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DDX56 cooperates with FMDV 3A to enhance FMDV replication by inhibiting the phosphorylation of IRF3

Shao-zu Fu, Wen-ping Yang, Yi Ru, Ke-shan Zhang, Yong Wang, Xiang-tao Liu, Dan Li*, Hai-xue Zheng*

State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, National Foot and Mouth Diseases Reference Laboratory, Chinese Academy of Agricultural Sciences, Lanzhou, China

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ABSTRACT

The components of foot-and-mouth disease virus (FMDV) interact with host cellular proteins to promote self-replication and evade the host immune response. Previous studies have shown that FMDV 3A, 2C and 2B proteins interact with host cellular proteins involved in FMDV replication. However, whether the other host proteins have an impact on FMDV replication is less understood. In this study, we identified DDX56 as a positive regulator of FMDV replication. DDX56 overexpression increased FMDV replication, whereas DDX56 knockdown had the opposite effect. DDX56 interacted and cooperated with FMDV 3A to inhibit the type I interferon by reducing the phosphorylation of IRF3. Moreover, the D166 site of DDX56 played a role in increasing FMDV replication and cooperating with FMDV 3A to inhibit the phosphorylation of IRF3. Additionally, knockdown of DDX56 or FMDV 3A results also showed that DDX56 cooperated with FMDV 3A to inhibit the phosphorylation of IRF3. These results suggest that the interaction between FMDV 3A protein and the host protein DDX56 is critical for FMDV replication.

1. Introduction

Foot-and-mouth disease virus (FMDV) belongs to the genus *Aphthovirus* in the family *Picornaviridae* [1,2]. The genome of FMDV is approximately 8400 nucleotides and contains a single open reading frame translated into a polyprotein. Upon cleavage by two virus-encoded proteases leader (L^{pro}) and $3C^{pro}$, the viral polyprotein is processed into precursors and individual structural as well as nonstructural proteins [2,3].

During FMDV infection, many host proteins can facilitate or inhibit virus replication by interacting with viral proteins. For instance, cellular beclin1 and vimentin bind with FMDV 2C, thereby promoting virus replication [4]. Conversely, dynactin subunit 3 (DCTN3) decreased FMDV replication by interacting with FMDV 3A in infected cells [5]. Moreover, FMDV VP1-induced suppression of type I interferon (IFN) was mediated by interacting with sorcin, which appeared to regulate the cellular response to viral infection [6]. Previous studies have showed that FMDV proteins regulate the innate immunity response by interacting with innate immunity molecules. For example, FMDV 3A and VP3 proteins interacted with virus-induced signaling adapter (VISA) and inhibited the production of IFN by decreasing the mRNA level of VISA [7,8]. In addition, FMDV VP3 protein interacted with Janus kinase 1 (JAK1) and inhibited the production of type II IFN by disrupting the assembly of JAK1 complex and degrading JAK1 [9,10]. Nevertheless, it remains unclear whether other host proteins interact with FMDV and affect the replication of FMDV.

In this study, we identified DEAD-Box Helicase 56 (DDX56) as a positive regulator of FMDV replication. We found that DDX56 interacted with FMDV 3A and interferon regulatory factor 3 (IRF3), and enhanced the capability of FMDV 3A to inhibit IRF3 phosphorylation. Our findings uncovered a new regulatory mechanism for DDX56-mediated FMDV replication.

2. Experimental procedure

2.1. Cell lines, viruses and antibodies

Human embryonic kidney (HEK293T) cells and porcine kidney (PK-15) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U penicillin/mL and 100 μg streptomycin/mL in a humidified incubator with 5% CO_2 at 37 °C. Rabbit monoclonal antibodies against DDX56, IRF3, p-IRF3

* Corresponding authors.
E-mail addresses: lidan@caas.cn (D. Li), haixuezheng@163.com (H.-x. Zheng).

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(Abcam) and mouse monoclonal antibodies against Flag (Sigma), HA (Origene), and β-actin (Sigma) were purchased from the indicated companies. Mouse anti-VP3 sera and rabbit anti-3A polyclonal antibody were prepared in our laboratory. Sendai virus (SeV) was previously described [11]. The FMDV type O strain O/BY/CHA/2010 was propagated in PK-15 cells, and the supernatant of infected cells was clarified and stored at −80 °C.

2.2. Constructs

IFN-β promoter and interferon stimulated response element (ISRE), the luciferase reporter plasmids, and mammalian expression plasmids for HA-tagged VISA, RIG-I, MDA5, TBK1, IKKε, IRF3, IRF3-5D and IRF7 were previously described [12]. Mammalian expression plasmids for porcine HA- or not-tagged DDX56 and its truncated mutants were constructed by standard molecular biology techniques. Mammalian expression plasmids for FMDV Flag-tagged VP0, VP1, VP2, VP3, 2B, 2C, 3A, 3C, and L proteins were constructed by standard molecular biology techniques.

2.3. Transfection and reporter assays

HEK293T cells were seeded in 48-well dishes and transfected by the standard calcium phosphate precipitation method. To normalize for transfection efficiency, the pRL-TK (Renilla luciferase) reporter plasmid was added to each transfection. Luciferase assays were performed using a dual specific luciferase assay kit (Promega). Firefly luciferase activities were normalized on the basis of Renilla luciferase activities. All reporter assays were repeated at least three times. Data shown are mean ± standard deviation (SD) from one representative experiment.

2.4. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from HEK293T or PK-15 cells using the TRIzol reagent (Invitrogen) and subjected to RT-PCR analysis to measure the expression of *3A*, *Ifnb1*, *Rantes*, *Isg56*, *Tnfa*, *Il8, Cxcl10* and *GAPDH* genes. Gene-specific primer sequences were as follows: Ifnb1, 5′-cagcaattttcagtgtcagaagct-3′ and 5′-cagtgactgtactccttggcctt-3′; Rantes, 5′-atgaaggtctccgcggcacgcct-3′ and 5′-ctagctcatctccaaagagttg-3′; Cxcl10, 5′-ggtgagaagagatgtctgaatcc-3′ and 5′-gtccatccttggaagcactgca-3′; Isg56, 5′-acggctgcctaatttacagc-3′ and 5′-agtggctgatatctgggtgc-3′; Il8, 5′-gagagtgattgagagtggaccac-3′ and 5′-cacaaccctctgcacccagttt-3′; human GAPDH, 5′-aaaatcaagtggggcgatgct-3′ and 5′-gggcagagatgatgacccttt-3′; 3A: 5′-ggcatggtccacgactctat-3′ and 5′-tcactgcatcatccaccatt-3′; and porcine GAPDH, 5′-actcactcttctacctttgatgct-3′ and 5′-tgttgctgtagccaaattca-3′.

Quantification of genome copies of FMDV was performed as previously described [13]. The viral genome copies in FMDV-infected (MOI = 0.1) PK-15 cells were quantified by RT-PCR. Total RNA of the FMDV-infected cells was extracted with TRIzol.

2.5. RNAi

Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper. RetroRNAi plasmid (Oligoengine Inc.). The following sequences were targeted for FMDV 3A: 3A-RNAi-#1: gccaacatgaggcagcaattg; 3A-RNAi-#2: ggcagcaattgaattctttga; 3A-RNAi-#3: gcatggtccacgactctatta. The following sequences were targeted for porcine DDX56: DDX56-RNAi-#1: cgaccagcccaggtcggtaa; DDX56-RNAi-#2: ctattgtgctcgagatatcc.

