Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.086

RESEARCH ARTICLE

The Avian Host lncGVRP1 Encodes a Novel Micro-Peptide that Facilitates Influenza A Virus Replication by Suppressing Antiviral Interferon Response

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ARTICLE HISTORY (26-203)

Received: February 26, 2026
Revised: April 25, 2026
Accepted: April 26, 2026
Published online: April 29, 2026

Key words:
Influenza A Virus
Interferon response
LncGVRP1
Long non-coding RNA
Micro-peptide

A B S T R A C T

Host long non-coding RNAs (lncRNAs) are emerging as critical regulators of influenza A virus (IAV) pathogenesis. Still, the functional landscape of avian host lncRNAs remains largely unexplored. In this study, we identified a novel H9N2-induced transcript lncRNA, lncGVRP1, which serves as a conserved positive regulator of IAV replication across H9N2, H1N1, and H3N2 subtypes. In addition, we discovered that lncGVRP1 acts as a functional lncRNA containing a hidden open reading frame (ORF). This ORF encodes a novel 74-amino acid micro-peptide, named GVRP1-ORF. Functional rescue experiments demonstrated that the enhancement of viral activity by the lncGVRP1 is strictly dependent on the peptide it encodes. Overexpression of the GVRP1-ORF recapitulated the pro-viral effect achieved by overexpressing the lncRNA. In contrast, an ORF-deleted mutant entirely failed to promote viral replication. Mechanistically, lncGVRP1 facilitates viral propagation by significantly suppressing the host type I interferon (IFN) response and downstream interferon-stimulated genes (ISGs). Furthermore, transcriptome-wide analysis indicated that lncGVRP1 modulates critical cellular machineries, including FoxO signaling and lysosomal trafficking. Collectively, our findings reveal a unique mechanism in which a lncRNA-derived micro-peptide hijacks host immunity to support viral persistence. Moreover, this micro-peptide may serve as a potential therapeutic target against influenza infection.

To Cite This Article: Fan M, Liu Z, Zeng Y, Qiao Y, Wang C, Rushanov A, Zhang Y, Su J and Ping J, 2026. The avian host lncGVRP1 encodes a novel micro-peptide that facilitates influenza a virus replication by suppressing antiviral interferon response. Pak Vet J, 46(4): 899-909. http://dx.doi.org/10.29261/pakvetj/2026.086

INTRODUCTION

Influenza A virus (IAV) continues to pose a significant threat to global public health and the poultry industry (Anum *et al*., 2021; Uyeki *et al*., 2022). Among the circulating avian influenza viruses (AIVs), the H9N2 subtype has become endemic in poultry populations (Liang *et al*., 2024). Although H9N2 viruses are generally low pathogenic in birds, they cause substantial economic losses due to co-infections and egg production drops (Flerlage *et al*., 2021; Bin Aslam *et al*., 2024). More alarmingly, H9N2 viruses serve as a dynamic internal gene donor for emerging zoonotic reassortants, such as the fatal H7N9 and H10N8 viruses, highlighting their pivotal role at the animal-human interface (Li *et al*., 2022; Su *et al*., 2021). Despite extensive vaccination efforts, the continuous evolution and reassortment of H9N2 viruses necessitate a deeper understanding of the host-pathogen interactions that facilitate their persistent replication (Cargnin Faccin *et al*., 2024; Ingrao *et al*., 2024).

The host innate immune system, particularly the IFN signaling pathway, constitutes the first line of defense against viral invasion (Anjum *et al*., 2020; Wang *et al*., 2026). Upon infection, pattern recognition receptors (PRRs) detect viral components to trigger the expression of IFNs and downstream ISGs, establishing an antiviral state (Stegeman *et al*., 2025). Nevertheless, the IAV infection is dictated by the dynamic interplay between viral immune evasion strategies and host antiviral defense mechanisms (Kambara *et al*., 2014). IAVs have evolved sophisticated strategies to evade immune surveillance or even hijack host cellular machinery to support their own life cycle (Chen *et*

al., 2024; Su *et al*., 2024). Emerging evidence suggests that the precise magnitude of the immune response is critical; while a robust response clears the virus, dysregulated or suppressed IFN signaling can create a cellular microenvironment conducive to efficient viral propagation (Tripathi *et al*., 2015; Varghese *et al*., 2022).

Long non-coding RNAs (lncRNAs), defined as transcripts longer than 200 nucleotides with limited protein-coding potential, have emerged as versatile regulators of gene expression during viral infections (Kesheh *et al*., 2022). Host lncRNAs, such as NRAV and lnc-ISG20, have been identified as critical modulators that can either inhibit or promote IAV replication by fine-tuning the antiviral immune response (Ouyang *et al*., 2014; Duan *et al*., 2021). Recently, classical lncRNA research has been challenged by the groundbreaking discovery that many annotated lncRNAs contain small open reading frames (sORFs) capable of encoding functional micro-peptides (Ho *et al*., 2025). These hidden peptides represent a novel layer of proteomic diversity and have been shown to regulate essential biological processes, including metabolism and muscle regeneration (Rai *et al*., 2025; Shu *et al*., 2026). However, whether avian host lncRNAs can regulate influenza virus replication through such encoded micro-peptides remain a largely unexplored frontier.

In this study, we investigated the transcriptomic landscape of host lncRNAs during H9N2 infection and identified a novel upregulated transcript, lncGVRP1. We demonstrate that lncGVRP1 is a functional RNA that encodes a previously uncharacterized micro-peptide. Our findings reveal that lncGVRP1 acts as a potent negative regulator of the host type I IFN response, thereby facilitating the replication of H9N2 and other IAV subtypes. This work uncovers a distinct mechanism of viral immune evasion mediated by host lncRNA, providing new insights into the complexity of avian antiviral immunity.

MATERIALS AND METHODS

Cells and viruses: Chicken Embryo Fibroblast Cell (DF-1), Madin Darby Canine Kidney (MDCK) cells and Human Embryonic Kidney 293T cells (HEK 293T) were purchased from ATCC and were cultured in Dulbecco' s Modified Eagle' s medium containing 10% fetal bovine serum (FBS), 0.2% $NaHCO_3$, 100 μg/mL streptomycin, and 100 IU/mL penicillin at 37°C with 5% CO_2. All cells were preserved in our laboratory.

Influenza viruses A/WSN/33 (H1N1) and A/Hong Kong/1/68 (H3N2) were propagated in MDCK cells; The H9N2 strain used in this study was A/chicken/Anhui/LH99/2017, which was isolated from diseased chickens collected at a farm in Jiangsu Province. It was purified by three consecutive limiting dilutions in SPF chicken embryos, propagated once in SPF chicken embryos, and stored at -80 °C for later use. The full gene sequences of this strain have been deposited in the NCBI database, with accession numbers ranging from MH489448.1 to MH489455.1.

Antibodies, plasmid and siRNA: Anti-GAPDH (Proteintech); anti-MYC (Abclonal); anti-NP antibodies generated by our lab. The lncGVRP1 sequence was inserted into the pCDNA3.1 vector. The ORFs of lncGVRP1 were cloned into the expression vector pcDNA3.1-MYC. The lncGVRP1-ORF sequence used for polyclonal antibody production was cloned into the prokaryotic expression vector pCold-TF. All plasmid constructs were confirmed by sequencing. The small interfering RNA (siRNA) targeting lncGVRP1 were purchased from GenePharma (Shanghai, China). Sense: 5'- GCAGGUGUCAUGCAAAUGUTT-3'; Antisense: 5'- ACAUUUGCAUGACACCUGCTT-3'.

