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The requirement of the DEAD-box protein DDX24 for the packaging of human immunodeficiency virus type 1 RNA

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Abstract

RNA helicases play important roles in RNA metabolism. Human immunodeficiency virus type 1 (HIV-1) does not carry its own RNA helicase, the virus thus needs to exploit cellular RNA helicases to promote the replication of its RNA at various steps such as transcription, folding and transport. In this study, we report that knockdown of a DEAD-box protein named DDX24 inhibits the packaging of HIV-1 RNA and thus diminishes viral infectivity. The decreased viral RNA packaging as a result of DDX24-knockdown is observed only in the context of the Rev/RRE (Rev response element)-dependent but not the CTE (constitutive transport element)-mediated nuclear export of viral RNA, which is explained by the specific interaction of DDX24 with the Rev protein. We propose that DDX24 acts at the early phase of HIV-1 RNA metabolism prior to nuclear export and the consequence of this action extends to the viral RNA packaging stage during virus assembly.

Keywords: HIV-1; RNA helicase; DDX24; Virus infectivity; Viral RNA packaging

Introduction

RNA helicases represent a large family of proteins that are able to hydrolyze nucleotide triphosphates and use the energy to alter RNA structure and remodel RNP complex (Cordin et al., 2006; Tanner and Linder, 2001). All RNA helicases contain the Walker A and Walker B motifs that are common to NTPase (Walker et al., 1982). On the basis of the conserved motifs, RNA helicases are grouped into five families named SF1 to SF5. The SF1 and SF2 members share five to seven conserved motifs. Their helicase core domain assumes two independent RecA-like folds (Caruthers and McKay, 2002). These helicases function as monomers or dimers (Tuteja and Tuteja, 2004a,b). Helicases of the SF3 to SF5 families are mostly of viral or bacterial origin (Patel and Picha, 2000). They each have one RecA-like motif and form hexamers. RNA helicases are involved in virtually every phase of RNA life ranging from transcription, splicing, nuclear export, translation to degradation (Rocak and Linder, 2004). Of particular importance are the essential roles of RNA helicases in the splicing of pre-mRNA and the biogenesis of ribosomes. Eight RNA helicases have been shown to participate in RNA splicing and up to twenty are involved in ribosomal assembly (Linder, 2006; Silverman et al., 2003; Tanner and Linder, 2001).

Viruses also utilize RNA helicases at various stages of their life cycle. Many viruses carry their own helicases to assist the synthesis of their genome. These include both DNA viruses such as herpesvirus (Chattopadhyay et al., 2006), poxvirus (Jankowsky et al., 2000) and parvovirus (Christensen and Tattersall, 2002) as well as RNA viruses such as alphavirus (Gomez de Cedron et al., 1999) and flavivirus (Utama et al., 2000). Viruses that synthesize their genome within the cell nucleus tend to exploit cellular helicases and thus do not encode

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their own. Human immunodeficiency virus type 1 (HIV-1) is one example of this latter virus group.

HIV-1 is a retrovirus and packages two copies of positive-strand full-length viral RNA per virion (Frankel and Young, 1998). Replication of HIV-1 RNA starts with its reverse transcription into cDNA followed by its integration into the host chromosomal DNA to form a provirus. HIV-1 RNA is then transcribed from proviral DNA by the cellular RNA polymerase II, matures like all cellular pre-mRNAs including 5′- and 3′-end modifications as well as splicing, and is exported into the cytoplasm for translation. It is noted that there are three processes pertaining to HIV-1 RNA that do not normally occur to cellular RNA: nuclear export of the intron-containing viral RNA, packaging of viral RNA into the space-limited interior of virus particles, and reverse transcription within the cytoplasm. To remodel the structure of viral RNA while going through these latter processes, HIV-1 encodes the nucleocapsid (NC) protein that bears RNA chaperone activity and has been shown to regulate HIV-1 RNA packaging and viral reverse transcription (Levin et al., 2005). Results of recent studies begin to reveal that HIV-1 also hijacks cellular RNA helicases to facilitate certain steps of viral RNA replication (Jeang and Yedavalli, 2006).

The first example is DDX3 that has been shown to be an integral component of the CRM1-Rev–RRE (Rev response element) complex and regulates the nuclear export of HIV-1 RNA (Yedavalli et al., 2004). DDX1 also interacts with Rev and RRE, and has an effect on HIV-1 RNA export (Fang et al., 2005, 2004). It is plausible that DDX1 and DDX3 act together to promote the translocation of viral RNA through the nuclear pore complex (NPC) by remodeling the viral RNP complex. RNA helicase A (RHA) has been reported to associate with HIV-1 particles and contributes to viral RNA reverse transcription (Roy et al., 2006). In addition, RHA promotes HIV-1 gene transcription by binding to the TAR (Tat transactivation region) RNA sequence (Fujii et al., 2001; Li et al., 1999). The function of RHA also extends to the regulation of the replication of other viruses. For example, RHA associates with the RNA sequence named the constitutive transport element (CTE) that has been identified in Mason Pfizer Monkey Virus (MPMV) (Tang et al., 1997). Together with Tap (Tip-associated protein) and HAP95 (RHA associated protein 95), RHA stimulates the nuclear export of the CTE-containing viral RNA (Tang and Wong-Staal, 2000; Yang et al., 2001). Furthermore, it has recently been reported that RHA binds to an RNA sequence named PCE (post transcriptional control element) in the avian spleen necrosis virus and enhances translation (Hartman et al., 2006). The multiple functions of RHA in a number of viral processes likely result from its association with a range of viral and cellular factors.

In addition to the aforementioned RNA helicases, a few others have also been suggested to play roles in HIV-1 life cycle. One example is RH116 whose overexpression leads to increased levels of unspliced and singly spliced HIV-1 RNA (Cocude et al., 2003). The Werner syndrome helicase has recently been shown to play an important role in the transactivation of HIV-1 RNA transcription by the viral Tat protein (Sharma et al., 2007). Results of microarray analysis reveal that HIV-1 infection changes the expression levels of a few RNA helicases including DDX9, DDX11, DDX18, DDX21, DDX24 (Krishnan and Zeichner, 2004a,b; van 't Wout et al., 2003). Moreover, results of proteomic analysis of the purified Gag complex implicate association of the RHA, DDX18, DDX2l, and DDX24 RNA helicases with HIV-1 Gag protein (Roy et al., 2006). However, interaction of these latter RNA helicases with HIV-1 RNA or proteins and their roles in specific steps of viral replication remains unclear. In the present study, we provide evidence that the DDX24 RNA helicase is required for efficient HIV-1 RNA packaging and thus plays an important role in HIV-1 replication. This function of DDX24 in viral RNA packaging has not been observed with the RHA helicase that also interacts with Gag (Roy et al., 2006), indicating to some extent the specificity of this role for DDX24.

Results

Knockdown of DDX24 significantly reduces HIV-1 infectivity

We first asked whether DDX24 has a role in HIV-1 replication. To this end, two siRNA oligos siDDX24-1 and siDDX24-2 were designed to knock down DDX24 (Fig. 1A). After siRNA treatment, 293T cells were transfected with HIV-1 proviral DNA clone BH10. Results of Western blots showed that knockdown of DDX24 led to a modest increase in both the expression of viral Gag protein in cells and the yield of viruses in the culture supernatants (Fig. 1A). We then measured viral infectivity by infecting the TZM-bl indicator cells (Wei et al., 2002). The results showed that viruses generated from DDX24-knockdown cells were 2- to 3-fold less infectious than those produced by cells treated with control siRNA (Fig. 1B).

