Silencing of HIV-1 co-factors

Eekels, J.J.M.

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Chapter 7
The RNA helicase DDX3 is involved in trans-activation of the HIV-1 Long Terminal Repeat promoter

Julia J.M. Eekels, Rienk E. Jeeninga and Ben Berkhout

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Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam
Abstract

The RNA helicase DDX3 has a plethora of cellular functions and has been implicated as a co-factor or inhibitor in some types of cancer. DDX3 can also influence the replication of human pathogenic viruses, including human immunodeficiency virus type 1 (HIV-1). We show that stable RNA interference (RNAi) mediated knockdown of DDX3 inhibits HIV-1 replication with minor effects on cell proliferation. As DDX3 is involved in the transcriptional control of different promoters, we studied the effect of DDX3 overexpression and knockdown on the activity of the HIV-1 Long Terminal Repeat (LTR) promoter. Knockdown of DDX3 reduces both the basal and Tat-induced LTR transcription levels. Overexpression of DDX3 has no effect on basal transcription, but enhanced Tat-induced transcription from the HIV-1 LTR. The DDX3 protein thus seems to facilitate optimal LTR transcription and may play a role as cellular co-factor of the viral Tat protein.

Introduction

The acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus type 1 (HIV-1) remains a serious global health threat, although combined anti-retroviral therapy (cART) is highly active against the virus. Despite the clinical success of cART, the emergence of drug-resistant viral strains is another major problem. To avoid viral drug resistance, it has been proposed to target cellular co-factors that HIV-1 requires for its replication cycle.

One of the many possible therapeutic targets is the RNA helicase DDX3. DDX3 is a member of the family of DEAD box RNA helicases (DEAD = AspGluAlaAsp) that possesses RNA-dependent ATPase and helicase activity (394). Members of this family are involved in a wide range of cellular processes that deal with RNA; e.g. splicing, mRNA export, transcription and translation (289). DDX3 has been implicated in many of these cellular functions as summarized in Figure 7.1 and reviewed in (308). DDX3 interacts with the transcription factor Sp1 and binds to several promoters, e.g. the interferon-β promoter to execute a transcriptional role (70;309). DDX3 shuttles between the cytoplasm and nucleus and nuclear export is mediated via interaction with the CRM1 protein, which also implicates DDX3 in the nuclear export of other RNAs (388). A function in mRNA translation is executed via an interaction with the translation initiation factor eIF3 (196). In most cases the exact molecular mechanism of DDX3 action remains unclear.

DDX3 also plays a role in innate immunity. DDX3 is involved in the intracellular signaling upon activation of pathogen recognition receptors (PPRs) such as Toll-like receptors and cytoplasmic viral RNA sensors like RIG-I. When PPRs sense the presence of a pathogen, several signaling pathways are triggered, eventually resulting in activation of the transcription factors NF-κB and interferon regulatory factors (IRF) 3 and 7. NF-κB activation leads to the expression of anti-inflammatory cytokines, IRF3 and 7 activation leads to the induction of type I interferons. Cytokines and type I interferons are known to be potent antiviral mediators. Several specific DDX3-virus interactions have been reported in literature as summarized in Figure 7.1. Vaccinia virus (VACV) inhibits the induction of interferon-β (IFNβ) by the viral protein K7, which binds to DDX3, to inhibit the DDX3-mediated induction of the IFNβ promoter (309). DDX3 plays a similar role in hepatitis B virus (HBV) replication. The HBV polymerase protein binds to DDX3 and blocks IFNβ
production (369;395). Interestingly, interaction with the viral polymerase leads to incorporation of DDX3 into the virion particle and DDX3 inhibits the first step of reverse transcription in the newly infected cell, thus augmenting the antiviral effect (368). In contrast, other viruses such as hepatitis C virus (HCV) use DDX3 as a cellular co-factor for its replication, although the precise mechanism remains unclear. DDX3 interacts with the HCV structural core protein and may modulate viral or cellular gene expression (17;23;265).

