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VIRUS-CELL INTERACTIONS

hnRNP K Degrades Viral Nucleocapsid Protein and Induces Type I IFN Production to Inhibit Porcine Epidemic Diarrhea Virus Replication

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ABSTRACT Porcine epidemic diarrhea virus (PEDV) is a re-emerging enteric coronavirus currently spreading in several nations and inflicting substantial financial damages on the swine industry. The currently available coronavirus vaccines do not provide adequate protection against the newly emerging viral strains. It is essential to study the relationship between host antiviral factors and the virus and to investigate the mechanisms underlying host immune response against PEDV infection. This study shows that heterogeneous nuclear ribonucleoprotein K (hnRNP K), the host protein determined by the transcription factor KLF15, inhibits the replication of PEDV by degrading the nucleocapsid (N) protein of PEDV in accordance with selective autophagy. hnRNP K was found to be capable of recruiting the E3 ubiquitin ligase, MARCH8, aiming to ubiquitinate N protein. Then, it was found that the ubiquitinated N protein could be delivered into autolysosomes for degradation by the cargo receptor NDP52, thereby inhibiting PEDV proliferation. Moreover, based on the enhanced MyD88 expression, we found that hnRNP K activated the interferon 1 (IFN-1) signaling pathway. Overall, the data obtained revealed a new mechanism of hnRNP K-mediated virus restriction wherein hnRNP K suppressed PEDV replication by degradation of viral N protein using the autophagic degradation pathway and by induction of IFN-1 production based on upregulation of MyD88 expression.

IMPORTANCE The spread of the highly virulent PEDV in many countries is still leading to several epidemic and endemic outbreaks. To elucidate effective antiviral mechanisms, it is important to study the relationship between host antiviral factors and the virus and to investigate the mechanisms underlying host immune response against PEDV infection. In the work, we detected hnRNP K as a new host restriction factor which can hinder PEDV replication through degrading the nucleocapsid protein based on E3 ubiquitin ligase MARCH8 and the cargo receptor NDP52. In addition, via the upregulation of MyD88 expression, hnRNP K could also activate the interferon (IFN) signaling pathway. This study describes a previously unknown antiviral function of hnRNP K and offers a new vision toward host antiviral factors that regulate innate immune response as well as a protein degradation pathway against PEDV infection.

KEYWORDS hnRNP K, PEDV, MARCH8, NDP52, N protein, selective autophagy, IFN-1

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric viral disease which causes vomiting, watery diarrhea, and a high mortality rate in suckling pigs. The causative agent of PED is porcine epidemic diarrhea virus (PEDV) (1, 2). In addition, it was found that PED was primarily documented on a swine farm in England in 1971 (2). Since then, adequate PED control has been achieved with the use of a few

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live-attenuated vaccines (3). Recently, in 2010, highly virulent PEDV strains emerged in China and disseminated rapidly to several other countries, leading to mortality rates as high as 100% among newborn piglets, which hit the economy tremendously (1, 4–6). Currently available drugs are evidently not sufficient to counter these newly emerging strains. Elucidating the association between the virus and the host innate responses is essential for the control of viral epidemics and the identification of new therapeutic targets.

PEDV, a member of the genus *Alphacoronavirus* in the *Coronaviridae* family (7), is a positive-sense, single-stranded RNA virus. Furthermore, its genome encompasses seven open reading frames (ORFs) which are responsible for encoding four structural proteins (envelope [E], membrane [M], spike [S], and nucleocapsid [N]), two polyproteins (pp1a and pp1ab), and one accessory protein (ORF3) (8, 9). Concerning viral replication, the N protein can shuttle between the nucleus and the cytoplasm as an RNA-binding protein (10). According to recent evidence, PEDV N protein interacts with TANK-binding kinase (TBK1) and blocks the phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF3), which contributes to inhibiting IRF3-mediated IFN production, implying that the PEDV N protein is essential for evasion of IFN response during PEDV infection (11, 12). PEDV N protein has also been reported to interact with p53 to trigger cell cycle arrest during S-phase and stimulate viral replication (13). As a result, N protein plays an important part in regulating the host innate immune system and viral replication.

Viruses have developed diverse tactics for neutralizing, eluding, and even taking control of the host defense during the infection phase. The host, on the other hand, fights viral infection through the initiation of versatile antiviral events, including intrinsic antiviral defense (14). In the case of PEDV infection, 11 viral proteins have been reported to inhibit IFN activity, thus neutralizing the congenital host immunity (15). The host, in turn, has been shown to employ various antiviral proteins to repress PEDV replication. A previous study reported that IRF1-induced BST2 (bone marrow stromal cell antigen 2) diminishes PEDV replication through targeting and deteriorating viral N protein based on selective autophagy (16). Through MARCH8-mediated upregulation of IRAV, the early growth response gene 1 (EGR1) mediates PEDV N protein deterioration, thereby suppressing viral replication (17). It has been reported that the host antiviral proteins cytoplasmic poly(A)-binding protein 4 (PABPC4), trans-active response DNA-binding protein (TARDBP), and far upstream element-binding protein 3 (FUBP3) utilize the cell protein degradation system to degrade PEDV N protein, which can lead to inhibition of viral replication (18–20). Exploring the continuous competition between the host and PEDV greatly contributes toward identifying novel approaches for preventing and treating PEDV infection.

We can regard heterogeneous nuclear ribonucleoprotein K (hnRNP K) as a highly conserved nucleic acid-binding protein. It is a member of the hnRNP complex and has crucial functions in chromatin remodeling, transcription, translation, pre-mRNA splicing, and ensuring RNA stability (21–23). Recently, a growing body of evidence shows that hnRNP K exerts vital functions in the modulation of viral infection (24). Bovine ephemeral fever virus (BEFV) and dengue virus (DENV) (25, 26) regulate hnRNP K to facilitate virus replication. hnRNP K has also been shown to bind miR-122, a mature liver-specific microRNA needed for hepatitis C virus replication (27). Through modulation of cell survival and cellular gene expression, hnRNP K can also suppress vesicular stomatitis virus replication (28). Some scholars have proved that hnRNP K serves as a novel internal ribosome entry site (IRES) trans-acting factor of the picornavirus. It negatively regulates the replication of foot-and-mouth disease virus (FMDV) by hindering viral IRES-dependent translation (24). Based on the findings obtained, hnRNP K modulates virus replication in diverse ways for different viral infections. This study found that overexpression of hnRNP K could efficiently inhibit PEDV replication. Further analysis revealed that hnRNP K upregulated the transcription factor KLF15 and inhibited PEDV replication via selective autophagy by targeting and degrading viral N protein, together with induction of MyD88-mediated activation of the IFN-1 signaling pathway.

RESULTS

PEDV infection downregulates hnRNP K expression via the transcription factor KLF15. We used mass spectrometry to search for proteins which interacted with PEDV N protein to regulate PEDV replication. We found that the hnRNP K protein interacted with PEDV N protein. To analyze the influence of PEDV infection on the level of hnRNP K protein, we first infected LLC-PK1 cells with PEDV (JS-2013 strain) at a multiplicity of infection (MOI) of 1 as previously described (29). Then, we assessed hnRNP K expression levels using Western blotting and reverse transcription-quantitative PCR (qRT-PCR) within the PEDV-infected cells. Our findings showed that compared to the uninfected cells, the infected cells showed lower protein and mRNA levels of hnRNP K (Fig. 1A and B), indicating that PEDV infection can downregulate endogenous hnRNP K expression in host cells.