To verify that the decreased viral infectivity results from the knockdown of DDX24 and not of other cellular proteins, we mutated the target site of the siDDX24-2 RNA oligo in the DDX24 cDNA and generated the DDX24-siM DNA construct. The DDX24-siM mRNA still encodes the wild type DDX24 protein but becomes resistant to the siDDX24-2 RNA oligo. Indeed, transfection of pDDX24-siM DNA into the siDDX24-2-treated cells led to increased levels of DDX24 protein and also restored viral infectivity to wild type levels (Fig. 1C).

We further assessed the effect of DDX24-knockdown on HIV-1 replication by infecting SupT1 cells. The microRNA30-based shRNAmir expression vector named LMP was employed to generate the DDX24-knockdown SupT1 cell line (Figs. 2A and B). The DDX24-knockdown SupT1 cells exhibited the same morphology and similar growth rate as the control cells. We infected the control and the DDX24-knockdwon SupT1 cells with wild type HIV-1 and monitored viral growth by measuring the levels of viral reverse transcriptase activity in the culture fluids. The results of Fig. 2C show lower levels of virus production by the DDX24-knockdown SupT1 cells throughout the infection period with a 5-fold decrease by day 13. This data supports the results of the one-round infection assay shown in Fig. 1B. Most of the cells died as a result of HIV-1 infection after day 15 and tissue culture was thus discontinued. Taken

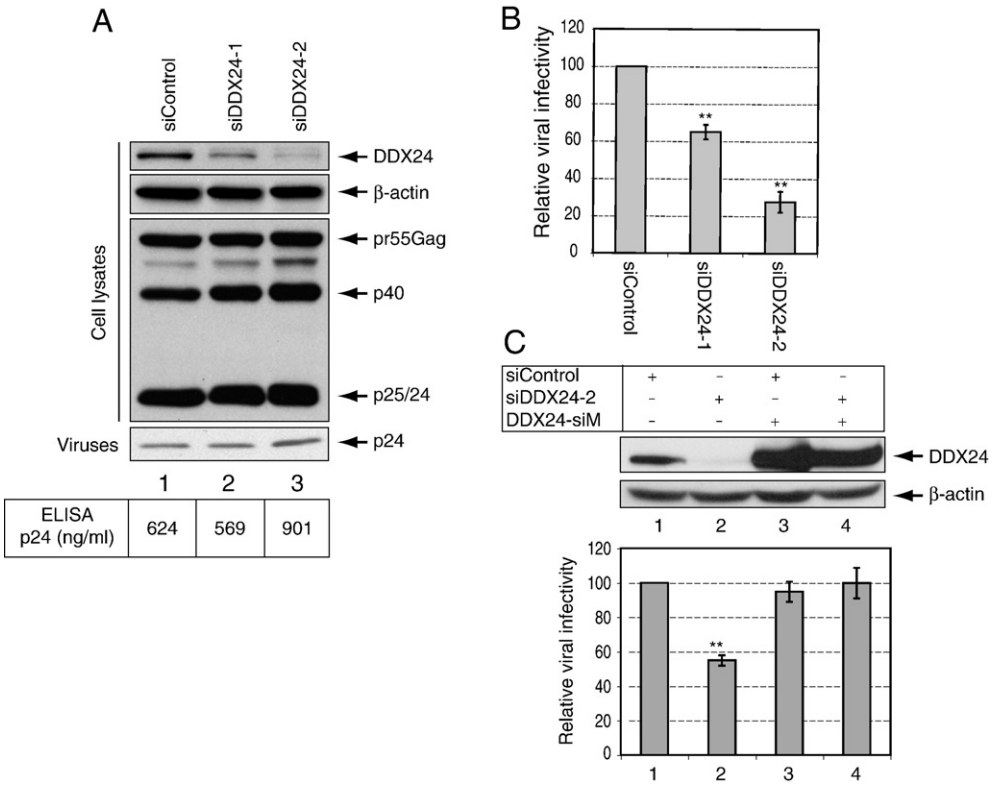

Fig. 1. DDX24-knockdown leads to decreased viral infectivity. (A). Effects of DDX24-knockdown on the expression of HIV-1 Gag protein and the production of virus particles. Levels of DDX24, Gag and β-actin in the lysates of 293T cells were assessed by Western blots. The amount of viruses in the culture supernatants was either determined in Western blots using anti-HIV p24 antibodies or by ELISA. Results shown represent one of the three independent transfection experiments. (B). Effect of DDX24-knockdown on viral infectivity. Viruses equivalent to 5 ng of p24(CA) were used to infect the TZM-bl indicator cells. Levels of firefly luciferase activity were measured 48 h after infection and the results are used to calculate viral infectivity. The infectivity of viruses from 293T cells that had been treated with control siRNA oligos was arbitrarily set as 100. The data shown represent three independent infections using virus stocks prepared by three independent transfection experiments. The standard deviations are shown. The p values were calculated using the T-Test program (two-sample, two-tail, assuming equal variances). The asterisks denote $p<0.001$. (C). Rescue of DDX24 expression in cells treated with the siDDX24-2 RNA oligos. The pDDX24-siM DNA was co-transfected with HIV-1 cDNA BH10 into 293T cells that had been treated with either the control siRNA or the siDDX24-2 RNA. The expression levels of DDX24 were monitored by Western blots. The infectivity of viruses was determined by infecting the TZM-bl indicator cells.

together, these results demonstrate that knockdown of DDX24 restricts HIV-1 replication.

DDX24-knockdown diminishes HIV-1 RNA packaging

We next measured the levels of virion-associated viral RNA in an attempt to identify the viral defects caused by DDX24-knockdown. Viral RNA was prepared from viruses containing 15 ng p24(CA) antigen and the RNA was further converted viral cDNA by reverse transcription using the p960-A primer that binds to the MA coding region, followed by regular PCR to amplify the viral DNA fragment spanning nucleotide positions 675 to 960. In the control experiment, no viral DNA signal was amplified directly from viral RNA samples (Fig. 3A, top, lanes 1–3), therefore ruling out contamination by viral plasmid DNA used in transfection. The results of RT-PCR revealed lower levels of viral RNA in the siDDX24-1 and siDDX24-2 virus samples than those in the control (Fig. 3A, lanes 4–6). This reduction was further quantified by real-time PCR to 3- to 4-fold for the DDX24-knockdown samples (Fig. 3A, bar graph).

HIV-1 genomic RNA is packaged into virus particles in the dimeric form (Paillart et al., 2004b; Russell et al., 2004). We were thus interested to know whether DDX24-knockdown also affects HIV-1 RNA dimerization in addition to the adverse effect on viral RNA packaging. To answer this question, viral RNA was extracted from virus particles, treated at a series of temperatures in a buffer containing 100 mM NaCl, followed by electrophoresis in 0.9% native agarose gels. The results of Northern blots showed that viral RNA was detected as dimers regardless of whether the samples were obtained from the control or the DDX24-knockdown experiments (Fig. 3B, lanes 1, 5, and 9). Furthermore, all viral RNA dimers were dissociated to monomers between 45 °C and 50 °C (Fig. 3B), suggesting that DDX24-knockdown does not change the thermo-stability of HIV-1 RNA dimers. It was also noted that the total levels of virion-associated viral RNA in the DDX24-knockdown samples were lower than those in the control (Fig. 3B), which is consistent with the real-time RT-PCR data in Fig. 3A.

