Cellular hnRNPAB binding to viral nucleoprotein inhibits flu virus replication by blocking nuclear export of viral mRNA

HIGHLIGHTS
HnRNPAB inhibits influenza A virus replication by repressing viral mRNA nuclear export
HnRNPAB interrupts viral mRNA transferring from ALY to NXF1
NP cooperates with hnRNPAB to further restrict viral mRNA nuclear export
The ALY-viral mRNA binding is restricted by NP-hnRNPAB complex
Cellular hnRNPAB binding to viral nucleoprotein inhibits flu virus replication by blocking nuclear export of viral mRNA

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SUMMARY

Heterogeneous nuclear ribonucleoproteins (hnRNPs) play critical roles in the nuclear export, splicing, and sensing of RNA. However, the role of heterogeneous nuclear ribonucleoprotein A/B (hnRNPAB) is poorly understood. In this study, we report that hnRNPAB cooperates with nucleoprotein (NP) to restrict viral mRNA nuclear export via inhibiting viral mRNA binding to ALY and NXF1. HnRNPAB restricts mRNA transfer from ALY to NXF1, inhibiting the mRNA nuclear export. Moreover, when cells are invaded by influenza A virus, NP interacts with hnRNPAB and interrupts the ALY-UAP56 interaction, leading to repression of ALY-viral mRNA binding, and then inhibits the viral mRNA binding to NXF1, leading to nuclear stimulation of viral mRNA. Collectively, these observations provide a new role of hnRNPAB to act as an mRNA nuclear retention factor, which is also effective for viral mRNA of influenza A virus, and NP cooperates with hnRNPAB to further restrict the viral mRNA nuclear export.

INTRODUCTION

Influenza A virus (IAV), a member of the Orthomyxoviridae family, poses a serious threat to public health. The genome of IAV consists of eight single-stranded negative-sense RNA segments, which are coated by a nucleoprotein (NP) to form viral ribonucleoprotein complex (vRNP) with viral polymerase (Julia et al., 2014). The vRNP comprises viral RNA (vRNA), NP, and heterotrimeric RNA-dependent RNA polymerase (Fodor, 2013; Resainfante et al., 2011). After IAV infection, the vRNP is transported into the nucleus of the host cell, followed by transcription and viral gene replication in the nucleus. NP is a core component of genomic vRNP and takes part in the organization of RNA packing, nuclear trafficking, and vRNA transcription and replication. RNA binding to NP is critical for the vRNP complex. NP is more than just a structural protein of IAV as it has been reported to interact with multiple cellular factors (Kawaguchi et al., 2011; Luo et al., 2018; Naito et al., 2007; Wang et al., 2009), which influences the replication of IAV. NP contains both a nuclear localization signal (NLS) and nuclear export signals (NES), which are critical for vRNP transporting through the nucleus during the virus life cycle (Li et al., 2015; Pemberton et al., 1998). However, whether NP is involved in modulating the host cell life cycle is still unknown.

Nuclear retention of mRNA is essential for gene expression in cells. Misprocessed RNAs or spurious transcripts from intergenic DNA regions are mostly retained in the nucleus and then degraded. Nuclear retention of mRNA can restrict misprocessed mRNA translation, which might be translated into toxic proteins and be harmful to the cell. Specialized cis-elements in certain RNAs and mistakes in splicing, polyadenylation, and RNA modifications all lead to RNA nuclear retention (Palazzo and Lee, 2018). Except for the misprocessed RNAs, the nuclear export of certain normally spliced and polyadenylated mRNA is also restricted to protect cytoplasmic transcript levels from transcriptional bursts (Halpern et al., 2015). Aberrant mRNA nuclear retention might lead to cell disability or disease. For example, an expansion mutation in polyadenylate RNA-binding protein 1 leadsto oculopharyngeal muscular dystrophy (Yotam and Raz, 2014); a mutation in COL1A1 (encoding collagen, type I, alpha 1) affects the splicing of intron 26 and leads to intron retention, which causes type I osteogenesis imperfecta (Carol Johnson et al., 2000). Whether there is a cell factor to restrict viral mRNA nuclear export during infection is still unknown.

The mRNA nuclear export is considered a key step for gene expression, and it requires the mRNA transport receptor NXF1-NXT1 (Bjork and Wieslander, 2014; Walsh et al., 2010). Once the exporting mRNA is...
Figure 1. HnRNPAB inhibits the replication of different subtypes of influenza A virus
(A) Dynamics of hnRNPAB mRNA in cells infected with influenza A virus. A549 cells were infected with H7N9, H5N1, H9N2, H3N2, or PR8 viruses (MOI = 1.0), respectively. The abundance of viral mRNA at the indicated time was detected using qRT-PCR. Expression was normalized to the mRNA level.
hnRNPAB plays an inhibitory role in flu A virus replication

HnRNPAB plays critical roles in nuclear export, splicing, and sensing of vRNA for IAV replication to trigger innate immune responses (Cao et al., 2019; Mor et al., 2017; Tsai et al., 2013; Wang et al., 2014). The hnRNPAB (AB), a member of hnRNP family, is an abundant RNA-binding protein first isolated in 1987 from 40S hnRNP particles (Akindahunsi et al., 2005; Dean et al., 2002). HnRNPAB is involved in transcription, alternative splicing, mRNA transport and localization, translation, mRNA instability, and telomere maintenance (Amit et al., 2012; Lampasona and Czaplinski, 2019; Nanaho et al., 2013; Raju et al., 2008; Yang et al., 2019). However, whether and how hnRNPAB is involved in mRNA nuclear export is not clear.

In this study, we demonstrated that hnRNPAB acts as an mRNA nuclear retention factor to regulate gene expression. In IAV-infected cells hnRNPAB interacts with NP via RNA to restrict viral mRNA nuclear export. Mechanistically, hnRNPAB interacts with the mRNA nuclear export factors, ALY and NXF1, and interrupts the mRNA transfer from ALY to NXF1, leading to mRNA nuclear retention. When cells were infected by IAV, hnRNPAB interacts with NP of IAV to interrupt the interaction of ALY and UAP56, leading to the repression of ALY-viral mRNA binding, and therefore the viral mRNA could not be transferred to NXF1 for nuclear export successfully.

RESULTS

Cellular hnRNPAB plays an inhibitory role in flu A virus replication

In primary cells, hnRNPAB interacts with NP to restrict mRNA nuclear export. In a transient transfection assay, 293T cells were transfected with myc-hnRNPAB (AB) for 12 h. The cells were counted and added into 96-well plate at 2,000 cells per well. After 12 h CCK-8 was measured at 450 nm. The Error bars: Mean ± SD of 3 independent tests. *p < 0.05; **p < 0.01; and ns (nonsignificant), p > 0.05 compared to control.

Figure 1. Continued

(B) Dynamics of hnRNPAB protein in cells infected with influenza A virus. Cell lysates collected from (A) at the indicated time were analyzed by immunoblotting with rabbit anti-hnRNPAB mAb, mouse anti-NP mAb, and rabbit anti-GAPDH pAbs. Quantitation of the immunoreactive protein bands on the blots was performed using ImageJ software, and the hnRNPAB band was normalized using the GAPDH control.

(C) Inhibition of H7N9 virus replication in hnRNPAB-overexpressing cells. 293T cells were transfected with myc-hnRNPAB. The myc vector was used as a control. At 24 h after transfection, cells were infected with H7N9, H5N1, H9N2, H3N2, or PR8 viruses (MOI = 1.0), respectively, and cultured for 24 h. The TCID50 was measured by IFA (immunofluorescence assay) with the mouse anti-M2 mAb.

(D) Enhancement of H7N9 virus replication in hnRNPAB knockdown cells. Cells were transfected with hnRNPAB small interfering RNA (siRNA) si-AB or control si-NC. At 36 h after transfection cells were infected with the H7N9, H5N1, H9N2, H3N2, or PR8 viruses (MOI = 1.0), respectively and cultured for 24 h. TCID50 was measured as described in (C).

(E) 293T cells transfected with myc vector, myc-tagged hnRNPAB, si-NC, or si-hnRNPAB for 12 h. The cells were counted and added into 96-well plate at 2,000 cells per well. After 12 h CCK-8 was measured at 450 nm. The Error bars: Mean ± SD of 3 independent tests. *p < 0.05; **p < 0.01; and ns (nonsignificant), p > 0.05 compared to control.
levels of four IAVs increased significantly (Figures 1D and S1B). Interestingly, in the early stage of infection, the M1 mRNA and vRNA alteration are not significant in both hnRNPAB-overexpressing and knockdown cells; however, in the late stage of infection the mRNA and vRNA levels of M1 have a significant decrease in hnRNPAB-overexpressing cells and a significant increase in hnRNPAB knockdown cells (Figure S1C and S1D). By the way, the cell proliferation assay via CCK-8 proved that expressing or knocking down hnRNPAB showed no significant effect on cell proliferation (Figure 1E). These results demonstrated that hnRNPAB inhibits the replication of different subtypes of IAV.