Next, to further elucidate the transcriptional regulation of hnRNP K, we cloned the 2,000-bp hnRNP K promoter and its truncated sequences (D1 to D7) into the pGL3-basic luciferase vector. Our findings showed that of all the truncated sequences, D4 (nucleotides from −550 to −1) triggered the highest luciferase activity. Next, we further truncated the D4 sequence, aiming to identify the boundaries of the minimal hnRNP K core promoter. The luciferase reporter assay demonstrated that the sequence comprising nucleotides from −75 to −25 triggered the highest luciferase activity, implying that the hnRNP K core promoter is located within positions −75 to −25 (Fig. 1C). Next, we used the JASPAR vertebrate database (http://jaspar.genereg.net/) (30) with the aim of determining the underlying transcription factor binding sites of the hnRNP K promoter. We observed that the hnRNP K core promoter region contained the binding sites for five transcription factors: ZBTB33, MAZ, KLF10, KLF14, and KLF15 (Fig. 1D). To determine the regulatory impacts of the transcription factors on the hnRNP K promoter, we assessed their mRNA expression in PEDV-infected LLC-PK1 cells. qRT-PCR analysis revealed significant downregulation of ZBTB33, MAZ, and KLF15 in PEDV-infected cells (Fig. 1E). Next, we transfected 293T cells with small interfering RNAs (siRNAs) specific to the ZBTB33, MAZ, and KLF15 mRNA sequences. The luciferase reporter assay proved that only the 293T cells transfected with KLF15 siRNA exhibited significantly enhanced hnRNP K expression (Fig. 1F). To immunoprecipitate the hnRNP K core promoter region, we also carried out a chromatin immunoprecipitation (ChIP) assay with Flag-KLF15. Based on the results obtained, KLF15 can absolutely bind to the hnRNP K promoter (Fig. 1G), proving that it is involved in controlling hnRNP K expression. These data suggested that PEDV infection leads to KLF15-mediated downregulation of hnRNP K expression.

hnRNP K inhibits PEDV replication. To illustrate the function of hnRNP K in PEDV infection, this study primarily evaluated the impact of hnRNP K on *in vitro* PEDV replication. We transfected Vero cells with Flag-hnRNP K plasmids. Then, with PEDV at a MOI of 0.01 at one day post-transfection, the cells were subject to infection. Samples of cells and supernatants were gathered at the expected time points for evaluation of PEDV N expression and PEDV viral loads. In addition, using Western blotting (Fig. 2A), qRT-PCR (Fig. 2B), and 50% tissue culture infective dose ($TCID_{50}$), we determined the viral yield at 20 and 24 h postinfection (hpi) (Fig. 2C). hnRNP K was shown to suppress PEDV proliferation in Vero cells. In addition, the viral titers in the supernatants of the Vero cell cultures also revealed that hnRNP K overexpression inhibited PEDV replication (Fig. 2D). Furthermore, we observed that transfecting the cells with enhancing concentrations of Flag-hnRNP K plasmids generated a decrease in PEDV N mRNA and protein levels in a concentration-dependent manner (Fig. 2E and F). Next, in LLC-PK1 cells, we observed that hnRNP K overexpression contributed to a decrease in PEDV N mRNA and protein levels at 24 and 28 hpi (Fig. 2G and H). Next, we transfected LLC-PK1 cells with hnRNP K siRNA. In accordance with the previous results, silencing hnRNP K expression enhanced PEDV replication in LLC-PK1 cells (Fig. 2I and J). Overall, the findings suggested that hnRNP K hinders PEDV replication in Vero and LLC-PK1 cells.

hnRNP K can target and degrade PEDV N protein according to selective autophagy. To determine the molecular mechanisms underlying hnRNP K-mediated suppression of PEDV replication, we examined whether hnRNP K interacted with PEDV

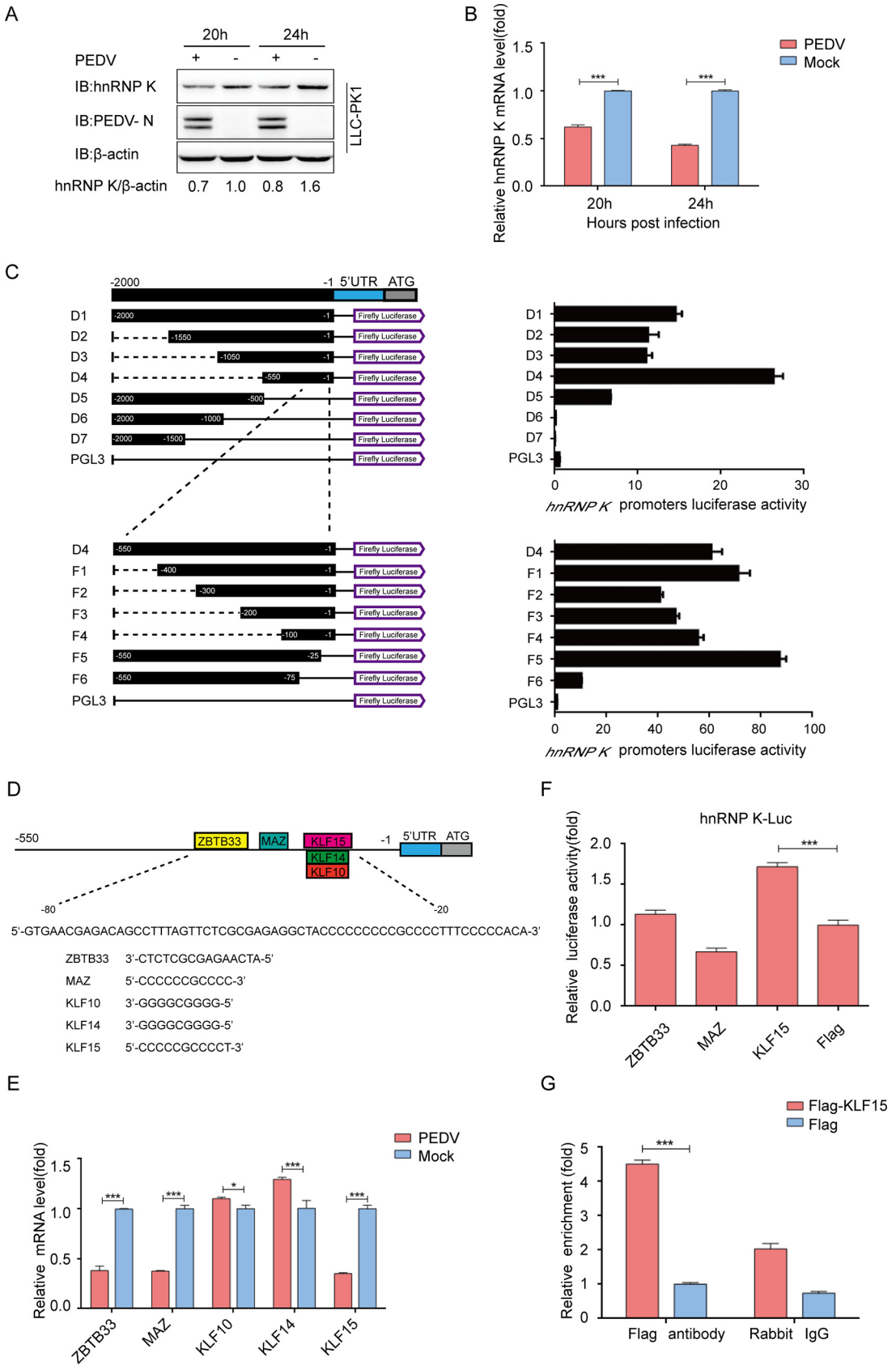

FIG 1 Porcine epidemic diarrhea virus (PEDV) infection leads to KLF15-mediated downregulation of heterogeneous nuclear ribonucleoprotein K (hnRNP K) expression. (A) When PEDV at a multiplicity of infection (MOI) reaches 1, we gathered LLC-PK1

