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Distinct DDX DEAD-box RNA helicases cooperate to modulate the HIV-1 Rev function

Mariko Yasuda-Inoue, Misao Kuroki, Yasuo Ariumi *

Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan

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ABSTRACT

RNA helicase plays an important role in host mRNA and viral mRNA transcription, transport, and translation. Many viruses utilize RNA helicases in their life cycle, while human immunodeficiency virus type 1 (HIV-1) does not encode an RNA helicase. Thus, host RNA helicase has been involved in HIV-1 replication. 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 distinct DDX RNA helicases cross-talk and cooperate to modulate the HIV-1 Rev function. In this study, we noticed that distinct DDX RNA helicases, including DDX1, DDX3, DDX5, DDX17, DDX21, DDX56, except DDX6, bound to the Rev protein and they colocalized with Rev in nucleolus or nucleus. In this context, these DEAD-box RNA helicases except DDX6 markedly enhanced the HIV-1 Rev-dependent RNA export. Furthermore, DDX3 interacted with DDX5 and synergistically enhanced the Rev function. As well, combination of other distinct DDX RNA helicases cooperated to stimulate the Rev function. Altogether, these results suggest that distinct DDX DEAD-box RNA helicases cooperate to modulate the HIV-1 Rev function.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus of the lentivirus genus with a positive strand RNA genome of 9 kb which encodes nine polypeptides, three structural proteins, Gag (group-specific antigen), Pol (polymerase) and Env (envelope), the accessory proteins, Vif, Vpu, Vpr, and Nef, and the regulatory proteins, Tat and Rev. The gene expression of 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]. Both Tat and Rev interact with several host factors in their transcriptional and post-transcriptional functions [1–6].

Since the intron-containing host RNA cannot leave the nucleus before it is completely spliced, HIV-1 needs to evade this form of host surveillance to export unspliced or partially spliced viral RNA into cytoplasm and produce HIV-1 structural proteins and accessory proteins. For this, Rev contains a classical leucine-rich nuclear export signal (NES) that recruits nuclear export receptor CRM1 [3–5]. Upon binding to the RRE together with the GTP-bound form of Ran, CRM1 forms the export complex and Rev/CRM1/RanGTP complex exports unspliced or partially spliced viral RNA from the nucleus to the cytoplasm [3–5].

Helicases are enzymes that hydrolyze nucleotide triphosphates (NTPs) and use the energy to unwind nucleic acid duplexes or to translocate along the nucleic acid strand. Depending on whether helicases unwind RNA or DNA duplexes, helicases are classified into RNA helicases and DNA helicases. Exceptionally, RNA helicase A (RHA) can unwind both RNA and DNA. DEAD (D-E-A-D: Asp-Glu-Ala-Asp)-box RNA helicases, which are ATPase-dependent RNA helicases and are found in all organisms from bacteria to humans, are involved in various RNA metabolic processes, including transcription, translation, RNA splicing, RNA transport, and RNA degradation [7,8]. Many viruses utilize RNA helicases in their life cycle. Indeed, we recently found that DDX3 and DDX6, DEAD-box RNA helicases, are required for hepatitis C virus (HCV) RNA replication [9,10]. In addition to DDX3 and DDX6, DDX5 binds to HCV NS5B RNA-dependent RNA polymerase and it is involved in the HCV replication [11,12]. Furthermore, DDX21 plays an important role in the translational control of a Borna disease virus (BDV) polycistronic mRNA [13]. Moreover, the capsid-binding nucleolar helicase DDX56 is important for infectivity of West Nile virus [14]. On the other hand, several viruses carry their own RNA helicases to assist the synthesis of their genome, such as HCV, flavivirus, severe acute respiratory syndrome (SARS) coronavirus, rubella virus, and alphavirus, however, HIV-1 does not encode an RNA helicase [15,16]. Thus, host RNA helicases may be involved in HIV-1 replication [15,17]. In fact, DDX1 and DDX3 have been implicated in the rep-

* Corresponding author. Address: Center for AIDS Research, Kumamoto University, 2-2-1, Honjo, Chuo-ku, Kumamoto 860-0811, Japan. Fax: +81 96 373 6834.
E-mail address: ariumi@kumamoto-u.ac.jp (Y. Ariumi).