We also studied whether the defective HIV-1 RNA packaging is a result of altered expression of viral RNA in cells. The Northern blot data showed similar levels of viral

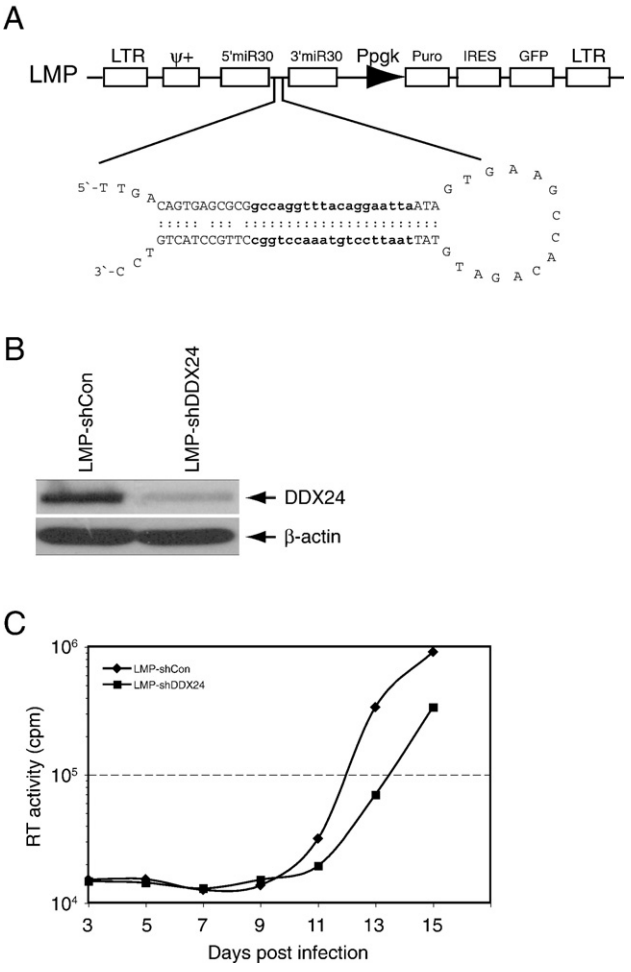

Fig. 2. HIV-1 replication is attenuated in the DDX24-knockdown SupT1 cells. (A). Illustration of the LMP vector and the hairpin sequence of the LMP-shDDX24 DNA. This microRNA is designed to target the DDX24 mRNA at nucleotide positions from 1330 to 1348. The mature miRNA sequence is shown in lowercase letters. (B). Western blots measuring levels of DDX24 protein in the LMP-shCon and the LMP-shDDX24 SupT1 cells. (C). Growth of HIV-1 in the LMP-shCon and the LMP-shDDX24 SupT1 cells. The culture fluid samples were collected at various time intervals, levels of reverse transcriptase activity were measured to monitor viral growth. Similar results were obtained in two independent infection experiments.

RNA (including the full-length and the spliced forms) in the control and in the DDX24-knockdown 293T cells that had been transfected with the BH10 DNA (Fig. 3C). Taken together, we conclude that knockdown of DDX24 adversely affects HIV-1 RNA packaging and as a result, restricts viral replication.

DDX24 interacts with HIV-1 Gag and Rev proteins

With the aim to gain insight into the mechanism underlying the involvement of DDX24 in HIV-1 RNA packaging, we studied the interaction of DDX24 with viral factors. Results of our previous proteomic analysis of the Gag complex reveal DDX24 as a potential Gag-associated cellular factor (Roy et al., 2006). We have now immunoprecipitated the Gag-Flag protein and assessed the DDX24 protein in the precipitates by Western blots using anti-DDX24 antibodies. The results showed that DDX24 was co-immunoprecipitated with Gag (Fig. 4A). Using a panel of Gag deletions, we were able to show that the NC sequence was required for Gag–DDX24 interaction (Fig. 4A). When cell lysates were treated with RNase A prior to the immunoprecipitation procedure, DDX24 was not co-precipitated with Gag, indicating the RNA-mediated nature of Gag–DDX24 interaction (Fig. 4B). It is known that the yeast homolog of DDX24 named MAK5 participates in the biogenesis of the 60S large ribosome subunit and is located within the nucleoli (Bernstein et al., 2006; Zagulski et al., 2003). Consistently, DDX24 was also seen within the nucleoli by indirect immunofluorescence staining (data not shown). Although HIV-1 Gag protein was mainly located within the cytoplasm, a minute amount was frequently seen within the nucleus (data not shown). We speculate that the interaction between the DDX24 and the Gag protein takes place within the nucleus.

The nucleolus-localization of DDX24 prompted us to investigate whether DDX24 interacts with the HIV-1 Rev protein that is also predominantly located within the nucleoli (Pollard and Malim, 1998). As expected, the endogenous DDX24 protein displayed significant co-localization with Rev (Fig. 4C). The interaction between DDX24 and Rev was further investigated by immunoprecipitation experiments. We first transfected 293T cells with the Rev-His DNA that expresses the Rev protein with the 6xhistidine tag (Invernizzi et al., 2006). The Rev-His protein was purified from cell lysates using the nickel-charged resin and assessed by Western blots. The results showed that the endogenous DDX24 was co-purified with the Rev-His protein (Fig. 4D). In accordance with this observation, immunoprecipitation of the DDX24-Flag protein also led to co-precipitation of the Rev protein (Fig. 4E). Interestingly, when the cell lysates were first treated with RNase A prior to immunoprecipitation, a significant amount of Rev protein was still pulled down together with DDX24-Flag (Fig. 4F). Thus, interaction of DDX24 and Rev is partially resistant to RNase treatment. Together, these data suggest the association of DDX24 with both Gag and Rev proteins, indicative that this RNA helicase is incorporated into the Rev–viral RNA or the Gag–viral RNA complexes likely at different stages of viral RNA metabolism, thus has the opportunity to actively modify the viral RNA conformation and regulate viral RNA functions including encapsidation into virus particles.

