AUTOPHAGY
2023, VOL. 19, NO. 8, 2338–2352
https://doi.org/10.1080/15548627.2023.2181615

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RESEARCH PAPER

N protein of PEDV plays chess game with host proteins by selective autophagy

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ABSTRACT

Macroautophagy/autophagy is a cellular degradation and recycling process that maintains the homeostasis of organisms. The protein degradation role of autophagy has been widely used to control viral infection at multiple levels. In the ongoing evolutionary arms race, viruses have developed various ways to hijack and subvert autophagy in favor of its replication. It is still unclear exactly how autophagy affects or inhibits viruses. In this study, we have found a novel host restriction factor, HNRNPA1, that could inhibit PEDV replication by degrading viral nucleocapsid (N) protein. The restriction factor activates the HNRNPA1-MARCHF8/MARCH8-CALCOCO2/NDP52-autophagosome pathway with the help of transcription factor EGR1 targeting the *HNRNPA1* promoter. HNRNPA1 could also promote the expression of IFN to facilitate the host antiviral defense response for antagonizing PEDV infection through RIGI protein interaction. During viral replication, we found that PEDV can, in contrast, degrade the host antiviral proteins HNRNPA1 and others (FUBP3, HNRNPK, PTBP1, and TARDBP) through its N protein through the autophagy pathway. These results reveal the dual function of selective autophagy in PEDV N and host proteins, which could promote the ubiquitination of viral particles and host antiviral proteins and degradation both of the proteins to regulate the relationship between virus infection and host innate immunity.

Abbreviations: 3-MA: 3-methyladenine; ATG: autophagy related; Baf A1: bafilomycin A_1; CALCOCO2/NDP52: calcium binding and coiled-coil domain 2; ChIP: chromatin immunoprecipitation; Co-IP: co-immunoprecipitation; CQ: chloroquine; DAPI: 4′,6-diamidino-2-phenylindole; GPI: glycosyl-phosphatidylinositol; hpi: hours post infection; MARCHF8/MARCH8: membrane-associated ring-CH-type finger 8; MOI: multiplicity of infection; N protein: nucleocapsid protein; PEDV: porcine epidemic diarrhea virus; siRNA: small interfering RNA; $TCID_{50}$: 50% tissue culture infectious doses.

ARTICLE HISTORY
Received 7 December 2022
Revised 12 February 2023
Accepted 13 February 2023

KEYWORDS
HNRNPA1; IFN; nucleocapsid protein; PEDV; selective autophagy

Introduction

Porcine epidemic diarrhea virus (PEDV) belonging to the family *Coronaviridae* (CoVs) causes infection in pigs of all ages and induces enteric diseases. The viral infection includes acute watery diarrhea, vomiting, dehydration, and high mortality in neonatal piglets [1]. Ever since the identification of the classic strain in 1978, the annual occurrence of infections of PEDV has been reported globally, leading to severe financial losses affecting the porcine industry [2–6]. PEDV is a positive single-stranded RNA virus with a genome size of around 28 kb, including one 5' cap and one 3′poly (a) tail [7]. Its genome includes more than seven open reading frames (ORFs) encoding 16 nonstructural proteins (nsp1-nsp16) and four structural proteins. These proteins include spike (S), envelope (E), membrane (M), nucleocapsid (N), and accessory (ORF3) proteins [7]. Of all the known structural proteins of CoVs, the N protein of PEDV is the only alkaline phosphorylated nucleocapsid protein. The multifunctional property of the N protein includes (1) binding to viral RNA, providing a helix nucleocapsid structure, and then working with the M protein for being packaged into viral particles attaining the core of CoVs; (2) promoting PEDV replication and transcription associated with the regulation of the host cell cycle due to their localization in the nucleus; (3) contributing in the biological processes of PEDV survival. Despite these known functionalities' the mechanism of action and their precision metabolism have not been fully explicated.

Autophagy is a conserved intracellular degradation process triggered under cellular stresses like starvation, hypoxia, endoplasmic reticulum stress, pathogen-associated molecular patterns (PAMPs), and pathogen invasion [8]. Autophagy involves selective mechanisms for the degradation of viral proteins, inhibiting virus replication. In this process, the viral proteins were ubiquitinated by E3-ubiquitin ligase and

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also recognized by cargo-specific autophagy receptors for degradation that resist virus replication [9–12]. Numerous studies have demonstrated that autophagy is a vital host defense mechanism closely associated with viral life cycles and negatively influences viral replication by removing virions through lysosomal degradation [13]. However, many viruses often hijack autophagy to replicate effectively within infected cells [14]. When the host antiviral proteins activate the autophagy that degrades viral proteins, it inhibits viral replication. The viral proteins also might utilize autophagy to degrade host antiviral proteins, suppressing the innate immune response and benefiting virus replication. Therefore, balancing autophagy's antiviral and pro-viral functions is important during virus infection.

HNRNPA1 is the most abundant and universally conceded heterogeneous nuclear ribonucleoprotein, which can regulate RNA splicing, stability, transport through nuclear pores, and translation of transcripts [15,16]. Additionally, it performs essential roles in the metabolism of mRNA and pre-mRNA, binding immature pre-mRNA in a sequence-specific manner and inducing the annealing of cRNA strands [17,18]. Recent evidence has emerged that HNRNPA1 interacts with many viral proteins and regulates viral replication. HNRNPA1 interacts with the genomic and subgenomic RNA promoters of the Sindbis virus and increases the synthesis of genomic and subgenomic RNA, promoting virus replication in both the infected cells and in vitro conditions [19]. However, HNRNPA1 negatively affects hepatitis C virus (HCV) replication in a subgenomic HCV replication system [20]. HNRNPA1 that interacts with the nucleoprotein of the Influenza A Virus impedes the virus replication [21]. In human colorectal cancer, HNRNPA1 also increases the levels of BECN1; its depletion induces peroxisomal autophagy by regulating the expression of PEX1, a peroxin [16,22]. The present study explored the HNRNPA1 antiviral function that triggered the MARCHF8-CALCOCO2/NDP52-autophagosome pathway and IFN-induced antiviral immunity to protect the host from virus damage. Moreover, PEDV also conversely degraded the host HNRNPA1 protein and other factors (FUBP3, HNRNPK, PTBP1, and TARDBP) through its N protein with the host autophagy pathway to facilitate PEDV replication, suggesting that the selective autophagy plays a dual role in PEDV infection and host innate immunity.

Results

HNRNPA1 expression can be influenced in PEDV-infected cells through transcription factor EGR1

The Co-IP and peptide sequencing by mass spectrometry (MS) assays were used to screen the host proteins interacting with PEDV N protein. These results showed that the host protein HNRNPA1 was coimmunoprecipitated by PEDV N protein. To test the effect of PEDV infection on HNRNPA1, we first examined HNRNPA1 expression variations in LLC-PK1 cells with PEDV infection at the multiplicity of infection (MOI) = 1 [23]. With the western blot results, the PEDV infection notably suppresses the HNRNPA1 protein (Figure 1A). In contrast, mRNA levels of *HNRNPA1* were observed with a rise in LLC-PK1 cells after the PEDV infection (Figure 1B) compared to the expressions in uninfected cells. These results indicated that PEDV infection might deteriorate the HNRNPA1 protein, and the cells attempt to compensate for the virus's downregulation of HNRNPA1 protein abundance by upregulating *HNRNPA1* mRNA level.

To determine the promoter and transcriptional regulations of HNRNPA1, a 1737 bp promoter gene of *HNRNPA1* and truncated promoter sequences (HA-HF) was amplified, and inserted in pGL3-Basic luciferase vector to test direct luciferase activities in HEK 293 T cells. The outcomes showed that the highest levels of luciferase activity were stimulated in promoter *HNRNPA1* gene sequences by fragments containing nucleotides from −550 to −1 (HF) (Figure 1C). This fragment was also truncated again (H1-H9) and inserted in the pGL3-Basic luciferase vector to detect the *HNRNPA1* core promoter sequence range. The truncated fragments with nucleotides from – 180 to – 100 induced the highest luciferase activity, manifesting the *HNRNPA1* core promoter at positions – 180 to – 100 (Figure 1C).

Based on the acquired results, the probable transcription factor-binding sites (TFBS) in gene promoters were then predicted with JASPAR (http://jaspar.genereg.net/) [24]. SP1-, SP2-, SP4-, NFYA-, NFYB- and EGR1-binding sites were held within the minimal *HNRNPA1* key promoter region (Figure 1D). Further analyses with the qRT-PCR on all the predicted TFBS revealed that only the *EGR1* mRNA level was upregulated among other predicted transcription factors in PEDV-infected LLC-PK1 cells. These results also coincided with *HNRNPA1* mRNA endogenous expression tendency (Figure 1E). We went a step further and verified the effect of EGR1 on HNRNPA1. The western blot and qRT-PCR results shown in Figure 1(F–H) confirmed that *HNRNPA1* mRNA level increased along with EGR1 over-expression and decreased when siRNAs targeting *EGR1* mRNA sequences were subject to transfection in LLC-PK1 cells 24 h post-PEDV infection. We also performed the luciferase reporter assay, which showed that overexpressed EGR1 could stimulate a significantly higher HNRNPA1 luciferase activity (Figure 1I). A chromatin immunoprecipitation (ChIP) assay using Flag-EGR1 was also done to immunoprecipitate the *HNRNPA1* core promoter. EGR1 is directly bound to the *HNRNPA1* promoter, regulating its expression, as shown in Figure 1J. All of these findings imply that the transcription factor EGR1 can affect the expression of HNRNPA1 in PEDV-infected cells.

