotransfected with Δ8.9 packaging plasmids and pVSV-G plasmid into 293T cells. After 48 h, the viruses were harvested to infect A549 cells. At 48 h post-infection, the cells were screened with puromycin (4 μg/mL). Immunoblotting was performed to detect the knockdown efficiency of C1QTNF5 (unscreened monoclonal knockout cells, belonging to knockdown cell mix). sgRNAs sequences are shown in Supplementary Table S1.

2.5. Immunofluorescence assay

Immunofluorescence assay as described previously (Wang et al., 2019), and there was a slight change in this study. Briefly, cells were infected with PR8 virus for 24 h, discarded the supernatant and washed three times with PBS. Fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin (BSA) for 30 min. Cells were incubated overnight at 4 °C with specific primary antibodies. Subsequently, the Alexa Fluor 488-conjugated secondary antibodies were incubated at room temperature in the dark for 1 h. The nucleus was stained with 0.1% 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime, C1002) and incubated for 10 min at room temperature. Images were captured by fluorescence microscopy.

2.6. Coimmunoprecipitation assay

293T cells were transiently transfected with the indicated plasmids, cells were lysed with RIPA buffer (supplemented protease inhibitor cocktail and PMSF) at 4 °C for 1 h. The protein supernatant was collected by centrifugation at 12,000 rpm for 15 min at 4 °C. One-tenth of the cell lysates to prepare the input, the remaining lysates were immunoprecipitated with anti-Flag M2 beads or anti-Myc antibodies overnight at 4 °C. The beads were washed with RIPA buffer for three times, resuspended with 2 × SDS loading buffer and denatured at 100 °C for 10 min. The protein levels were analyzed by Western blotting.

2.7. Pull down assay

GST-C1QTNF5 and His-HA proteins were expressed in BL21 cells and purified. The purified His or His-HA proteins were incubated with 60 μL Ni-NTA resin beads at 4 °C for 4 h. Subsequently, centrifuge at 1000 rpm for 2 min and discard the supernatant. The same quality GST-C1QTNF5 protein was added and incubated at 4 °C overnight, followed by washing the beads three times. Finally, pull-down eluates were analyzed by Western blotting.

2.8. qPCR

RNA isolation and qPCR as described previously (Zheng et al., 2022). Total RNA from the cells were extracted using TRIzol reagent (Ambion, USA), and reverse transcription was performed with HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, China). RNA levels of target genes were analyzed by qPCR with ChamQ SYBR qPCR Master Mix (Vazyme, China). Data shown were the relative abundance of the indicated mRNA or vRNA derived from human or mouse cells normalized to human GAPDH or mouse Actin. All qPCR primer sequences are shown in Supplementary Table S1.

2.9. Plaque assay

Plaque assay as described previously (Zhang et al., 2016). MDCK cells were seeded in 12 or 24-well plates and infected when the cell density reached 100%. The cells were washed once with PBS and infected with a series of 10-fold dilutions of viruses at 37 °C for 1 h, the viruses were removed and the cells were covered with agarose medium (2 × MEM containing 1% low melting point agarose, 0.2% BSA, and 2 μg/mL of TPCK-trypsin). The well plate was cooled at 4 °C for 5–10 min to coagulate the agarose, and then the cells were incubated for 72 h in 37 °C, 5% CO_2 incubator. After fixing the cells with 4% paraformaldehyde and staining with 1% crystal violet, the clearly visible plaques were counted to determine the virus titers.

2.10. IAV minigenome system for polymerase activity

IAV minigenome system for polymerase activity assay was previously described (Yang et al., 2018). In detail, seeding 293T or A549-C1QTNF5 overexpressing cell lines in 12-well plates, cells were transfected with pCAGGS-PB1, -PB2, -PA (100 ng each), -NP (300 ng), luciferase reporter pPolI-NP-Luc (100 ng), and pTK-RL (10 ng). After 24 h, cells were lysed and analyzed with a dual luciferase reporter assay kit (Promega).

2.11. IAV entry assay

IAV entry assays were previously described (Chen et al., 2022; Wang et al., 2020). Briefly, cells (prechilled in advance) were incubated with IAV (MOI = 1) at 4 °C, and washed three times with cold PBS to remove unattachment virus. Fresh DMEM medium was added and infected at 37 °C for 1 h to allow the virus to enter the cells. Next, rinsed three times with PBS to remove the attached but not yet internalized virions. Cells were lysed with TRIzol and the viral NP vRNA levels were determined by qPCR to assess viral entry or cells were fixed for electron microscope observation.

2.12. Infection inhibition by anti-C1QTNF5 antibody and recombination protein C1QTNF5

To further examine the effect of C1QTNF5 protein on PR8 entry, A549 cells were blocked with anti-C1QTNF5 antibody (10 μg/mL) for 2 h at 4 °C (IgG antibody as control) or PR8 virus was incubated with different concentrations (100 μg/mL, 200 μg/mL, 400 μg/mL) of purified recombinant C1QTNF5 protein at 37 °C for 1 h, and then PR8 virus (MOI = 1) was added to cells for attachment at 4 °C, cells were washed with PBS for three times and to continue infection at 37 °C for another 1 h. The cells were lysed by TRIzol after the attached but not yet internalized virions were washed away with PBS. Viral entry was assessed by detecting influenza NP vRNA levels in cell lysates by qPCR.

As described above, immunoblotting analysis, viral titers determination of cell supernatants, and immunofluorescence assay were also performed. For infection inhibition by anti-C1QTNF5 antibody treatment, A549 cells were blocked with anti-C1QTNF5 antibody (10 μg/mL) for 2 h at 4 °C, and then PR8 virus (MOI = 1) was added to cells for attachment at 4 °C for 30 min. Cells were washed with PBS for three times and to continue infection at 37 °C for another 1 h. Next, the cells were washed to remove the attached but not yet internalized virions, fresh medium was added to the infected cells and infection was allowed to proceed for 24 h at 37 °C. For infection inhibition by recombination protein C1QTNF5 treatment, PR8 virus was incubated with recombinant C1QTNF5 protein (200 μg/mL) at 37 °C for 1 h, GST-Vector was used as a control. Protein-virus complex (MOI = 1) added into chilled A549 cells for 1 h at 4 °C, then cells were washed with PBS and to continue infected at 37 °C for another 1 h. Next, the cells were washed to remove the attached but not yet internalized virions, fresh medium was added to the infected cells and infection was allowed to proceed for 24 h at 37 °C.

2.13. Preparation and genotyping of C1qtnf5 knockout mice

C57/BL6J mice were used as the background model, and *C1qtnf5* knockout mice were prepared utilizing CRISPR/Cas9 system by Nanjing GemPharmatech (Nanjing, China). *C1qtnf5* gene contains three exons, ATG translation start site is located in exon 2, TAA translation stop site is located in exon 3, exon 2 of this gene is used as the target and screening mice with open reading frame frameshift mutation of genes. Briefly, Cas9 and gRNA (5′-GGGATCTTGTTGTCGTCCAG-3′ or 5′-CCATCAC GGCCGTCACGGCC-3′) were microinjected into the zygotes of C57BL/6J mice. Positive F0 generation mice were confirmed by PCR, and crossed with wild type C57/BL6J mice to obtain $C1qtnf5^{+/-}$ mice. The genome of $C1qtnf5^{-/-}$ mice was sequenced, and it was found that 86 bp deletion caused frameshift mutation. The genome of $C1qtnf5^{-/-}$ mice by PCR (Forward primer: 5′-AGTTCTTGGTTTGGGG CACCC-3’; Reverse primer: 5′-ACTCCAGCCCGACTGGAATGGA-3′) also confirmed the sequencing deletion results.

2.14. Mouse infections and histology

The mouse-adapted influenza A/Puerto Rico/8/1934 (PR8) strain was propagated in 10 day embryonated chicken eggs, and the virus titer was determined by plaque assay in MDCK cells. After 6–8 weeks mice were anesthetized by isoflurane, they were intranasally infected with 835 PFU mouse-adapted PR8 virus (in 50 μL PBS), and the mock group was infected with an equal volume of PBS. The weight and survival of mice were monitored for several consecutive days. On the 7th day after infection, the mice were euthanized and the lung tissues were collected for virus titer detection. On the 8th day after infection, the lung tissues were collected for pathological observation by HE staining. Histological observation was performed as previously described (Lei et al., 2019), lung tissue from experimental mice was washed with PBS and immediately fixed with 4% paraformaldehyde for 24 h. Lung tissue was dehydrated and embedded in paraffin and sectioned, paraffin-embedded sections were subjected to HE staining and pictures were acquired using Nikon upright optical microscope.

