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Respiratory virus mRNA as competitive viral RNA increases host factors expression and facilitates viral infection and replication

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Abstract

Respiratory viruses, mostly RNA viruses, generally express a large amount of viral RNA and mRNA upon infection. Here, we discovered that the PA RNA/mRNA of the H1N1 influenza virus competes for binding and sequesters miR-10a-5p, thereby relieving the repression of a host factor CAMK2B. CAMK2B phosphorylates M1 protein, which facilitates the export of vRNPs from the nucleus, thereby enhancing viral replication. Additionally, the 3' UTR of SARS-CoV-2 was found to competitively bind with miR-1307-3p, which alleviates repression of a host factor CCZ1B. This process promotes the transport of the viral receptor ACE2 from the endosome to the cell membrane. The quantitative formula between viral RNAs and host factor mRNAs shows that viral RNAs regulate the levels of host RNAs in a power-law manner. These findings provide new insights into the competitive viral RNA mechanisms of replication capabilities of different influenza subtypes and the evolutionary patterns of infection of SARS-CoV-2 variants.

Introduction

Respiratory viruses primarily infect the human respiratory system and cause various respiratory symptoms and diseases[1, 2]. Influenza A Virus (IAV) contains a single-stranded negative RNA genome consisting of eight segments[3]. Each of viral RNA (vRNA) segments binds to nucleoprotein (NP) and RNA-dependent RNA polymerase (including PA, PB1, and PB2) to form ribonucleoprotein (vRNP), which is the basic unit of IAV for transcription and replication[4]. During the early stage of IAV infection, M1 protein binds to the host factor TNPO1 mediate the nuclear translocation of vRNPs to finish transcription and replication at nucleus[5]. Then in the late stage of infection, the nuclear export signal (NES) on M1 and NEP protein is recognized by CRM1 and the newly replicated vRNP is exported from the nucleus[6–8]. Multiple host factors can limit or promote viral infection, which may affect virus entry, genomic replication, translation and virus release[5, 9].

Another respiratory virus, the SARS-CoV-2, is a single stranded, positive sense RNA virus with the protein envelope[10]. Coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 had brought serious threat to public health worldwide[11, 12]. SARS-CoV-2 proteins mainly include non-structural proteins (NSPs) and four structural proteins that is the spike (S) protein, the membrane (M) protein, the envelope (E) protein and the nucleocapsid (N) protein[13, 14]. The angiotensin-converting enzyme 2 (ACE2) is the major receptor that mediates the cell entry of SARS-CoV-2[15]. Both Rab7A and CCZ1 are involved in SARS-CoV-2 infections via the endosomal pathway[16, 17].SARS-CoV-2 infection may lead to cytokine storm, with abnormal release of pro-inflammatory cytokines and chemokines by immune effector cells[18].

MicroRNAs (miRNAs) are small, non-coding RNA molecules, typically about 21–23 nucleotides in length. They function primarily by binding to complementary sequences in target mRNAs, leading to mRNA degradation or inhibition of translation. MiRNAs can also interact with viral RNAs during viral infections, regulating viral infection, replication, and pathogenicity[19, 20]. Viral mRNAs and host mRNAs may

contain common miRNA-binding sequences. This intricate interplay between miRNAs, host mRNAs, and viral RNAs highlights the complex roles that these small RNAs play in both cellular and viral biology. We previously found that HBV mRNAs could regulate expression of host gene with the shared miRNA-binding sequences by binding to the common miRNAs, which was named as competitive viral RNAs (cvRNA) hypothesis[21, 22].

RNA viruses including influenza and SARS-CoV-2 replicate by expressing large amounts of viral RNA within infected host cells, enabling the production of viral proteins necessary for assembling new viral particles[23]. This extensive replication of viral RNA is crucial for the infection cycle, as it not only promotes the spread of the virus within the host but also enhances its capacity to infect neighboring cells. By binding to specific host miRNAs, viral RNAs may likely sequester these miRNAs, preventing them from regulating the host's mRNA[24, 25]. Understanding this mechanism sheds light on the intricate strategies employed by respiratory viruses to manipulate host cellular environments for their advantage. Furthermore, it underscores the potential implications for therapeutic interventions, as targeting these interactions could enhance our ability to control viral infections and restore normal cellular functions. This research opens new avenues for exploring the complex dynamics of host-pathogen interactions and their impact on gene regulation.

Results

Influenza viral RNA shared a common hsa-miR-10a-5p binding site with a cell host factor CAMK2B.

We previously found that hsa-miR-10a-5p inhibited IAV H1N1 more strongly than H3N2[26]. To determine if hsa-miR-10a-5p directly targets the viral genome, luciferase reporters with 8 viral RNA segments in the 3’-UTR were constructed. As shown in Figure. S1A, only the reporter activities of PA in H1N1 were reduced, while the activities of other segments in H1N1 were not changed upon treatment with hsa-miR-10a-5p. Whereas no obvious changes of reporter activities were observed for all 8 viral RNA segments of H3N2 under hsa-miR-10a-5p treatment. Perfect matches were observed between the PA segment (298–323 nt) of H1N1 but not H3N2 and hsa-miR-10a-5p by Miranda software. PA of H1N1 is confirmed as the target of hsa-miR-10a-5p (Fig. 1A). H1N1 and PA-mut H1N1 with the mutant hsa-miR-10a-5p binding sites that does not change the amino acid sequence, have the same ability to replicate under minimal hsa-miR-10a-5p (Fig. S1B-S1E). However, besides H1N1, hsa-miR-10a-5p also showed inhibitory effect on expression and replication of PA-mut H1N1 or H3N2 with no hsa-miR-10a-5p binding sites (Fig. 1B and Fig. 1C), indicating that host factors by which hsa-miR-10a-5p suppresses viral replication may be involved.

As a result, CAMK2B (Calcium/Calmodulin-dependent Protein Kinase II Beta) was identified according to the predicted targets of hsa-miR-10a-5p and the 295 known host factors that enhance IAV replication (Fig. 1D and Table S1)[27]. A putative hsa-miR-10a-5p complementary region in the CAMK2B (4084 to 4106 nt) is confirmed as the target of hsa-miR-10a-5p by the luciferase assay (Fig. 1E), real time PCR and western blotting (Fig. 1F and 1G). We found overexpression of CAMK2B pronouncedly elevated, while its

knockdown by RNAi reduced expression and replication of H1N1 (Fig. 2H and 2I). Furthermore, CAMKII inhibitors KN-93 or KN-62[28] suppressed viral replication in a dose-dependent manner (Fig. S1F-S1I). These data validate that CAMK2B as a host factor promoting expression and replication of H1N1.

CAMK2B phosphorylates threonine of viral M1 protein, which promotes binding of CRM1 and M1

CAMK2B, with a substrate preference for the Hyd-X-Arg-X-X-Ser*/Thr*-Hyd motif, was predicted to phosphorylate M1 and PB1 of IAV (Fig. 2A). Co-immunoprecipitation in H1N1 infected A549 cells showed that M1 protein interacts with CAMK2B (Fig. 2B). Subsequent experiments demonstrated that CAMK2B overexpression increased phosphorylated levels of wild type but not mutant M1 (mutation at T_{108}), identifying T_{108} of M1 as the specific phosphorylation site for CAMK2B (Fig. 2C).

