Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.184

RESEARCH ARTICLE

Induction of HAMP by Influenza A Virus in the LncRNA-155-Deficient Host Contributes to Suppression of Antiviral Innate Immunity

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ARTICLE HISTORY (26-107)

Received: February 06, 2026
Revised: May 23, 2026
Accepted: August 8, 2026
Published online: August 25, 2026

Key words:
Antiviral response
HAMP
Influenza virus
Innate immunity
lncRNA-155

ABSTRACT

Influenza A virus (IAV) is a major zoonotic respiratory pathogen with persistent pandemic potential. Long non-coding RNAs (lncRNAs) are known to play a critical role in antiviral immunity. Our previous study has identified lncRNA-155, a product of MIR155HG, as a positive regulator of antiviral innate immunity, but the molecular mechanism by which it potentiates immune response remains unclear. Here, RNA-sequencing analysis revealed that lncRNA-155 is critical for interferon-β (IFN-β)-mediated innate immunity, as lncRNA-155-deficient mice exhibits a unique transcriptional signature characterized by the downregulation of IAV-induced expression of IFN-β and a set of interferon-stimulated genes compared to other genotypes. Furthermore, we found that lncRNA-155 functions as a negative regulator of HAMP expression, as evidenced by a robust upregulation of HAMP expression in response to IAV infection in lncRNA-155-deficient animal tissues and cells. Functional analysis showed the profound impact of HAMP on IAV replication. Knockdown of HAMP results in reduced viral replication by enhancing IAV-induced antiviral responses. Conversely, HAMP overexpression promotes IAV replication by suppressing the antiviral responses, as characterized by the significant inhibition of IAV-induced IFN-β production and key interferon-stimulated genes expression. HAMP overexpression also causes the impaired activation of the transcription factors NF-κB and IRF3 following IAV infection. Importantly, forced HAMP expression abrogates the antiviral responses potentiated by lncRNA-155. Together, this study identifies a new lncRNA-155/HAMP axis wherein lncRNA-155 augments antiviral immunity by suppressing HAMP.

To Cite This Article: Shrestha P, Zhang Q, Gao S, Wang Y, Wen F, Li Y, Sajjad N, Huang S, Rai KR and Chen JL, 2026. Induction of HAMP by influenza a virus in the LncRNA-155-deficient host contributes to suppression of antiviral innate immunity. Pak Vet J, 46(8): 1906-1919. http://dx.doi.org/10.29261/pakvetj/2026.184

INTRODUCTION

Influenza A virus (IAV) is a major respiratory pathogen in animals and poses a considerable zoonotic risk to humans (Kang *et al.*, 2024). Ability of IAV to adapt and spread across species fosters the emergence of pandemic strains (Ahad *et al*., 2013; Kang *et al.*, 2024). While vaccines and antivirals exist, they are often undermined by the virus's high mutation rate and antigenic diversity, leading to persistent control challenges (Becker *et al.*, 2021). Consequently, research is focused on both innovative strategies that modulate the innate immune response to the viral infection and targeting the virus directly (Rai *et al.*, 2021). Particularly, studies of long non-coding RNAs (lncRNAs) are important for better understanding of IAV pathogenesis,

since they are crucial modulators of antiviral signaling (Chen *et al.*, 2024). LncRNAs regulate immune gene expression, signaling pathways, and feedback loops through diverse mechanisms (Mattick *et al.*, 2023). However, their complex role in antiviral immunity remains largely unexplored. Understanding this interplay could enable broad-spectrum, mutation-resistant antiviral strategies.

Innate immunity is the first antiviral defense that employs host pattern recognition receptors to detect viral and host-derived damage-associated molecular patterns (Carty *et al*., 2021). This detection triggers signaling cascades that activate key transcription factors, including interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Carty *et al*., 2021). These factors subsequently induce the expression of type I interferons (IFNs) and interferon-stimulated genes (ISGs) to establish an antiviral state. IAV-triggered innate immunity also regulates immunomodulatory lncRNAs, such as MIR155HG (Rai *et al.*, 2022). The highly conserved MIR155HG (also known as BIC/lncRNA-155) is robustly induced by immune stimuli like viral infections, TLR ligands (e.g., LPS, poly(I:C)), and IFN-β (Tam, 2001; Maarouf *et al.*, 2019; Rai *et al.*, 2022). Upon induction, MIR155HG potentiates antiviral and inflammatory responses by enhancing the activation of transcription factors IRF3, NF-κB, and AP-1, thereby upregulating type I interferons such as IFN-β (Wang *et al*., 2010; Elton *et al.*, 2013; Maarouf *et al.*, 2019; Rai *et al.*, 2022). Interestingly, MIR155HG locus is complex and produces multiple immune-regulatory products, including lncRNA-155, miRNA-155, miPEP155, and ceRNA (Wu *et al.*, 2019; Niu *et al.*, 2020; Zhang *et al*., 2021; Rai *et al.*, 2022). Our prior studies in cells and animals have demonstrated that lncRNA-155 activates IRF3 and boosts IFN-β production independently of miRNA-155 (Maarouf *et al.*, 2019; Rai *et al.*, 2022). However, how lncRNA-155 integrates complex antiviral innate immune responses remains unclear.

Hepcidin antimicrobial peptide (HAMP), originally identified as an antimicrobial peptide produced primarily by hepatocytes (Krause *et al.*, 2000; Park *et al.*, 2001), is a key gene regulating systemic iron homeostasis (Nemeth *et al*., 2003; Galy *et al.*, 2024). While HAMP is highly conserved across species, mice possess two hepcidin genes: *hepcidin-1* (the functional homolog of human hepcidin, here called HAMP) and the more divergent *hepcidin-2*, unlike the single gene found in humans (Ganz, 2011). Recently, HAMP has garnered attention for its emerging immunoregulatory roles (Bessman *et al*., 2020; Frost *et al.*, 2021; Crisell *et al.*, 2025). Its expression is dynamically modulated during infection and inflammation (Lee *et al*., 2005; Armitage *et al.*, 2011; Navidifar *et al.*, 2025). For example, certain viral pathogens upregulate HAMP in hepatocytes and increase its circulating levels (Armitage *et al.*, 2011; Rodriguez *et al*., 2014; Navidifar *et al.*, 2025), while others downregulate it (Girelli *et al.*, 2009; Hörl and Schmidt, 2014) or induce minimal change (Armitage *et al.*, 2014), indicating a complex, context-dependent interplay with innate immune signaling (Navidifar *et al.*, 2025). In hepatocytes, HAMP upregulation by immune stimuli is primarily driven by interleukin 6 (IL-6) signaling (Rodriguez *et al.*, 2014; Navidifar *et al.*, 2025). However, some cytokines, including IFN-β, have been reported to suppress HAMP expression (Nemeth *et al.*, 2003, 2004; Lee *et al.*, 2005). Moreover, LPS-induced HAMP upregulation in murine alveolar macrophages is independent of interleukin 1 (IL-1) and IL-6 signaling (Nguyen *et al.*, 2006). Apart from regulating iron homeostasis, HAMP exerts immunomodulatory role by modulating cytokine production (De Domenico *et al.*, 2010; Zhang and Rovin, 2013; Michels *et al.*, 2015; Frost *et al.*, 2021; Krepuska *et al.*, 2024), and dysregulation of HAMP is linked to inflammatory disease and infection outcomes (Zhang and Rovin, 2013; Michels *et al.*, 2015). While most studies focus on systemic, hepatocyte-derived HAMP (Armitage *et al.*, 2014; Rodriguez *et al.*, 2014; Michels *et al.*, 2015; Bonitz *et al.*, 2025), the regulation of non-hepatocyte-derived hepcidin remains poorly defined. Moreover, the regulatory network connecting immunomodulatory lncRNAs, HAMP, and virus-sensing pathways is uncharacterized.

To identify lncRNA-155 targets, we performed RNA-sequencing (RNA-seq) analysis on wild-type (WT), miRNA-155 knockout (KO), and lncRNA-155KO mice infected with IAV. This screen identified HAMP as one of the most upregulated genes following IAV infection in lncRNA-155-deficient mice. Further experiments revealed that lncRNA-155 negatively regulates HAMP, and HAMP suppresses antiviral immunity to facilitate IAV replication. Therefore, this study unveils a new lncRNA-155/HAMP axis regulating innate immunity.

MATERIALS AND METHODS

Ethics statement: All animal experiments were approved by Fujian Agriculture and Forestry University Research Ethics Committee (Permit No. PZCASFAFU2019009) and complied with national experimental animal regulations.