2.15. Statistical analysis

In this study, Unpaired Student's *t*-test is used for data statistical analysis with software Prism GraphPad 8. For the mouse survival study, statistical analysis was performed by Log-Rank test. The data were shown as mean ± standard deviations (SD) from three independent experiments. P value > 0.05 means no significant (NS), and P value < 0.05 means significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

3. Results

3.1. C1QTNF5 promotes influenza A virus infection in vitro

To identify whether the C1QTNF5 protein plays a functional role in influenza virus infection, two stable C1QTNF5 overexpressing cell lines were constructed with HEK293T cells and A549 cells (Supplementary Fig. S1A and S1B). Influenza Renilla luciferase reporter virus (PR8-Rluc) was used to infect the C1QTNF5-overexpressing 293T cell line. The results showed that C1QTNF5 overexpression promoted IAV infection compared with the empty vector control (Fig. 1A). Meanwhile, influenza PR8-Renilla luciferase reporter virus was also used to infect the C1QTNF5-overexpressing A549 cell line. The luciferase reporter activity of cell supernatants indicated that the overexpression of C1QTNF5 also promoted IAV infection in A549 cells (Fig. 1B). Next, we evaluated the effect of C1QTNF5 overexpression on authentic IAV infection by detecting the viral NP protein levels. C1QTNF5 overexpression significantly increased the viral NP protein levels under different multiplicity of infection (MOI), indicating that C1QTNF5 promoted IAV infection (Fig. 1C). Besides, C1QTNF5-overexpressing 293T cells were infected with PR8 strain at a low multiplicity of infection (MOI = 0.005). Cell proteins, RNAs and supernatants were harvested at the indicated time points, and their levels were measured. C1QTNF5 overexpression increased the IAV NP protein levels at the indicated time points (Fig. 1D). A qPCR analysis showed that C1QTNF5 overexpression also increased the IAV NP mRNA abundance (Fig. 1E). Plaque assay was performed to assess the effect of C1QTNF5 overexpression on the production of mature IAV particles, and overexpression of C1QTNF5 was shown to increase the viral titer in cell supernatants (Fig. 1F). In addition, we repeated these results in A549 cells. C1QTNF5-overexpressing A549 cells were infected with influenza PR8 strain (MOI = 0.2), the cell proteins, RNAs and supernatants were harvested and their levels were measured. The NP protein levels were detected by western blotting 24 h after influenza infection, the result showed that C1QTNF5 overexpression increased the NP protein levels (Fig. 1G). qPCR analysis confirmed that C1QTNF5 overexpression increased the viral NP mRNA abundance at the indicated time points in A549 cells (Fig. 1H). Furthermore, the overexpression of C1QTNF5 protein also increased the number of mature virus particles in cell supernatants (Fig. 1I). Finally, we explored the effect of endogenous C1QTNF5 on influenza A virus infection by the small interfering RNA (siRNA), viral NP protein levels were significantly reduced in C1QTNF5 knockdown cells compared to control cells (Fig. 1J), and the titer of PR8 was decreased in C1QTNF5 knockdown cell supernatants (Fig. 1K). In conclusion, these results indicated that C1QTNF5 promoted IAV infection *in vitro*.

3.2. Influenza A virus replicates at lower levels in *C1qtnf5*$^{-/-}$ mice

To investigate whether C1QTNF5 protein promotes IAV infection *in vivo*, *C1qtnf5* gene knockout mice were generated by the CRISPR/Cas9 system, and the genotypes of the mice were identified by PCR (Supplementary Fig. S2). Mice were infected with the mouse-adapted PR8 strain of influenza virus and then observed for 13 days to monitor the body weight and death of the mice (Fig. 2A). From the results of weight change, it was found that the weight of *C1qtnf5*$^{-/-}$ mice decreased more slowly than the wild-type mice under the same infectious virus dose and infection time (Fig. 2B). All the mice in the mock group survived, the wild-type mice in the infection group died starting on the 7th day and all died after 2 days, while the *C1qtnf5*$^{-/-}$ mice in the infection group died starting on the 9th day and some mice survived until the 13th day, demonstrating that the death time of *C1qtnf5*$^{-/-}$ mice was delayed and some mice survived after PR8 infection (Fig. 2C). To determine why the *C1qtnf5*$^{-/-}$ mice lost weight more slowly and some survived, on the 7th day of infection, the mice were euthanized, and lung tissues were removed under sterile conditions to measure the viral load in the lungs. The results showed that the viral load in the lungs of *C1qtnf5*$^{-/-}$ mice was lower than wild-type mice (Fig. 2D). Subsequently, the lungs of mice were stained with Hematoxylin-eosin (HE) and the pathological characteristics were observed. The lungs of mice in the mock group showed no obvious signs of injury, while the lungs of wild-type mice showed more severe lung injury than the *C1qtnf5*$^{-/-}$ mice after viral infection, which was caused by more severe hemorrhagic pleural effusion and pulmonary edema (Fig. 2E). The results of HE staining further indicated that, compared with those in the mock infection and *C1qtnf5*$^{-/-}$ mice, the pathological changes of lung tissue in the WT mice were more severe, with more cells and proteins debris infiltrating into alveoli and obvious alveolar necrosis (Fig. 2F). All these results illustrated that knocking out the C1QTNF5 protein *in vivo* inhibited influenza A virus infection and reduced the damage caused by influenza virus in mice.

3.3. Overexpression of C1QTNF5 protein promotes the entry of IAV

The above results suggest that the C1QTNF5 protein promotes influenza A virus infection. To determine the mechanism by which the C1QTNF5 protein promotes influenza A virus infection, we investigated whether the C1QTNF5 protein affects influenza A virus polymerase activity to promote viral infection by performing a mature miniature replicon assay. Similar RdRp activity was observed with or without C1QTNF5 protein overexpression in 293T cells and A549 cells, suggesting that the C1QTNF5 protein exerted no obvious effect on influenza virus polymerase activity (Fig. 3A). Therefore, C1QTNF5 protein promoting influenza A virus infection is not due to the polymerase activity. Considering that the C1QTNF5 protein is a membrane-associated protein and we also detected C1QTNF5 expression in the cell membrane (Fig. 3B). Therefore, we performed viral entry experiments and used electron microscopy to directly observe the entry of IAV virions. According to the results of the pictures taken, C1QTNF5 promoted the entry of influenza A virus (Fig. 3C). To further verify the role of C1QTNF5 in the entry process of influenza A virus, we performed virus entry assays again. C1QTNF5 stably overexpressing 293T cells were incubated with PR8 for 15 min or 30 min at 4 °C, washed three times with PBS to remove the unattached virus and incubated at 37 °C for another 1 h. PBS washed the cells three times again to remove the attached but not internalized