During life cycle of IAV, M1 plays important roles in regulating nuclear import and export of vRNP[8]. TNPO1 and CRM1, are the transporter molecules essential for nuclear import and export of vRNP respectively[29, 30]. We found that overexpression of CAMK2B enhanced CRM1 but not TNPO1 levels bound to M1 (Fig. 2D). CAMK2B overexpression promoted the binding of CRM1 to wild type M1, while interaction of mutant M1 and CRM1 was not affected (Fig. 2E). The AlphaFold3-predicted modeling indicates that M1 phosphorylation facilitates its binding with CRM1 whether in the absence or presence of NEP, and the intermolecular interaction energies is shown in Table S2. Phosphorylated Thr_{108} of M1 formed hydrogen bonds with either Arg_{44} (with NEP) or Gln_{47} (without NEP) of CRM1, whereas M1 without phosphorylated Thr_{108} or mutant M1 failed to establish these interactions (Fig. 2F and 2G).

H1N1 PA mRNA upregulates the expression of CAMK2B through competitively binding to hsa-miR-10a-5p to promote vRNPs nuclear exporting

A very low ratio (1.98%) of hsa-miR-10a-5p vs. PA mRNAs or (9.88%) of CAMK2B mRNAs vs. hsa-miR-10a-5p was displayed (Fig. 3A) in A549 cells infected with H1N1, indicating that PA mRNAs can be effectively engaged with hsa-miR-10a-5p in terms of quantity. Treatment with wild-type but not mutant PA mRNA abruptly reduced hsa-miR-10a-5p levels by around 72% (Fig. 3B), and subsequently increased CAMK2B mRNA (Fig. 3C) and protein levels (Fig. 3D), and enhanced the CAMK2B luciferase activity (Fig. 3E) (all $P < 0.001$). The upregulation of CAMK2B by PA mRNA was blocked by hsa-miR-10a-5p inhibitor, suggesting that this effect is dependent on hsa-miR-10a-5p (Fig. 3F).

We further explored the influence of H1N1 infection on CAMK2B expression. H1N1 infection only enhanced the luciferase activity of wild-type but not the mutated CAMK2B in hsa-miR-10a-5p binding sites (Fig. 3G). After infection of wild-type H1N1, CAMK2B expression was continuously increased and showed a high degree of temporal correlation with PA expression (Fig. 3H). In contrast, no obvious increases of CAMK2B expression were observed under PA-mut H1N1 infection. A known hsa-miR-10a-5p target PIK3CA was also upregulated by H1N1 infection, while Akt that was not a hsa-miR-10a-5p target remained unaffected[31] (Fig. S2A-C). CLIP assays demonstrated that wt-PA mRNA directly binds hsa-

miR-10a-5p within the RNA-induced silencing complexes (RISCs), while much less CAMK2B mRNA was found in RISCs of cells infected with wild-type H1N1 than PA-mut H1N1 (Fig. 3I, 3J and Fig. S2D). In addition, more colocalization in the cytoplasm of the CRM1 and M1 was observed in wild-type H1N1, than PA mutant H1N1-infected cells (Fig. 3K). Wild-type H1N1 exhibited significantly higher replication capacity than the mutant type ($P < 0.001$) (Fig. 3L).

Altogether, the results indicate that in H1N1 infection, numerically dominant PA mRNA directly interacts with hsa-miR-10a-5p, and competitively deprived its binding to CAMK2B mRNA. This leads to de-suppression of the supportive host factor CAMK2B, facilitating the phosphorylation of M1 protein and its binding to CRM1, which promotes the nuclear export of vRNPs and accelerates viral replication (Fig. 3M).

SARS-CoV-2 3’ UTR up-regulates CCZ1B expression through competitively binding to hsa-miR-1307-3p

We further selected another respiratory virus SARS-CoV-2 to investigate the effect of cvRNA on viral infection. A549-ACE2 cells were transfected with plasmids expressing viral S, E, M, N mRNAs without expressing proteins, and viral 3’UTR, and scRNA-seq was performed to screen out the upregulated inflammatory factors and host factors associated with SARS-CoV-2 infection (Fig. S4-S6)[17, 32–37]. Real-time-PCR results validated that S and N mRNAs significantly upregulated multiple inflammatory cytokines and chemokines, including IL6, TNF-α, CCL2, CCL3, CCL5, CXCL10 and TNF-α, while 3’UTR and E mRNAs increased host factor CCZ1B expression. Highly expressed miRNAs in A549-ACE2 cells that were predicted to bind to both mRNAs of SARS-CoV-2 S (Fig. S7A) or N (Fig. S7B) and respective inflammatory factors were screened out, indicating that cvRNA mechanism may be involved in immunopathology during SARS-CoV-2 infection (Table S7-S9). Similarly, hsa-miR-1307-3p was predicted to bind both SARS-CoV-2 3’UTR and host factor CCZ1B mRNA[38], and hsa-miR-186-5p was predicted to bind SARS-CoV-2 E and CCZ1B mRNA (Fig. S7C) .

Transfection with SARS-CoV-2 3’UTR, significantly decreased hsa-miR-1307-3p (Fig. 4A). Matches between the SARS-CoV-2 3’UTR or CCZ1B and hsa-miR-1307-3p are shown (Fig. 4B). Luciferase assay reveals that SARS-CoV-2 3’UTR and CCZ1B are both the targets of hsa-miR-1307-3p. Furthermore, hsa-miR-1307-3p mimic decreased while its inhibitor increased CCZ1B expression (Fig. 4C). Treatment with wild type but not mutant SARS-CoV-2 3’UTR within the hsa-miR-1307-3p binding sites reduced hsa-miR-1307-3p levels and thereby increased CCZ1B expression (Fig. 4D and Fig. 4E). The downregulation effect of CCZ1B by hsa-miR-1307-3p was reversed under co-transfection with SARS-CoV-2 3’UTR (Fig. 4F). Whereas the upregulation effect of CCZ1B by SARS-CoV-2 3’UTR was mostly weakened under co-transfection with hsa-miR-1307-3p inhibitor (Fig. 4G).

As shown in Figure. 4H, SARS-CoV-2-3’UTR has the highest abundance, followed by hsa-miR-1307-3p, while CCZ1B has the lowest in 3’UTR transfected A549-ACE2 cells. RIP results demonstrated that

compared to mutant SARS-CoV-2-3’UTR, treatment with wild type 3’UTR led to much higher of 3’UTR and less CCZ1B mRNA detected in RISCs (Fig. 4I), indicating functional engagement to hsa-miR-1307-3p.