Transient transfection: Cells were transiently transfected with siRNA or DNA plasmids in 24-well plates using Lipofectamine™ 2000 Reagent (Invitrogen) according to the manufacturer's protocol. For functional analysis, cells were transfected with either overexpression plasmids (500 ng per well) or control vectors (500 ng per well), alongside siRNA (50 nM per well) in culture medium.

Sample preparation and total RNA isolation: DF-1 cells cultured in 6-well plates were subjected to mock infection or infected with H9N2 IAV at a multiplicity of infection (MOI) of 3 for 12 h. Subsequently, cells were harvested for RNA sequencing. Total RNA was extracted using the Total RNA Extraction Reagent (Vazyme, China) following the manufacturer's protocol.

Differential expression analysis: Differential expression analysis of lncRNAs with absolute fold change >2 and false discovery rate (FDR)-adjusted $P<0.05$ in comparisons between infected and control groups were classified as significantly differentially expressed.

Virus infection: DF-1 cells were cultured in 24-well plates, and were infected with H9N2 IAV and incubated at 37°C for 1 hour. Then the cells were washed with PBS buffer and replaced with fresh culture medium containing 1 μg/ml TPCK-trypsin and 0.3% BSA. Cell supernatants were collected 12, 24, and 36 hours post-infection. Viral titers were detected by plaque assay.

Plaque assay: The infectious titer of influenza virus was determined by plaque assay. Briefly, viral supernatants were serially diluted 10-fold in DMEM, and then inoculated onto MDCK cells grown to a dense monolayer. After incubation at 37°C for 1 hour, the cells were overlaid with 2 × concentrated DMEM containing 2% agarose and 1 μg/mL TPCK-trypsin. The culture plates were then inverted and incubated at 37°C. Plaque numbers were counted at 36-48 hours post-infection. Three biological replicates were set for each sample group, and the unit of viral titer was PFU/mL.

Western blotting: Cells were washed with PBS and lysed in RIPA lysis buffer. Supernatants were collected, and protein concentrations were quantified using the BCA assay, and separated by SDS-PAGE on 10-12% resolving gels. Proteins were electrophoresis transferred to 0.22μm PVDF membranes pre-wetted with methanol. Transfer conditions included wet transfer at 100 V for 90-120 min. Membranes were blocked with 5% non-fat milk in TBST for 1h at room temperature, followed by incubation with primary antibodies (1:1000) overnight at 4°C. After three

10 min TBST washes, membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 1h at room temperature. Following additional washes, protein bands were visualized using ECL substrate and detected by a chemiluminescence imaging system. GAPDH served as loading controls for normalization.

Quantitative real-time PCR (qPCR): RT-qPCR was performed with SYBR Green Premix on a Roche LightCycler 96 following the manufacturer's protocol. Relative RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH or U6 served as internal controls, and relative RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All primer sequences information were provided in Table 1.

Table 1: The primer sets for RT-qPCR

Gene (chicken)	Primers
Gallus-GAPDH	Forward: 5'- GAGGGTAGTGAAGGCTGCTG -3'
	Reverse: 5'- CACAACACGGTTGCTGTATC-3'
Gallus-U6	Forward: 5'- GATAATACTGGGGACAAGCAG-3'
	Reverse: 5'- CACCCAAATTCGTATGTCATCC-3'
LncGVRP1	Forward: 5'- TCCGTTCTGGTGCTTGGC-3'
	Reverse: 5'- CAACCGCTGCTTGCTCAC-3'
Gallus-IFN-α	Forward: 5'-CCAGCACCTCGAGCAAT-3'
	Reverse: 5'- GGCGCTGTAATCGTTGTCT -3'
Gallus-IFN-β	Forward: 5'-CCAGCTCCTTCAGAATACGG-3'
	Reverse: 5'-TGGCTGCTTGCTTCTTGTCC-3'
Gallus-Mx1	Forward: 5'- GCTGTTGCGATGCTGAACAA-3'
	Reverse: 5'- AACCAATTCTGGCCTGAGCA-3'
Gallus-OAS1	Forward: 5'- CAGCATCACCAGTCCGCGCTA-3'
	Reverse: 5'- GCGGGCATCCTGAATCACCT-3'
H9N2-NP	Forward: 5'-GGCAACGAACCCGATCGTG-3'
	Reverse: 5'-CCAGATCGTTCGAGTCGT-3'
ENSGALT00000	Forward: 5'- TCAGGGTGATTCCTTTGGAGC-3'
092760	Reverse: 5'- GCAGGAACCCACACGACTTA -3'
ENSGALT00000	Forward: 5'- GCCTGGGTGGCTATGAATGA-3'
101623	Reverse: 5'- AGACAGACACAGGCCGAGTA -3'
ENSGALT00000	Forward: 5'- GTCCAAATGCACGTTCCTCC-3'
103562	Reverse: 5'- ACAGAGTGGGTCCTGTAGGT -3'
MSTRG.15667.1	Forward: 5'- CAGTGATGGCTTTTGTCTTGTCT-3'
	Reverse: 5'- AGGTAAAACGGCCTACAAAGGT -3'
MSTRG.22911.3	Forward: 5'- ACACACCCACCCACCTACG-3'
	Reverse: 5'- CCGCTGCGCTATGATTGGTT-3'
MSTRG.23130.1	Forward: 5'- CGCTCCAATTTGCGCTCGTA -3'
	Reverse: 5'- AGCACAGAGTGCCGTGAGC-3'
MSTRG.28664.1	Forward: 5'- AGGGCGAAGTGAAGGAGACA-3'
	Reverse: 5'- TCTCGTGTCCCCGTTCTGG-3'

Immunofluorescence assays: DF-1 cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100. After incubated with primary and secondary antibodies, the images were acquired using a confocal microscope. (Nikon, Japan).

Cell fractionation: Nuclear and cytoplasmic fractions were isolated using the PARIS™ Kit following the manufacturer's protocol.

Preparation of polyclonal antibodies: The GVRP1-ORF sequence was chemically synthesized to produce an antigenic peptide, which was then conjugated to a carrier protein to enhance immunogenicity. The conjugate was thoroughly emulsified with an adjuvant, and mice were immunized multiple times. Blood samples were collected at regular intervals, and antiserum was isolated to determine antibody titers. After purification, polyclonal antibodies were obtained and subsequently subjected to specificity testing.

Statistical analysis: The data are shown as mean values ± standard deviations, all the data presented are representative results of at least three independent biological replicates. Statistics were analyzed by two-tailed Student's t test or one-way or two-way analysis of variance (ANOVA) as indicated. Significance was indicated by asterisks: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; "ns" indicates no significance.

RESULTS

Identification and functional landscape of avian host lncRNAs induced by H9N2 infection: To elucidate the impact of H9N2 AIV infection on the host non-coding transcriptome, we performed systematic identification and classification of lncRNAs in H9N2-infected and MOCK-control groups. Genomic characterization revealed that intergenic lncRNAs (lincRNAs) constituted the largest proportion of identified transcripts, and a substantial number of novel lncRNAs were predicted across all biotypes (Fig. 1A). Differential expression (DE) analysis demonstrated a profound shift in the lncRNA expression profile following H9N2 infection. A total of 1,985 DE lncRNAs were identified, characterized by a striking bias toward upregulation, 1,838 upregulated and 147 downregulated, suggesting that H9N2 infection predominantly triggers the activation of host non-coding genomic elements (Fig. 1B and 1C). To further explore the functional repertoire of these DE lncRNAs, we constructed lncRNA-mRNA regulatory networks and performed KEGG pathway enrichment analysis across three distinct regulatory modes: antisense, cis-, and trans-regulation. Targets of antisense lncRNAs were primarily enriched in fine-tuned signaling and secretory pathways, such as the cGMP-PKG signaling pathway and the inflammatory mediator regulation of TRP channels (Fig. 1D). This suggests that antisense lncRNAs may act as precision modulators of specific transmembrane signaling events during infection. Cis-acting lncRNAs predominantly modulated their neighboring genes involved in structural reorganization and localized immune recognition, specifically focusing on the regulation of the actin cytoskeleton and C-type lectin receptor signaling (Fig. 1E). Trans-acting lncRNAs exhibited the most robust and extensive enrichment profiles. The most significant pathways identified were cytokine-cytokine receptor interaction and viral protein-related immune responses (Fig. 1F). This highlights trans-regulation as the primary mechanism driving the massive "cytokine storm" and systemic antiviral defense programs.