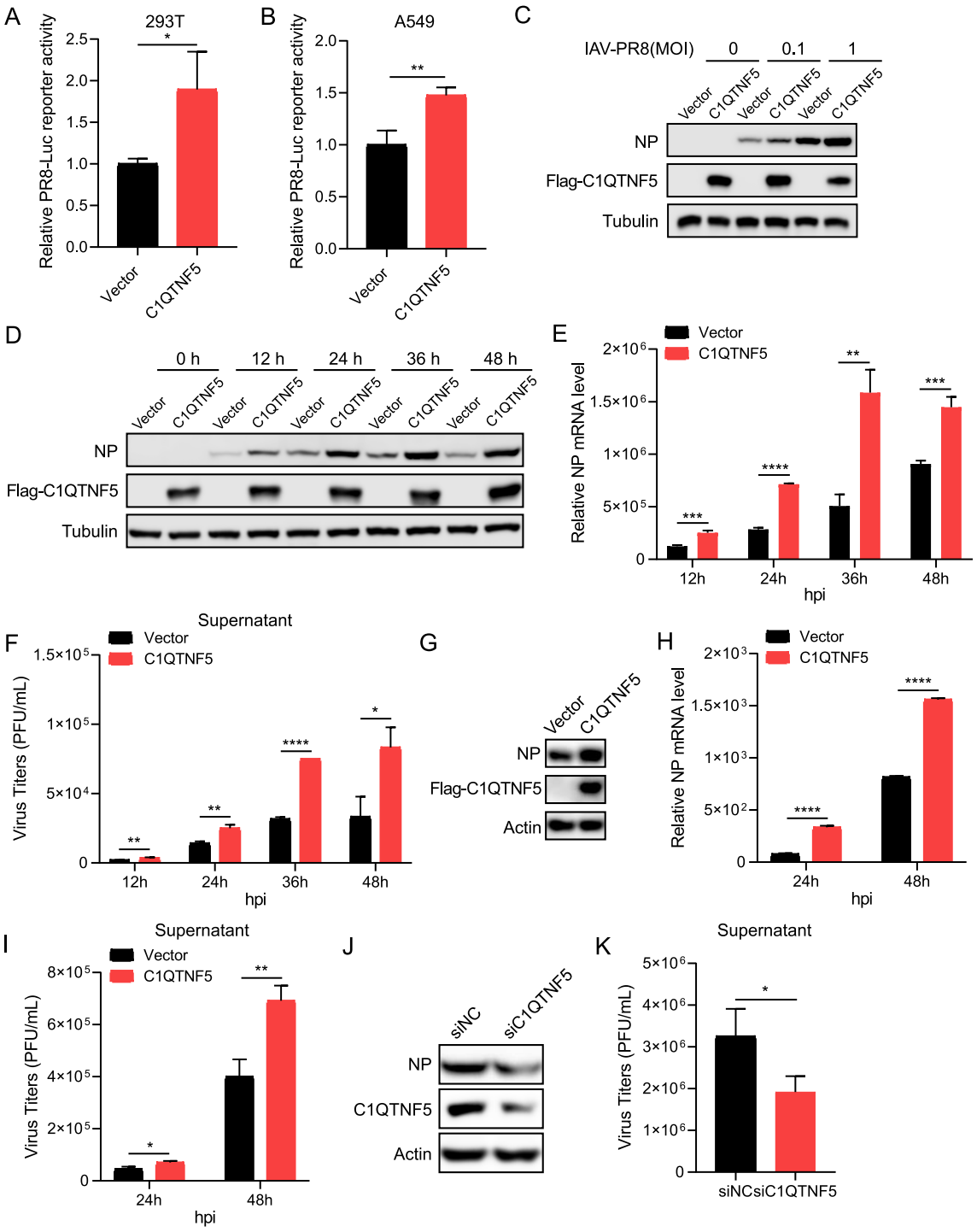

Fig. 1. C1QTNF5 promotes influenza A virus infection *in vitro*. **A, B** Overexpression of C1QTNF5 increased the luciferase activity of PR8-Luc viruses. C1QTNF5 stable overexpressing 293T cells (**A**) or A549 cells (**B**) were infected with PR8-Luc for 48 h, and then the luciferase activity was examined. **C**1QTNF5 stable overexpressing 293T cells were infected at the indicated MOI with PR8 for 48 h, and cell lysates were detected using the indicated antibodies. **D–F** Effect of C1QTNF5 overexpression on PR8 infection in 293T cells. C1QTNF5 stable overexpressing 293T cells were infected with PR8 strain (MOI = 0.005). The RNA levels, protein levels, and cell supernatants were measured at different time points postinfection. The protein levels were detected by Western blotting (**D**). NP mRNA levels were detected by qPCR and normalized to the level of GAPDH (**E**). Viral titer in cell supernatants were determined by plaque assay (**F**). **G–I** Effect of C1QTNF5 overexpression on PR8 infection in A549 cells. C1QTNF5 stable overexpressing A549 cells were infected with PR8 virus (MOI = 0.2), viral NP protein levels were measured at 24 h (**G**). NP mRNA levels were detected by qPCR at the indicated time points (**H**). Viral titer in cell supernatants were determined by plaque assay (**I**). **J, K** Effect of knocking down C1QTNF5 on PR8 infection. MDCK cells were transfected with siRNAs (siNC or siC1QTNF5) for 48 h and then infected with PR8 for 12 h. Viral NP protein levels were measured at 12 h by Western blotting (**J**). Viral titer in cell supernatants were determined by plaque assay (**K**). The data were shown as mean ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. MOI, multiplicity of infection; PFU, plaque-forming units.

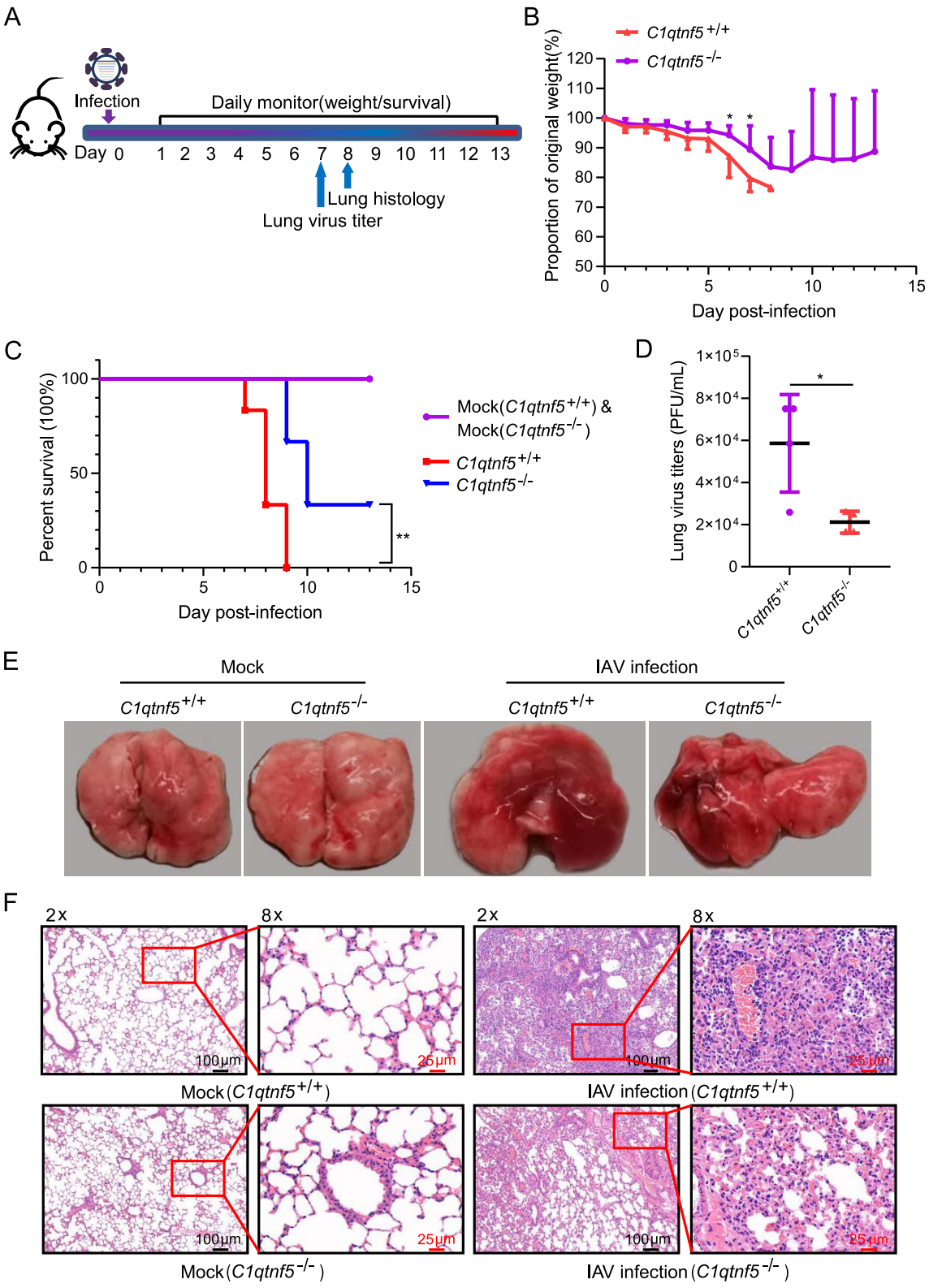

Fig. 2. Influenza A virus replicates at lower levels in *C1qtnf5*$^{-/-}$ mice. **A** Diagram of the WT and KO mice infection model. **B, C** Morbidity and mortality are reduced in *C1qtnf5*$^{-/-}$ mice after influenza A virus infection. WT (n = 6) and KO (n = 6) mice were inoculated intranasally with the 835 PFU mouse adapted PR8 strain. The mice were monitored daily for weight loss (**B**) and survival rate (**C**) until 13 days post-infection. The mouse weight on Day 0 (day of inoculation) was considered to be 100% to determine the mouse weight on subsequent days. **D** Detection of viral titers in the lungs of infected mice. The WT (n = 4) and KO (n = 4) mice were euthanized on the 7th day, and the lung tissues were removed for virus titer detection by plaque assay. **E** A direct view of the infected mouse lungs. Mice were euthanized on the 8th day, and the lung tissues were removed, a whole lung is shown. **F** A pathological examination of infected lungs was performed. Pathology of mouse lungs as visualized by HE staining on the 8th day of infection (2-fold: scale bar represents 100 μm; 8-fold: scale bar represents 25 μm). The data were shown as mean ± SD. *, $P < 0.05$; **, $P < 0.01$. WT, wild type; KO, knock out; HE, hematoxylin-eosin.