CCZ1B promotes ACE2 transport from early to late endosome and facilitates infection of JN.1 pseudovirus

Maturation of early to late endosomes is crucial for maintaining endocytosis and recycling of intracellular membrane proteins in eukaryotic cells. One hallmark of endosomal maturation is the transition from the early endosomal Rab5 to the late endosomal Rab7. During the transition process, one key regulator is the conserved RAB7-GEF (guanine nucleotide exchange factor) complex MON1-CCZ1B[39]. The third subunit of the MON1-CCZ1 complex is RMC1 protein in human cells[40]. RAB7A-GTP, the active form of RAB7A mediated by RAB7-GEF, is crucial for the proper functioning of late endosomes and for the transport of vesicles to lysosomes. As seen in Fig. 5A, CCZ1B knocking down reduced and overexpression increased RAB7A-GTP levels. Co-immunoprecipitation results confirmed the interaction between MON1, CCZ1B, RMC1, and RAB7A(Fig.S8A). Further Structural modeling through the AlphaFold2 suggested that human MON1-CCZ1-RMC1 could resemble the trimer of D.melanogaster, and LD1 of CCZ1B, LD1 and LD2 of MON1 formed the RAB7A binding sites (Fig. S8B).

Next, we determine whether CCZ1B-mediated endosomal maturation vesicle transport and the trafficking affect ACE2 localization and recycling in cells. Knockdown or overexpression of CCZ1B in A549-ACE2 cells did not change total ACE2 protein levels (Fig.S8C). In cells with CCZ1B knockdown, ACE2 mainly localized at in EEA1-positive early endosomes, while CCZ1B overexpression increased its colocalization with LAMP1 + compartment, suggesting that CCZ1B promotes ACE2 trafficking from early to late endosomes (Fig. 5B). A similar phenomenon was observed for SARS-CoV-2 3'UTR (Fig. 5C). Additionally, we found that CCZ1B had no effect on the stability of ACE2 (Fig. 8D).

As seen in Fig. 5D, CCZ1B siRNA reduced, whereas its overexpression enhanced cell membrane ACE2 levels by flow cytometry. RAB7A inhibitor largely blocked the CCZ1B-mediated increase of cell membrane ACE2 (Fig. 5E), indicating that regulation of ACE2 transport by CCZ1B is dependent on RAB7A. Treatment with hsa-miR-1307-3p decreased cell membrane ACE2 levels (Fig. 5F). Transfection with SARS-CoV-2 3’UTR but not mutant 3’UTR significantly upregulated ACE2 level on the cell membrane ($P < 0.001$) (Fig. 5G). Hsa-miR-1307-3p inhibitor almost blocked the ability of SARS-CoV-2 3’UTR to increase ACE2 (Fig. 8E). The VSV-M protein levels and the green fluorescence in JN.1 pseudovirus infected cells represented the ability of virus infection. CCZ1B knockdown inhibited infection of JN.1 pseudovirus, whereas CCZ1B overexpression or wt-3'UTR potentiate the infection (Fig. 5H and 5I, Fig. S8F).

Collectively, SARS-CoV-2 3’UTR up-regulates the expression of CCZ1B by exclusionary binding to endogenous hsa-miR-1307-3p. CCZ1B forms a complex with MON1 and RMC1 to activate RAB7A, which promotes the transport of ACE2 from early to late endosome and to plasma membrane, and thereby enhancing SARS-CoV-2 infection (Fig. S8G and Fig. S8H).

The cvRNAs regulation mechanism may be prevalent in respiratory viral infection, replication and evolution

To clarify the quantitative relationship between cvRNAs and host factors during early respiratory viral infection, a plasmid containing the SARS-CoV-2 3'UTR was transfected into cells. The copy numbers of CCZ1B mRNA (A) and SARS-CoV-2 3'UTR RNA (C) at different time points were measured by qPCR. The copy numbers at 0 h and 24 h were taken as the initial (A0 and C0) and final concentrations (Ae and Ce) of CCZ1B mRNA and the SARS-CoV-2 3'UTR RNA, respectively. Here, A0 = 161.2, C0 = 47.94, Ae = 521.00, Ce = 17320.00. According to the derived functional formula: $Ae/A0 = (Ce/C0)^{(k_A/k_C)}$, the relationship between CCZ1B mRNA and SARS-CoV-2 3'UTR RNA is finally obtained as: $A = 74.4 * C^{0.199}$ (Fig. 6A). Similarly, the relationship between CAMK2B mRNA (A) and H1N1 PA RNA/mRNA (C) is finally obtained as: $A = 0.569 * C^{0.511}$. The curve graph indicates that SARS-CoV-2 3'UTR or H1N1 PA RNA/mRNA is involved in positive regulation of CCZ1B or CAMK2B expression (Fig. 6B).

According to the seed sequence of PA in H1N1 complementary to hsa-miR-10a-5p (Fig. 6C), we analyzed the sequence in different subtypes of IAV and found that this sequence was relatively conserved in IAV (Fig. 6D and Fig. 6E), suggesting a common regulation of host CAMK2B via the cvRNA mechanism during IAV infection. This sequence had much higher alignment particularly in H1N1 and H7N9 (Fig. 6F). Furthermore, the phosphorylation substrate sequence of CAMK2B in M1 protein is highly conserved among different IAVs (Fig. S3A).

Similarly, the binding sequences of hsa-miR-1307-3p in SARS-CoV-2 variants were analyzed. As shown in Fig. 6G, the seed sequence of hsa-miR-1307-3p in Hu1.1, Beta, Gamma and Omicron BA.1.1 were completely consistent, reflecting a high degree of conservation in this sequence. In contrast, later Omicron subvariants show more mutations or mostly deletions in these target sequences. Besides, except for SARS-CoV-2 and SARS-CoV, other coronaviruses are basically lack of the binding sequences of hsa-miR-1307-3p (Fig. 6H).

In addition to miR-10a-5p, we also predicted the binding of 64 miRNAs (Table S4) among the 163 miRNAs (Table S3) with high expression levels in human lung epithelial cells[26] that could bind to the 3'UTRs of 258 host factors[27], and all of them may interact with at least one of the 8 mRNA segments of H1N1 (Table S5). The interaction features of host factors, viral genes and miRNAs is visualized by the Sankey diagram (Fig. S3B). This indicates that cvRNA-miRNA-host factor axes seem to be a widespread regulation mechanism in influenza virus infections and replication.

Discussion

Respiratory viruses, mostly belonging to RNA viruses, generally produce substantial amounts of redundant RNA and mRNA after infection. In this study, we found that H1N1 PA mRNA competitively binds to hsa-miR-10a-5p to relieve its inhibition of the host factor CAMK2B. Once freed from miRNA suppression, CAMK2B phosphorylates viral M1 protein, which facilitates the nuclear export of vRNPs.

This process is crucial for the efficient replication of the virus, as it enables the viral components to exit the nucleus and assemble, ultimately leading to the production of new viral particles. Indeed, PA-mut H1N1 with mutant hsa-miR-10a-5p binding sites exhibited significantly lower replication ability compared to wildtype H1N1 (Fig. 3L). Sequence alignment show that the seed sequences of H1N1 and H7N9 PA mRNA have a higher match with hsa-miR-10a-5p (Fig. 6E), which may contribute to their high replication capabilities.