Knockdown of DDX24 moderately increases the nuclear export of unspliced HIV-1 Gag-Pol RNA

The interaction with Rev suggests a role of DDX24 in the function of Rev. In order to test this possibility, we assessed the effect of DDX24-knockdown on Rev-dependent expression of chloramphenicol acetyltransferase (CAT) from the pDM128 reporter DNA (Hope et al., 1990). The pGL3-Luc plasmid DNA was also co-transfected and levels of firefly luciferase activity were used as an internal control to correct transfection efficiency. The results showed that the Rev protein enhanced the level of CAT expression by 21-fold in the control cells as opposed to a 32-fold increase in the DDX24-knockdown cells (Fig. 5A). However, statistical analysis of the data indicates that

the difference is not significant ($p=0.1$). Therefore, DDX24-knockdown promotes the function of Rev only to a marginal extent if there is any. We further investigated this subject by measuring the Rev/RRE-dependent expression of Gag from the GPV-RRE DNA in the DDX24-knockdown cells. The results of Fig. 5B show that the GPV-RRE DNA produced higher levels of Gag protein in the DDX24-knockdown cells than in the control, which is consistent with a 2-fold increase of viral RNA expression in the knockdown cells (Fig. 5C, lanes 1 and 2). To answer whether DDX24-knockdown affects the nuclear export of viral RNA, RNA was extracted from the cytosol and the nuclear fractions followed by Northern blot analysis. As compared to the 2-fold increase of total viral RNA due to DDX24-knockdown, a 2.9-fold increase was measured for the levels of viral RNA in the cytoplasm (Fig. 5C, lanes 3 and 4). These data together suggest that knockdown of DDX24 moderately increases the Rev-dependent nuclear export of HIV-1 Gag-Pol RNA. Similar experiments were performed with the GPV-CTEx4 DNA that expresses viral RNA via the CTE/Tap mediated nuclear export pathway (Wodrich et al., 2000). The results showed that knockdown of DDX24 modestly inhibited the CTE-mediated nuclear export of HIV-1 RNA (Fig. 5C). Therefore, DDX24 exhibits a specific though moderate effect on Rev-mediated nuclear export of HIV-1 RNA.

Knockdown of DDX24 restricts packaging of viral RNA that is exported by the Rev-dependent pathway

It has recently been reported that, in addition to the export of viral RNA from the nucleus, Rev also warrants efficient packaging of viral genomic RNA into virus particles (Brandt et al., 2007). We speculate that this latter function of Rev may benefit from the associated cellular factors such as DDX24 that was shown herein to interact with Rev and its knockdown led to reduced viral RNA packaging. To further test this hypothesis, we measured the packaging of viral RNA that is expressed from the GPV-RRE and the GPV-CTEx4 DNA. Since both DNA vectors bear the intact 5′ UTR of HIV-1 RNA (Fig. 5B), the RNA transcribed from both vectors should be recognized by viral Gag protein with equal efficiency. Viral RNA was extracted from viruses of equal amounts of p24(CA) and quantified by real-time RT-PCR. The results showed that knockdown of DDX24 inhibited packaging of viral RNA into the GPV-RRE viruses by approximately 2-fold (Fig. 5D), which supports the results obtained with the full-length HIV-1 proviral DNA (Fig. 3). The GPV-CTEx4 viruses carried 2- to 3-fold less viral RNA than the GPV-RRE viruses (Fig. 5D). This result is consistent with the previous finding that Rev-mediated nuclear

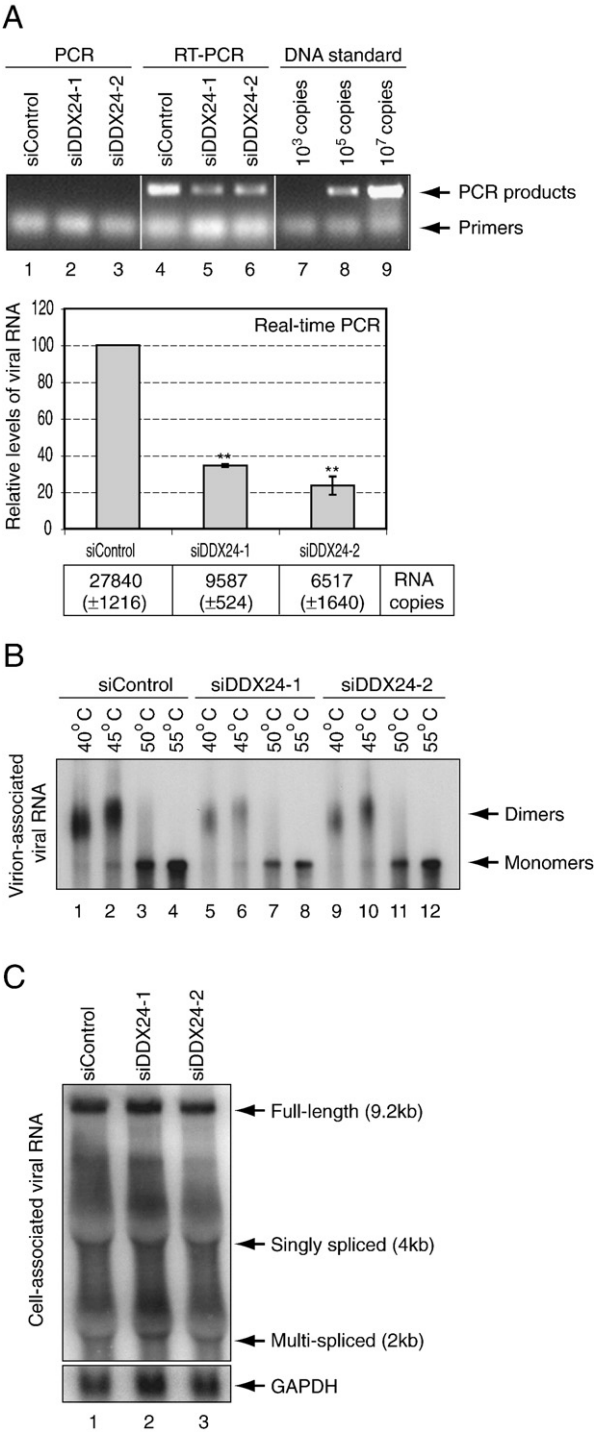

Fig. 3. DDX24-knockdown inhibits HIV-1 RNA packaging. (A). Viral RNA was extracted from viruses equivalent to 15 ng of p24(CA), followed by treatment with DNase I and reverse transcription using the p960-A primer. To rule out the possible contamination by plasmid DNA, the viral RNA samples were directly assessed by PCR (lanes 1–3). The reverse transcribed samples were analyzed by regular PCR using primers p675-S/p960-A (lanes 4–6). Dilutions of the BH10 DNA were amplified as standards (lanes 7–9). The reverse transcription products were further quantified by real-time PCR with the same primers. Levels of viral RNA in the control experiments are arbitrarily set as 100. Data shown in the bar graph are the average of three real-time RT-PCR assays measuring levels of viral RNA prepared from three independent virus stocks. Copies of the HIV-1 full-length RNA from one real-time PCR experiment are also shown. The asterisks denote $p<0.001$. (B). DDX24-knockdown does not affect HIV-1 RNA dimerization. Viral RNA was extracted from viruses equivalent to 200 ng of p24 (CA) and incubated at 40 °C, 45 °C, 50 °C or 55 °C prior to electrophoresis and Northern blots. (C). Expression of viral RNA in the control and DDX24-knockdown 293T cells. Total cellular RNA was extracted and an amount of 20 μg was analyzed in Northern blots using the [α-^{32}P] labeled HIV-1 DNA probes (nucleotide positions 1 to 2000). GAPDH mRNA was detected as an internal control (Ferraiuolo et al., 2005).

