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DDX3 RNA helicase is required for HIV-1 Tat function

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ABSTRACT

Host RNA helicase has been involved in human immunodeficiency virus type 1 (HIV-1) replication, since HIV-1 does not encode an RNA helicase. Indeed, DDX1 and DDX3 DEAD-box RNA helicases are known to be required for efficient HIV-1 Rev-dependent RNA export. However, it remains unclear whether DDX RNA helicases modulate the HIV-1 Tat function. In this study, we demonstrate, for the first time, that DDX3 is required for the HIV-1 Tat function. Notably, DDX3 colocalized and interacted with HIV-1 Tat in cytoplasmic foci. Indeed, DDX3 localized in the cytoplasmic foci P-bodies or stress granules under stress condition after the treatment with arsenite. Importantly, only DDX3 enhanced the Tat function, while various distinct DEAD-box RNA helicases including DDX1, DDX3, DDX5, DDX17, DDX21, and DDX56, stimulated the HIV-1 Rev-dependent RNA export function, indicating a specific role of DDX3 in Tat function. Indeed, the ATPase-dependent RNA helicase activity of DDX3 seemed to be required for the Tat function as well as the colocalization with Tat. Furthermore, the combination of DDX3 with other distinct DDX RNA helicases cooperated to stimulate the Rev but not Tat function. Thus, DDX3 seems to interact with the HIV-1 Tat and facilitate the Tat function.

1. Introduction

The gene expression of human immunodeficiency virus type 1 (HIV-1) is regulated transcriptionally by Tat through its binding to a nascent viral *trans*-activation responsive (TAR) RNA [1,2], and post-transcriptionally by Rev through its association with Rev-responsive element (RRE) RNA in the *env* gene [3–5]. Tat binds to TAR RNA and recruits transcription factors, such as p300/CREB-binding protein (CBP), p300/CBP-associated factor (PCAF), chromatin remodeling factors Brahma (BRM), Brahma-related gene 1 (BRG1), integrase interactor 1 (INI1), and positive transcription elongation factor (P-TEFb), a complex of cyclin T1 and cyclin-dependent kinase 9 (CDK9), to stimulate both transcription initiation from the HIV-1 long terminal repeat (LTR) and transcription elongation [1,2,6,7]. CDK9 hyperphosphorylates the C-terminal domain (CTD) of RNA polymerase II and activates transcription elongation.

DEAD-box RNA helicases are involved in various RNA metabolic processes, including transcription, translation, RNA splicing, RNA transport, and RNA degradation in the ATPase-dependent manner [8–10]. Host RNA helicases may be involved in HIV-1 replication [11,12], since HIV-1 does not encode an RNA helicase. In fact, DDX1 and DDX3 have been implicated in the replication of HIV-1 replication [13–15]. Both DDX1 and DDX3 interact with HIV-1 Rev and enhance Rev-dependent HIV-1 nuclear export [13–15]. In addition, we recently demonstrated that several DDX, including DDX1, DDX3, DDX5, DDX17, DDX21, and DDX56 interact with HIV-1 Rev and enhance the Rev-dependent nuclear export [16]. However, the role of these DDX DEAD-box RNA helicases in HIV-1 Tat function is still unknown. To address this issue, we first examined the interaction of these DDX RNA helicases with HIV-1 Tat and the potential role of DDX in the Tat function.

2. Material and methods

2.1. Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS).

2.2. Plasmids

We used pcDNA3-HA, pcDNA3-FLAG, pHLV-1-LTR-Luc [17], pHA-DDX3 [13,18,19], pcRev, pDM628 [14,15,20], pcDNA3-Tat101-FLAG [21], pcDNA3-HA-DDX1, pcDNA3-HA-DDX5, pcDNA3-HA-DDX17, pcDNA3-HA-DDX21, and pcDNA3-HA-DDX56 [16].