Meanwhile, our study reveals another cvRNA regulation mechanism by which numerically dominant 3' UTR of SARS-CoV-2 binds to and sequesters hsa-miR-1307-3p, thereby alleviating its inhibition of CCZ1B. CCZ1B interacted with MON1 and RMC1 to form a complex, which activates RAB7A and drives late endosomal maturation [48]. ACE2 may be retrieved and recycled back to the plasma membrane from late endosomes, facilitating infection by SARS-CoV-2. This process involves endosomal sorting and specific recognition mechanisms [49]. Notably, we observed 3'UTR of earlier SARS-CoV-2 strains including alpha, beta, gamma, and Omicron BA.1 harbor the conserved hsa-miR-1307-3p binding sequences, whereas most Omicron subvariants including BA.2, XBB.1.5 and JN.1.7, etc. exhibit mutations or deletions in these sequences. Most Omicron variants exhibit lower airborne transmission efficiency than Delta in hamsters, although human epidemiological data indicate that these Omicron variants spread rapidly around the globe[41]. This suggests potential role of cvRNA mechanism in the virus's infectivity and transmission. Besides SARS-CoV-2 3'UTR, SARS-CoV-2 E mRNA was also found to upregulate CCZ1B, but no shared miRNA was validated (see Fig. 4A, Fig. S6A-S6C and and Fig. S7C). There may be possibly other miRNAs that simultaneously bind to SARS-CoV-2 E and CCZ1B mRNAs in a non-classical manner.

Phosphorylation at T_{108} is required for IAV M1 nuclear localization, nuclear vRNP export, M1 multimerization, and the binding of M1 to the cellular striatin-interacting phosphatases and kinases (STRIPAK) complex[42]. However, the kinase responsible for T_{108} phosphorylation was unknown. In the present study, T_{108} of M1 protein was identified as the phosphorylated site of CAMK2B (Fig. 2), which phosphorylates various target proteins, including other kinases, transcription factors, and cytoskeletal proteins, leading to diverse cellular responses[43].We speculate that M1 protein might bind to CRM1 protein directly, and CAMK2B-mediated T_{108} phosphorylation may facilitate M1-CRM1 binding and vRNP nuclear export. This is consistent with an earlier research showing that M1 amino acid residues 76 to 115 are necessary for vRNP nuclear export[44]. The T_{108} of M1 may be connected to the 101 RKLKR 105 sequence of M1, which has been reported to mediate binding of M1 to RNA[45] and function as a nuclear-localization signal (NLS)[46].

The upregulation of inflammatory factors and the occurrence of a cytokine storm are prominent features of COVID-19 infection. SARS-CoV-2 infection elevates IL-6, IL-1, and IFN-γ, which causes activation of JAK/STAT pathway[47]. In addition, cytokines such as IL-1, IL-18, and TNF-α may activate p38 MAPK signal pathway, and thus more chemokines and pro-inflammatory cytokines are released[48]. Our study demonstrates that SARS-CoV-2 S and N mRNAs function as cvRNAs, and enhance the expression of

inflammatory factors such as CCL2, TNF-α, IL6, and CXCL10 (Fig.S7), which may contribute to the hyper-inflammatory responses observed in viral infection. This deserves further investigation.

To conclude, our study demonstrates that reciprocal regulation among viral RNA-miRNA-host factor mRNA plays a significant role in promoting influenza virus replication and enhancing SARS-CoV-2 infection. This not only reveals how viral RNA/mRNA alone can influence infection, replication and pathogenesis, but also may help to develop targeted strategies to disrupt the interaction between viral RNAs and the host's cellular machinery. In addition, mutations in the non-coding regions or nonsense mutations in coding regions of virus may also influence viral infection and replication through cvRNA mechanisms, which will provide epidemiological insights in predicting outbreak patterns and the emergence of new viral strains of respiratory viruses.

Declarations

Authors' contributions

S.M, and C.L concepted and designed the study. Z.W, L.Q, Z.Z, R.L, Z.L and S.P performed the experiments. F.C, and J.W analyzed data. Z.W, L.Q, Z.Z, and C.L made the statistical analysis and wrote the manuscript. C.L. and S.M supervised this work. All authors read and approved the final manuscript.

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Figures

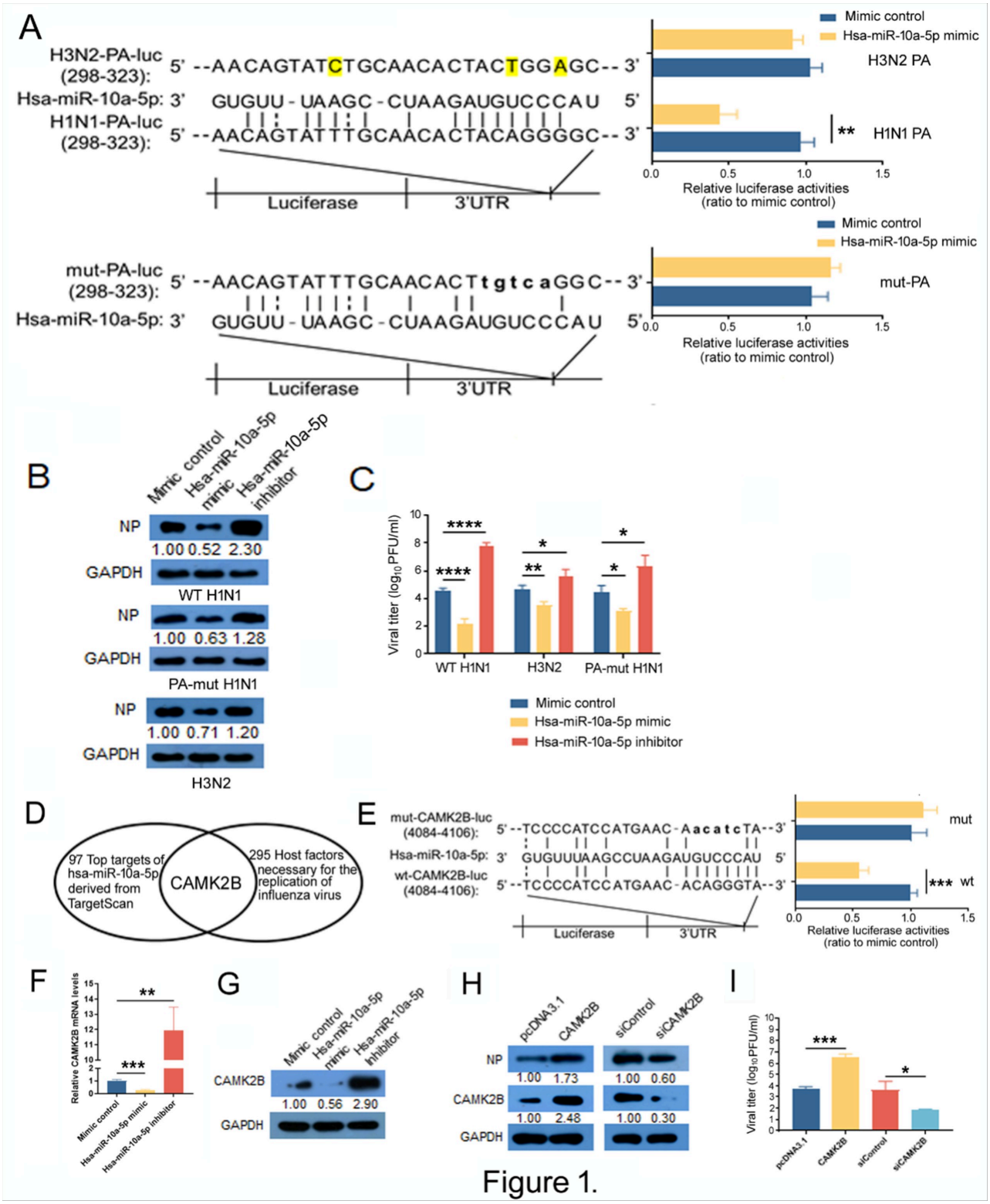


Figure 1.