In summary, H9N2 infection induces a complex lncRNA-mediated regulatory network where trans-regulation serves as the central driver of the systemic inflammatory response, while cis- and antisense regulations provide auxiliary support by maintaining cellular structural integrity and fine-tuning local signaling cascades.

Validation and characterization of the candidate lncRNA GVRP1: To validate the reliability of our transcriptomic data, we selected eight differentially expressed lncRNAs for experimental verification using RT-qPCR and RT-PCR. The expression patterns of these

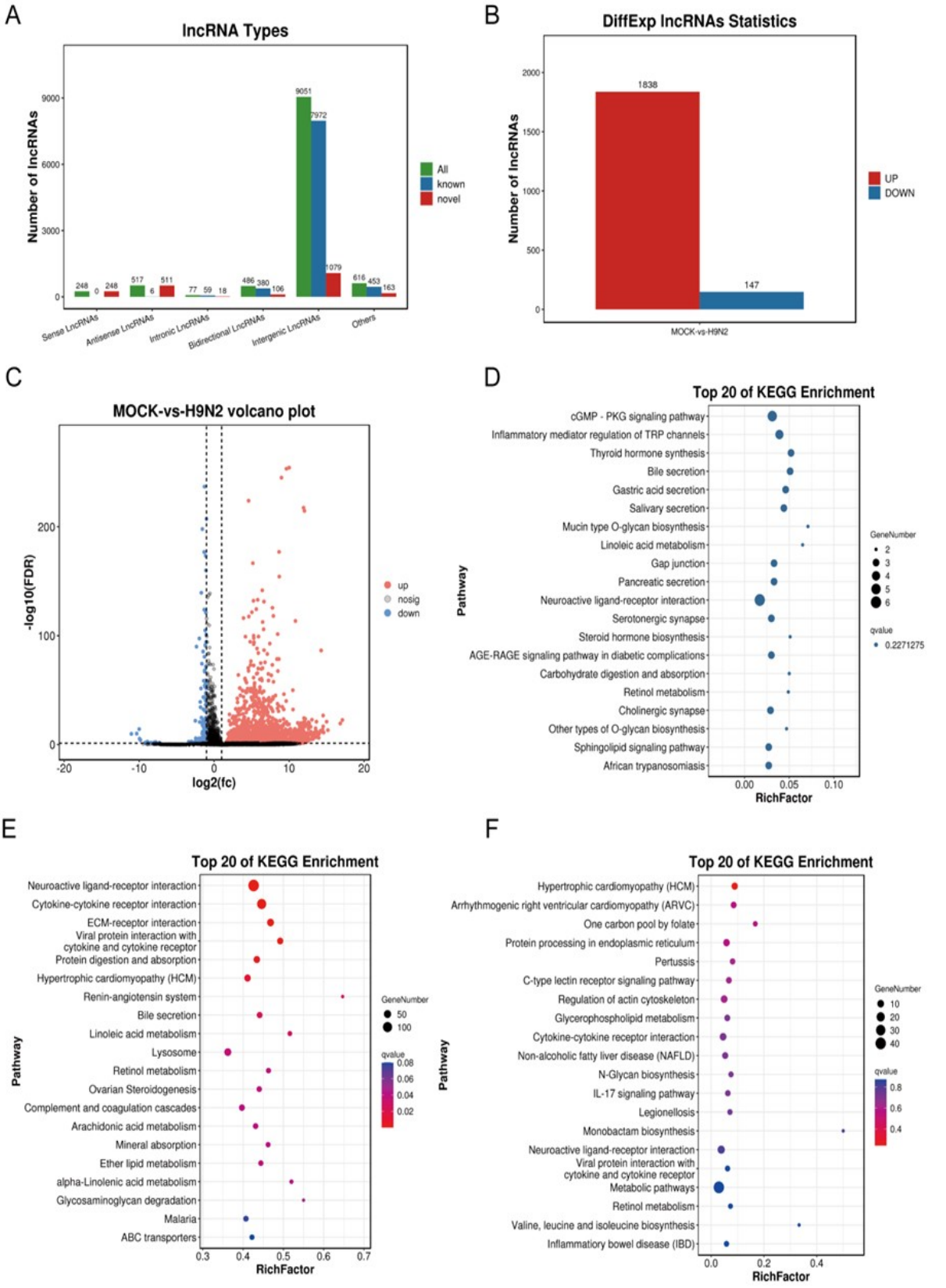


Fig. 1: Transcriptomic landscape of lncRNAs in H9N2-infected DF-1 cells. (A) Classification of identified lncRNAs based on their genomic location relative to protein-coding genes. (B) Statistical analysis of differentially expressed lncRNAs between H9N2-infected and MOCK groups. Red indicates upregulation, and blue indicates downregulation. (C) Volcano plot displaying the distribution of DE-lncRNAs. The vertical lines correspond to a 2-fold change threshold, and the horizontal line represents P<0.05. (D-F) KEGG pathway enrichment analysis of the antisense-, cis- and trans-target genes of DE-lncRNAs, highlighting top enriched pathways involved in the host response to viral infection.

candidates were highly consistent with the high-throughput sequencing results, with most showing significant upregulation following H9N2 infection (Fig. 2A, B). Among these, the transcript ENSGALT00000106466 exhibited the most dramatic increase in expression, with its expression level increased by approximately 40-fold. We then named it lncGVRP1 (Gallus virus-responsive proviral lncRNA 1) for further functional characterization. Detailed kinetic analysis revealed that the induction of lncGVRP1 is tightly regulated by the viral infection process. Specifically, lncGVRP1 expression increased in a time-dependent manner, reaching its peak at 36 hours post-infection (hpi). Similarly, its transcript levels displayed a dose-dependent response to increasing multiplicities of infection (MOI), significantly rising as the viral load intensified from 0.01 to 3 MOI (Fig. 2C, D). Next, we examined the lncGVRP1 expression levels in various types of avian cells, as well as its expression patterns following infection with IAV. The results showed that LncGVRP1 was detected in both LMH and CEF cells, and its expression levels increased after H9N2 infection at an MOI of 1 (Fig. 2E). Furthermore, lncGVRP1 was found to be significantly upregulated not only by H9N2 but also by other IAV subtypes, including H3N2 and H1N1, indicating that its induction is a conserved host response to IAV infection (Fig. 2F). To gain insight into its potential regulatory mechanism, we investigated the subcellular localization of lncGVRP1. Nuclear-cytoplasmic fractionation assays showed that while lncGVRP1 was predominantly localized in the nucleus under mock conditions, its cytoplasmic distribution significantly increased following H9N2 infection (Fig. 2G). This shift in localization suggests that lncGVRP1 may execute its functions through trans-regulatory mechanisms in the cytoplasm, such as modulating mRNA stability or protein translation. Finally, secondary structure prediction revealed that lncGVRP1 possesses complex hairpin and loop motifs, providing a structural basis for its potential interaction with viral or host proteins (Fig. 2H).