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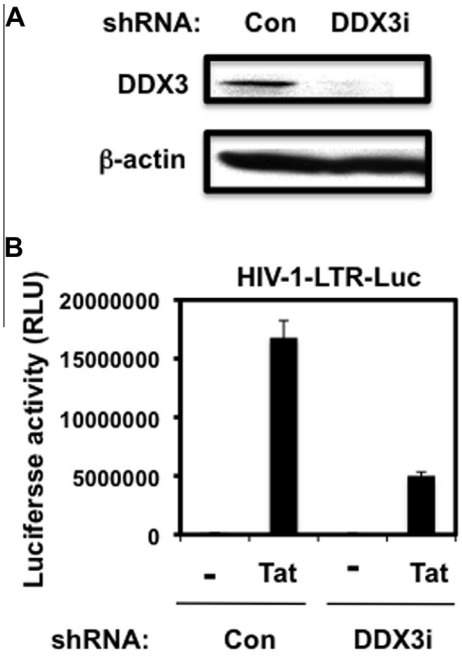

Fig. 1. Requirement of DDX3 for HIV-1 Tat function. (A) Inhibition of DDX3 expression by shRNA-producing lentiviral vector. The results of Western blot analysis of cellular lysates with anti-DDX3 or anti-β-actin antibody in the cells expressing shRNA targeted to DDX3 (DDX3i) as well as in the cells transduced with a control lentiviral vector (shCon) are shown. (B) HIV-1 Tat-mediated transcription in the DDX3 knockdown cells. Cells (2×10^4 cells) were cotransfected with pHIV-1-LTR-Luc [7,17,20] (100 ng) and/or pcDNA3-Tat101-FLAG [21] (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured. Results are from three independent experiments.