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structural proteins (S1, S2, E, M, and N). We used a co-immunoprecipitation (co-IP) assay in HEK 293T cells exposed to co-transfection with individual plasmids and Flag-hnRNP K plasmids. We observed precipitation of PEDV N in cells transfected with Flag-hnRNP K (Fig. 3A). Since hnRNP K is a highly conserved nucleic acid-binding protein (21), we treated cell lysates with RNase and found that it did not interfere with the affinity of hnRNP K for PEDV N (Fig. 3A), suggesting that the association of hnRNP K and PEDV N protein was RNA-independent. Further, we observed efficient co-immunoprecipitation of the PEDV N protein with the endogenous hnRNP K protein (Fig. 3B). In addition to the co-IP assay, we performed a glutathione *S*-transferase (GST) pulldown assay to confirm whether hnRNP K binds to PEDV N protein. Although GST-fused PEDV N (GST-N) was found to be in combination with hnRNP K, no such binding was observed between GST and hnRNP K (Fig. 3C). These findings indicated that PEDV N directly binds to hnRNP K. Next, we used confocal microscopy to assess whether hnRNP K colocalized with PEDV N. It was shown that PEDV N protein colocalized with hnRNP K in the cytoplasm (Fig. 3D).

PEDV N protein plays several roles in viral replication and immune evasion (31) and can be degraded by several antiviral proteins, such as BST2, IRAV, and PABPC4 (16–18). We hypothesized that hnRNP K might inhibit PEDV replication by regulating PEDV N protein levels. To confirm our hypothesis, the cells were exposed to co-transfection by adding concentrations of Flag-hnRNP K expression plasmids along with HA-N plasmids. In addition, PEDV N protein levels were found to decreases in a Flag-hnRNP K concentration-dependent manner (Fig. 3E). Next, we hypothesized that PEDV N protein levels might be regulated via two primary intracellular protein degradation pathways in eukaryotic cells: the ubiquitin-proteasome system and the autolysosome pathway (32). To detect the degradation pathway predominantly involved in hnRNP K-mediated PEDV N protein degradation, we co-transfected HEK 293T cells with plasmids encoding Flag-hnRNP K and HA-N and treated them with the protease inhibitor MG132 and the autophagy inhibitors bafilomycin A1 (Baf A1), 3-methyladenine (3-MA), and chloroquine (CQ). Using Western blotting, we quantified intracellular PEDV N protein expression. Our findings demonstrated that hnRNP K-mediated PEDV N protein degradation could be reversed through the autophagy inhibitors, BafA1, 3MA, and CQ, whereas MG132 failed to exert such an effect (Fig. 3F). Overall, these findings indicated that hnRNP K triggered PEDV N degradation via the autophagy pathway.

hnRNP K can degrade PEDV N protein via the hnRNP K-MARCH8-NDP52-autophagosome pathway. Through selective autophagy, the substrate proteins can be ubiquitinated by the E3 ubiquitin ligase, with the ubiquitinated complexes being subsequently known by cargo receptors. Then, cargo receptors can carry the substrates to the ATG8 family proteins, resulting in the formation of autophagosome degradation substrates which ultimately degrade the substrate proteins (33). Based on recent studies, some host antiviral factors, including FUBP3 and TARDBP, can recruit E3 ubiquitin ligase MARCH8 with the aim of catalyzing the ubiquitination of PEDV N protein. Subsequently, through the cargo receptor NDP52, the ubiquitinated PEDV N protein can be transported to the lysosome for degradation (19, 20). To explore the mechanisms underlying hnRNP K-mediated degradation of PEDV N protein using the autophagy pathway, we performed a co-IP assay. We observed that Flag-hnRNP K co-immunoprecipitated with MARCH8 and NDP52 in HEK 293T cells (Fig. 4A). A GST affinity-isolation assay was used to confirm whether hnRNP K was directly bound to MARCH8 and NDP52 (Fig. 4B and C). According to immunofluorescence confocal microscopy analysis, hnRNP K was

FIG 1 Legend (Continued)
cells at 20 and 24 h postinfection (hpi). Additionally, to verify the expression of hnRNP K and PEDV nucleocapsid (N) proteins, we used Western blotting. β-Actin was used as the sample loading control. (B) Reverse transcription-quantitative PCR (qRT-PCR) was used to quantify hnRNP K mRNA expression levels in the samples. (C) To explore dual luciferase activity, HEK 293T cells were transfected with truncated constructs of hnRNP K promoter comprising Renilla luciferase reporter vector (pRL-TK-luc). (D) The JASPAR vertebrate database was used to predict the transcription factor binding sites (TFBSs) of the hnRNP K promoter. (E) To assess the relative mRNA levels of predicted genes, we exposed PEDV-infected LLC-PK1 cells to qRT-PCR. (F) 293T cells were transfected with hnRNP K promoter-driven luciferase vector and plasmids encoding ZBTB33, MAZ, or KLF15. Samples were later gathered one day post-transfection, followed by assessment of dual luciferase activity. (G) LLC-PK1 cells were transfected with Flag-KLF15 plasmid or empty vector. Next, the cells were subsequently collected and subjected to chromatin immunoprecipitation (ChIP) analysis. Data are shown as means ± standard deviation (SD) of triplicate samples. *, $P < 0.05$; ***, $P < 0.001$ by two-tailed Student's *t* test.