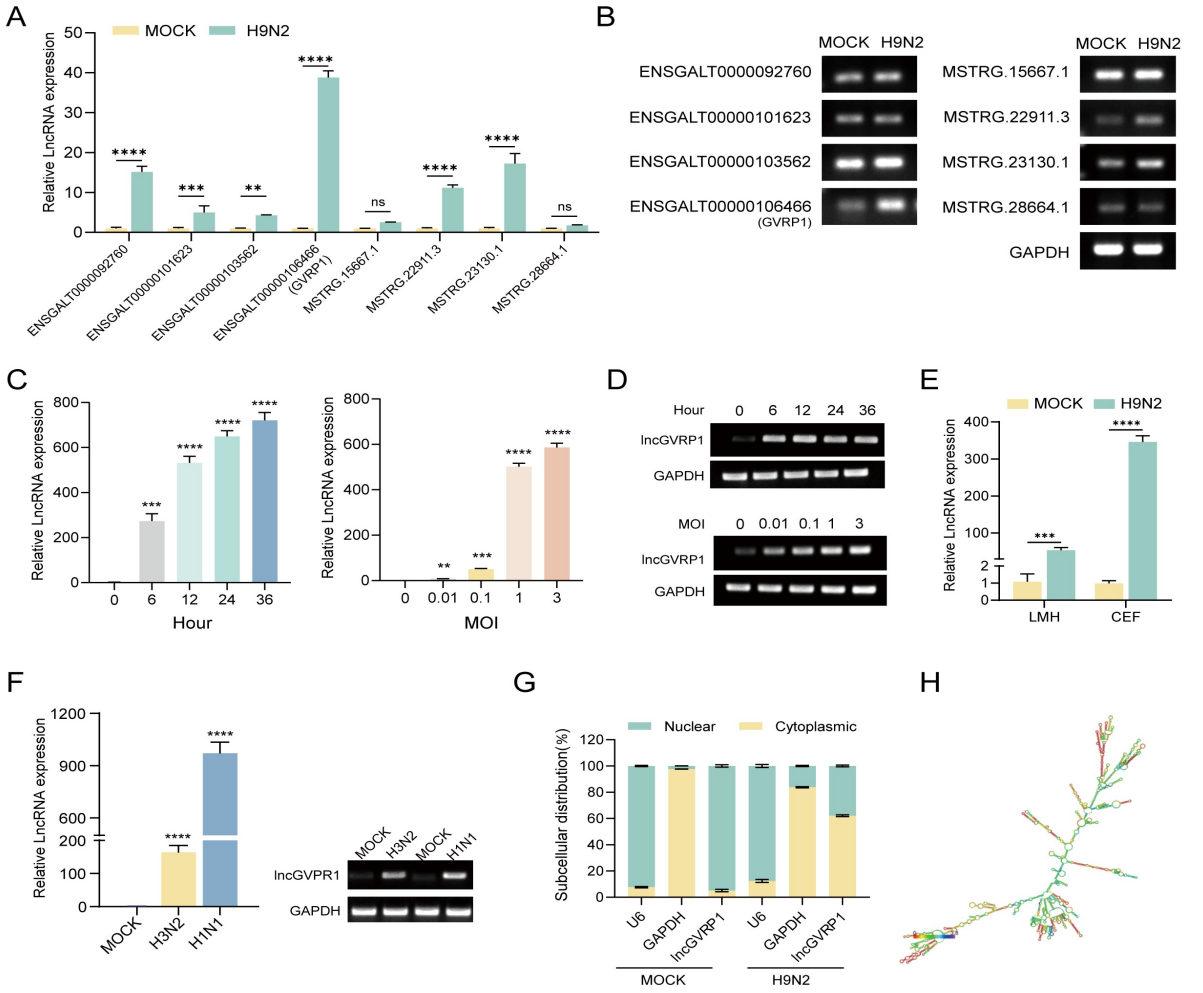


Fig. 2: Validation and characterization of the lead candidate lncGVRP1. (A) RT-qPCR validation of eight selected differentially expressed lncRNAs. (B) Semi-quantitative RT-PCR visualization of the candidate lncRNAs. (C) RT-qPCR validation of time-course expression analysis of lncGVRP1 in DF-1 cells infected with H9N2 (MOI=1) at 6, 12, 24, and 36 hpi (left). RT-qPCR validation of dose-dependent expression of lncGVRP1 at different multiplicities of infection (MOI=0, 0.01, 0.1, 1, and 3) (right). (D) RT-PCR validation of time-course expression analysis or dose-dependent expression of lncGVRP1 in DF-1 cells infected with H9N2 (E) RT-qPCR analysis of lncGVRP1 expression in LMH or CEF cells infected with H9N2 (MOI=1) at 24 h. (F) RT-qPCR (left) and RT-PCR (right) validation expression levels of lncGVRP1 upon infection with other influenza A virus subtypes (H3N2 and H1N1). (G) Subcellular fractionation assay followed by RT-qPCR determining the nuclear and cytoplasmic distribution of lncGVRP1. GAPDH and U6 were used as cytoplasmic and nuclear markers, respectively. (H) Predicted secondary structure of lncGVRP1 by RNAFold. Statistical differences between groups were marked according to one-way or two-way analysis of variance. Data are presented as the mean ± SD of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; "ns" indicates no difference.

LncGVRP1 acts as a positive regulator of IAV replication: To determine the biological function of lncGVRP1 during H9N2 infection, we performed gain- and loss- of function assays to assess its impact on viral replication. First, we constructed an overexpression model by transfecting cells with the pcDNA3.1-lncGVRP1 plasmid. RT-qPCR confirmed a robust upregulation of lncGVRP1 expression compared to the empty vector control (Fig. 3A). Compared with the control group, the virus titer was approximately 3-fold higher in DF-1 cells with lncGVRP1 overexpression (Fig. 3B, upper panel). Consistent with this, western blot analysis revealed elevated levels of the viral nucleoprotein (NP) (Fig. 3B, lower panel). Immunofluorescence assays (IFA) further corroborated these findings, displaying enhanced and more widespread viral NP signals in lncGVRP1-overexpressing cells compared to controls (Fig. 3C). Conversely, to verify the necessity of lncGVRP1 for viral replication, we silenced its expression using specific small interfering RNAs (si-lncGVRP1). Following effective knockdown, approximately 75% reduction, we observed a significant attenuation in viral replication (Fig. 3D). Plaque assay results indicated that knockdown of lncGVRP1 led to an approximately 2-fold reduction in viral titer. Western blot analysis yielded consistent results, revealing a decrease in the expression level of the NP protein in the si-lncGVRP1 group. (Fig. 3E). Furthermore, IFA imaging showed a substantial decrease in NP-positive cells in the knockdown group, confirming that endogenous lncGVRP1 is required for efficient H9N2 propagation (Fig. 3F). Finally, to investigate whether this pro-viral effect is conserved across other IAV subtypes, we examined the impact of lncGVRP1 modulation on H1N1 and H3N2 strains. Overexpression of lncGVRP1 consistently facilitated the replication of both subtypes (Fig. 3G), whereas it's silencing significantly impaired their viral titers (Fig. 3H). Collectively, these data demonstrate that H9N2-induced lncGVRP1 functions as a broad-spectrum positive regulator, promoting the efficient replication of multiple IAV subtypes in DF-1 cells.