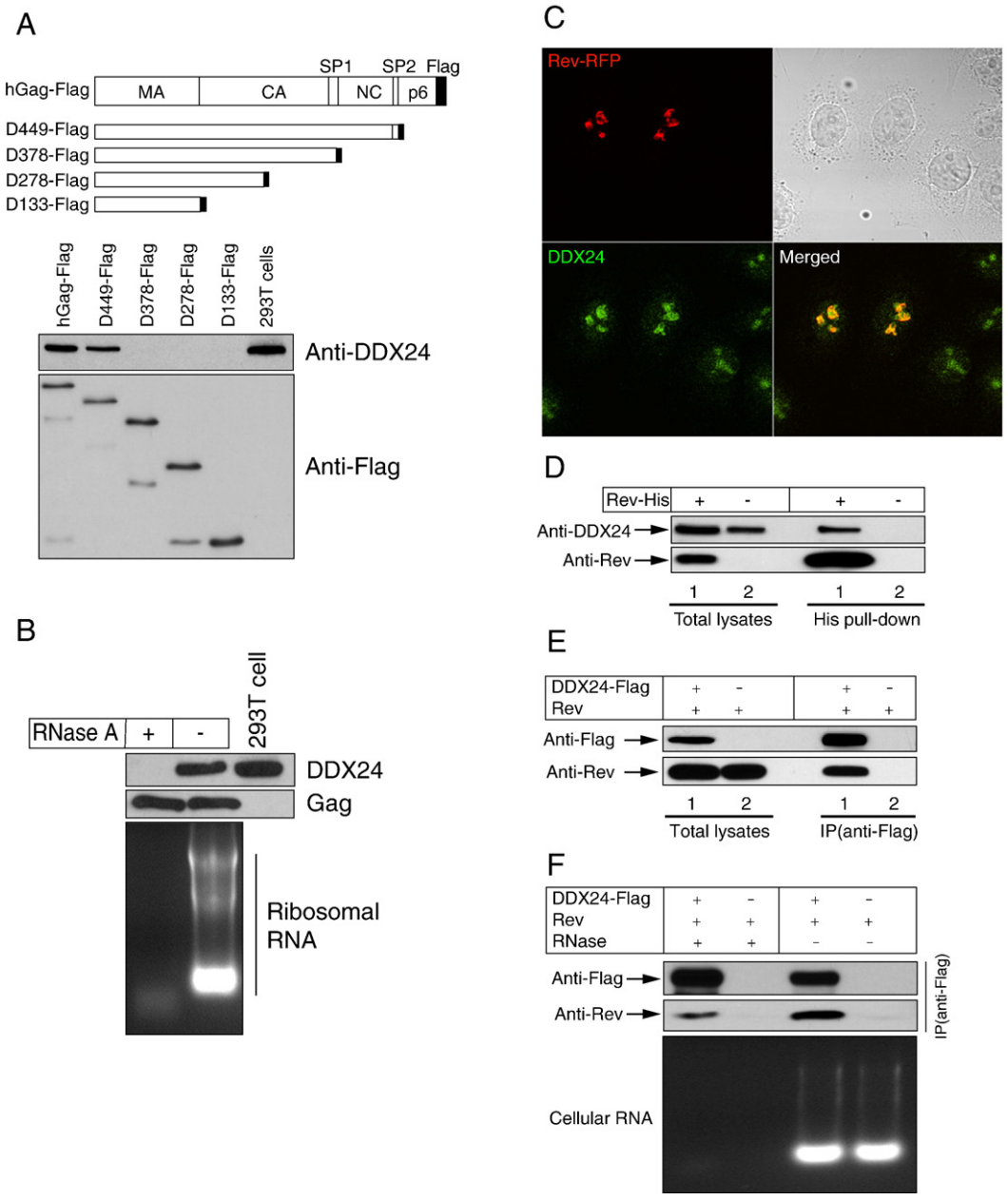

Fig. 4. Interaction of DDX24 with HIV-1 Gag and Rev proteins. (A). DDX24 was co-immunoprecipitated with Gag. Domain structures of the wild type hGag-Flag and the hGag mutants are illustrated. D449-Flag, D378-Flag, D278-Flag, and D133-Flag lack the p6, NC-p6, CA(CTD)-NC-p6, and CA-NC-p6 domains, respectively. The 293T cells were transfected with the wild type or the truncated versions of the Gag-Flag DNA followed by purification with the anti-Flag affinity gel. The eluted samples were subjected to Western blots using anti-DDX24 or anti-Flag antibodies. (B). DDX24-Gag interaction is sensitive to RNase A treatment. Cell lysates were treated with RNase A (100 μg/ml) prior to immunoprecipitation with anti-Flag affinity gel. The effectiveness of RNase A treatment was assessed by determining the levels of ribosomal RNA. (C). DDX24 colocalizes with Rev. HeLa cells were transfected with the Rev-RFP DNA. The endogenous DDX24 was immuno-stained with anti-DDX24 antibody. (D). The endogenous DDX24 is pulled down with Rev-His. The Rev-His protein was purified from 1 mg cell lysates with the nickel-charged agarose. The total cell lysates (50 μg) and the purified samples were subjected to Western blots using antibodies either against DDX24 or Rev. (E). Rev is co-immunoprecipitated with DDX24-Flag. The 293T cells were transfected with the Rev and the DDX24-Flag DNA. The DDX24-Flag protein was immunoprecipitated with the anti-Flag M2 affinity gel. The Rev and DDX24-Flag proteins were detected with anti-Rev and anti-Flag antibodies, respectively. (F). The interaction of DDX24-Flag and Rev is partially resistant to RNase A treatment. The total cell lysates with DDX24-Flag and Rev proteins were treated with RNase A (100 μg/ml) for 30 min at 37 °C prior to the immunoprecipitation procedure using anti-Flag antibodies. Levels of the DDX24-Flag and Rev proteins in the precipitated samples were assessed by Western blots. Total cellular RNA was extracted from cell lysates that had been treated or not treated with RNase A and separated by electrophoresis in 1% agarose gels.

export pathway confers higher packaging efficiency to HIV-1 RNA than the CTE-mediated pathway (Brandt et al., 2007). Interestingly, knockdown of DDX24 did not further reduce the levels of viral RNA associated with the GPV-CTEx4 viruses (Fig. 5D). These data suggest that the DDX24 protein exerts its effect on HIV-1 RNA packaging only in the context of the Rev-

