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Article

Keywords: DDX58, RIG-I-like receptor, Enterovirus A71, Gene Knockdown

Posted Date: October 28th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11209151/v1>

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Additional Declarations: No competing interests reported.

Effects of DDX58 Gene Knockdown on EV-A71 Replication and Modulation of RIG-I-Mediated Antiviral Signaling

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Supported by: Natural Science Basic Research Program of Shaanxi Province (No. 2025JC-QYXQ-046)

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Abstract

Objective: The aim of this study was to investigate the role of DDX58 (RIG-I) in EV-A71 replication and its impact on RIG-I-mediated antiviral signaling responses. **Methods:** DDX58 knockdown was achieved using siRNA transfection in RD cells, followed by EV-A71 infection. Viral replication and host antiviral responses were assessed by qRT-PCR and Western blotting. The secretion of interferons (IFN- α and IFN- β) and CXCL-10 was measured by ELISA. **Results:** DDX58 knockdown significantly reduced EV-A71 replication, as indicated by decreased viral RNA levels and VP1 protein expression. Additionally, the expression of key antiviral signaling molecules, including IRF3, IRF7, and CXCL-10, showed a transient increase at 24 hours post-infection, followed by a decline at 36 hours. Consistently, IFN- α and IFN- β levels exhibited a similar time-dependent pattern, with early elevation and subsequent reduction. **Conclusion:** These findings suggest that DDX58 may exert a context- and time-dependent regulatory effect on EV-A71 replication and antiviral signaling. The observed transient activation of downstream pathways despite DDX58 knockdown indicates potential involvement of compensatory innate immune mechanisms. Further studies are required to elucidate the underlying molecular interactions.

Key words: DDX58; RIG-I-like receptor; Enterovirus A71; Gene Knockdown

Core Tip: DDX58 knockdown unexpectedly suppresses EV-A71 replication while inducing transient antiviral activation, suggesting a non-canonical and time-dependent role of DDX58 in host–virus interactions, potentially involving compensatory innate immune mechanisms.

Forward

Enterovirus A71 (EV-A71), a member of the Picornaviridae family, along with Coxsackievirus, is among the primary pathogens responsible for hand, foot, and mouth disease (HFMD) and its associated neurological complications [1, 2]. Severe HFMD, particularly that caused by EV-A71, often leads to significant neurological damage, such as encephalitis, meningitis, and even death, especially in infants and young children [1, 3]. In 2023, more than 1.68 million HFMD cases were reported in mainland China, with an incidence rate of 0.6/1,000,000, making it one of the most prevalent infectious diseases in China. Over the past decade, from 2008 to 2024, nearly 28 million cases of HFMD were reported, with a total of 3,704 deaths [1, 4, 5].

The innate immune system serves as the first line of defense against EV-A71 invasion. The balance between antiviral immune responses and immune evasion is crucial in determining the outcome of infection [4]. Pattern-recognition receptors (PRRs) are central to the innate immune response, detecting viral RNA intermediates and activating a cascade of antiviral signals. Upon EV-A71 infection, PRRs such as RIG-I (retinoic acid-induced gene I), which is encoded by the DDX58 gene, recognize viral RNA species, particularly the 5'-triphosphate RNA and short double-stranded RNA structures, triggering the production of type I interferons (IFN-I) and inflammatory cytokines [6, 7]. This activation contributes to the establishment of an antiviral immune state in host cells and controlling viral replication.

Genetic variations in DDX58 are associated with increased susceptibility to severe viral infections, such as influenza, and play a pivotal role in antiviral immunity [11, 12]. In the context of EV-A71 infection, RIG-I activation initiates an inflammatory response, facilitating the release of cytokines such as interferons, which contribute to the suppression of viral replication [7]. Notably, studies

have indicated that low expression of DDX58 is significantly correlated with more severe cases of HFMD, highlighting its important role in regulating the severity of the disease [13]. However, whether DDX58 exerts context-dependent or non-canonical regulatory effects during EV-A71 infection remains unclear.

Thus, the proper regulation of RIG-I signaling, particularly through downstream factors such as IRF3 and IRF7, is essential for mounting effective antiviral responses. However, excessive or dysregulated activation of this pathway may also contribute to inflammatory damage and disease progression [14, 15]. Notably, whether DDX58 exerts context-dependent or non-canonical regulatory effects during EV-A71 infection remains unclear. Therefore, this study aims to investigate the role of DDX58 in regulating EV-A71 replication and modulating the RIG-I signaling, focusing on its dynamic impact on antiviral immune responses. These findings may provide new insights into host-virus interactions and reveal potential regulatory mechanisms beyond the classical antiviral paradigm.