HnRNPAB binding to viral nucleoprotein enhances the inhibition of IAV replication

The NP of IAV was reported to be the major component of vRNP complexes and to bind single-stranded RNA without sequence specificity (Klumpp et al., 1997). We wanted to know whether viral NP associates with hnRNPAB-induced inhibition of IAV. For the purpose we investigated the interaction of hnRNPAB and NP during infection of IAV. A549 cells were infected with H7N9 virus as a model virus, and 12 h later the cells were harvested. Confocal microscopy analysis revealed that hnRNPAB overlapped with the NP protein (Figure 2A). Furthermore, co-immunoprecipitation assay showed that the viral protein NP had an interaction with hnRNPAB and the interaction disappeared after RNase A treatment (Figure 2B). RNA immunoprecipitation (RIP) assay further proved that hnRNPAB binds mainly to viral mRNA instead of vRNA or cRNA (Figure S2A). In the GST pull-down experiment, NP was pulled down by GST-hnRNPAB but not GST alone, and the interaction between hnRNPAB and NP disappeared after RNase A treatment and was recovered when RNA was added (Figure 2C). By GST pull-down assay, we further identified the critical region(s) for the interaction between hnRNPAB and NP. The results showed that both of RRMs and Gly domains are critical for hnRNPAB interaction with NP (Figure S2B) and that the interaction between Gly and NP is dependent on RNA (Figure S2C). These data confirmed that RNA mediates the interaction between hnRNPAB and NP.

To analyze the impact of hnRNPAB-NP interaction on virus replication, the 293T cells transfected with myc-hnRNPAB and/or FLAG-NP, individually or together, were inoculated with H7N9 virus. The levels of viral protein NS1 were downregulated significantly in hnRNPAB-overexpressing cells, especially in hnRNPAB and NP co-expressing cells (Figure 2D, p < 0.01); however, the alteration of viral protein NS1 was not significant in NP-overexpressing cells. Meanwhile, we tested the viral mRNA and vRNA levels of M1 when hnRNPAB and/or NP were overexpressed. Results showed that in the early stage of infection overexpression of hnRNPAB and/or NP showed no significant effect on the viral mRNA and vRNA levels. However, in the late stage of infection, the viral mRNA and vRNA levels were both inhibited significantly when hnRNPAB was expressed individually or expressed with NP (Figure S2D). Taken together, the results demonstrated that hnRNPAB binding to NP via RNA exacerbates the inhibition of virus replication.

The Gly motif of hnRNPAB is critical to inhibit IAV replication

Whether the hnRNPAB from different species has identical function during virus infection is unknown. We constructed myc-tagged hnRNPAB from mouse (m-AB) and chicken (c-AB). Then, 293T cells were transfected with m-AB or c-AB and infected with the H7N9 virus for 16 h. The immunoblotting results showed that both m-AB and c-AB could restrict the expression of viral proteins NP and NS1 significantly during H7N9 infection (Figures 3A and 3B), indicating that hnRNPABs from different species have a similar function during virus replication. To further characterize the critical domain of hnRNPAB for inhibition of virus replication, we constructed myc-tagged CBFNT fragment (CARG-binding factor N-terminus), RRMs fragment (RNA recognition motif), Gly fragment (Gly) of hnRNPAB, and CBFNT-deficient hnRNPAB (∆CBFNT), RRMs-deficient hnRNPAB (∆RRMs), and Gly-deficient hnRNPAB (∆Gly) (Figure 3C). The 293T cells were transfected with these constructs for 24 h and then infected with H7N9 virus (multiplicity of infection [MOI] = 1.0) for 16 h. The results showed that only the Gly domain of hnRNPAB, but not the CBFNT and RRMs, inhibited virus replication. However, in the ∆Gly-transfected cells, the expression of viral proteins showed no significant change during infection (Figures 3D and 3E). The TCID₅₀ assay of the cells’ supernatant that was transfected with myc-tagged Gly and infected by H7N9 virus showed that Gly inhibited H7N9 virus replication significantly (Figure S2E). These data suggested that the Gly motif was critical for hnRNPAB to inhibit virus replication.

To finely locate the critical sites of the Gly motif, we analyzed the Gly motif of hnRNPAB, including the N-terminal Arg-rich domain (245–254 aa), the N-terminal Arg and Gly-rich domain (241–263 aa), and the C-terminal conservative sequence (312–332 aa). Subsequently, we constructed myc-tagged 245–254 aa-
deficient hnRNPAB (Δ245–254 aa), 241–263 aa-deficient hnRNPAB (Δ241–263 aa), and 312–332 aa-deficient hnRNPAB (Δ312–332 aa). 293T cells were transfected with these constructs for 24 h and then infected with H7N9 virus (MOI = 1.0) for 16 h. The results showed that in Δ241–263 aa- and Δ312–332 aa-, rather than Δ245–254 aa-, transfected cells the expression of viral proteins NP and NS1 showed no significant change during infection (Figure 3F). These data suggested that residues 241–263 aa and 312–332 aa within the Gly motif of hnRNPAB are the critical regions to inhibit virus replication.

**Nuclear export of virus mRNA is limited by interacting with Gly motif of hnRNPAB**

HnRNPAB was reported to bind to poly(A)⁺ RNA (Piergiorgio et al., 2002). To investigate the role of hnRNPAB in the cellular mRNA nuclear export, we checked the distribution of poly(A)⁺ RNA in
Figure 3. Identification of the Gly motif of hnRNPAB for the inhibition of flu virus replication

(A and B) Inhibition of H7N9 virus replication in mouse and chicken hnRNPAB-overexpressing cells. 293T cells were transfected with myc-m-AB (A) or myc-c-AB (B) for 24 h and infected with H7N9 virus at MOI = 1.0 for 16 h. Cell lysates were analyzed using western blotting with the indicated antibody.

(C) Schematic representation of the CBFNT, RRM1, RRM2, and Gly domains of hnRNPAB. The numbers indicate amino acid (aa) positions.

(D and E) Gly domain of hnRNPAB is critical to inhibit H7N9 virus replication. 293T cells were transfected with myc-tagged hnRNPAB or the hnRNPAB-truncated mutants CBFNT, RRM1, and Gly (D), or hnRNPAB-deleted mutants D254–254 aa, D241–263 aa, and D312–332 aa (E) for 24 h, and these cells were infected with H7N9 virus for 16 h. Cell extracts were analyzed by western blotting using anti-NP mAb, anti-NS1 mAb, and anti-GAPDH pAbs. The Error bars: Mean + SD of 3 independent tests. *p < 0.05; **p < 0.01; and ns (nonsignificant), p > 0.05 compared to control.
hnRNPAB-overexpressing and knockdown cells using a cy3-labeled oligo(dT)$_{40}$ probe. Confocal microscopy observation showed that significant amounts of poly(A)$^+$RNA remained in the nucleus of hnRNPAB-overexpressing cells, which contrasted with poly(A)$^+$RNA being mainly distributed in the cytoplasm of wild-type 293T cells (Figure 4A, 4B, and 4D). However, poly(A)$^+$RNA levels decreased significantly in the nucleus of hnRNPAB knockdown A549 cells (Figures 4C and 4D). The results demonstrated that hnRNPAB limited the nuclear export of poly(A)$^+$RNA. To verify if the intranuclear retention of poly(A)$^+$RNA is involved in the interaction with hnRNPAB, 293T cells were transfected with GFP-tagged CBFNT, RRMs, and Gly of hnRNPAB and the Gly mutants D245–254 aa, D241–263 aa, and D312–332 aa. 293T cells were transfected with these constructs and stained with the cy3-labeled oligo(dT)$_{40}$ probe. The overlapping analysis showed that the Gly domain overlapped with poly(A)$^+$RNA in the nucleus of the transfected cells; however, CBFNT and RRMs were mainly distributed in the cytoplasm of the transfected cells and were not colocalized with poly(A)$^+$RNA (Figures 4E, S3A, and S3B). However, an RNA pull-down assay demonstrated an interaction between full-length hnRNPAB, Gly, RRMS, and poly(A)$^+$RNA, respectively (Figure 4F), confirming that hnRNPAB interacts with poly(A)$^+$RNA by its Gly and RRM motifs. The colocalization experiment using the Gly mutants revealed that the intranuclear accumulation of poly(A)$^+$RNA decreased significantly in the D312–332 aa-transfected cells, but not in D245–254 and D241–263 aa-transfected cells (Figures 4G and 4H), indicating that the C-terminal residues 312–332 aa within the Gly motif are the NLS involved in the interaction of hnRNPAB and the N-terminal residues 245–254 aa and 241–263 aa of the Gly motif are crucial for hnRNPAB binding to poly(A)$^+$RNA.

