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Cellular DDX3 regulates Japanese encephalitis virus replication by interacting with viral un-translated regions

Chen Li a,b,* , Ling-ling Ge a , Peng-peng Li a , Yue Wang a , Juan-juan Dai c , Ming-xia Sun a , Li Huang a , Zhi-qiang Shen b , Xiao-chun Hu a , Hassan Ishag a , Xiang Mao a,b,**

a College of Veterinary Medicine, Nanjing Agricultural University, 1 Weigang, Nanjing, Jiangsu Province 210095, China
b Shandong Binzhou Animal Science and Veterinary Medicine Institute, 169 Yellow River Road 2, Binzhou, Shandong Province 256600, China
c Shandong Lvdu Ante Veterinary Drug Industry, 169 Yellow River Road 2, Binzhou, Shandong Province 256600, China

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Japanese encephalitis virus is one of the most common causes for epidemic viral encephalitis in humans and animals. Herein we demonstrated that cellular helicase DDX3 is involved in JEV replication. DDX3 knockdown inhibits JEV replication. The helicase activity of DDX3 is crucial for JEV replication. GST-pulldown and co-immunoprecipitation experiments demonstrated that DDX3 could interact with JEV non-structural proteins 3 and 5. Co-immunoprecipitation and confocal microscopy analysis confirmed that DDX3 interacts and colocalizes with these viral proteins and viral RNA during the infection. We determined that DDX3 binds to JEV 5′- and 3′- un-translated regions. We used a JEV-replicon system to demonstrate that DDX3 positively regulates viral RNA translation, which might affect viral RNA replication at the late stage of virus infection. Collectively, we identified that DDX3 is necessary for JEV infection, suggesting that DDX3 might be a novel target to design new antiviral agents against JEV or other flavivirus infections.

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Introduction

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is one of the most common causes for epidemic viral encephalitis in humans and animals (Solomon, 2003; Tsai, 2000). Its genome encodes a single polyprotein composed of three structural proteins [core, membrane (prM/M), and envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The mature viral proteins are produced via proteolytic processing of the single polyprotein by viral serine protease (NS2B–NS3) and cellular proteases (Lindenbach and Rice, 2001, 2003). The viral replication is initiated by the replication complex through a process of RNA-dependent RNA polymerization in the perinuclear endoplasmic reticulum membranes (Westaway et al., 2003). Nonstructural proteins 3 and 5 are components of the replication complex, which associates with the 3′ noncoding region of genomic RNA to initiate viral replication (Chen et al., 1997; Edward and Takegami, 1993). NS5, the largest and most conserved viral protein, contains methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) domain. The MTase is involved in methylation of the 5′-cap structure of genomic RNA, while RdRp is important for viral genome replication (Ackermann and Padmanabhan, 2001; Egloff et al., 2002; Guyatt et al., 2001; Koonin, 1993). Until now there is no specific and effective treatment available for Japanese encephalitis virus infection; vaccination is the only effective control measure. Although vaccination is widely used, some public health concerns exist (Takahashi et al., 2000).

Cellular DEAD-box helicases reside in both nucleus and cytoplasm (Chao et al., 2006; Vedavalli et al., 2004); they are involved in a variety of cellular processes related to RNA, such as splicing, mRNA export, transcriptional and translational regulation, RNA decay and ribosome biogenesis (Beckham et al., 2008; Choi and Lee, 2012; Garbelli et al., 2011; Rocak and Linder, 2004; Shih et al., 2007; Soulat et al., 2008). DDX3(X), identified in 1997, is a member of the DEAD (Asp–Glu–Ala–Asp)-box helicase family (Park et al., 1998). It is ubiquitously expressed in a wide range of tissues (Kim et al., 2001). DDX3 has orthologous candidates in many eukaryotic organisms from yeast and plants to animals; they share significant identities between each other (Garbelli et al., 2011). DDX3 is a nucleo-cytoplasmic shuttling protein characterized by nine conserved motifs. It displays RNA-dependent ATPase and ATP-dependent RNA helicase activities (Rocak and Linder, 2004; Schröder, 2010).
Recently, DDX3 attracts great interest because several studies have shown that it is involved in the replication of several viruses that have major global health threats, such as Human Immuno-deficiency Virus (HIV) (Owsianka and Patel, 1999), Hepatitis B Virus (Wang et al., 2009), Hepatitis C virus (HCV) (Ariumi et al., 2007; Mamiya and Worman, 1999; Owsianka and Patel, 1999; You et al., 1999) and poxviruses (Kalverda et al., 2009). The other cellular helicases such as DDX1, DDX5, DHX15, DDX28, DDX56 and DDX42 are the cofactors for the replication of HIV, coronavirus, hepatitis B virus, HCV, JEV and WNV (Ariumi et al., 2007; Fang et al., 2005, 2004; Li et al., 2013; Wang et al., 2009; Xu et al., 2010, 2011; Xu and Hobman, 2012). Two recently published papers report that the novel DDX3 inhibitors possess the anti-HIV activity which highlights DDX3 as a novel target for the development of antiviral agents (Maga et al., 2011; Radi et al., 2012).

In order to determine whether DDX3 is involved in the JEV infection, we first silenced the endogenous DDX3 expression and found that JEV replication was significantly reduced. We then determined that the helicase activity is crucial for JEV replication by studying the effects of the helicase-dead DDX3 mutants and DDX3 inhibitor on virus replication. Furthermore, we also demonstrated that DDX3 could interact with JEV NS3, NS5-MTase and NS5-RdRp proteins. Meanwhile, we found that DDX3 could bind to the JEV 5’ and 3’ un-translated regions (UTR) and colocalize with viral RNA during viral infection. Finally, we showed that DDX3 plays an important role in viral protein translation, which might affect the viral RNA replication at the late stage of viral infection. Collectively, our studies demonstrated that cellular DDX3 plays critical roles in regulating JEV infection.