Overexpression of HNRNPA1 Inhibits PEDV Infection

Of the known structural proteins of CoVs, the N protein is the only one that is phosphorylated. During PEDV viral infection, the N protein and its mRNA levels reached high, executing various functionalities. Therefore, we decided to examine the HNRNPA1 effects of PEDV infection. HNRNPA1 plasmids or control plasmids were transfected into Vero cells, and PEDV was infected into cells at the MOI = 0.01 after 24 h post-transfection (hpt). Supernatants from the cell culture of infected cells were gathered at 20 and 24 h post-infection (hpi). Western blotting (Figure 2A), qRT-PCR (Figure 2B)

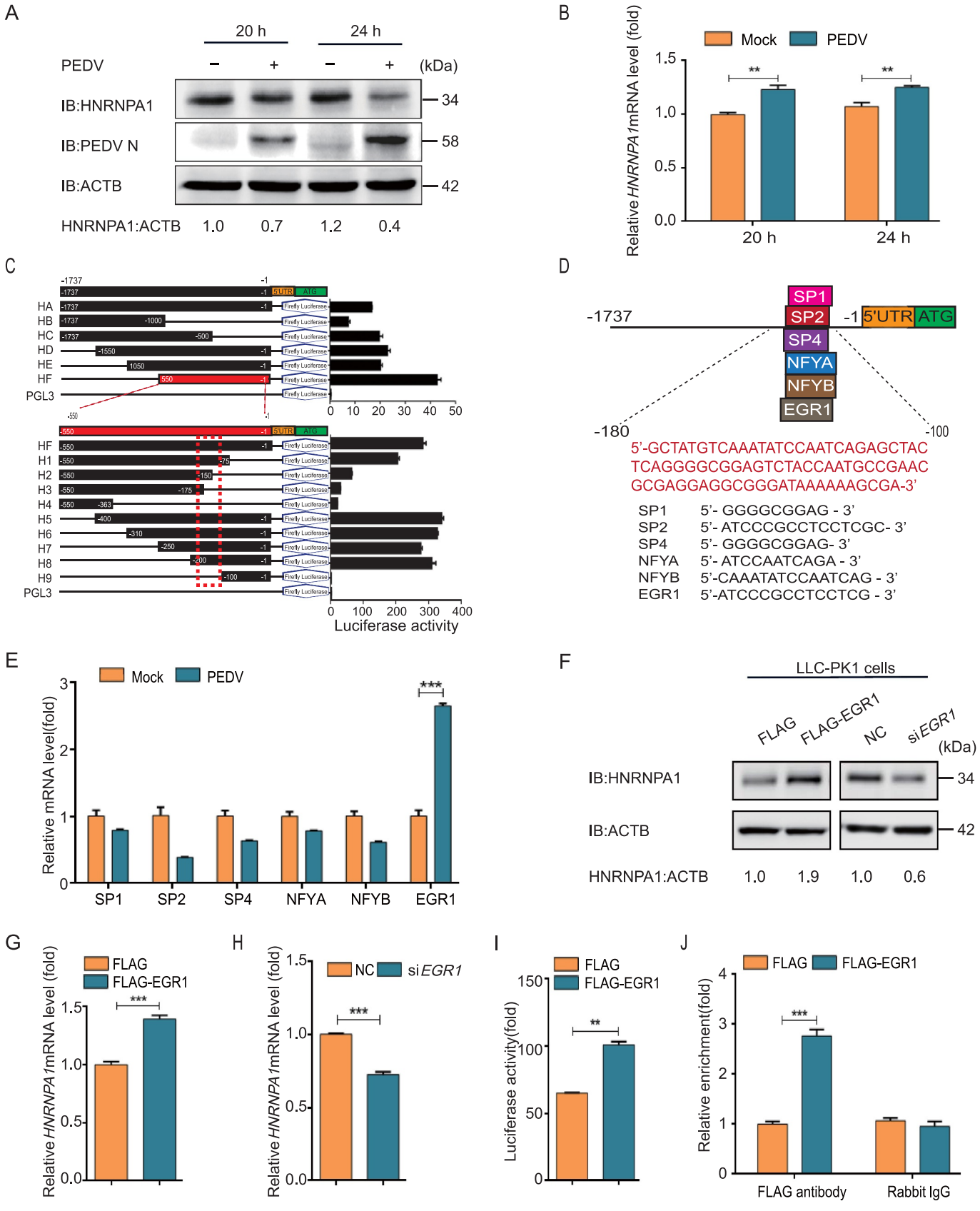

Figure 1. HNRNPA1 expression can be influenced in PEDV-infected cells through transcription factor EGR1. (A) PEDV infected or negatively infected LLC-PK1 cells (strain JS-2013) at MOI = 1 were collected at 20 and 24 hpi. Western blotting was applied to detect the expression levels of HNRNPA1 and PEDV N proteins with ACTB as a loading control. (B) qRT-PCR was employed to evaluate the *HNRNPA1* relative mRNA expression levels. (C) HEK 293 T cells transfected with pGL3-Basic luciferase vector carrying truncated constructs (−1737 to − 1) of *HNRNPA1* promoter were analyzed for the luciferase activity. (D) The TFBS of the *HNRNPA1* promoter expected with JASPAR. (E) Relative mRNA levels of predicted genes explored using qRT-PCR in LLC-PK1 cells infected with PEDV 24 hpi. (**F, G**, and **H**) *HNRNPA1* relative mRNA levels in the EGR1 over-expressed or EGR1 knockdown LLC-PK1 cells explored using western blotting and qRT-PCR. (I) The luciferase activity was analyzed in EGR1 over-expressing HEK 293 T cells. (J) Following transfection with Flag-EGR1 plasmid or blank vector, ChIP analysis for LLC-PK1 cells subjected to harvesting and treatment. Data presented are means ±SD from triplicate experiments. *, ** and ***, respectively indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ (two-tailed Student's *t*-test).

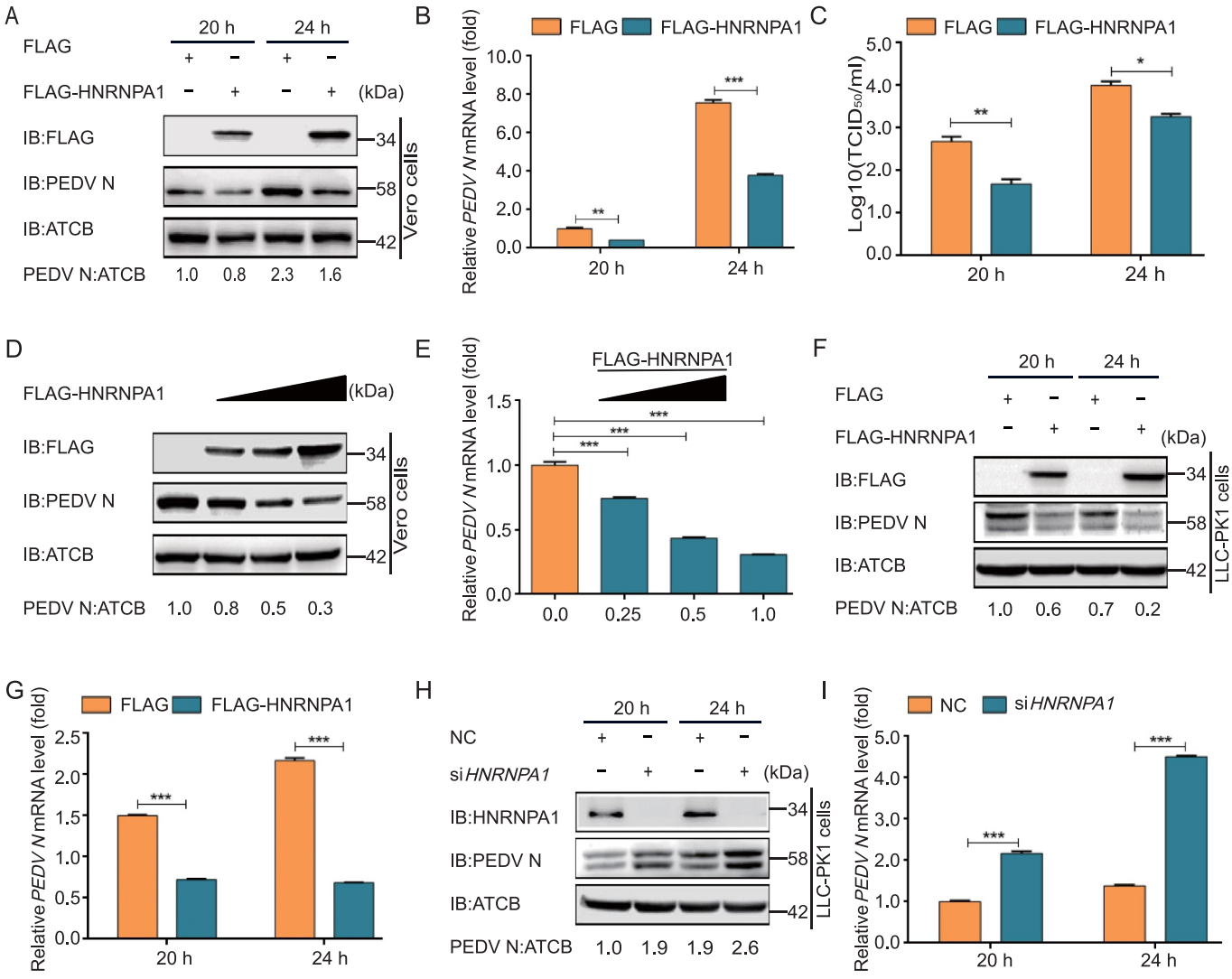

Figure 2. Overexpression of HNRNPA1 Inhibits PEDV Infection. (**A, B**, and **C**) Vero cells transfected with HNRNPA1 plasmid and infected with PEDV at an MOI of 0.01 at 24 h post-transfection. The PEDV titers were explored with western blotting, qRT-PCR, and $TCID_{50}$. (D and E) Gradient concentrations of HNRNPA1 plasmids transfected into Vero cells infected with PEDV at an MOI of 0.01 at 24 h post-transfection. (**F, G, H**, and **I**) HNRNPA1 plasmids or *HNRNPA1* siRNA transfected into LLC-PK1 cells and infected with PEDV at 24 h post-transfection at the MOI of 1. The cell lysates were detected with western blotting and qRT-PCR. Data are presented as means ±SD of triplicate samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's *t*-test).