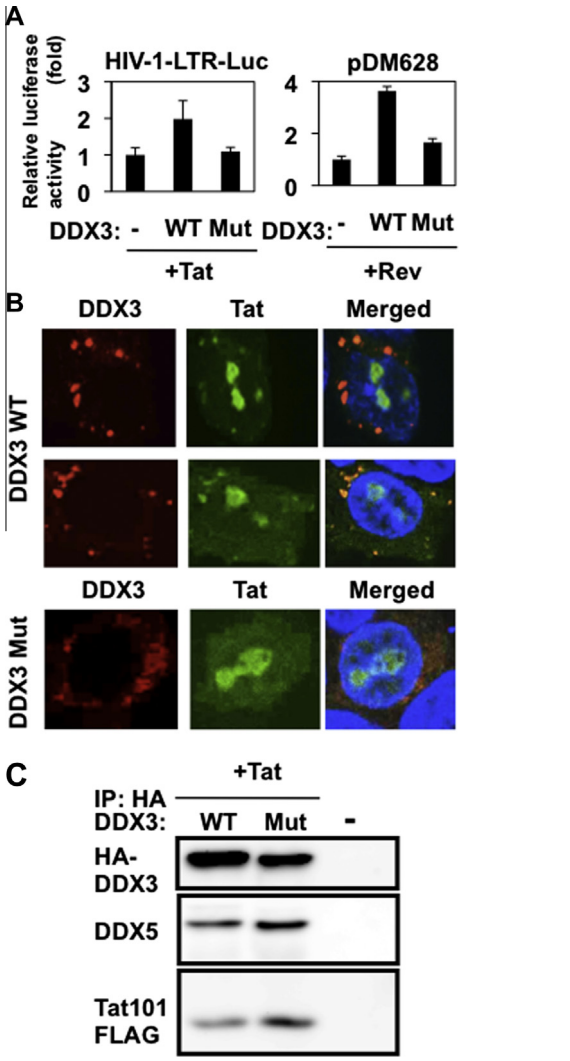

Fig. 2. DDX3 interacts with HIV-1 Tat in cytoplasmic foci. (A) The ATPase-dependent RNA helicase activity of DDX3 is required for the Tat function. 293FT cells (2×10^4 cells) were cotransfected with pHIV-1-LTR-Luc (100 ng), pHA-DDX3 WT (wild-type) or pHA-DDX3 Mut (ATPase-defective mutant) [13], and/or pcDNA3-Tat101-FLAG (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured. (B) DDX3 colocalizes with Tat. 293FT cells cotransfected with pHA-DDX3 WT or pHA-DDX3 Mut (200 ng) and pcDNA3-Tat101-FLAG (200 ng) were examined by confocal laser scanning microscopy. Cells were stained with anti-DDX3 (LS-C64576) and Cy3-conjugated anti-mouse IgG antibody followed by staining with FITC-conjugated anti-FLAG antibody, and then visualized with Cy3 (DDX3) or FITC (Tat). Nuclei were stained with DAPI. Images were visualized by using confocal laser scanning microscopy. The right panels exhibit the two-color overlay images (Merged). (C) 293FT cells were cotransfected with pcDNA3-Tat101-FLAG (2 μg) and either pHA-DDX3 WT or pHA-DDX3 Mut (2 μg). The cell lysates were immunoprecipitated with an anti-HA antibody, followed by immunoblot analysis using anti-HA, anti-DDX5, or anti-FLAG antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Lentiviral vector production

pLV-DDX3i and the vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [18,22,23]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 [22,23] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pRDI292 into 293FT cells with FuGENE 6 (Promega, Madison, WI, USA).

2.4. Luciferase assay

Plasmids were transfected into 293FT cells (2×10^4 cells) using the FuGENE 6 transfection reagent. Luciferase assays were performed 24 h after transfection using luciferase assay reagent according to the manufacturer's instructions (Promega). All transfections utilized equal total amounts of plasmid DNA quantities owing to the addition of empty vector into the transfection mixture. Results were obtained through three independent transfections. A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect the luciferase activity.

2.5. Western blot analysis

Cells were lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P (NP)-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Supernatants from these lysates were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-HA (HA-7; Sigma, Saint Louis, MI, USA), anti-DDX3 (54257 [NT] and 5428 [IN]; Anaspec, San Jose, CA, USA), anti-DDX5 (A300-523A; Bethyl Laboratories, Montgomery, TX, USA) anti-β-actin (A5441, Sigma), or anti-FLAG antibody (M2, Sigma).

2.6. Immunoprecipitation

Cells were lysed in buffer containing 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.1% NP-40, 10 mM NaF, 1 mM DTT and 1 mM PMSF. Lysates were pre-cleared with 30 μl of protein-G-Sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden). Pre-cleared supernatants were incubated with 5 μg of anti-HA