2.6. Overexpressed or knockdown cell lines

HEK293T cells were transfected with two packaging plasmids, pGag-Pol (10 μg) and pVSV-G (3 μg), empty vector, RNAi or overexpression retroviral plasmid (10 μg) by calcium phosphate precipitation. Cells were washed after 12 h transfection and new medium without antibiotics was added for an additional 24 h. The recombinant virus-containing medium was filtered and used to infect cells in the presence of polybrene (4 μg/mL). The infected cells were selected with puromycin (5 μg/mL) for 14 days before additional experiments.

2.7. Co-immunoprecipitation (Co-IP) and immunoblot analysis

HEK293T cells were lysed in 1 mL Nonidet P-40 lysis buffer (20 mM Tris·HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation, a 0.4-mL aliquot of lysate was incubated with 0.5–2 μg of the indicated antibody or control IgG and 25 μL of a 1:1 slurry of Protein-G Sepharose (GE Healthcare) for at least 2 h. The Sepharose beads were washed three times with 1 mL of lysis buffer containing 500 mM NaCl. The precipitates were fractionated on SDS-PAGE, and immunoblot analysis was performed.

2.8. Statistical analysis

The significance of differences between samples was assessed using an unpaired two-tailed Student's *t*-test. The variance was estimated by calculating the standard deviation (SD) and represented by error bars. All experiments were performed independently at least three times, with a representative experiment being shown.

3. Results

3.1. DDX56 potentiates FMDV replication

We first sought to assess the role of DDX56 in FMDV replication by evaluating viral replication in DDX56-overexpressed PK-15 cells. FMDV genomic copies were quantified by RT-PCR experiments, and the results showed that there was a significantly higher viral replication in DDX56-overexpressed cells than that in control cells (Fig. 1A). Likewise, we observed that the expression levels of FMDV structural protein VP3 and nonstructural protein 3A were markedly higher in DDX56-overexpressed PK-15 cells than those in control cells (Fig. 1B). These results indicate that overexpression of DDX56 results in the increased virus replication in PK-15 cells.

We next investigated the function of endogenous DDX56 in FMDV replication with two RNAi plasmids targeting DDX56. Both RNAi plasmids could markedly reduce the expression of endogenous DDX56 in PK-15 cells (Fig. 1C) and lead to the reduction of FMDV replication (Fig. 1D). Consistently, we found that knockdown of DDX56 inhibited the expression of FMDV VP3 and 3A proteins (Fig. 1E). Collectively, these results suggest that knockdown of DDX56 inhibits FMDV replication.

3.2. DDX56 overexpression inhibits virus-triggered induction of IFN-β

Innate immune response is an important mechanism that protects the host from microbial infection by triggering a series of signaling events that lead to induction of type I IFN [14,15]. We therefore determined whether DDX56 has an effect on virus-triggered induction of IFN-β. In reporter assays, DDX56 overexpression inhibited SeV-triggered activation of IFN-β promoter and ISRE in a dose-dependent manner in HEK293T cells (Fig. 2A–B). In RT-PCR experiments, we observed that SeV-triggered transcription of *Ifnb1*, *Tnfa*, *Il8*, *Cxcl10*, *Rantes* and *Isg56* genes were markedly inhibited in DDX56-overexpressed HEK293T cells in comparison with wild-type cells (Fig. 2C–H). Collectively, these results suggest that overexpression of DDX56 inhibits virus-triggered induction of IFN-β.

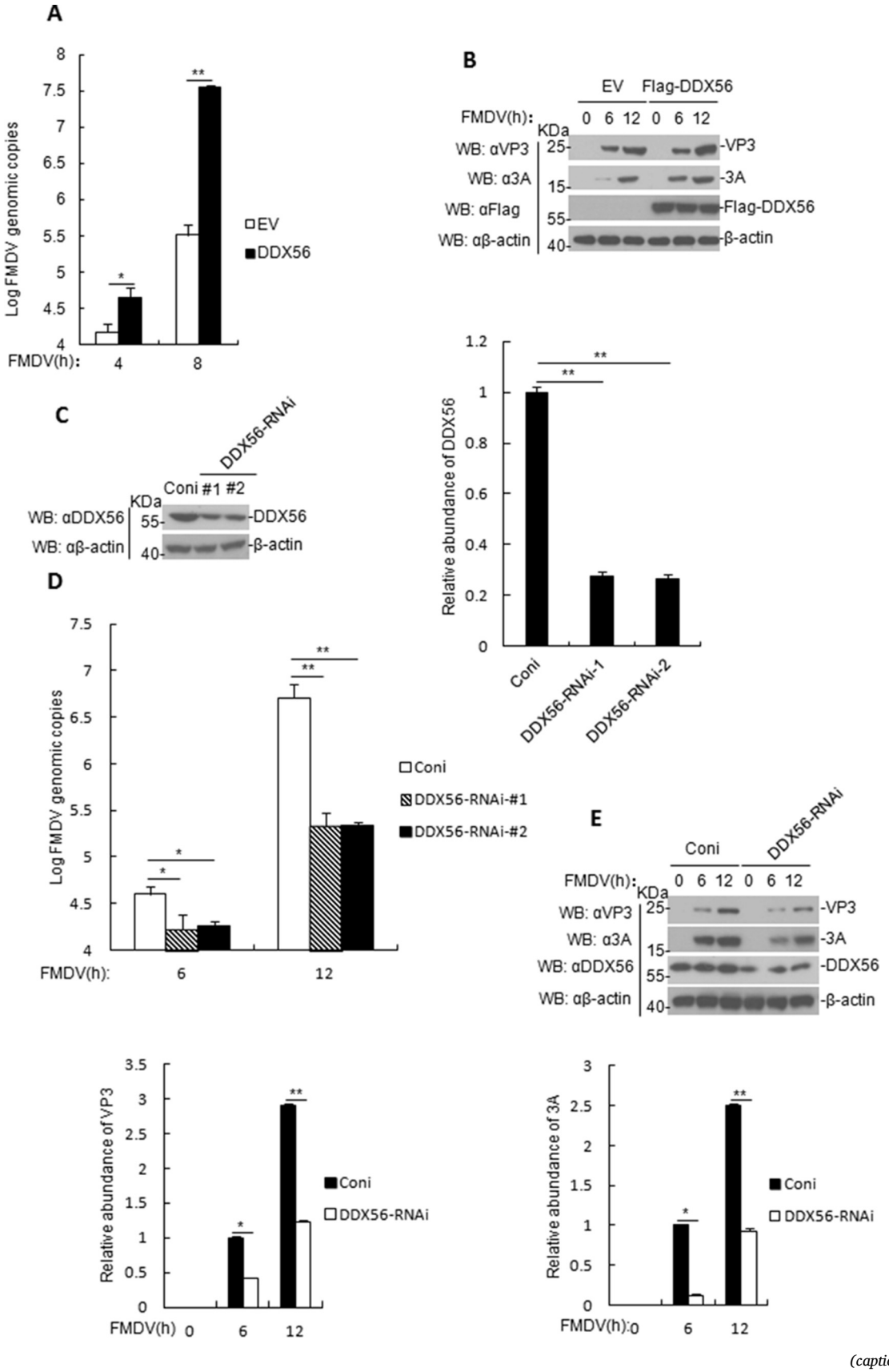

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Fig. 1. Effect of DDX56 on FMDV replication in DDX56-overexpressed PK-15 cells.
(A) Overexpression of DDX56 increases FMDV replication. DDX56-overexpressed PK-15 cells (2×10^5) were infected with FMDV (MOI = 0.1) at the indicated time points. FMDV replication was evaluated in PK-15 cells by RT-PCR. (B) DDX56 overexpression increases the expressions of FMDV VP3 and 3A proteins. Infection conditions were as described for panel A. FMDV VP3 and 3A proteins expression levels were analyzed by immunoblot analysis. (C) Immunoblot analysis of DDX56 protein levels in DDX56-knockdown PK-15 cells. The change of endogenous DDX56 was determined by densitometric analysis using ImageJ software and normalized to β-actin (right panel). (D) Knockdown of DDX56 inhibits FMDV replication. The experiments were similarly performed as in A. (E) Knockdown of DDX56 reduces the expressions of FMDV VP3 and 3A proteins. Experiments were similarly performed as in B. All of the above mentioned experiments were repeated three times. EV, empty vector; coni, control-RNAi; RT-PCR, Real-time polymerase chain reaction. *, $P < .05$, considered significant; **, $P < .01$, considered highly significant.