**Results**

**Requirement of endogenous DDX3 for JEV replication**

It has been reported that DDX3 plays important roles in the replication of several viruses (Garbelli et al., 2011; Schröder, 2010). To determine whether DDX3 is required for JEV replication, we...
first used DDX3 shRNA to reduce the expression level of endogenous DDX3 before the cells were infected with JEV (MOI=0.01). Western blot analysis using anti-DDX3 antibody demonstrated that 90% reduction of protein expression level was achieved upon gene silencing compared with the control (Fig. 1A). A reduction of the endogenous DDX3 expression resulted in more than 15-fold decrease in viral titers (Fig. 1B) and a significant decrease in JEV genomic RNA level (Fig. 1C). The viral protein expression level was also decreased after DDX3 silencing as detected by Western blot analysis using anti-JEV-envelope monoclonal antibody and anti-HEV-N55 polyclonal antibody (Fig. 1D).

As knockdown of DDX3 can influence the replication of JEV at 48 hpi, we further determined the effect of knockdown of DDX3 on JEV replication at different time points. The cells were infected with JEV (MOI=0.01) after being transfected with DDX3 shRNA plasmid; the virus loads were determined at different time points. As shown in Fig. 1E, the titers of JEV in DDX3 knockdown cells were lower than that in the control cells (p < 0.01). At the same time, we determined whether the effect of DDX3 knockdown on JEV replication is dose-dependent; we transfected the BHK-21 cells with different amounts of DDX3 shRNA plasmid before infecting the cells with JEV (MOI=0.01). The virus load analysis showed that DDX3 knockdown reduced JEV titers in a dose-dependent manner. The DDX3 shRNA interference efficiency of different amounts of DDX3 shRNA plasmid was detected using anti-DDX3 polyclonal antibody (Fig. 1F).

**DDX3 knockdown does not affect JEV assembly or release**

In order to determine whether cellular DDX3 is involved in virus assembly or release, the BHK-21 cells were transfected with DDX3 shRNA plasmid before being infected with JEV (MOI=0.01). 48 h later, the JEV RNA copy numbers between the supernatants and the cell lysates were detected; although the RNA copy numbers in the cell lysates were nearly 15-fold higher than that in the supernatants, the ratios between them were almost the same between the DDX3 knockdown cells and the control cells (p > 0.05), demonstrating that DDX3 silencing did not affect JEV assembly (Fig. 2A). Meanwhile, the ratios of the viral titers in the supernatants and cell lysates were also similar between DDX3 knockdown cells and the control cells (p > 0.05) as determined by plaque formation assay (p > 0.05) (Fig. 2B), indicating that DDX3 knockdown did not affect the virus release.

**The helicase activity of DDX3 is crucial for JEV infection**

In order to determine whether the helicase activity of DDX3 was important for JEV replication, the DDX3 helicase-dead mutant lacking either ATPase activity (K230E) or RNA unwinding activity (S382L) (Cordin et al., 2006; Garbelli et al., 2011) was used. BHK-21 cells were first transfected with DDX3 shRNA plasmid to decrease the endogenous DDX3 expression level. 24 h later, the cells were transfected with RNA interference-resistant plasmid (to exclude the influence of DDX3 shRNA plasmid) pCDNA3.1-DDX3r-HA, pCDNA3.1-DDX3r-K230E-HA or pCDNA3.1-DDX3r-S382L-HA before the cells were infected with JEV (MOI=0.01). The virus titers were detected 2 days later by plaque formation assay, the results showed that overexpression of DDX3r-K230E, DDX3r-S382L and the control plasmid pCDNA3.1 after DDX3 knockdown resulted in the reduction of JEV replication for 13-fold (p < 0.01), 12-fold (p < 0.01) and 15-fold (p < 0.01). Meanwhile overexpression of DDX3r after DDX3 knockdown did not reduce JEV replication when compared with the control cells (Fig. 3A), suggesting that overexpression of the exogenous DDX3r could restore JEV replication. The Q-PCR results of the DDX3r, DDX3r-K230E or DDX3r-S382L transfected cells after DDX3 knockdown were consistent with the results of plaque formation assay (Fig. 3B) (p < 0.01). The expression of endogenous DXX3 or DXX3 mutants was confirmed by Western blot analysis using anti-HA monoclonal antibody and anti-DDX3 polyclonal antibody (Fig. 3C). Both of the results demonstrated that DDX3 helicase activity was crucial for JEV infection. The cell viability of all DDX3 constructs and control plasmids transfected BHK-21 cells was not significantly affected as determined by using the cytotoxicity test using CytoTox 96® NonRadioactive Cytotoxicity Assay Kit (Fig. 3D).