Materials and Methods

1.1 Cell Lines and Virus Strain

Human rhabdomyosarcoma (RD) cells were obtained from the Department of Microbiology and Pathogenic Biology at the Air Force Military Medical University (Xi'an, China). EV-A71 (strain 87-2008 Xi'an Shaanxi, GenBank accession no. HM003207.1) was obtained from the Xi'an Centre for Disease Control and Prevention (Xi'an, Shaanxi, China). The EV-A71 strain used in this study was previously isolated and characterized in the laboratory and stored at -80°C until use.

1.2 Reagents

RNA extraction was performed using the E.Z.N.A.[®] MicroElute[®] Total RNA Kit (Omega Bio-tek, USA). qRT-PCR was conducted using the 2 $\times$ qRT-PCR SmArt Mix (SYBR Green) from Dr. D Medical Biological Technology Co., Ltd. (China). Reverse transcription was carried out with reagents from Shanghai Yisheng Bioengineering (China). Lipofectamine RNAi MAX Reagent (Invitrogen, USA) was used for cell transfection. Primary antibodies against RIG-I/DDX58 [EPR18629] (Abcam, UK) and β -actin (Proteintech, USA) were used for Western blotting. EV-A71 VP1 antibody was prepared and stored by the Department of Microbiology and Pathogenic Biology at the Air Force Military Medical University.

1.3 Cell Culture and Transfection

RD cells were cultured in complete medium (1% penicillin-streptomycin, 10% fetal bovine serum, and 90% DMEM) at 37°C with 5% CO_2 . Cells were passaged when they reached 70%-80% confluence. After being washed with PBS twice to remove serum, the cells were incubated with trypsin for 2-3 minutes, and the digestion process was terminated by the addition of complete medium. The cells were then resuspended in fresh medium and cultured further. For DDX58 knockdown, RD cells were transfected with DDX58-specific siRNA (final concentration: 20 μM) using Lipofectamine RNAi MAX Reagent. After 6 hours of transfection, the medium was replaced with fresh complete medium.

1.4 EV-A71 Infection

RD cells transfected with DDX58-specific siRNA were infected with EV-A71 at a multiplicity of infection (MOI) of 0.1. After 1 hour of infection, the cells were washed, and the medium was replaced with fresh complete medium. Cells were collected at 0-, 12-, 24- and 36-hours post-infection for subsequent analyses.

1.5 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cell samples using the E.Z.N.A.® Total RNA Kit, and cDNA was synthesized according to the manufacturer's instructions. qRT-PCR was performed to measure the mRNA expression levels of DDX58, EV-A71, MAVS, IRF3, IRF7, and CXCL-10 using SYBR Green chemistry. β -actin was used as the internal control. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

1.6 Western Blotting

Total protein was extracted from RD cells using RIPA lysis buffer, and protein concentrations were determined using a BCA assay kit. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk and incubated overnight at 4 °C with primary antibodies against RIG-I/DDX58 (Abcam, clone EPR18629; 1:1,000), MAVS (Proteintech, 14341-1-AP; 1:3,000), IRF3 (Affinity Biosciences, DF6895; 1:1,000), IRF7 (Proteintech, 22392-1-AP; 1:3,000), or EV-A71 VP1 (prepared in-house by the Department of Microbiology and Pathogenic Biology at the Air Force Military Medical University, Xi'an, China; 1:1,000). For MAVS, IRF3, and IRF7 analysis, β -actin (Affinity Biosciences, T0022; 1:20,000) served as the loading control. After incubation with appropriate HRP-conjugated secondary antibodies, bands were visualized using an ECL substrate (Servicebio, China).

1.7 ELISA

The culture supernatants were collected and centrifuged at 2,500 rpm for 5 minutes at 2–8 °C to remove cell debris. The clarified supernatants were used for ELISA. The OD450 values were immediately read using a microplate reader at 450 nm. The concentrations of IFN- α , IFN- β , and CXCL-10 in the clarified supernatant were measured using commercially available ELISA kits (Fine Biotech EH3252; Fine Biotech EH0485; Reed Biotech, RE1028H) according to the manufacturer's instructions.

1.8 Statistical Analysis

All experiments were performed in triplicate. Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using GraphPad Prism software. Differences between groups were evaluated using Student's t-test or one-way ANOVA, with $p < 0.05$ considered statistically significant.