As in the early stage of IAV infection, hnRNPAB showed its significant effect on the viral protein expression instead of viral mRNA and vRNA levels, which proved that hnRNPAB did not affect the virus RNA synthesis, we then determined whether the inhibition of hnRNPAB-induced virus replication is involved in the nuclear export limitation of viral mRNA. HnRNPAB-transfected cells were infected with H7N9 virus and then stained with the cy3-labeled probes specific for the M1, HA, NP, and NS1 mRNA and the cy3-labeled M1 vRNA probes. Confocal microscopy observations showed that after H7N9 virus infection, the level of intranuclear M1, HA, NP, and NS1 mRNA increased markedly in hnRNPAB-overexpressing cells when compared with that in mock-transfected cells, and overexpression of hnRNPAB showed no significant effect on the cellular distribution of vRNA (Figures 4I and 4J and S3C). However, the hnRNPAB knockdown A549 cells with ActD treatment displayed that intranuclear distribution of M1 mRNA decreased significantly (Figure S3D). Subsequently, the nuclear-cytoplasmic separation was carried out in hnRNPAB-overexpressing or knockdown cells with H7N9 virus infection, and total RNA was extracted. The mRNA levels of the H7N9 virus in the cytoplasm and nucleus were analyzed using qRT-PCR with M1 mRNA primers, which showed that when

Figure 4. HnRNPAB blocks nuclear export of viral mRNA
(A and B) FISH analysis of the distribution of poly(A)$^+$RNA in cells transfected with hnRNPAB. 293T cells were transfected without (A) or with (B) GFP-hnRNPAB for 12 h and then probed with cy3-labeled oligo(dT)$_{40}$ for poly(A)$^+$RNA. Nuclei were stained with DAPI. Scale bars, 10 μm.

(C) FISH analysis of the distribution of poly(A)$^+$RNA in A549 cells that were transfected with si-hnRNPAB or si-NC as control. Scale bars, 10 μm.

(D) Fluorescence intensity for the nuclear/cytoplasmic ratio of poly(A)$^+$RNA in hnRNPAB-overexpressing and knockdown cells was analyzed by counting 100 cells using ImageJ software.

(E) FISH analysis of poly(A)$^+$RNA distribution in cells transfected with the Gly domain of hnRNPAB. 293T cells were transfected with GFP-Gly of hnRNPAB for 12 h and probed with cy3-labeled oligo(dT)$_{40}$. Scale bars, 10 μm.

(F) RNA pull-down assay of hnRNPAB. 293T cells were transfected with GFP-tagged hnRNPAB and indicated truncation mutants for 24 h and were then subjected to biotin-oligo(dT)$_{40}$ and western blotting.

(G) Poly(A)$^+$RNA-distributed FISH analysis in cells transfected with the Gly domain-truncated mutants. 293T cells were transfected with GFP-tagged Gly-truncated mutants D241–263 aa, D245–254 aa, and D312–332 aa and stained with cy3-labeled oligo(dT)$_{40}$ and DAPI. Scale bars, 10 μm.

(H) Fluorescence intensity for the nuclear/cytoplasmic ratio of poly(A)$^+$RNA in hnRNPAB mutant-overexpressing cells by ImageJ software.

(I) The viral mRNA-distributed FISH analysis in H7N9 virus-infected cells. 293T cells were transfected with myc vector or myc-tagged hnRNPAB for 24 h and then infected with H7N9 virus for 12 h. Cells were stained with cy3-labeled M1 mRNA probes or cy3-labeled M1 vRNA probes. Scale bars, 10 μm.

(J) Fluorescence intensity for the nuclear/cytoplasmic ratio of M1 mRNA or vRNA in hnRNPAB-overexpressing cells was analyzed by counting 100 cells using ImageJ software.

(K and L) The nuclear-cytoplasmic ratio of viral mRNA. 293T cells were transfected with the indicated plasmids (K) or siRNA (L) for 24 h and were infected with H7N9 at MOI = 1.0 for 6 and 12 h. Cells are subjected to nuclear-cytoplasmic separation and qRT-PCR measurements of the M1 mRNA. The Error bars: Mean + SD of 3 independent tests. *p < 0.05, **p < 0.01, and ns (nonsignificant), p > 0.05 compared to control.
Figure 5. HnRNPAB inhibits viral mRNA binding to NXF1

(A) The impact of NXF1, UAP56, and ALY on H7N9 virus replication. 293T cells were transfected with the indicated siRNAs for 36 h and then infected with H7N9 virus for 12 h. Cell lysates were analyzed with mAbs recognizing ALY, UAP56, NXF1, and NS1 in western blotting.

(B) The nucleo-cytoplasmic ratio of H7N9 viral mRNA in NXF1, UAP56, or ALY knockdown cells. Cells from (A) were subjected to nuclear-cytoplasmic separation. RT-qPCR assays were performed for the H7N9 M1 mRNA analysis.

(C) Co-IP assay of FLAG-ALY and myc-hnRNPAB. 293T cells were co-transfected with myc-hnRNPAB and FLAG-ALY. Cell lysates were added with or without RNase as indicated and then subjected to immunoprecipitation with anti-FLAG mAb and western blotting with anti-FLAG mAb and anti-myc pAb. FLAG was used as control.
were solubilized with RIP lysis buffer and subjected to immunoprecipitation with anti-myc mAb. The NXF1-bound RNAs were analyzed using qRT-PCR.

(O) RIP assay of viral mRNA binding to NXF1. 293T cells were transfected with myc-NXF1 or FLAG-hnRNPAB for 24 h. Cells lysates were subjected to biotin-oligo (dT) pull-down and western blotting assays using anti-NXF1 and anti-hnRNPAB mAbs and anti-myc and anti-GAPDH pAbs. The quantitation of ALY was analyzed using ImageJ software.

(G) Identification of hnRNPAB interaction with endogenous NXF1. Co-IP assay of myc-hnRNPAB and endogenous NXF1 in 293T cells. 293T cells were transfected with myc-hnRNPAB and subjected to immunoprecipitation and western blotting assays using anti-myc pAbs and anti-NXF1 mAb.

(H) Pull-down assay of NXF1 and hnRNPAB. Pull-down assay of FLAG-gst-hnRNPAB and myc-NXF1. 293T cells were transfected with myc-NXF1 and FLAG-gst-hnRNPAB individually. Pull-down assays were performed as in (D).

(I) Mapping of the NXF1-interacting domain within hnRNPAB. FLAG-gst tagged hnRNPAB and truncated mutants were transfected with myc-NXF1 into 293T cells individually for 24 h. Cell lysates were mixed and were then pulled down, and western blotting were conducted using anti-FLAG mAb and anti-myc pAbs.

(J) The residues 241–263 aa and 312–332 aa of the hnRNPAB Gly domain are critical for the interaction with NXF1. 293T cells were transfected with FLAG-NXF1 and myc-tagged Gly domain of hnRNPAB 241–263 aa or 312–332 aa, individually or in combination. Cell lysates were subjected to immunoprecipitation and western blotting with anti-FLAG mAb and anti-myc pAbs.

(K) hnRNPAB and NP do not interrupt the interaction of ALY with NXF1. 293T cells were transfected with myc-NXF1, FLAG-hnRNPAB, FLAG-NP, or FLAG-ALY individually for 24 h. Cell lysates were immunoprecipitated using anti-myc mAb and analyzed using western blotting with anti-FLAG mAb and anti-myc pAbs.

(L) Identification of the hnRNPAB-binding domain of NXF1. 293T cells were transfected with FLAG-gst-hnRNPAB, myc-tagged NXF1, and NXF1 deletion mutants ABDo, ΔA2R, ΔLRR, ΔNTF2L, and ΔNTF2L + UBA for 24 h. Cell lysates were subjected to pull-down and western blotting assays using anti-FLAG mAb and anti-myc pAbs.

(M and N) RNA pull-down assay of NXF1 in hnRNPAB-overexpressing or knockdown cells. 293T cells were transfected with myc-hnRNPAB (M) or its siRNA (N). Cell lysates were subjected to RNA pull-down and western blotting assays using anti-NXF1 and anti-hnRNPAB mAbs and anti-myc and anti-GAPDH pAbs.

(O) RIP assay of viral mRNA binding to NXF1. 293T cells were transfected with myc-NXF1 or FLAG-hnRNPAB for 24 h or infected with H7N9 virus for 24 h. Cells were solubilized with RIP lysis buffer and subjected to immunoprecipitation with anti-myc mAb. The NXF1-bound RNAs were analyzed using qRT-PCR.