It has been shown that DDX3 is involved in HIV-1 replication by exporting un-spliced or partially spliced viral RNAs from the nucleus to the cytoplasm (Yedavalli et al., 2004), and one recently published paper reported that two chemical compounds (Cmp6 and Cmp8) can specifically inhibit the helicase activity of DDX3, and therefore inhibit HIV replication in PBMCs (Radi et al., 2012). We synthesized Cmp6 to further confirm whether DDX3 helicase activity is critical for JEV infection. We infected BHK-21 cells with JEV virus (MOI=0.01) at 37 °C for 2 h before Cmp6 was added (0–50 μM). The plaque formation assay at 48 hpi showed that Cmp6 significantly inhibited JEV replication by 20-fold at the concentration of 50 μM (Fig. 4A). The Q-PCR also confirmed the results (Fig. 4B), and the results were consistent with the DDX3 helicase-dead mutants experiment. Meanwhile, we confirmed that Cmp6 did not stimulate significant cytotoxicity in BHK-21 cells at the concentration of 50 μM, while significant cytotoxicity was detected at the concentration of 100 or 200 μM by LDH assay (Fig. 4C). Therefore, 0–50 μM of Cmp6 was used in our study. In summary, our results suggested that the helicase activity of DDX3 is necessary for JEV infection.
DDX3 can interact with JEV NS3 and NS5 proteins

It has also been shown that both JEV NS3 and NS5 are components of the replication complex (Uchil and Satchidanandam, 2003). We next determined whether DDX3 was involved in JEV replication by interacting with JEV proteins responsible for viral RNA replication. We used GST-pulldown assay to monitor the interactions between NS3, NS5-MTase, NS5-RdRp and DDX3 or vice versa. The Western blot analysis showed GST-NS3, GST-NS5-MTase or GST-NS5-RdRp could interact with DDX3 (Fig. 5A), and GST-DDX3 could also interact with His-tagged JEV NS3, NS5-Mtase or NS5-RdRp (Fig. 5B).

NS5 and NS3 has been hypothesized to seed the formation of the replication complex, and they may form a replication complex together with 3′ noncoding region of JEV genomic RNA (Uchil and Satchidanandam, 2003). Therefore, we examined the interactions between DDX3 and viral proteins in BHK-21 cells. We first infected the BHK-21 cells with JEV (MOI = 0.01). 48 h later, the anti-DDX3 polyclonal antibody was used to co-immunoprecipitate the proteins from the whole cell lysate. The results showed JEV NS3 and NS5 proteins could be co-immunoprecipitated by the endogenous DDX3 (Fig. 5C). Since DDX3 is a nucleic acid-binding protein, it is possible that the interactions might be mediated by RNA or DNA, and then we determined the interactions between DDX3 and viral proteins in the presence of RNase (100 μg/ml) and DNase (100 μg/ml). Western blot analysis showed that the interaction between DDX3 and JEV NS3 or NS5 proteins was not mediated by RNA or DNA (Fig. 5D).

Several studies reported that DDX3 can be incorporated into HBV, Herpes simplex virus type 1 (HSV-1) and Human cytomegalovirus (HCMV) (Stegen et al., 2013; Varnum et al., 2004; Wang et al., 2009), so we also determined whether DDX3 was incorporated into the JEV virions. We infected the BHK-21 cells with JEV (MOI = 0.01) for 48 h, the anti-E monoclonal antibody was used to co-immunoprecipitate the mature virions in the medium, the virions were then detected using anti-DDX3 polyclonal antibody by western blot analysis. We did not detect the presence of DDX3 in our experiment, suggesting that DDX3 is not incorporated into the JEV virions (Fig. 5E).

DDX3 colocalized with the JEV NS3 and NS5 proteins and viral RNA during virus infection

As previously reported, DDX3 is primarily localized in the cytoplasm (Choi and Lee, 2012; Lai et al., 2008). To find out whether...
the cellular localization of the endogenous DDX3 was changed during JEV infection and whether DDX3 colocalized with JEV NS3 and NS5 proteins, we infected BHK-21 cells with JEV (MOI=0.01) for 48 h, and studied the localization of DDX3 and viral proteins by confocal microscopy. The results demonstrated that DDX3 colocalized with JEV NS3 and NS5 proteins in the cytoplasm (Fig. 6A). We did not observe any changes in the distribution of DDX3 in the cells infected with JEV (MOI=0.01) compared with mock-infected cells. The monoclonal antibody against JEV envelope protein was used to confirm that the cells were not infected. Meanwhile, we also found that endogenous DDX3 colocalized with JEV RNA in the cytoplasm (Fig. 6B), implying that DDX3 might be involved in JEV RNA replication.

**DDX3 interacts with JEV 5′ and 3′ UTR and positively regulate JEV protein translation and viral RNA replication**

Since JEV NS3 and NS5 bind to the 3′ UTR of the viral RNA during virus replication (Chen et al., 1997), we next determined whether DDX3 could bind to JEV 5′/or/and 3′-UTR. We labeled JEV 5′ and 3′ UTR with Tobramycin Affinity Tag to enable the RNAs to bind to tobramycin matrix beads to pull down the endogenous DDX3. The Western blot results showed that DDX3 could bind to both JEV 5′ and 3′ UTRs (Fig. 7A), which was consistent with the fact that DDX3 colocalized with viral RNA during the infection (Fig. 6B).

It has been suggested that 5′ and 3′ UTR of JEV are both involved in the first round of viral protein translation and also in the virus RNA replication (Chien et al., 2011), so we constructed a JEV replicon reporter system to determine whether DDX3 could regulate viral translation and/or RNA replication according to the previous study (Chien et al., 2011). BHK-21 cells were first transfected with DDX3 shRNA to knockdown the expression of DDX3, the non-targeting shRNA was used as the control. The cells were then transfected with in vitro-transcribed JEV replicon and firefly luciferase RNA was used as the internal control. The cells were harvested for dual luciferase assays at 3, 6, 9, 24, 48, and 72 h post transfection. The results showed that there were two peaks of replication luciferase activity, which represented the first translation and the second replication peak. In DDX3 knockdown cells, lower luciferase activities were observed for all of the time points in both peaks (Fig. 7B), indicating that viral protein translation and viral RNA replication were all hampered by the knockdown of DDX3.