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lication of HIV-1 replication [18–22]. Both DDX1 and DDX3 interact with HIV-1 Rev and enhance Rev-dependent HIV-1 nuclear export [18–22]. However, the role of cross talk of these DDX or other DDX DEAD-box RNA helicases is still unknown. To address this issue, we first examined whether distinct DDX RNA helicases cooperate to modulate the HIV-1 Rev function and these DDX interact with Rev.

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. Plasmid construction

To construct pcDNA3-HA-DDX1, pcDNA3-HA-DDX5, pcDNA-HA-DDX6, pcDNA3-HA-DDX17, pcDNA3-HA-DDX21, pcDNA3-HA-DDX56, or pcDNA3-FLAG-DDX5, a DNA fragment encoding DDX1, DDX5, DDX6, DDX17, DDX21, or DDX56 was amplified from total RNAs by reverse transcription (RT)-PCR using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) and the following pairs of primers: DDX1, 5′-CGGGATCCAAGATGGCGGCCTTCTCCGAGAT GGGTGTAATG (Forward), 5′-CCGCTCGAGTCAGAAGGTTCTGAA-CAGCTGGTTAGGAAG-3′ (Reverse); DDX5, 5′-CGGGATCCAAGAT-GTCGGGTTATTCGAGTGACCGAGACCGC-3′ (Forward), 5′-CCGCT CGAGTTATTGGGAATATCCTGTTGGCATTGGATA-3′ (Reverse); DDX6, 5′-CGGGATCCAAGATGAGCACGGCCAGAACAGAGAACCC TGTT-3′ (Forward), 5′-CCGCTCGAGTTAAGGTTTCTCATCTTCTA-CAGGCTCGCT-3′ (Reverse); DDX17, 5′-CGGGATCCAAGATGCCC ACCGGCTTTGTAG-3′ (Forward), 5′-CCGGCGGCCGCTCATTTACGT-GAAGGAGGA-3′ (Reverse); DDX21, 5′-CGGGATCCAAGATGCCGG-GAAAACTCCGTAGTGACGCTGGT-3′ (Forward), 5′-CCGCTCGA GTTATTGACCAAATGCTTTACTGAAACTCCG-3′ (Reverse); DDX56, 5′-CGGGATCCAAGATGGAGGACTCTGAAGCACT-3′ (Forward), 5′-CCGGCGGCCGCTCAGGAGGGCTTGGCTGTGGGTC-3′ (Reverse).

The obtained DNA fragments were subcloned into either *Bam*HI-*Xho*I or *Bam*HI-*Not*I site of the pcDNA3-HA or pcDNA3-FLAG vector [23], and the nucleotide sequences were determined. We previously described pHA-DDX3 [9,18].

2.3. Luciferase assay

Plasmids were transfected into 293FT cells (2×10^4 cells) using the FuGENE 6 transfection reagent (Promega, Madison, WI, USA). 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.

2.4. 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-HIV Rev (A2-832; Icosagen, Tartu, Estonia), anti-HA (HA-7; Sigma, Saint Louis, MI, USA), anti-DDX5 (A300-523A; Bethyl), or anti-FLAG antibody (M2, Sigma).

2.5. 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 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 2X Laemmli sample buffer. The proteins were then subjected to SDS–PAGE, followed by immunoblotting analysis using anti-HA, anti-FLAG, or anti-HIV-1 Rev antibody.