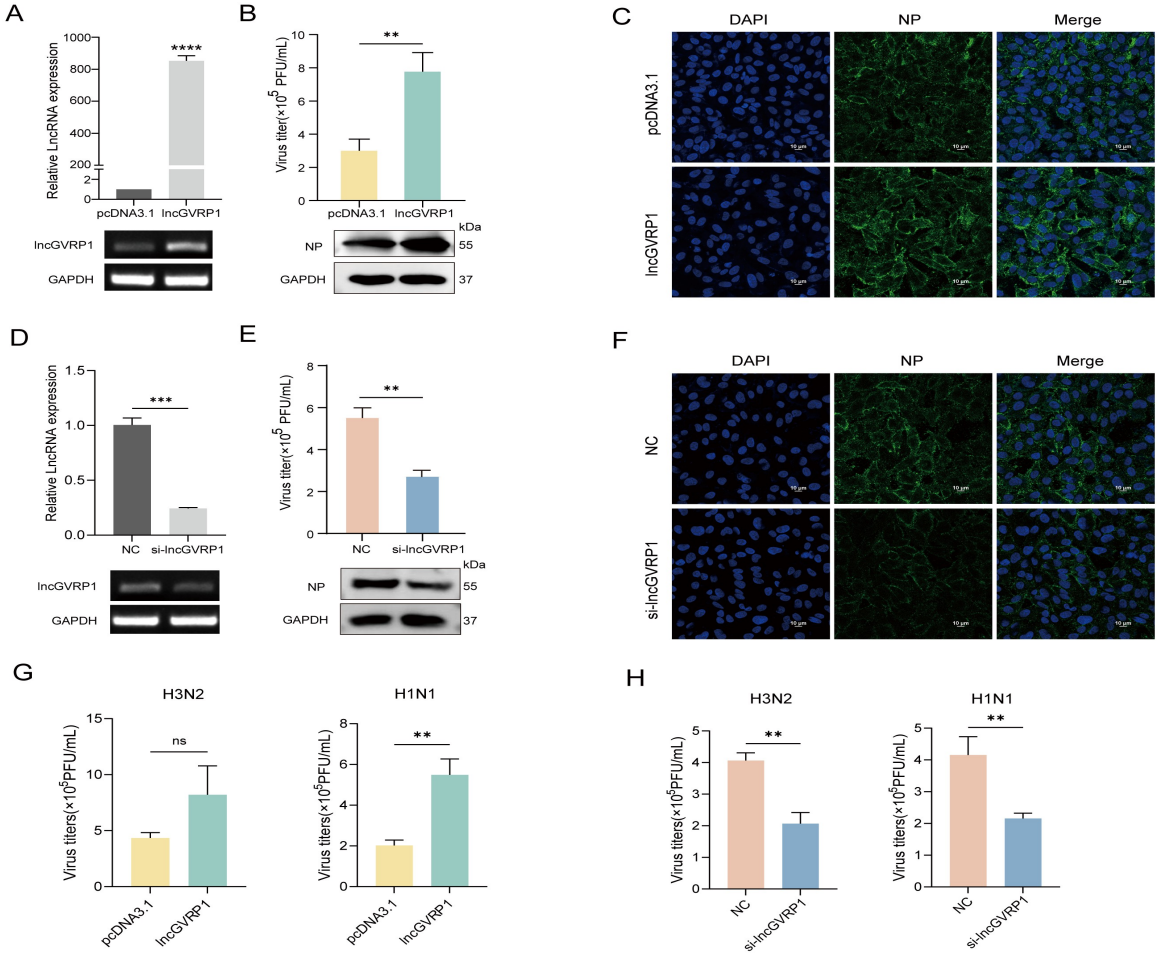


Fig. 3: LncGVRP1 acts as a positive regulator of influenza A virus replication. (A) Validation of lncGVRP1 overexpression efficiency in DF-1 cells transfected with pcDNA3.1-lncGVRP1 by RT-qPCR (up) and RT-PCR (down). (B) Determination of viral titers and NP protein expression levels by plaque assay (up) and western blot (down) in lncGVRP1-overexpressing DF-1 cells infected with H9N2 (MOI=1). (C) Immunofluorescence assay showing NP expression (green) in lncGVRP1-overexpressing DF-1 cells. Nuclei were stained with DAPI (blue). (D) Validation of lncGVRP1 knockdown efficiency using siRNA (si-lncGVRP1) by RT-qPCR (up) and RT-PCR (down). (E) Determination of viral titers and NP protein expression levels by plaque assay (up) and western blot (down) in lncGVRP1 knockdown DF-1 cells infected with H9N2 (MOI=1). (F) Immunofluorescence assay showing NP expression (green) in lncGVRP1 knockdown DF-1 cells. Nuclei were stained with DAPI (blue). (G) Plaque assay to determinate the impact of lncGVRP1 overexpression on the replication of H3N2 and H1N1 subtypes. (H) Plaque assay to determinate the impact of lncGVRP1 knockdown on the replication of H3N2 and H1N1 subtypes. Statistical differences between groups were marked according to one-way or two-way analysis of variance. Data are presented as the mean ± SD of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; "ns" indicates no difference.

LncGVRP1 negatively regulates the host type I interferon response: Having established that lncGVRP1 facilitates influenza virus replication, we next investigated whether this pro-viral effect was mediated through the modulation of the host innate immune system. We examined the expression of Type I interferons (IFNs) and key interferon-stimulated genes (ISGs) under both gain- and loss- of function conditions. To evaluate the inhibitory effect of lncGVRP1, cells were transfected with the pcDNA3.1-lncGVRP1 plasmid. While H9N2 infection naturally induced a robust immune response, the overexpression of lncGVRP1 suppressed the mRNA levels of IFN-α, IFN-β, and the downstream antiviral effectors Mx1 and OAS1 compared to the empty vector control (Fig. 4A). These results demonstrated that high levels of lncGVRP1 interfere with the potentiation of the interferon signaling pathway during viral challenge. Conversely, silencing endogenous lncGVRP1 (si-lncGVRP1) led to a heightened immune state. Upon H9N2 infection, cells with lncGVRP1 knockdown exhibited higher expression levels of IFN-α, IFN-β, Mx1, and OAS1 compared to the negative control (NC) group (Fig. 4B). Taken together, these findings reveal that H9N2 induced lncGVRP1 serves as a negative regulator of the IFNs response. By dampening the host's antiviral defenses, lncGVRP1 creates a cellular environment conducive to efficient viral propagation, providing a mechanistic explanation for its pro-viral activity observed in previous experiments.

LncRNA GVRP1 has potential coding capacity: To explore whether lncGVRP1 possesses coding potential beyond its regulatory role as an RNA molecule, we performed a systematic screening of its sequence for open reading frames (ORFs). Using the ORF Finder tool, we scanned the full-length sequence of lncGVRP1 and identified several potential ORFs. Following the criterion of selecting sequences with a predicted length exceeding 40 amino acids (aa), we identified eight candidate ORFs (ORF1-ORF8) distributed across both the sense and antisense strands. Notably, ORF1, located on the sense strand, was predicted to encode a 74 aa micro-peptide with a molecular weight consistent with functional proteins (Fig. 5A and Table 2). To experimentally verify the translation of these candidates, the eight predicted ORFs were cloned into MYC-tagged expression vectors and transfected into HEK293T cells. Western blot analysis using an anti-MYC antibody revealed that only ORF1 yielded a distinct protein band, confirming its translation in avian cells. The stability and expression of the ORF1-MYC fusion protein were further corroborated in independent replicates in DF-1 cells (Fig. 5B, C). To definitively confirm that ORF1 utilizes its own start codon for translation initiation, we employed a GFP reporter system in DF-1 cells. We constructed a fusion plasmid where the ORF1 sequence was placed upstream of a start-codon-deficient GFP gene (ORF-GFPmut-pcDNA3.1, ATGGTG to ATTGTT). Cells transfected with the wild-type ORF1-GFP construct exhibited robust green fluorescence, whereas the mutation of the ORF1 start codon (ATG to ATT) completely abolished the fluorescence signa (Fig. 5D). Next, using PYMOL 3D structural modeling of the ORF1-encoded peptide predicted a stable structure characterized by prominent α-helices (Fig. 5E), suggesting it may function as a bioactive molecule through protein-protein interactions. To further detect the endogenous expression of GVRP1-ORF1, we subsequently constructed a prokaryotic expression plasmid for GVRP1-ORF1, prepared the GVRP1-ORF1 expression peptide, and immunized mice with it to obtain polyclonal antibodies. Western blot result revealed the presence of endogenous protein in DF-1 cells, and protein expression levels increased following overexpression of GVRP1-ORF1. These results further confirm the presence of GVRP1-ORF1 (Fig. 5F). Collectively, these results demonstrate that lncGVRP1 is a bifunctional RNA that encodes a novel micro-peptide.