Because JEV RNA replication depends on viral proteins, we next confirmed the roles of DDX3 on viral translation using a replication-deficient replicon, which contains a GDD→AAG mutation in NS5 polymerase (Blight et al., 2003). The results showed that luciferase activity derived from deficient-replicon RNA was lower in DDX3 knockdown cells when compared with the control cells (Fig. 7C). However, the luciferase activities derived from a control replicon, Renilla luciferase flanked by cellular GAPDH 5′ and 3′ UTRs were not significantly changed (Fig. 7D). In conclusion, all the data demonstrated that DDX3 could positively regulate JEV infection by promoting viral protein translation. Because DDX3 can bind to viral 3′ UTR and viral NS3 and NS5 proteins, DDX3 might promote viral RNA replication.

**Discussion**

The prevalence of the disease caused by JEV and the limited efficacy of therapies stimulate the search for safer and more effective antiviral agents against JEV infection. Recently, new compounds have been discovered to inhibit JEV replication by targeting the virus (Chen et al., 1997; Chien et al., 2008; Deas et al., 2005; Ishag et al., 2013a, 2013b; Lee et al., 2011; Li et al., 2012; Nawa et al., 2003; Ray and Shi, 2006; Yoo et al., 2009). However, the virus resistance to these kinds of inhibitors has been a problem. One possible solution to overcome virus resistance mutations is to target host cellular factors (Garbelli et al., 2011). Theoretically, a drug targeting the cellular factors important for the viral life cycle can be used to inhibit the viral infection (Kwong et al., 2005). Various host proteins are known to interact with DDX3, such as several translation initiation factors, transcription factor SP1, spliced mRNA in an exon junction complex, mRNP, and ile4E (Shih et al., 2007). Several viral proteins have also been reported to interact with DDX3, including hepatitis B virus DNA polymerase, hepatitis C virus core protein, vaccinia virus K7, HIV-1 and poxviruses proteins (Angus et al., 2010; Ariumi et al., 2007; Mamiya and Worman, 1999; Owlsanka and Patel, 1999; Schröder, 2010; Sun et al., 2010; Wang et al., 2009; Wang and Ryu, 2010). Given that DDX3 is required for replication of HBV, HCV,
HIV and poxviruses, it can be suggested that it is a promising target for drug development against these viruses that pose major global health threats (Garbelli et al., 2011; Kwong et al., 2005).

Many single-stranded positive-sense RNA viruses have been reported to use the host intracellular membranes as platforms for viral replication (Strauss and Strauss, 1994; Wimmer et al., 1993). The viral genome replication is thought to utilize viral proteins as well as host proteins (Kuo et al., 1996; Lindenbach et al., 2005; Lindenbach and Rice, 1997; Mackenzie et al., 1998; Uchil and Satchidanandam, 2003; Westaway et al., 1997). In our studies, we demonstrated that DDX3 was required for JEV replication, the infectious virus production was strongly inhibited by DDX3 knockdown. We also confirmed that helicase activity of DDX3 was crucial for JEV replication.

It has been predicted that JEV 5' and 3' UTR contain highly conserved RNA secondary structures involved in the regulation of viral translation and RNA replication (Brinton and Disposto, 1988; Song et al., 2008). The minus-strand RNA synthesis requires the presence of both the 5' and 3' UTR (You et al., 2001), and genomic RNA cyclization mediated by 5' and 3'–UTR is essential for viral replication in all mosquito-borne flaviviruses (Fan et al., 2011; Ivanji-Nagy and Darlix, 2012). DDX3 also has been reported to be involved in translation initiation (Geissler et al., 2012) and DDX3 can promote HIV proteins translation by binding to viral 5' UTR. It is most likely that DDX3 destabilizes the RNA structure locally to form a region large enough to allow the recognition of the m7GTP cap or loading onto the 40S subunit to initiate protein translation (Soto-Rifo et al., 2012). A recently published paper suggests that HCV 3' UTR retains ribosome complexes during translation termination to facilitate efficient initiation of subsequent rounds of translation (Bai et al., 2013). Our JEV replicon experiment confirmed that DDX3 positively regulates JEV protein translation.

JEV NS3 and NS5 proteins have been shown to play important roles in viral genome replication (Chen et al., 1997). Although we confirmed the role of DDX3 in JEV protein translation, we could not rule out its role in viral RNA replication in our studies. Since DDX3 could bind to JEV 5' and 3' UTR and the helicase activity was necessary for JEV replication, we suspected that DDX3 might help the unwinding of the secondary structure viral RNA to enable viral RNA replication more efficiently; especially our GST-pulldown,
Fig. 6. The colocalization analysis of endogenous DDX3, JEV NS3, NS5 proteins and viral RNA. (A) The location of endogenous DDX3 in JEV infected BHK-21 cells and the colocalization of DDX3 with JEV NS3 and NS5 proteins. BHK-21 cells were infected with JEV (MOI = 0.01), 48 h later, DDX3, JEV NS3 and NS5 proteins were detected using their polyclonal antibodies and the colocalization study was performed under confocal microscopy. (B) The colocalization of DDX3 and viral RNA. BHK-21 cells were infected with JEV (MOI = 0.01), endogenous DDX3 was detected by anti-DDX3 polyclonal antibody and the viral RNA was detected (red) using the Click-iT® RNA Imaging Kit at 24 hpi under confocal microscopy. The mock-infected cells were also included as a control.
co-immunoprecipitation and confocal microscopy analyses demonstrated that DDX3 could interact with JEV NS3, NS5 and viral RNA. Collectively, our results suggested that DDX3 regulates JEV replication by modulating JEV protein expression. Therefore, the drugs targeting DDX3 might be used for treatment of Japanese encephalitis virus or other flaviviruses infection.