Cell culture, virus propagation, and infection: 293T and A549 cells (ATCC) were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), 2mM glutamine, and antibiotics 100U/mL penicillin, 100μg/mL streptomycin at 37°C with 5% CO_2. IAV strains (PR8, WSN, H9N2: Fujian/MQ01/2015) were propagated in chicken embryos; Sendai virus (SeV) was prepared as described (Chi *et al.*, 2024). For infections, cells were washed with PBS, inoculated with the indicated multiplicity of infection (MOI) for 1h at 37°C, and maintained in DMEM containing 2μg/mL TPCK-trypsin (Gibco) and antibiotics. All experiments were performed under BSL-2 conditions as previously described (Maarouf *et al.*, 2019).

Mouse experiments: Wild-type, miRNA-155KO, and lncRNA-155KO mice on a C57BL/6 background. Female and male C57BL/6J mice (5-6 weeks old, sex matched across groups, 11-13 mice per group) (Maarouf *et al.*, 2019; Rai *et al.*, 2022) were anesthetized and intranasally inoculated with 5×10^4PFU of WSN IAV or mock control in 50μl PBS under enhanced BSL-2 conditions. At

indicated times post-infection, mice were euthanized and lung tissues were collected for further analysis as previously described (Maarouf *et al.*, 2019).

RT-PCR, quantitative real-time PCR (qPCR) and Western blotting (WB): RNA was extracted with Trizol (Invitrogen). For cDNA synthesis, 1μg RNA was reverse transcribed with oligo-dT/random primers using Reverse Transcriptase (Promega). PCR used Taq polymerase; qPCR employed SYBR Premix Ex TaqII (TaKaRa) on a LightCycler (Roche). Results were analyzed by ΔΔCT using actin as a reference gene (Supplementary data). For WB, cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Whatman; MilliporeSigma), and immunoblotted using anti-IAV NP polyclonal antibody (raised against GST-NP; described in Chi *et al.*, 2024), and other antibodies listed in supplementary data.

Plaque forming assay (PFA) and hemagglutination (HA) assay: MDCK cells (ATCC) were infected with serial virus dilutions (37°C, 1h), washed, and overlaid with α-MEM/3% agarose (Gibco) containing 2μg/mL TPCK-trypsin. After 72h, plaques were fixed (4% formaldehyde), stained with crystal violet, and counted. For HA assays, infected supernatants were mixed 1:1 with 1% chicken erythrocytes in PBS, and titers were determined as described (Rai *et al.*, 2022).

Vector construction and generation of stable cell lines: Myc-tagged HAMP (NR_002819.4) was PCR-amplified, cloned into a PNL vector, and verified by sequencing before transfection into target cells. Stable HAMP overexpression cell lines were generated as described (Maarouf *et al.*, 2019). ShRNA-based knockdown cell lines were generated as described (Chen *et al.*, 2024). LncRNA-155 and control siRNAs (RiboBio) were transfected using Lipofectamine 3000 (Invitrogen) per manufacturer's instructions. Primers and all sequences are listed in supplementary data.

RNA-sequencing: RNA-seq was performed by Novogene (Beijing, China) as described previously (Chen *et al.*, 2024). In brief, total RNA was extracted from mouse lung tissue using Trizol. The RNA integrity was then assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Following this, sequencing libraries were generated and run on a Novaseq 6000 system (Illumina, San Diego, CA, USA). Differential expression between infected and control groups was analyzed using DESeq2, defining DEGs as having an adjusted p-value < 0.05 and $\log_2FC \geq 1$.

Dual-luciferase reporter assay: Following establishment of HAMP-knockdown or overexpressing 293T cells (lentiviral) with vector controls, triplicate wells were transfected with NF-κB/ISRE/IFN-β luciferase reporters and *Renilla* plasmid (MiaoLing, Wuhan, China) using Lipofectamine 3000. Cells were then mock-infected or infected with SeV (16h) and assayed using the dual-luciferase system (Promega).

Statistical analysis: Comparisons used Student's t test on data from three independent experiments (mean ± SD). Differences were considered statistically significant with $P<0.05$.

RESULTS

RNA sequencing analysis reveals impaired interferon expression and antiviral response in lncRNA-155KO mice: Our previous work has shown that IAV-inducible lncRNA-155 potentiates antiviral innate immunity by activating the IRF3 and NF-κB pathways and upregulating IFN-β expression (Maarouf *et al.*, 2019; Rai *et al.*, 2022). However, the complex molecular interplay by which lncRNA-155 potentiates antiviral innate immunity remains unclear. For this, we performed RNA-seq on lung tissues from three mouse genotypes (WT, miRNA-155KO and lncRNA-155KO) to elucidate the gene expression networks modulated by lncRNA-155 after IAV infection. RNA-seq analysis identified 1727, 3060 and 2723 differentially expressed genes (DEGs) in WT, miRNA-155KO and lncRNA-155KO, respectively, in response to IAV infection compared to their respective controls ($|\log_2$ fold change$| \geq 1$, adj. p-value ≤ 0.05) (Fig.1A-C; S1A). Principal component analysis (PCA) of transcriptional profiles following WSN infection demonstrated distinct clustering of the three groups, indicating that loss of either miRNA-155 or lncRNA-155 results in unique transcriptional changes compared to WT controls (Supplementary data).

We next performed Gene Set Enrichment Analysis (GSEA) across the IAV-infected three mouse genotypes. We found that immune signaling pathways were significantly enriched in WT mice compared to both miRNA-155KO and lncRNA-155KO groups (Fig. 1D-E). Notably, as shown in Fig. 1F, when comparing the miRNA-155KO group to the lncRNA-155KO group, the most significantly enriched pathway was "cellular response to IFN-β" followed by other antiviral pathways including "defense response to virus" and "negative regulation of viral replication". Moreover, lncRNA-155KO exhibited an impaired expression of IFN-β and several antiviral genes compared to other genotypes following IAV infection (Fig. 1G).

Expression of HAMP is significantly enhanced in IAV-infected host deficient of lncRNA-155: To identify potential immunomodulatory genes regulated by IAV in the context of lncRNA-155 deficiency, we compared the most strongly dysregulated transcripts from WT, miRNA-155KO, and lncRNA-155KO mice during the IAV infection. In IAV-infected mice, lncRNA-155 deficiency resulted in a unique transcriptional signature characterized by the downregulation of numerous antiviral genes and the strong upregulation of a specific gene subset, most notably HAMP, as visualized by RNA-seq heatmap (Fig. 2A). HAMP expression exhibited minimal change in miRNA-155KO mice, but a significant upregulation of HAMP expression was observed in lncRNA-155KO mice compared with WT mice ($\log_2$ fold change = 8.038) following IAV infection. Given its strong induction in lncRNA-155KO mice and its potential role in innate immune responses, HAMP was prioritized for further investigation.

To validate the RNA-seq findings, we further performed both *in vivo* and *in vitro* studies. In our experimental condition, RT-qPCR and RT-PCR analyses demonstrated that WSN IAV infection induced a sharp upregulation of HAMP specifically in lncRNA-155KO mouse lungs, compared with other genotypes (Fig. 2B; 2C; S1C-D). Interestingly, similar results were observed in mice infected with pseudorabies virus (PRV), a DNA virus (Fig. 2D). Next, we examined HAMP expression in 293T cells overexpressing lncRNA-155 full (full-length lncRNA-155) or miRD155-lncRNA155 (lncRNA-155 lacking the miRNA-155 region). Relative levels of lncRNA-155 were examined by RT-qPCR (Fig. 2E). Overexpression of lncRNA-155 full or miRD155-lncRNA155 significantly reduced HAMP expression compared to empty vector (EV) control cells with or without IAV infection (Fig. 2F-H). Additionally, we used siRNAs targeting lncRNA-155 to disrupt the expression of lncRNA-155 (Fig. 2I). A time-course analysis showed that lncRNA-155 knockdown caused a significant increase in HAMP and viral gene expression during IAV and PRV infection (Fig. 2J; S1E-G). These findings suggest that lncRNA-155 inhibits the expression HAMP during the viral infection.