and 50% tissue culture infectious doses ($TCID_{50}$) (Figure 2C) were performed to calculate viral yield. The findings showed that the PEDV N protein, mRNA level, and viral load significantly declined at 20 and 24 hpi than the control group. Furthermore, the PEDV N protein and mRNA expression levels were observed to be downregulated as the quantity of Flag-HNRNPA1 plasmids increased (Figure 2(D and E)). This result implies that HNRNPA1 inhibited the replication of PEDV in Vero cells. Eventually, we also tested the inhibition of HNRNPA1 with PEDV infection on LLC-PK1 cells. Western blotting (Figure 2F) and qRT-PCR (Figure 2G) showed that overexpressed HNRNPA1 significantly inhibited PEDV replication. To corroborate the inhibition relationship, we transfected *HNRNPA1* siRNA into LLC-PK1 cells infected with PEDV. The outcomes showed that the PEDV N protein and mRNA expression were upregulated in LLC-PK1 cells when HNRNPA1 expression was silenced compared to the placebo ones (Figure 2(H and I)). Based on the above findings, we deduced that the overexpression of HNRNPA1 could inhibit PEDV infection.

HNRNPA1 degrades PEDV N protein via autophagy

To detect the function of the molecular mechanisms of HNRNPA1 inhibiting PEDV replication, a co-immunoprecipitation (co-IP) assay was performed. HNRNPA1 was precipitated with PEDV-N protein in the assay, and RNase did not hamper their effective combination (Figure 3A). Co-IP also showed that the PEDV N protein could coimmunoprecipitate with the endogenous HNRNPA1protein in Vero cells (Figure 3B). This interaction of HNRNPA1 and PEDV N proteins was then validated through the GST affinity-isolation assay (Figure 3C). HNRNPA1 is predominantly a nuclear protein that can shuttle between the nucleus and cytoplasm, performing its function. We next detected the two

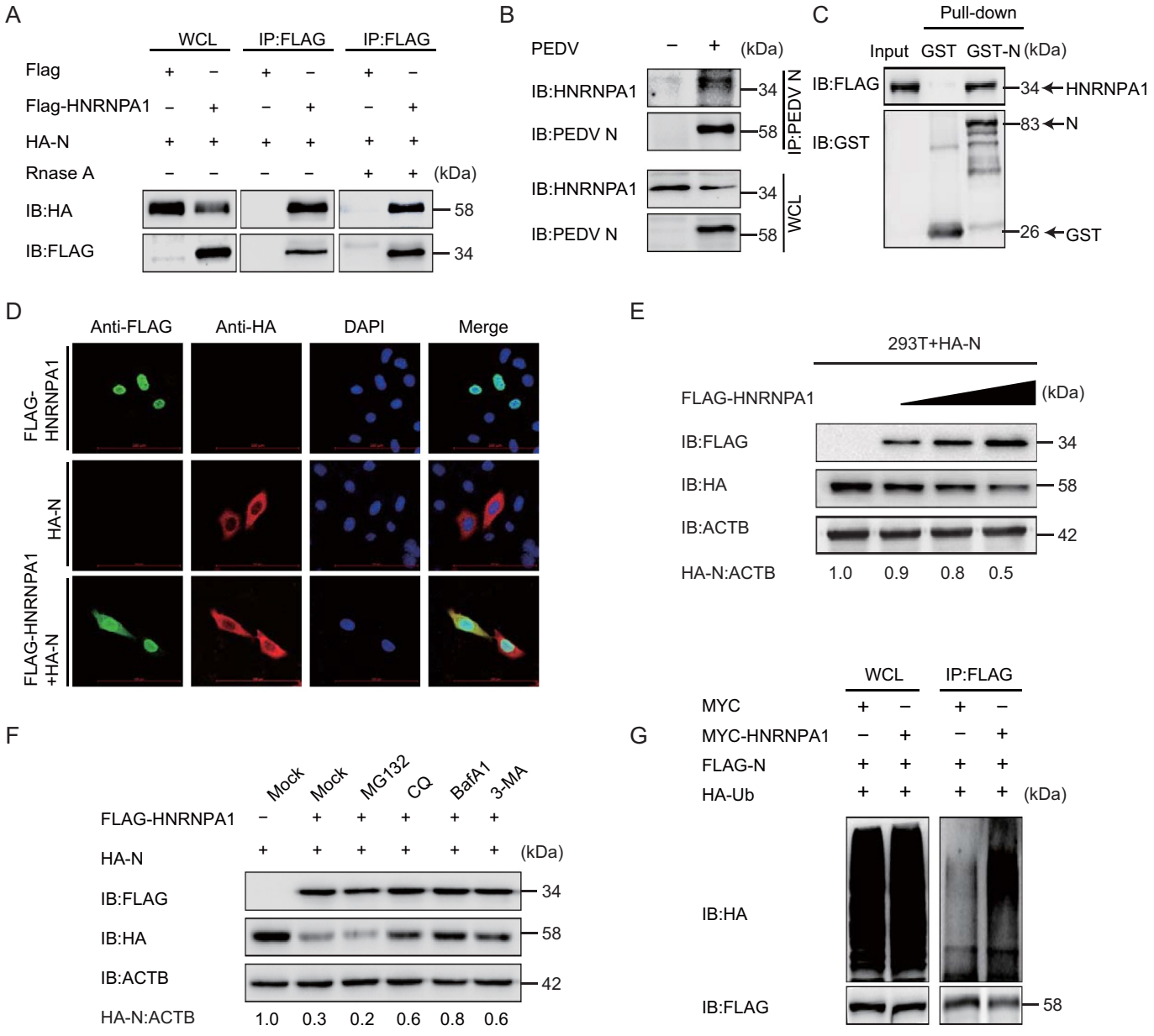

Figure 3. HNRNPA1 can degrade PEDV N protein via autophagy. (A) HEK 293 T cells transfected with the HA-N- and Flag-HNRNPA1-encoding plasmids or RNase and assayed for Co-IP with anti-Flag binding beads. The precipitated proteins were analyzed with western blotting. (B) Following negative infection or infection using PEDV at MOI = 0.01, the Vero cells gathered for the endogenous HNRNPA1 immunoprecipitation based on the antibody of N protein. (C) The pCold TF and pCold GST plasmids utilized for independent cloning of the HNRNPA1, PEDV N and subsequently denoted in the BL21 (DE3) bacterial strain for affinity isolation of GST. The eluted proteins were analyzed with western blotting. (D) HeLa cells co-transfected using HA-N- and FLAG-HNRNPA1-encoding plasmids and cellular labeled with specific antibodies. DAPI (4,6-diamidino-2-phenylindole) labeling for the cellular nuclei and fluorescent signals were monitored with a confocal immunofluorescent microscope (scale bars = 100 μm). (E) Gradient concentration of Flag-HNRNPA1 and HA-N-plasmids were co-transfected into HEK 293 T cells. 24 h later, the cellular lysates were detected by western blotting. (F) Flag-HNRNPA1 and HA-N-plasmids co-transfected into HEK 293 T cells, and then the cells treated with the MG132, CQ, Baf A1, and 3-MA. Western blotting was utilized to detect the cellular lysates. (G) Co-transfection of HEK 293 T cells with MYC-HNRNPA1 and Flag-N plasmids and immunoprecipitation of the ubiquitinated N proteins were with an anti-Flag antibody analyzed with western blotting.

proteins using confocal immunofluorescence assay to investigate how HNRNPA1 interacts with the PEDV N protein. The results showed that HNRNPA1 could primarily localize in the nucleus and shuttle to the cytoplasm to colocalize with N proteins upon PEDV infection (Figure 3D). These results showed that HNRNPA1 could interact with the PEDV N protein.

To elucidate the impact of HNRNPA1-N interaction in PEDV infection, we transfected the plasmids encoding Flag-HNRNPA1 and HA-N in HEK 293 T cells. The observed results showed that the HNRNPA1 gradient degraded the PEDV N protein in HEK 293 T cells (Figure 3E). To detect which degradation system mediates the degradation of HA-N by HNRNPA1, protease inhibitor MG132 and autophagy inhibitors chloroquine (CQ), bafilomycin A_1 (Baf A1), and 3-methyladenine (3-MA) were employed. Figure 3F demonstrates that the HNRNPA1 degradation impact on PEDV N protein was significantly enhanced through the autophagy inhibitors, indicating that HNRNPA1 could degrade PEDV N protein through autophagy process. HEK 293 T cells were

co-transfected with MYC-HNRNPA1, Flag-N, and the cellular lysates were investigated with ubiquitination assay. Western blotting demonstrated that the overexpressed HNRNPA1 notably promoted the ubiquitination of the N protein (Figure 3G). Together, the results demonstrate that HNRNPA1 can activate autophagy to cause the degradation of PEDV N.