Materials and methods

Viruses, cells and transfection

BHK-21 cells (C-13, American Type Culture Collection) were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO₂. SA14-14-2 strain of JEV was propagated in BHK-21 cells, and the viral titers were determined by plaque formation assay. The cells were transfected using polyethylenimine (25 kDa; Sigma-Aldrich). The amount of plasmid DNA with which the cells were transfected (1 μg per well of 24-well plate and 10 μg per 100 mm plate) was kept constant by the inclusion of empty expression vector. Transfection efficiencies around 70% were routinely obtained by using pEGFP-N2 (Clontech) plasmid to monitor the transfection efficiency.

Plasmids and RNA interference

To construct HA-tagged pcDNA3.1-DDX3, pGEX-4T-3-DDX3, pCold-I-DDX3, and DDX3 gene were amplified by PCR using HA-DDX3 plasmid (as a gift from Prof. K.T. Jeang) as the template with specific primers and cloned into pcDNA3.1-(+) (Invitrogen), pGEX-4T-3 (GE Healthcare) or pCold-I (Takara) vector respectively. The genes encoding JEV core, NS3, NS5-MTase (1–268) or NS5-RdRp (318–905) were also subcloned into pET-24a (+) vector (Novagen). The Oligonucleotides GTGCCGTCTTGGTTAGAAA was cloned into pGPU6/Neo vector as short hairpin RNA (shRNA) plasmid against DDX3 (Lai et al., 2008). The oligodeoxyribonucleotide encoding non-targeting shRNA (GTTCTCCGAACGTGTCACGT) was cloned into pGPU6/Neo vector by Genepharma (Shanghai, China), which was used as the negative control. pcDNA3.1-DDX3-K230E-HA, pcDNA3.1-DDX3-S382L-HA mutants were made with Quick-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions using pcDNA3.1-DDX3-HA as the template by PCR using specific primers. To construct RNA interference resistant pcDNA3.1-DDX3r, pcDNA3.1-DDX3r-K230E-HA or pcDNA3.1-DDX3r-S382L-HA mutant plasmid, the correspondent sequence to DDX3 shRNA in these DDX3 mutants was mutated into GTCCCATCATGGCTGGAG without introducing any residue change by using QuikChange site-directed mutagenesis kit (Stratagene) using pcDNA3.1-DDX3-HA, pcDNA3.1-DDX3-K230E-HA or pcDNA3.1-DDX3-S382L-HA as the template. The nucleotide sequences of all the plasmids were confirmed by DNA sequencing.

To construct the templates for JEV 5′ and 3′ UTR RNA transcription, the JEV 5′ or 3′ UTR gene was amplified by PCR with specific primers: JEV5′-sense primer: TAATAAGCAATCTATAGGCAGAGTGTGTTG-GAATGGTCGCTGGTAG, anti-sense primer: GGTAGCAGGTGAAGTCCTGGAAGCTGCGTTATACGACAGGAGGTCTTGCATGATCAGGAGCGGCTAACATGCAGTTAG; JEV 3′-sense primer: TAATAAGCAATCTATAGGCAGAGTGTGTTG-GAATGGTCGCTGGTAG, anti-sense primer: GGTAGCAGGTGAAGTCCTGGAAGCTGCGTTATACGACAGGAGGTCTTGCATGATCAGGAGCGGCTAACATGCAGTTAG; JEV 3′ anti-sense:GGCTAGAAGTCGGTATAGGTAAGAGGATAG, JEV 3′ anti-sense:GGCTAGAAGTCGGTATAGGTAAGAGGATAG. The JEV 5′ and 3′ UTR RNA templates were generated by PCR amplification of part of the ampicillin resistance gene from pcDNA3.1 (+) vector (1–250 bp) using ampicillin-sense
primer: TAATACGACTCACTATAGGGATGAGATTCAACTTTCCGTG-
TCG and ampicillin-anti-sense primer: GCCTCAGCAGACTGT-
AGCTAAAACCTCTGCTATGAACCGCTCATTACGGGATAATAC (Vashist et al., 2012). T7 promoter and tobramycin aptamer tag (Hartmuth et al., 2004) sequences underlined were included in sense and anti-
sense primer respectively. The PCR products were subcloned into pMD-18T vector (Takara, China). The nucleotide sequences of all the constructs were confirmed by DNA sequencing.

The JEV replicon with Renilla luciferase gene was generated by cloning JEV SA14-14-2 strain cDNA into pBluescript II KS (Agilent Technologies) under the control of a T7 promoter as the previous study (Chien et al., 2011). The Renilla luciferase gene was inserted after the first 102 bp of the JEV C gene, followed by the foot-and-
mouth disease virus 2A self-cleaving protease (FMDV-2A) to enable the cleavage of the luciferase away from downstream nonstructural proteins (Lo et al., 2003; Varnavski and Khromykh, 1999). FMDV 2A was fused to the last 90 bp of the E gene that is necessary for the proper topology of the following viral proteins. To ensure RNA stability and processing, a hepatitis delta virus ribozyme was placed immediately adjacent to the 3’ end of the JEV cDNA followed by a bovine growth hormone (BGH) polyadenylation sequence. The JEV-replication-deficient replicon was constructed by introducing a mutation (GDD → AAG) in NS5 gene (Bligh et al., 2003) using Quick-Change site-directed mutagenesis kit (Stratagene) with the specific primers. The control construct: GAPDH 5’ and 3’ UTR-flanking luciferase reporter gene, was cloned into pMD-18T Vector (Takara, China).