**Figure 7.1. Different functions of the DDX3 protein**

DDX3 has been implicated in many cellular processes and we grouped the specialized functions in innate immunity, virus replication and cancer. “↑” indicates a process positively influenced by DDX3, “↓” indicates processes negatively influenced by DDX3. Functions of DDX3 that are linked are depicted in the same colour. Red: nuclear RNA export. Blue: Upregulated transcription. Green: Downregulated transcription. Purple: Interferon-β induction. The effect of DDX3 on Tat-mediated transcription is the focus of this study (grey box).

DDX3 binds CRM1 and this interaction is important in HIV-1 replication as unspliced and incompletely spliced viral transcripts require nuclear export via the CRM1 pathway that is controlled by the viral Rev-RRE axis (388). Furthermore, DDX3 expression was found to be induced by the HIV-1 protein Tat. It has been reported that DDX3 does not influence transcription from the HIV-1 LTR promoter, but these experiments were performed exclusively in the absence of Tat (388). Two recent studies confirmed that DDX3 expression is enhanced by Tat and that the two proteins physically interact (62;129).

Opposing roles have also been described for DDX3 in cancer. DDX3 acts as an oncogene in breast cancer cells by reducing transcription from the E-cadherin promoter, which has been associated with progression to cancer and metastasis (44). DDX3 acts as a tumor suppressor in hepatocellular carcinoma cells, where it upregulates the expression of the tumor suppressor p21 via an interaction with the transcription factor Sp1 (70).

As DDX3 has been implicated in transcriptional control in diverse settings, we wanted to readdress a putative role in HIV-1 transcription (see box in Figure 7.1). We and others have previously shown that stable knockdown of DDX3 inhibits HIV-1 replication (103;158). This result was confirmed in this study, with additional experimentation to
document the mRNA knockdown level and the impact on cell growth. We subsequently tested the effect of DDX3 overexpression or knockdown on transcription from the HIV-1 LTR promoter. DDX3 overexpression enhances transcription from the LTR in the presence of the viral Tat protein, while knockdown of DDX3 has the opposite effect. The ATPase and helicase activities of DDX3 are critical for this role as HIV-1 co-factor. We thus propose that DDX3 functions at two steps of the HIV-1 replication cycle, via Tat in transactivation of the HIV-1 LTR promoter and via CRM1/Rev in the nuclear export of HIV-1 RNAs. This double action as co-factor makes DDX3 a promising target for anti-HIV-1 therapy, of which the impact may be broader as DDX3 is also a co-factor for HCV, which is frequently seen as co-infection in HIV-1 infected individuals.

Materials and methods

Plasmids

ShRNA-expressing constructs (pLKO.1) are from the MISSION™ TRC-Hs 1.0 library (Sigma-Aldrich) and were obtained as bacterial clones (293). Target sequences can be found on the website of Sigma-Aldrich [http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/individual-genes.html]. Plasmid DNA was isolated with Nucleobond Midiprep columns according to the manufacturer’s protocol (Macherey-Nagel). For the competitive cell growth assay it was necessary to replace the gene for the puromycin selection marker in pLKO.1 for the GFP gene, as described earlier (Eekels et al, Chapter 4). pCMV-Myc-DDX3 encodes DDX3 with a Myc tag and pCMV-Myc-K230E encoding a mutant form of DDX3. Both plasmids were kindly provided by dr. M. Schröder (National University of Ireland, Maynooth). pSuper-shDDX3 was constructed as described (292). pBluescript3’LTRLuc (164) was used to study HIV-1 LTR activity and the empty vector pBluescript KS(-) was used to control for input DNA amounts in transfections. pTat-exon contains the Tat coding sequence under control of the constitutive CMV promoter (360).