Results

2.1 Verification of the Effect of DDX58 Knockdown

qRT-PCR and Western blot analyses confirmed that DDX58 expression was significantly reduced in RD cells transfected with siDDX58 at both 12 and 24 h post-transfection, with a more pronounced reduction observed at 24 h (Fig. 1). These results demonstrate efficient knockdown of DDX58 expression.

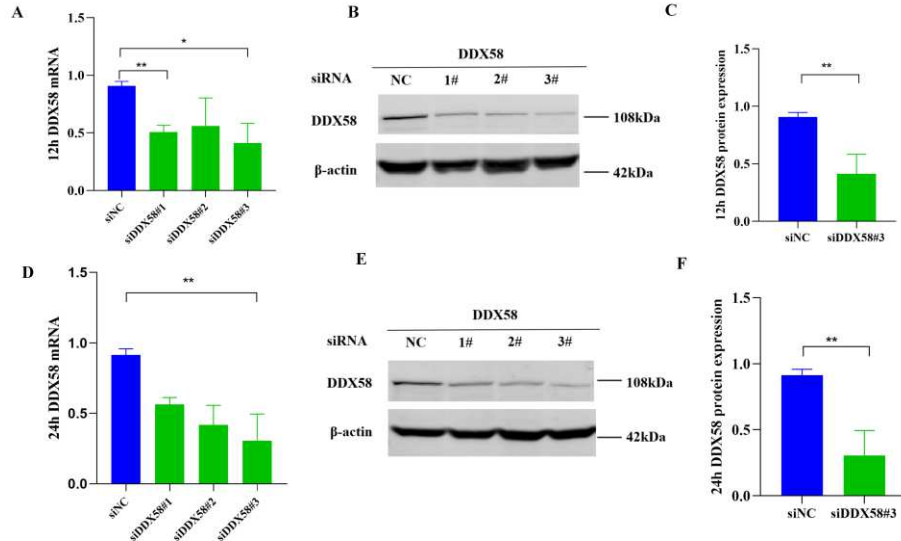


Figure 1. Verification of DDX58 knockdown efficiency in RD cells.

(A-C) DDX58 mRNA and protein expression at 12h post-transfection. (D-F) DDX58 mRNA and protein expression at 24 h post-transfection. Data are presented as mean $\pm$ SD. ns, not significant; * $p < 0.05$; *** $p < 0.001$.

2.2 DDX58 Knockdown Suppresses EV-A71 Replication

Following EV-A71 infection, both viral RNA levels and VP1 protein expression were significantly reduced in siDDX58-transfected RD cells compared with the siNC group at 12 and 24 h post-infection (Fig. 2). At both time points examined, EV-A71 mRNA levels in the siDDX58 group were consistently lower than those in the siNC group. Western blot analysis further confirmed that VP1 protein expression was markedly decreased in siDDX58-transfected cells compared with controls. These results consistently indicate that DDX58 knockdown suppresses EV-A71 replication.

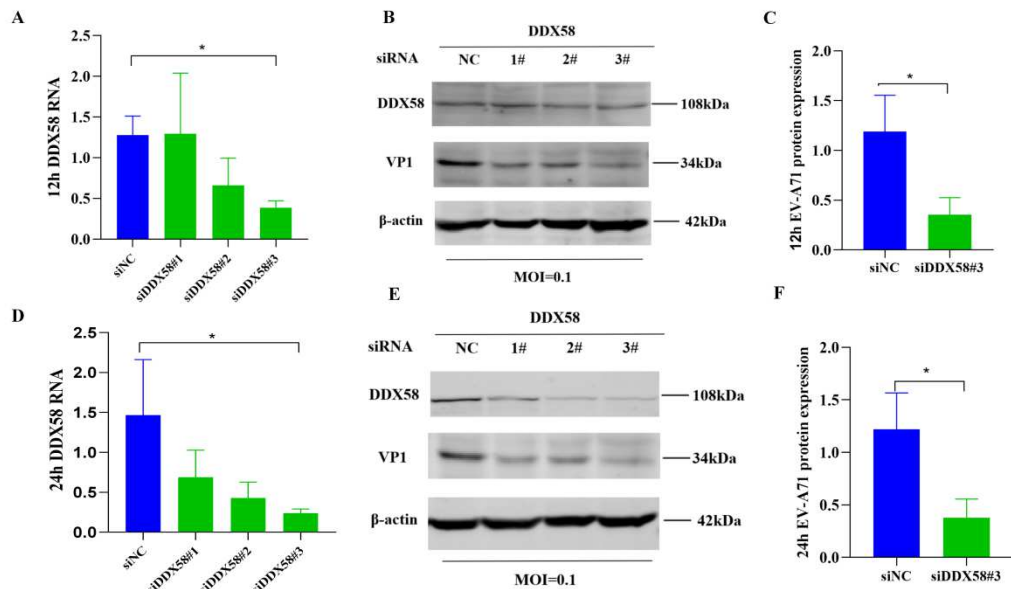


Figure 2. Effect of DDX58 knockdown on EV-A71 replication.