To further analyze whether hnRNPAB interaction with viral protein NP affects virus replication via viral mRNA nuclear export, we transfected myc-tagged hnRNPAB and FLAG-tagged NP into 293T cells, and the cells were then infected with H7N9 virus. Fluorescence in situ hybridization (FISH) analysis with cy3-labeled M1 mRNA probes indicated that the viral M1 mRNA showed more distribution in the nucleus of cells co-expressing hnRNPAB and NP in contrast to that of the cells expressing hnRNPAB individually (Figure S4A). RNA cytoplasm and nuclear isolation assay proved that expression of NP and hnRNPAB inhibited viral M1 mRNA nuclear export (Figure S4B). The data demonstrated that the NP interaction with hnRNPAB inhibited viral mRNA nuclear export.

HnRNPAB inhibits the interaction of NXF1 with viral mRNA via binding to the NXF1 and TREX complex

In eukaryotic cells, the nuclear export of mRNA is mediated by the transport receptor NXF1. The TREX complex acts as the key adaptor of NXF1. The TREX complex is mainly composed of ALY, UAP56, and THO complex, and UAP56 is required for the assembly of the complex and recruitment of ALY to mRNA (Luo et al., 2001). To investigate whether the cellular mRNA nuclear export factors are involved in the nuclear export of viral mRNA, the expression of ALY or UAP56 or NXF1 was knocked down, and these cells were then infected with the H7N9 virus. These cells were subjected to immunoblotting and nuclear-cytoplasmic fraction separation for qRT-PCR. In the immunoblotting assay, H7N9 virus protein NS1 levels were significantly reduced in ALY, UAP56, or NXF1 knockdown cells (Figure S5A, p < 0.01). FISH and qRT-PCR showed that the intranuclear concentration of viral mRNA was significantly increased in the ALY, UAP56, or NXF1 knockdown cells in contrast to the control cells (Figure S5B and S5A-B, p < 0.01), especially in the NXF1 knockdown cells. These results showed that the cellular mRNA export pathway participates in regulating the nuclear export of viral mRNA (Read and Digard, 2010). Whether hnRNPAB is related to nuclear export of viral mRNA compared with that in mock-transfected cells, the intranuclear levels of the mRNA transcript of M1 increased significantly in hnRNPAB-transfected cells (Figures 4K and S3E, p < 0.01) and decreased significantly in hnRNPAB knockdown cells (Figure 4L and S3F, p < 0.01). These data demonstrated that hnRNPAB blocks the nuclear export of viral mRNA during infection. Taken together, these data allowed us to conclude that nuclear export of viral mRNA is inhibited by interaction with the motif Gly of hnRNPAB.
Figure 6. Viral protein NP interaction with HnRNPAB strengthens inhibition of ALY-viral mRNA binding

(A) Detection of viral protein NP interaction with ALY and UAP56. 293T cells were cotransfected with myc-UAP56, FLAG-hnRNPAB, FLAG-NP, or FLAG-ALY for 24 h. Cell lysates were subjected to immunoprecipitation and western blotting with anti-FLAG mAb and anti-myc pAbs.
regulated by NXF1 and TREX complex is unknown. Therefore, we performed immunoprecipitation to detect the interaction of hnRNPAB with ALY or UAP56 in overexpressing cells. In the co-immunoprecipitation (co-IP) assay, hnRNPAB could bind to ALY in ALY and hnRNPAB co-expressing cells without RNase treatment but not with RNase treatment; however, hnRNPAB bound to UAP56 in UAP56 and hnRNPAB co-expressing cells with or without RNase treatment (Figures 5C and 5D), confirming that RNA mediated the interaction of hnRNPAB with ALY, and that hnRNPAB interacts with UAP56 directly. Meanwhile, we also tested hnRNPAB’s interaction with both UAP56 and ALY by artificially mixing the myc-hnRNPAB, FLAG-ALY, and myc-UAP56. An anti-FLAG co-IP assay showed that hnRNPAB bound to ALY and UAP56 to form a trimer complex, and that binding of UAP56 to ALY and hnRNPAB in the ALY-UAP56-hnRNPAB mixture decreased when compared with that in the ALY-UAP56 mixture (Figure 5E; p < 0.001), showing that hnRNPAB decreased UAP56 binding to ALY. A biotin-oligo(dT) pull-down assay further showed that the binding of ALY to mRNA decreased in hnRNPAB-overexpressing cells (Figure 5F; p < 0.01), indicating that overexpression of hnRNPAB repressed the binding of ALY to mRNA. Taken together, these results demonstrated that hnRNPAB weakened mRNA transfer from UAP56 to ALY by binding to ALY.

Next, we investigated if the NXF1’s RNA binding is also restricted by hnRNPAB to limit viral mRNA nuclear export. In co-IP and pull-down assays, fine location experiments showed that NXF1 is directly bound to the N-terminal residues 241–263 aa and C-terminal residues 312–332 aa within the Gly motif of hnRNPAB (Figures 5G–5J). NXF1 binds to ALY and exposes its RBD (Viphakone et al., 2012), and viral mRNAs are exported via the NXF1-dependent pathway (Read and Digard, 2010). Therefore, we wanted to know whether NXF1 binding to hnRNPAB blocks the exposing of its RBD and whether the NXF1-viral mRNA binding is restricted. For the purpose, we performed co-IP, GST, and RNA pull-down assays. The co-IP assay showed that hnRNPAB binding to NXF1 did not interrupt NXF1’s interaction with ALY when hnRNPAB was incubated with ALY and NXF1 (Figure 5K), suggesting that the hnRNPAB-NXF1 interaction did not interfere with the NXF1-ALY interaction. In the GST pull-down assay, hnRNPAB was only detected to interact with the RBD of NXF1 by incubating separately with NXF1 RBD (ΔRBD), ψRRM (ΔψRRM), LRR (ΔLRR), NTF2L (ΔNTF2L), or NTF2L + UBA (ΔNTF2L + UBA) deletion mutants (Figure 5L). Consistently, in the biotin-oligo(dT) pull-down assay, the RNA-binding activity of NXF1 was upregulated in hnRNPAB knockdown cells, but not in hnRNPAB-overexpressing cells (Figures 5M and 5N). RNA pull-down assay with biotin-labeled M1 mRNA probes further showed that overexpression of hnRNPAB inhibited the NXF1 and ALY binding to M1 mRNA; however, knocking down of hnRNPAB promoted the NXF1 and ALY binding to M1 mRNA (Figures S6A and S6B). Meanwhile, in the RIP assay, we proved that hnRNPAB markedly repressed NXF1 binding to viral mRNA (Figures 5O and S6C), confirming that hnRNPAB binding to the RBD of NXF1 inhibits the NXF1 RNA-binding activity and mRNA transfer from ALY to NXF1. Taken together, the results suggested that hnRNPAB binding to NXF1 and the core components, UAP56 and ALY, of the TREX complex inhibits the transfer of RNA from ALY to NXF1.

The interaction of viral protein NP with hnRNPAB enhances the inhibition of ALY binding to viral mRNA

To analyze how viral protein NP binding to hnRNPAB inhibits H7N9 virus replication, we tested whether the NP-hnRNPAB complex disrupts the mRNA nuclear export pathway. Strikingly, the NP-hnRNPAB complex markedly repressed the interaction between ALY and UAP56 (Figure 6A; p < 0.01). To test whether the
NP-hnRNPA1 interacts with the mRNA export receptor NXF1, and the binding of NXF1 to the Gly domain of hnRNPA1 weakens the interaction between ALY and NXF1 by linking to NXF1 and UAP56 (Figures 5K, 5M, and 5N). Meanwhile, hnRNPA1 directly interacts with the RBD of NXF1 via its N-terminal 241–263 residues and C-terminal 312–332 residues. More importantly, during influenza virus infection, hnRNPA1 binding to viral protein NP enhances the inhibition of mRNA nuclear export (Figures 4I–4L). Taken together, these results demonstrated that the Gly domain of hnRNPA1 is the binding domain of poly(A)+RNA and that hnRNPA1 acts as a negative regulator of influenza virus replication by inhibiting mRNA nuclear export.