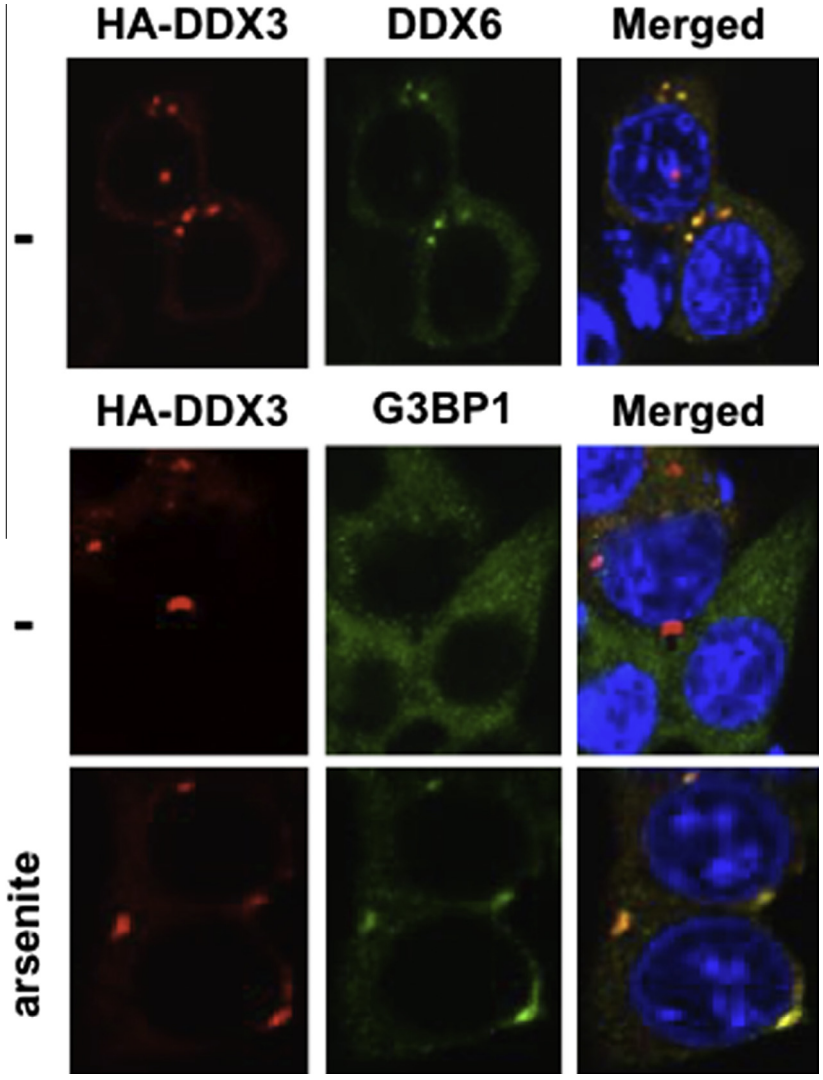

Fig. 3. DDX3 localizes in P-bodies and stress granules. 293FT cells expressing HA-DDX3 were treated with or without 0.5 mM arsenite for 45 min. Cells were stained with anti-HA and anti-DDX6, or anti-G3BP1 antibodies and were examined by confocal laser scanning microscopy. Nuclei were stained with DAPI.

antibody (3F10; Roche Diagnostics, Mannheim, Germany) at 4 °C for 1 h. Following absorption of the precipitates on 30 μl of protein-G-Sepharose resin for 1 h, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 2× Laemmli sample buffer. The proteins were then subjected to SDS–PAGE, followed by immunoblotting analysis using anti-HA, anti-DDX5, or anti-FLAG antibody.

2.7. Immunofluorescence and confocal microscopic analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% Nonidet P-40 in PBS at room temperature, and incubated with anti-HA (HA-7; Sigma), anti-DDX6 (A300-460A; Bethyl), anti-DDX3X (LS-C64576; LifeSpan BioSciences, Seattle, WA, USA), and/or anti-FLAG (M2) antibody at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37 °C for 30 min. They were then stained with Cy3-conjugated anti-mouse antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:300 dilution in PBS containing BSA at 37 °C for 30 min. Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole). Following extensive washing in PBS, the cells were mounted on slides using a mounting media of SlowFade Gold antifade reagent (Invitrogen) added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan).

3. Results

3.1. DDX3 facilitates HIV-1 Tat function

DDX3 DEAD-box RNA helicase is known to be required for efficient HIV-1 Rev-dependent RNA export [13]. However, it remains unclear whether DDX3 modulates the HIV-1 Tat function. To address this issue, we first used lentivirus vector-mediated RNA interference to stably knockdown DDX3. To express shRNA targeted to DDX3, we used a VSV-G-pseudotyped HIV-1-based vector system [22,23]. Western blot analysis of the lysates demonstrated very effective knockdown of DDX3 (Fig. 1A). In this context, the Tat-induced transcription from the HIV-1-long terminal repeat (LTR) within a transfected LTR-luciferase reporter plasmid [7,17,20] was significantly impaired to compared the control cells (Fig. 1B), suggesting that DDX3 is required for the Tat function. Reciprocally, overexpression of DDX3 enhanced the Tat-induced transcription as well as the Rev-dependent nuclear export function (Fig. 2A). Thus, DDX3 seems to facilitate the Tat function.