3.3. Knockdown of DDX56 potentiates Poly I:C-triggered induction of IFN-β

We next investigated the function of endogenous DDX56 in Poly I:C-triggered type I IFN production in DDX56-knockdown PK-15 cells. The reporter assays results showed that knockdown of DDX56 potentiated Poly I:C-triggered activation of IFN-β promoter and ISRE. (Fig. 3A–B). As shown in Fig. 3C–H, Poly I:C-triggered transcription of the *Ifnb1*, *Tnfα*, *Il8*, *Rantes*, *Cxcl10* and *Isg56* genes was increased in DDX56-knockdown PK-15 cells in comparison with control cells by real-time PCR assay. Collectively, these results suggest that knockdown of DDX56 potentiates the Poly I:C-triggered induction of IFN-β.

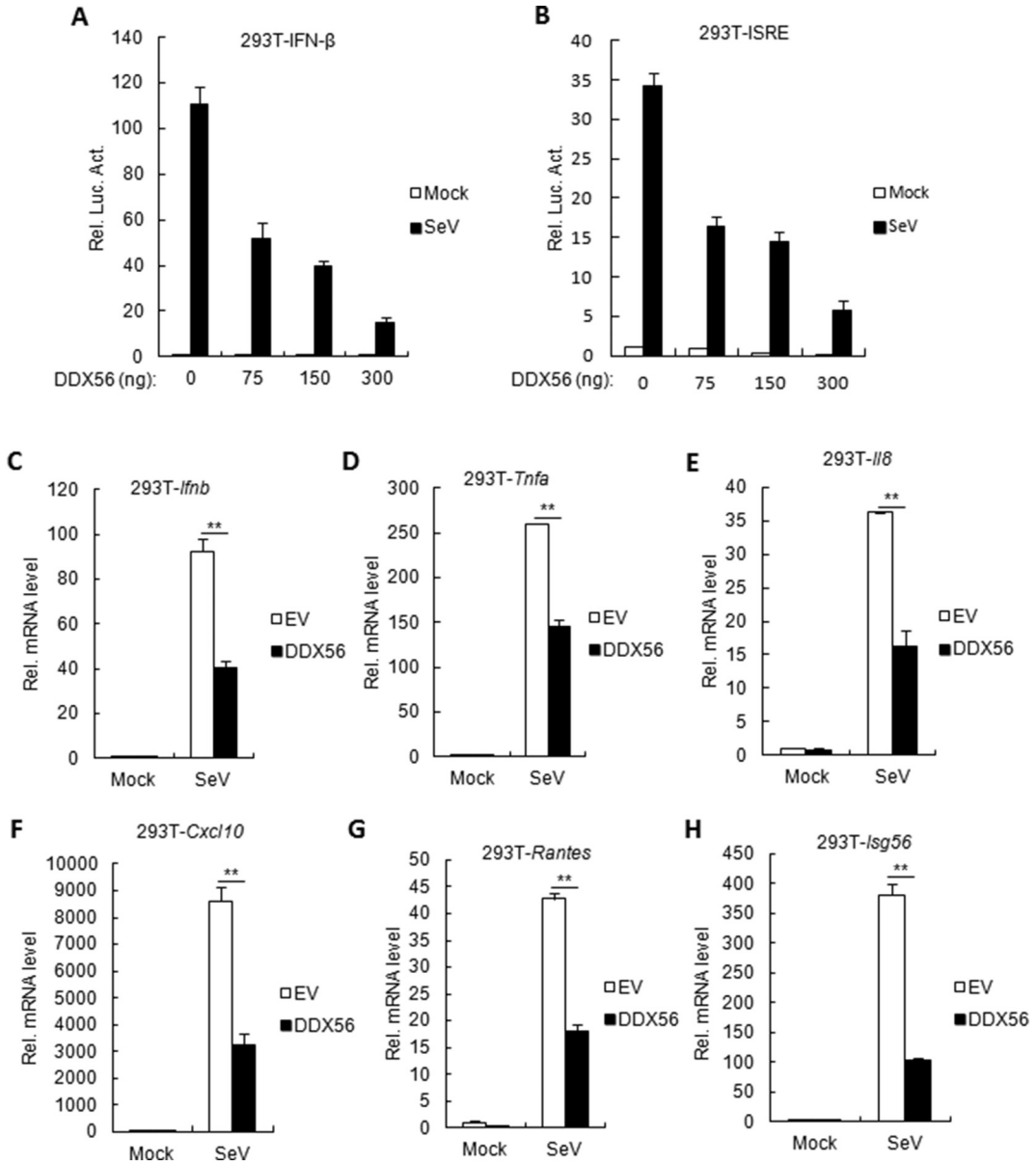

Fig. 2. DDX56 suppresses SeV-triggered signaling.
(A–B) Dose-dependent effects of DDX56 on SeV-triggered activation of IFN-β promoter and ISRE. HEK293T cells (1×10^5) were transfected with IFN-β reporter or ISRE (0.1 μg) and the indicated expression plasmids for 24 h. Cells were then infected with SeV or uninfected for another 12 h before luciferase assays. (C–H) Effects of DDX56 on SeV-triggered transcription of *Ifnb1*, *Tnfa*, *Il8*, *Rantes*, *Cxcl10* and *Isg56* genes. HEK293T cells (2×10^5) were transfected with expression plasmids containing DDX56 for 24 h. Cells were then uninfected or infected with SeV for another 12 h before RT-PCR analysis. All of the above mentioned experiments were repeated three times. EV, empty vector; Luc, luciferase; RT-PCR, Real-time polymerase chain reaction. **, $P < .01$, considered highly significant.

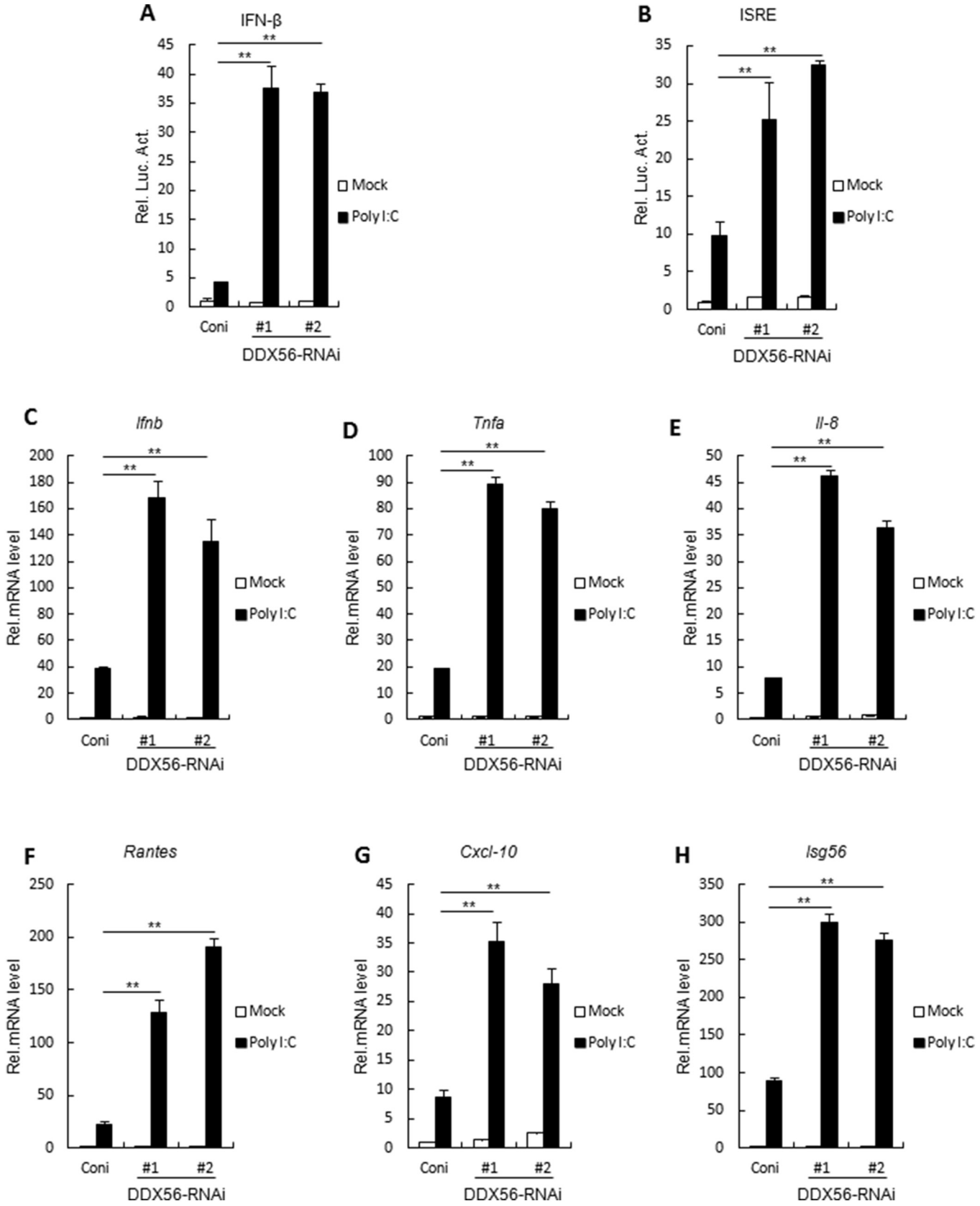

Fig. 3. Knockdown of DDX56 potentiates Poly I:C-triggered signaling.

(A–B) Effects of DDX56-RNAi plasmids on Poly I:C-triggered activation of the IFN-β promoter and ISRE. The stable DDX56-knockdown PK-15 cells (1×10^5) were transfected with the IFN-β promoter or ISRE (100 ng) for 24 h. Then the cells were treated or untreated with Poly I:C (1 μg/mL) for another 18 h before reporter assays. (C–H) Effects of DDX56-RNAi plasmids on Poly I:C-triggered transcription of *IFNB1*, *TNFα*, *Il8*, *Rantes*, *Cxcl10* and *Isg56* genes in PK-15 cells. The stable DDX56-knockdown PK-15 cells (2×10^5) were treated or untreated with Poly I:C (1 μg/mL) for 18 h before RT–PCR. All of the above mentioned experiments were repeated three times. Coni, control-RNAi; Luc, luciferase; RT-PCR, Real-time polymerase chain reaction. **, $P < .01$, considered highly significant.

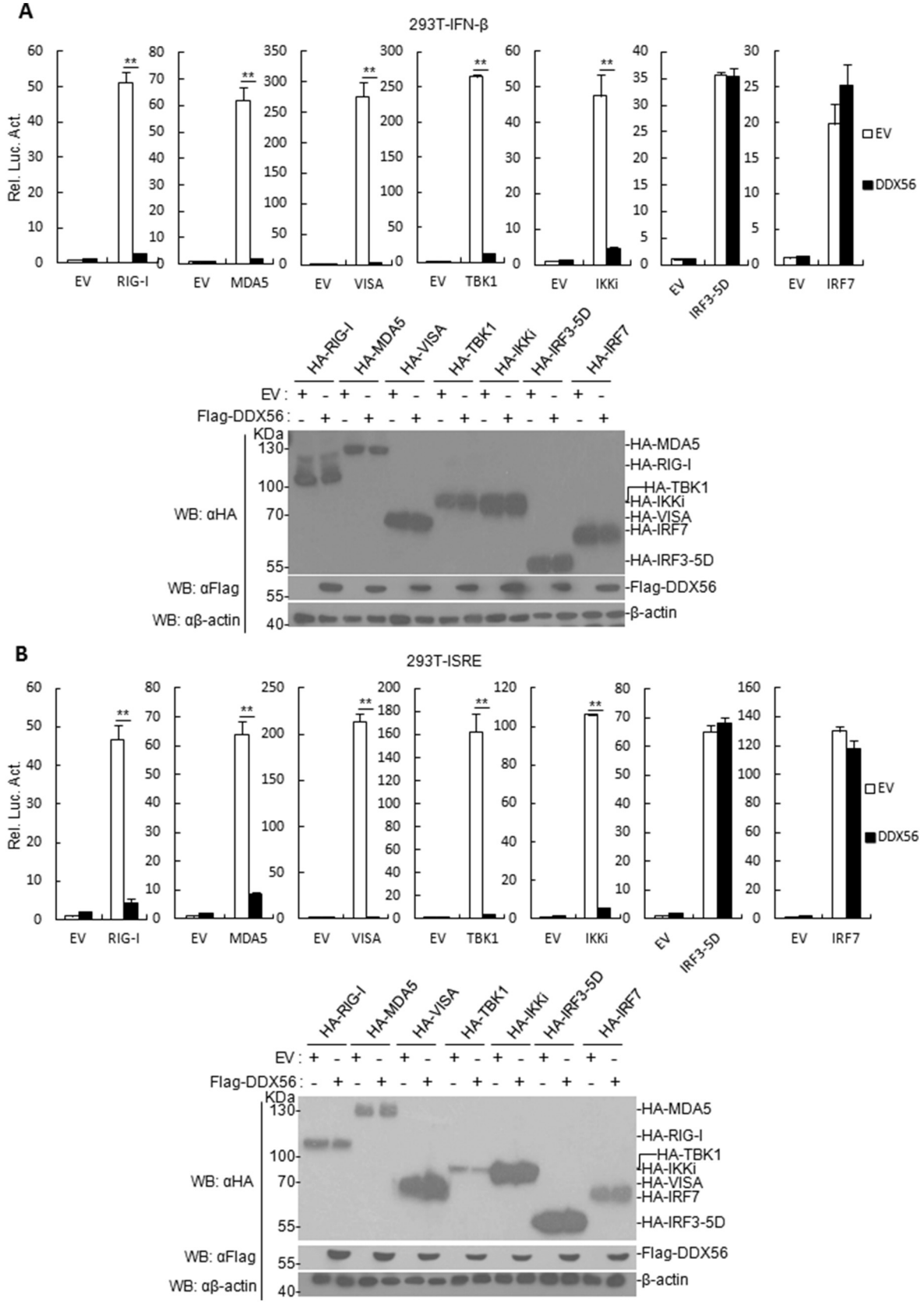

Fig. 4. DDX56 targets at or upstream of IRF3.

(A) Effects of DDX56 on IFN-β promoter activation by various signaling components. HEK293T cells (1×10^5) were transfected with the IFN-β promoter (0.1 μg), and the plasmids expressing DDX56 and the indicated plasmids (0.1 μg each) for 24 h and then luciferase assays were performed. Immunoblot analysis was performed with the indicated antibodies. (B) Effects of DDX56 on ISRE activation by various signaling components. The experiments were similarly performed as in A. All of the above mentioned experiments were repeated three times. Data are shown mean ± SD. EV, empty vector; Luc, luciferase; Rel, relative; SD, standard deviation. **, $P < .01$, considered highly significant.

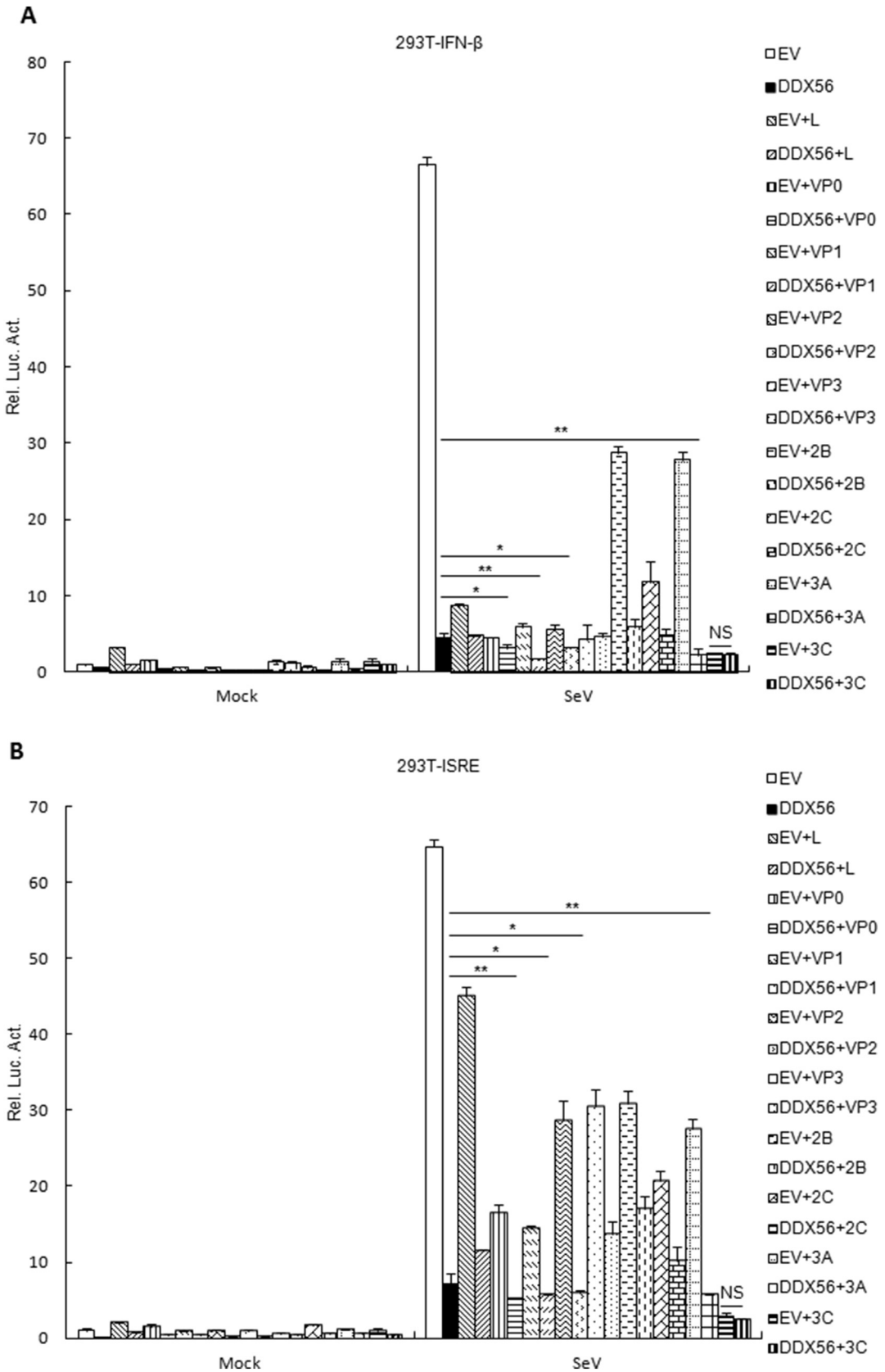

Fig. 5. FMDV VP0, VP1, VP2 and 3A cooperate with DDX56 to inhibit the virus-trigged type I IFN production.
(A) Effects of co-expression FMDV proteins and DDX56 on SeV-triggered activation of IFN-β promoter. HEK293T cells (1×10^5) were transfected with the IFN-β reporter (0.1 μg) and the indicated expression (0.1 μg) plasmids for 24 h. Cells were then infected with SeV or uninfected for another 12 h before luciferase assays. (B) Effects of co-expression FMDV proteins and DDX56 on SeV-triggered activation of ISRE. The experiments were similarly performed as in A. All of the above mentioned experiments were repeated three times. EV, empty vector; Luc, luciferase; NS, no significance; *, $P < .05$, considered significant; **, $P < .01$, considered highly significant.

3.4. DDX56 regulates virus-triggered signaling at IRF3 level

Various components are involved in virus-triggered signaling pathways. As shown in Fig. 4A–B, DDX56 inhibited the IFN-β promoter and ISRE activation by targeting IRF3 upstream components RIG-I, MDA5, VISA, IKKε, and TBK1 but not IRF3-5D or IRF7. Furthermore, DDX56 had no effect on the expression of these proteins (Fig. 4A–B). These results suggest that DDX56 targets IRF3 or an upstream signaling step of IRF3.

3.5. DDX56 cooperates with FMDV VP0, VP1, VP2 and 3A to inhibit virus-triggered induction of IFN-β

The aforementioned results suggested that DDX56 inhibited virus-triggered IFN-β induction and increases FMDV replication. These findings prompted us to examine whether FMDV proteins cooperate with DDX56 to inhibit virus-triggered induction of IFN-β. In reporter assays, FMDV VP0, VP1, VP2 and 3A proteins, but not FMDV VP3, 2B, 2C, 3C and L proteins, cooperated with DDX56 to inhibit SeV-triggered activation of IFN-β promoter and ISRE (Fig. 5A–B). These data suggest that DDX56 cooperates with FMDV VP0, VP1, VP2 and 3A proteins to inhibit virus-triggered induction of IFN-β.

3.6. DDX56 interacts with FMDV VP0, VP1, VP2 and 3A and cooperates with FMDV 3A to inhibit the phosphorylation of IRF3

Since DDX56 cooperates with FMDV VP0, VP1, VP2 and 3A proteins to inhibit SeV-triggered activation of IFN-β promoter and ISRE, we further investigated whether DDX56 interacts with these proteins by Co-IP experiments. The data indicated that DDX56 was directly associated with FMDV VP0, VP1, VP2 and 3A, but not with negative control FMDV L in PK-15 cells (Fig. 6A).

It is well known that the phosphorylation plays a role in activation of IRF3 before it translocated into the nucleus [16,17]. Since DDX56 regulates virus-triggered signaling at the IRF3 level, we next explored whether FMDV VP0, VP1, VP2 and 3A proteins cooperate with DDX56 to inhibit phosphorylation of IRF3. We observed that co-expression of DDX56 and FMDV 3A significantly inhibited the phosphorylation of IRF3 in SeV-infected HEK293T cells; however, this phenomenon was not observed in FMDV VP0, VP1 and VP2 proteins (Fig. 6B–C). Intriguingly, we found that the phosphorylation level of IRF3 was markedly decreased in cells co-expressed DDX56 and FMDV 3A in comparison with cells expressed DDX56 or FMDV 3A individually (Fig. 6D). Therefore, the following study focused on study the role of cellular protein DDX56 and FMDV 3A. Taken together, these results suggest that co-expression of DDX56 and FMDV 3A inhibits the phosphorylation level of IRF3.

3.7. Identification of DDX56 site for enhancing FMDV replication

The DEAD box motif is highly conserved in a subgroup of RNA helicases [18], and mutagenesis studies have shown that substitution of asparagine for aspartic acid or glutamine for glutamic acid results in complete loss of helicase activity [19]. To investigate the role of DDX56 DEAD box in innate immune response, we created DDX56 mutants D166N and E167Q and then carried out reporter assays. The result indicated that DDX56 and DDX56 (E167Q), but not DDX56 (D166N), inhibited SeV-triggered activation of IFN-β promoter and ISRE (Fig. 7A–B). Additional domain-mapping analysis indicated that both DDX56 (1–190) and DDX56 (191–547) recovered SeV-triggered activation of IFN-β promoter and ISRE (Fig. 7A–B). Collectively, these data indicate that the D166 site of DDX56 and a complete DDX56 protein are crucial for inhibiting the virus-triggered IFN-β induction.

We next assessed the significance of the association between DDX56 and IRF3. We first mapped the domains or sites of DDX56 that are required for its interaction with IRF3. As shown in Fig. 7C and D, full-length DDX56, DDX56 (D166N), DDX56 (E167Q), DDX56 (1–190) and DDX56 (191–547) interacted with IRF3.

Additionally, we also found that the D166 site of DDX56 was important for inhibition of virus-triggered IFN-β induction. We next examined whether the D166 site of DDX56 is required for promoting FMDV replication. RT-PCR experiments results indicated that DDX56 and DDX56 (E167Q), but not DDX56 (D166N), enhanced FMDV replication (Fig. 7E). These data suggest that the D166 site of DDX56 is essential for the increase of FMDV replication.

We next determined whether the D166 site of DDX56 plays an important role in FMDV 3A inhibition of IRF3 phosphorylation. In the transient transfection and immunoblot experiments, we observed that the co-expression of DDX56 or DDX56 (E167Q) and FMDV 3A, but not DDX56 (D166N), reduced the phosphorylation level of IRF3 (Fig. 7F).

Furthermore, we investigated the interaction between DDX56, DDX56 mutants and FMDV 3A by Co-IP experiments. The data indicated that DDX56 and DDX56 (E167Q) were associated with FMDV 3A, but not DDX56 (D166N) (Fig. 7G). These results suggest that D166 site in DDX56 is important for FMDV 3A to inhibit the phosphorylation level of IRF3.

3.8. Co-expression DDX56 and FMDV 3A are required for inhibiting IRF3 phosphorylation

To further investigate the effect of endogenous DDX56 on FMDV 3A inhibition of IRF3 phosphorylation, we constructed RNAi plasmids for DDX56 in PK-15 cells. The DDX56-knockdown PK-15 cell lines were co-transfected with HA-IRF3 and plasmids expressing FMDV 3A. Immunoblot analysis confirmed that overexpression of FMDV 3A did not affect IRF3 phosphorylation in the DDX56-knockdown PK-15 cell lines (Fig. 8A). We next investigated the function of endogenous FMDV 3A in inhibition of IRF3 phosphorylation. We constructed three RNAi plasmids for FMDV 3A, and 3A-RNAi-#3 markedly reduced the transcription and expression of endogenous FMDV 3A (Fig. 8B–C) in FMDV-infected PK-15 cells. In addition, immunoblot analysis results showed that IRF3 phosphorylation did not change in FMDV 3A-knockdown PK-15 cells (Fig. 8D). Because co-expression of DDX56 and FMDV 3A could reduce the phosphorylation level of IRF3, we next detected the effect of 3A on the interaction between DDX56 and IRF3. Co-IP results showed that FMDV 3A increased the interaction between DDX56 and IRF3 (Fig. 8E). These findings demonstrate that the co-expression of DDX56 and FMDV 3A plays a critical role in inhibiting IRF3 phosphorylation.

4. Discussion

FMDV is capable of manipulating host cell machinery for viral replication and evading the host immune response, but the mechanisms are not fully understood. To manipulate the host cells, FMDV interacts with cellular proteins. For example, FMDV nonstructural protein 2C is able to bind cellular beclin1 to prevent autophagosome-lysosome fusion, favoring virus survival [20]. FMDV 2C has also been reported to interact with cellular vimentin, which is a protein that forms a cage-like structure approximately 2C during early infection but later can be resolved for virus replication to progress [4]. Furthermore, FMDV nonstructural protein 3A binds to DCTN3, a subunit of the dynactin complex that acts as a co-factor for the microtubule-base motor dynein [5]. Here, we report that DDX56 interacted and cooperated with the FMDV nonstructural protein 3A to inhibit the phosphorylation of IRF3, which increased FMDV replication.

DDX56 belongs to the DEAD-(Asp-Glu-Ala-Asp) box protein family [21]. Xu et al. reported that DDX56 and capsid form stable complexes in West Nile Virus (WNV)-infected cells and that this interaction is important for infectious WNV virion production [22,23]. Furthermore, DDX56 translocated to the site of virus assembly during WNV infection, and interaction of DDX56 with the WNV capsid in the cytoplasm may occur transiently during virion morphogenesis [24]. Furthermore, it

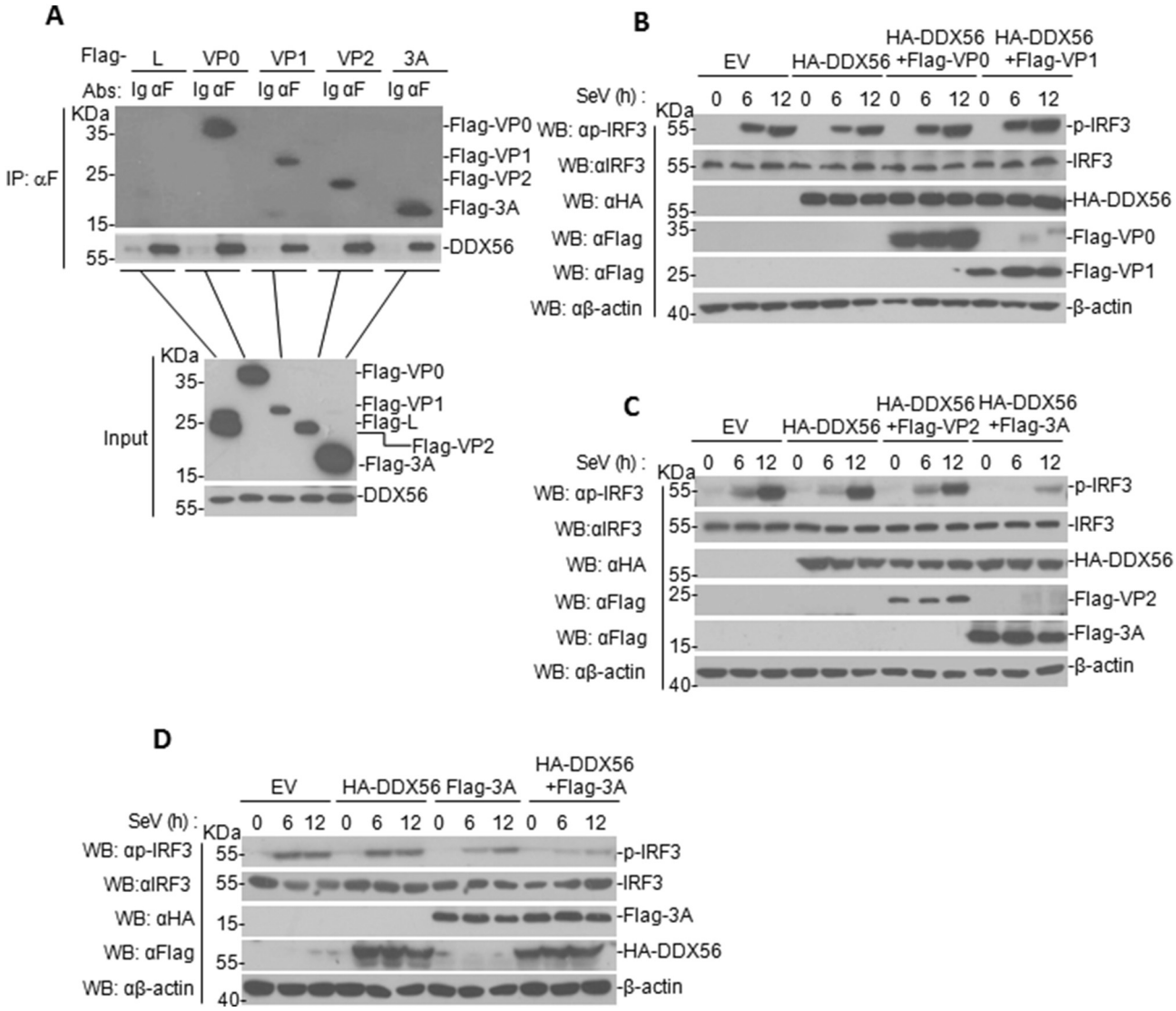

Fig. 6. Co-expression FMDV 3A and DDX56 inhibits the phosphorylation of IRF3.
(A) FMDV VP0, VP1, VP2 and 3A proteins interact with DDX56. HEK293T cells (2×10^6) were transfected with the FMDV L, VP0, VP1, VP2 and 3A plasmids (4 μg each). Co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (B–D) Effects of co-expression FMDV proteins and DDX56 on the phosphorylation of IRF3. HEK293T cells (2×10^5)were transfected with the indicated plasmids (1 μg) for 24 h. Cells were then uninfected or infected with SeV for the indicated time points. Immunoblot analysis was performed with the indicated antibodies. EV, empty vector. αF, anti-Flag antibody.

was reported that DDX3, DDX5 and DDX56 enhance Rev. function during HIV-1 infection [25]. Here, we reported that DDX56 may promote FMDV replication by cooperating with FMDV 3A to inhibit IRF3 phosphorylation.

The D166 and E167 residues of DDX56 play a role in its function. DDX56 (D166N) and DDX56 (E167Q) mutants did not interfere with the replication of WNV [23], which indicates that the D166 residue is as important as the E167 residue in DDX56. In this study, we found that DDX56 (E167Q), but not DDX56 (D166N), increased FMDV replication. Moreover, DDX56 (E167Q), but not DDX56 (D166N), inhibited virus-trigged IFN-β induction. Furthermore, co-expression of DDX56 (E167Q) with FMDV 3A, but not DDX56 (D166N), inhibited the phosphorylation level of IRF3. In addition, FMDV 3A interacted with DDX56 and DDX56 (E167Q), but not with DDX56 (D166N). These results indicate that there are differences between the D166 and E167 residues of DDX56, however, the mechanism still needs to be elucidated in the future.

FMDV 3A is a partially conserved protein of 153 amino acids (aa) [26]. It has been reported that FMDV 3A plays important roles in virus replication, virulence and host range [5,27–31]. We have also previously reported that FDMV 3A is able to bind to cellular VISA and inhibit virus-induced IFN induction through reducing the mRNA level of VISA [8]. Here, we found that DDX56 may promote FMDV replication by cooperating with FMDV 3A to inhibit IRF3 phosphorylation. Moreover, FMDV 3A increased the interaction between DDX56 and IRF3, which may explain the mechanism of FMDA 3A cooperates with DDX56 to inhibit the phosphorylation of IRF3. These data are consistent with our previous observations that FMDV 3A inhibits virus-induced IFN-β induction.

The results reported here identified a novel cellular host protein DDX56, which interacted with FMDV 3A protein. Although DDX56 overexpression did not significantly inhibit the phosphorylation of IRF3, co-expression DDX56 and FMDV 3A inhibited IRF3 phosphorylation. We hypothesized that DDX56 may inhibit the IFN-β signaling by other mechanisms. Co-expression of DDX56 and FMDV 3A may change the function of DDX56 so that DDX56 cooperates with FMDV 3A to inhibit IRF3 phosphorylation. Therefore, further studies are needed to be performed in the future to explain the aforementioned hypothesis. Further understanding of host protein-viral protein relationships will