Figure 1

Analysis of the effect of hsa-miR-10a-5p or CAMK2B, a target of hsa-miR-10a-5p on replication of influenza virus

(A) Matches between the H1N1, H3N2 PA or mutant PA (H1N1) segment (298-323nt) and hsa-miR-10a-5p were shown (Left). HEK293T cells were co-transfected with wt-PA-luc or mut-PA-luc, pRL-TK and hsa-

miR-10a-5p mimic. At 48 hours post-transfection, the firefly luciferase activity was measured (Right). (B, C) A549 cells were transfected with hsa-miR-10a-5p mimic or inhibitor for 24 hours, followed by infection with H1N1, H3N2 or PA-mut H1N1 (MOI = 0.1) for 24 hours. NP protein levels (B) and viral titers (C) were detected. (D) Venn diagram representing CAMK2B that was a predicted target of hsa-miR-10a-5p and as a host factor to enhance the replication of H1N1. (E) Complementary pairing of CAMK2B 3'UTR (4084-4106 nt) and hsa-miR-10a-5p seed sequences (Left). HEK293T cells were co-transfected with wt-CAMK2B-luc or mut-CAMK2B-luc, pRL-TK and hsa-miR-10a-5p mimic. At 48 hours post-transfection, the firefly luciferase activity was detected (Right). (F, G) A549 cells were transfected with hsa-miR-10a-5p mimic or hsa-miR-10a-5p inhibitor. At 24 hours post-transfection, CAMK2B mRNA (F) and protein levels (G) were determined. (H, I) A549 cells were transfected with CAMK2B expression vector or CAMK2B siRNA, and infected with H1N1 (MOI = 0.1). At 24 hours, NP, CAMK2B protein levels (H) and viral titers (I) were detected. Data are presented as mean ± sd from three independent experiments. P value: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

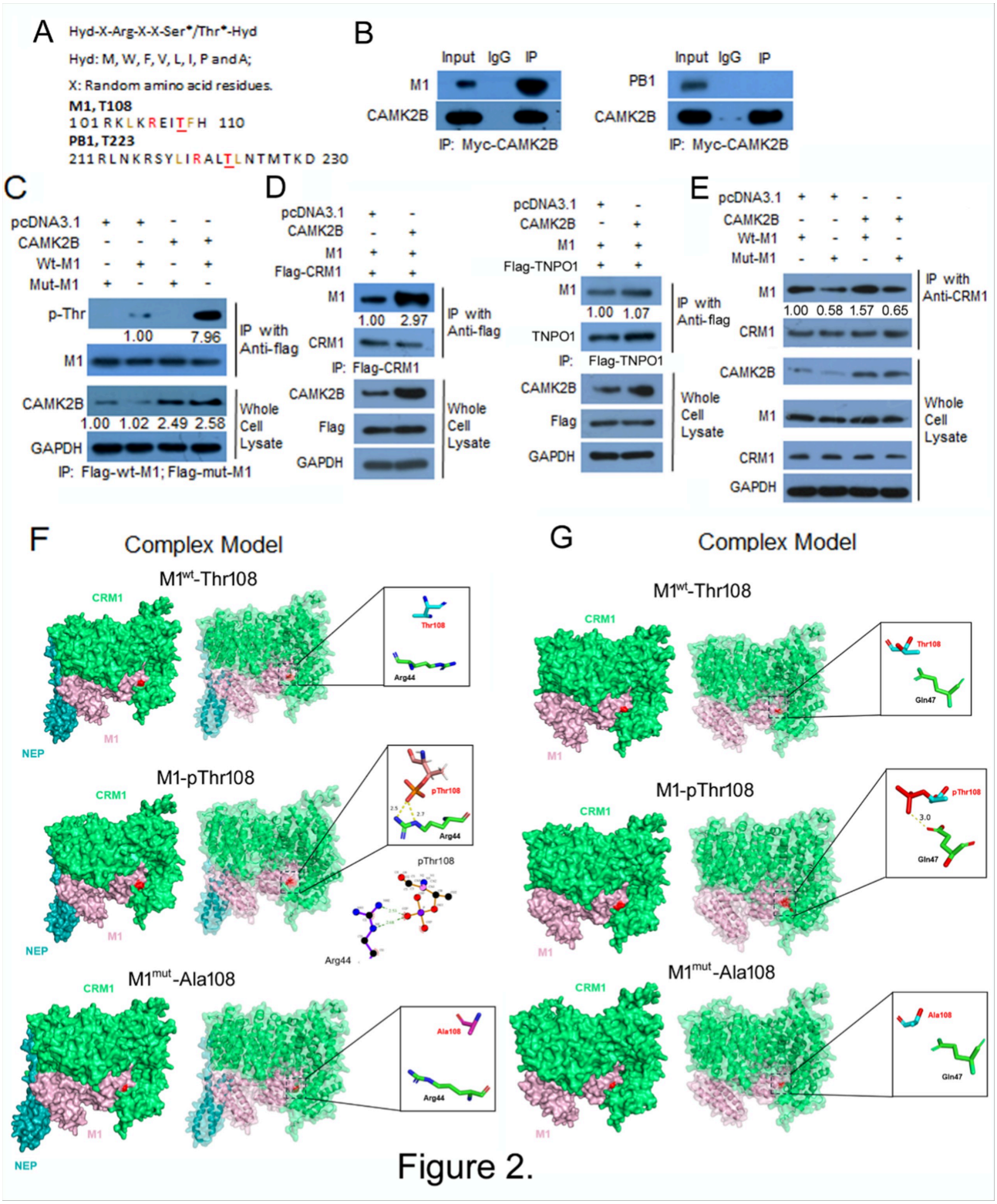


Figure 2.

Figure 2

CAMK2B promotes nuclear export of H1N1 vRNP by phosphorylates M1 protein

(A) Phosphorylated substrate preference sequences of CAMK2B in M1 and PB1 protein were shown. (B) A549 cells were transfected with CAMK2B expression vector and infected with H1N1. Immunoprecipitates were analyzed for CAMK2B, M1 and PB1. (C, E) A549 cells were co-transfected with

wt-M1 or mut-M1 and CAMK2B expression vector for 24 hours. The protein levels of M1, CAMK2B and phosphorylated threonine (C) or CRM1(E) were detected in the immunoprecipitates. (D) A549 cells were co-transfected with the CRM1 or TNPO1, M1 and CAMK2B expression vector for 24 hours. Immunoprecipitates were analyzed for M1, CRM1 or TNPO1 protein levels. (F) The complexes containing NEP were modeled using AlphaFold3, and the CRM1-M1 ($M1^{wt}$-Thr_{108}, M1-$pThr_{108}$ or $M1^{mut}$-Ala_{108}) binding interfaces were analyzed. (G) AlphaFold3 models the binding of CRM1 with phosphorylated M1, wt-M1 or mut-M1.