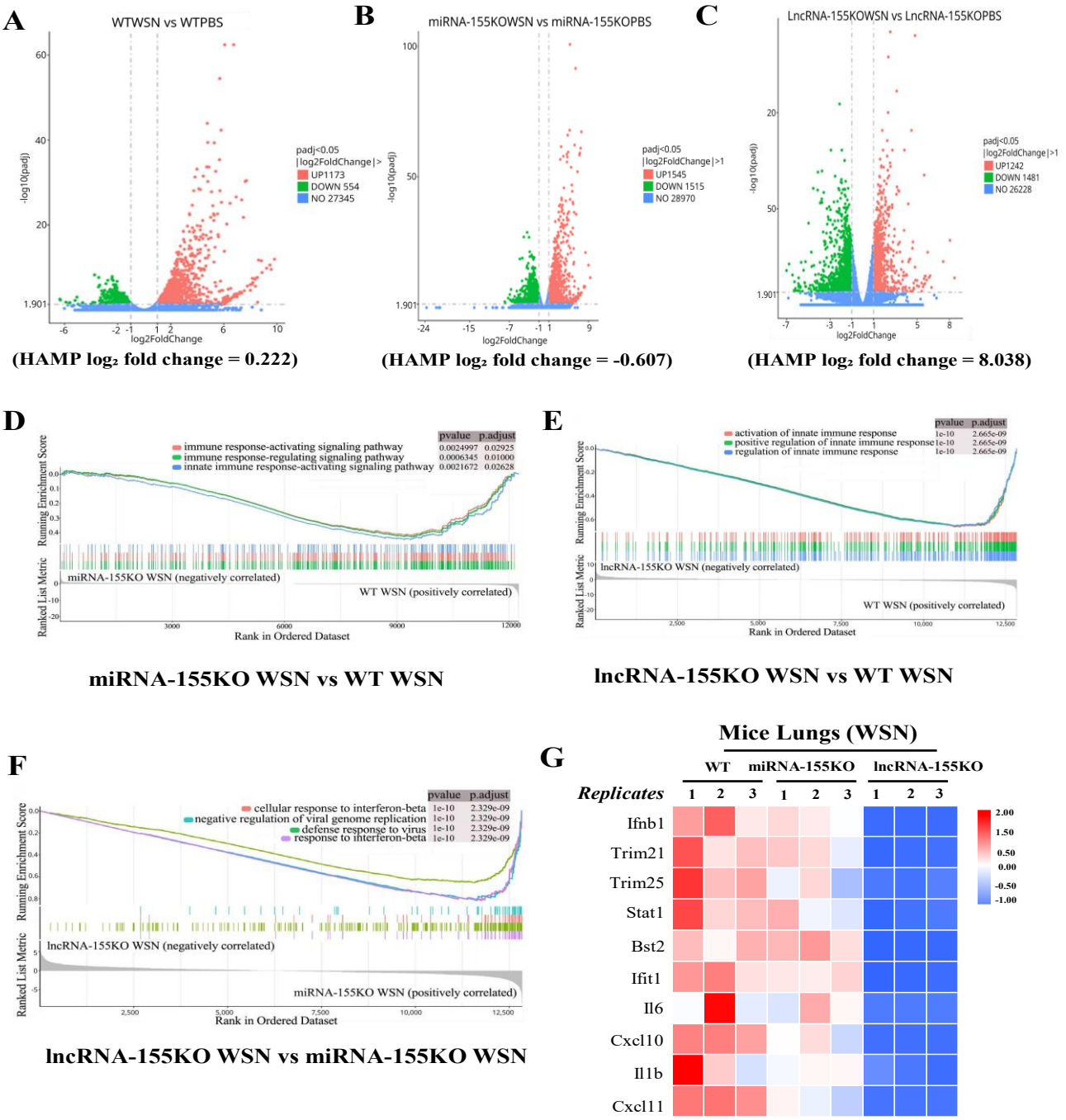

Fig. 1: RNA-seq reveals an impaired cellular response to IFN-β in lncRNA-155KO mice during IAV infection. (A-C) Differential gene expression in response to IAV infection. Volcano plots show DEGs between WSN-infected and PBS mock-treated (control) mice for the (A) WT, (B) miRNA-155KO, and (C) lncRNA-155KO genotypes. n=3 biological replicates per group. Genes with a $|\log_2$ (fold change)$| \geq 1$ and an adjusted p-value ≤ 0.05 are considered significant. (D-F) Plots depict the leading enriched biological pathways identified from comparisons between the indicated IAV-infected mouse genotypes. Gene sets were ranked by their normalized enrichment score (NES), and those with a false discovery rate (FDR) q-value < 0.25 were considered statistically significant. (G) Expression of several antiviral genes of lncRNA155KO compared to other genotypes following IAV infection.

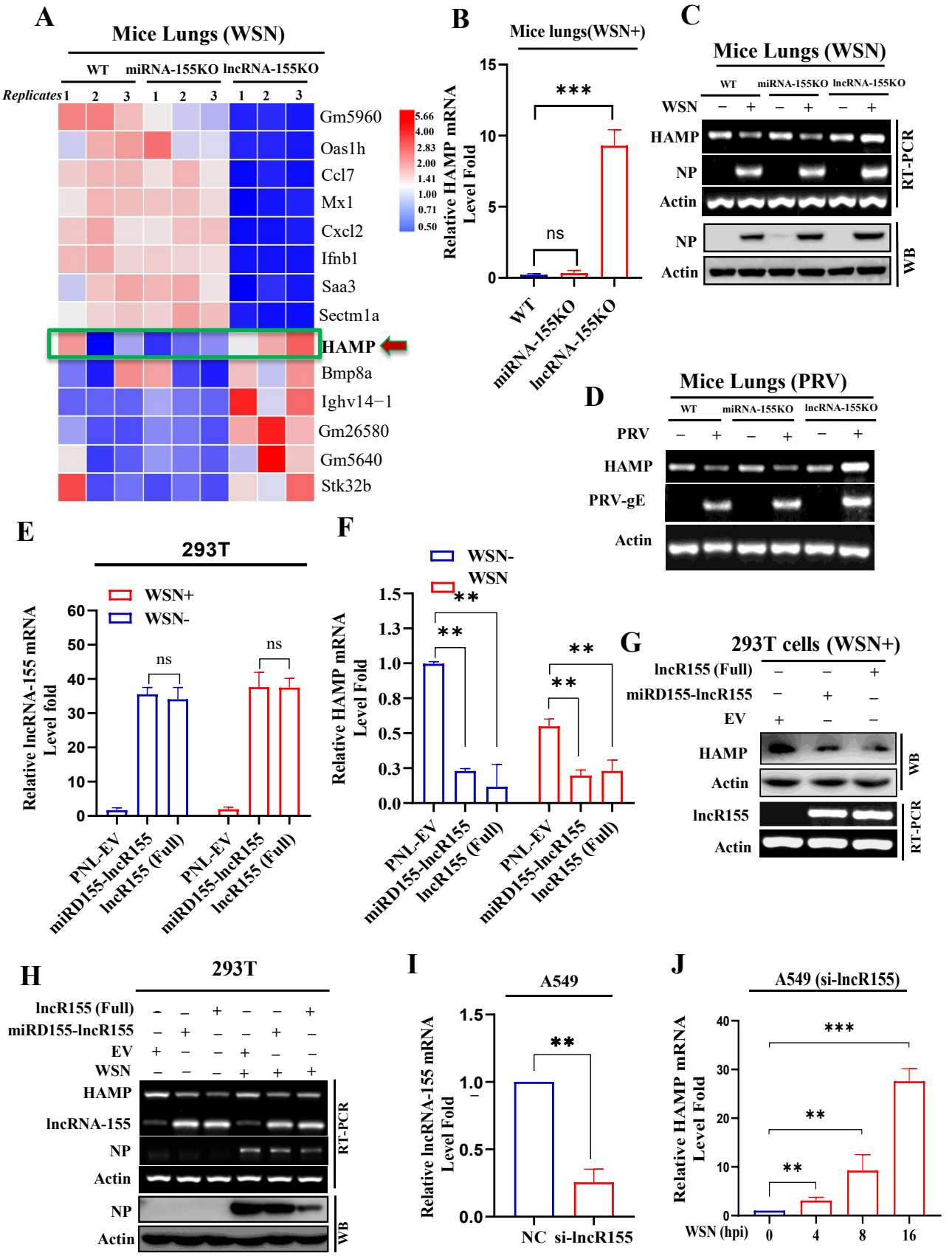

Fig. 2: HAMP is induced by IAV in lncRNA-155-deficient mice and cells. (A) Heatmap depicts the top upregulated and downregulated genes overlapping among IAV-infected three genotypes (n = 3 per group). (B-C) 5 to 6 weeks old mice were intranasally inoculated with 5 × 10^4PFU WSN for 48h, and the expression of HAMP was examined by RT-qPCR (B) and RT-PCR (C). (D) The expression of HAMP was examined in the indicated group by RT-PCR following 24h of PRV infection. (E-H) 293T cells were transfected with the plasmid expressing lncR155 (full) or miRD155-lncR155 and EV control for 24h and infected with WSN IAV for 16h. The expression of lncRNA-155 and HAMP was examined by RT-qPCR (E and F), RT-PCR and Western blotting (G and H). (I-J) siRNAs specifically targeting lncRNA-155 were used to disrupt the expression of lncRNA-155 in A549 cells, followed by infection with WSN at indicated hours of post infection (hpi). The expression of lncRNA-155 (I) and HAMP (J) in lncRNA-155-depleted cells was examined by qRT-PCR. Data are shown as means ± standard deviation (SD); **, P<0.01; *n*=3.