2.6. 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-DDX1 (IHC-00132; Bethyl, Montgomery, TX, USA), anti-DDX5 (A300-523A; Bethyl), anti-DDX6 (A300-460A; Bethyl), anti-DDX17 (A300-509A; Bethyl), anti-DDX21 (A300-627A; Bethyl), anti-DDX56 (A302-978A; Bethyl), anti-DDX3 (IN and NT; AnaSpec, San Jose, CA, USA), anti-DDX3X (LS-C64576; LifeSpan BioSciences, Seattle, WA, USA), and anti-HIV-1 Rev antibody (A2-832; Icosagen) 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 either fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 647 anti-rabbit IgG (Molecular Probes, Invitrogen) 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. Distinct DDX RNA helicaes interact with HIV-1 Rev and enhance the Rev function

To investigate the potential role of distinct DDX DEAD-box RNA helicases in HIV-1 Rev function, we first used the Rev-dependent luciferase-based reporter plasmid pDM628 [19,20,24] (Fig. 1A). As previously described, luciferase production was markedly stimulated by Rev, which induced a 15-fold increase in reporter signal (Fig. 1A). Then, 293FT cells were cotransfected with several HA-tagged DDX, including DDX1, DDX3, DDX5, DDX6, DDX17, DDX21, DDX56, pDM628, and/or Rev expression plasmid. While each DDX alone had no effect or marginal effect on the Rev-dependent luciferase-based reporter pDM628 in the absence of Rev (data not shown), all DDX synergized to stimulate the luciferase levels with Rev, respectively (Fig. 1B). Indeed, DDX1, DDX3, or DDX5 markedly enhanced the Rev function, while DDX6 had a marginal effect. Thus, distinct DDX RNA helicases seemed to regulate HIV-1 Rev function. Since both DDX1 and DDX3 interact with HIV-1 Rev and enhance Rev-dependent HIV-1 nuclear export [18–22], other DDX RNA helicases also might bind to Rev. To probe this possibility, we performed co-immunoprecipitation analyses on extracts of 293FT cells expressing Rev and HA-tagged DDX3, DDX5,

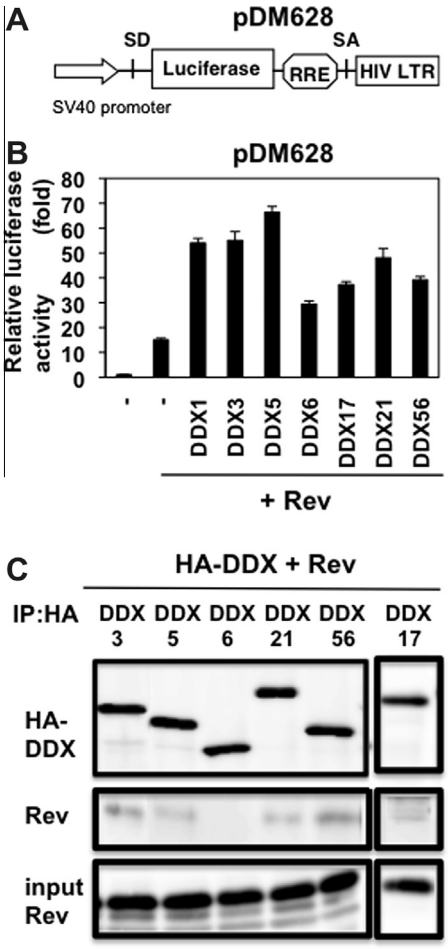

Fig. 1. Distinct DDX DEAD-box RNA helicases enhance HIV-1 Rev function. (A) Schematic representation of the Rev-dependent luciferase-based reporter plasmid pDM628 harboring a splice donor (SD), splice acceptor (SA), and Rev-responsive element (RRE). (B) 293FT cells (2×10^4 cells) were cotransfected with 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. Results are from three independent experiments. (C) 293FT cells were cotransfected with pHA-DDX3 (4 μg), pcDNA3-HA-DDX5, pcDNA3-HA-DDX6, pcDNA3-HA-DDX17, pcDNA3-HA-DDX21, or pcDNA3-HA-DDX56, and pcRev (4 μg). The cell lysates were immunoprecipitated with an anti-HA antibody, followed by immunoblot analysis using either anti-HA or anti-Rev antibody.