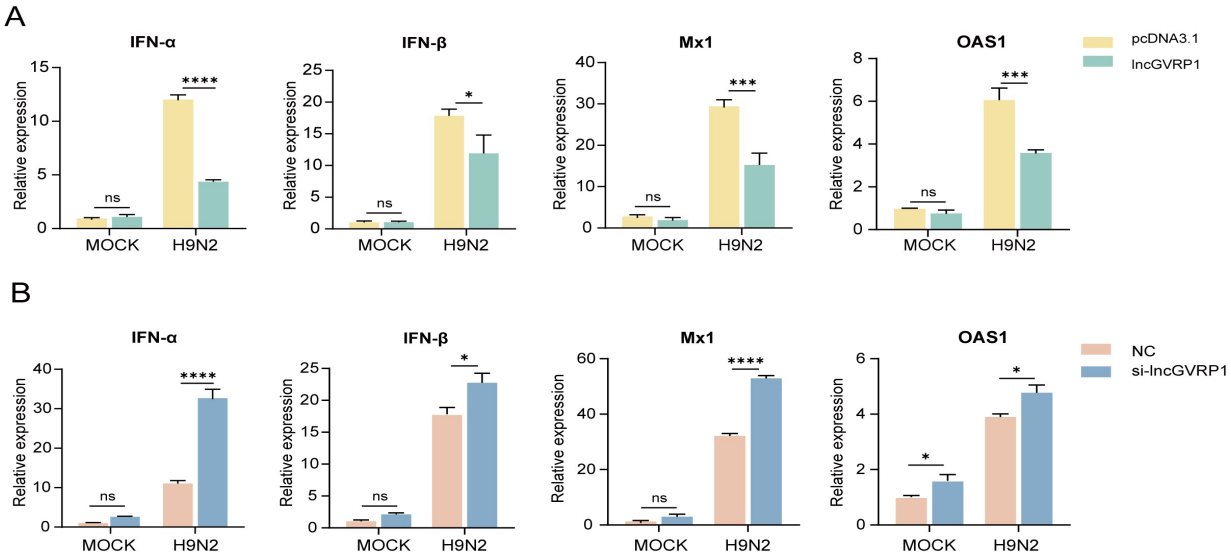
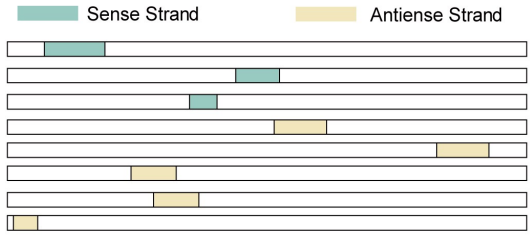
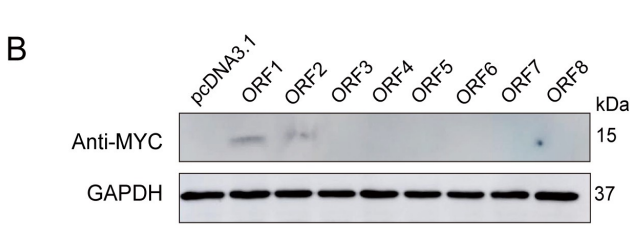


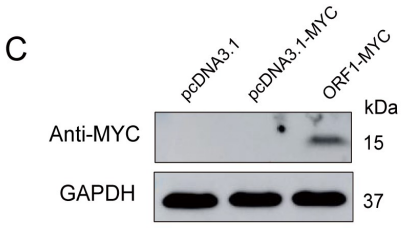
Fig. 4: LncGVRP1 negatively regulates the avian host type I interferon response. (A) RT-qPCR analysis of type I interferons (IFN-α, IFN-β) and interferon-stimulated genes (Mx1, OAS1) in DF-1 cells transfected with pcDNA3.1-lncGVRP1 or empty vector, followed by H9N2 infection. (B) RT-qPCR analysis of the same panel of immune genes in cells transfected with si-lncGVRP1 or negative control (NC) siRNA under H9N2 infection. Statistical differences between groups were marked according to one-way or two-way analysis of variance. Data are presented as the mean ± SD of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; "ns" indicates no difference.

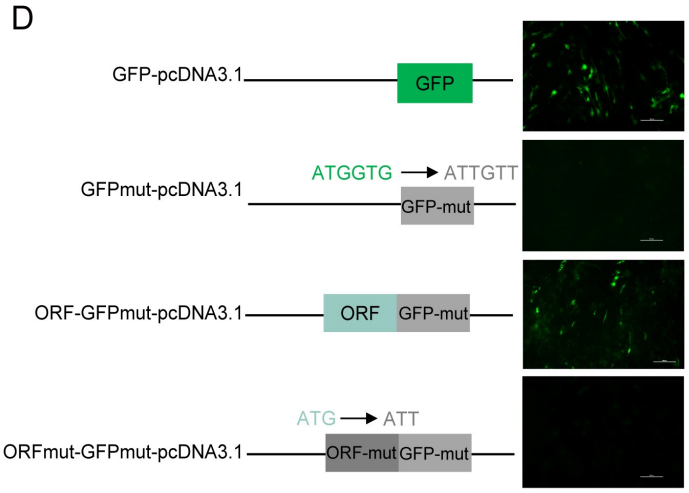
A

Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF1	+	1	289	513	225 | 74
ORF2	+	3	1524	1694	171 | 56
ORF3	+	3	1329	1475	147 | 48
ORF4	-	1	1837	1622	216 | 71
ORF5	-	2	2573	2354	210 | 69
ORF6	-	1	985	782	204 | 67
ORF7	-	3	1142	954	189 | 62
ORF8	-	1	232	86	147 | 48









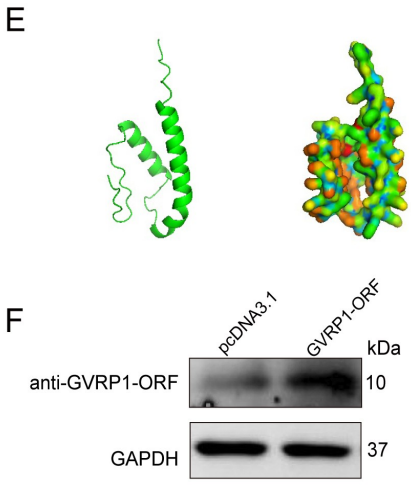


Fig. 5: LncGVRP1 encodes a novel functional micro-peptide. (A) Schematic representation and table of potential open reading frames predicted within lncGVRP1 using ORF Finder. (B) Western blot screening of HEK293T cells transfected with MYC-tagged constructs for all eight predicted ORFs. (C) Western blot of DF-1 cells transfected with MYC-tagged constructs for ORF1. (D) Evaluation of translation initiation using a GFP reporter system. (E) PYMOL predicted 3D structural model of the peptide encoded by ORF1 (GVRP1-ORF). (F) Overexpress pcDNA3.1 or GVRP1-ORF in DF-1 cells respectively, and use a polyclonal antibody against GVRP1-ORF to detect the expression of endogenous GVRP1-ORF protein.