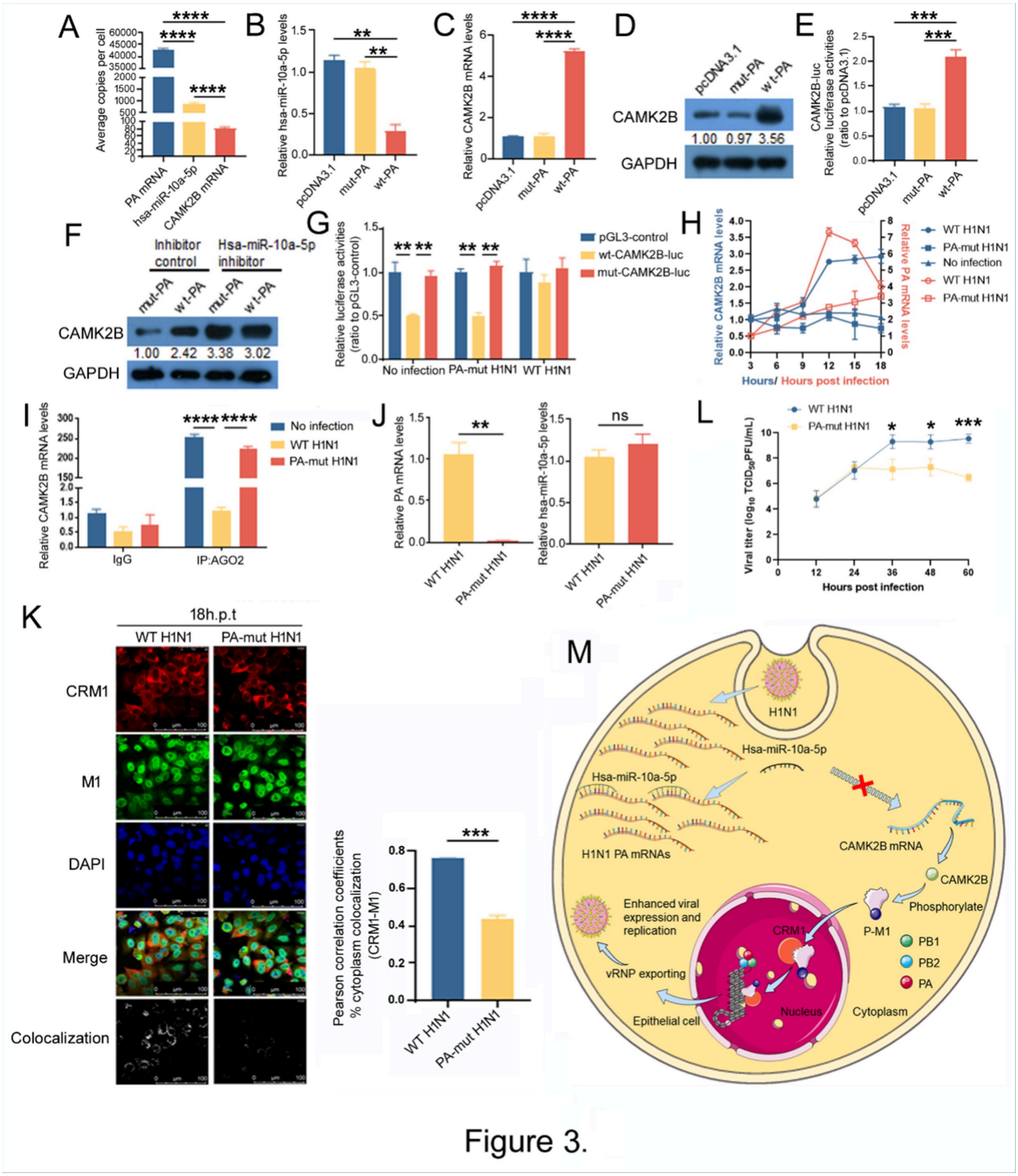


Figure 3.

Figure 3

PA mRNA upregulates expression of CAMK2B by miR-10a-5p

(A) The copy numbers of PA, hsa-miR-10a-5p and CAMK2B mRNAs in A549 cells infected with H1N1. (B, C) At 24 hours post-transfection with wt-PA or mut-PA, levels of hsa-miR-10a-5p (B), CAMK2B mRNA (C) and protein (D) were measured. (E) HEK293T cells were co-transfected with wt-PA or mut-PA and wt-

CAMK2B-luc. At 48 hours, the firefly luciferase activity was measured. (F) A549 cells were co-transfected with wt-PA or mut-PA and hsa-miR-10a-5p inhibitor for 24h, and CAMK2B protein levels were detected. (G) A549 cells were co-transfected with wt-CAMK2B-luc or mut-CAMK2B-luc and pRL-TK, then, cells were infected with the H1N1 or PA-mut H1N1 at 24 h.p.t. The firefly luciferase activity was measured and standardized at 48 h.p.t. (H) The mRNA levels of CAMK2B and PA in A549 cells infected with H1N1 or PA-mut H1N1 (MOI = 3.0). (I, J) A549 cells infected with H1N1 or PA-mut H1N1 were cross-linked, followed by immunoprecipitation with anti-AGO2. The mRNA levels of CAMK2B (I), PA or hsa-miR-10a-5p (J) were detected in RISC. (K) At 18 hours after 293T cells were transfected with the plasmids CRM1 and reverse genetics system expressing mutant PA or wild type PA, cells were incubated with anti-CRM1 and anti-M1 antibody. Images shown are representative of two independent experiments, and the colocalization of M1 and CRM1 in cytoplasm was quantified with the Pearson correlation coefficients. (L)A549 cells were infected with H1N1, or PA-mut H1N1 (MOI = 0.01). At indicated times, viral titers were detected. (M) Diagrammatic drawing of the cvRNA mechanism caused by H1N1 infection. Data are presented as mean ± sd from three independent experiments. P value: ns (not significant) > 0.05, *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.