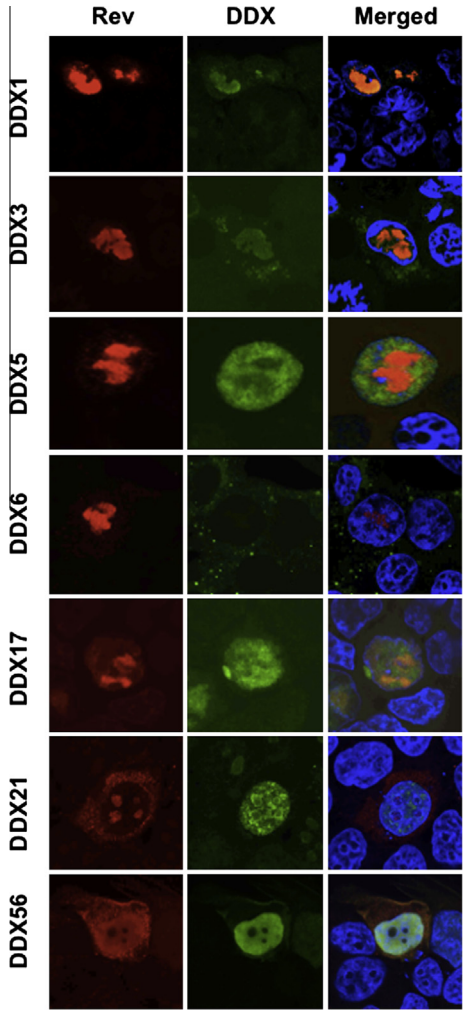

Fig. 2. Subcellular localization of DDX and Rev in 293FT cells. 293FT cells were cotransfected with pcDNA3-HA-tagged DDX1, DDX3, DDX5, DDX17, DDX21, or DDX56 (200 ng), and pcRev (200 ng). Cells were stained with anti-Rev and anti-DDX1, anti-DDX3, anti-DDX5, anti-DDX6, anti-DDX17, anti-DDX21, or anti-DDX56 antibodies 24 h after transfection and then visualized with Cy3 (Rev) or FITC (DDX). Nuclei were stained with DAPI. Images were visualized by using confocal laser scanning microscopy. The right panels exhibit the two-color overlay images (Merged).

DDX6, DDX17, DDX21, or DDX56 (Fig. 1C). Consequently, Rev and HA-DDX3, HA-DDX5, HA-DDX21, or HA-DDX56 could be immunoprecipitated with anti-HA antibody, indicating that Rev formed a complex with DDX3, DDX5, DDX17, DDX21, or DDX56, whether directly or indirectly. Importantly, Rev and DDX6 could not be immunoprecipitated, suggesting that Rev does not bind to DDX6 (Fig. 1C). Thus, we confirmed that several DDX DEAD-box RNA helicases except DDX6 could bind to Rev.

Then, we examined subcellular localization of Rev and HA-tagged DDX1, DDX3, DDX5, DDX6, DDX17, DDX21, or DDX56 in 293FT cells using confocal laser scanning microscopy (Fig. 2). Consequently, we observed that Rev predominantly localized in nucleolus. Both DDX1 and DDX3 colocalized with Rev in nucleolus and DDX3 also distributed in the cytoplasm. DDX5 mostly localized in nucleus and seemed to be partially colocalized with Rev in nucleus and nucleolus, while DDX6 predominantly localized in cytoplasmic speckles termed processing (P)-bodies. Consistent with the finding by immunoprecipitation analysis (Fig. 1C), DDX6 did not colocalize with Rev. Furthermore, DDX17 localized in both nucleus and nucleolus and DDX17 partially colocalized with Rev in the nucleolus. Moreover, DDX21 localized in nucleolus and DDX21 partially colocalized with Rev in the perinucleolar region. Notably, DDX56 altered the subcellular localization of Rev from nucleolus to nucleus and it mostly colocalized with Rev in the nucleus. Thus, several DDX RNA helicases except DDX6 could mostly or partially colocalize with Rev in the nucleolus or nucleus, suggesting that distinct DDX RNA helicases interact with Rev.