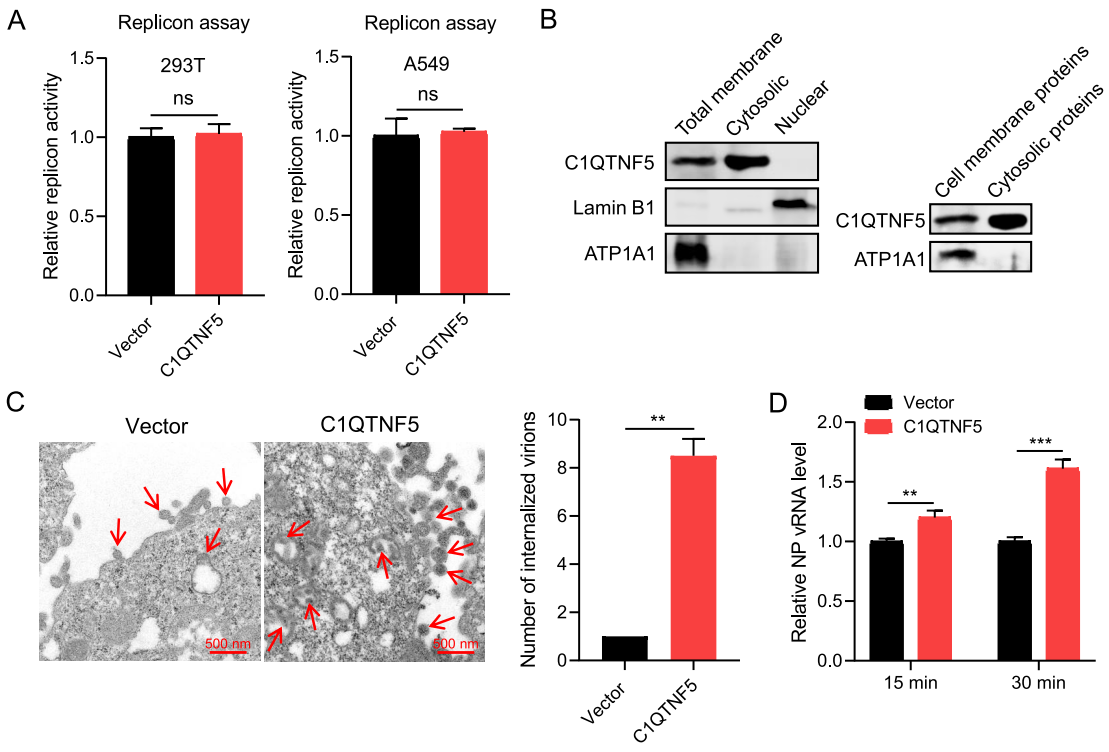

Fig. 3. Overexpression of C1QTNF5 protein promotes the entry of IAV. **A** Effect of C1QTNF5 protein on polymerase activity of influenza A virus. C1QTNF5 overexpressing 293T cells (left) or A549 cells (right) were cotransfected with the viral protein expression plasmids PB1, PB2, PA, and NP, the luciferase reporter pPolI-NP-Luc, and pTK-RL. The luciferase activity was measured after 24 h of transfection. **B** Subcellular protein extraction from A549 cells. Total membrane, cytoplasmic, and nuclear components of A549 cells were isolated, and the localization of C1QTNF5 was analyzed (left). The total membrane components were further separated into cell membrane and organelle membrane fractions, and C1QTNF5 localization was analyzed in the cell membrane components (right). **C** Effect of C1QTNF5 protein on entry of influenza A virus. C1QTNF5 overexpressing 293T cell line was incubated with PR8 (MOI = 1) at 4 °C for 30 min, washed three times with PBS and infected at 37 °C for another 15 min. And then fixed the cells for electron microscope observation. Scale bar represents 500 nm. The red arrows represent virus particles (left). Statistical analysis on the number of internalized virions in two visual fields (right). **D** C1QTNF5 overexpression 293T cells were incubated with PR8 (MOI = 1) for 15 min or 30 min at 4 °C, immediately washed three times with PBS and infected at 37 °C for another 1 h, PBS washed the cells three times again to remove the attached but not internalized virions. Cells were lysed by TRIzol reagent and viral NP vRNA levels were assessed by qPCR. The data were shown as mean ± SD from three independent experiments. NS, not significant; **, $P < 0.01$; ***, $P < 0.001$.

virions and the viral NP vRNA levels were detected by qPCR. In the presence of C1QTNF5 protein, viral NP vRNA levels increased regardless of the virus was allowed to attach for 15 min or 30 min, which further indicated that C1QTNF5 promoted the entry of influenza A virus (Fig. 3D).

3.4. Knocking down of C1QTNF5 or blocking A549 cells with anti-C1QTNF5 antibody inhibits IAV entry

The above results showed that overexpression of C1QTNF5 promoted the entry of IAV. To further confirm these results, endogenous C1QTNF5 was knocked down in A549 cells (monoclonal knockout cells were not screened). The knockdown effect of C1QTNF5 was confirmed (Fig. 4A). Previous viral entry assay was also performed in C1QTNF5 knockdown cell line, and consistent with the prior result, the knockdown of endogenous C1QTNF5 protein significantly inhibited the ability of IAV to enter A549 cells compared with the control (Fig. 4B). As blocking cell surface proteins with specific antibodies is the most common method used in the study of viral entry (Cantuti-Castelvetri et al., 2020; Wang et al., 2020; Zhu et al., 2020). A549 cells were blocked with anti-C1QTNF5 antibody (10 μg/mL, with IgG as the control) for 2 h at 4 °C, and then, the cells were incubated with PR8 for 30 min at 4 °C, washed three times with PBS to remove the unattached virus and continued to be infected at 37 °C for another 1 h. The viral NP vRNA abundance was detected by qPCR to assess the entry of influenza A virus. As expected, blocking the C1QTNF5 protein on the surface of A549 cells with anti-C1QTNF5 antibody reduced viral entry (Fig. 4C). Pretreatment with aiti-C1QTNF5 antibody also reduced the viral NP protein levels compared with the IgG treated control (Fig. 4D). Furthermore, the viral titers of cell supernatants were determined by plaque assay, and the results showed that pretreatment with anti-C1QTNF5 antibody reduced virions production (Fig. 4E). Consistently, after treatment of A549 cells with anti-C1QTNF5 antibody, the number of PR8 positive cells was found to be decreased in the immunofluorescence assay (Fig. 4F). Taken together, these results confirmed that C1QTNF5 plays a role in IAV entry.

3.5. Recombinant C1QTNF5 protein blocks the entry of IAV

To corroborate the above results, we also performed a recombinant C1QTNF5 protein infection inhibition assay to prove this conclusion. First, the purified recombinant GST-C1QTNF5 protein was obtained (Fig. 5A). Next, the different concentrations of GST-C1QTNF5 protein were incubated with influenza virus PR8 at 37 °C for 1 h, and then, the viral entry assay was performed. Intracellular RNA was collected and the viral NP vRNA levels in different groups were measured by qPCR to determine differences in the rate of entry. The results showed that the recombinant protein C1QTNF5 inhibited the entry of influenza virus in a dose-dependent manner (Fig. 5B). In addition, we incubated the PR8 strain with 200 μg/mL recombinant C1QTNF5 protein as described above, and detected the influenza NP protein levels and viral titers in cell supernatants after 24 h infection. These results showed that 200 μg/mL recombinant C1QTNF5 protein restricted the entry of influenza virus and