(A-C) EV-A71 mRNA and VP1 protein expression at 12 h post-infection. (D-F) EV-A71 mRNA and VP1 protein expression at 24 h post-infection. Data are presented as mean $\pm$ SD. * $p < 0.05$;

**** $p < 0.01$; *** $p < 0.001$.**

2.3 Impact of DDX58 Knockdown on IRF3, IRF7, IFN α , IFN β , and CXCL-10 Expression

Sequence 3 of siDDX58 was selected for subsequent experiments based on its highest knockdown efficiency. The observation period was extended to 36 h post-infection. MAVS expression gradually decreased over time in both groups, with no significant differences observed between the siNC and siDDX58 groups (Fig. 3A, F). IRF3 expression increased over time in the siNC group, whereas in the siDDX58 group it peaked at 24 h and declined at 36 h, with a significant difference observed at 36 h (Fig. 3B, G). IRF7 expression showed a similar trend in both groups, peaking at 24 h and decreasing thereafter, with significant differences between groups at 24 and 36 h (Fig. 3C, H). CXCL-10 mRNA levels were significantly lower in the siDDX58 group than in the siNC group at 24 and 36 h (Fig. 3D). ELISA analysis showed that IFN- α , IFN- β , and CXCL-10 levels increased over time in the siNC group. In contrast, in the siDDX58 group, these cytokines peaked at 24 h and decreased at 36 h, with significant differences observed at 24 and 36 h (Fig. 3I–K).

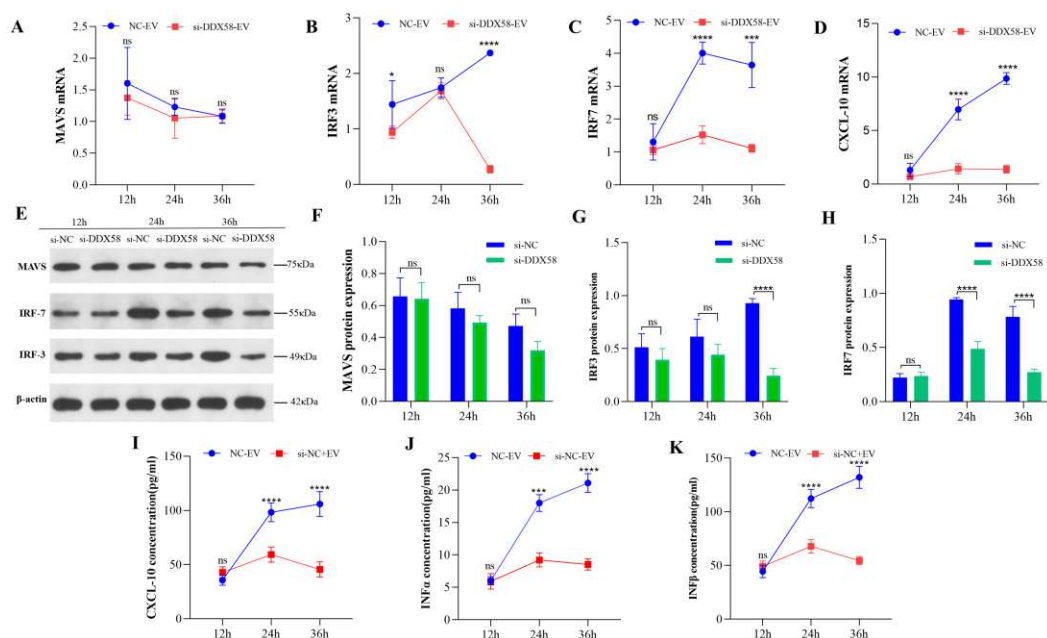


Figure 3 Effects of DDX58 knockdown on antiviral signaling pathways.

(A–D) mRNA expression levels of MAVS, IRF3, IRF7, and CXCL-10. (E–H) Protein expression levels detected by Western blot. (I–K) ELISA analysis of IFN- α , IFN- β , and CXCL-10 levels in culture supernatants. Data are presented as mean $\pm$ SD. ns, not significant; * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

Discussion

This study presents an investigation of the role of DDX58, also known as RIG-I, in regulating EV-A71 replication through the RIG-I-like receptor (RLR) signaling pathway. Our findings revealed an unexpected finding: knockdown of DDX58 not only attenuated antiviral signaling but also suppressed viral replication. This observation contrasts with the classical view that RIG-I functions predominantly as an antiviral sensor that restricts viral replication through induction of type I interferons [7, 8, 16].