**Protein expression and purification**

To express the proteins used in the experiments, the plasmid pCold-1-DDX3, pGEX-4T-3-DDX3, pET-24a (+)-core, pET-24a (+)-NS3, pET-24a (+)-NS5-Mtase, pET-24a (+)-NS5- RdRp, pGEX-4T-
3-NS3, pGEX-4T-3-NS5-Mtase or pGEX-4T-3-NS5-RdRp was transformed into E. coli expression strain Rosetta 2 cells. A single colony was used to inoculate 10 ml of Luria-Bertani (LB) media containing either ampicillin (100 μg/ml) or kanamycin (50 μg/ml), and the culture was grown at 37 °C overnight. The cultures were then diluted into the LB media containing either ampicillin (100 μg/ml) or kanamycin (50 μg/ml) and grown to an A600 of 0.6 at 37 °C; protein expression was then induced with 0.1 mM IPTG for 16–20 h at 22 °C. The cells were harvested by centrifugation at 5000g for 20 min at 4 °C and re-suspended in lysis buffer (100 mM NaCl, 20 mM Tris–HCl, pH 8.0, 0.1 mM PMSF). The cells were disrupted by sonication, and the supernatant was collected by centrifugation. The supernatant was then subjected to affinity purification using Bio-Rad Profinity™ IMAC or Profinity™ GST column (Bio-Rad). The proteins were then eluted, pooled together and further purified using size exclusion chromatography (Superdex75, GE Healthcare). The concentration of the purified protein was determined by Bradford assay.

**Antibodies**

The DDX3 rabbit polyclonal antibody, anti-β-actin, anti-GST, anti-His mononclonal antibodies and HRP conjugated secondary antibody were purchased from Abmart Company (Shanghai, China). The mononclonal antibody against the JEV envelope glyco-
protein was described previously (Ishag et al., 2013b). To produce anti-core, NS3 and NS5 polyclonal antibodies of mice source, the BALB/c mice free of pathogens was inoculated with 50 μg of NS5-
RdRp protein emulsified with equal amount of Freund’s complete adjuvant (Sigma-Aldrich) via subcutaneous injection. Booster doses were subsequently given at 1, 2 and 3 weeks later with 50 μg of NS3, NS5-RdRp emulsified with incomplete adjuvant. Finally, the serum of the mice was collected. The serum of the mice without immunized with any protein was collected as the negative serum. Both immunoblot and ELISA were performed to evaluate the immunoreactivity between JEV NS3 and NS5 proteins and their polyclonal antibodies. Animal use was in compliance with the Nanjing Agricultural University Institutional Animal Care and Use Committee.

**Plaque formation assay**

The viral culture supernatants with 10-fold dilutions (from 10^2 to 10^5) were added into 6-well plate with confluent monolayer of BHK-21. The plate was then incubated at 37 °C for 2 h, with gentle agitation at every 15-min interval. The excess virus inocula were removed by rinsing the cells with PBS for three times. Subse-
quently, overlay medium (2% low melting-point agarose with DMEM medium containing 2% FBS) was added to each well and further incubated at 37 °C with 5% CO₂ for 3–5 days. The cells were stained with 0.5% crystal violet.

**Cytotoxicity assay**

The cell viability of BHK-21 cells transfected with the plasmids was detected by the lactate dehydrogenase (LDH) activity, a stable cytosolic enzyme that is released upon cell lysis using CytoTox 96® NonRadioactive Cytotoxicity Assay (Promega) at 72 h after transfection. The Cytotoxicity of cmpl6 on BHK-21 cells was detected by CytoTox 96® NonRadioactive Cytotoxicity Assay at 48 h after being added to the cells with different the concentrations (0–200 ng/ml).

**GST pulldown assay**

50 μl glutathione-Sepharose 4B beads (GE Healthcare) were resuspended in binding buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.5% NP-40, and 0.1 mM PMSF). 10 μg purified GST-DDX3, GST-NS3, GST-NS5-Mtase or GST-NS5-RdRp was incubated with the beads for 2 h at 4 °C under gentle agitation. Unbound proteins were washed away using washing buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.1 mM PMSF). The purified NS3, NS5-Mtase, NS5-RdRp or DDX3 protein was incubated with the beads overnight with gentle agitation at 4 °C respectively. The beads were washed four times with 1 ml of washing buffer. Finally the beads were resuspended in 200 μl of SDS-PAGE sample buffer and boiled. 15 μl protein sample was separated on 12% or 15% SDS-
PAGE gel and then transferred to PVDF membrane. The presence of the targeted protein was detected with either anti-DDX3 polyclonal antibody or anti-His mononclonal antibody. Glutathione-Sepharose 4B beads incubated with GST proteins were used as control.

**Co-immunoprecipitation assay**

BH1-21 cells were infected with JEV for 48 h. The cells were rinsed twice in cold phosphate-buffered saline and incubated for 3 h at 4 °C in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM PMSF, 1% NP-40) with gentle agitation. Cell debris was removed by centrifugation at 10,000g for 10 min at 4 °C. The protein A/G (Abmart) agarose beads coupled with DDX3 polyclonal antibody were incubated with the supernatant either in the absence or presence of RNase (100 μg/ml) and DNase (100 μg/ml) for 2 h at 4 °C. The beads were washed five times with 1 ml washing buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40), resuspended in 200 μl SDS-PAGE sample buffer and boiled. The protein samples were then sub-
tected to Western blot analysis. The targeted proteins were analyzed by western blot analysis using anti-DDX3, anti-NS3 or anti-NS5 polyclonal antibody.
Western blot analysis and Q-PCR analysis

The protein mixtures were separated on 12% or 15% SDS-PAGE gel and transferred to PVDF membrane (Millipore, USA) in a Trans-Blot SD semi-dry transfer cell (Bio-Rad, USA). The membrane was blocked with 5% non-fat milk powder in TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), and then probed with anti-JEV-envelope monoclonal antibody, anti-β–antibody to the bound antibody was detected using HRP-conjugated secondary antibody and visualized using enhanced chemiluminescence (GE Healthcare). The intensity band ratio of grayscale for JEV envelope, NS5 and DDX3 proteins were analyzed by ImageJ software using β-actin as the internal control.