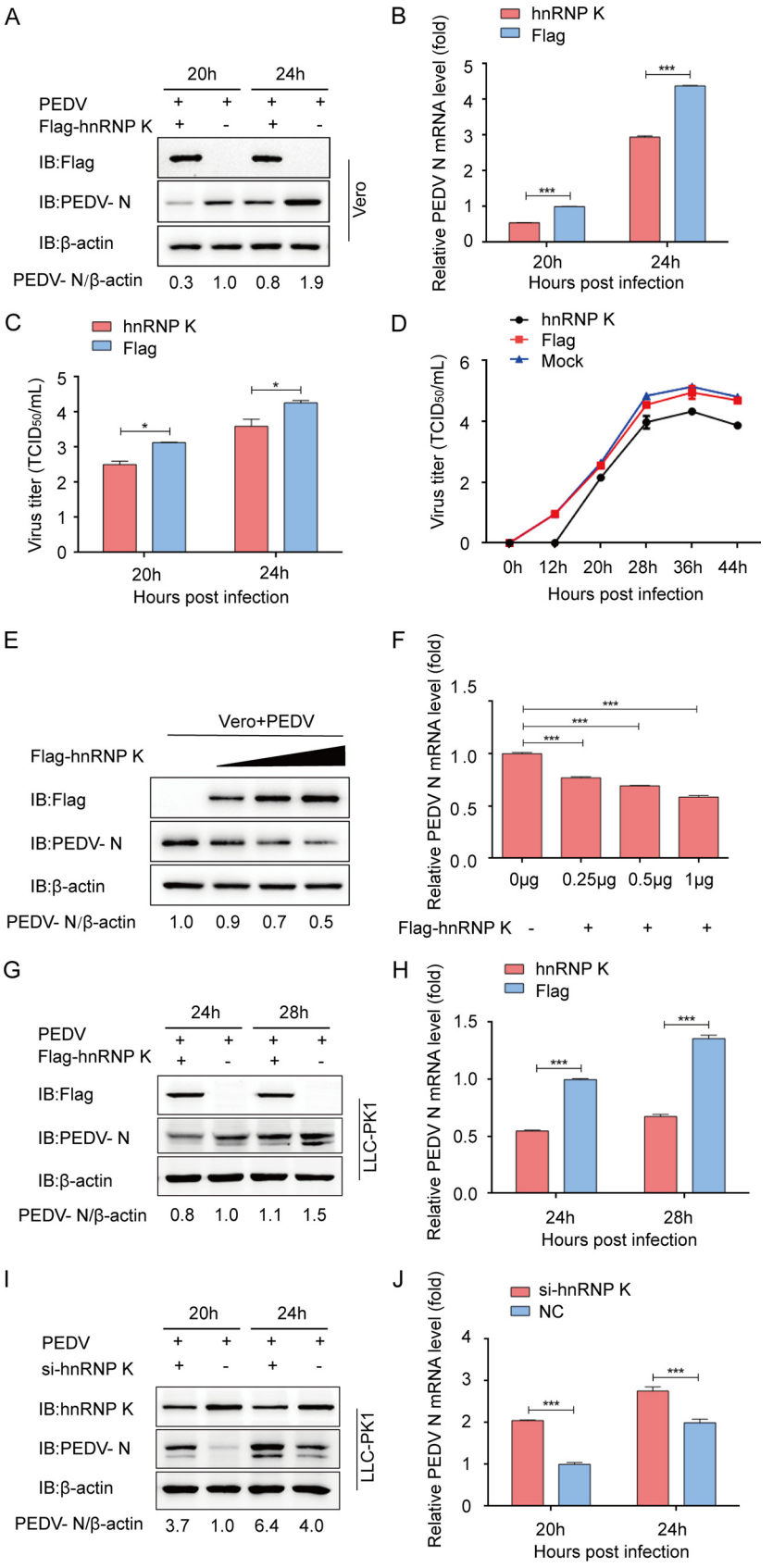

FIG 2 hnRNP K can inhibit PEDV replication. (A to D) Vero cells were transfected with plasmid encoding Flag-hnRNP K and exposed to PEDV infection (MOI = 0.01) with cell samples taken at the

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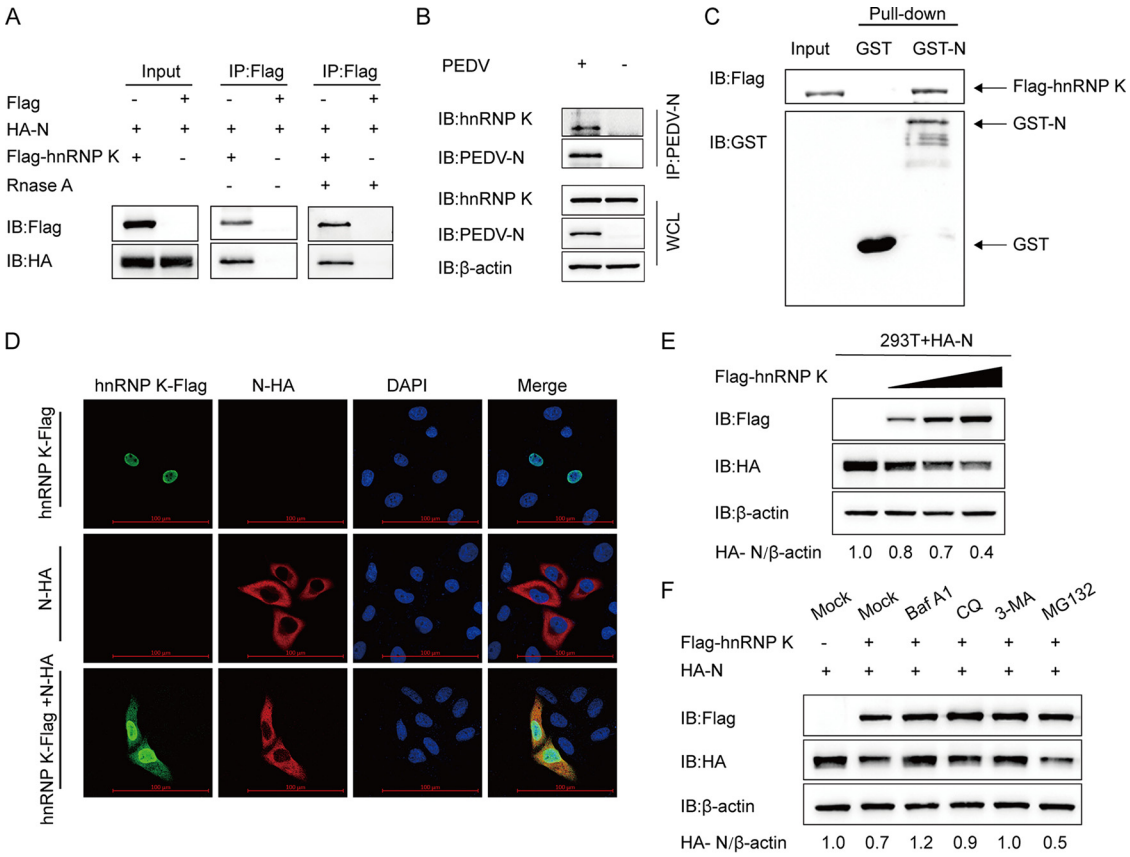

FIG 3 hnRNP K degrades PEDV N protein through selective autophagy. (A) HEK 293T cells were co-transfected with plasmids encoding Flag-hnRNP K and HA-N. Lysates were incubated with RNase, and the correlation between hnRNP K and N proteins was investigated using co-immunoprecipitation (co-IP) assays. (B) Vero cells were exposed to either mock-infection or infection with PEDV (MOI = 0.01). We collected samples at one day postinfection. Then, we performed co-IP assays based on anti-PEDV N protein antibody. (C) GST-N and hnRNP K proteins were denoted in bacterial strain BL21(DE3). Glutathione *S*-transferase (GST) pulldown analysis was used to explore the correlation between hnRNP K and N proteins. (D) HeLa cells were co-transfected with plasmids encoding Flag-hnRNP K and HA-N for a whole day. In addition, cells incubated with the indicated primary and fluorescently labeled secondary antibodies. Confocal immunofluorescence microscopy was used to observe the cells. Scale bar = 100 μm. (E) HEK 293T cells were transfected with an expression vector encoding HA-N and increasing doses of an expression vector encoding Flag-hnRNP K (wedge). Western blotting was used to analyze cell lysates. (F) HEK 293T cells were transfected with plasmids encoding Flag-hnRNP K and HA-N for 24 h, and subsequently treated with bafilomycin A1 (Baf A1; 50 μM), chloroquine (CQ; 50 μM), 3-methyladenine (3-MA; 1 mM), or MG132 (5 μM) for 9 h. Western blotting was used to analyze cell lysates.

translocated from the nucleus to the cytoplasm and effectively colocalized with MARCH8 and NDP52 (Fig. 4D). To study whether the MARCH8-NDP52-autophagosome pathway was engaged during hnRNP K-induced PEDV N protein degradation, HEK 293T cells were co-transfected with HA-N and Flag-hnRNP K encoding plasmids as well as either MARCH8 siRNA or NDP52 siRNA. According to Western blot analysis, interfering with MARCH8 or NDP52 suppressed hnRNP K-triggered degradation of PEDV N protein (Fig. 4E). Then, to determine whether the MARCH8-NDP52-autophagosome axis was indispensable for hnRNP K-mediated suppression of PEDV replication, we co-transfected PEDV-infected Vero cells with Flag-hnRNP

FIG 2 Legend (Continued)
desired times. Western blotting, qRT-PCR, and 50% tissue culture infective dose ($TCID_{50}$) were used for the cell lysates and supernatants. β-Actin was used as the sample loading control. (E and F) Higher concentrations of Flag-hnRNP K plasmids were enhanced to transfect the Vero cells. Subsequently, we infected the cells with PEDV (MOI = 0.01) with samples taken for Western blotting and qRT-PCR. (G and H) LLC-PK1 cells were transfected with Flag-hnRNP K plasmid and exposed to PEDV infection at an MOI of 1. Cells were subsequently gathered for Western blotting and qRT-PCR. (I and J) LLC-PK1 cells were transfected with hnRNP K small interfering RNA (siRNA) or negative-control siRNA and subjected to PEDV infection (MOI = 1). Samples were taken at specific times. We used Western blotting and qRT-PCR to investigate PEDV N. Data are shown as means ± SD of triplicate samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by two-tailed Student's *t* test.