3.2. Distinct DDX RNA helicases cooperate to enhance the Rev function

To examine whether DDX3 and DDX5 cooperate to modulate the HIV-1 Rev function, 293FT cells were cotransfected with HA-tagged DDX3, HA-DDX5, pDM628, and/or Rev expression plasmid. When both DDX3 and DDX5 were co-expressed, they synergistically enhanced the Rev function (Fig. 3A), suggesting that DDX3 and DDX5 cooperate to enhance the Rev function. To probe the mechanism of this phenomenon, we performed co-immunoprecipitation analyses on extracts of 293FT cells expressing HA-tagged

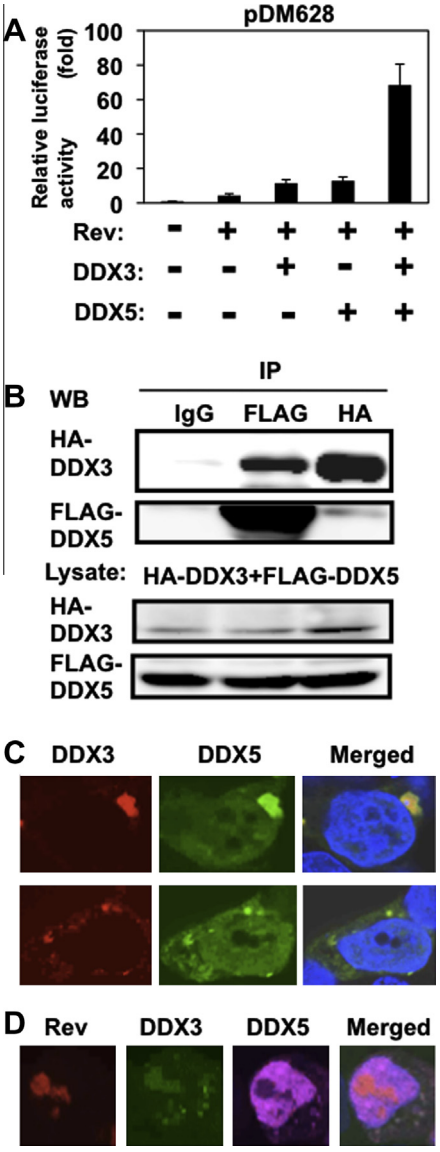

Fig. 3. DDX3 and DDX5 cooperates to enhance Rev function. (A) 293FT cells (2×10^4 cells) were cotransfected with pDM628 (100 ng), pcRev (50 ng), pHA-DDX3 (100 ng), and/or pcDNA3-HA-DDX5 (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured. Results are from three independent experiments. (B) 293FT cells were cotransfected with pHA-DDX3 (4 μg) and pcDNA3-FLAG-DDX5 (4 μg). The cell lysates were immunoprecipitated with an anti-HA, anti-FLAG antibody, or normal mouse IgG, followed by immunoblot analysis using either anti-HA or anti-FLAG antibody. (C) DDX3 colocalizes with DDX5. 293FT cells cotransfected with pHA-DDX3 (200 ng) and pcDNA3-FLAG-DDX5 (200 ng) were examined by confocal laser scanning microscopy. Cells were stained with anti-DDX3 (LS-C64576; LifeSpan) and anti-DDX5 antibodies and then visualized with Cy3 (DDX3) or FITC (DDX5). (D) 293FT cells cotransfected with pcRev (200 ng), pHA-DDX3 (200 ng), and pcDNA3-FLAG-DDX5 (200 ng) were stained with FITC-conjugated anti-HA, anti-Rev, or anti-DDX5 antibody and then visualized with Cy3 (Rev), FITC (DDX3) or Far-red/Alexa Fluor 647 (DDX5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