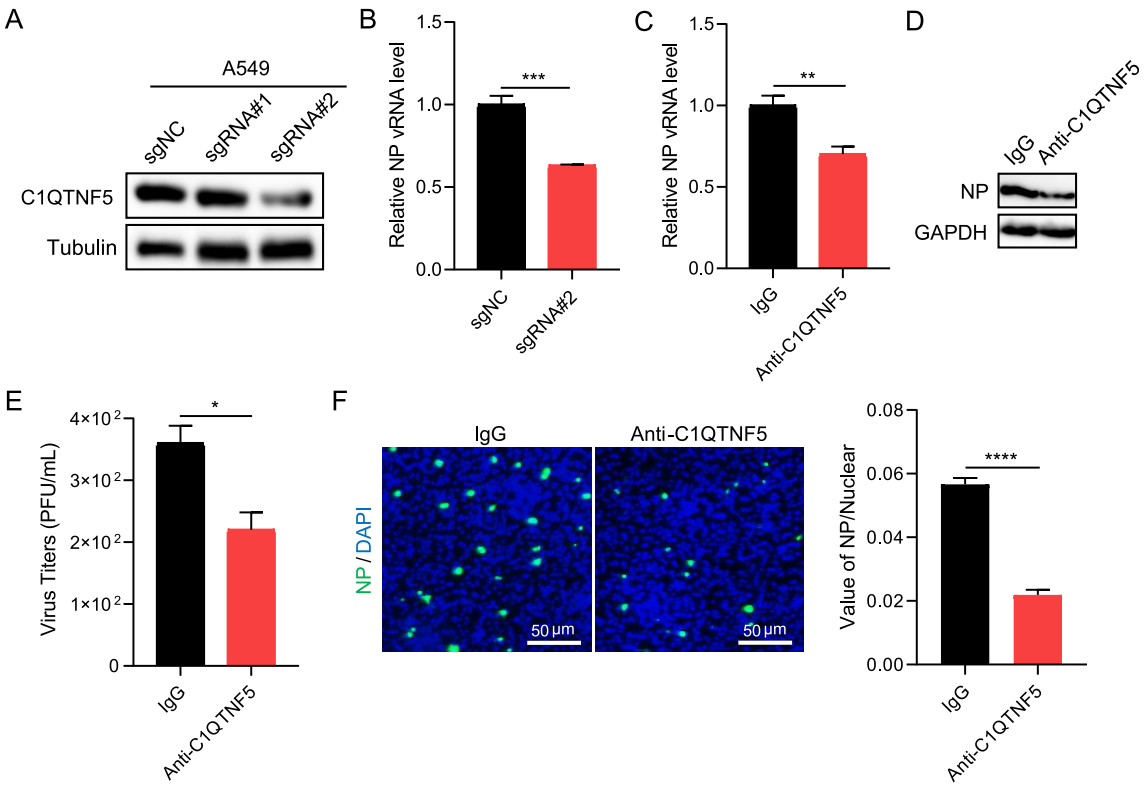

Fig. 4. Knocking down of C1QTNF5 or blocking A549 cells with anti-C1QTNF5 antibody inhibits IAV entry. **A** Knockdown of endogenous C1QTNF5 in A549 cells, the knockdown efficiency was determined by Western blotting. **B** C1QTNF5- knockdown A549 cells were infected with PR8 (MOI = 1) for 1 h at 4 °C. Immediately washing three times with PBS, and the infected cells were cultured at 37 °C for another 1 h. Then, PBS washed the cells three times again to remove the attached but not internalized virions. The cells were lysed and the entry difference of PR8 was assessed by detecting viral NP vRNA levels. **C** A549 cells were incubated with anti-C1QTNF5 antibody or control IgG at 4 °C for 2 h, and then were incubated with PR8 (MOI = 1) for 30 min at 4 °C. Immediately cells were washed three times with PBS and were cultured at 37 °C for another 1 h, the entry difference of PR8 was assessed by detecting viral NP vRNA levels after PBS washed the cells again. **D–F** As described in **C**, infected A549 cells were cultured for another 24 h at 37 °C after remove the attached but not internalized virions. The lysates and cell supernatants were collected, cells lysates were analyzed by Western blotting (**D**). Viral titers of cell supernatants were determined by plaque assay (**E**). A immunofluorescence assay was performed, and then visualized by fluorescence microscopy (NP: green; Nuclear: blue), scale bar represents 50 μm, the fluorescence area was analyzed using image J software to perform statistical analysis (**F**). The data were shown as mean ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

inhibited viral infection (Fig. 5C and D). Finally, the immunofluorescence assays also verified these results (Fig. 5E).

3.6. C1QTNF5 interacts with the HA protein of PR8

The influenza virus glycoprotein hemagglutinin (HA) binds to the sialic acid receptor on the cell surface to initiate infection and entry (Chu and Whittaker, 2004; Gamblin et al., 2021). The aforementioned results demonstrated that C1QTNF5 protein promotes the entry of influenza virus. Whether C1QTNF5 protein interacts with the influenza virus envelope glycoprotein HA like sialic acid receptor is unknown. Because there is no commercial C1QTNF5 protein specific antibody with immunoprecipitation function, we constructed protein expression plasmids and conducted exogenous coimmunoprecipitation (Co-IP) assays. The results showed that C1QTNF5 interacted with the PR8 virus hemagglutinin (HA) protein (Fig. 6A and B). In order to further explore whether C1QTNF5 directly interacts with viral HA protein, GST-C1QTNF5 and His-HA were purified in *Escherichia coli* cells for His pull down. The results showed that C1QTNF5 could directly interact with HA protein of PR8 (Fig. 6C). To detect the interaction between C1QTNF5 and PR8-HA upon virus infection, C1QTNF5 stable overexpressing 293T cells were infected with PR8 (MOI = 0.5) for 24 h. The immunoprecipitation result indicated that C1QTNF5 interacts with PR8-HA upon virus infection (Fig. 6D). The influenza virus HA protein is encoded by fragment 4, and its mRNA is transcribed in the nucleus and exported to the cytoplasm, where the precursor protein HA0 is formed through translation (Gething et al., 1986). HA0 is transported to the plasma membrane via the Golgi complex and cleaved by intracellular proteases to produce HA1 and HA2 (Copeland et al., 1986; Steinhauer, 1999). The HA domain is shown in Fig. 6E, and it is mainly composed of three different regions: fusion domain (F), vestigial esterase domain (VE) and receptor binding domain (RBD) (Sriwilaijaroen and Suzuki, 2012). To identify which part of the influenza virus HA protein interacts with C1QTNF5, we constructed HA1 and HA2 truncation mutants with Flag tags and performed Co-IP assays. The results indicated that influenza virus HA protein interacted with C1QTNF5 through the HA1 region (Fig. 6F). To further define the C1QTNF5 region that interacts with the influenza virus HA protein, two C1QTNF5 truncation mutants were designed and constructed (Fig. 6G). The results showed that both the N103 and 103C regions of C1QTNF5 protein interacted with influenza HA protein (Fig. 6H).

3.7. C1QTNF5 is an attachment factor for IAV

The above results demonstrated that the C1QTNF5 protein located in plasma membrane can promote the entry of influenza A virus and interacts with viral hemagglutinin (HA). We next sought to determine whether C1QTNF5 is an IAV receptor that promotes viral entry. First, sialic acid on the surface of A549 cells was digested with sialidase (200 mU/mL), and then, the cells were infected with PR8 strain. After 24 h, the NP mRNA levels were analyzed. In this assay, the ability of influenza A