PEDV replication can be hindered by HNRNPA1 through the MARCHF8-CALCOCO2-autophagosome pathway

During autophagy, pathogens can be ubiquitinated and delivered by cargo receptors forming autophagosome degradation substrates [25]. The host antiviral factors can recruit E3 ubiquitin ligase MARCHF8 to ubiquitinate pathogens like PEDV N protein. Subsequently, they also transmit the ubiquitinated proteins to the lysosome for degradation by the cargo receptor CALCOCO2 [8,26]. Co-IP assays were conducted in HEK 293 T cells co-transfected with MARCHF8 and Flag-HNRNPA1 plasmids and found that MARCHF8 could be coimmunoprecipitated with overexpressed HNRNPA1 (Figure 4A). Cell lysis with RNase did not interfere with this effective combination (Figure 4A). Furthermore, the endogenous MARCHF8 was also coimmunoprecipitated by the HNRNPA1 protein (Figure 4B). The direct binding of MARCHF8 to HNRNPA1 was affirmed by the GST affinity-isolation assay (Figure 4C). Similarly, the interaction of CALCOCO2 and HNRNPA1 protein was also examined with co-IP and the GST affinity-isolation assays (Figure 4D, E, and F). Confocal immunofluorescence microscopy supported the evidence that HNRNPA1 shuttled from the nucleus to the cytoplasm and efficiently colocalized with MARCHF8 and CALCOCO2 in HeLa cells (Figure 4G). HEK 293 T cells were co-transfected with Flag-HNRNPA1 and HA-N together with *MARCHF8* siRNA or *CALCOCO2* siRNA to validate that induced HNRNPA1/MARCHF8/CALCOCO2 autophagy facilitated HNRNPA1 degradation of PEDV N protein. Western blotting indicated that interfering with MARCHF8 or CALCOCO2 could reverse the HNRNPA1-induced N protein degradation compared to the controls (Figure 4H). Interrupting the autophagy process of cells could reduce the inhibitory effect of HNRNPA1 on PEDV replication (Figure 4I). These studies show that the HNRNPA1-MARCHF8-CALCOCO2-autophagosome pathway can be used by HNRNPA1 to obstruct PEDV replication.

HNRNPA1 promotes IFN expression by its interaction with RIGI

As the crucial member in host antiviral immunity, interferons (IFNs) can regulate immunocyte subpopulations to help antiviral immune response [27]. Viruses have evolved aggressive strategies against IFNs response during their invasion, so the host must exploit new roles with its antiviral proteins to handle the intruder [8]. We hypothesized HNRNPA1 as a host antiviral proteins family that might stimulate IFNs expression to antagonize PEDV. To explore this, we carried out an IFN-β promoter and IFN-stimulated response element (ISRE)-driven luciferase reporter assay. As shown in Figure 5 (A and B), overexpressed HNRNPA1 increased the luciferase activity, which gradually increased along with the concentration of the HNRNPA1 protein. The upstream regulation factors of the IFN signal pathway were transfected, and the HNRNPA1 showed increased luciferase reporter activity induced by IFIH1/MDA5, RIGI, IKK, MAVS, and IRF3 (Figure 5C). Therefore, HNRNPA1 should induce interferon signaling by regulating the upstream regulation factors MDA5 or RIGI. Then we found the interference of RIGI lowered the stimulated luciferase activity in HEK 293 T cells overexpressing HNRNPA1 (Figure 5D), and the HNRNPA1 increased the mRNA level of RIGI (Figure 5E), suggesting that HNRNPA1 may have stimulated RIGI expression to induce IFN activity during PEDV infection. The co-IP and confocal immunofluorescence assay also demonstrated the HNRNPA1 interaction and localization with RIGI in the cytoplasm (Figure 5F, G, and H). We also found that HNRNPA1 efficiently upregulated the RIGI, TRAF3, phosphorylated TBK1, and phosphorylated IRF3 proteins in HEK 293 T cells in a dose-dependent way. The up-regulation effect brought by HNRNPA1 lost its efficacy in cells when co-transfected with *RIGI* siRNA (Figure 5(I and J)). From all these outcomes, it can be seen that HNRNPA1 interacts with the RIGI protein to activate an IFN signal pathway that prevents PEDV infection.

PEDV N protein exploits cellular autophagic machinery to degrade HNRNPA1 protein

Viruses have evolved and have developed strategies to improve or hijack the host autophagy machinery for virus replication and pathogenesis [13]. Our study showed that the PEDV infection significantly suppressed the expression of HNRNPA1 protein but promoted *HNRNPA1* mRNA (Figure 1(A and B)). Based on this, we hypothesized that HNRNPA1 degrades PEDV N protein through the HNRNPA1-MARCHF8- CALCOCO2-autophagosome pathway, and conversely, the PEDV infection may also degrade HNRNPA1 protein through N protein. Western blot assay showed that PEDV N protein significantly suppressed the over-expressed HNRNPA1 protein and endogenous HNRNPA1 protein from LLC-PK1 cells in a dose-dependent way (Figure 6(A and B)), and the degradation of HNRNPA1 by PEDV N protein could be significantly interrupted by the autophagy inhibitors Baf A1 and 3-MA (Figure 6C), which supports HNRNPA1 degradation by PEDV N through the autophagy process. Also, the abundance of endogenous HNRNPA1 could be decreased by PEDV infection and restored by the autophagy inhibitors (Figure 6D). Furthermore, the PEDV N protein promoted the ubiquitination of HNRNPA1 (Figure 6E). Taken together, PEDV infection can degrade the HNRNPA1 protein with its N protein through the host autophagy pathway.

PEDV N protein can degrade host antiviral proteins by selective autophagy

From the above results, we studied if PEDV infection could degrade other host antiviral proteins with its N protein through cellular selective autophagy. Host factors such as