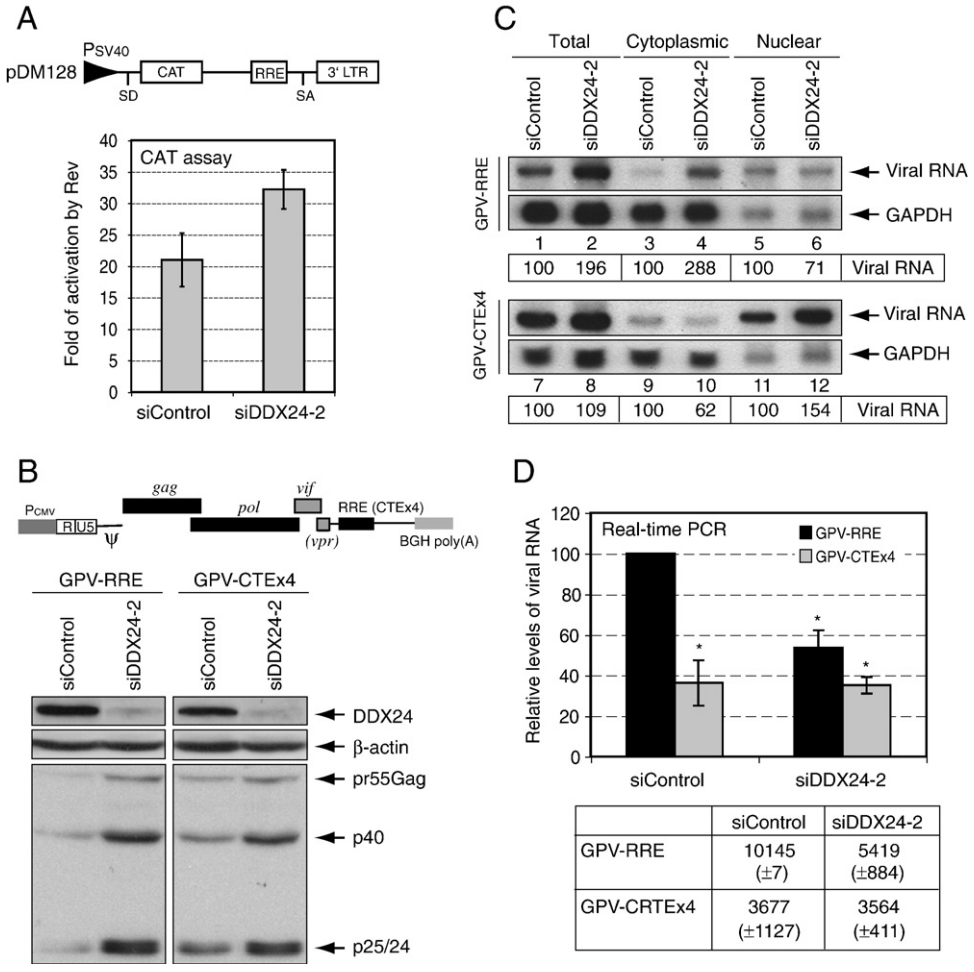

Fig. 5. Effects of DDX24-knockdown on the Rev-dependent gene expression and HIV-1 RNA packaging. (A). DDX24-knockdown stimulates Rev-dependent expression of the CAT enzyme. On the top is the schematic illustration of the pDM128 DNA (Hope et al., 1990). The pGL3-Luc plasmid DNA, which expresses firefly luciferase from the SV40 promoter, was included in the co-transfection. The levels of firefly luciferase were used as internal control to adjust the CAT activity. Levels of CAT activity in the presence of Rev expression were divided by the levels that were expressed from the pDM128 DNA alone. The results are used to represent the fold of activation by the Rev protein. The standard deviations are shown, the p value is calculated as 0.1. (B). Structures of the GPV-RRE and GPV-CTEx4 DNA are depicted. Both DNA constructs contain HIV-1 RNA sequence spanning nucleotide positions 1 to 5337 (referring to the transcription initiation site). The RRE or the CTEx4 cassette is inserted immediately downstream of the HIV-1 sequence. Levels of viral Gag proteins were assessed by Western blots using anti-HIV p24 antibodies. (C). Knockdown of DDX24 promotes the nuclear export of HIV-1 RNA. RNA was extracted from the total cell lysates, the cytosol or the nucleus, followed by Northern blots probing for HIV-1 RNA and GAPDH mRNA. The relative levels of viral RNA from each cellular fraction were calculated and are shown with those from the control cells as 100. (D). DDX24-knockdown inhibits viral RNA packaging into the GPV-RRE viruses. Viral RNA was extracted from viruses equivalent to 15 ng of p24(CA), and quantified in real-time RT-PCR. Levels of viral RNA associated with the GPV-RRE viruses produced from control siRNA-treated cells are arbitrarily set as 100 and the results are shown in the bar graph. The calculated p values are indicated (*denotes <0.01). Copies of viral RNA from one real-time RT-PCR experiment are also presented.

mediated nuclear export pathway likely by virtue of its direct association with the Rev protein.

Discussion

We previously attempted to identify Gag-associated cellular factors by proteomic analysis and the results unveiled four RNA helicases named RHA, DDX18, DDX21 and DDX24 (Roy et al., 2006). We now demonstrate that DDX24 contributes to HIV-1 replication by promoting the packaging of viral RNA into virus particles. This role of DDX24 in viral RNA packaging is likely specific since we did not observe any effect from RHA-knockdown in this regard (Roy et al., 2006). The function of DDX18 and DDX21 in viral RNA packaging was not pursued mainly because of the significantly reduced levels of viral RNA expression in cells with knockdown of either DDX18 or DDX21 (data not shown), which renders it difficult to accurately calculate the efficiency of viral RNA packaging.

DDX24 bears the nine motifs that are conserved in all DEAD-box proteins and is thus recognized as a putative RNA helicase. In light of this feature, DDX24 is speculated to regulate the function of viral RNA by remodeling the viral RNP complexes. One question is how DDX24 gains access to HIV-1 RNP, an issue that applies to almost all RNA helicases in terms of how their substrate specificity is determined. It is currently unclear whether DDX24 directly binds to HIV-1 RNA, but we did observe that DDX24 interacted with HIV-1 Rev protein that specifically recognizes a stretch of 234 nucleotides viral RNA

sequence within the *env* coding region termed RRE and directs the nuclear export of unspliced and singly spliced viral RNA (Pollard and Malim, 1998). In addition, DDX24 was also co-immunoprecipitated with viral Gag protein (Fig. 4A). Considering the specific interactions of Rev and Gag with viral RNA, we propose that DDX24 represents a component of the viral RNP complex and is able to rearrange viral RNA structures by exercising its helicase activity. In line with the predominant nucleolus-localization of DDX24, this RNA helicase is expected to exert its effect on viral RNA prior to the nuclear export event. Molecular details of this function of DDX24 remain to be investigated.

Knockdown of DDX24 affects two steps in viral RNA replication: the Rev-dependent nuclear export and the packaging into virus particles. As opposed to the requirement of DDX1 and DDX3 for Rev's function (Fang et al., 2005, 2004; Yedavalli et al., 2004), depletion of DDX24 moderately promotes the export of the Gag-Pol viral RNA (Fig. 5C). One explanation is that DDX24 reinforces the sequestration of Rev within the nucleoli. With decreased levels of DDX24, more Rev can shuttle between the nucleus and the cytoplasm and export more viral RNA. However, considering the moderate effect of DDX24-knockdown on HIV-1 RNA export, DDX24 may not be considered as an important helicase player in the Rev-Crm1 pathway.

The specific HIV-1 RNA packaging is determined by the high affinity of the NC domain of Gag precursor for viral RNA packaging signal termed ψ that is located within the 5′ UTR (Berkowitz et al., 1996; D'Souza and Summers, 2005). Up to date, little is known regarding where and when HIV-1 RNA packaging starts in cells. A recent study showed that HIV-1 Gag protein interacts with viral RNA at the perinuclear/centrosomal site, indicating that the selection of HIV-1 RNA by Gag initiates immediately following the export of viral RNA from the nucleus (Poole et al., 2005). In addition, the nuclear history of HIV-1 RNA also has an impact on its packaging into virus particles. This latter idea is highlighted by the involvement of Rev in this packaging process (Swanson and Malim, 2006; Swanson et al., 2004). It has been reported that the RRE-containing lentivector RNA is spread among cells at a much higher frequency upon HIV-1 infection than lentiviral vector RNA that does not bear the authentic RRE sequence (Lucke et al., 2005). Recent studies demonstrated that this is because the RRE-containing RNA is far more efficiently incorporated into HIV-1 particles (Brandt et al., 2007; Lucke et al., 2005). Yet, the underlying mechanism is still unclear. DDX24 may represent one important element involved in this mechanism considering that DDX24 binds to Rev and DDX24-knockdown inhibits the packaging of RRE-containing but not the CTE-containing viral RNA (Fig. 5).