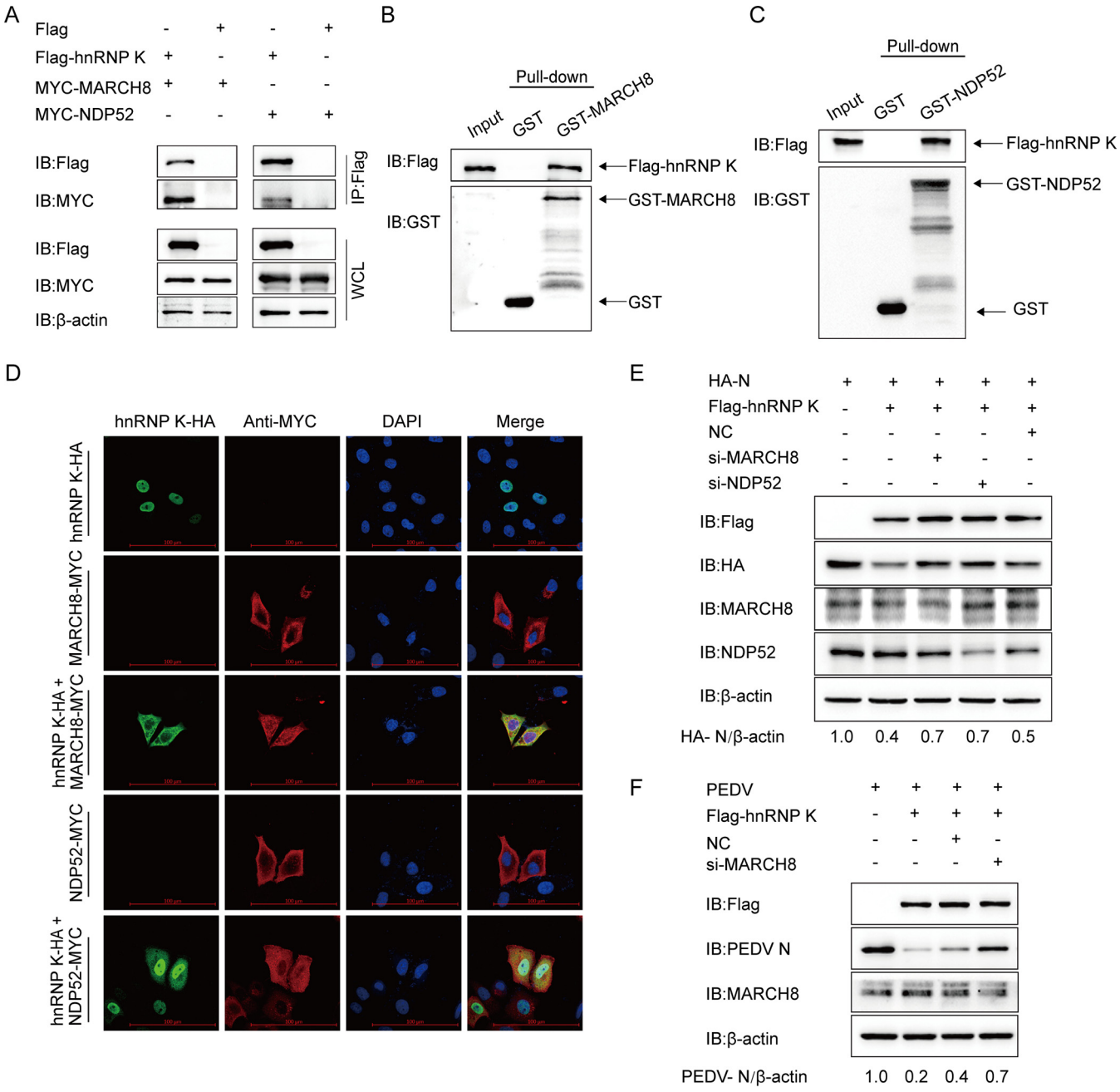

FIG 4 hnRNP K degrades N protein via the MARCH8-NDP52-autophagosome pathway. (A) We co-transfected HEK 293T cells with plasmids encoding Flag-hnRNP K and either MYC-MARCH8 or MYC-NDP52, and immunoprecipitated them with anti-Flag binding beads. We then examined the samples using Western blotting. (B and C) GST-MARCH8, GST-NDP52, and hnRNP K were denoted in bacterial strain BL21(DE3). A GST pulldown analysis was used to investigate the correlation between hnRNP K and MARCH8 or NDP52. (D) HeLa cells were co-transfected with HA-hnRNP K and either MYC-MARCH8 or MYC-NDP52 for a whole day. Cells were then incubated with the indicated antibody. We used confocal immunofluorescence microscopy to determine the co-localization of hnRNP K and MARCH8. Scale bar = 100 μm. (E) HEK 293T cells were co-transfected with plasmids encoding Flag-hnRNP K, HA-N, and either MARCH8 siRNA or NDP52 siRNA. Western blotting was used to quantify N protein. (F) Vero cells were co-transfected with plasmids encoding Flag-hnRNP K and MARCH8 siRNA for one day and infected with PEDV (MOI = 0.01). Finally, Western blotting was performed using monoclonal antibody against PEDV N protein.

K-encoding plasmid and MARCH8 siRNA. We found that hnRNP K-mediated inhibition of PEDV infection could be reversed by interfering with MARCH8 (Fig. 4F). Thus, blocking the autophagy pathway could reverse the hnRNP K-mediated inhibition of PEDV proliferation. Overall, these findings showed that hnRNP K could degrade PEDV N protein via the hnRNP K-MARCH8-NDP52-autophagosome pathway.

hnRNP K upregulates IFN production via increasing the expression of MyD88. During viral infection, the host's innate immune response is activated, leading to the

release of interferons, establishing an antiviral state against viral infection (11). To tackle rapidly evolving or newly emerging viruses, antiviral proteins would also need to evolve and acquire some novel functions (34). To explore whether hnRNP K induces IFN production, we performed an interferon β (IFN-β) promoter-driven luciferase reporter assay and discovered that hnRNP K triggered IFN-β activation in a dose-dependent manner (Fig. 5A). Furthermore, IFN-β mRNA levels constantly increased in the hnRNP K-transfected HEK 293T cells (Fig. 5B). To elucidate the mechanism underlying hnRNP K-mediated induction of IFN-β production, we co-transfected hnRNP K expression plasmids with plasmids encoding key signaling proteins engaged in the innate antiviral response. As shown in Fig. 5C, hnRNP K enhanced the luciferase reporter activity triggered by MyD88, TRAF3, and TRAF6. Afterwards, we attempted to co-transfect the HEK 293T cells with hnRNP K-expressing plasmid and either MyD88, TRAF3, or TRAF6 siRNA. In addition, a dual-luciferase reporter assay showed significant downregulation of IFN-β expression after the suppression of MyD88, TRAF3, and TRAF6 expression (Fig. 5D). This suggests that MyD88 may exert a key function in hnRNP K-mediated induction of IFN-β production. Next, we induced hnRNP K protein overexpression and observed a consistent upregulation of MyD88 expression (Fig. 5E and F). Furthermore, a Western blot assay showed that reducing MyD88 expression effectively prevented the activation of IFN-1 by hnRNP K (Fig. 5G). Overall, our findings revealed that hnRNP K induced MyD88-mediated upregulation of IFN expression to trigger the host antiviral innate immune response.