Regulation of HAMP by viral infection and innate immune stimuli: It has been shown that regulation of HAMP expression in response to viral infection is pathogen-specific (Girelli *et al.*, 2009; Armitage *et al.*, 2014; Hörl and Schmidt, 2014; Rodriguez *et al.*, 2014; Bonitz *et al.*, 2025; Navidifar *et al.*, 2025). These studies on HAMP regulation by pathogenic infection is exclusively limited to hepatocytes (Michels *et al.*, 2015; Bonitz *et al.*, 2025). Based on this, we utilized two other cell models, including A549 (lung epithelial cells) and 293T (kidney epithelial-like cells), to investigate HAMP expression following viral infection and stimulation with innate immune factors. The cells were infected with IAV WSN and examined for the expression of HAMP over time. We found that IAV infection significantly downregulated endogenous HAMP expression in both 293T and A549 cells (Fig. 3A-C; S2A). We also investigated whether infection with other strains of IAV affects HAMP expression. To this end, we tested additional IAV strains, including PR8 and H9N2, and observed a similar reduction in HAMP expression (Fig. 3D-E; S2B). Moreover, infection of WSN at varying MOI revealed that HAMP downregulation was significant across different infection doses (Fig. 3F).

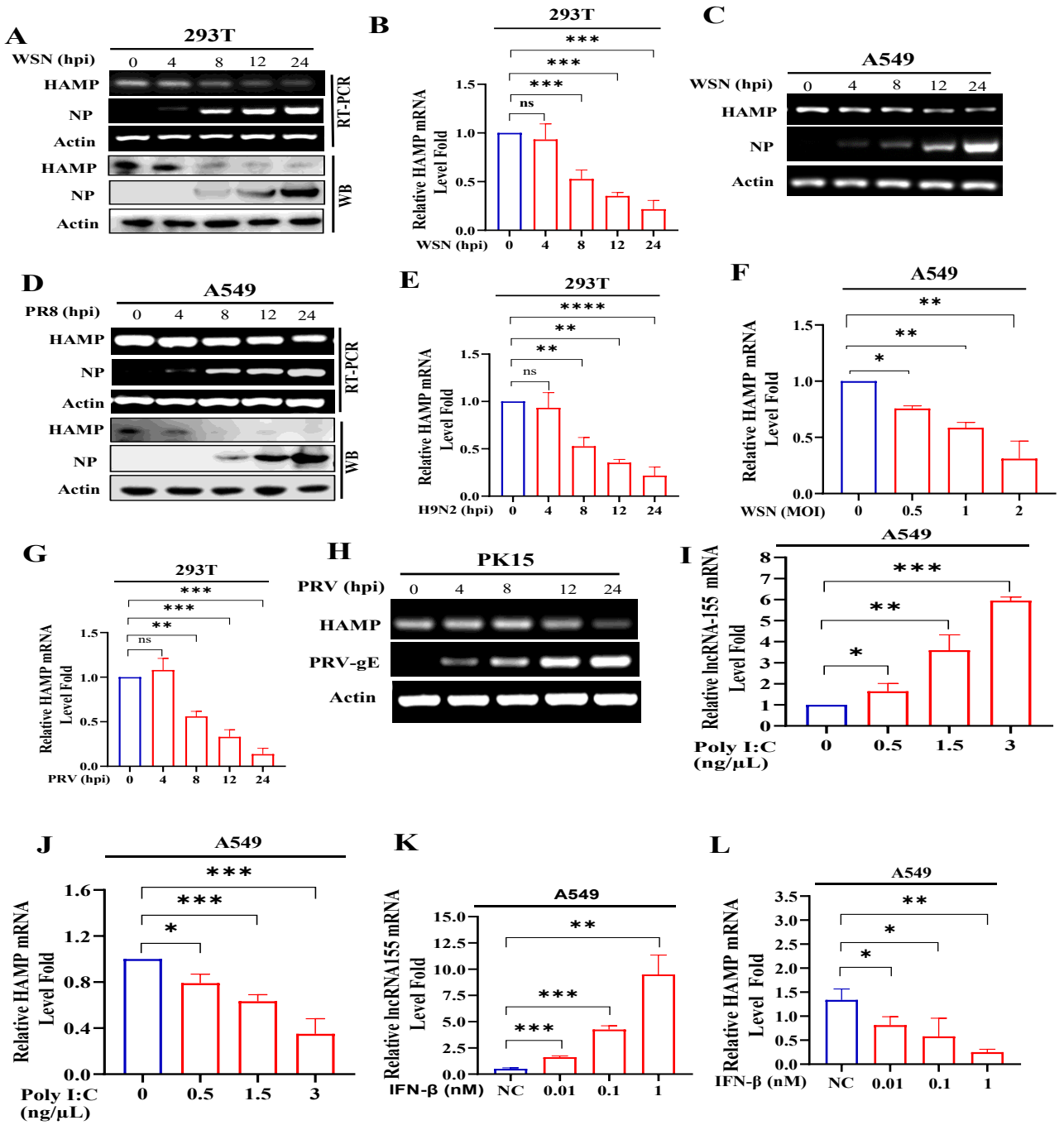

Fig. 3: HAMP is regulated by virus infection and innate immune stimuli. (A-C) Expression levels of HAMP in different cells were examined following infection with WSN IAV strains (MOI = 0.5) by RT-PCR (A and C), Western blotting (A) and RT-qPCR(B) in cells at the indicated hpi. (D-F) Expression levels of HAMP in indicated cells were examined following infection with various IAV strains (MOI = 0.5) by RT-qPCR (E, F), Western blotting (D) and RT-PCR (D) in indicated cells at the indicated hpi. (G-H) Expression levels of HAMP (G and H) and PRV-gE (H) in indicated cells were examined following infection with PRV. (I-L) Expression levels of lncRNA-155 and HAMP were examined by RT-qPCR by treating cells with indicated concentration of Poly(I:C) for 4h (I and J) and IFN-β (K and L) for 2h. Data are presented as means ± SD, *P<0.05, **P<0.01, and ***P<0.001 (*n*=3).

Next, we employed pseudorabies virus (PRV) as a DNA virus model to investigate its effects on HAMP expression in 293T and A549 cells (Fig. 3G; S2C). Following PRV infection, expression of viral gene (PRV-gE) and HAMP were quantified by RT-qPCR. The time-course experiments revealed a significant downregulation of HAMP expression in both 293T and A549 cell types (Fig. 3G; S2C-E). To validate this observation, we extended our analysis to PK15 cells, a host (pig)-derived kidney epithelial cell line for PRV. Consistent with the findings in human cell lines, PRV infection resulted in a marked reduction of porcine HAMP expression in PK15 cells (Fig. 3H).

We also treated cells with polyinosinic:polycytidylic acid (poly I:C), a structural analog of dsRNA. The results showed that poly(I:C) treatment dose-dependently reduced the expression of HAMP, while increasing the expression of lncRNA-155 (Fig. 3I-J). To confirm cytokine-specific effects, A549 cells were treated with IFN-β, a main cytokine induced by IAV infection. Similarly, IFN-β treatment dose-dependently decreased the expression of HAMP, but increased the expression of lncRNA-155 (Fig. 3K-L), which was consistent with the observations in hepatocytes (Nemeth *et al.*, 2004; Lee *et al.*, 2005). In addition, other cytokines such as IL-1, IL-6, TNF-α, and IFN-γ have been reported not to influence HAMP expression in alveolar macrophages (Nguyen *et al.*, 2006). Together, these data suggest that downregulation of HAMP expression by IAV and PRV might be mediated by IFN-β induced by the viral infection.

HAMP promotes viral replication: To investigate the role of HAMP in IAV replication, we generated HAMP-knockdown 293T cells using lentiviral shRNAs. Knockdown efficiency was confirmed by GFP expression (Fig. 4A) and RT-PCR (Fig. 4B). Compared to controls, HAMP knockdown impaired IAV replication, reducing viral nucleoprotein (NP) expression (Fig. 4B, 4C), viral load (Fig. 4D), and replication kinetics (Fig. 4E). We then generated HAMP knockdown A549 cells using lentiviral shRNAs. In these cells, HAMP knockdown also reduced the expression of viral genes from both IAV WSN and PRV (Fig. 4G; 4H).