DDX24 likely exerts its effect on HIV-1 RNA packaging by modifying viral RNA structures such as assisting the HIV-1 5′ UTR RNA to switch between two mutually exclusive conformations called the branched multi-hairpin (BMH) and the long-distance interaction (LDI) (Abbink and Berkhout, 2003; Berkhout et al., 2002; Ooms et al., 2004a,b). The BMH conformation properly presents the dimerization initiation signal and the RNA packaging signal, and thus is competent for dimerization and packaging (Berkhout et al., 2002; Ooms et al., 2004a). In contrast, these viral RNA signals are hidden in the LDI conformation. Interestingly, in an attempt to decipher the secondary structures of HIV-1 5′ UTR RNA by chemical modification assays, it has been discovered that in infected cells, HIV-1 RNA is not engaged in long range interactions including the LDI model (Paillart et al., 2004a). It is further suggested that HIV-1 RNA may initiate the dimerization process as early as right after transcription in the nucleus (Sinck et al., 2007). Considering the tendency of HIV-1 RNA to assume either the BMH or LDI conformation *in vitro*, it is arguable that cellular factors promote the folding of the BMH structure. DDX24 may represent a cellular factor of this kind. It will not be surprising if additional nuclear RNA helicases are discovered to modulate the function of HIV-1 RNA considering that the majority of cellular RNA helicases are located within the nucleus and that HIV-1 RNA needs to assume the right conformation when it leaves the nucleus. In support of this view, we recently reported the multiple effects of the overexpression of the DHX30 helicase on HIV-1 gene expression and viral RNA packaging (Zhou et al., in press). In contrast to the positive role of DDX24 in HIV-1 RNA packaging, overexpression of DHX30 leads to decreased levels of virion-associated viral RNA and hence blocks viral replication.

In summary, our data reveal an important role of DDX24 in HIV-1 RNA packaging. This function of DDX24 is attributed to its interaction with HIV-1 Gag and Rev proteins. Our finding further strengthens the influence of the nuclear history of HIV-1 RNA on its subsequent activities within the cytoplasm.

Materials and methods

Plasmid DNA constructs

The BH10 infectious HIV-1 cDNA clone was used in the following experiments. The pRev and pRev-His DNA constructs were kindly provided by Dr. Cedric F Invernizzi (Invernizzi et al., 2006). The pGL3-Luc plasmid DNA is a gift from Dr. Chris M. Brown (Rackham and Brown, 2004). To generate the pGPV-RRE and the pGPV-CTEx4 DNA, the RRE and the CTEx4 cassettes were amplified from HIV-1 cDNA BH10 and the MPMV cDNA using primer pairs RRE-S (5′-GAC TTC TAG ACC TCG AGG CGG CCG CGT AGC ACC CAC CAA GGC AAA G-3′) and RRE-A (5′-GAC TGG GCC CTA GCA TTC CAA GGC ACA GCA G-3′), CTE-S (5′-GAC TTC TAG ACC TCG AGG CGG CCG CGA TCC ACT AGT ACT AGA CCA C-3′) and CTE-A (5′-GAC TGG GCC CGC CGC TCT AGC TGA TCA ACA C-3′), respectively. These DNA fragments were first inserted into the XbaI/ApaI sites of pcDNA3.1, followed by insertion at the BamHI/XhoI sites of the HIV-1 sequence spanning nucleotide positions 1 to 5337 (referring to the first nucleotide of the 5′ R region) that was amplified using primer pair Bam-454-S (5′-GAT CGG ATC CGG TCT CTC TGG TTA GAC CAG-3′) and Sal-A (5′-CTA TTC TGC TAT GTC GAC ACC C-3′).

The hGag sequence was amplified by PCR from the pVRC_3900 vector (Huang et al., 2001) (kindly provided by

Dr. Gary J. Nabel) using primer pair hGag-S (5′-GAC TGA TAT CAT GGG CGC CCG CGC CAG CGT G-3′)/hGag-A (5′-GAC TTC TAG ATT ACT TAT CGT CGT CAT CCT TGT AAT CTT GTG ACG AGG GGT CGC TGC C-3′). The hGag-A primer contains the Flag sequence that is attached to the C-terminus of hGag. The amplified DNA fragment was inserted at the EcoRV/XbaI sites of pcDNA3.1 (Invitrogen), and the plasmid thus generated is named phGag-Flag. The D449-Flag, D378-Flag, D278-Flag and D133-Flag mutants were created using the following primers:

D449: 5′-GCA TCT CGA GAA AAT TCC CTG GCC TTC CCT TG-3′;
D378: 5′-GCA TCT CGA GCA TGA TGG TGG CGC TGT TGG-3′;
D278: 5′-GCA TCT CGA GGT ACA TGC GCA CGA TCT TGT TC-3′;
D133: 5′-GCA TCT CGA GGT AGT TCT GGC TCA CCT GG-3′.

The DDX24 cDNA clone was purchased from Invitrogen (clone ID 4303448). Primers DDX24-S (5′-CAC GCG GCC GCA TGA AGT TGA AGG ACA CAA AAT CAA GG-3′) and DDX24-A (5′-CCA TCT AGA TTA AAG AGC GTA ATC TGG AAC ATC GTA TGG GTA ATT TGC ACT TGT ACT TGG CTG TGG-3′) were used to amplify the cDNA of DDX24 and the PCR product was inserted into the expression vector pcDNA3.1. The DDX24-siM DNA contains the mutations G2280A/A2283T/C2286A/T2289C/T2295A at the siDDX24-2 target site (nt 2277 to 2295). It was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with primers 5′-GTG GTC AAG GAA CGT ATA CGC TTA GCA CGA CAG ATT GAG-3′ and 5′-CTC AAT CTG TCG TGC TAA GCG TAT ACG TTC CTT GAC CAC-3′.

The LMP-shDDX24 DNA was engineered by inserting the XhoI–EcoRI DNA fragment of 110 bp from vector pSM2c (clone ID V2HS_244226, Open Biosystems) into the LMP vector (Open Biosystems). The Rev-RFP DNA was constructed by inserting the Rev and RFP coding sequences into the pcDNA3.1 (Invitrogen) expression vector. The Rev sequence was amplified with primers 5′-GAC TGA TAT CAC CAT GGC AGG AAG AAG CGG AGA CAG CG-3′ (with the EcoRV site) and 5′-GAC ACT CGA GTT CTT TAG TTC CTG ACT CCA ATA C-3′ (with the XhoI site), RFP was amplified with 5′-GAC TGG GCC CTA CAG GAA CAG GTG GTG GCG-3′ (with the XbaI site) and 5′-GAC TGG GCC CTA CAG GAA CAG GTG GTG GCG-3′ (with the ApaI site).