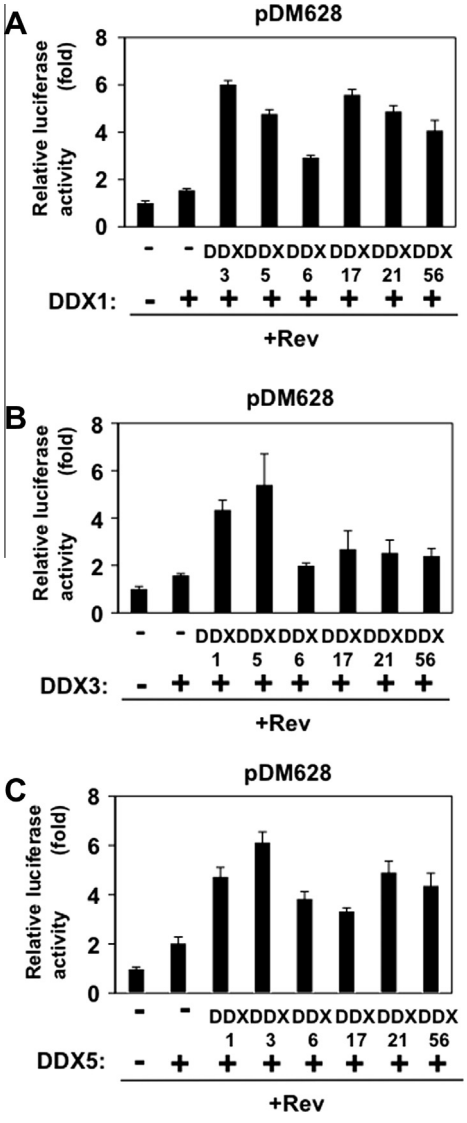

Fig. 4. Combination of distinct DDX RNA helicases cooperate to enhance the Rev function. (A–C) 293FT cells (2×10^4 cells) were cotransfected with pDM628 (100 ng), pcRev (100 ng) and/or combination with pcDNA3-HA-DDX1, pHA-DDX3, pcDNA3-HA-DDX5, pcDNA3-HA-DDX6, pcDNA3-HA-DDX17, pcDNA3-HA-DDX21, and/or pcDNA3-HA-DDX56 (100 ng). 24 h after transfection, luciferase activity in the cellular lysates was measured. Results are from three independent experiments.

DDX3 and FLAG-tagged DDX5 (Fig. 3B). Consequently, HA-DDX3 and FLAG-DDX5 could be co-immunoprecipitated with anti-HA or anti-FLAG antibody, indicating that DDX3 formed a complex with DDX5 whether directly or indirectly. Furthermore, DDX3 mostly colocalized with DDX5 in cytoplasmic speckles in the perinuclear region (Fig. 3C). Moreover, DDX3 relocalized and colocalized with Rev in nucleolus and DDX3 also colocalized with DDX5 in the cytoplasmic speckles, when DDX3, DDX5, and Rev were co-expressed in 293FT cells (Fig. 3D). Although DDX5 mostly localized in the nucleus, it faintly localized in the nucleolus as well as in the cytoplasmic speckles (Fig. 3D). Thus, Rev could interact with both DDX3 and DDX5.

To further confirm the cooperation of distinct DDX DEAD-box RNA helicases in the Rev function, we examined the luciferase assays with various combination series of distinct DDX, including DDX1, DDX3, DDX5, DDX6, DDX17, DDX21, and DDX56 (Fig. 4). In this context, we found that several combination of DDX (DDX1 and DDX3; DDX1 and DDX5; DDX3 and DDX5; DDX5 and DDX21) could synergistically enhance the Rev function (Fig. 4A–C).