To determine if HAMP overexpression affects IAV replication, we generated stable 293T cells expressing myc-tagged HAMP, and overexpression was confirmed by Western blotting (Fig. 4I). Expectedly, cells overexpressing HAMP showed a marked increase in IAV NP protein expression compared to control cells (Fig. 4I-J). Analysis of HA and PFA further demonstrated that HAMP overexpression significantly enhanced replication kinetics (Fig. 4K) and increased viral load (Fig. 4L).

To substantiate the above observation, we performed transient transfections with increasing concentrations of HAMP-expressing plasmid while maintaining equivalent empty vector controls. Indeed, HAMP overexpression led to a concentration-dependent increase in IAV NP, suggesting a dose-responsive effect (Fig. 4M). Moreover, to assess whether this effect extends beyond IAV, HAMP-overexpressing 293T cells were infected with PRV. We observed a notable increase in the expression of PRV-gE levels in HAMP-overexpressing 293T cells infected with PRV (Fig. 4N). Collectively, these findings demonstrate that HAMP promotes viral replication, indicating a pro-viral role for HAMP.

HAMP overexpression inhibits antiviral response: Next, we sought to investigate how HAMP promotes viral replication. 293T cell lines stably overexpressing HAMP, along with an empty vector control, were utilized. SeV-induced promoter activities of IFN-β, NF-κB, and ISRE were assessed using dual-luciferase reporter assays in these cells. Under our experimental conditions, we observed a significant reduction in SeV-induced promoter activities in cells overexpressing HAMP, compared to control cells (Fig. 5A-C).

Furthermore, we tested IAV-induced IFN-β and other important ISGs in these cells. As expected, overexpression of HAMP significantly decreased the levels of IFN-β and key ISGs such as ISG-15 and MX1 as compared to control (Fig. 5D-F).

Phosphorylation of IRF3 and NF-κB-p65 is critical for upregulating antiviral responses during IAV infection (Chen *et al.*, 2024).To determine the involvement of IRF3 and NF-κB in HAMP-mediated viral replication, next, we co-transfected with increasing concentrations of a HAMP-expressing vector along with the empty vector (EV) in 293T cells, while maintaining equal total DNA amounts in each case. Levels of HAMP overexpression and indicated genes were examined by Western blotting (Fig. 5G-H). Interestingly, HAMP overexpression resulted in a concentration-dependent inhibition of IAV-induced phosphorylation of both IRF3 and NF-κB-p65 (Fig. 5G-H). These findings suggest that HAMP overexpression impairs antiviral responses likely through suppression of the key transcription factors.

HAMP knockdown significantly promotes the antiviral response: To further examine effect of HAMP knockdown in IAV-induced antiviral responses, we utilized dual-luciferase reporter assays to test the activity of major elements of antiviral innate immunity, such as IFN-β, NF-κB, and ISRE. The results showed that HAMP knockdown significantly enhanced the activation of these promoters following SeV infection (Fig. 6A-C). Next, we quantified the expression of critical antiviral genes such as IFN-β and ISG-15, by RT-qPCR and RT-PCR in HAMP knockdown cells. Notably, depletion of HAMP resulted in a significant increase in the expression of virus-induced IFN-β and indicated ISGs (Fig. 6D-G), and also clearly upregulated the IAV-induced IRF3 phosphorylation (Fig. 6H). These data reveal that HAMP knockdown significantly enhances the IAV-induced antiviral response.

Forced expression of HAMP suppresses lncRNA-155-potentiated antiviral response: We previously reported that lncRNA-155 positively regulates innate immune signaling that governs IFN expression (Rai *et al.*, 2022). Here, we examined SeV-induced luciferase activities of IFN-β and ISRE in 293T cells overexpressing lncR155 (Full length of lncRNA-155) and miRD155-lncR155 in which coding sequences of miRNA-155 was removed from lncRNA-155. Consistent with our previous findings, overexpression of both lncR155 (Full) and

miRD155-lncR155 led to a significant increase in SeV-induced IFN-β promoter activity compared to EV control cells with no significant difference between effects of lncR155 (Full) and miRD155-lncR155 on the promoter activity (Fig. 7A). Indeed, lncR155 (Full) induced significantly higher ISRE activity compared to miRD155-lncR155 (Fig. 7B). These data imply that lncRNA-155 alone accelerates SeV-induced IFN-β activity independently of miRNA-155, but miRNA-155 contributes to ISRE activation.