DISCUSSION

PEDV is a re-emerging enteric coronavirus spreading across several nations and causing huge losses to the swine industry (35). Although antiviral vaccines are usually an efficient tool to counter virus infection, currently available commercial PEDV vaccines have proven ineffective in resisting the newly emerging epidemic strains (36, 37). Understanding the relationship between the virus and host antiviral factors can help us comprehend effective antiviral mechanisms. This information can, in turn, be highly beneficial in the development of novel efficient vaccines and the identification of novel drug targets for treatment of PEDV infection. This study demonstrated the previously unknown role of hnRNP K in suppressing PEDV infection. Our results showed that in PEDV-infected cells, the transcription factor KLF15 regulated the expression of hnRNP K. We also observed that using the hnRNP K-MARCH8-NDP52 autophagosome pathway, hnRNP K suppresses PEDV replication by degrading PEDV N protein. Furthermore, hnRNP K was also shown to stimulate the MyD88-mediated activation of the IFN signaling pathway (Fig. 6).

hnRNP K, a DNA/RNA-binding protein, is associated with a broad scope of biological processes, including translation, long noncoding RNA (lncRNA) regulation, cancer development, and bone homoeostasis (22). It has previously been reported that hnRNP K expression can be controlled via the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway in chronic myelogenous leukemia. MAPK-mediated phosphorylation leads to overexpression and enhanced stability of hnRNP K (38). However, the role of hnRNP K in cases of virus infection is still poorly understood. This study observed that hnRNP K expression was associated with the suppression of PEDV infection. While determining the transcription factors that regulate hnRNP K expression, the results obtained showed that the minimal hnRNP K core promoter was located within positions–75 to −25. Investigation of the regulatory elements of hnRNP K revealed that its expression is regulated by the transcription factor KLF15.

The continuous clashes between viruses and hosts have led to the evolution of host antiviral proteins, which are considered essential for the suppression of rapidly evolving or newly emerging viruses. In addition, viral infections can even foster the evolution of antiviral activities in proteins which were previously known to only exert functions that had no association with host immunity (34). Several studies have revealed that hnRNP K exerts a vital function in virus infection, apart from other regulatory functions. The interaction between the BEFVα3 gene and host hnRNP K gene in BEFV-induced apoptosis facilitates

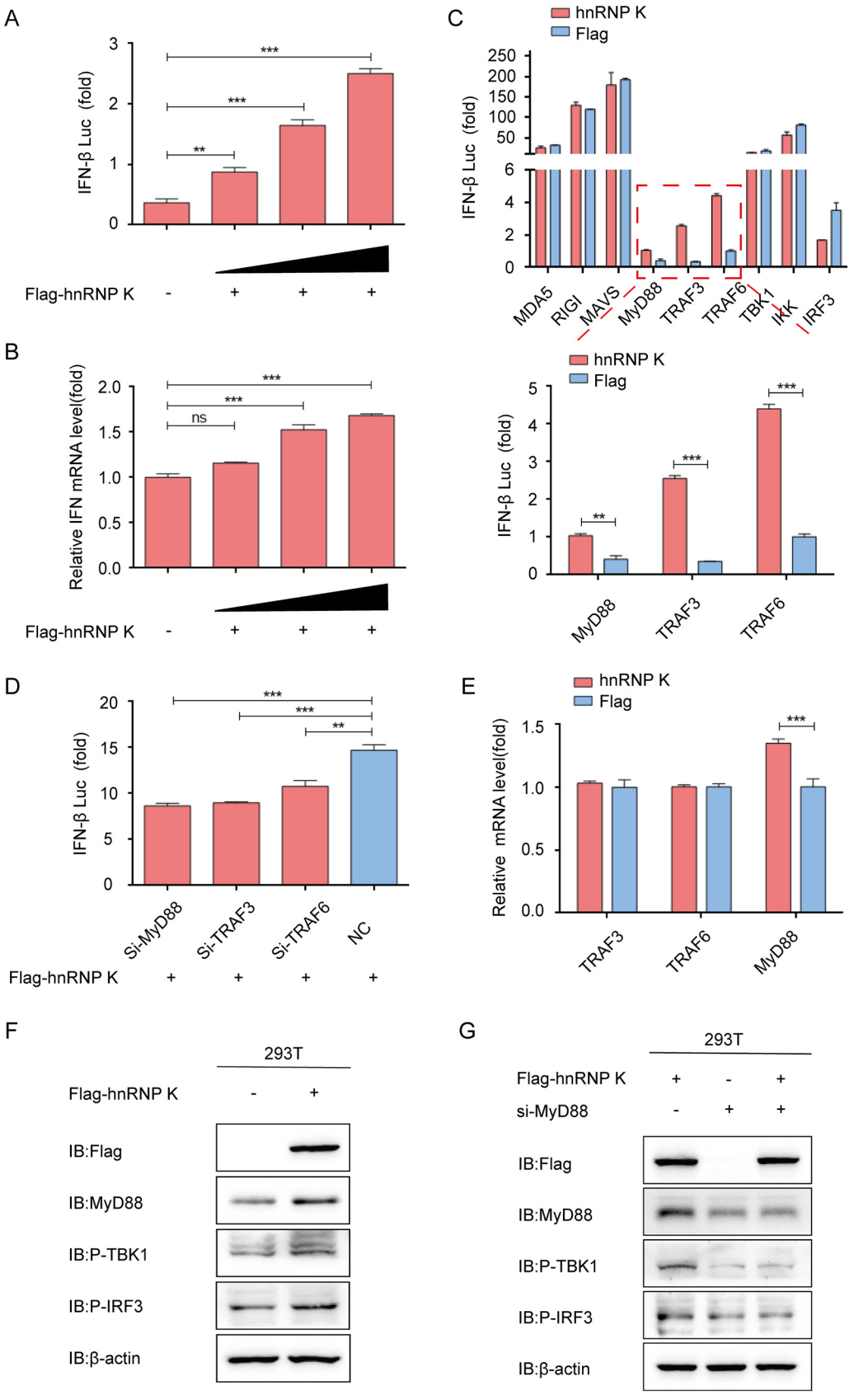

FIG 5 hnRNP K activates interferon (IFN) signaling pathway via MyD88 upregulation. (A) HEK 293T cells were transfected with an IFN-β luciferase reporter and increasing doses of an expression vector encoding Flag-hnRNP K to

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BEFV replication (26). During DENV infection, the cytoplasmic translocation of hnRNP K has been shown to favor viral replication (25). Additionally, hnRNP K has been shown to be involved in host cell apoptosis and to participate in the regulation of vesicular stomatitis virus-induced apoptosis (28). However, FMDV replication is inhibited by hnRNP K via suppression of viral IRES-mediated translation (24). Here, we discovered that hnRNP K also inhibits PEDV replication. Further analysis revealed that hnRNP K interacted with the PEDV N protein and that the association between hnRNP K and PEDV N protein was RNA-independent.