### DISCUSSION

HnRNP A/B is the most abundant sub-family of hnRNPs (He and Smith, 2009) and is involved in regulating the virus life cycle in different ways. HnRNPA1’s interaction with nucleocapsid protein of porcine epidemic diarrhea virus impairs viral replication (Li et al., 2018). HnRNPA2B1 inhibits IAV replication by suppressing NS1 mRNA nuclear export (Wang et al., 2014), inhibits DNA virus replication by initiating the innate immune response (Wang et al., 2019), and supports hepatitis E virus replication by interacting with the promoter regions of the hepatitis E virus genome (Pingale et al., 2020). HnRNPA1, as an RNA-binding protein, is considered as a distantly related member of hnRNPs A/B and is involved in the regulation of RNA biological processes. Structurally, hnRNPA1 comprises a CARG-binding factor-A domain in the N terminus (CBFNT), two RBDs (RRM1 and RRM2), and a C-terminal glycine-rich region (Gly domain) (Khan et al., 1991). The Gly domain contains a motif characterized by closely spaced clusters of Arg-Gly-Gly tripeptide repeats with interspersed aromatic amino acids and is responsible for RNA binding (Geuens et al., 2016). In the present study, infection with different subtypes of IAV continuously upregulated both mRNA and protein levels of hnRNPA1, and overexpression of hnRNPA1 and its Gly domain inhibited IAV replication (Figures 3D and 3E). This led to nuclear accumulation of poly(A)+RNA and enhanced nuclear retention of viral mRNA (Figures 4I–4L). Taken together, these results demonstrated that the Gly domain of hnRNPA1 is the binding domain of poly(A)+RNA and that hnRNPA1 acts as a negative regulator of influenza virus replication by inhibiting mRNA nuclear export.

The TREX complex, composed of ALY, UAP56, and THO complexes, is a key player in mRNA biogenesis and participates in almost all stages of mRNA processing, linking transcription, and export (Katja et al., 2002; Viphakone et al., 2012). During the maturation and nucleo-cytoplasmic transport of poly(A)+RNA, UAP56 binding to pre-mRNAs is required for the recruitment of ALY to form the TREX complex (Hautbergue et al., 2008), and the RNA bound to UAP56 is transferred to ALY by UAP56 binding to ALY (Klumpp et al., 1997). ALY also interacts with the mRNA export receptor NXF1 and the binding between ALY and NXF1 triggers the transfer of RNA from ALY to NXF1 (Ben-Shem et al., 2011; Ming-Lung et al., 2010). The mRNAs of HA, M1, and NS1 of PR8 virus were shown to be dependent on NXF1 and UAP56 for their nuclear export (Read and Digard, 2010). HIV-1 ANP32A and ANP32B mediate the nuclear export of HIV-1 unspliced or partially spliced mRNA via interactions with Rev and CRM1 (Branche and Falsey, 2016). The limited studies on viral mRNA nuclear export have shown that nuclear export of viral mRNA from different viruses depends on different nuclear export pathways. In the present study, we demonstrated that hnRNPA1 markedly repressed NXF1-poly(A)+RNA binding and did not interrupt the interaction of ALY and NXF1 by directly linking to NXF1 and UAP56 (Figures 5K, 5M, and 5N). Meanwhile, hnRNPA1 weakens the interaction between ALY and UAP56 by linking to ALY, resulting in poly(A)+RNA not transferring from UAP56 to ALY or from ALY to NXF1. Moreover, we also found that the Gly domain of hnRNPA1 directly interacts with the RBD of NXF1 via its N-terminal 241–263 residues and C-terminal 312–332 residues. More importantly, during influenza virus infection, hnRNPA1 binding to viral protein NP enhances the interference with the UAP56-ALY interaction (Figure 6A). Therefore, we proposed that hnRNPA1 interaction with NP of IAV strengthens the inhibition of flu virus replication by limiting the nuclear export of viral mRNA.

Amino acid sequence analysis revealed that hnRNPA1 is conserved among different species and has 91.10% homology between human and mouse and 79.05% homology between human and chicken (data not shown). We also proved that hnRNPA1 from different species has the ability to inhibit virus replication (Figures 3A and 3B), indicating that hnRNPA1 from different species has a similar function during influenza virus infection. Members of hnRNPs A/B subfamily, such as hnRNPA1 and hnRNPA2/B1, contain a nuclear
targeting sequence in their C termini (He and Smith, 2009; Jacques et al., 2013). In the present study, we also found that the C-terminal residues 312–332 in the Gly domain represent the NLS of hnRNPAB and are critical for hnRNPAB to interact with the NXF1 and viral mRNA. Interestingly, C-terminal residues 312–332 aa within the Gly motif are also the binding site for NXF1 (Figure 5J). Combined with the fact that hnRNPAB binds to poly(A)^+RNA, our findings suggest that in the nucleus, hnRNPAB, via its NLS, participates in inhibiting IAV replication.

Our data indicated that hnRNPAB acts as a negative regulator of gene expression by inhibiting mRNA nuclear export. In this process, hnRNPAB acts as a valve to calm down the protein expression of both exogenous and endogenous genes when cells are subjected to external stimuli like virus infection for maintaining cell homeostasis. This is consistent with the viewpoint that normal nuclear retention of mRNA enables cells to rapidly respond to stress. Therefore, we inferred that when cells were infected with viruses or invaded by exogenous genes, hnRNPAB regulated negatively the expression of the exogenous genes to gain time for cells to prepare innate immunity or other response and keep the cells in a stable station.

In summary, our study indicated that hnRNPAB could inhibit the replication of IAV via repression of viral mRNA nuclear export. HnRNPAB binding to viral protein NP enhances its repression of viral mRNA nuclear export by interrupting the transfer of poly(A)^+RNA from UAP56 to ALY and NXF1 (Figure 6E).

**Limitations of the study**

First, we demonstrated that hnRNPAB interacts with viral mRNA and inhibits the viral mRNA nuclear export. However, whether the viral mRNA is decayed in the nucleus or is transferred into cytoplasm later is still unclear. Second, we demonstrate that the IAV NP cooperates with hnRNPAB to repress the interaction of UAP56 and ALY, further inhibiting viral mRNA transfer from UAP56 to ALY. However, the mechanism of NP-hnRNPAB complex inhibiting the interaction of UAP56 and ALY is unknown.

**Resource availability**

**Lead contact**

Further requests and information should be directed to and will be fulfilled by Min Liao (liaomin4545@zju.edu.cn).

**Materials availability**

Materials are available upon reasonable request.

**Data and code availability**

All data are included in the published article and the Supplemental Information, and any additional information will be available from the lead contact upon request.

**METHODS**

All methods can be found in the accompanying Transparent methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102160.

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**AUTHOR CONTRIBUTIONS**

X.W., J.Z., and M.L. designed the experiments. X.W., L.L., and Y.Z. equally performed the experiments with the help of M.F., T.Y., Y.Y., and M.L., X.W., and J.Z. wrote the paper.
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Supplemental information

Cellular hnRNPAB binding to viral nucleoprotein inhibits flu virus replication by blocking nuclear export of viral mRNA

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Figure S1

A

|               | PR8 | H3N2 | H9N2 | H5N1 | H7N9 |
|---------------|-----|------|------|------|------|
| N-myc        | +   | +    | -    | +    | +    |
| myc-AB       | -   | -    | +    | -    | -    |
| PB1          |     |      |      |      |      |
| PA           |     |      |      |      |      |
| NP           |     |      |      |      |      |
| M1           |     |      |      |      |      |
| NS1          |     |      |      |      |      |
| myc-AB       |     |      |      |      |      |
| GAPDH        |     |      |      |      |      |

B

|               | PR8 | H3N2 | H9N2 | H5N1 | H7N9 |
|---------------|-----|------|------|------|------|
| si-NC        | +   | +    | -    | +    | +    |
| si-AB        | -   | -    | +    | -    | -    |
| PB2          |     |      |      |      |      |
| NP           |     |      |      |      |      |
| NS1          |     |      |      |      |      |
| M2           |     |      |      |      |      |
| AB           |     |      |      |      |      |
| GAPDH        |     |      |      |      |      |

C

|               | PR8 vRNA | H3N2 vRNA | H9N2 vRNA | H5N1 vRNA | H7N9 vRNA |
|---------------|----------|-----------|-----------|-----------|-----------|
| N-myc        | +        | +         | -         | +         | +         |
| myc-AB       | -        | -         | +         | -         | -         |

D

|               | PR8 mRNA | H3N2 mRNA | H9N2 mRNA | H5N1 mRNA | H7N9 mRNA |
|---------------|----------|-----------|-----------|-----------|-----------|
| si-NC        | +        | +         | -         | +         | +         |
| si-AB        | -        | -         | +         | -         | -         |

Relative mRNA abundance
Figure S3

A
GFP-CBFNT
dpoly(A)+RNA
DAPI
GFP
50 μm

B
GFP-RRMs
dpoly(A)+RNA
Merge
DAPI
GFP
50 μm

C
HA mRNA
10μm
myc-AB
DAPI
Merge

NP mRNA
10μm
myc-AB
DAPI
Merge

NS1 mRNA
10μm
myc-AB
DAPI
Merge

HA mRNA Intensity (nuc/cyt)