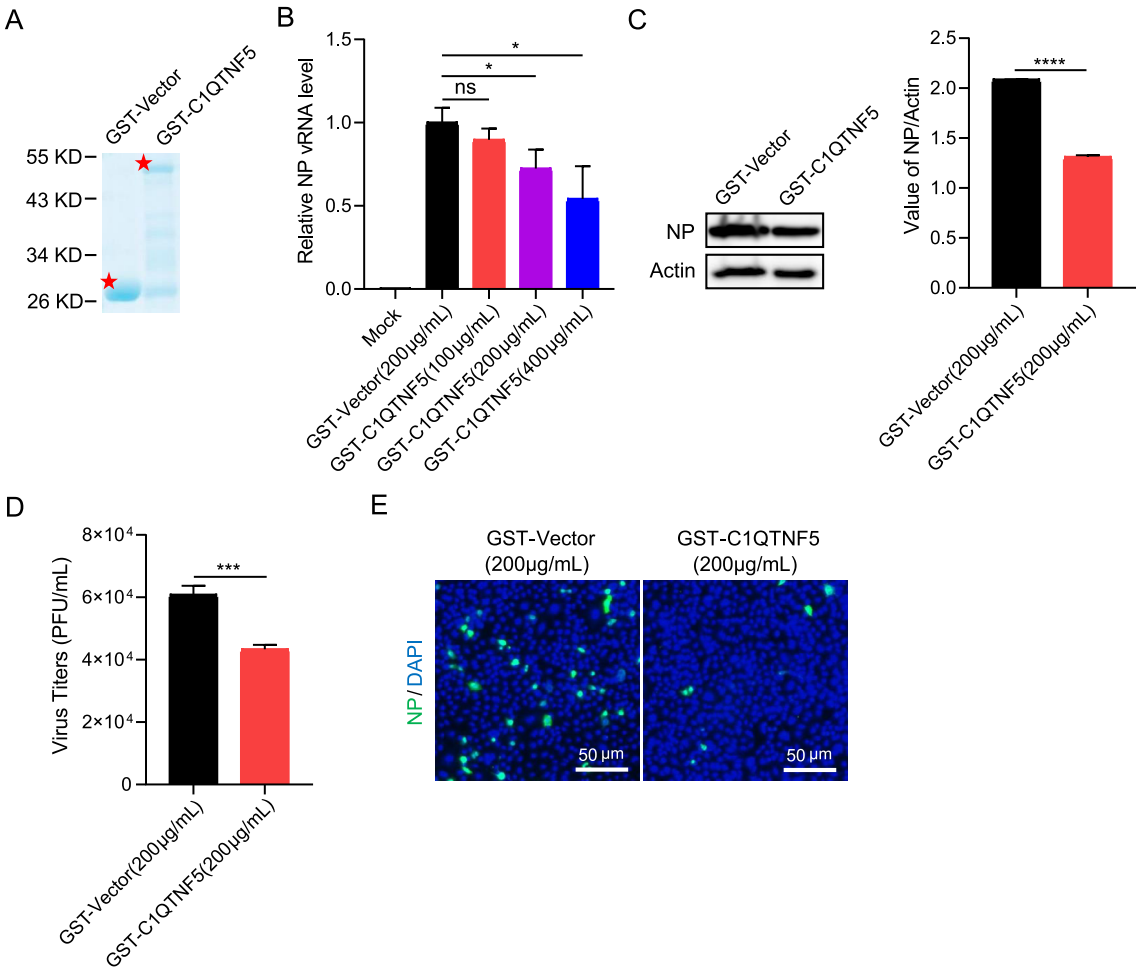

Fig. 5. Recombinant C1QTNF5 protein blocks the entry of IAV. **A** The GST-C1QTNF5 protein was expressed, purified and separated using SDS-PAGE and stained with Coomassie brilliant blue. **B** PR8 virus was pretreated with different concentrations of GST-C1QTNF5 or GST-Vector at 37 °C for 1 h and then added to chilled A549 cells (MOI = 1) and incubated at 4 °C for 1 h. Cells were washed with PBS and incubated at 37 °C for 1 h. PBS washed the cells three times again to remove the attached but not internalized virions, cells were lysed with TRIzol and qPCR was performed to measure viral NP vRNA levels and analyze viral entry differences. **C–E** As described in **B**, PR8 virus was pretreated with 200 μg/mL GST-C1QTNF5 or GST-Vector at 37 °C for 1 h, and then, infected A549 cells were cultured for another 24 h at 37 °C after remove the attached but not internalized virions. The lysates were analyzed by Western blotting (**C**), the virus titer of cell supernatants was determined by plaque assay (**D**). Immunofluorescence assay was performed, and then visualized by fluorescence microscopy (NP:green; Nuclear: blue), scale bar represents 50 μm (**E**). The data were shown as mean ± SD from three independent experiments. NS, not significant; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

virus to infect A549 cells was significantly reduced after sialidase digestion compared with untreated control (Fig. 7A). Next, we carried out identical infection assays in C1QTNF5 overexpressing A549 cells after sialic acid digestion, and found that the NP mRNA levels were increased when sialic acid was mostly digested compared with the empty vector control (Fig. 7B), indicating that the C1QTNF5 protein could still promote influenza A virus infection. This phenomenon may be due to the fact that the C1QTNF5 protein can act as an independent receptor to mediate the entry of some influenza viruses or the sialidase failed to digest sialic acid completely. The C1QTNF5 protein enables more virus to attach to the cell surface, and the virus then enters host cells through the residual sialic acid. In summary, C1QTNF5 still promotes IAV infection when sialic acid receptor function is disrupted.

To determine whether C1QTNF5 truly promotes the entry of influenza A virus as an independent receptor, we tested the hypothesis in CHO-Lec2 cells with sialic acid deficiency. CHO-Lec2 cells, a sialic acid deficient Chinese hamster ovary (CHO) cell line, and due to the lack of effective cytidine monophosphate (CMP)-sialic acid Golgi transporter caused the CMP-sialic acid cannot be transported to Golgi (Eckhardt et al., 1998; Londrigan et al., 2011). To verify whether influenza A virus can infect CHO-Lec2 cells, the cells were infected with PR8 strain. The intracellular proteins and RNAs were collected, and their levels were measured. The results showed that compared with CHO-K1 wild-type cells, the viral NP mRNA and protein levels were completely undetectable in the CHO-Lec2 cells, suggesting that influenza A virus easily infects wild-type CHO-K1 cells, but cannot infect sialic acid deficient CHO-Lec2 cells (Fig. 7C and D). The endogenous expression of the C1QTNF5 protein is very low (nondetectable) in CHO-Lec2 cells (Fig. 7E). Therefore, CHO-Lec2 cells were transiently transfected with C1QTNF5 protein expression plasmid and infected with influenza A virus to determine whether C1QTNF5 can truly function as a receptor to mediate the entry of virus under sialic acid deficiency conditions. The results of immunoblotting and qPCR assays showed that the C1QTNF5 protein cannot function as an independent receptor to mediate the entry and infection of influenza A virus (Fig. 7F and G). Subsequently, we performed virus attachment assays in CHO-Lec2 cells and found that the C1QTNF5 protein promoted the attachment of influenza A virus, despite the sialic acid deficiency of these cells, indicating that C1QTNF5 is an important attachment factor for IAV, enabling more viruses to attach to the cell surface, thereby promoting viral entry (Fig. 7H).