The autolysosome and proteasome pathways can be regarded as the two primary protein degradation pathways found in eukaryotic cells (39). Here, we demonstrated that via the autophagy pathway, hnRNP K could promote the degradation of PEDV N protein. During autophagy, targeting substrates and engulfed cargo are associated with specific selectively autophagy during virus infection (40). In selective autophagy, the substrates are ubiquitinated by E3 ubiquitin ligase, with the ubiquitinated complexes being identified by cargo receptors. According to previous studies, the host antiviral proteins BST2 and PABPC4 can target the E3 ubiquitin ligase MARCH8 and the cargo receptor NDP52 to trigger the degradation of PEDV N protein and inhibition of PEDV replication (16, 18). This study found that hnRNP K could recruit E3 ubiquitin ligase MARCH8 with the aim of catalyzing the PEDV N protein. In addition, the ubiquitinated PEDV N protein was also recognized and degraded by NDP52-dependent selective autophagy. As a result, based on the obtained findings, hnRNP K stimulated the degradation of PEDV N protein according to selective autophagy with the purpose of inhibiting PEDV replication.

During virus infection, the host rapidly detects viral components or replication intermediates using pattern recognition receptors (PRRs) to induce antiviral responses to inhibit virus replication. IFN and IFN-induced cellular antiviral responses are the primary defense mechanisms against viral infection (31). Upon sensing viral RNAs in the cytoplasm, the PRRs interact with the mitochondrial antiviral signaling (MAVS) protein to activate the downstream TBK1 and an important transcription factor, IRF3, with the aim of triggering type 1 IFNs (41). In this study, we found that hnRNP K upregulates MyD88 expression, subsequently regulating the phosphorylation of TBK1 and IRF3 and inducing IFN-1 production. In addition, this function also contributes to the antiviral activity of hnRNP K.

Overall, it was demonstrated that hnRNP K hinders PEDV replication through the degradation of PEDV N protein and induction of type 1 IFN expression. hnRNP K can recruit MARCH8, an E3 ubiquitin ligase, aiming to trigger PEDV N protein ubiquitination. Subsequently, NDP52 (a cargo receptor) is found to detect the ubiquitin complex (PEDV N-hnRNP K-MARCH8), triggering degradation of PEDV N by delivering it into autolysosomes. In addition, hnRNP K also activates the IFN signaling pathway by enhancing the MyD88 expression. In this study, we presented an innovative mechanism of hnRNP K-regulated viral restriction and proposed a potential anti-PEDV target.

MATERIALS AND METHODS

Antibodies and reagents. Anti-Myc-tag antibody (no. 2276s) and Baf A1 (no. 54645) were purchased from Cell Signaling Technologies. In addition, anti- hemagglutinin (HA) antibody (no. H6908), anti-Flag M2 antibody (F1804), MG132 (M7449), CQ (PHR1258), and 3-MA (M9281) were obtained from Sigma-Aldrich. Anti-GST antibody (no. 10000-0-AP), anti-β-actin (66009-1-Ig), anti-hnRNP K antibody

FIG 5 Legend (Continued)
examine dual luciferase activity. (B) HEK 293T cells were transfected with increasing doses of an expression vector encoding Flag-hnRNP K. qRT-PCR was used to determine IFN mRNA levels. (C) We attempted to co-transfect HEK 293T cells with plasmids encoding hnRNP K and IFN-β luciferase reporter together with plasmids encoding MDA5, RIG-I, MAVS, MyD88, TRAF3, TRAF6, TBK1, IKK, or IRF3. We investigated dual luciferase activity. (D) HEK 293T cell lysates were transfected with the expression vector encoding Flag-hnRNP K. MyD88 siRNA, TRAF3 siRNA, and TRAF6 siRNA expression was examined using a luciferase assay. (E) HEK 293T cells were transfected with plasmids encoding Flag-hnRNP K, and levels of MyD88, TRAF3, and TRAF6 mRNA were quantified using qRT-PCR. (F) HEK 293T cells were transfected with the expression vector encoding Flag-hnRNP K for 24 h. Western blotting was used to assess cell lysates. (G) Flag-hnRNP K and MyD88 siRNA were co-transfected into HEK 293T cells for 24 h. Cell lysates were analyzed with a Western blot.

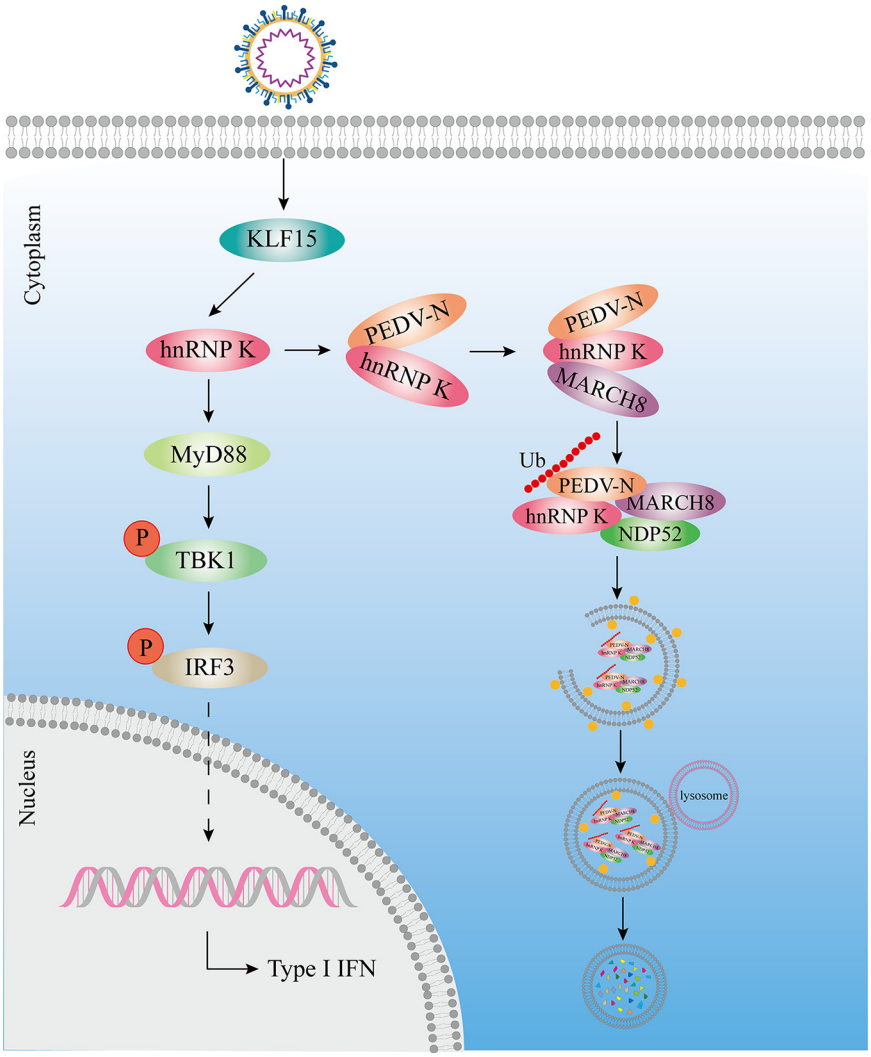

FIG 6 hnRNP K mediates antiviral function by inhibiting PEDV replication. During PEDV infection, the transcription factor KLF15 upregulated hnRNP K expression. hnRNP K ubiquitinated the viral N protein by E3 ubiquitin ligase MARCH8, and the ubiquitinated N protein was recognized and transported to the autophagosome for degradation by the cargo receptor NDP52. hnRNP K activates type 1 IFN signaling by upregulating MyD88 expression, triggering the phosphorylation of TBK1 and IRF3.