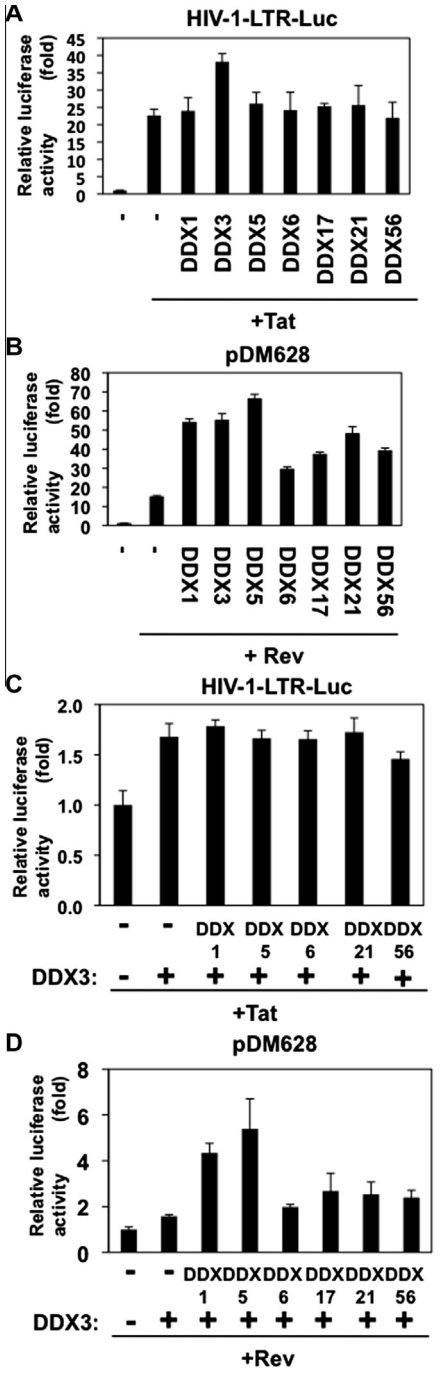

Fig. 4. Combination of distinct DDX RNA helicases cooperate to enhance the Rev but not the Tat function. (A) 293FT cells (2×10^4 cells) were cotransfected with pHIV-1-LTR-Luc (100 ng), pcDNA3-HA-DDX1, pHA-DDX3, pcDNA3-HA-DDX5, pcDNA3-HA-DDX6, pcDNA3-HA-DDX17, pcDNA3-HA-DDX21, or pcDNA3-HA-DDX56 (100 ng) [16], and/or pcDNA3-Tat101-FLAG (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured. Results are from three independent experiments. (B) 293FT cells (2×10^4 cells) were cotransfected with pDM628 (100 ng), pcDNA3-HA-DDX1, pHA-DDX3, pcDNA3-HA-DDX5, pcDNA3-HA-DDX6, pcDNA3-HA-DDX17, pcDNA3-HA-DDX21, or pcDNA3-HA-DDX56 (100 ng), and/or pcRev (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured. (C) 293FT cells (2×10^4 cells) were cotransfected with pHIV-1-LTR-Luc (100 ng), pcDNA3-Tat101-FLAG (100 ng), pHA-DDX3 (100 ng), and/or pcDNA3-HA-DDX1, pcDNA3-HA-DDX5, pcDNA3-HA-DDX6, pcDNA3-HA-DDX21, or pcDNA3-HA-DDX56 (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured. (D) 293FT cells (2×10^4 cells) were cotransfected with pDM628 (100 ng), pcRev (100 ng), pHA-DDX3, and/or pHA-DDX1, pcDNA3-HA-DDX5, pcDNA3-HA-DDX6, pcDNA3-HA-DDX17, pcDNA3-HA-DDX21, or pcDNA3-HA-DDX56 (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured.

3.2. DDX3 colocalized and interacted with HIV-1 Tat in cytoplasmic foci

To clarify the mechanism how DDX3 enhances the Tat function, we used an ATPase-defective mutant of DDX3 (Mut) [13]. Consequently, this mutant of DDX3 did not enhance the Tat-induced transcription as well as the Rev-dependent nuclear export function (Fig. 2A). Since this ATPase-defective mutant of DDX3 is known to abolish DDX3's RNA-unwinding activity, the ATPase-dependent RNA helicase activity of DDX3 seemed to be required for both Tat and Rev function. Recently, DDX3 was reported to associate with stress granules in translational regulation [24]. Consistently, wild-type (WT) DDX3 colocalized with Tat in the cytoplasmic foci, whereas ATPase-defective mutant of DDX3 (Mut) was dispersed in the cytoplasm and it did not colocalize with Tat (Fig. 2B). In fact, we observed that DDX3 localized in P-bodies and stress granules (Fig. 3). Moreover, HA-DDX3 (both WT and Mut), endogenous DDX5, and Tat could be co-immunoprecipitated with anti-HA antibody by the immunoprecipitation analysis, indicating that DDX3 forms a complex with Tat and DDX5 whether directly or indirectly (Fig. 2C).