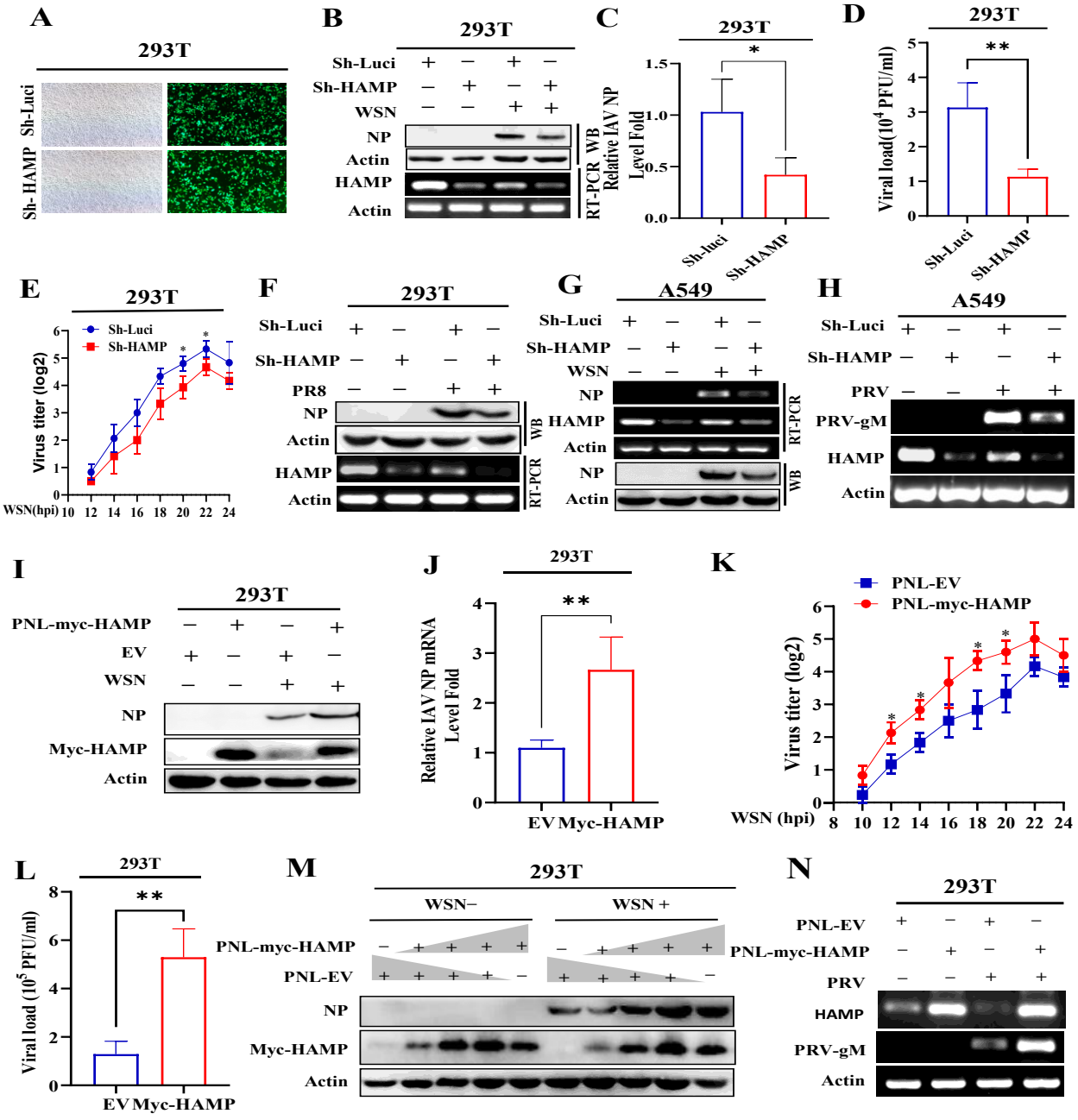

Fig. 4: HAMP knockdown inhibits IAV replication. (A-H) 293T cells stably expressing shRNA targeting endogenous HAMP or luciferase control were generated by lentivirus-transfection, and the efficiency of HAMP knockdown was examined by microscopic examination of green fluorescent protein expression (A). Cells were mock-infected or infected with influenza WSN (MOI=1) or PR8 IAV (MOI=1) for 16h. Total protein and RNA were extracted to examine HAMP knockdown efficiency and expression of indicated viral genes by RT-PCR and Western blotting (B) and RT-qPCR (C). The cell culture supernatants were harvested from WSN-infected HAMP knockdown and control cells at 16 hpi for plaque assay to measure viral load (D) and at the indicated time points for HA assay (E). (F) 293T knockdown and control cells infected with IAV PR8 (MOI=1) and expression NP and HAMP were examined by Western blotting and RT-PCR. (G and H) A549 cells stably expressing shRNA targeting endogenous HAMP or luciferase control generated by lentivirus-transfection. Cells were mock-infected or infected with influenza WSN (MOI=1) or PRV for 16h. Total protein and RNA were extracted to examine HAMP knockdown efficiency and expression levels of indicated viral genes by Western blotting (G) and RT-PCR (H). (I-L) 293T cells stably expressing a myc-tagged HAMP generated by lentivirus-transfection. Control and HAMP overexpressed cells were mock-infected or infected with influenza WSN (MOI=1) for 16h. Total protein and RNA were extracted to examine HAMP expression and expression levels of indicated viral genes by Western blotting (I) and RT-qPCR (J). The cell culture supernatants were harvested from WSN-infected HAMP overexpressed and control cells at the indicated time points for HA assay (K) and at 16 hpi for plaque assay to measure virus load (L). (M) 293T cells were transfected with increasing amounts of PNL-myc-HAMP plasmid, using PNL-EV as both negative control and for maintaining equivalent total DNA amounts across transfection conditions. After 24h of post-transfection, cells were infected by WSN (MOI=1) for 16h. Total proteins were harvested and subjected to Western blotting to detect indicated proteins. (N) HAMP overexpressed and control cells were infected with PRV for 16h. Total RNA was extracted and expression levels of indicated genes were examined by RT-PCR. Data are presented as means ± SD, *P<0.05, and **P<0.01 (*n*=3).

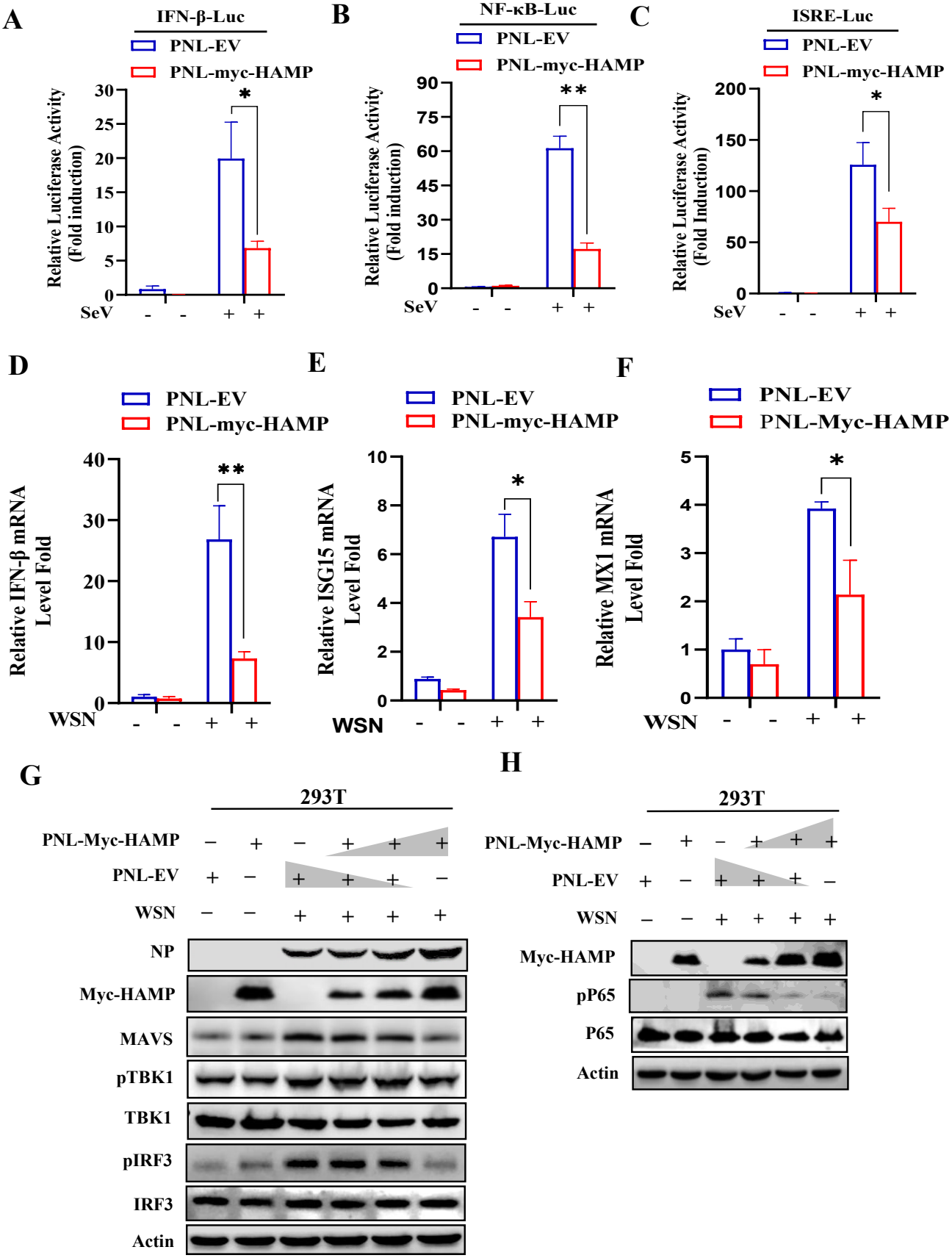

Fig. 5: HAMP overexpression suppresses antiviral immune responses. (A-C) Control and HAMP overexpressed 293T cells were transfected with the indicated luciferase reporter vector for 24h, followed by SeV (MOI=0.5) infection for 16h. The cells were harvested for luciferase assay. (D-F) Control and HAMP overexpressed 293T cells were infected with WSN (MOI=1) for 16h and expression levels of indicated antiviral genes were examined by RT-qPCR (D-F). (G and H) 293T cells were co-transfected with increasing amounts of PNL-myc-HAMP plasmid, using PNL-EV as both negative control and for maintaining equivalent total DNA amounts across transfection conditions. After 24h of post-transfection, cells were infected by WSN (MOI=1) for 16h. Total proteins were harvested and subjected to Western blotting to detect indicated proteins. Data are presented as means ± SD, *P<0.05, and **P<0.01 (*n*=3).