JEV-specific RNA copy number was quantified using quantitative PCR (Q-PCR). The total RNA was extracted from JEV-infected BHK-21 cells with TRIzol reagent (Invitrogen), and purified according to the manufacturer’s recommendations. For cDNA preparation, total RNA (1 μg) was reverse transcribed with first strand cDNA synthesis kit (Takara, China). cDNA samples were amplified with the RT-PCR kit. Intracellular JEV genome levels were quantified with the SYBR Green Probe 3-step Q-PCR kit (Takara, China) and fluorescent quantization machine (ABI PRISM 7300 sequence detection system, Applied Biosystems). The primer for the JEV NS1 and β-actin were the following: NS1 sense primer: 5′-acatcctgctagatcaggtc-3′; antisense primer: 5′-gccagaaatcctaccagaag-3′; β-actin sense primer: 5′-catcctgaagactctgctgacac-3′; antisense primer, 5′-atgagcagcaccatca-3′ and cellular β-actin mRNA from the same RNA extract was used as an internal control in BHK-21 cells as the previous study (Ishag et al., 2013b).

In vitro RNA transcription and the interaction of JEV 5′ and 3′ UTR with DDX3

To synthesize RNA in vitro, purified plasmid was first linearized with BamHI or PspI enzyme. In vitro transcription of JEV replicon, replication-deficient replicon, GAPDH-luciferase, JEV 5′, 3′ UTR and control RNAs were performed using Riboprobe® System-T7 Kit (Promega, China) in a 20 μl reaction mixture containing 4 μl 5 × transcription buffer, 2 μl RNasin® RNA Inhibitor (40 U/μl), 1 μl each NTP (10 mM), 1 μl of linearized DNA template and 1 μl T7 RNA polymerase (20 U/μl). The mixture was incubated at 37 °C for 2 h. 10 μl of DNasel (1 U/μl) was added to the mixture and incubated at 37 °C for 30 min. The synthesized RNAs were purified using the RNeasy mini kit (Qiagen) and analyzed by 1% agarose gels. JEV 5′, 3′ UTR and control RNAs were added to the matrix beads conjugated with tobramycin (Hartmuth et al., 2004) and 2 h. The mixture was subjected to Western blot analysis using anti-bound antibody was detected using HRP-conjugated secondary antibody and visualized using enhanced chemiluminescence (GE Healthcare). The intensity band ratio of grayscale for JEV envelope, NS5 and DDX3 proteins were analyzed by ImageJ software using β-actin as the internal control.

Luciferase reporter assay

For the replicon luciferase reporter assay, BHK-21 cells were seeded in 24-well plates and transfected with DDX3 shRNA plasmid. 24 h later, the cells were transfected with 0.6 μg in vitro-transcribed JEV replicon RNA plus 0.2 μg of a firefly luciferase RNA in vitro-transcribed as an internal control using TransFast™ (Promega). At the indicated time points post transfection, cell lysates were collected for the dual-luciferase assay using Dual-Luciferase® Reporter Assay System Kit and GloMax® 20/20 Luminometer instrumentation (Promega).

Confocal microscopy analysis

BHK-21 cells were plated onto cover slips in a 6-well plate. In the following day, the cells were infected with JEV. 48 h later, the cells were fixed with ethanol for 30 min at 4 °C. Viral proteins and endogenous DDX3 were firstly stained with their polyclonal antibodies. The cells were then washed with PBS and treated for 60 min at room temperature with Rhodamine-conjugated or FITC-conjugated secondary antibody (Invitrogen, China). The coverslip was mounted onto the slide glass using PBS containing 50% glycerol. The cell nucleus was stained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, China). For viral RNA staining, cells were first infected with JEV (MOI=0.01). 3 h later, the cells were treated with actinomycin D (Sigma, China) for 4 h (15 μg/ml) (Xu et al., 2010) to inhibit cellular RNA transcription before 5-ethylvin uridine (EU) was supplied. The viral RNA was detected using the Click-IT® RNA Imaging Kits (Invitrogen, China) according to the manufacturer’s instructions. The coverslip was mounted onto the slide glass using PBS containing 50% glycerol. The cell nucleus were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, China). Confocal images were obtained using a Zeiss LSM 710 scanning confocal microscope.

Statistical analysis

All data were determined in triplicate and were representative of at least two separate experiments. The results represented the means ± standard deviations of triplicate determinations. The differences between means were considered significant at *p < 0.05 and very significant **p < 0.01. All statistical analyses were performed by one-way ANOVA using a SPSS 16.0 software package (version 16.0, SPSS Inc., Chicago, IL, USA).

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Ivanyi-Nagy, R., Darlix, J.L., 2012. Core protein-mediated inhibition of flavivirus RNA polymerase activity. Virology 425, 270–275.

Ishag, H.Z., Li, C., Huang, L., Sun, M.X., Wang, F., Ni, B., Malik, T., Chen, P.Y., Mao, X., 2013a. Inhibition of Japanese encephalitis virus infection in vitro and in vivo by pokeweed antiviral virus (PAV) drug, chlorpromazine. J. Gen. Virol. 84, 1737–1741.