NP mRNA Intensity (nuc/cyt)

NS1 mRNA Intensity (nuc/cyt)

D
M1 RNA
10μm
si-NC
si-AB

DAPI
Merge

M1 mRNA Intensity (nuc/cyt)

E
N-myc myc-AB
NS1 si-AB
si-NC
si-AB
GAPDH
H3
Input (kDa)

F
N-myc myc-AB
si-NC
si-AB
GAPDH
H3
Input (kDa)

M1 mRNA (Input %)

N-myc myc-AB
si-NC
si-AB

M1 mRNA Intensity (nuc/cyt)

GFP-CBFNT
GFP-RRMs
GAPDH
H3

M1 mRNA
poly(A)+RNA
DAPI
GFP
GFP-RRMs
poly(A)+RNA
DAPI
GFP
Figure S4

A

B

Figure S4
Figure S5

A

GAPDH %

150

100

50

0

nuc
cyt

si-NC

si-ALY

si-UAP56

si-NXF1

GAPDH %

150

100

50

0

nuc
cyt

si-NC

si-ALY

si-UAP56

si-NXF1

B

si-NC

si-ALY

si-UAP56

si-NXF1

M1 mRNA DAPI Merge
Figure S6

A  myc  +  -  -  -  
mymc-AB  -  +  -  +  
H7N9  -  -  -  +  

Input  myc-AB  ALY  NXF1  

Biotin M  Pull down  (kDa)  

B  myc  +  -  -  -  
mymc-AB  -  +  -  +  
H7N9  -  -  +  +  

Input  myc-AB  ALY  NXF1  

Biotin M  Pull down  (kDa)  

C  M1 mRNA  RIP/Input  

H7N9  -  +  +  +  

NXF1  +  +  -  -  
AB  +  +  -  -  
NS1  +  +  -  -  
GAPDH  +  +  -  -  

**  35  70  40  55  25  35  40  55  70  (kDa)
Figure S7

A

|          | Flag  | Flag-NP | myc   | myc-AB |
|----------|-------|---------|-------|--------|
|          | +     | +       | -     | -      |
| H7N9     | +     | +       | +     | +      |

B

|          | Flag  | Flag-NP | myc   | myc-AB |
|----------|-------|---------|-------|--------|
|          | +     | +       | -     | -      |
| H7N9     | +     | +       | +     | +      |
Figure S1. The proteins, mRNA and vRNA levels of influenza A virus in hnRNPAB overexpressed or knocked down cells, Related to Figure 1. (A and B) The expression of flu viral proteins, mRNA and vRNA in hnRNPAB overexpressing cells. 293T cells were transfected with myc-hnRNPAB for 24 h and were then infected with the indicated influenza A viruses at MOI = 1.0, respectively. (A) Cell lysates were analyzed by western blotting with the indicated antibodies. (B) The whole RNA was isolated at the indicated time post-infection and the vRNA and mRNA of influenza viruses were analyzed by qRT-PCR. (C and D) The expression of flu virus proteins, mRNA and vRNA in hnRNPAB knocked down cells. 293T cells were transfected with si-hnRNPAB 36 h and were then infected with the indicated influenza A viruses at MOI = 1.0. (C) Viral protein levels were analyzed by western blotting with the indicated antibodies. si-NC was used as control siRNA. (D) The whole RNA was isolated at the indicated time post-infection and the vRNA and mRNA of influenza viruses were analyzed by qRT-PCR.

Figure S2. Gly motif of hnRNPAB interacts with NP via RNA, Related to Figures 2 and 3. (A) 293T cells were infected with H7N9 (MOI=1.0) for 12 h and were then lysed with RIP lysis buffer. RIP assay was performed with anti-hnRNPAB mAb and the viral mRNA, vRNA and cRNA were analyzed by qRT-PCR. (B) The Flag tagged NP, Flag-gst tagged hnRNPAB or its motifs (CBFNT, RRM and Gly) was transfected into 293T cells individually and 24 h later the cells were collected and solubilized with NP-40 lysis buffer. The cell lysates of Flag tagged NP were incubated with that of Flag-gst tagged hnRNPAB or its motifs as indicated and were then subjected to gst pull down assay. (C) 293T cells transfected with myc tagged Gly and Flag tagged NP were lysed with or without RNase A added as indicated and were then subjected to co-IP assay and western blotting analysis with myc pAb and Flag mAb. (D) 293T cells transfected with myc tagged hnRNPAB and/or Flag tagged NP as indicated were infected with H7N9 (MOI=1.0) for 6 h or 24 h and were then subjected to whole RNA isolation. The mRNA or vRNA of segment 7 (M) was analyzed by qRT-PCR. E. 293T cells were transfected with myc tagged Gly or myc vector as control for 24 h and were then infected with H7N9 (MOI=1.0) for 16 h. The TCID50 of the supernatant was measured by IFA with the mouse anti-M2 mAb.

Figure S3. CBFNT and RRM motifs have no effect on cellular distribution of poly(A)+RNA and hnRNPAB blocks the nuclear export of different viral mRNA, Related to Figure 4. (A and B) 293T cells were transfected with GFP-CBFNT (A) or GFP-RRMs (B) for 12 h. Cells were probed with cy3-oligo(dT) and stained with DAPI for the nuclei. (C and D) The viral mRNA-distributed FISH
analysis in H7N9 virus infected cells. (C) 293T cells were transfected with myc vector or myc tagged hnRNPAB for 24 h, and then infected with H7N9 virus for 12 h. Cells were stained with a cy3 labeled HA, NP or NS1 mRNA probe. Fluorescence intensity for the nuclear/cytoplasmic ratio of viral mRNA in hnRNPAB overexpressing cells was analyzed by counting 100 cells with Image J. (D) A549 cells were transfected with si-AB or si-NC as control and 24 h later the cells were infected with H7N9 and incubated with ActD for 90 min. 8 h post-infection the cells were fixed and stained with the cy3-labeled probe of M1 mRNA. (E and F) The protein samples from Figure 4K-L were subjected to western blotting analysis with the GAPDH, Histone 3 (H3), NS1 and hnRNPAB (AB) antibody and the total mRNA levels of M1 mRNA of the RNA samples in 6 h post-infection was analyzed by qRT-PCR.

**Figure S4. Viral protein NP cooperates with hnRNPA B to strengthen inhibition of viral mRNA nuclear export, Related to Figure 4.** (A) 293T cells were transfected with myc tagged hnRNPA B and/or Flag tagged NP as indicated for 24h and were then infected with H7N9 for 12 h. The cells were fixed and stained with cy3-labeled probes of M1 mRNA. (B) 293T cells were transfected with myc tagged hnRNPA B and/or Flag tagged NP as indicated for 24h and were then infected with H7N9 for 12 h. The cells were subjected to RNA cytoplasm and nuclear isolation. The M1 mRNA level of different fractions was analyzed by qRT-PCR.

**Figure S5. Down-regulating the NXF1, UAP56 or ALY restricts the nuclear export of influenza A virus mRNA, Related to Figure 5.** (A) The GAPDH and snRNA U6 (U6) of the sample in Figure 5B were analyzed by qRT-PCR and the GAPDH and Histone 3 (H3) of the sample were analyzed by western blot. (B) A549 cells were transfected with si-NXF1, si-UAP56, si-ALY or si-NC as control for 24 h and were then infected with H7N9 (MOI=1.0) for 12 h. The cells were fixed and were stained with cy3-labeled probes of M1 mRNA.

**Figure S6. HnRNPA B inhibits the NXF1 and ALY binding to viral mRNA, Related to Figure 5.** (A and B) 293T cells were transfected with (A) myc tagged hnRNPA B, myc vector, (B) si-NC or si-AB. 24 h later the cells were infected with H7N9 (MOI=1.0) for 24 h and were then collected and subjected to RNA pull down assay with biotin-labeled M1 mRNA probes. (C) RIP assay of viral mRNA binding to NXF1. The hnRNPA B knocked down cells were transfected with myc-NXF1 or infected with H7N9 virus for 24 h and were then solubilized with RIP lysis buffer. Meanwhile the mock normal 293T cells and hnRNPA B knocked down cells were also solubilized with RIP lysis buffer. The cell lysates were mixed as indicated and were then subjected to immunoprecipitation with anti-myc mAb. The NXF1
bound RNAs were analyzed using qRT-PCR.