4. Discussion

So far, it has been indicated that host RNA helicases may be involved in HIV-1 replication at multiple stages, such as the reverse transcription of HIV-1 RNA, HIV-1 mRNA transcription, the nu-

cleus-to-cytoplasm transport of HIV-1 mRNA, and HIV-1 particle assembly, since HIV-1 does not encode own RNA helicase [15,17]. Indeed, DDX1 and DDX3 have been implicated in post-transcriptional regulation of HIV-1 [18–22]. Both DDX1 and DDX3 interact with HIV-1 Rev and enhance the Rev-dependent HIV-1 nuclear export [18–22]. In addition to DDX1 and DDX3, we have demonstrated that DDX5, DDX17, DDX21, and DDX56, interacted with Rev and stimulated the Rev function (Figs. 1 and 2). Quite recently, Naji et al. employed the proteomics and statistical analysis to identify candidate host cell factors that interact with Rev/RRE [25]. In addition to above DDX RNA helicases, they also identified DHX36, DDX24, DHX9, and DDX47 as the Rev-binding DEAD/H-box proteins [25]. Their interactome analysis of HIV-1 Rev supports and compensates our and their primary findings each other, since they did not demonstrate the direct evidence of these DDX RNA helicases modulate on the Rev function as well as their subcellular localization.

Lorgeoux et al. proposed that DDX1 and DDX3 act sequentially in the Rev-dependent RNA export [17]. DDX1 first binds to Rev and promotes Rev oligomerization on the RRE RNA. Then, the oligomerized Rev recruits the CRM1/DDX3 complex that subsequently exports the RRE-containing HIV-1 RNA into the cytoplasm. Accordingly, we have found that combination of DDX1 and DDX3 cooperated to synergistically enhance the Rev-dependent nuclear export function (Fig. 4). We have demonstrated an interaction of DDX3 with DDX5 (Fig. 3), indicating the cross-talk among distinct DDX RNA helicases. In this context, other combinations of distinct DDX RNA helicases, such as DDX3 and DDX5 or DDX1 and DDX21, synergistically cooperated to stimulate the HIV-1 Rev function (Fig. 4). In addition to DDX, RHA also plays a role in Rev/RRE-dependent post-transcriptional regulation of HIV-1 [26]. RHA binds weakly to HIV-1 RRE independently of Rev. Thus, various RNA helicases seemed to be involved in the post-transcriptional regulation of HIV-1, however, it remains to be clarified when and where these distinct RNA helicases sequentially or hierarchically orchestrate the Rev-dependent RNA export.

Although we failed to observe the prominent effect of DDX6 on the HIV-1 Rev function (Fig. 1), DDX6 was reported to affect the viral genome packaging of foamy virus, a spumaretrovirus [27]. Relocation of DDX6 from P-bodies and stress granules to virus assembly sites at the perinuclear region was seen in foamy virus infected cells. However, DDX6 did not interact with Gag proteins and was not incorporated into the virion. In contrast, Reed et al. recently reported that DDX6 and Ago2 bind to HIV-1 Gag and DDX6 facilitates Gag assembly independent of HIV-1 RNA packaging [28]. Notably, DDX6 and Ago2, that are major components of P-body and act as microRNA effectors, suppress the HIV-1 replication [29,30].

Finally, Van't Wout et al. and Krishnan and Zeichner reported that the expression of several DDX RNA helicases, including DDX10, DDX18, DDX21, DDX23, DDX39, and DDX52 is modulated during HIV-1 infection [31–33]. Intriguingly, several DDX RNA helicases were upregulated during HIV-1 infection from latency toreactivation [33], however, the biological significance of these phenomenon was not understood. Altogether, several distinct DDX DEAD-box RNA helicases could cross-talk and contribute to the HIV-1 life cycle at multiple stages.

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