Table 2: LncGVRP1-ORF amino acid sequence information

Gene	Sequence Information
LncGVRP1-ORF	MAIRFVAGSVMVPDLLLSPSQHHFCHSLLSTFVPGGIISVAQGWKLNKEISMFFSLWLRTWAYSCFLPKPSCFV*

The micro-peptide encoded by lncGVRP1 is essential for its pro-viral function: To determine whether the pro-viral effects of lncGVRP1 were mediated by the RNA transcript itself or its encoded micro-peptide, we performed a series of functional rescue and mutation assays targeting the identified ORF. We first synthesized the specific coding sequence, designated as GVRP1-ORF, and assessed its independent impact on viral replication. Overexpression of the standalone GVRP1-ORF in DF-1 cells resulted in a dramatic increase in H9N2 progeny titers (Fig. 6A). This promotion of viral replication was further confirmed by Western blot analysis, which showed that NP protein levels were higher in the GVRP1-ORF group compared to the control (Fig. 6B, C). Immunofluorescence microscopy revealed that the GVRP1-ORF peptide (green) localized predominantly within the cytoplasm, appearing to associate with viral replication (Fig. 6D). To definitively establish the necessity of this peptide, we constructed various mutants and truncations of lncGVRP1 (Fig. 6E). While both the full-length lncGVRP1 and the standalone GVRP1-ORF significantly boosted viral titers, the pro-viral activity of the lncRNA was completely abolished upon the deletion of the ORF region (lncGVRP1-△ORF), with titers returning to baseline levels (Fig. 6F). Furthermore, only the lncRNA fragment containing the ORF (lncGVRP1-A) retained the ability to facilitate viral replication, whereas the fragment lacking the ORF (lncGVRP1-B) showed no significant effect (Fig. 6F). Collectively, these results demonstrate that the micro-peptide GVRP1-ORF is the primary functional moiety through which lncGVRP1 exerts its pro-viral influence.

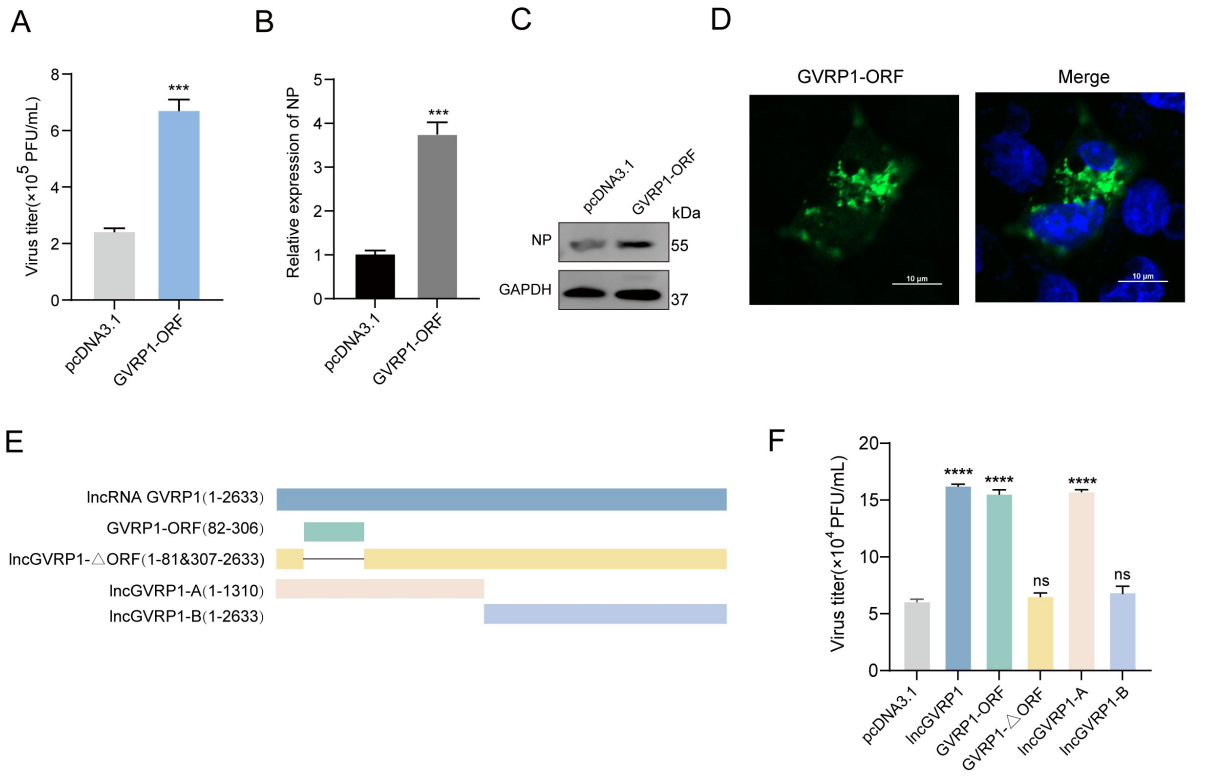


Fig. 6: The GVRP1-ORF micro-peptide is the functional moiety driving viral replication. (A) Plaque assay to determinate the impact of GVRP1-ORF peptide overexpression on the replication of H9N2 (MOI=1). (B) RT-qPCR analysis of viral NP mRNA expression in GVRP1-ORF overexpressing cells. (C) Western blot analysis of viral NP protein level in GVRP1-ORF overexpressing cells. (D) Immunofluorescence showing the subcellular localization of the GVRP1-ORF peptide (green). Nuclei were stained with DAPI (blue). (E) Schematic diagram of full-length lncGVRP1, the isolated GVRP1-ORF, the ORF-deletion mutant (lncGVRP1-△ORF), and truncated fragments (Fragment A and B). (F) Functional rescue assay measuring viral titers in cells transfected with the various constructs. Statistical differences between groups were marked according to one-way or two-way analysis of variance. Data are presented as the mean ± SD of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; "ns" indicates no difference.

Transcriptome-wide trans-regulatory landscape of lncGVRP1: To further elucidate the systemic influence of lncGVRP1 on the host cellular environment, we performed a comprehensive KEGG enrichment analysis of its trans-regulated target genes. The Sankey diagram reveals that these targets are distributed across three primary functional domains: Cellular Processes, Environmental Information Processing, and Genetic Information Processing, indicating that lncGVRP1 exerts broad-spectrum control over host homeostasis during H9N2 infection (Fig. 7). A significant number of targets were enriched in the FoxO signaling pathway, featuring key inflammatory and stress-response genes such as STAT3, IL6, and MAPK8. This confirms that lncGVRP1 acts as a central hub in modulating the host cytokine response. The Lysosome pathway emerged as a major focus, with multiple cathepsin family genes (e.g., CTSL, CTSD) being trans-regulated. This suggests lncGVRP1 may facilitate viral uncoating and cytoplasmic entry by optimizing lysosomal enzymatic activity. Enrichment in Protein processing in endoplasmic reticulum (ER), involving chaperones like HSPA5, highlights the role of lncGVRP1 in managing ER stress and supporting the massive synthesis of viral proteins. The Apoptosis pathway was also significantly targeted (e.g., FASLG, MAPK10), potentially reflecting the viral strategy to manipulate cell survival for maximal replication efficiency. In conclusion, these trans-regulatory profiles demonstrate that lncGVRP1 functions as a regulatory factor, may participate in critical host cellular mechanisms ranging from lysosomal trafficking to inflammatory signaling pathways, thereby mediating IAV replication.