**Figure S7. Viral protein NP interaction with hnRNPAB strengthens inhibition of ALY-viral mRNA binding, Related to Figure 6.** (A and B) 293T cells were infected with H7N9 virus for 12 h and were then transfected with myc tagged hnRNPAB and/or Flag tagged NP for 12h. Cell lysates were subjected to RNA pull down with biotin-labeled M1 mRNA probes. (A) The beads were analyzed by western blotting with anti-ALY mAb, anti-myc pAbs and anti-Flag mAb. (B) The beads were analyzed by western blotting with anti-NXF1 mAb, anti-myc pAbs and anti-Flag mAb.
| Gene      | Forward                  | Reverse                  |
|-----------|--------------------------|--------------------------|
| M1 mRNA   | TTCTAACCGAGGTCGAAAC      | CGTCTACGCTGCAGTCC        |
| hnRNPA    | TCAAAGATGCAGCCAGGTG      | TCTTCACCAGGTCTCTCTTCT    |
| GAPDH     | CATGAGAGTGATGACAACAGCCT  | AGTCTTTCCACAGATACAAAAAG  |
| U6        | CTCGCTTCGGCAGCACA        | AACGCTTCAGGAATTGCGT      |
| GFP       | GGTGAACTTCAAGATCCGCC     | CTTGTACAGCTCGTCCATG      |
| ΔCBFNT    | GCCCGAATTCGGAATGTTCGTTG  | ACGAACATTTTCCAGATTCGG    |
|           | GTGGC                    | GCTCCATG                 |
| ΔRRMs     | GAGGAGGACGCGGACAGCCAAA   | GACTTCTTTGGGCTGGTCCCCGC  |
|          | GAAGTC                   | GTCCTCTTC                |
| ΔGly      | GAGATCAAGGTTGCCGCTACCGCG| CGCGCCGCCGGTACCGGACCAC   |
|           | GCCGCG                   | CTGTGATCTC               |
| Δ245-254aa| TATGGCTCTTGGGGCAGCGAGGT  | ACCACACCCTTCCGCTCCCCCC  |
|          | GTGGTGGAGGT              | AGAGCCATACTGCTG          |
| Δ241-263aa| CAGCAGCAGCATCAGAGTCAG    | CCAACTCTGACTCTGATAGC     |
|          | AGTGGGAATCAG             | TGCTGATGATAGAC           |
| Δ312-332aa| CTACGACTACGTACGTOACGCTAC| GGCGCGGTTACCTGACTGACT    |
|           | GCCGCCGCCGGGG            | GTAGTCTGAGCCGGG          |
**Supplementary Table S2. Probes used in this study. Related to Figures 4-6.**

| mRNA | 5’-CCTCAAGTCTCTGTGCAGATCTCGGC-3’ |
|------|----------------------------------|
|      | 5’-CCACTCCATGAGAGCCCTCAGATCTCTG-3’ |
|      | 5’-CCTGGGACGGTGAGCGTGACAC-3’ |
|      | 5’-GGACAAACCGTCTACGTGCAGTCC-3’ |
|      | 5’-CCTTGTCCATGTGTTTGGGTCTCC-3’ |

| mRNA | 5’-GCCGAGATGCACAGAGACTTGAGG-3’ |
|------|--------------------------------|
|      | 5’-CAGATCTCGAGGCTCTCATGAGTGGG-3’ |
|      | 5’-GTGGTACGGCTACCGTGCCAGCAGTG-3’ |
|      | 5’-GGACTTGACGGTGAGCGTTGTGCTCC-3’ |
|      | 5’-GGAGACCCAAACACATGGACAGGGG-3’ |

| mRNA | 5’-ACAGACATCACCACCTTCTCAGCCTC-3’ |
|------|----------------------------------|
|      | 5’-ATTACCGATTTGCTTCTCTACCTC-3’ |
|      | 5’-TGGCATCAACCTTCTACCCTCTC-3’ |
|      | 5’-GGTCTCGTCTCCTGAACCTCGTACAA-3’ |
|      | 5’-GACTCCTTTGCTAAACATATCTCGGC-3’ |

| mRNA | 5’-GCTTGTGCCAATTCTCTCTCT-3’ |
|------|--------------------------------|
|      | 5’-CTATCCCTTCTCAGCTGCA-3’ |
|      | 5’-CCATCTCTTGTCTGTCTACCT-3’ |
|      | 5’-CAGTCAAGTCCGTACACAC-3’ |
|      | 5’-CTAAGGTCCCTCAAACGCTGC-3’ |

| mRNA | 5’-AGAGAAGGTAATGGTGAGATTTC-3’ |
|------|--------------------------------|
|      | 5’-CTCCAAGCAGAATCTCTGAGTTT-3’ |
|      | 5’-GGCTAAGTTCCTCTCCATTTCCC-3’ |
|      | 5’-GGACATGCAAAGAAAGCAGTCTAC-3’ |
|      | 5’-ATCATTCCATTCAAATCTCCGATG-3’ |
**Transparent Methods**

**Cell, virus, antibodies, and reagents.**

A549 and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, 1616756; Biological Industries, Israel) and MDCK cells were cultured in minimum Eagle’s medium (MEM, Gibco) with 5% FBS. Different subtypes of influenza A virus including A/Swine/Zhejiang/04/2009 (H3N2), A/Hangzhou/1/2013 (H7N9), A/Chicken/Jiande/09/2009 (H9N2), A/Puerto Rico/8/1934 (H1N1), and A/pika/Qinghai/B1/2007 (H5N1) were isolated and stored in our laboratory (Zhou et al., 2006; Zhou et al., 2009). Mouse anti-M2/ NS1/ M1/ NP/ PA/ PB1/ PB2 monoclonal antibodies (mAbs) were prepared in the Key Laboratory of Animal Virology of Ministry of Agriculture, Zhejiang University (Feng et al., 2018). Rabbit anti-hnRNPAB (ab199724; Abcam, Cambridge, UK). Rabbit anti-NXF1 mAb (12735) was purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-labeled goat anti-mouse or anti-rabbit IgG, fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit and Alexa Fluor™ 546 labeled donkey anti-mouse antibodies, were purchased from KPL (Millford, MA, USA). Actinomycin D (ActD) was purchased from APEX BIO (A4448) and solubilized in Dimethyl sulfoxide (DMSO) to a stock of 5 mg/ml.

**Virus infection and titer determination**

The cells were infected with influenza A virus diluted in DMEM without FBS and incubated at 37 °C for 1 h. Then the DMEM was moved from the cells. The cells were washed with phosphate-buffered saline (PBS) and added with DMEM containing 1 μg/ml N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (LS003750, Worthington Biochemicals). 50% tissue culture infective dose (TCID₅₀) assay was measured to determine the virus titer. The MOCK cells were seeded into 96-well plates the day before inoculation. The cells were washed with cold and sterile PBS. Influenza A virus was suffered to gradient dilution in DMEM with 1 μg/ml TPCK-treated trypsin and 2% FBS and were then added into the cells. The assay was carried with eight parallels each dilution and the last column of a 96-well plate was added with DMEM as a control. The data was calculated according to the Reed-Muench method.

**Plasmids and transfection**

To make myc-m-hnRNPAB and myc-c-hnRNPAB constructs, the mouse hnRNPAB and chicken hnRNPAB were cloned and inserted into pcmv-myc-N (635689; Clontech, Mountain View, CA, USA).
The coding region of human hnRNPAB, myc-UAP56, and myc-NXF1 were cloned and inserted into pcmv-myc-N to make the myc-hnRNPAB, myc-UAP56, and myc-NXF1 constructs. To make the Flag-NP, Flag-ALY and Flag-hnRNPAB constructs, the open reading frame (ORF) of the NP from A/HangZhou/1/2013 (H7N9), and human ALY and hnRNPAB were cloned and inserted into pcmv-flag-N (635688; Clontech). To make the flag-gst-vc constructs, the coding region of GST was cloned from pGEX-4T-1 (27-4580-01; GE healthcare, Chicago, IL, USA) and inserted into the BamHI enzyme site of pcmv-flag-N. The human hnRNPAB ORF the was inserted into the flag-gst-vc to make flag-gst-hnRNPAB construct. HnRNPAB truncation mutants (CBFNT, RRM, Gly) were cloned from myc-hnRNPAB and inserted into pcmv-myc-N, EGFP-C3 (#6082-1; Biosciences Clontech) or flag-gst-vc. The deletion mutants ΔCBFNT, ΔRRMs, ΔGly, Δ245–254 aa, Δ241–263 aa and Δ312–332 aa were cloned using deletion primers (Supplementary Table S1) from hnRNPAB. To make the GST-hnRNPAB construct, the human hnRNPAB ORF was cloned and inserted into pGEX-4T-1. The coding region of NP from A/HangZhou/1/2013 (H7N9) was cloned and inserted into pET-28a-C (69864-3; Novagen, Madison, WI, USA) to make His-NP construct. The constructed plasmids were transfected into cells using ExFect transfection reagent (T101-01/02; Vazyme Biotechnology, Nanjing, China) according to the manufacturer’s instructions.