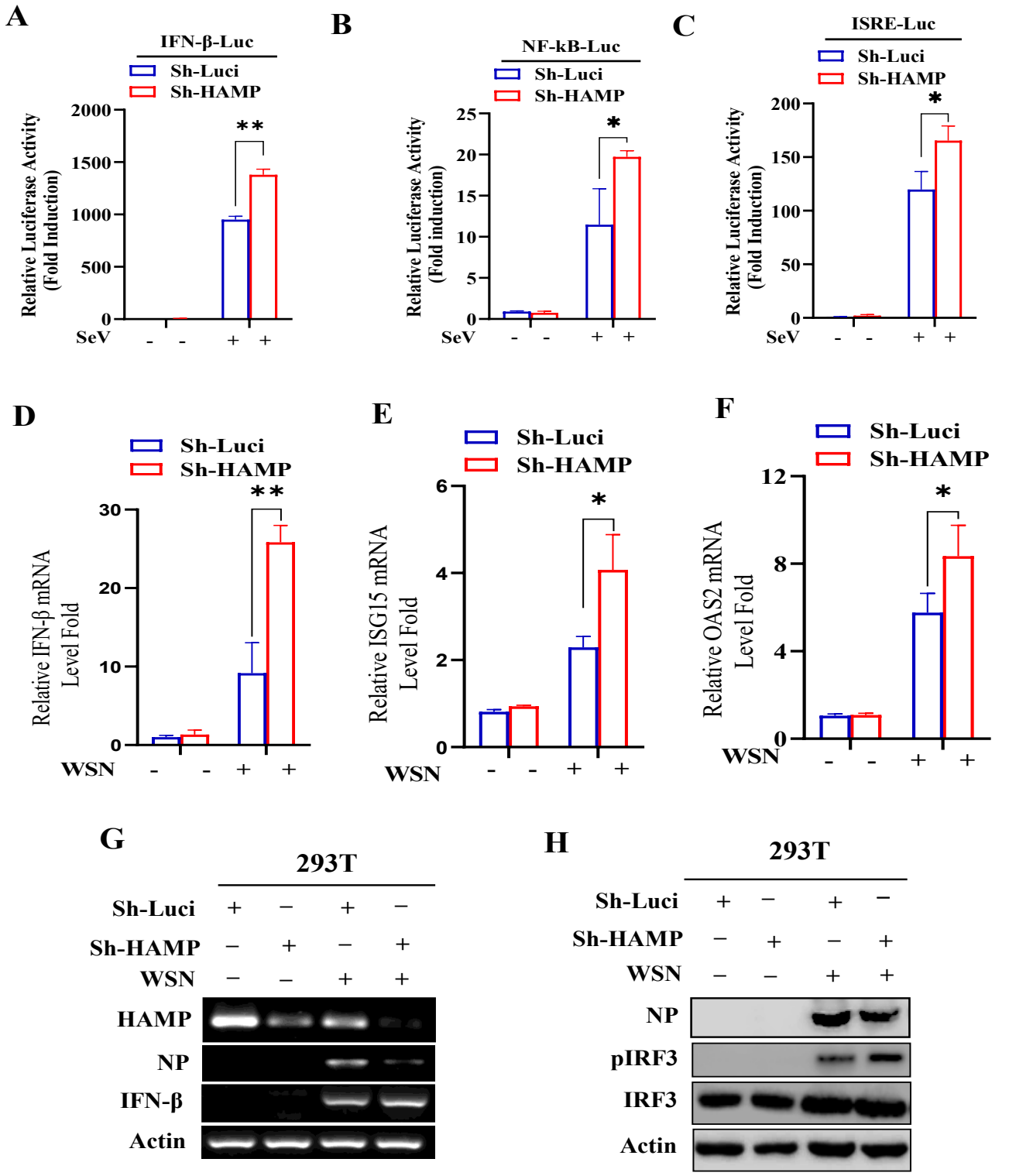

Fig. 6: HAMP knockdown enhances antiviral immune responses. (A-C) Control and HAMP knockdown 293T cells were transfected with the indicated luciferase reporter vector for 24h, followed by SeV (MOI=0.5) infection for 16h. The cells were harvested for luciferase assay. (D-H) Control and HAMP knockdown 293T cells were infected with WSN (MOI=1) for 16h. Total RNA and protein were extracted. The expression levels of indicated antiviral genes and protein levels were examined by RT-qPCR (D-F) and RT-PCR (G), and Western blotting (H). Data are presented as means ± SD, *P<0.05, and **P<0.01 (*n*=3).

Given that HAMP disrupts immune responses to both vaccination and infection in mice, a preclinical model, including immunity against influenza viruses (Frost *et al.*, 2021), we sought to test whether this effect extends to lncRNA-155-mediated antiviral innate immunity. For this, we utilized 293T transiently transfected either with lncR155 (Full), or lncR155 (Full) and HAMP. Indeed, lncR155 (Full) overexpression significantly enhanced IAV-induced antiviral responses, but which was reversed by co-transfection with HAMP (Fig. 7C; 7F-H). Overexpression of lncRNA-155 and HAMP were confirmed by RT-qPCR and RT-PCR (Fig 7D; 7E; 7H). Together, these results suggest that forced expression of HAMP suppresses lncRNA-155-mediated enhancement of antiviral responses triggered by the viral infection.

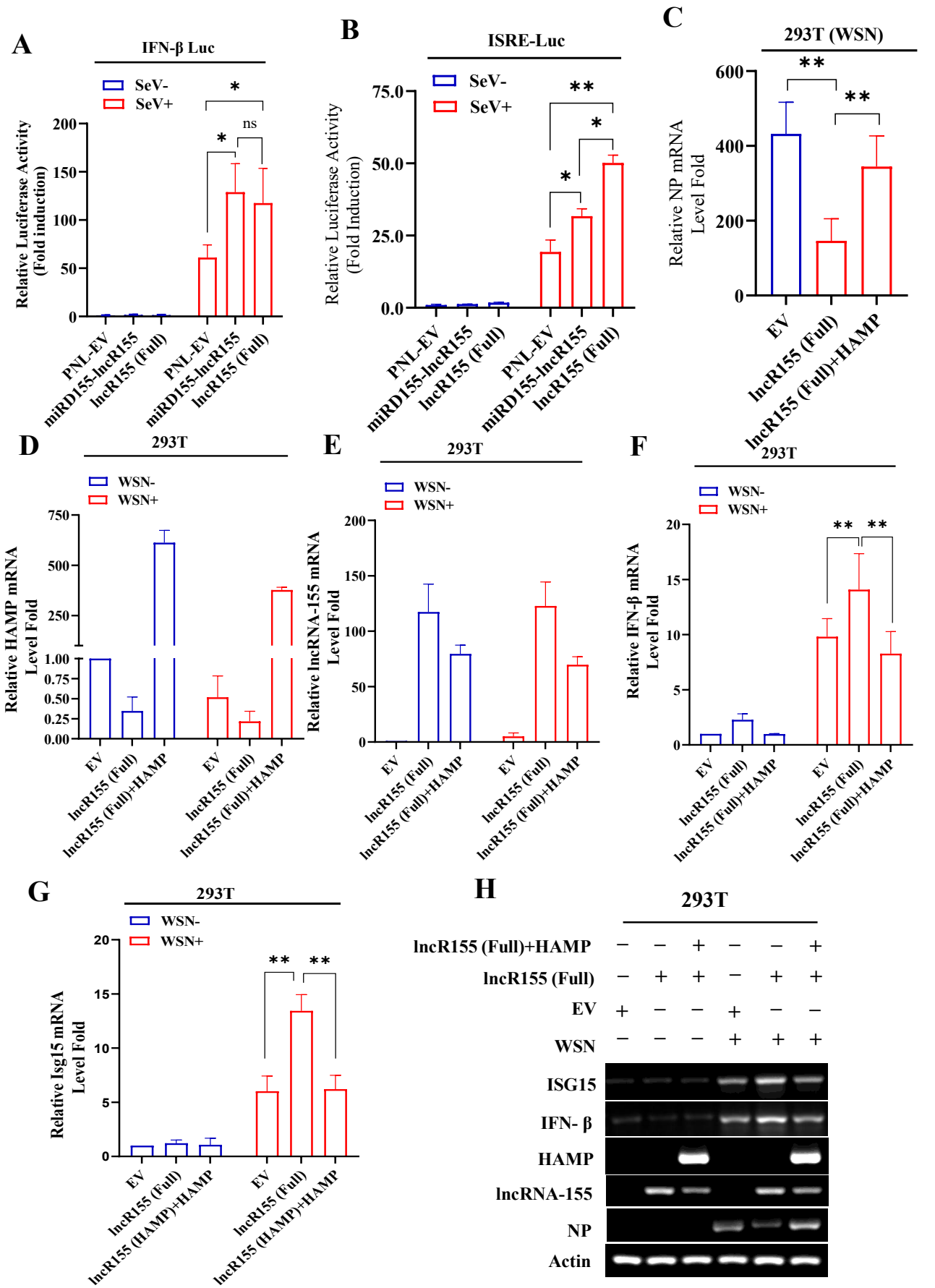

Fig. 7: Forced expression of HAMP suppresses lncRNA-155-potentiated IAV-induced antiviral responses. (A-B) Control and lncRNA-155 (lncRNA-155 (Full) or miRD155-lncR155) overexpressed 293T cells were transfected with the indicated luciferase reporter vector for 24h, followed by SeV (MOI=0.5) infection for 16h. The cells were harvested for luciferase assay (A and B). (C) 293T transiently transfected either with lncRNA-155 or HAMP co-transfected with lncRNA-155 with empty vector control for 24h, followed by WSN (MOI=1) infection for 16h. Total RNA was extracted and subjected for the examination of indicated genes by RT-qPCR (C-G) and RT-PCR (H). Data are presented as means ± SD, *P<0.05, and **P<0.01.