Cell culture, DNA transfection, lentiviral vector production and transduction

Human embryonic kidney 293T (HEK293T) adherent cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Human SupT1 suspension T cells were grown in Rosewell Park Memorial Institute medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. For puromycin selection the final concentration in the culture medium was 1 μg/ml. Cell lines were cultured in a humidified chamber at 37°C and 5% CO2.

DNA transfections were performed with Lipofectamine 2000 according to manufacturer’s instructions (Invitrogen). In short, 2 x 10⁵ cells per well (24-wells) were seeded a day before transfection. The next day cells were transfected, where necessary the empty plasmid pBluescript KS (-) was used to obtain an equal amount of DNA per well. Cells were harvested 24 hours post transfection. All transfections were performed in four-fold, of which two wells were analyzed for luciferase activity and two wells were harvested for RNA isolation. Luciferase activity was measured using the DualGlo Luciferase kit (Promega).
Lentiviral vectors were produced as previously described (292). Briefly, HEK293T cells were co-transfected with the shRNA-construct and the packaging plasmids (pRSV-Rev, pMDLg/pRRE and pVSV-G) using Lipofectamine 2000 (Invitrogen). One day after transfection the medium was refreshed and the supernatant was harvested the next day. The virus containing supernatant was centrifuged and aliquots were stored at −80°C. A sample was taken for CA-p24 enzyme-linked immunosorbent assay (ELISA) to monitor lentiviral particle production. SupT1 T cells were seeded in a 24-wells plate (1 x 10^5 cells/well), lentiviral vector was added and incubated overnight. Excess virus was washed away the next day. Stably transduced cells were selected for either by addition of 1 µg/ml puromycin to the culture medium or by FACS sorting based on GFP expression, depending on the selection marker encoded by the lentiviral vector.

**CA-p24 ELISA**

Culture supernatant was heat-inactivated at 56°C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem). The CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom) as the capture antibody and the alkaline phosphatase-conjugated anti-CA-p24 monoclonal antibody EH12-AP (International Enzymes) as the detection antibody. Detection was performed with the Lumiphos plus system (Lumigen) in a LUMistar Galaxy luminescence reader (BMG Labtechnologies). Recombinant CA-p24 produced in a baculovirus system was used as reference standard.

**CCG assay and FACS analysis**

To study the effects of shRNA expression on cell proliferation, the CCG assay was used as described earlier (Eekels et al, Chapter 4). Briefly, cells were transduced with 1 or 10 µl of lentiviral vector encoding a shRNA and the GFP selection marker. The percentage of GFP-expressing cells was monitored over time with FACS analysis. To do so, a sample was taken from the cell culture and cells were collected by centrifugation (4 min, 4000 rpm, Eppendorf centrifuge). The cell pellet was resuspended in FACS solution (Phosphate buffered saline (PBS) + 2% FCS). FACS analysis was performed with the FACScanto (BD Biosciences). Live cell population was based on forward and side scatter parameters and GFP-positive population was determined based on unstained and untransduced cells.

**HIV-1 replication and single cycle infection experiments**

HIV-1 was produced by transfection of HEK293T cells with the molecular clone HIV-1 LAI (270) and virus production was measured by CA-p24 ELISA. SupT1 T cell cultures (5 ml in T25 format, 2.5 x 10^6 cells/flask) were infected with HIV-1 (0.5 ng of CA-p24). Every day virus replication was monitored by scoring syncytia formation and supernatant samples were taken for CA-p24 ELISA. For single cycle infection experiments, 1.5 x 10^5 SupT1 cells were incubated with HIV-1 for 4 hours, after which excess virus was washed away. Cells were subsequently cultured in complete medium supplemented with 0.1 µg/ml fusion inhibitor T1249 to block new infections (Eggink et al., 2008). Cells were analyzed 48 hours post infection for intracellular CA-p24 by FACS. Therefore cells were collected by centrifugation (as above) and fixated in 500 µl 4% formaldehyde for 5 minutes at room temperature. 500 µl Perm/Wash (BD Biosciences) was added and cells were again pelleted by centrifugation. