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DEAD-box RNA helicase DDX3X inhibits DENV replication via regulating type one interferon pathway

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ABSTRACT

Dengue virus (DENV) is a mosquito-borne virus that threatens approximately 2.5 billion people worldwide. Vaccines against DENV are currently unavailable. DEAD-box RNA helicases (DDXs) have been reported to participate in viral replication and host innate immune response. In the present study, we analyzed the role of 40 DDX proteins during DENV replication. Among these proteins, DDX3X showed antiviral effect against DENV infection. Viral replication significantly increased in DDX3X-silenced cells compared with the controls. The interferon (IFN)-β transcription level decreased during the early stage of DENV infection in DDX3X-silenced cells compared with that in the controls. DDX3X could stimulate IFN-β transcription through the IRF3 and the NFκB branches in DENV-infected cells. Our data imply that DDX3X, a member of DEAD-box RNA helicase, is necessary for IFN production and could inhibit DENV replication.

1. Introduction

Dengue virus (DENV) is a mosquito-borne virus of the *Flaviviridae* family and the etiological agent of dengue fever in humans. Up to 400 million DENV infections occur each year, and the severity can range from asymptomatic to life-threatening dengue hemorrhagic fevers (DHF) or dengue shock syndromes (DSS) [1–3]. DENV vaccine development is challenging because four serotypes of DENV exist [4]. Therefore, the antiviral mechanisms of the host that underlie the control of DENV infection and vaccine development must be determined [5–7].

Asp-Glu-Ala-Asp (DEAD)-box RNA helicases are a part of DexD/H-box helicase that belongs to the splicing factor 2 helicase superfamily. These helicases are involved in various RNA metabolic processes, including transcription, splicing, transport, translation, decay, and ribosome biogenesis [8–11]. A number of DEAD-box helicases, including DDX58, DDX3, DDX60, DDX41, and DDX1, are involved in anti-viral immunity [12–15]. A recent study identified DDX5, DDX17, and DDX21 as human immunodeficiency virus (HIV) Rev-binding proteins [16,17]. DDX10, DDX18, DDX21, DDX23, DDX39, and DDX52 are modulated during HIV-1 infection [18,19]. DDX6 is required for HIV-1, foamy virus, hepatitis C virus (HCV), and West Nile viral replication [20–23]. Moreover, DDX3 and DDX5 participate in HCV replication [24].

DDX3X (also named DBX, DDX3) is a member of putative RNA helicases that comprise DEAD-box family [25]. DDX3X is ubiquitously expressed in most tissues and resides in both nucleus and cytoplasm [26]. Similar to other DEAD-box helicases, DDX3X has been implicated in various aspects of RNA metabolism, including transcriptional regulation, splicing, mRNA export, ribosome biogenesis, and translational regulation [9]. DDX3X is reported necessary for IFN production induction as a critical effector of TANA-binding kinase 1 (TBK1) or by involving in TBK1/IKKepsilon-mediated activation of interferon (IFN)-regulatory factor (IRF) [27,28]. However, DDX3X has different functions in the life cycles of different viruses [12,29–32]. DDX3X is an essential host factor for HCV [24,33,34], inhibits HBV [35] and VSV infections [12] while contributing to HIV [29] infections. The distinct role of DDX3X during DENV infection needs to be addressed.

We recently screened 40 genes of DEAD-box family using the RNAi approach to investigate the host factors in DENV infection. DDX3X has been revealed to inhibit the DENV infection. In the present study, we elaborated the involvement of DDX3X in DENV infection using an *in vitro* model. We confirmed that DDX3X inhibits DENV replication by modulating the IFN pathway.

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2. Materials and methods

2.1. Virus and cell culture reagents

DENV-2 virus (DENV New Guinea C stain) was propagated in mosquito C6/36 cells as described previously [36]. The HEK293T cells were cultured in Dulbecco's modified eagle medium supplemented with fetal bovine serum (10%) and penicillin/streptomycin (1%). Cells were maintained at 37 °C in a 5% CO_2 laboratory incubator that was routinely cleaned and decontaminated.

2.2. Transfections and viral infections

Transfections of HEK293T cells with plasmid DNA and siRNA were conducted using Lipofectamine 2000 (Invitrogen, USA) according to the manual of the manufacturer. The siRNA library for 40 genes of DDX family was purchased from RiboBio Co., China. The siRNA sequences for human DDX3X gene were 5′-GUGCCGUC UUGGUUAGAAA dTdT-3′ and 3′-dTdT CACGGCAGAACCAAUCUUU-5′. RNAi efficiency was confirmed through quantitative reverse transcription polymerase chain reaction (qRT-PCR). The HEK293T cells were infected with DENV at a multiplicity of infection (MOI) of 1.0 for 48 h (except for the cases noted in the text).

2.3. Plasmid constructs

Recombinant plasmid for DDX3X expression was constructed using standard protocols by inserting the DDX3X open reading frame into the pcDNA3.1 vector. Reporter plasmids NFκB-luc and pRL-TK were purchased from Clontech (USA) and used for dual luciferase reporter assays. Reporter plasmids IFN-β-luc, IRF3-luc, and ISRE-luc were obtained from the laboratory of Professor ChunFu Zheng in Soochow University.

2.4. Real-time PCR

Total RNA was isolated using an RNA extraction kit (Omega, Netherlands); cDNA synthesis was performed using random primers with 500 ng of total RNA (Takara, Japan). Real-time PCR was performed using a SYBR Green with gene-specific primers (Applied Biosystems, USA) and normalized with human β-actin gene. The intracellular viral loads, in terms of transcript levels of the DENV-2 envelop gene (E), were quantified through quantitative PCR and normalized to human β-actin gene. The primer sequences of the DENV-2 gene are 5′-CATTCCAAGTGAGAATCTCTTTGTCA-3′ and 5′-CAGATCTCTGATGAATAACCAACG-3′.

2.5. Luciferase reporter assays

For luciferase reporter assays, 70% confluent HEK293T cells were transfected with 10 ng of pRL-TK reporter (herpes simplex virus thymidine kinase promoter driving *Renilla* luciferase, internal control), 100 ng of NFκB, IRF3, and IFN-β luciferase reporter (firefly luciferase, experimental reporter) plasmid, as well as either 100 ng of recombinant expressing plasmids (Vector, DDX3X) or 50 nM siRNAs (N.C. or DDX3X siRNA). At 24 h post-transfection, cells were infected with DENV-2 at an MOI of 1.0 and incubated further for 24 h. Luciferase activity was measured using a Dual Glow kit according to the instructions of the manufacturer (Promega, USA).

2.6. Immunofluorescence microscopy

HEK293T cells were transfected with siRNA (negative control or DDX3X siRNA) using Lipofectamine 2000 and the effect of DDX3X silencing on DENV infection were examined by immunofluorescence assay. At 24 h post-transfection, cells were infected with DENV-2 at an MOI of 1. Cells were fixed in 1% paraformaldehyde and permeabilized with 1% Triton X100 at 48 h post-infection. DENV envelope proteins were probed with mouse anti-DENV (Santa Cruz, USA) and stained with FITC-labeled anti-mouse IgG (Jackson ImmunoResearch, USA). Cell nuclei were stained with DAPI. Cells were then examined using a fluorescence microscope.

2.7. Western blot

HEK293T cells were transfected with pcDNA-DDX3X (or pcDNA3.1 vector) and infected with DENV-2 at an MOI of 10. At 24 h post-infection, cell lysates were subjected to SDS–PAGE and transferred onto a PVDF membrane for Western blotting. Non-reducing native PAGE was performed to detect the dimerization of IRF3. Briefly, cell lysates were prepared in a native sample buffer without SDS and electrophoresed on a 10% non-reducing polyacrylamide gel without SDS.

The following primary antibodies were used for Western blotting: anti-human Actin polyclone Ab (Proteintech, USA), His-tag polyclone antibody (GenScript, USA), IRF3 polyclonal antibody (Biolegend, USA), anti-IRF3 (phospho S386) Ab (Cell Signaling, USA). HRP-conjugated donkey anti-rabbit IgG and rabbit anti mouse IgG mAb (Biolegend, USA) were used as secondary antibodies. The signals were detected using an ECL detection system (Merck Millipore Ltd, USA).

2.8. Statistical analysis

Statistical significance was calculated with an unpaired two-tailed Student's *t*-test using Prism 5 software (GraphPad).

3. Results

3.1. Role of DEAD-box family during DENV infection

The mechanism of host antiviral innate immune response against DENV infection is not well understood. The DEAD-box family of RNA helicase (DDXs) is important for virus infection [37]. We performed RNAi screening of 40 human DDX genes on DENV-infected HEK293T cells to investigate whether DDXs are involved in DENV replication. The viral replication in DDX siRNA-treated HEK293T cells were analyzed by measuring the DENV Envelope (E) gene copies through qRT-PCR method and normalized with the copy of an endogenous control (β-actin) gene. Among the genes studied, DENV replication was significantly upregulated in DDX3X and DDX50 siRNA-treated cells, but downregulated in DDX25 and INTS6 siRNA-treated cells (in which the differences were statistically significant in three independent experiments. *t*-test, $p < 0.05$.) (Fig. 1). In this study, we focused on the association between DDX3X and DENV.

3.2. DDX3X is upregulated upon DENV infection

By using qRT-PCR, we showed that the transcription level of DDX3X was upregulated in human epithelial cell line HEK293T and A549 during DENV infection (Fig. 2A). Western Blot result confirmed that the protein level of DDX3X also increased in HEK293T and A549 cells upon DENV infection. This suggested that DDX3X may involve in DENV infection of multiple cells.

3.3. DDX3X inhibits DENV replication

We silenced endogenous DDX3X expression using RNAi to confirm the role of endogenous DDX3X in DENV replication.

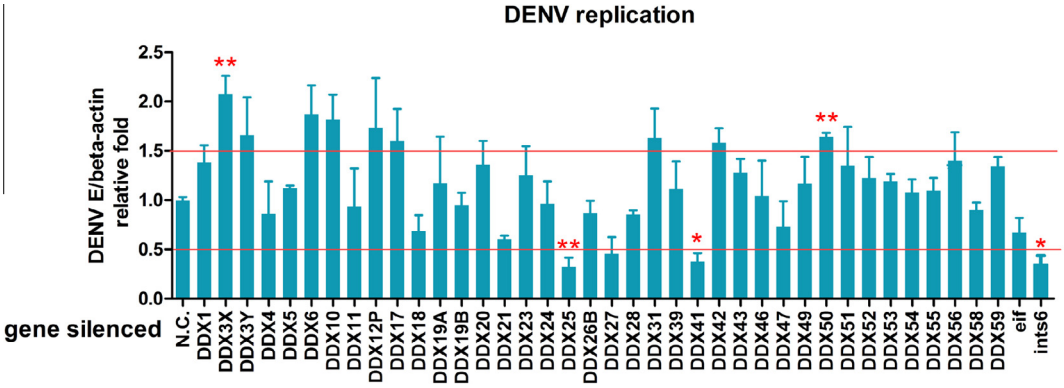

Fig. 1. Role of DEAD-box RNA helicase family during DENV infection: an RNAi screening. HEK293T cells were transfected with siRNAs for specific genes from DDX RNA helicase family, and infected with DENV at an MOI = 1.0. The viral loads were analyzed through measuring the virus E gene copy using qRT-PCR and normalized to a human beta actin gene. The relative replication level of DENV in nonsense siRNA (N.C.) transfected cells was set to 1. Results are expressed as mean + SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ (*t*-test). Representative results are from at least three independent experiments.

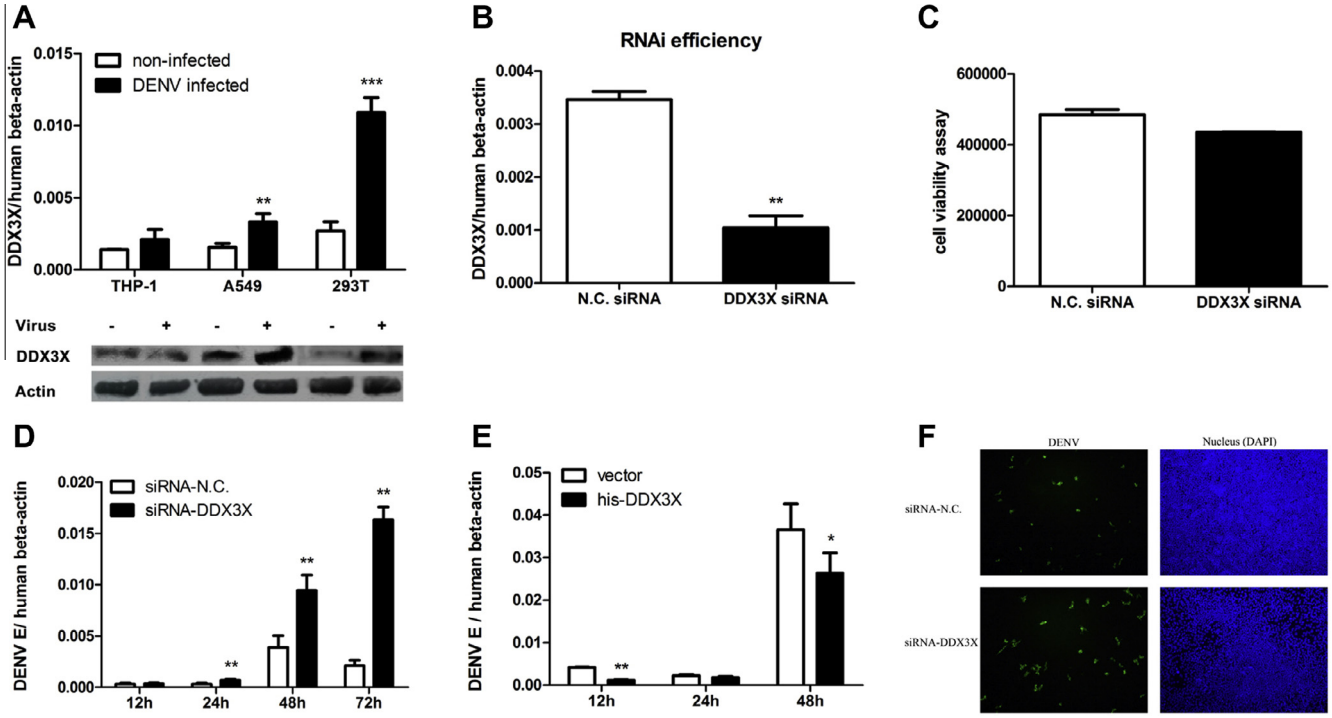

Fig. 2. DDX3X is upregulated upon DENV infection and exerts antiviral effect. (A) DDX3X mRNA and protein level increased in human A549 and HEK293T cells upon DENV infection. (MOI = 1.0, 48 h post-infection). (B) RNAi efficiency for DDX3X, as shown in qRT-PCR analysis. (C) Cell viability after transferring siRNA into HEK293T cells. (D) siRNA-mediated DDX3X silencing-induced DENV replication. The viral loads were analyzed through measuring the virus E gene copy using qRT-PCR and normalized to human beta actin gene. (E) Viral loads in DDX3X-overexpressed cells decreased compared with that in control cells. Results are expressed as mean + SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$(*t*-test). Representative results are from at least three independent experiments. (F) Intracellular viral loads were significantly higher in DDX3X-silenced cells than in the controls, as shown in immunofluorescence assay.

Oligonucleotides specifically targeting DDX3X significantly reduced DDX3X mRNA expression compared with cells transfected with a control oligonucleotide, as shown in the qRT-PCR analysis (Fig. 2B). Cell viability was not affected after siRNA transfection, as tested through Promega Cell Titer-Glo® substrate assay (Fig. 2C). The intracellular viral loads, in terms of the transcript levels of the DENV envelop gene (E), increased by 2.4-fold and 7.6-fold ($p < 0.05$) in DDX3X-silenced cells compared with control cells after DENV infection for 48 and 72 h, respectively (Fig. 2D). Immunofluorescence assay further confirmed the increased DENV burden in DDX3X silenced cells compared with control cells (Fig. 2F). Conversely, DDX3X overexpression by transfecting HEK293T cell with a plasmid that encodes his-tagged DDX3X substantially decreased DENV production (Fig. 2E). Overall, these data suggest that DDX3X could inhibit DENV replication.

3.4. DDX3X regulates IFN-β production

To evaluate the role of DDX3X in regulation of innate immune signaling, we measured the transcription level of IFN-β in DDX3X silenced cells during DENV infection. Unlike the increased DENV replication at all time points in DDX3X silenced cells (Fig. 2D),

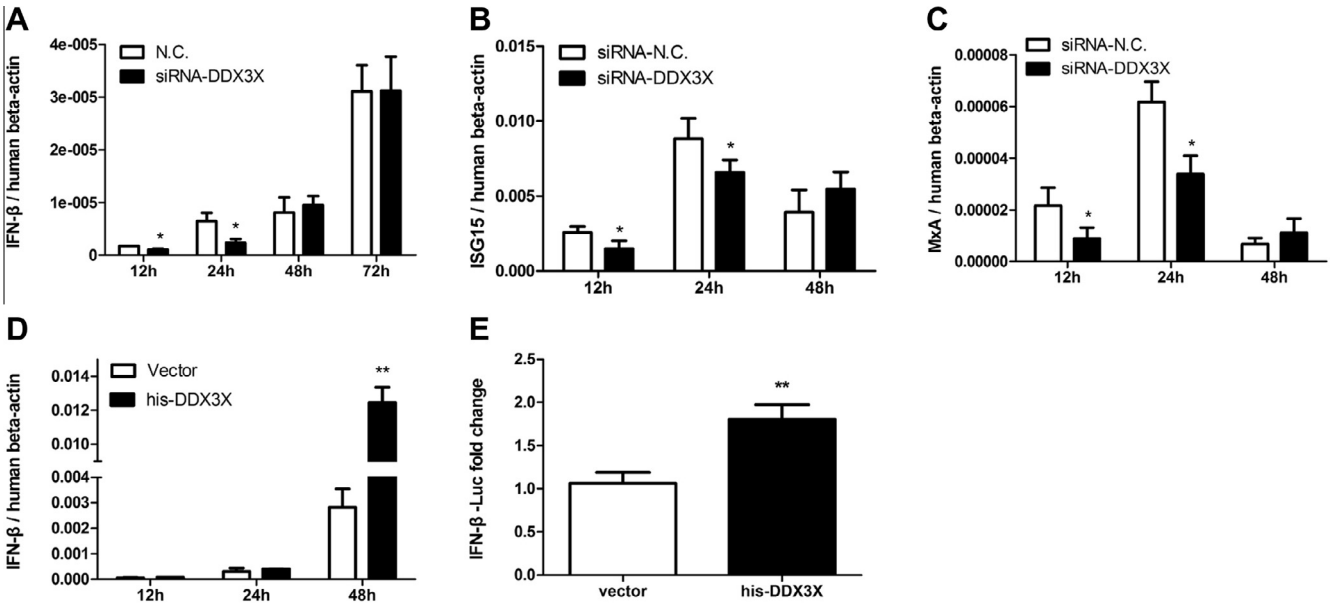

Fig. 3. DDX3X promotes IFN-β production in DENV-infected HEK293T cells. (A–C) Knocking down DDX3X suppressed the expression of genes from IFN pathway at the early stage of DENV infection. (D) DDX3X overexpression activates IFN-β production in DENV-infected HEK293T cells. (E) DDX3X overexpression promoted IFN-β reporter activities on DENV stimulation. The results were normalized with the use of an internal control (pRL-TK *Renilla* luciferase value). The mean value of activities from DENV-infected control cells was set to 1.0. Results are expressed as mean + SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ (*t*-test). Representative results are from at least three independent experiments.

the transcription levels of IFN-β decreased by approximately 1.6- and 2.9-fold at 12 and 24 h, respectively, and finally increased at 48 h post-infection (Fig. 3A). The transcription level of ISG15 and MxA, two representative interferon stimulated genes, were consistent with IFN at these specific time points (Fig. 3B and C). These results suggests that DDX3X may contribute to IFN-β production during the early stage of DENV infection, thereby inhibiting DENV replication.

Furthermore, we analyzed the IFN-β level in DDX3X-overexpressed HEK293T cells infected with DENV. Overexpression of DDX3X increased the transcription level of IFN-β (IFN transcription level increased by 4.4-fold ($p < 0.01$) at 48 h) (Fig. 3D). Consistent with this, overexpression of DDX3X could increase the transcription of an IFN-β promoter-driven luciferase reporter after DENV infection (Fig. 3E). Similar results were also obtained when IFN-β reporter was activated through over-expression of RIG-I or MAVS (data not shown). Thus, DDX3X may positively regulate innate immune-signaling processes and activate type I IFN production during DENV infection. While, some of the difference are minor, which implied that regulation of interferon signaling may not be the only function of DDX3X in DENV infection. This multiple function RNA helicase may also participate in other cellular events during DENV replication, which need to be further investigated.

3.5. DDX3X involved in DENV-induced activation of IRF3 and NFκB

To investigate whether DDX3X activates DENV-induced activation of IRF3, we performed reporter assays in HEK293T cells transfected with the luciferase reporter plasmid PRD(III–I)4-Luc, in which an IRF3 specific binding elements (positive regulatory domains III [PRD (III–I)] from the IFN-β promoter) was used to drive the reporter gene expression. Overexpression of DDX3X activated the IRF3-responsive PRD (III–I) promoter activity by 1.7-fold when compared with controls during DENV infection (Fig. 4A).

Western blotting was performed to address the role of DDX3X in IRF3 phosphorylation (or dimerization) during DENV infection. Phosphorylation of IRF3 significantly increased in DDX3X the overexpressed group compared with vector control in DENV infected cells, consistent with the results from the reporter assay (Fig. 4B). We also found that the IRF3 dimer, another feature for IRF3 activation, increased in the DDX3X overexpressed group of the DENV infected cell through a non-reducing gel Western blotting (Fig. 4B).

NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) has a crucial function in antiviral innate immune response because NFκB promotes transcription of numerous antiviral or pro-inflammatory genes. DDX3X was co-expressed with NFκB reporter plasmid in HEK293T cells, which significantly increased DENV-induced activation of NFκB (Fig. 4C). Similar results were obtained when NFκB reporter is activated by RIG-I over-expression (Fig. 4D). These data suggest that DDX3X contributes to production of IFN-β during DENV infection through both branches of IFN-β pathway: IRF3 and NFκB.

4. Discussion

DENV can cause severe disease in humans by interacting with host cell factors to create a more favorable environment for replication. However, few linkages between DENV and human proteins have been reported as of this writing. High-throughput yeast two-hybrid assays were used to screen DENV-human protein interactions, and disrupting of DDX3X was reported to cause a significant decrease in the replication of a DENV replicon after 48 h, but the underlying mechanism is unclear [38]. In that report, Khadka and colleagues tested the role of DDX3X using a modified DENV replicon with luciferase activity in Huh-7.5 cells, a cell line defective in interferon production. Using this interferon defective cell line may lead to the missing of the role of DDX3X in regulation of interferon pathway during DENV infection. In our present study, we used a live DENV to infect a DENV sensitive cell line HEK293T cells. HEK293T cells contain an intact RIG-I directed interferon pathway, and also are responsive to interferon (more than 80 ISGs were upregulated in HEK293T cells upon DENV infection, from unpublished RNAseq data of our group). We tested the DENV replication at 12, 24, 48, and 72 h after infection, and found that the replication of DENV increased in DDX3X silenced cells, while decreased in DDX3X overexpressed cells. We further confirmed that DDX3X inhibits DENV replication via amplifying interferon production at early time point of infection.

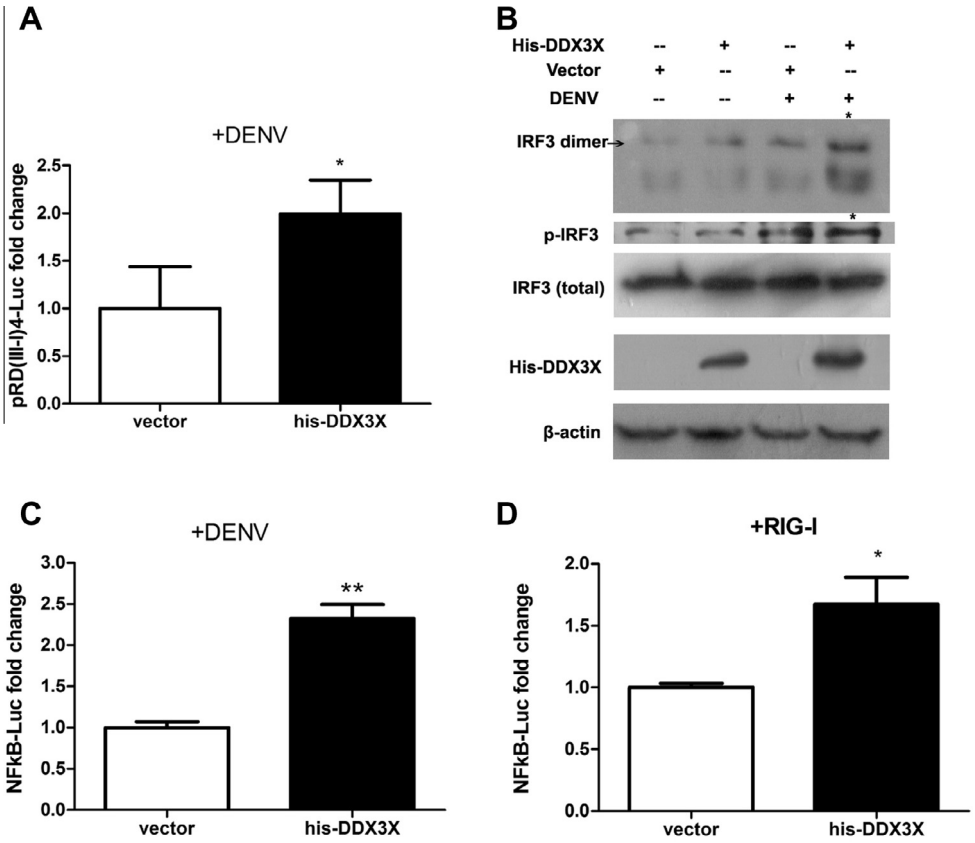

Fig. 4. DDX3X overexpression activated IRF3 and NFκB in DENV-infected HEK293T cells. (A) DDX3X overexpression activated IRF3 reporter activity in DENV-infected HEK293T cells. (B) DDX3X overexpression enhanced IRF3 phosphorylation and dimerization in DENV-infected HEK293T cells. (C) DDX3X overexpression activated NFκB reporter activity in DENV-infected HEK293T cells. (D) DDX3X overexpression enhanced RIG-I-directed NFκB reporter activity. Results are expressed as mean + SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ (*t*-test). Representative results are from at least three independent experiments.

DDX3X is an alias for DDX3, representing the copy of DDX3 gene on the X chromosome [39]. As reported, DDX3 is a multiple function protein that involved in protein translation, cell cycle, apoptosis and, most importantly, in innate immune regulation. Schroder et al. first demonstrated that DDX3 contributes to antiviral innate immunity [27]. Since then, more works have focused on the roles of DDX3 in different virus infection models, but the results are conflicting. DDX3 shows antiviral functions against VACV and HBV. DDX3 could inhibit the initial step of reverse transcription of HBV via incorporating into nucleocapsids together with HBV pol [35]. VACV K7 protein interacts with DDX3, interrupting the association of DDX3 and IRF3, which is required for interferon production during VACV infection [27]. Meanwhile, other works have suggested that DDX3 is an important host factor required for HCV and HIV infection. Three original studies suggested that HCV core protein targets DDX3 to manipulate splicing, transcriptional or translational regulation [24,40,41]. These studies implicated that the RNA helicase activity of DDX3 may be utilized by HCV for its infection. Consistent with this, the DDX3's helicase activity was also required for HIV RNA export [29]. All these reports suggested that, as a multiple function RNA helicase, DDX3 plays distinct role in a virus specific manner. Our current study, using RNA interference and overexpression approach, described the role of DDX3X during DENV infection. We found that DDX3 (DDX3X) inhibits DENV infection and is required for early interferon production during DENV infection, similar with the results obtained in VACV and HBV models. Although DENV shares similar genomic structure and protein organization with HCV, these two viruses have different reactions to DDX3X for their infections. This result indicates that targeting DDX3 (DDX3X) cannot be a general approach against *flavivirus* infection.

DDX3X belongs to the same family of helicases as RIG-I and MDA5; unlike RIG-I and MDA5, its mode of action in innate immunity fails to exert a PRR effect, but situated downstream in the signaling cascade that controls IFN-β production [28]. Several studies have described DDX3 as a protein that constantly shuttles between the cytoplasm and the nucleus [26,27,29]. However, we failed to observe the cytoplasm to nucleus shuttling of DDX3X during DENV infection (data not shown). Thus, DDX3X may modulate IFN production indirectly via influencing the activity of molecules of IFN-β pathway during DENV infection.

DDX3X reportedly interacts with IPS-1 to induce signaling in the early stages of viral infection [12]. In the present study, we also found that DDX3X stimulated the transcription of the IFN-β gene, especially in the early stages of DENV infection, through both IRF3 and NFκB branches. Furthermore, we confirmed that DDX3X increased MAVS-activated IFN-β, NFκB, and IRF3 reporter activation in HEK293T cells (data not shown). The mechanism underlying such effect is currently unclear and will be the subject of future studies. The anti dengue virus function of DDX3X may help the development of strategies to suppress viral replication.

Competing interests

None.

Authors contribution

T.F., X.S. and J.D. designed the experiments and prepared the manuscript. G.L., W.P. and T.F. performed all the experiments. All authors read and approved the final manuscript.

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