3.3. Combination of distinct DDX RNA helicases cooperate to enhance the Rev but not the Tat function

So far, several DDX RNA helicases have been implicated in the HIV-1 Rev function [13–16], while the role of DDX DEAD-box RNA helicases on the HIV-1 Tat function was not fully understood. Finally, we further investigated the potential role of other DDX DEAD-box RNA helicases on the Tat function. For this, we examined whether various DDX DEAD-box RNA helicases, including DDX1, DDX3, DDX5, DDX6, DDX17, DDX21, and DDX56, modulates Tat-mediated *trans*-activation function. Consequently, only DDX3 could facilitate the Tat-induced transcription (Fig. 4A). In contrast, other DDX RNA helicases did not affect the Tat-induced transcription, while all tested DDX could stimulate the Rev-dependent nuclear export function using the Rev-dependent luciferase-based reporter plasmid pDM628 [14,15,20] (Fig. 4A and B), indicating that DDX3 specifically modulates the Tat function. As well, we found that various combination series of DDX3 with other DDXs, including DDX1, DDX5, DDX6, DDX21, and DDX56, did not synergize to enhance the Tat function compared to that of DDX3 alone (Fig. 4C). Conversely, the combination of DDX3 with other DDXs could synergistically enhance the Rev function (Fig. 4D). Thus, DDX3 specifically enhanced the HIV-1 Tat function.

Altogether, DDX3 RNA helicases seemed to interact with HIV-1 Tat and modulated the Tat function.

4. Discussion

Host RNA helicases have been involved in HIV-1 mRNA transcription [11,12]. Indeed, the Werner syndrome helicase (WRN) and RNA helicase A (RHA) were reported to act as cofactors of Tat and enhance HIV-1 gene expression [25,26]. WRN, a member of the RecQ helicase family, participates in DNA replication, DNA recombination, double strand break repair, telomere maintenance, and p53 activation. Mutations of *wrn* gene cause the Werner syndrome (WS), an autosomal recessive premature aging disorder associated with cancer disposition. WRN also promotes RNA polymerase II-dependent transcription. Thus, WRN was recently shown to interact with HIV-1 Tat and promote HIV-1 LTR transactivation [25]. Indeed, WRN participates in the recruitment of PCAF/P-TEFb-containing transcription complex to HIV-1 LTR. In addition to WRN, RHA also promotes the TAR-dependent HIV-1 gene expression [26]. RHA increases both basal level transcription from

HIV-1 LTR and Tat-induced transcription. Furthermore, Cocude et al. reported that a novel DExH RNA helicase RH116 was found localized in the nucleus of HIV-1 infected cells and augmented the transcription of unspliced HIV-1 transcript [27], while it remains unclear whether RHA and RH116 directly interact with Tat and provide direct role of the Tat-induced transcription. On the other hand, we have demonstrated that DDX3 bound to Tat and modulated the Tat function (Figs. 1 and 2). Atleast, the ATPase-dependent RNA helicase activity of DDX3 was required for the modulation of Tat function (Fig. 2A). Accordingly, DDX3 was reported to associated with translational regulation through interaction with eIF4E/poly(A)-binding protein 1 (PABP1) in stress granules [24]. In fact, DDX3 WT but not DDX3 Mut colocalized with Tat in cytoplasmic foci (Fig. 2B) and DDX3 localized in stress granules (Fig. 3). In this regard, Soto-Rifo et al. recently reported that DDX3 associates with eIF4F complex through an eIF4G and PABP1 double interaction to promote translation initiation of HIV-1 genomic (g) RNA [28]. Similarly, RHA also enhances HIV-1 RNA translation [29] like DDX3. This function of RHA depends on its binding to the R-U5 sequence of HIV-1 RNA termed post-transcriptional control element (PCE). Thus, DDX3 seems to be involved in HIV-1 translation through an interaction with Tat and HIV-1 5′ untranslated region (UTR) in stress granules.

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