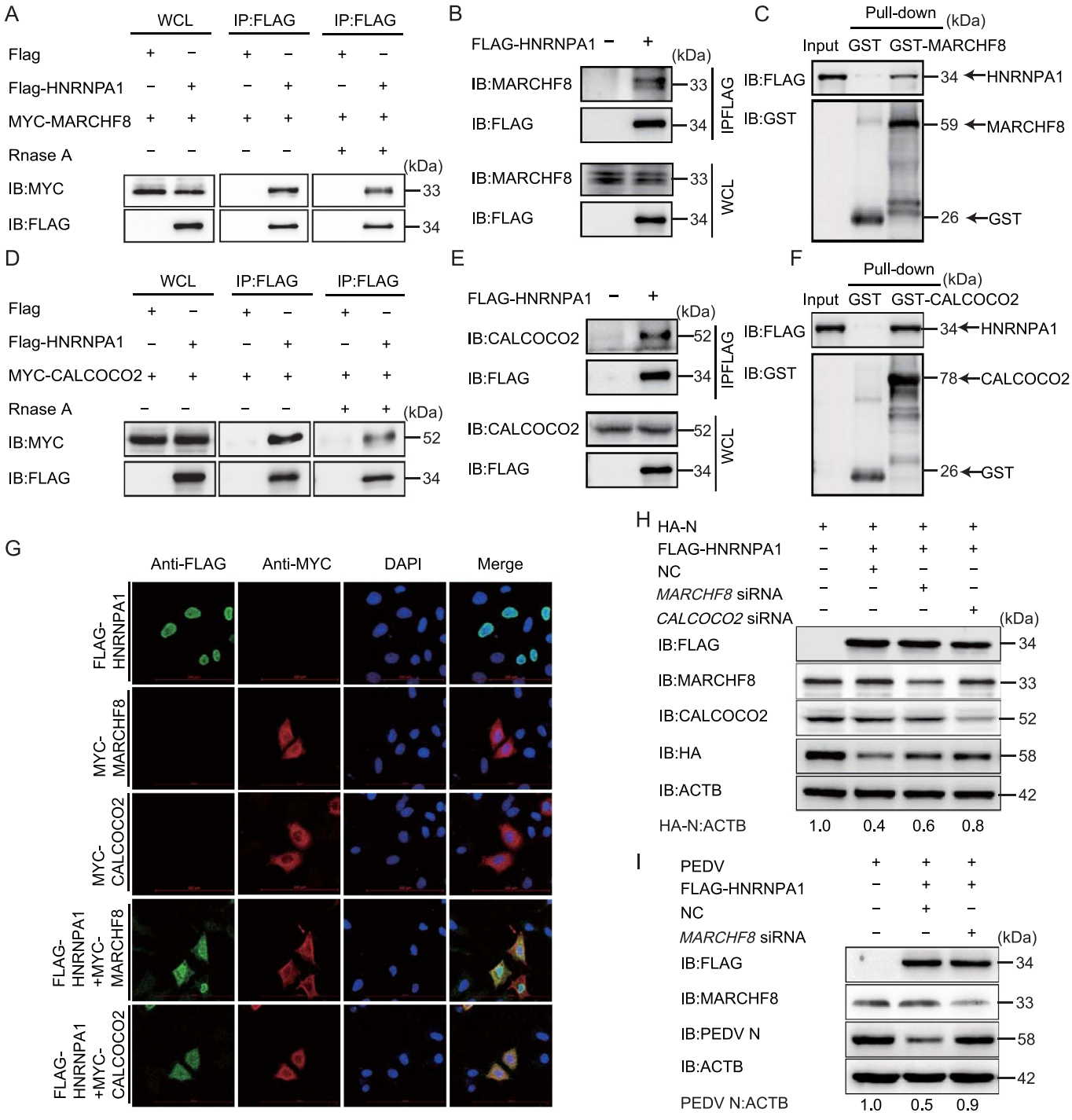

Figure 4. PEDV replication can be hindered by HNRNPA1 through the HNRNPA1-MARCHF8-CALCOCO2-autophagosome pathway. (A) HEK 293 T cells treated with Flag-HNRNPA1- and MYC-MARCHF8-encoding plasmids, anti-Flag binding beads were utilized for the Co-IP procedure 24 h post-transfection. Western blotting analysis was adopted for analyzing the precipitated proteins. (B) Following transfected with Flag-HNRNPA1 plasmids or negative controls, the HEK 293 T cells were collected for the endogenous MARCHF8 immunoprecipitation. (C) The GST affinity-isolation assay detected the GST-MARCHF8 and HNRNPA1. (D) HEK 293 T cells treated with Flag-HNRNPA1- and MYC-CALCOCO2-encoding plasmids were precipitated, and proteins were analyzed using anti-Flag binding beads. (E) Following transfected with Flag-HNRNPA1 plasmids or negative controls, the Vero cells were collected for the endogenous CALCOCO2 immunoprecipitation. (F) The GST affinity-isolation assay detected the interaction of GST-CALCOCO2 and HNRNPA1. (G) Flag-HNRNPA1 and MYC-MARCHF8 or MYC-CALCOCO2 plasmids transfected into Hela cells were subsequently labeled with antibodies for confocal immunofluorescence microscopy. Scale bars: 100 μm. (H) HA-N, Flag-HNRNPA1 plasmids, and siRNA (*MARCHF8* siRNA or *CALCOCO2* siRNA) co-transfected into HEK 293 T cells. (I) Flag-HNRNPA1 plasmid and *MARCHF8* siRNA transfected into the Vero cells and, after 24 h, infected with PEDV at MOI = 0.01. Western blotting analysis was applied to detect N protein.

FUBP3, HNRNPK, PTBP1, and TARDBP could prevent the PEDV replication by autophagosome pathway. The expression levels of these host antiviral proteins were found to be decreased during PEDV infection [28,29]. We hypothesized that the PEDV N protein could degrade host antiviral proteins by selective autophagy to facilitate virus replication. Western blot assay showed that LLC-PK1 cells and HEK 293 T cells' levels of endogenous FUBP3, HNRNPK, PTBP1, and

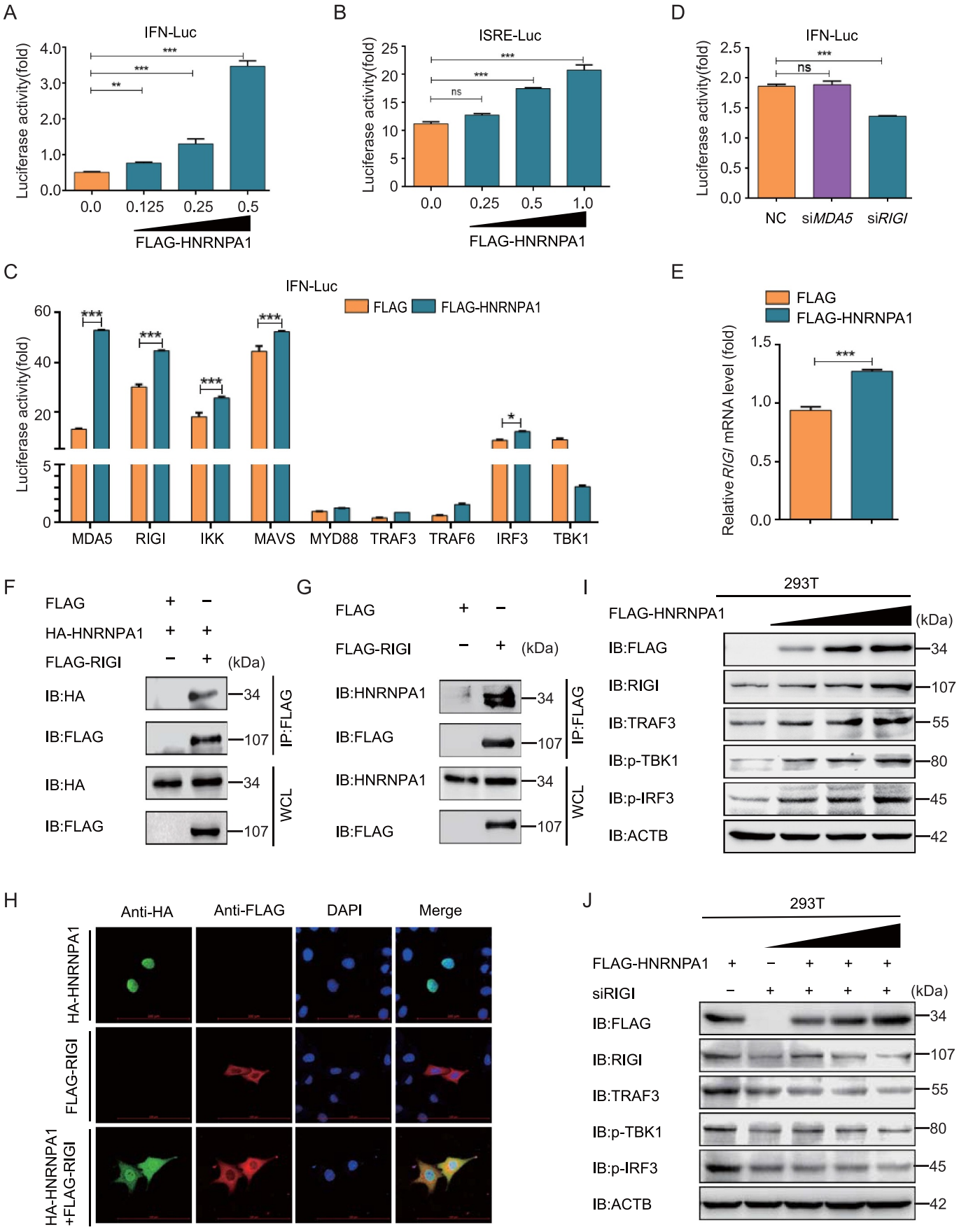

Figure 5. HNRNPA1 promotes IFN expression by its interaction with RIGI. (A and B) HEK 293 T cells transfected with IFN-β or ISRE luciferase reporter along with the increasing amounts (wedge) of Flag-HNRNPA1, and dual luciferase activity was detected. (C) HNRNPA1, IFN-β luciferase reporter, plasmids encoding MDA5, RIGI, IKK, MAVS, MYD88, TRAF3, TRAF6, IRF3 or TBK1, co-transfected into HEK 293 T cells to detect dual luciferase activity. (D) HEK 293 T cell lysates were transfected with Flag-HNRNPA1, *MDA5* siRNA, or *RIGI* siRNA to detect the dual luciferase activity. (E) HEK 293 T cells transfected with FLAG-HNRNPA1. The qRT-PCR was employed to value