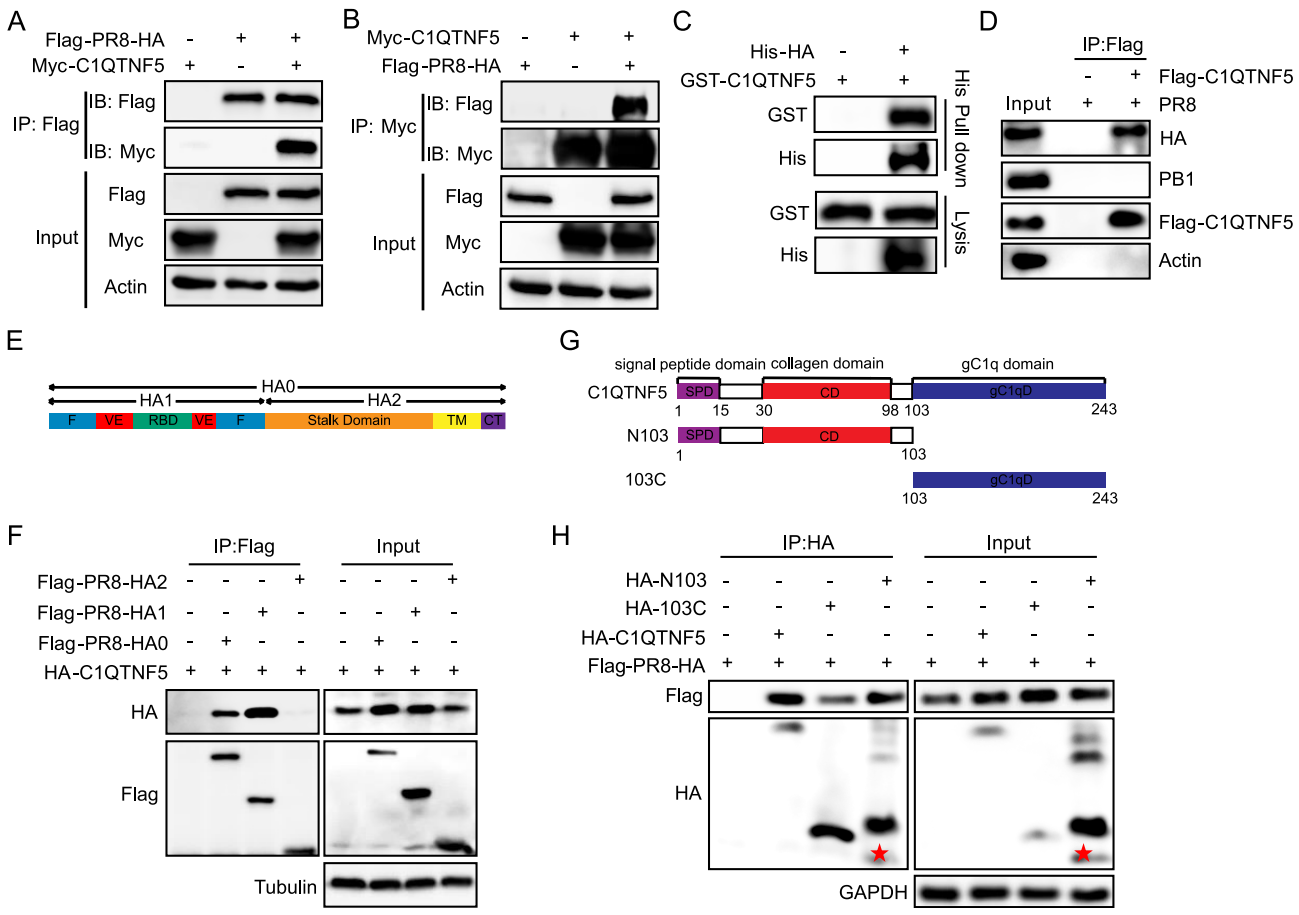

Fig. 6. C1QTNF5 interacts with the HA protein of PR8. **A, B** 293T cells were cotransfected with Flag-tagged PR8-HA plasmid and Myc-tagged C1QTNF5 plasmid or empty vector controls. After 48 h, a coimmunoprecipitation (Co-IP) assay was performed with anti-Flag antibody (**A**) or anti-Myc antibody (**B**). **C** His pull down assay of C1QTNF5 and PR8-HA protein. GST-C1QTNF5 and His-HA were expressed in BL21 cells and purified. The two purified proteins were incubated together for His pull down. **D** C1QTNF5 stable overexpressing 293T cells were infected with PR8 (MOI = 0.5) for 24 h, and then cells were lysed and immunoprecipitated with anti-Flag antibody (PB1 was used as a negative control). **E** Schematic diagram of the PR8 hemagglutinin (HA) protein domain, mainly including HA1 and HA2 region. **F** Defining the C1QTNF5-interacting domain of hemagglutinin. 293T cells were cotransfected with HA-tagged C1QTNF5, Flag-tagged full length PR8-HA plasmid and truncations or empty vector control. A Co-IP assay was performed (IP: with anti-Flag). **G** Schematic diagram of C1QTNF5 and C1QTNF5 mutants. SPD, signal peptide domain; CD, collagen domain; gC1qD, globular C1q domain; The truncation mutants mainly included N103 (amino acids 1 to 103), 103C (amino acids 103 to 243). **H** Defining the hemagglutinin-interacting domain of C1QTNF5. 293T cells were cotransfected with Flag-tagged PR8-HA plasmid, HA-tagged full length C1QTNF5 and truncations or empty vector control. A Co-IP assay was performed (IP: with anti-HA).

4. Discussion

Sialic acid, an acidic nine-carbonic amino sugar, was the first viral receptor to be confirmed (Karakus et al., 2020; Matrosovich et al., 2015). The viewpoint that influenza virus initiates infection via viral glycoprotein hemagglutinin binding to cell-surface sialic acid has been widely accepted (Gottschalk, 1959). In addition to influenza viruses, many other viruses also use sialic acid as an entry receptor, including parainfluenza, norovirus and rotavirus (Matrosovich et al., 2015). In our study, we found that C1QTNF5 promotes the entry of IAV. Disrupting the binding of C1QTNF5 to the viral hemagglutinin protein significantly reduced viral entry. However, when we overexpressed C1QTNF5 in CHO-Lec2 cells with sialic acid deficiency, we found that it failed to mediate IAV infection, but still promoted the attachment of virions, suggesting that C1QTNF5 is an attachment factor that promotes IAV entry. Specifically, C1QTNF5 enables more virions to attach to the cell surface, facilitating virions binding to sialic acid and eventually entry into the cells via sialic acid receptor pathway. Our results establish a working model to clarify the function of C1QTNF5 in IAV entry (Fig. 8).

The human C1QTNF5 protein belongs to the C1q and tumor necrosis factor-related superfamily (C1q/TNF), which consists of 15 members (Schaffler and Buechler, 2012). Through sequence alignment, we found that the amino acid sequence similarity between other members of this family and C1QTNF5 is very low (Supplementary Fig. S3). C1QTNF2 is showed the closest homologue, but only 33.33% amino acid sequence similarity with C1QTNF5. The sequence similarity of C1QTNF12 showed the lowest homologue to C1QTNF5, only 17.49%. Therefore, we speculated that among the C1q/TNF family members, only C1QTNF5 promotes the entry of IAV, or that C1QTNF5 is the main factor in this family related to the IAV entry. This may be consistent with the fact that most other members of the C1q/TNF family are not expressed in lung tissues. It also further indicates that cell localization and tissue distribution determine whether proteins can function as entry factors of viruses. Moreover, compared with human C1QTNF5, we found that the similarity of the chicken C1QTNF5 amino acid sequence was 72.47%, while that of the pig and horse was 91.54% and 96.30%, respectively. This suggests that the role of C1QTNF5 in promoting the entry of influenza A viruses may be conserved in different host species. In fact, IAV mainly infects the lung, but can also infect the heart, liver, kidney and spleen in a mouse model (Fislova et al., 2009; Kenney et al., 2019). In addition to high expression in the lung, C1QTNF5 is also expressed in extrapulmonary tissues, such as the heart, liver, kidney and spleen (Lei et al., 2016). This may indicate that C1QTNF5 plays a role in