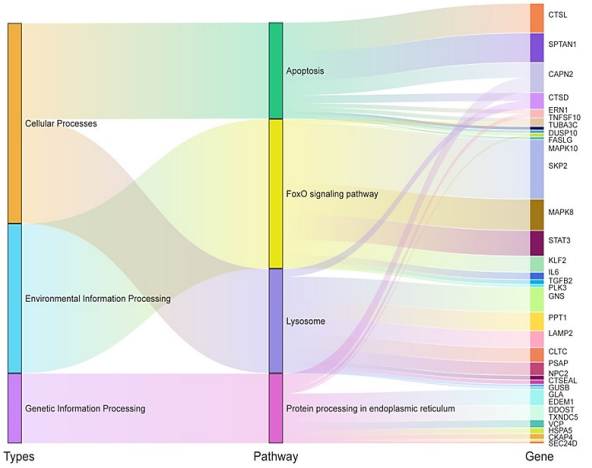


Fig. 7: Transcriptome-wide trans-regulatory network of lncGVRP1. Sankey diagram illustrating the functional enrichment of genes trans-regulated by lncGVRP1. The flow represents the mapping of target genes (right) to specific KEGG signaling pathways (middle) and their broader biological classifications (left).

DISCUSSION

The interaction between influenza A virus (IAV) and host cells is an intricate process involving extensive modulation of host factors and signaling cascades (Carter and Iqbal, 2024). In the past studies, long non-coding RNAs (lncRNAs) have transitioned from being dismissed as "genomic junk" to being recognized as pivotal components of the regulatory landscape (Statello *et al*., 2021). In this study, we identified a novel avian host transcript, lncGVRP1, which is significantly upregulated upon H9N2 infection. Our results demonstrate that lncGVRP1 functions as a bifunctional RNA that encodes a novel micro-peptide, designated GVRP1-ORF. Critically, lncGVRP1 facilitates the replication of multiple IAV subtypes, including H9N2, H1N1, and H3N2, by suppressing the type I interferon (IFN) response, thereby establishing a cellular microenvironment conducive to efficient viral propagation. These findings provide novel molecular evidence for the strategy by which avian influenza viruses hijack host factors to achieve immune evasion.

The widespread application of transcriptomic technologies has profoundly expanded our understanding of host responses during influenza infection. High-throughput RNA sequencing (RNA-seq) offers a holistic perspective for dissecting complex virus-host interactions (Briscoe *et al*., 2022). During IAV infection, a vast array of differentially expressed lncRNAs are generated, which often exhibit high degrees of tissue and species specificity (Liao *et al*., 2021). While classical differential analysis typically focuses on identifying antiviral factors, accumulating evidence suggests that viruses exploit host non-coding RNAs to suppress innate immunity or reprogram cellular metabolism, thereby creating a beneficial intracellular milieu for IAV replication (Vierbuchen and Fitzgerald, 2021). By performing deep sequencing of H9N2-infected DF-1 cells, we not only constructed a comprehensive response landscape of avian lncRNAs but also revealed that these differentially expressed lncRNAs are extensively involved in processes ranging from signal transduction to protein processing. This highlights the necessity of focusing on host factors that synergistically regulate IAV replication.

Influenza viruses have evolved sophisticated mechanisms to circumvent host innate immune surveillance. For instance, the viral non-structural protein 1 (NS1) acts as a primary antagonist by binding host proteins to block IFN induction and signaling, serving as a core determinant of viral pathogenicity (Gao *et al*., 2012; Wolf *et al*., 2021). Recent studies have highlighted the unique advantages of lncRNA-encoded micro-peptides in immunomodulation; due to their small size and rapid diffusion, they can swiftly bind to and modulate the activity of protein complexes (Rai *et al*., 2025). Host micro-peptides have been shown to block downstream antiviral signaling cascades by interacting with key sensors such as RIG-I or MAVS (Shi *et al*., 2023). This direct supportive role for host peptides in viral infection is consistent with findings for PCBP1-AS1 and other regulatory transcripts (Chi *et al*., 2024). Our data strongly indicate that overexpression of lncGVRP1 significantly reduces the mRNA levels of IFN-α, IFN-β, and ISGs. Nevertheless, the underlying mechanism responsible for this inhibitory effect remains poorly investigated, and its specific regulatory mechanism requires further elucidation. In addition, given that GVRP1-ORF localizes to the cytoplasm and significantly promotes viral Nucleoprotein (NP) expression, it is plausible that this peptide acts not only by modulating IFN molecules but also through direct physical interaction with viral components (such as NS1 or vRNP subunits) to assist in viral genome replication or assembly, a hypothesis that warrants further investigation.

Our transcriptome-wide trans-regulatory analysis revealed that the target gene network of lncGVRP1 is primarily enriched in the FoxO signaling pathway, lysosome, and endoplasmic reticulum (ER). Transcription factors such as FoxO1 have been proven to directly influence IAV replication efficiency by regulating the expression of interferon-stimulated genes (ISGs) (Wu *et al*., 2019), while the enrichment of STAT3 and IL6 in our study suggests that lncGVRP1 may participate in the virus-induced "cell cytokine storm". Furthermore, the significant enrichment of lysosomal pathway genes (e.g., CTSL) is noteworthy, as cathepsins serve as rate-limiting enzymes during membrane fusion and viral uncoating following entry (Benton *et al*., 2020; Rice *et al*., 2022). These findings suggest that lncGVRP1 serves as a pleiotropic regulator that potentially facilitates viral entry via the lysosomal pathway in the early stage while blunting the interferon response through its encoded micro-peptide in the later stages. Such coordinated regulation across multiple physiological systems underscores the complexity of host factors in viral pathogenesis. Nevertheless, several limitations of this study should be acknowledged. In vivo validation using chicken infection models was not performed in the present study, and all functional and mechanistic data were obtained from in vitro cell line models. Although DF-1 cells and primary chicken cells are a classic and widely used in vitro model for avian influenza virus research, they differ from primary chicken cells and intact poultry tissues in physiological characteristics, immune activation status and tissue microenvironment. Despite these constraints, the current in vitro evidence strongly supports our conclusion that lncGVRP1 and its encoded micro-peptide GVRP1-ORF promote influenza A virus replication by suppressing the type I interferon response. Further verification using animal infection models is needed in subsequent studies to better clarify the biological function of lncGVRP1 during H9N2 infection.

In conclusion, this study identifies and characterizes the central role of the avian host lncGVRP1 and its encoded micro-peptide GVRP1-ORF in IAV infection. By inhibiting the type I interferon response and potentially orchestrating trans-systemic cellular pathways, it functions as a potent driver of viral replication. These findings not only enrich the field of avian immunogenomics but also identify potential molecular targets for the development of novel antiviral therapeutics or the generation of disease-resistant poultry lines through genome editing.

Authors contribution: Jihui Ping conceptualized the study, secured funding support, and revised the

manuscript. Menglu Fan drafted the manuscript, performed experimental validation, and conducted data visualization. Zhiyuan Liu, Yiran Zeng, Yixuan Qiao and Chenbin Wang provided support for the preparation of experimental materials. Yijia Zhang, Asef Rushanov and Jaun Su offered critical advice on data analysis. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work.

Fundings: This work was supported by Xinjiang Uygur Autonomous Region Major Science and Technology Project-Xinjiang Animal Disease Prevention and Control System Quality Improvement Project [2023A02007] and Postgraduate Research and Practice Innovation Program of Jiangsu Province [KYCX25_1012]

Conflicts of interest: The authors declare no conflict of interest.

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