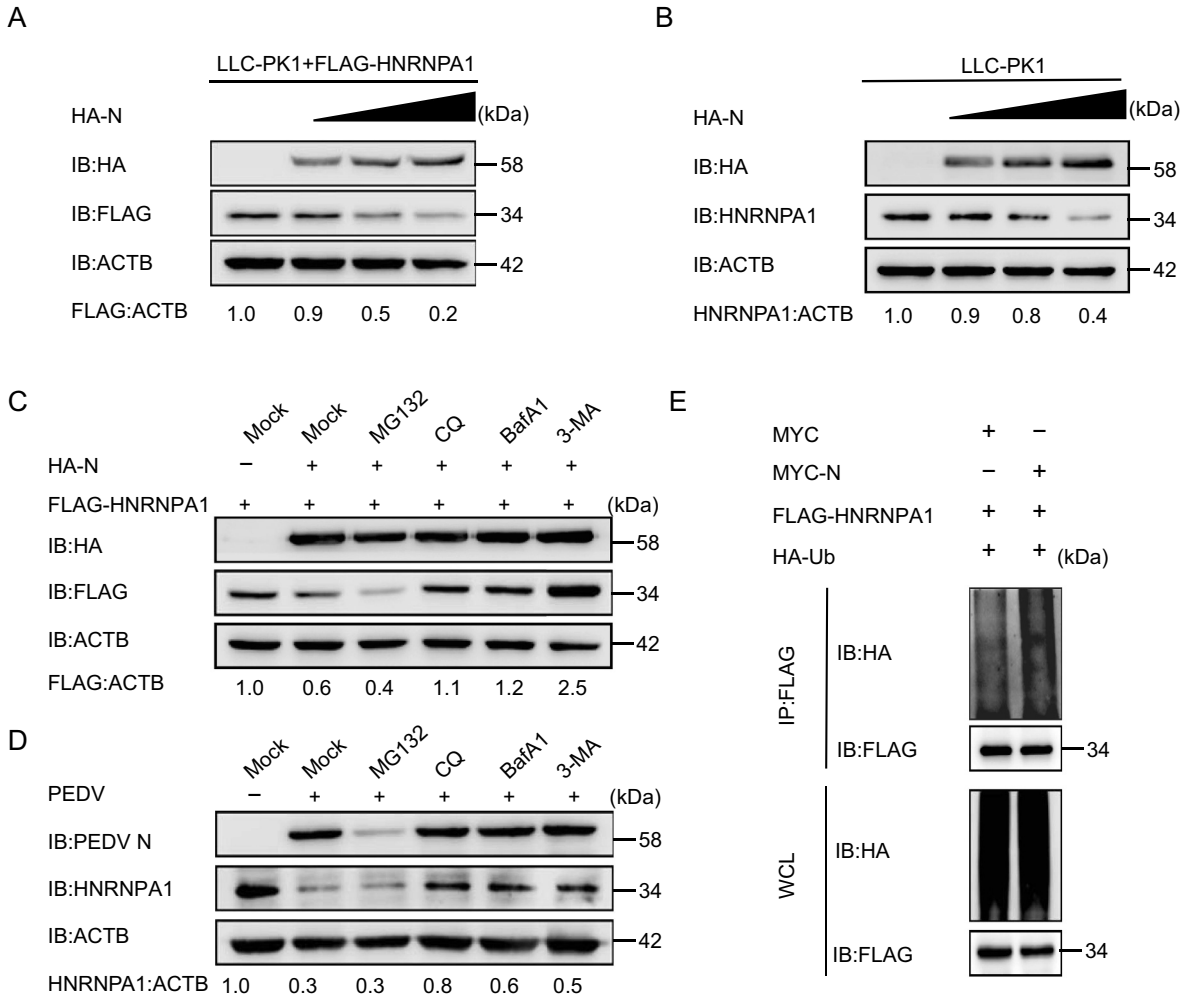

Figure 6. PEDV N protein can exploit the cellular autophagic machinery to degrade HNRNPA1 protein. (A) LLC-PK1 cells co-transfected with the increasing amounts of HA-N and over-expressed Flag-HNRNPA1. (B) LLC-PK1 cells transfected with increasing amounts of HA-N. (C) Flag-HNRNPA1 and HA-N plasmids co-transfected into HEK 293 T cells, and then the cells were treated with the MG132, CQ, Baf A1, and 3-MA. (D) LLC-PK1 cells infected with PEDV and treated with the protease inhibitor or autophagy inhibitor. (E) Co-transfection of HEK 293 T cells with FLAG-HNRNPA1 and MYC-N plasmids and cellular lysates collected following a 24 h post-transfection. The ubiquitinated N proteins were immunoprecipitated with an anti-Flag antibody. All samples were analyzed with western blotting.

TARDBP proteins are dose-dependently suppressed by the overexpression of the PEDV N protein (Figure 7A and 7B). We also found that the PEDV N protein degraded FUBP3, HNRNPK, PTBP1, or TARDBP proteins (Figure 7C, D, E, and F). Similarly, HEK 293 T cells were co-transfected with Flag-FUBP3 plasmid, HA-N plasmid, and treated with Baf A1, 3-MA, or MG132 to deduce which degradation system mediates the degradation of the FUBP3, HNRNPK, PTBP1 or TARDBP by PEDV N protein. Based on the western blot results, the degradation of FUBP3 protein by PEDV N was enhanced significantly by autophagy inhibitor Baf A1 and 3-MA, but not the proteasome inhibitor MG132. This suggests that the PEDV N protein can degrade the host antiviral proteins: FUBP3, HNRNPK, PTBP1, and TARDBP through autophagy (Figure 7G, H, I, and J). Combined with our earlier findings, PEDV infection is not only prevented by host antiviral proteins but also reversed by the virus, which uses its N protein to degrade host antiviral factors through the autophagy pathway.

Discussion

PEDV has been a threatening risk factor to the swine industry globally. The currently available commercial vaccines cannot fully protect the host against PEDV. This study aimed to explore the novel potential host factors that antagonize PEDV infection. Here, we demonstrated that PEDV infection

the RIGI relative mRNA expression. (F) HEK 293 T cells co-transfected with the Flag-RIGI and HA-HNRNPA1-encoding plasmids to conduct the Co-IP procedure. The precipitated proteins were analyzed with western blotting. (G) HEK 293 T cells were transfected with the Flag-RIGI for the endogenous HNRNPA1 immunoprecipitation. (H) HeLa cells were transfected with Flag-RIGI and HA-HNRNPA1 plasmids and labeled with specific antibodies. The confocal immunofluorescent microscope was utilized to monitor fluorescent signals (scale bars = 100 μm). (I and J) HEK 293 T cells co-transfected with increasing amounts of Flag-HNRNPA1 plasmids and *RIGI* siRNA.

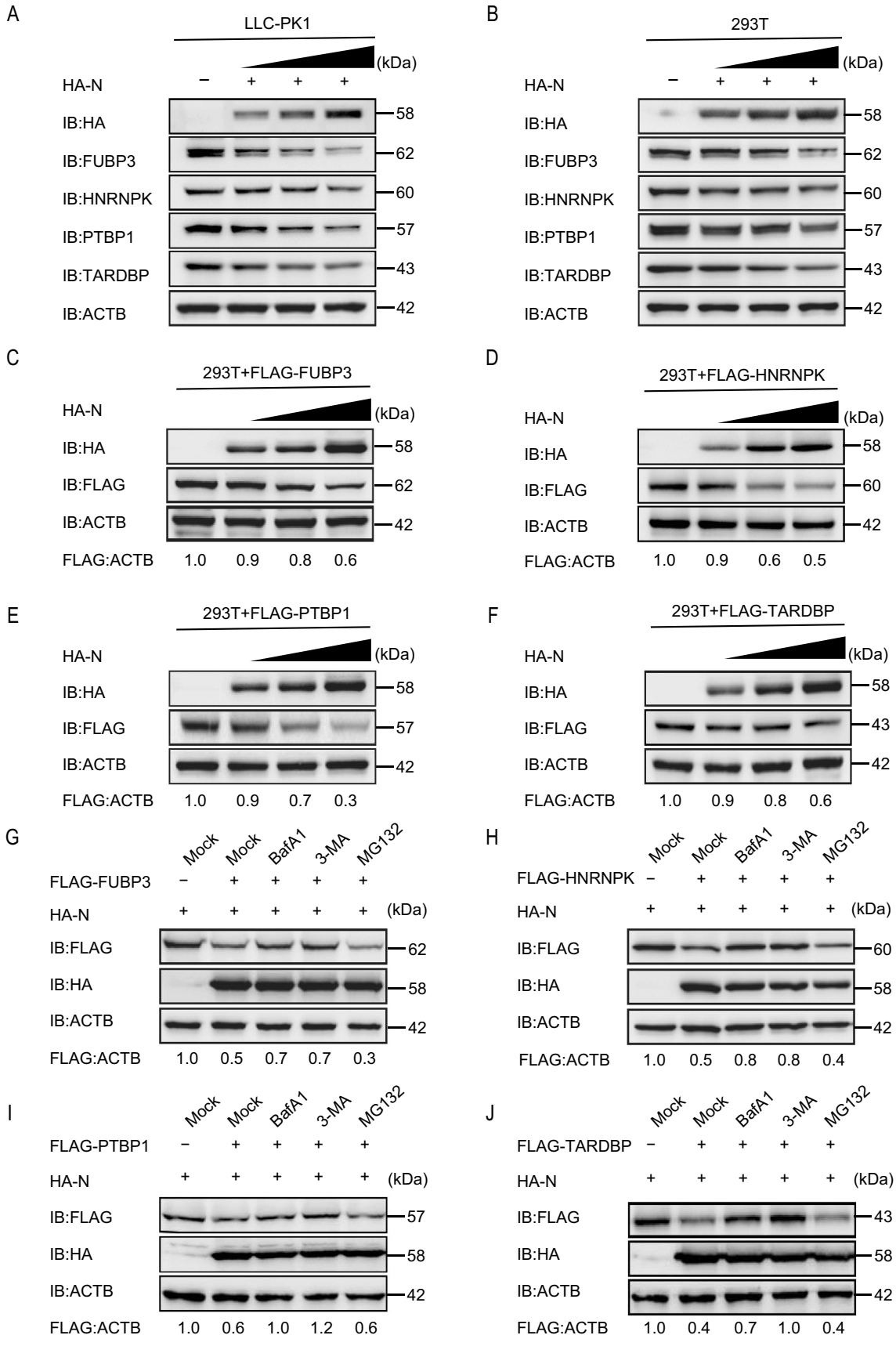

Figure 7. PEDV N protein can degrade host antiviral proteins by selective autophagy. (A and B) LLC-PK1 cells and HEK 293 T cells transfected with increasing amounts of HA-N. Western blotting was used to detect and analyze the cell lysates. (**C, D, E**, and **F**) HEK 293 T cells co-transfected with increasing amounts of HA-N and over-expressed Flag-FUBP3, Flag-HNRNPK, Flag-PTBP1, or Flag-TARDBP. (**G, H, I**, and **J**) Flag-FUBP3, Flag-HNRNPK, Flag-PTBP1, or Flag-TARDBP plasmids and HA-N-plasmids were co-transfected into HEK 293 T cells; then the cells were treated with the Baf A1, 3-MA and MG132. All samples were analyzed with western blotting.

stimulated *HNRNPA1* mRNA expression and triggered the HNRNPA1-MARCHF8-CALCOCO2-autophagosome pathway to prevent viral replication. Meanwhile, PEDV infection could also degrade the HNRNPA1 protein through its N protein through the host autophagy pathway. We have also revealed that HNRNPA1 induces the IFN signal pathway to antagonize PEDV infection by RIGI protein interaction. The study also demonstrated that the PEDV N protein could selectively cleave host proteins like FUBP3, HNRNPK, PTBP1, and TARDBP through autophagy (Figure 8).