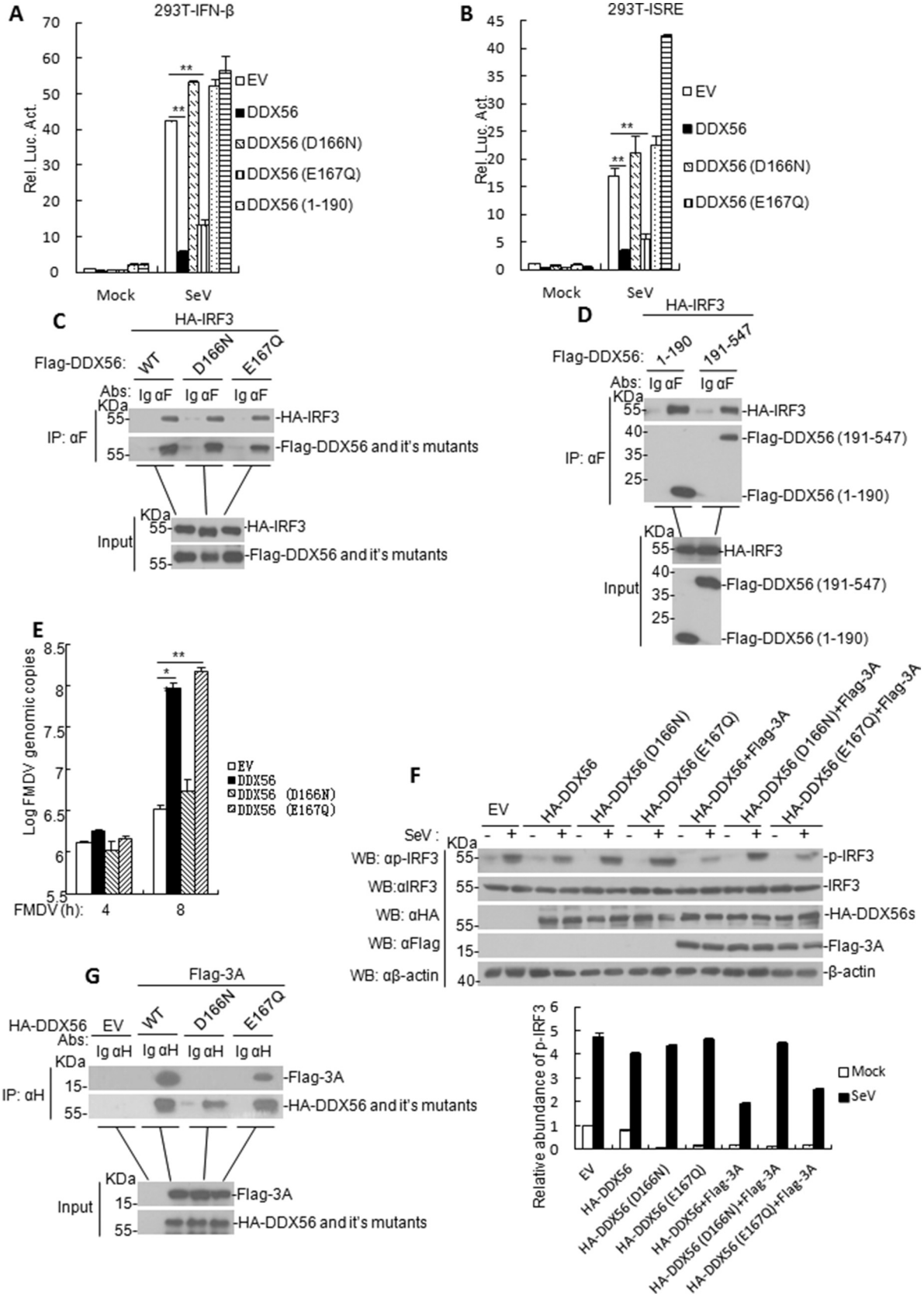

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Fig. 7. The key sites of DDX56 increase FMDV replication.
(A–B) Effects of DDX56 mutants on SeV-triggered activation of IFN-β promoter and ISRE. HEK293T cells (1×10^5) were transfected with the IFN-β reporter or ISRE (0.1 μg) and the indicated expression (0.1 μg) plasmids for 24 h. Cells were then infected with SeV or uninfected for another 12 h before luciferase assays. (C–D) DDX56 mutants interact with IRF3. HEK293T cells (2×10^6) were transfected with the indicated plasmids (5 μg each). Co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (E) Effects of DDX56 point mutants on FMDV replication. PK-15 cells overexpressing DDX56 or DDX56 point mutants were infected with FMDV (MOI = 0.1) at the indicated time points. FMDV replication was assessed in PK-15 cells by RT-PCR. (F) Effects of co-expression DDX56 or DDX56 point mutants and FMDV 3A on the phosphorylation of IRF3. HEK293T cells (2×10^5) were transfected with the indicated plasmids (1 μg) for 24 h and then cells were uninfected or infected with SeV for the indicated time points. Immunoblot analysis was performed with the indicated antibodies. The change of phosphorylated IRF3 was determined by densitometric analysis using ImageJ software and normalized to β-actin (below panel). (G) FMDV 3A interacts with DDX56 and DDX56 (E167Q) not DDX56 (D166N). HEK293T cells (2×10^6) were transfected with the indicated plasmids (5 μg each). Co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. All of the above mentioned experiments were repeated three times. EV, empty vector; αF, anti-Flag antibody; WT, wild type; Luc, luciferase; RT-PCR, Real-time polymerase chain reaction. *, $P < .05$, considered significant; **, $P < .01$, considered highly significant.

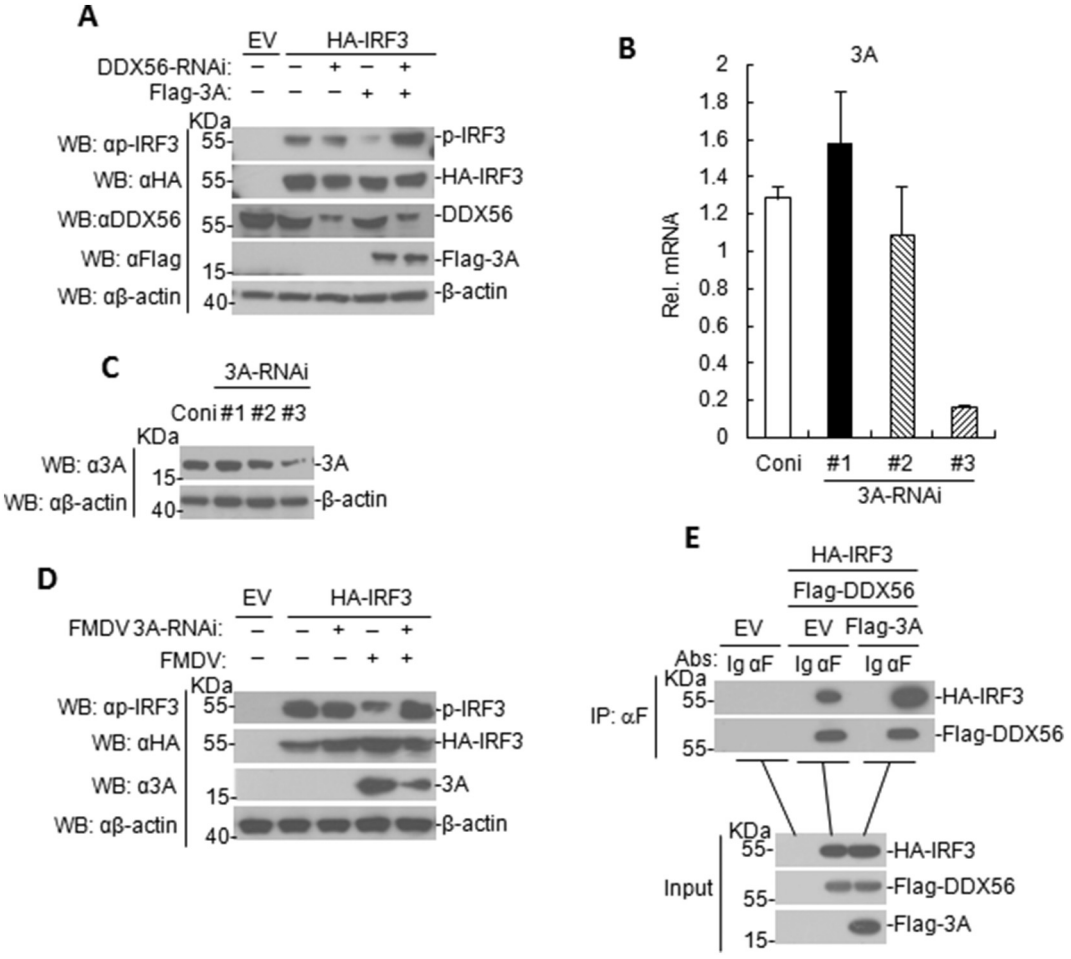

Fig. 8. Co-expression of DDX56 and FMDV 3A plays a role in the inhibition of IRF3 phosphorylation.
(A) Effects of FMDV 3A overexpression on IRF3 phosphorylation in DDX56-knockdown PK-15 cells. DDX56-knockdown PK-15 cells (2×10^5) were transfected with the indicated plasmids (2 μg each) for 24 h and then immunoblot analysis was performed with the indicated antibodies. (B) Effects of 3A-RNAi plasmids on the transcription of endogenous FMDV. FMDV 3A-knockdown PK-15 cells (2×10^5) were infected with FMDV (MOI = 0.1) for 12 h before RT–PCR experiments. (C) Immunoblot analysis of 3A protein levels in FMDV-knockdown PK-15 cells. FMDV-knockdown PK-15 cells (2×10^5) were infected with FMDV (MOI = 0.1) for 12 h before immunoblot analysis. (D) Effects of FMDV 3A knockdown on the phosphorylation of IRF3. FMDV 3A-knockdown PK-15 cells (2×10^5) were transfected with HA-IRF3 (2 μg) for 24 h and then the cells were infected or uninfected with FMDV (MOI = 0.1) for another 12 h before immunoblot analysis. (E) Effects of FMDV 3A on the interaction DDX56 and IRF3. HEK293T cells (2×10^6) were transfected with the indicated plasmids (5 μg each). Co-immunoprecipitations were performed with anti-Flag or control IgG. Immunoblot analysis was performed with anti-HA and anti-Flag (upper panels). Expression levels of the proteins were analyzed by immunoblot analysis with anti-HA and anti-Flag (lower panels). All of the above mentioned experiments were repeated three times. EV, empty vector; Con, control; RT-PCR, Real-time polymerase chain reaction.

encourage the design of novel therapeutic strategies that disrupt viral-host interactions.

In conclusion, we demonstrated that DDX56 increased FMDV replication. In addition, DDX56 was confirmed to cooperate with FMDA 3A to further inhibit IRF3 phosphorylation.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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