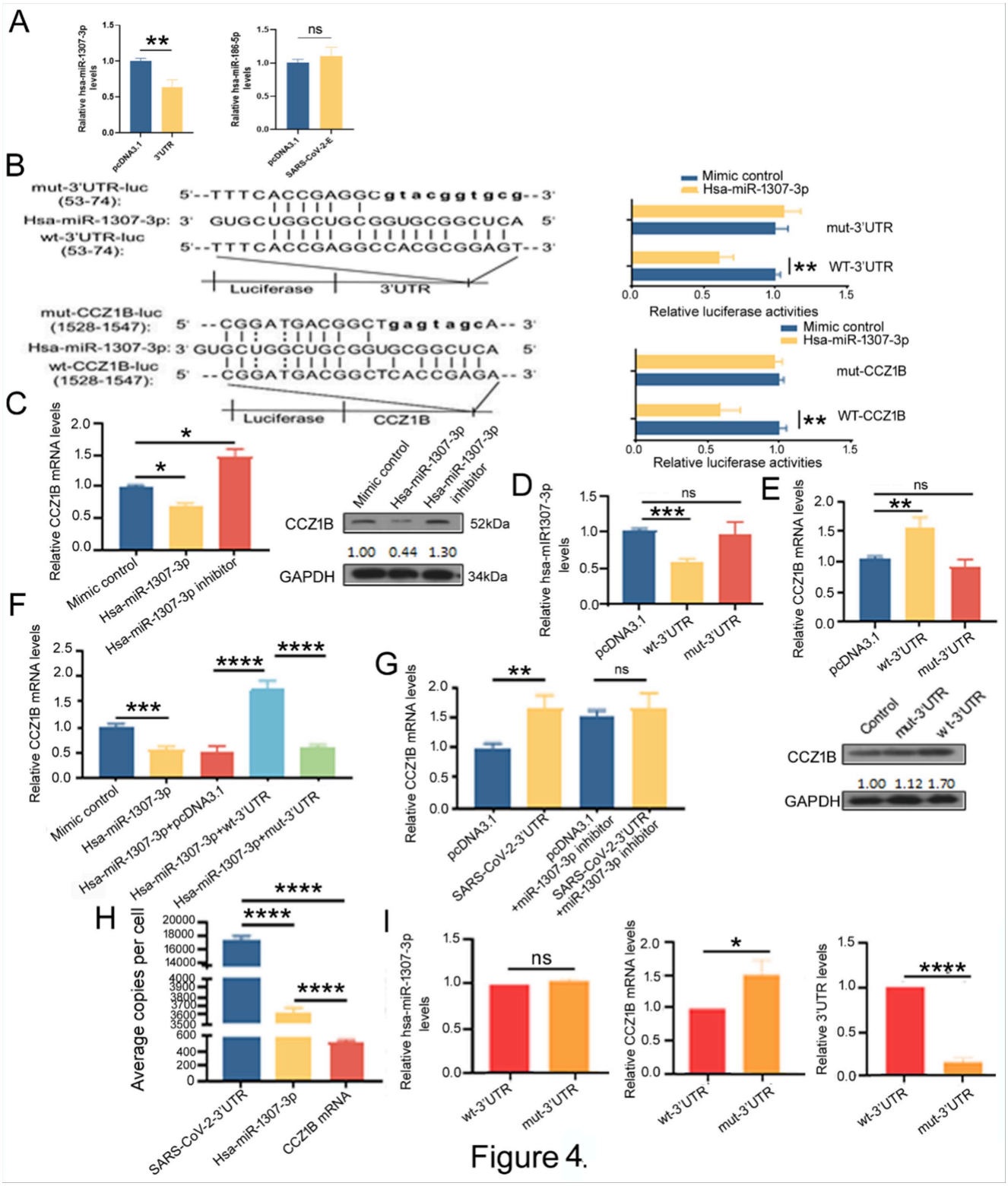


Figure 4

SARS-CoV-2 enhances the expression of CCZ1B via cvRNA.

(A) hsa-miR-1307-3p levels in cells transfected with the SARS-CoV-2 3'UTR or hsa-miR-186-5p levels in cells transfected with the SARS-CoV-2 E. (B) Matches of SARS-CoV-2 3'UTR or CCZ1B 3'UTR (1528-1547nt) and hsa-miR-1307-3p seed sequences. The firefly luciferase activity in HEK293T cells co-

transfected with wt-3’UTR-luc or mut-3’UTR-luc or (wt-CCZ1B-luc or mut-CCZ1B-luc) and hsa-miR-1307-3p. (C) mRNA and protein levels of CCZ1B in cells transfected with hsa-miR-1307-3p or inhibitor. (D, E) levels of hsa-miR-1307-3p (D) and CCZ1B (E) in A549-ACE2 cells transfected with wt-3’UTR or mut-3’UTR. (F, G) CCZ1B mRNA of A549-ACE2 cells transfected with hsa-miR-1307-3p or miR-1307-3p-inhibitor and wt-3’UTR or mut-3’UTR. (H) Copy numbers of SARS-CoV-2 3'UTR, hsa-miR-1307-3p or CCZ1B mRNAs in A549-ACE2 cells transfected with the SARS-CoV-2 3'UTR. (I) A549-ACE2 cells transfected with wt-3’UTR or mut-3’UTR underwent immunoprecipitation with anti-Ago2 antibody. Hsa-miR-1307-3p, CCZ1B or SARS-CoV-2 3'UTR levels were detected. Data are presented as mean ± sd from three independent experiments. P value: ns (not significant) > 0.05, *, $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

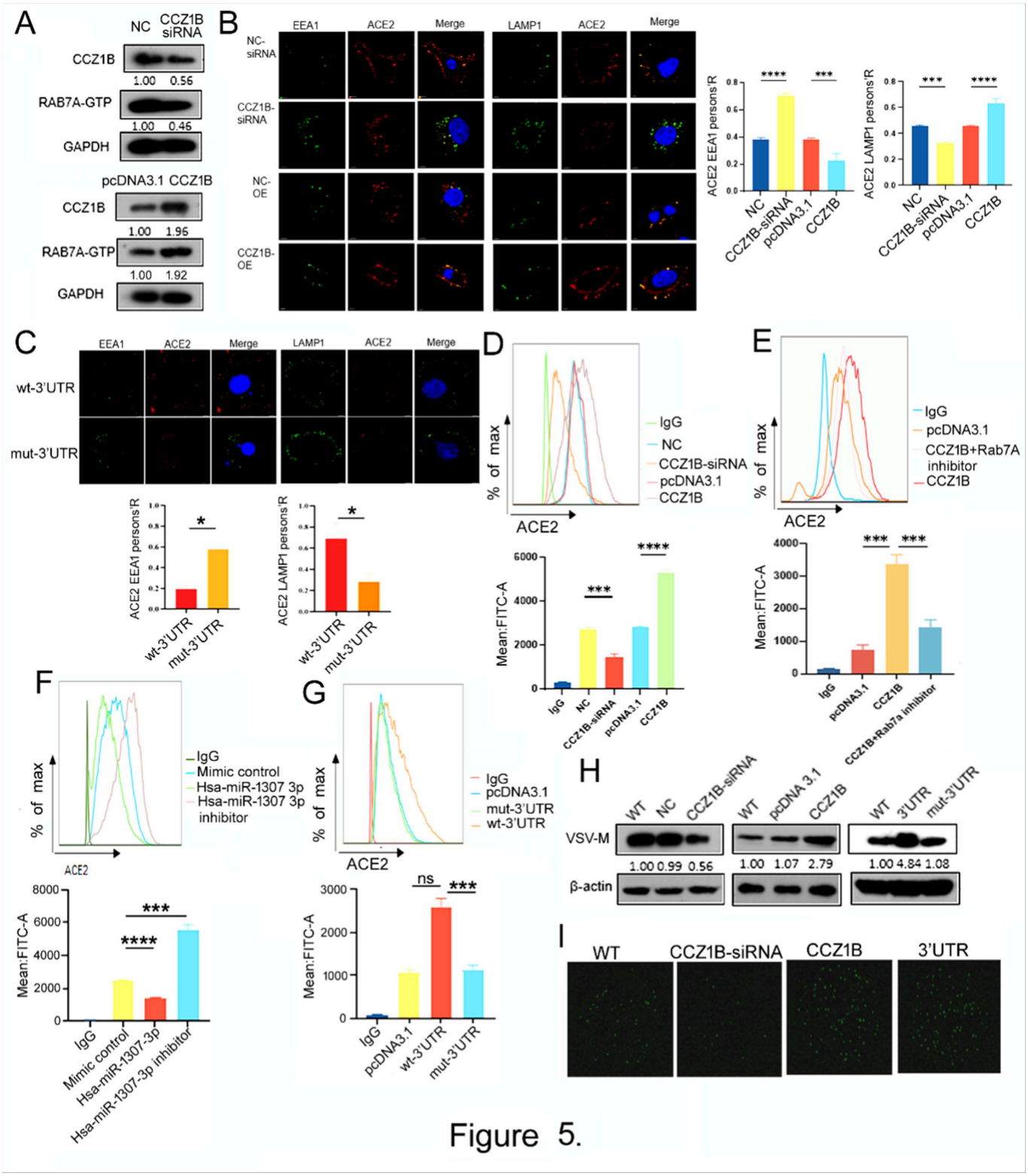


Figure 5.