It has been shown that HNRNPA1 participates in mechanisms that control the gene expression and replication of various viruses, including the human rhinovirus [30], enterovirus 71 [31], Sindbis virus [32], hepatitis C virus [33], human papillomavirus [34,35], and HIV-1 [36,37]. It is reported that HNRNPA1 interacts with the N protein of mouse hepatitis virus (MHV), one of the Coronavirus, in vitro and in vivo to facilitate virus replication [38]. However, this study demonstrates the novel antiviral function of HNRNPA1 in PEDV infection in LLC-PK1 cells. Also, we identified the region that spanned nucleotides – 180 to – 100 upstream from the transcription start site as an HNRNPA1 gene core promoter with an apparent transcription activity and its core promoter EGR1. The previous study showed that HNRNPA1, as an RNA-binding protein, regulates the process and expression of the nascent transcripts, modulating their localization, RNA

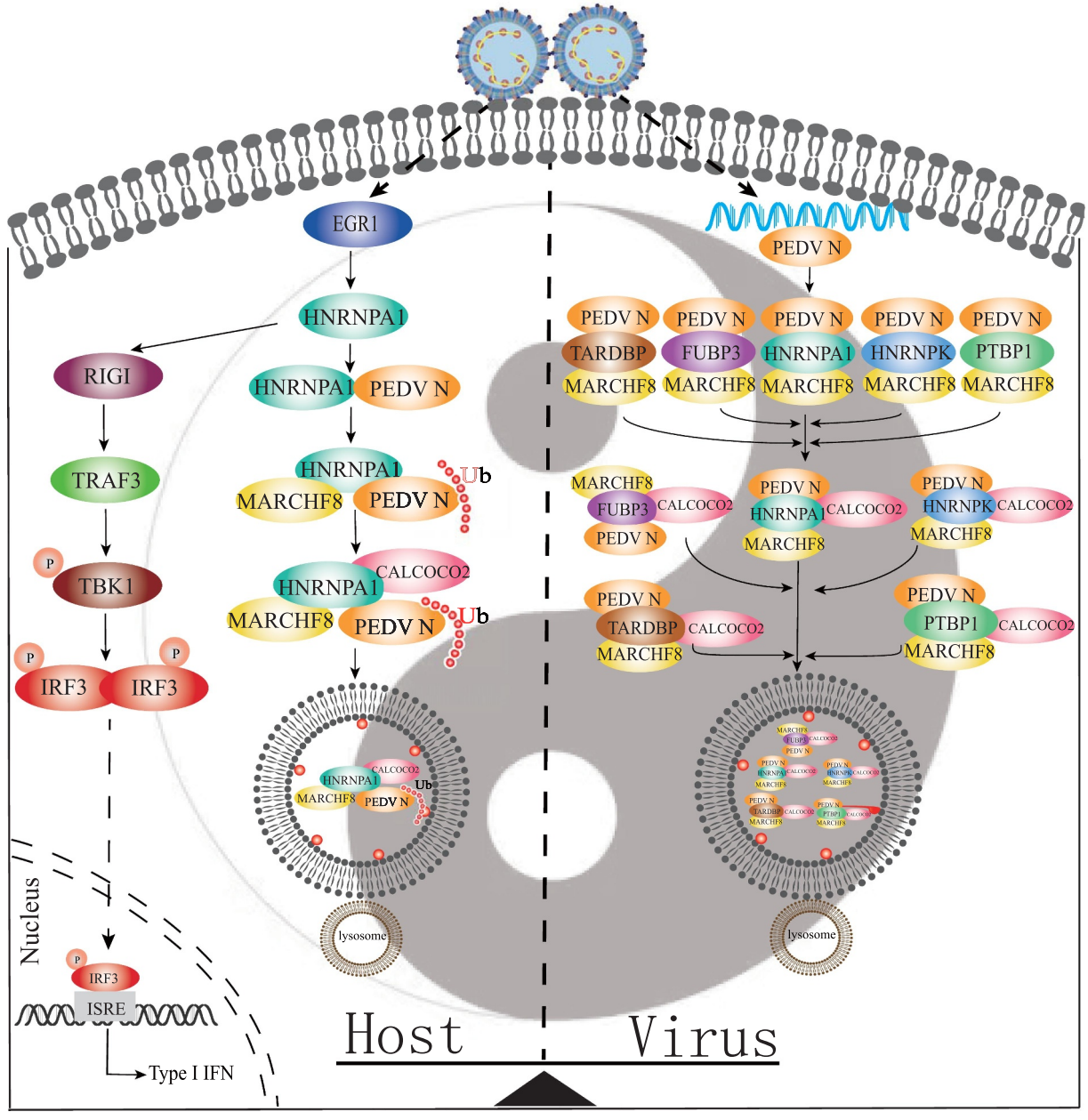

Figure 8. N protein of PEDV plays a chess game with HNRNPA1. During PEDV infection, the host antiviral protein HNRNPA1 triggered the MARCHF8-CALCOCO2-autophagosome pathway to thwart viral replication. Moreover, HNRNPA1 promoted IFN expression to stimulate the host antiviral defense response to antagonize PEDV infection by RIGI protein interaction. Meanwhile, PEDV also utilized viral N protein to degrade the host antiviral protein HNRNPA1, FUBP3, HNRNPK, PTBP1, and Flag-TARDBP through the host autophagy process to facilitate virus replication. All samples were analyzed with western blotting.

binding specificity, and interaction with other cellular factors [39]. We demonstrated that HNRNPA1 could target and degrade the PEDV N protein to inhibit virus replication.

Autophagy is a ubiquitous intracellular degradation process that involves the formation of autophagosomes, the encapsulation of cytoplasmic cargo, and its delivery to lysosomes for degradation. Upon virus infection such as vesicular stomatitis virus (VSV), human parainfluenza virus type 3, and Sindbis virus, autophagy is vital in the host defense manner by delivering invading microorganisms to the restricted lysosomal. From the findings of our previous study, PEDV infection notably triggered LC3-I conversion, suggesting strong upregulation of autophagy [8]. Moreover, the host factors involving BST2, PABPC4, RALY, and HNRNPA1 can induce the selective autophagy pathway decreasing PEDV replication [8,26,28,40]. In this study, we discovered that HNRNPA1 overexpression stimulated PEDV N protein ubiquitination and that HNRNPA1 recruited MARCHF8, an E3 ubiquitin ligase, for the catalysis of PEDV N ubiquitination which was then detected by cargo receptors CALCOCO2 and transmitted to the lysosome for degradation.

After the viral infection, it would be recognized as the pathogen-associated molecular pattern (PAMP) through pattern recognition receptors (PRR), including MDA5 and RIGI [41]. When RIGI-MDA5 is activated, it forms homo-oligomers and recruits mitochondrial antiviral signal proteins (MAVS). These proteins then activate TBK1/IB kinase (IKK) by TRAFs and phosphorylate IRF3/IRF7 to increase the production of type I IFN [42]. This study found that HNRNPA1 interacts with RIGI, which upregulates RIGI, TRAF3, phosphorylated TBK1 and IRF3 proteins in HEK 293 T cells to induce the IFN signal pathway. Notably, the PEDV N protein has been detected to interact with TBK1 to obstruct TBK1-induced IRF3 phosphorylation directly and the activation of the IFNB/IFN-β to hinder the host's innate immune response [43]. Our hypothesis was confirmed that HNRNPA1 inhibited PEDV replication by activating IFN pathways through RIGI interaction and PEDV N protein degradation, which could antagonize IFNB production.

Host antiviral proteins are vital in limiting viral infections, and the virus have evolved redundant mechanisms to counteract the host's innate immunity for optimal viral adaptation and replication. Certain viruses, like the herpes virus, influenza A virus (IAV) [44], and human immunodeficiency virus [45,46], have evolved effectively in developing various strategies for immune evasion and escape or undermining autophagy [47,48]. Moreover, some RNA viruses aggressively trigger or develop the autophagy machinery to benefit their replication [13]. For instance, poliovirus and coxsackievirus B3 could induce the autophagosome formation, but not promote lysosome-mediated protein degradation, suggesting that the virus could activating the incomplete autophagy process to support the viral RNA replication [49]. In this study, we observed that PEDV infection inhibited HNRNPA1 protein expression and other host antiviral proteins, such as FUBP3, HNRNPK, PTBP1, and TARDBP, which were previously reported. Moreover, this study demonstrated the bifunctional autophagy activities during PEDV infection. Besides the degradation of PEDV N protein, the virus also utilize the autophagy to degrade the host antiviral proteins. In contrast to autophagic antiviral activity, viruses could also enhance autophagy to destroy antiviral proteins when they encounter the host. It was reported that the PEDV nsp6 protein could be activate the autophagy process to promote virus replication [44,45]. A study in porcine intestinal epithelial cells showed that upregulated autophagy pathways negatively affect viral replication [12]. These conflicting reports prompt that host cellular reaction can be a double-edged sword when PEDV encounters autophagy. Therefore, a careful balance between autophagy's pro-viral and antiviral functions is essential.

In conclusion, the current study explored that HNRNPA1 thwarts PEDV replication by degrading viral nucleocapsid protein and boosting IFN expression. On the one hand, the transcription factor EGR1, which can directly bind to the core promoter of *HNRNPA1*, upregulates HNRNPA1 protein expression. HNRNPA1 also combines with E3 ubiquitin ligase MARCHF8 for the catalysis of PEDV N protein ubiquitination. This ubiquitin complex (PEDV N- HNRNPA1-MARCHF8) could be recognized by the cargo receptor CALCOCO2 that delivers it into autolysosomes. Moreover, HNRNPA1 promotes IFN expression with support from the host-autonomous defense response to antagonize PEDV infection by interacting with the RIGI protein. Meanwhile, PEDV infection can conversely degrade the HNRNPA1 protein with its N protein through the host autophagy pathway. Furthermore, the PEDV N protein with selective autophagy could also degrade other host proteins, including FUBP3, HNRNPK, PTBP1, and TARDBP. This study illustrates the mechanism underlying host antiviral protein HNRNPA1-regulated viral restriction. Meanwhile, PEDV also used autophagy to degrade host antiviral proteins to facilitate virus replication. Our finding reveals a novel antiviral mechanism of HNRNPA1 and adds to our knowledge of the dual roles of autophagy in viral replication.