RNAi and transfection

Short interfering RNAs (siRNA) targeting hnRNPAB (5’-GGUAGUACAAACUACCGCATT-3’), ALY (5’-GAUAUUCAGGAACUCUUUGTT-3’), UAP56 (5’-GGGCUUGGCUAUCACAUUUTT-3’) and NXF1 (5’-UGACAUAGUCUAGCAUGUUTT-3’) were designed and synthesized by GenePharma (Shanghai, China) as described previously (Viphakone et al., 2012). siRNAs were transfected using jetPRIME (21Y0910L3; Polyplus, France) transfection reagent, as recommended by the manufacture. After 24 h or 36 h, cells transfected with siRNAs were harvested or infected with viruses.

Cell proliferation assay

Cell proliferation was determined using Cell Counting Kit-8 (CCK-8) (C0037, Beyotime, China). The assay was carried according to instructions. The 293T cells were transfected with myc-AB, myc, si-AB or si-NC for 12 h and were then seeded into 96-well plate with 2×10^3 cells/well. 12 h later cells were added with CCK-8 with 10 μl and were incubated at 37 °C for 2 h. The absorbance was finally determined at 450 nm using a microplate reader.

Western blotting

Protein samples were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk that was diluted with phosphate buffer saline
containing 0.1% Tween 20 (PBST) at 37 °C for 1 h. Membranes were washed with PBST three times and then incubated with appropriately diluted primary antibodies at 4 °C overnight. After washing with PBST three times, the membranes were incubated with corresponding HRP-labeled secondary antibodies for 1 h at room temperature. Blots were visualized using the AMI600 system (GE Healthcare, USA).

**RNA isolation, reverse transcription, and real-time PCR.**

Total RNA was isolated from A549 or 293T cells with RNAiso Plus (9109; Takara, Shiga, Japan) according to the manufacturer’s instructions. PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A; Takara) and RevertAid First Strand cDNA Synthesis Kit (00251950; Thermo Scientific, Waltham MA, USA) were used for reverse transcription, performed according to the manufacturer’s instruction. Quantitative real-time PCR (qPCR) was carried out using TB Green Premix Ex Taq (Tli RNaseH Plus) (RR420; Takara) according to the manufacturer's instructions. Reactions were performed in triplicate, and fold changes were calculated using the $2^{-\Delta\Delta C_{T}}$ method, where $\Delta C_{T}$ is the difference between the amplification fluorescence threshold of the mRNA of interest and the mRNA encoding actin used as an internal reference (available upon request). The primers used for qPCR are listed in Supplementary Table S1.

**Co-Immunoprecipitation (co-IP)**

293T cells were transfected with the indicated plasmids for 24 h and then lysed with NP-40 lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were immunoprecipitated with the indicated antibodies and protein A/G (J1817; Santa Cruz biotechnology, Santa Cruz, CA, USA) and then incubated at 4 °C for 4 h. Protein A/G beads were washed with cold PBS four times. The beads were boiled with SDS lysis buffer and loading buffer for 10 min. The immunoprecipitates and cell whole lysates were analysis using western blotting with the indicated antibodies.

**GST pull down**

293T cells were separately transfected with flag-gst-vc, flag-gst-hnRNPAB, flag-gst tagged truncation mutants of hnRNPAB, myc-UAP56, myc tagged NXF1, or truncation mutants of NXF1. At 36 h post-transfection, cells were solubilized with NP-40. The cell lysates with flag-gst transfection were mixed with Pierce Glutathione Agarose (16101; Thermo Scientific) at 4 °C for 4 h and washed with cold PBS three times. The resultant agarose was incubated with lysates of cells transfected with the indicated plasmids at 4 °C for 4 h for western blotting.

*Escherichia* BL21 cells were separately transformed GST, GST-hnRNPAB, and His-NP and induced with 0.1 mM IPTG at 16 °C for 24 h. The GST and GST-hnRNPAB proteins were purified using Pierce
Glutathione Agarose. The His-NP protein was purified using Ni-NTA Agarose (163014747; QIANGEN, Hilde, Germany). The purified GST and GST-hnRNPAB (100 μg) were mixed with His-NP (100 μg) at 4 °C for 2 h and then added with Pierce Glutathione Agarose and rocked at 4 °C for 4 h. The bound proteins were analyzed using western blotting.

**In situ hybridization**

In situ hybridization was performed as previously reported (Mor et al., 2017). The DNA probe 5′ cy3-labeled oligo(dT) (cy3-oligo(dT)) and H7N9 M1, HA, NP and NS1 mRNA probes (Table S2) used in this study were synthesized by Sangon Biotech (Shanghai, China). Cells were fixed with 4% polyformaldehyde (PFA) for 15 min at room temperature and then incubated in ethanol for 12 h at 4 °C. Cells were permeabilized in 0.5% Triton-100 for 5 min and then treated with the probe at 37 °C for 16 h. Cells were incubated with the indicated primary antibodies at 37 °C for 1 h and with secondary antibodies at 37 °C for 1 h. Nuclei were stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI). Confocal microscopy observation was performed using a Zeiss LSM 510 laser confocal microscope (Zeiss, Oberkochen, Baden-Württemberg, Germany).

**Confocal microscopy**

A549 cells were infected with H7N9 for 24 h, fixed with 4% PFA for 15 min at room temperature and permeabilized with 0.5% Triton-X-100 for 15 min. The cells were then blocked with 5% bovine serum albumin for 1 h, and then incubated with primary antibodies (mouse anti-NP mAb, 1:500; rabbit anti-hnRNPAB mAb, 1:500) at 37 °C for 2 h and then with the appropriate secondary antibodies (FITC labeled goat anti-rabbit IgG(H+L), 1:1000; Alexa Fluor™ 546 donkey anti-mouse IgG (H+L), 1:1000) at 37 °C for 1 h. DAPI was used to stain the nucleus.

**Isolation of Nuclear and Cytoplasmic RNAs or proteins**

Isolation of nuclear and cytoplasm RNAs and proteins was performed as described previously (Jiang et al., 2018). Cells that were transfected with the indicated plasmids or infected with influenza A virus were harvest with PBS or Diethyl pyrocarbonate (DEPC)-treated PBS. To isolate RNA, the lysis buffer was added with RNase inhibitor (B600008; Sangon Biotech) and the RNA was isolated with RNAiso Plus from cytoplasmic and nuclear fractions. To isolate proteins, the whole lysates, and the cytoplasmic and nuclear fractions were added with loading buffer and boiled for 10 min, followed by western blotting analysis.

**RNA Immunoprecipitation (RIP)**
Cells were washed with cold DEPC-treated PBS twice and centrifuged at 1,000 \( \times g \) for 5 min. Pellets were resuspended with RIP lysis buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT (A100281; Sangon Biotech), 0.5% NP40, 100 U/ml RNAase inhibitor, 1 mM PMSF) and then incubated at 4 °C for 1 h. After centrifugation at 12,000 \( \times g \) for 10 min, the supernatants were incubated with the indicated antibodies at 4 °C for 4 h and then protein A/G was added and incubation continued at 4 °C for 4 h. The beads were then washed with RIP lysis buffer four times and then subjected to RNA isolation and reverse transcription to cDNA. The cDNA was analyzed by qPCR using the indicated primers.

**RNA Pull Down Assay**

Biotin-labeled oligo(dT) (DNA, 40 nt) and biotin-labeled M1 mRNA probes (Table S2) were synthesized by Sangon Biotech. The synthesized biotin-oligo(dT) was heated at 95 °C for 5 min and immediately put on ice for 5 min. The 293T cells were transfected with the indicated plasmids or infected with influenza A virus. The cells were UV-crosslinked in PBS with 300 mJ/cm² and were then lysed using RIP lysis buffer and then centrifuged at 12000 \( \times g \) for 10 min. The total proteins (1-2 mg) were denatured with SDS lysis buffer (0.5 % SDS, 150 mM KCl, 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.5 mM DTT, 100 U/ml RNAase inhibitor (add fresh each time), Protease inhibitors (add fresh each time)) and incubated with biotin-oligo (dT) at 4 °C for 4 h. Streptavidin M280 beads (11205D; Thermo Fisher Scientific) were added into the mix and incubated at 4 °C for 4 h. The beads were washed with SDS lysis buffer for five times and analyzed by western blotting with the indicated antibodies.

**Statistical analysis**

Student's t test was used for data analysis with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA. If the P value was < 0.05, the mean difference was considered statistically. The p values in figures are defined as follows: **, \( p < 0.01 \); *, \( p < 0.05 \); and ns (nonsignificant), \( p > 0.05 \).
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