Knockdown of DDX24 protein

siRNA oligos 5′-CCU GGU CCA AAC UGC UCC ATT-3′ (siDDX24-1, targeting nt positions 537 to 555 (NM_020414)), 5′-GGA GCG AAU CCG UUU AGC UTT-3′ (siDDX24-2, targeting nt positions 2277 to 2295 (NM_020414)), and control siRNA oligos (catalogue number AM4635) were purchased from Ambion. Transfection of siRNA (at 40 nM) was performed with Lipofectamine 2000 (Invitrogen). To generate stable DDX24-knockdown cell lines, the LMP-shDDX24 DNA was first transfected into the LinX packaging cells (Open Biosystems). Viruses in the supernatants were used to infect SupT1 cells. Puromycin (Sigma) was added to the culture at a final concentration of 2 μg/ml 24 h after infection. The positively infected cells were selected in three weeks following six successive passages. Puromycin-resistant cells were further assessed with flow-cytometry for GFP expression to ascertain the stable integration of the LMP vector. Knockdown efficiency was evaluated in Western blots using rabbit anti-DDX24 antibodies (Novus Biologicals).

Immunofluorescence staining

HeLa cells were transfected with the Rev-RFP DNA. Twenty-four hours after transfection, cells were fixed with 3.7% formaldehyde at room temperature for 10 min, followed by permeabilization with 0.1% Triton-X100 for 10 min. After washing with 1× PBS, cells were incubated in 5% milk at room temperature for 1 h prior to adding the Rabbit anti-DDX24 antibodies (1:150 dilution, Novus Biologicals). Cells were subsequently stained with Alexa fluor 488-conjugated goat anti-rabbit secondary antibodies (1:500 dilution, Invitrogen). Images were recorded using LSM 5 PASCAL laser scanning confocal microscope (Carl Zeiss).

Immunoprecipitation

The DDX24-Flag or Gag-Flag proteins were immunoprecipitated with anti-Flag antibodies. After washing with 1× phosphate buffered saline, transfected cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktails (Roche Diagnostics), followed by three times freeze-thaw treatment. The cell debris was pelleted by spinning at 13,000 rpm in an eppendorf centrifuge at 4 °C. One mg of the clarified cell lysates was incubated with 30 μl of the anti-Flag M2 affinity gel (Sigma) at 4 °C for 16 h. After washing in TBS (50 mM Tris (pH 7.4), 150 mM NaCl) for three times, the bound DDX24-Flag proteins were eluted with 100 μg/ml Flag peptides in TBS. The Rev-His proteins were purified using the nickel-charged agarose. 293T cells were transfected with the pRev-His DNA and lysed in the buffer containing 50 mM NaH_2PO_4, 5 mM Tris–HCl (pH 8.0), 300 mM NaCl, 0.05% Tween-20, 10 mM β-mercaptoethanol, and protease inhibitor cocktails (Roche Diagnostics). After clarifying at 13,000 rpm in an eppendorf centrifuge, the cell lysates were incubated with 30 μl of the Ni-NTA agarose (Qiagen) at 4 °C for 2 h. After extensive washing with 50 mM NaH_2PO_4, 5 mM Tris–HCl (pH 8.0), 300 mM NaCl and 10 mM imidazole, the bound Rev-His proteins were eluted with 50 mM NaH_2PO_4, 5 mM Tris–HCl (pH 8.0), 300 mM NaCl and 250 mM imidazole.

Measuring viral infectivity in one-round infection assay

Amounts of HIV-1 were determined by measuring levels of viral p24(CA) antigen using the Vironostika HIV-1 Antigen

Microelisa System (Biomerieux, The Netherlands). Viruses equivalent to 5 ng p24(CA) were used to infect 4×10^4 TZM-bl cells in a 24-well plate. TZM-bl are HeLa cells that bear the CD4, CXCR4, and CCR5 cell surface markers and express firefly luciferase upon HIV-1 infection (Wei et al., 2002). Cells were collected 48 h post infection and luciferase activity was measured using the Luciferase Assay System (Promega).

CAT (chloramphenicol acetyltransferase) assay

The 293T cells were seeded in 6-well plates one day prior to transfection with 200 ng of the pDM128 DNA alone or together with 50 ng of the Rev DNA. Cells were collected 48 h after transfection and lysed in 250 mM Tris–HCl (pH 7.5) by freeze and thaw. The CAT activity was measured in a reaction mixture containing 50 μl cell lysates, 30 μM [^{14}C] chloramphenicol (at 50 μCi/ml, Amershan), 0.3 mg/ml *n*-butyryl coenzyme-A (Sigma) at 37 °C for 1 h. The reaction products were extracted with ethyl acetate, separated on the TLC plate, detected by exposure to phosphor screen and quantified using the Storm840 phosphor imager (Amersham).

Viral RNA analysis

Levels of viral RNA in cells were assessed by Northern blots as previously described (Roy et al., 2006). Briefly, transfected cells were washed with 1× phosphate buffered saline and directly solubilized by Trizol (Invitrogen). RNA was extracted in accordance with the manufacturer's instruction. To prepare the cytoplasmic and nuclear fractions, cells were first lysed in a buffer containing 0.5% Nonidet P-40, 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 5 mM $MgCl_2$. After spinning at 5000 rpm in an eppendorf centrifuge at 4 °C for 30 min, the supernatants were collected and represented the cytoplasmic fraction. The pellets were washed twice with lysis buffer and represented the nuclear fraction. RNA was extracted from these two fractions using the Trizol-LS reagent (Invitrogen).

Levels of virion-associated viral RNA were determined by real-time RT-PCR or by native Northern blots. RNA was first extracted from viruses using Trizol-LS (Invitrogen), followed by DNase I digestion at 37 °C for 15 min to remove any contaminating plasmid DNA. DNase I was inactivated by incubation at 65 °C for 10 min. The effectiveness of DNase I treatment is tested by amplifying the treated RNA samples in PCR with the primers p675-S (5′-CCA GAG GAG CTC TCT CGA CGC AG-3′) and p960-A (5′-GTA TTT GTC TAC AGC CTT CTG-3′). This primer pair amplifies HIV-1 cDNA sequence spanning nucleotides 675 to 960. Subsequent to reverse transcription of viral RNA with primer p960-A, levels of viral cDNA were measured by real-time PCR with primers p675-S/p960-A using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics) in the light cycler real-time PCR machine (Roche Diagnostics). The standard curve was created with a series of diluted HIV-1 cDNA including 10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4 and 10^5 copies.

Native Northern blots were performed as previously described (Russell et al., 2003). Briefly, HIV-1 particles were harvested by ultracentrifugation at 35,000 rpm for 1 h using the SW41 rotor. The virus pellets were suspended in the TN buffer (10 mM Tris–HCl (pH 7.5) and 100 mM NaCl). The amounts of viruses were determined by measuring viral p24 levels in ELISA. After treatment with protease K at 37 °C for 20 min in a buffer with 100 μg/ml protease K, 0.1% SDS, 2 mg/ml yeast tRNA, 10 mM Tris–HCl (pH 7.5) and 100 mM NaCl, viral RNA was prepared by sequential extraction with phenol: chlorophorm:isoproponal (25:24:1) and chlorophorm. Following precipitation in 70% ethanol with 0.3 M NaOAc (pH 5.2), viral RNA was suspended in RNA storage buffer containing 1 mM sodium citrate (Ambion). After incubation for 10 min at a series of temperatures (40 °C, 45 °C, 50 °C and 55 °C) in the presence of 100 mM NaCl, viral RNA was subjected to electrophoresis in 0.9% agarose gels at 4 °C, 100 V for 4 h. Viral RNA was subsequently transferred onto the nylon membranes and detected with [α-^{32}P] labeled HIV-1 DNA probes.

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