Oswianska, A.M., Patel, A.H., 1999. Hepatitis C virus core protein interacts with a flavivirus RNA helicase. Biochem. Biophys. Res. Commun. 260, 330–340.

Park, S., Lee, S.G., Kim, Y., Song, K. 1998. Assignment of a human putative RNA helicase gene, DDX3, to human X chromosome bands p11.3–p11.3. Cytotherapy. Gene 11, 79–178.

Rafi, M., Falchi, F., Garbelli, A., Samuele, A., Bernardo, V., Paolucci, S., Baldanti, F., Schenone, S., Manetti, F., Maga, G., Botto, A., 2012. Discovery of the first small molecule inhibitor of human DDX3 specifically designed to target the RNA binding site: towards the next generation HIV-1 inhibitors. Bioorg. Med. Chem. Lett. 22, 2094–2098.

Ray, D., Shi, P.Y., 2006. Recent advances in flavivirus antiviral drug discovery and vaccine development. Recent Pat. Antifung. Drug Discov. 1, 45–55.

Rock, S., Linder, P., 2004. DEAD-box proteins: the driving forces behind RNA metabolism. Nat. Rev. Mol. Cell Biol. 5, 232–242.

Schröder, M., 2010. Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation. Bioorg. Med. Chem. 18, 59–69.

Shih, J., Tsai, T., Chao, C., Lee, Y.H.W., 2007. Candidate tumor suppressor DDX3 RNA helicase specifically represses cap-dependent translation by acting as an eIF4E inhibitor. Oncogene 26, 700–714.

Solomon, T., 2003. Recent advances in Japanese encephalitis. J. Neurovirol. 9, 274–283.

Song, B.H., Yun, S.I., Choi, Y.J., Kim, J.Y., Lee, C.H., Lee, Y.M., 2008. A complex RNA motif defined by three discontinuous S-nucleotide-long strands is essential for Flavivirus RNA replication. RNA 14, 1791–1811.

Soto-Rifo, R., Rubilar, P.S., Limousin, T., de Breyne, S., Decimo, D., Ohlmann, T., 2012. Analysis of antiviral aminoglycosides targeting mammalian ribosome 30S factors. Virology 428, 241–246.

Varnavski, A.N., Khromykh, A.A., 1999. Noncytopathic JEV replication in Neuro2A astrocytes. Virology 336, 299–307.

Wang, D., Camp 2nd, D.G., Rodland, K., Wiley, S., Britt, W., Shenk, T., Smith, R.D., 1994. Identification of a cellular protein that binds to the flavivirus NS3 RNA helicase by a novel strategy to inhibit viral replication. Curr. Med. Chem. 21, 1091–1100.

Wang, H., Ryu, S., 2009. DDX3 DEAD-Box RNA helicase inhibits hepatitis B virus reverse transcription by incorporation into nucleocapsid. J. Virol. 83, 5815–5824.

Wang, H., Ryu, W.S., 2010. DDX3 DEAD-Box RNA helicase inhibits hepatitis C virus core protein recruitment and blocks pattern recognition receptor signaling via interaction with DDX3. Implications for immune evasion. PLoS Pathog. 6, e1000896.

Westaway, E.G., Mackenzie, J.M., Kenney, M.T., Jones, M.K., Khromykh, A.A., 1997. Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS3 with virus-induced membrane structures. J. Virol. 71, 6560–6561.

Westaway, E.G., Mackenzie, J.M., Khromykh, A.A., 2003. Kunjin RNA replication and applications of Kunjin replication. Adv. Virus Res. 59, 99–140.
Wimmer, E., Hellen, C.U.T., Cao, X., 1993. Genetics of poliovirus. Annu. Rev. Genet. 27, 353–436.
Xu, L., Khadijah, S., Fang, S., Wang, L., Tay, F.P.L., Liu, D.X., 2010. The cellular RNA helicase DDX1 interacts with coronavirus nonstructural protein 14 and enhances viral replication. J. Virol. 84, 8571–8583.
Xu, Z., Anderson, R., Hobman, T.C., 2011. The capsid-binding nucleolar helicase DDX56 is important for infectivity of West Nile virus. J. Virol. 85, 5571–5580.
Xu, Z., Hobman, T.C., 2012. The helicase activity of DDX56 is required for its role in assembly of infectious West Nile virus particles. Virology 433, 226–235.
Yedavalli, V.S.R.K., Neuveut, C., Chi, Y., Kleiman, L., Jeang, K.T., 2004. Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. Cell 119, 381–392.

Yennamalli, R., Subharao, N., Kampmann, T., McGeary, R.P., Young, P.R., Kobe, B., 2009. Identification of novel target sites and an inhibitor of the dengue virus E protein. J. Comput. Aided Mol. Des. 23, 333–341.
Yoo, J.S., Kim, C.M., Kim, J.H., Kim, J.Y., Oh, J.W., 2009. Inhibition of Japanese encephalitis virus replication by peptide nucleic acids targeting cis-acting elements on the plus- and minus-strands of viral RNA. Antivir. Res. 82, 122–133.
You, L.R., Chen, C.M., Yeh, T.S., Tsai, T.Y., Mai, K.T., Lin, C.H., Lee, Y.H.W., 1999. Hepatitis C virus core protein interacts with cellular putative RNA helicase. J. Virol. 73, 2841.
You, S., Falgout, B., Markoff, L., Padmanabhan, R., 2001. In vitro RNA synthesis from exogenous dengue viral RNA templates requires long range interactions between 5′- and 3′-terminal regions that influence RNA structure. J. Biol. Chem. 276, 15581–15591.