DISCUSSION

The MIR155HG gene is characterized by generating multiple immune regulators (Elton *et al.*, 2013; Maarouf *et al.*, 2019; Wu *et al.*, 2019; Niu *et al.*, 2020; Zhang *et al.*, 2021; Rai *et al.*, 2022). It encodes both lncRNA-155 and miRNA-155-5p, which play distinct roles in innate antiviral immunity (Rai *et al.*, 2022). To further elucidate the function of lncRNA-155, we performed RNA-seq for lung tissues from three genotypes (WT, miRNA-155KO, and lncRNA-155KO) of mice. WT mice showed significant enrichment of IAV-induced innate immune pathways compared to both miRNA-155KO and lncRNA-155KO groups, consistent with our earlier finding that miRNA-155 enhances STAT signaling, while lncRNA-155 promotes IFN-β production by activating IRF3 (Rai *et al.*, 2022).

RNA-seq revealed that lncRNA-155 deficiency profoundly reshapes the transcriptional landscape during IAV infection. Following IAV infection, HAMP expression was minimally altered in WT and miRNA-155KO mice. However, lncRNA-155KO mice exhibited strong HAMP induction. This phenotype was replicated with PRV infection *in vivo*. Experiments using lncRNA-155 knockdown A549 cells showed that lncRNA-155 deficiency leads to a strong upregulation of HAMP upon IAV infection, indicating lncRNA-155 functions as a negative regulator of virus-induced HAMP transcription.

Reports on regulation of HAMP expression in hepatocytes by viral infection are inconsistent. Some pathogens are reported to upregulate the expression of HAMP (Armitage *et al.*, 2011; Rodriguez *et al.*, 2014; Navidifar *et al.*, 2025) while others downregulate it (Girelli *et al.*, 2009; Hörl and Schmidt, 2014) or induce minimal change (Armitage *et al.*, 2014), reflecting a complex, context-dependent mechanism. Here, we found that IAV infection consistently downregulates HAMP in non-hepatocyte cell models (A549, 293T, and PK15). HAMP downregulation was observed across multiple IAV strains (WSN, PR8, H9N2) and infection doses, and extended to PRV, indicating that HAMP downregulation may be a conserved response across certain RNA and DNA viruses in non-hepatocyte systems. Mechanistically, IFN-β, a central antiviral cytokine, significantly suppressed HAMP expression while simultaneously increasing lncRNA-155 expression, which is consistent with prior observations in hepatocytes (Nemeth *et al.*, 2003; Lee *et al.*, 2005).

Some studies have shown that certain IAV strains can induce HAMP expression in hepatocytes under certain conditions. For example, a significant upregulation of liver HAMP occurs with a highly pathogenic dose of Influenza A/PR/8/34 virus only after 3 days of post infection (Armitage *et al.*, 2011). Here, our study demonstrates that in non-hepatocyte models (A549, 293T cells, and mouse lung tissues), IAV consistently downregulates HAMP expression. Of note, we used a low-dose IAV infection model because lncRNA-155KO mice are highly sensitive to IAV challenge, as discussed in our previous work (Rai *et al.*, 2022). Thus, the findings of this study expand our understanding of virus-induced HAMP expression to non-hepatocyte cell systems, highlighting both cell-type specificity and the distinct regulatory roles of lncRNA-155 in the host response to IAV infection.

Systemic HAMP is predominantly synthesized by hepatocytes (Bonitz *et al.*, 2025). In hepatocytes, IL-6 has been shown to upregulate HAMP expression by activating the JAK-STAT3 signaling cascade (Lee *et al.*, 2005; Armitage *et al.*, 2011; Navidifar *et al.*, 2025). More importantly, this IL-6 response is not autonomous and requires a functional BMP-SMAD pathway; the absence of SMAD4 or a mutated BMP-response element in the HAMP promoter abolishes IL-6-induced transcription (Armitage *et al.*, 2011; Krepuska *et al.*, 2024; Bonitz *et al.*, 2025). Moreover, cytokines such as IL-1, IL-6, TNF-α, and IFN-γ do not regulate HAMP expression in alveolar macrophages (Nguyen *et al.*, 2006). Conversely, hypoxia is a key and potent negative regulator of HAMP (Chaston *et al.*, 2011; Liu *et al.*, 2012). Given that IAV infection can induce hypoxic conditions across diverse cell types (Zhao *et al.*, 2020), and lncRNA155HG is a hypoxia-responsive lncRNA (Qiu *et al.*, 2024), further research is warranted to investigate the impact of hypoxia/lncRNA155HG axis in HAMP expression in non-hepatocyte cells.

This study identifies HAMP as a proviral factor during IAV infection, an effect that can also be recapitulated in the DNA virus PRV. Mechanistically, HAMP appears to enhance IAV replication by impairing IAV-induced innate immune responses. Indeed, HAMP has immunomodulatory functions, including cytokine modulation, which extends beyond its canonical role in the regulation of iron homeostasis (Zhang and Rovin, 2013; Michels *et al.*, 2015; Frost *et al.*, 2021; Krepuska *et al.*, 2024). This is evidenced by a recent preclinical study showing that HAMP disrupts immune responses to both vaccination and infection in mice, including immunity against influenza viruses (Frost *et al.*, 2021). Moreover, a murine model study showed that HAMP knockout induces hypercytokinemia, while HAMP pretreatment rescues HAMP knockout mice from LPS-induced toxicity (De Domenico *et al.*, 2010). Building on our previous finding that the MiR155HG gene product, i.e. lncRNA-155, potentiates antiviral innate immunity (Rai *et al.*, 2022), the present study demonstrates HAMP as a suppressor of lncRNA-155-potentiated antiviral responses.

This study showed that overexpression of lncRNA-155 reduced HAMP levels both at baseline and during IAV infection, indicating that lncRNA-155 regulates HAMP expression. In certain eukaryotic models, genome-wide identification of antisense lncRNAs has shown long noncoding natural antisense transcripts (lncNATs) complimentary to HAMP (Ali and Salem, 2022). HAMP has also been reported to be trans-regulated by many lncRNAs (Ali and Salem, 2022). A recent study observed that lncRNA GAS5 level was inversely correlated with HAMP expression (Al-Sanabra *et al.*, 2025). It remains unclear whether lncRNA-155 directly targets HAMP or acts indirectly through an immune signaling pathway. While our findings identify HAMP as a negative regulator of type I interferon responses during IAV infection, some limitations must be acknowledged. The direct molecular mechanism by which lncRNA-155 regulates HAMP remains unresolved. Due to time constraints, we could not

perform promoter assays, RIP, ChIRP, or transcription factor mapping, and this mechanistic gap limits interpretation of the regulatory axis. Additionally, while some important experiments were validated in A549 cells, the extensive use of 293T cells; which are not ideal for immune or primary cell modeling; represents an additional limitation. We therefore emphasize that follow-up investigations in primary airway epithelial cells and other immune-relevant cell types, alongside mechanistic and iron-focused studies, are importantly needed to fully establish the biological significance of HAMP-mediated immunomodulation.

Similarly, how HAMP antagonizes the function of lncRNA-155 remains to be determined. As a secreted hormone, intracellular HAMP overexpression may mimic autocrine/paracrine signaling to disrupt iron homeostasis. Since many innate immune processes are iron-dependent, HAMP-induced iron sequestration could create an environment suboptimal for lncRNA-155. Alternatively, HAMP signaling via ferroportin may activate pathways that impair lncRNA-155 stability, localization, or protein-protein interactions. These possibilities warrant future investigation. Elucidating this regulatory interplay may help advance ongoing therapeutic studies involving HAMP, which has recently been the subject of increasing investigation in preclinical trials (Frost *et al.*, 2021; Kremyanskaya *et al.,* 2024).

Conclusions: This study identifies a novel regulatory circuit in which IAV infection induces a robust upregulation of HAMP in the host deficient of lncRNA-155. HAMP then functions as a proviral factor by suppressing the antiviral responses that are normally enhanced by lncRNA-155. This mutual inhibition forms a self-limiting feedback loop. Disruption of this loop via lncRNA-155 deficiency leads to excessive HAMP expression and consequent immunosuppression (Supplementary data), positioning HAMP as a promising target for antiviral therapy.

Acknowledgment: This work was supported by National Natural Science Foundation of China (U23A20235 and 32030110). We thank all the members of Prof. Chen laboratory for helpful discussions and assistance.

Generative AI statement: No generative AI or AI assisted figures and texts were used in the preparation of this manuscript.

Authors contribution: J-LC conceptualized and supervised the project. PS, KRR, QXZ, SCG, YMW, FXW, YXL, NS, and SH, designed and performed experiments. PS, KRR, QXZ, and YXL analyzed the data. PS and KRR wrote the original manuscript draft. J-LC, and SH reviewed and revised the manuscript. All authors read the article and approved the final manuscript.

Declaration of interests: The authors declare that they have no competing interests.

Data availability: Supplementary data supporting the findings of this study are available from the corresponding author upon reasonable request.

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