(11426-1-AP), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (SA00001-2), and HRP-conjugated anti-mouse IgG antibody (SA00001-1) were obtained from Proteintech Group, Inc. Alexa Fluor 488 goat anti-rabbit IgG(H+L) cross-adsorbed secondary antibody (no. A-11008) and Alexa Fluor 594 goat anti-mouse IgG(H+L) cross-adsorbed secondary antibody (no. A-11032) were obtained from Invitrogen.

siRNAs were constructed and obtained from GenePharma (Table 1). In addition, human MARCH8 siRNA (no. sc-90432) and human NDP52 siRNA (no. 93738) were acquired from Santa Cruz Biotechnology.

Cell culture and virus. With the supplementation of 10% fetal bovine serum (FBS) (no. 10099141; Gibco), African green monkey kidney cells (Vero cells, CCL-81; American Type Culture Collection [ATCC]), and 293T human embryonic kidney cells (CRL-11268; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (no. 12430–054; Gibco). Porcine kidney cells (LLC-PK1) donated by Rui Luo from Huazhong Agricultural University were cultivated in modified Eagle's medium (no. 11095-080; Gibco). All cells used in this study were nurtured at 37°C in an atmosphere with 5% CO_2.

PEDV variant strain JS-2013 was separated and kept *in vitro* as previously described. In addition, Vero cells were used to propagate and titrate JS-2013 as previously described (29). Viral titers were computed using the methods of Karber and denoted as $TCID_{50}$.

Plasmids and transfection. We used a ClonExpress II One-Step kit (no. C112–02; Vazyme Biotech) to perform homologous recombination cloning of hnRNP K-and other protein-encoding recombinant plasmids into a p3×Flag-CMV-7.1 vector (no. P1118; Sigma-Aldrich). Upon reaching a confluence of 80% to 90%, the Vero or HEK 293T cells were transfected with the recombinant plasmids based on the application of the Lipofectamine 3000 reagent (no. L3000-015; Invitrogen). Furthermore, cells which reached a

TABLE 1 siRNA sequences used in this study[a]

Name	Sequence (5′–3′)
si-hnRNP K	
sense	GCCUCCAUCUAGAAGAGAUTT
antisense	AUCUCUUCUAGAUGGAGGCTT
si-MyD88	
sense	GUACAAGGCAAUGAAGAAATT
antisense	UUUCUUCAUUGCCUUGUACTT
si-TRAF3	
sense	GGCCGUUUAAGCAGAAAGUTT
antisense	ACUUUCUGCUUAAACGGCCTT
si-TRAF6	
sense	GCGCUGUGCAAACUAUAUATT
antisense	UAUAUAGUUUGCACAGCGCTT
NC	
sense	UUCUCCGAACGUGUCACGUTT
antisense	ACGUGACACGUUCGGAGAATT

[a]siRNA, small interfering RNA; hnRNP K, heterogeneous nuclear ribonucleoprotein K; NC, negative control.

confluence of around 50% to 60% were subjected to siRNA transfection with the help of Lipofectamine RNAiMAX (no. 13778-150; Invitrogen) per the instructor's guidelines.

RNA extraction and qRT-PCR. Phosphate-buffered saline (PBS) was used to wash cells. They were then lysed, and total RNA was extracted using an RNeasy minikit (no. 74106; Qiagen) per the manufacturer's protocol. Using the PrimeScript RT reagent kit (no. RRO47A; TaKaRa Bio), one microgram of the extracted RNA was subject to reverse transcription to cDNA. Per the instructor's guidelines, we used SYBR Premix *Ex Taq* (no. q711-02; Vazyme Biotech) for qRT-PCR assessment of synthesized cDNA.

Western blotting. Cells were subjected to a 5-min RIPA (radioimmunoprecipitation assay) lysis on ice with extraction buffer (no. 89901; Thermo Fisher) following washing in cold PBS. The next step was protein isolation via SDS-PAGE and the subsequent shifting of the bands onto nitrocellulose membranes for the Western blot analysis (no. 10600001; Cytiva). Next, we blocked the membranes with dry skim milk (5%; no. 232100; BD) in 0.2% PBST (0.2% Tween 20 with PBS; no. 0777-1L; VWR International) for an hour. The membranes were then nurtured with primary antibody for one hour at ambient temperature, followed by PBST (0.1%) washing. Next, the membranes were deeply incubated for one hour with HRP-labeled secondary antibody, and finally, the ECL (enhanced chemiluminescence) substrate (no. P10300; New Cell & Molecular Biotech) was used to assay the proteins.

Co-immunoprecipitation assay. Based on a 24-h transfection using the designated plasmids, the cells were subjected to 30-min lysis with NP40 buffer (no. FNN0021; Invitrogen) at 4°C. The next step was centrifugation-based clearing of the lysates and a subsequent 30-min incubation with protein G (Dynabeads; no. 10004D; Invitrogen)-bound affine antibodies at 25°C. After PBST (0.02%) washing, Dynabead suspension was performed in an elution buffer (50 mM glycine [pH 2.8]). Immunoblotting was used to assay the proteins with the aid of designated antibodies.

Dual-luciferase reporter assay. We cultured cells were in a 24-well microplate. Lipofectamine 3000 was used to transfect the 293T cells with the luciferase reporter plasmid (pRL-TK) plus either the designated expression plasmid or the empty control plasmid. After a whole day, the cells were lysed. With the aid of a dual luciferase reporter assay system (no. DL101-01; Vazyme Biotech), the activities of firefly and Renilla luciferases were assessed per the manufacturer's guidelines. Luciferase values were normalized against the internal control, Renilla luciferase. Data from triplicate experiments were collected.

GST pulldown. Full-length cloning of the *PEDV N*, *hnRNP K*, *NDP52*, and *MARCH8* genes into the pCold TF plasmid (no. 3365) or the pCold GST plasmid (no. 3372) (both Clontech Laboratories) was performed. For the expression of recombinant proteins, we utilized the BL21 competent cells (no. C504-03; Vazyme Biotech). Protein interactions were evaluated per the instructions of the GST Protein Interaction Pull-Down kit (no. 21516; Thermo Fisher Scientific). Reduced glutathione was utilized for the elution of proteins, followed by Western blot analysis.

Fluorescence microscopy. Based on a 24-h co-transfection using the designated plasmids, HeLa cells were immobilized for 15 min in paraformaldehyde (4%; no. P6148; Sigma-Aldrich) and then subjected to 10-min permeabilization using Triton X-100 (0.1%; no. T9284; Sigma-Aldrich) at ambient temperature. In addition, after three washes with PBS, the cells were nurtured with the designated primary antibody and then incubated with the secondary antibody labeled with fluorescent dye. A confocal immunofluorescent microscope (Carl Zeiss) was adopted for observing the cells.

Statistical analysis. We used Student's *t* test (two-tailed) to process the data via Prism 5 (GraphPad Software), and data were expressed in terms of mean ± standard deviation (SD). Differences were considered significant when $P < 0.05$.

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We declare that we have no potential conflicts of interest.

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