Meanwhile, our results suggest that the role of DDX58 in EV-A71 infection may be context- and time-dependent. Although DDX58 knockdown led to a reduction in IRF3, IRF7, IFN- α/β , and

CXCL-10 expression, viral replication was concurrently decreased. This apparent paradox may reflect the involvement of compensatory innate immune pathways or alternative RNA sensing mechanisms, such as MDA5 or other pattern-recognition receptors, which can be activated in response to increased viral RNA burden under conditions of impaired RIG-I signaling [17, 18, 19]. In addition, the suppression of RIG-I signaling may alter the intracellular antiviral equilibrium, potentially reducing excessive immune activation while simultaneously limiting viral exploitation of host signaling pathways [16, 20]. Such a shift may create a transient cellular environment that is less favorable for efficient viral replication. Such compensatory activation may transiently enhance antiviral responses, as reflected by the peak of cytokine expression at 24 h post-infection, followed by a decline at later time points.

Interestingly, MAVS expression remained largely unchanged despite DDX58 knockdown, further suggesting that EV-A71-induced signaling may not rely exclusively on canonical RIG-I–MAVS activation. Instead, EV-A71 infection may engage parallel or redundant pathways to regulate downstream antiviral responses. Previous studies have shown that EV-A71 can modulate host innate immunity through viral proteases, such as 2Apro and 3Cpro, which target key signaling molecules to suppress interferon production [7, 21, 22]. Our findings extend this concept by suggesting that disruption of RIG-I signaling does not necessarily lead to enhanced viral replication, highlighting the complexity of host–virus interactions. This observation further suggests that EV-A71 may selectively engage non-canonical signaling routes or partially bypass classical RIG-I–MAVS signaling under certain conditions [23, 24].

Moreover, emerging evidence indicates that RIG-I signaling may exert dual roles depending on the stage of infection and the cellular context [16, 25]. In early infection, RIG-I activation promotes antiviral responses, whereas in later stages, dysregulated or excessive signaling may contribute to altered immune states that could influence viral persistence or host responses [16, 25]. In this study, the transient activation pattern of antiviral signaling observed in the siDDX58 group supports a dynamic regulatory model rather than a strictly linear antiviral pathway.

Consistent with previous clinical observations, alterations in DDX58, including DNA methylation and genetic polymorphisms, have been associated with disease severity in HFMD [13], highlighting the importance of RIG-I signaling in antiviral defense [16, 22]. However, our in vitro findings suggest that the relationship between DDX58 expression and viral replication is not strictly linear and may vary depending on cellular context and immune status. This discrepancy highlights the importance of considering temporal dynamics and system-level regulation when interpreting antiviral signaling pathways.

Despite these findings, several limitations should be acknowledged. First, this study was performed in an in vitro cell model, which may not fully capture the complexity of in vivo immune responses. Second, the precise molecular mechanisms underlying the observed non-canonical effects of DDX58 remain to be fully elucidated. Future studies should investigate the interaction between RIG-I and other RNA sensors, as well as the role of EV-A71 viral proteins in modulating these pathways. A deeper understanding of these mechanisms may provide new insights into host–virus interactions and identify potential targets for antiviral intervention.

Conclusion

In conclusion, this study highlights the complex role of RIG-I in regulating EV-A71 replication and immune responses. These findings suggest that RIG-I signaling is not only crucial for antiviral

immunity but also plays a dual regulatory role that can influence the outcome of infection. These insights provide valuable information for the development of therapeutic strategies aimed at modulating the RIG-I pathway to enhance antiviral immunity and mitigate the immune evasion mechanisms of EV-A71.

Acknowledgements and Author contributions

we thank for Ya-Ping Li, Guo-E Gou and Mei Li: Manuscript writing and study design; Guo-E Gou, Meng Zhang and Mei Li: Experimental operations; Xin-Yu Wang, Mu-Qi Wang, Wen Zhang, and Chen-Rui Liu: Data collection and statistical analysis; Ya-Ping Li and Song Zhai: Research supervision, manuscript review, and funding support.

Conflict of interest statement:

All authors declare that they have no conflicts of interest.

Funding

Natural Science Basic Research Program of Shaanxi Province (No. 2025JC-QYXQ-046)

Data availability

The original Western blot images are provided in the supplementary PDF file accompanying this article. Other datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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