Figure 5

CCZ1B promotes the transport of ACE2 from early to late endosome and JN.1 pseudovirus infection.

(A) CCZ1B and RAB7A-GTP levels in A549-ACE2 cells transfected with CCZ1B siRNA or CCZ1B plasmid (pCCZ1B). (B, C) Co-localization of ACE2 (Red) with EEA1 or LAMP1 (Green) in cells transfected with CCZ1B siRNA, pCCZ1B (B) or wt-3'UTR, mut-3'UTR (C) and quantification of colocalization with the

Pearson correlation coefficients. (D, E) FACS analysis of ACE2 on the cell membrane of cells transfected with CCZ1B siRNA or pCCZ1B with or without RAB7A inhibitor for 12 hours. (F, G) ACE2 on the cell membrane of cells transfected with hsa-miR-1307-3p, hsa-miR-1307-3p inhibitor (F) or wt-3'UTR, mut-3'UTR(G). (H, I) VSV-M levels in cells transfected with CCZ1B siRNA or pCCZ1B, wt-3'UTR, mut-3'UTR (H) and the green fluorescence of JN.1 pseudovirus with GFP in cells transfected with CCZ1B siRNA, pCCZ1B or SARS-CoV-2 3'UTR (I). Data are presented as mean ± sd from three independent experiments. P value: ns (not significant) > 0.05, *p < 0.05, ***p < 0.001, ****p < 0.0001.

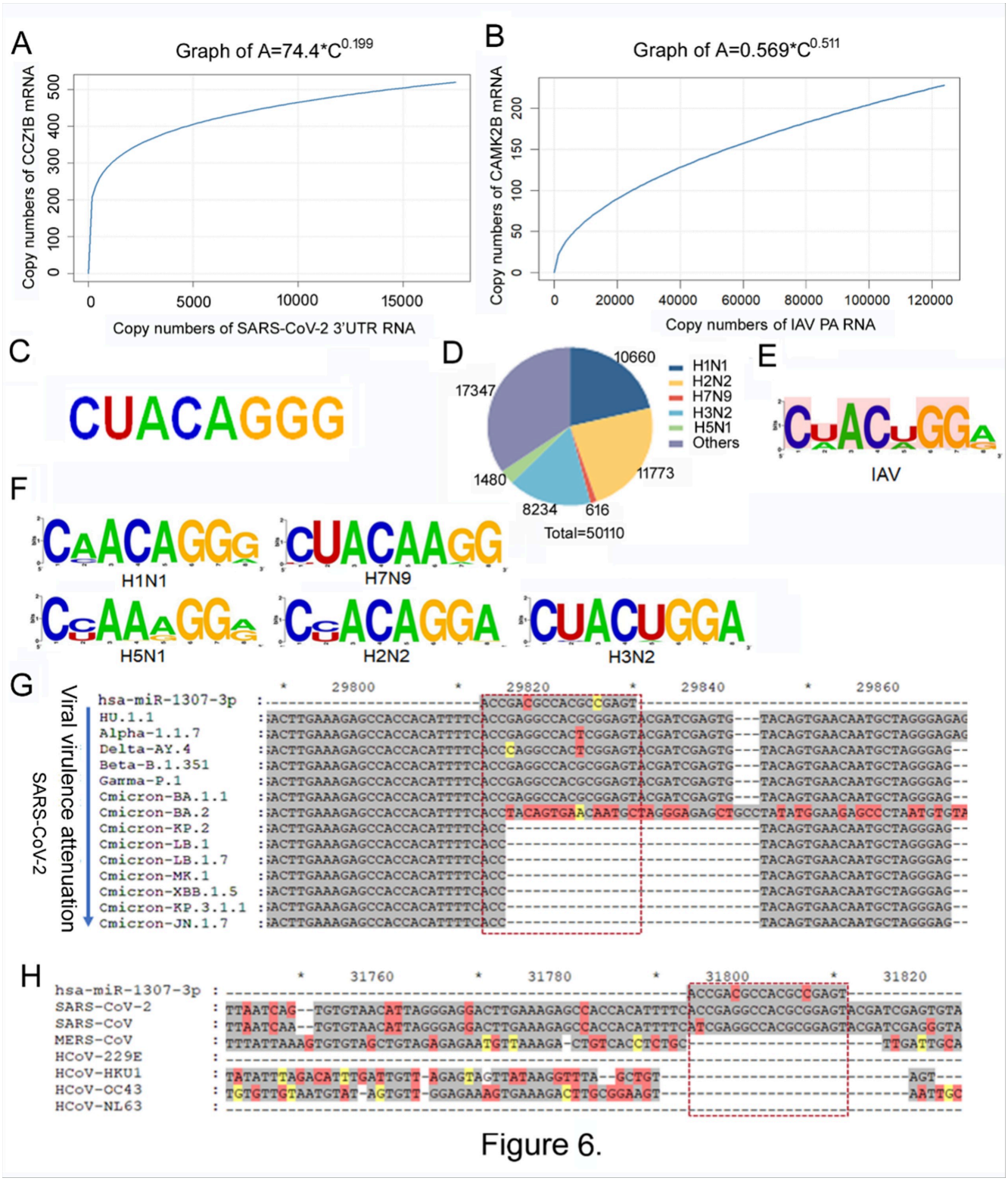


Figure 6.

Figure 6

The analysis of homology and quantitative relationship of cvRNAs

(A, B) The curve graph of SARS-CoV-2 3'UTR or IAV PA and CCZ1B or CAMK2B mRNA. (C) The seed sequence complementary to hsa-miR-10a-5p of PA segments in H1N1. (D) Total number of sequences, the breakdown of contributing strains H1N1, H2N2, H7N9, H3N2, H5N1 and others are indicated in the pie charts. (E) Publicly available and unique full-length sequences of the PA segments (314- 321 nt) were aligned. (F) The variations of the seed sequence complementary to hsa-miR-10a-5p of PA segments in different subtypes of IAV. (G, H) The binding sequences of hsa-miR-1307-3p on various strains of SARS-CoV-2 (G) or different coronavirus 3 'UTR (H) were analyzed.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS4The64miRNAsbindingtothe3UTRsof258hostfactors.csv
- TableS5The64miRNAsbindingtothe8H1N1viralmRNAs.csv
- supplementalmaterials.docx