Materials and methods

Antibodies and reagents

In this study, anti-HNRNPA1 antibody (11,176-1-AP), Anti-FUBP3 antibody (10,623-1-AP), Anti-HNRNPK antibody (11,426-1-AP), Anti-TARDBP antibody (10,782–2-AP), anti-ACTB/β-actin antibody (66,009-1-lg), anti-GST-tag antibody (10,000–0-AP), anti-MARCHF8/MARCH8 antibody (14,119-1-AP), anti-CALCOCO2/NDP52 antibody (12,229-1-AP), anti-TRAF3 antibody (66,310-1-lg), anti-TRAF6 antibody (66,498-1-lg), anti-MYD88 antibody (23,230-1-lg), anti-RIGI antibody (67,556-1-lg), horseradish peroxidase (HRP)-labeled anti-mouse (SA00001-1) and anti-rabbit (SA00001–2) IgG antibodies, were obtained from Proteintech Group. In addition, bafilomycin A_1 (Baf A1; 54,645), anti-HA-tag antibody (3724), anti-p-TBK1 antibody (5483S), and anti-p-IRF3 antibody (29047S) were acquired from Cell Signaling Technology. Anti-ubiquitin antibody (SC-8017), human *CALCOCO2/NDP52* siRNA (sc-93,738), and human *MARCHF8* siRNA (SC-90432) were acquired from Santa Cruz Biotechnology. The design and synthesis

Table 1. List of primer and siRNA sequences used in this study.

Purpose	names	Sequence (5′-3′)
Real-time PCR Primers	PEDV *N* forward	GAGGGTGTTTTCTGGGTTG
	PEDV *N* reverse	CGTGAAGTAGGAGGTGTGTTAG
	pHNRNPA1 forward	ATGAGGGACCCAAACACCAA
	pHNRNPA1 reverse	TTAAGTGGGCACCAGGTCTTT
	ACTB forward	TCCCTGGAGAAGAGCTACGA
	ACTB reverse	AGCACTGTGTTGGCGTACAG
siRNA sequences	*si-HNRNPA1* sense	GGCAAUUACAACAAUCAAUTT
	si-HNRNPA1 antisense	AUUGAUUGUUGUAAUUGCCTT
	si-RIGI sense	GCCCAUUUAAACCAAGAAATT
	si-RIGI antisense	UUUCUUGGUUUAAAUGGGCTT
	si-EGR1 sense	GCACAGUGGUUUCCCAUCATT
	si-EGR1 antisense	UGAUGGGAAACCACUGUGCTT
	NC sense	UUCUCCGAACGUGUCACGUTT
	NC antisense	ACGUGACACGUUCGGAGAATT

of siRNAs in this study were done by GenePharma (Table 1). 3-Methyladenine (3-MA; M9281), anti-Flag-tag antibody (F1804), anti-PTBP1 antibody (WH0005725M1), MG132 (M7449) and chloroquine phosphate (CQ; PHR1258) were from Sigma-Aldrich. 4',6-diamidino-2-phenylindole (DAPI; C1002) from Beyotime Biotechnology, ClonExpress II One Step Cloning Kit (C112–02) and Dual-Glo Luciferase Assay System (DL101) was obtained Vazyme Biotech Co., Ltd. In addition, we provided an anti-PEDV (JS-2013) N protein monoclonal antibody [46].

Cell culture and transfection

Human embryonic kidney cells (HEK 293 T cells; CRL-11268, ATCC) and African green monkey kidney cells (Vero cells; CCL-81, ATCC) were cultured in media composition containing DMEM (Sigma-Aldrich, D6429) with 10% fetal bovine serum (FBS; Gibco, 10,099,141). LLC-PK1 cells were offered by Dr. Rui Luo from Huazhong Agricultural University and were cultured in MEM (Invitrogen, 11,095,080). All the cell lines were inoculated and maintained at 5% CO_2 and 37°C. The cells were inoculated in 6-well plates and subjected to transfection with plasmids at 80–90% density by Lipofectamine 3000 (Invitrogen, L3000015). Moreover, siRNA was transfected into cells by Lipofectamine RNAiMAX at 50–60% density (Invitrogen, 13,778,150), and the interference efficiency of siRNA was analyzed by real-time PCR assay.

PEDV infection

For the current study, the PEDV variant strain JS-2013 (MH910099) used was separated and preserved in our laboratory [23]. For PEDV to be infected, Vero cells were grown with over 90% adherence in culture plates, washed with PBS (Gibco, C20012500BT), and then infected at a 1 or 0.01 MOI together with 4 μg/mL trypsin treatment (Invitrogen, 15,050,065). Before collecting, cells were rinsed with PBS and analysis the virus replication by western blot and qPCR. In addition, Kaerber's approach was also adopted for determining viral titers, which was shown to reach 50% of tissue culture infective dose $TCID_{50}$.

Quantitative real-time PCR (qRT-PCR)

According to the protocol, RNeasy Mini Kit (Qiagen, 74,104) or QIAamp Viral RNA Mini Kit (Qiagen, 52,906) was used for extracting total RNA. PrimeScript™ RT reagent Kit (Takara, RRO47A) was used for preparing cDNA, and the qRT-PCR was performed with the application of SYBR Premix Ex Taq TM (Vazyme Biotech Co., Ltd, q711–03). GAPDH or ACTB was used as reference genes in this qRT-PCR. In Table S1, all primer sequences involved in qPCR are shown.

Western blotting

Cells were lysed on ice with RIPA Lysis and extraction buffer (Thermo Fisher Scientific, 89,901) containing protease/phosphatase inhibitor cocktail (Bimake, B14001, B15001). In 5 × SDS-PAGE sample loading buffer, lysates were collected and denatured for 10 min. Proteins isolated by SDS-PAGE were transferred onto nitrocellulose membranes (GE Healthcare, 10,600,001) and blocked by 5% defatted milk powder (BD, 232,100) and 0.2% Tween 20 (Sigma-Aldrich, P1379). These proteins were probed based on primary antibodies and HRP-labeled secondary antibodies. Subsequently, the protein was measured with enhanced chemiluminescence (ECL) (Share-bio, SB-WB012).

ChIP assay

LLC-PK-1 cells were inoculated into the 6-well plates and transfected with Flag or other coding plasmids. After 24 h, cells were harvested, and a ChIP assay was done with a SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology, 9003). The chromatin fragments were immunoprecipitated with anti-Flag antibody-coupled protein G magnetic beads (Cell Signaling Technology, 9006).

Co-immunoprecipitation assay

Cells were transfected with plasmids for 24 h and lysed with NP40 cell lysis buffer (Life Technologies, FNN0021) containing a protease inhibitor Cocktail. Collected lysates were centrifuged and incubated on Dynabeads Protein G coupled with anti-Flag-antibody (Life Technologies, 10004D). This mix was washed with 0.02% PBST resuspension in 50 mM glycine

elution buffer (pH 2.8). Using specific antibodies, immunoblotting was done to examine the proteins.

GST affinity-isolation assay

Full-length sequences of PEDV *N, MARCHF8, HNRNPA1*, and *CALCOCO2* were inserted in pCold TF plasmid (Clontech Laboratories, Inc, 3365) and pCold GST plasmid (Clontech Laboratories, Inc, 3372). Furthermore, these genes were denoted in the BL21 competent cells (Vazyme Biotech, C504–03), and protein interactions were evaluated using GST Protein Interaction Pull-Down Kits (Thermo, 21,516).

Confocal immunofluorescence assay

Following the transfection, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, P6148) and subjected to permeabilization in 0.1% Triton X-100 (Sigma-Aldrich, T9284). After blocking with 5% bovine serum albumin (BSA; Cell Signaling Technology, 9998), cells were incubated with primary antibodies and washed thrice with PBS. The fluorescently-labeled secondary antibody was incubated in the dark for one hour [8]. Eventually, nuclei were also stained for 5 min with DAPI. Finally, the laser scanning confocal immunofluorescence microscope (Carl Zeiss, Oberkochen, Germany) was used to view the fluorescence images.

Luciferase reporter assay

Target-encoding plasmids were transfected into HEK 293 T cells cultivated in the 24-well plates with Lipofectamine 3000. The cells were collected after 24 h to measure their luciferase activities with Dual-Glo Luciferase Assay System (Vazyme Biotech Co., Ltd, DL101), and *Renilla* luciferase functioning was used as reference.

Statistical analysis

To compare two groups based on a two-tailed Student's *t*-test, GraphPad Prism 5 software (GraphPad Software, USA) was used. In addition, significance levels were detected at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, whereas ns is considered insignificant. Moreover, the data represent means from 3 separate assays.

Disclosure statement

The authors declare that they have no potential conflicts of interest.

Funding

This study was supported by the National Key Research and Development Programs of China (no. 2021YFD1801102), the National Natural Science Foundation of China (no. 32102665 and 32272999), and Central Public-interest Scientific Institution Basal Research Fund (no. Y2022QC28).

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