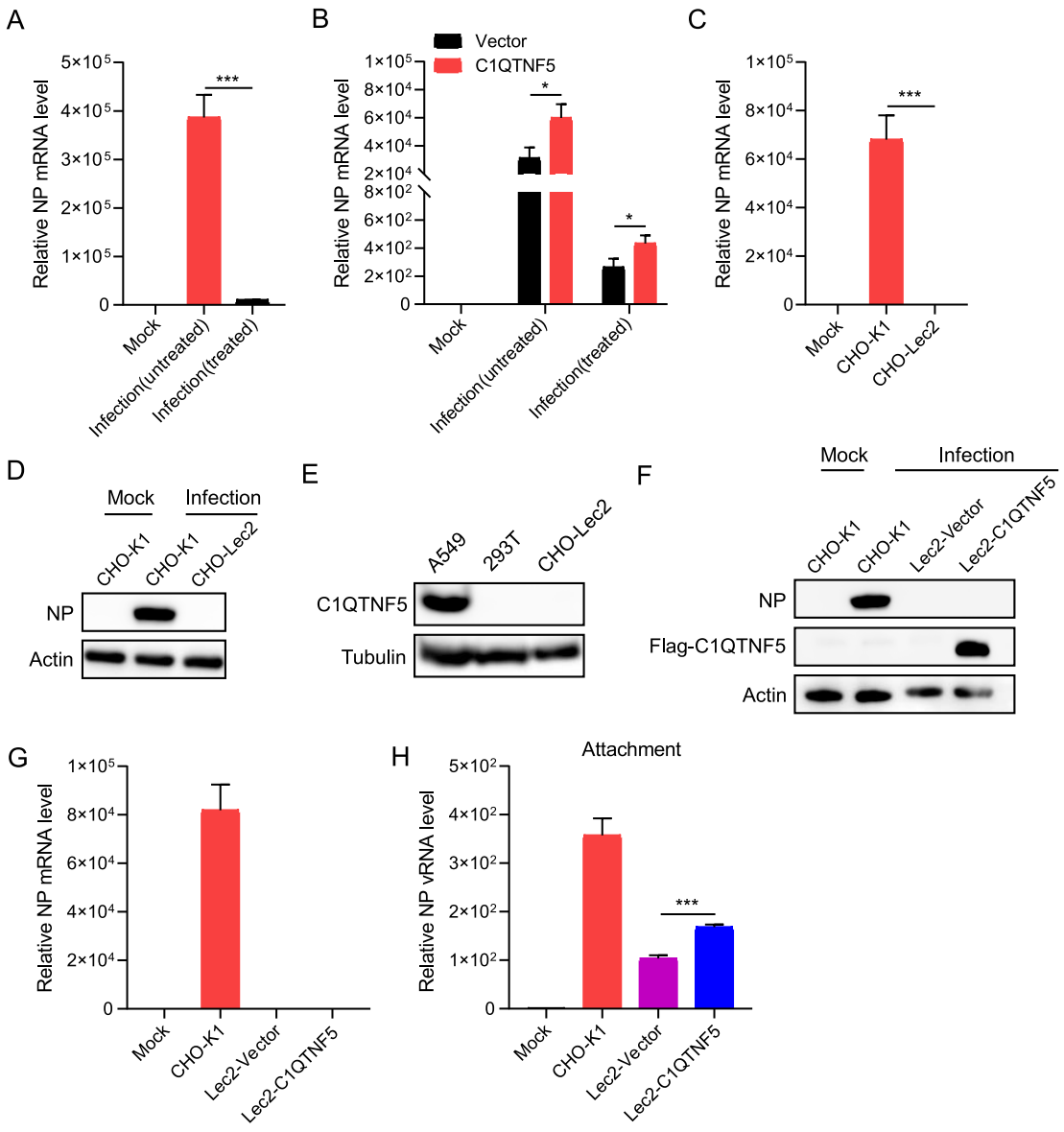

Fig. 7. C1QTNF5 is an attachment factor for IAV. **A** Sialidase treatment inhibited the ability of influenza virus to infect A549 cells. A549 cells were treated with or without 200 mU/mL sialidase at 37 °C for 3 h, and then cells were infected with PR8 (MOI = 1) for 24 h. The NP mRNA levels were analyzed by qPCR. **B** C1QTNF5-overexpressing A549 cell line was treated with 200 mU/mL sialidase, cells were infected with PR8 (MOI = 1) for 24 h, the NP mRNA levels were analyzed by qPCR. **C, D** CHO-K1 and CHO-Lec2 cells were infected with PR8 (MOI = 0.25) for 24 h, and intracellular NP RNA level (**C**) and protein level (**D**) were analyzed. **E** Endogenous expression of C1QTNF5 in CHO-Lec2 cells was detected by Western blotting. **F, G** The Flag-C1QTNF5 plasmid or empty vector (2 μg) was transfected into CHO-Lec2 cells and expressed. After 36 h, CHO-K1 and CHO-Lec2 cells were infected with PR8 (MOI = 0.25) for 24 h. Then the samples were collected, NP protein levels were analyzed by Western blotting (**F**), NP mRNA levels were measured by qPCR (**G**). **H** The Flag-C1QTNF5 plasmid or empty vector (2 μg) was transfected into CHO-Lec2. After 48 h, CHO-K1 and CHO-Lec2 cells were incubated with PR8 (MOI = 0.25) for 1 h at 4 °C, cells were washed three times with PBS and then lysed. The attachment of PR8 was assessed by detecting the viral NP vRNA levels. The data were shown as mean ± SD from three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

broad tissue tropism during IAV infection, ultimately resulting in greater harm to many tissues. Influenza virus produces many virus subtypes through antigen drift or continuous variation. In this work, we found that C1QTNF5 promotes the entry of influenza PR8 virus, thus enhancing the infection and pathogenicity of PR8 *in vitro* and *in vivo*. Besides, we found that C1QTNF5 can also promote the infection of influenza H3N2 subtype (Supplementary Fig. S4A-C). Blocking A549 cells with anti-C1QTNF5 antibody inhibits the entry of H3N2 subtype in the single life cycle (Supplementary Fig. S4D). Finally, it was found that C1QTNF5 promoted the attachment of H3N2 by attachment assay (Supplementary Fig. S4E). In summary, we revealed that C1QTNF5 has a positive regulatory effect on at least two subtypes of influenza virus. In order to explore whether C1QTNF5 is specific in promoting the entry of influenza virus, we tested this hypothesis with another RNA virus (EV71, which is unrelated to influenza virus) for verification. The results showed that C1QTNF5 did not affect the attachment and entry of EV71 virus (Supplementary Fig. S5A and B), suggesting that C1QTNF5 is relatively specific in promoting the entry of influenza virus.

In fact, according to related research reports, we can find that the influenza virus entry process is more complicated. This means that the entry of influenza virus is not a single process by viral hemagglutinin binding to host sialic acid. In addition to the main receptor, many host entry factors are also involved. For example, the previously mentioned HSP90AA1 protein promotes IAV entry into A549 cells (Wang et al., 2020). DC-SIGN mediates avian influenza H5N1 infection (Wang et al., 2008). Entry factors are important targets for antiviral research. In the development of antiviral drugs with the virus-host interface as the target,

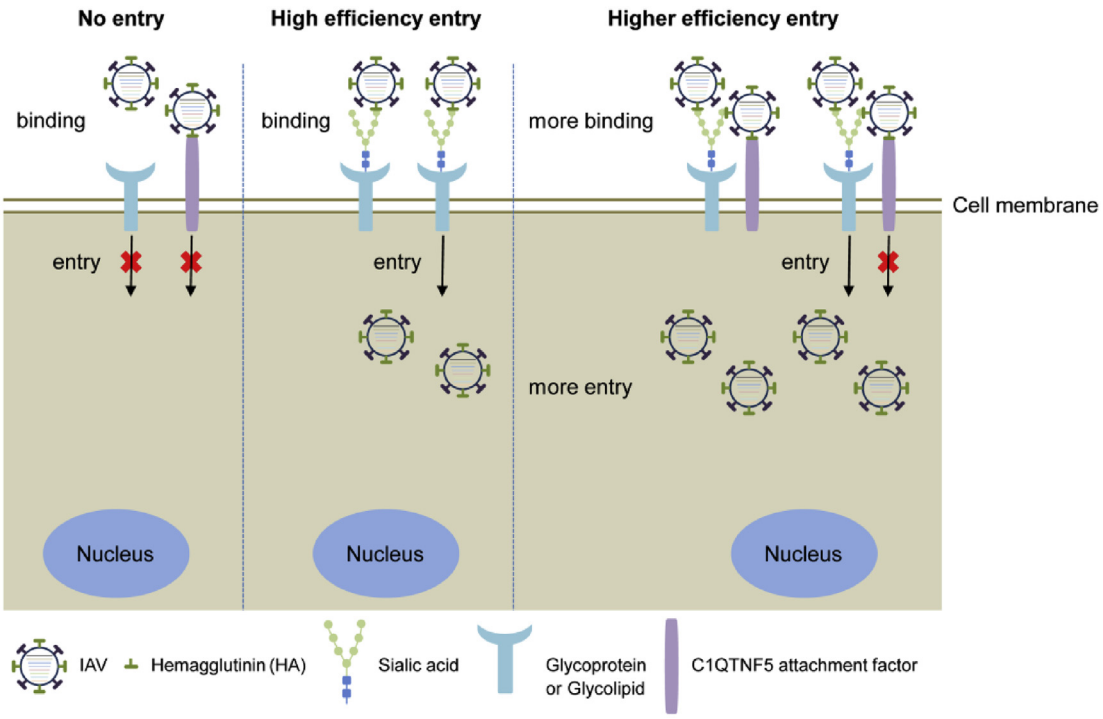

Fig. 8. Working model for C1QTNF5 functions as an attachment factor to promote IAV entry. No entry: without sialic acid, influenza A virus cannot enter host cells. High efficiency entry: influenza A virus binds to the sialic acid receptor on the cell surface via its surface glycoprotein hemagglutinin (HA), and then efficiently endocytoses into the cells under normal circumstances. Higher efficiency entry: the C1QTNF5 protein functions as an attachment factor rather than an endocytic receptor, which enables more viruses bind to the cell surface, and thus allows more IAV virions to enter host cells through the sialic acid pathway, thereby promoting the entry and infection of influenza A virus.

compared with the stages of virus uncoating, replication and transcription, assembly and release, the viral entry stage is the ideal target, because it is the fastest and most effective way to weaken the infectivity and pathogenicity of virus in the beginning.

In this study, the most key binding region of C1QTNF5 is not clear. Future studies will be performed to identify the key residues in the interaction between influenza A virus hemagglutinin and C1QTNF5 via structural biology analysis. The key residues will be targets for the development of anti-influenza virus strategies. Taken together, our results revealed a novel viral protein-binding factor that acts as an attachment factor to promote IAV entry. This work elucidated the mechanism of viral entry into cells at the molecular level, which is of great significance for clarifying IAV entry events and providing antiviral development targets.

5. Conclusions

In summary, we demonstrate that the host protein C1QTNF5, a novel binding partner for the IAV envelope protein hemagglutinin, significantly promotes IAV infection *in vitro* and *in vivo*. Mechanistically, C1QTNF5 promotes the cell entry of IAV through functioning as an important attachment factor instead of a receptor. Our results provide a new clue for better understanding of viral entry and may shed light on the development of antivirals for IAV.

Data availability

All the data generated during the current study are included in the manuscript or supplementary information.

Ethics statement

All the animal experiments were approved by the College of Life Sciences Animal Care and Use Committee of Wuhan University, Wuhan, China (approval number: SKLV-AE2021-009).

Author contributions

Lei Yu: investigation, data curation, methodology, software, validation, writing – original draft. Xinjin Liu: methodology. Xiaoqin Wei: methodology, resources. Junrui Ren: visualization. Xueyun Wang: formal analysis. Shuwen Wu: methodology, project administration, supervision, writing - review & editing. Ke Lan: funding acquisition, project administration, supervision, validation, writing - review & editing. All authors read and approved the manuscript.

Conflict of interest

Professor Ke Lan is an editorial board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article. We declare that we have no conflict of interest.

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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.2024.01.003.

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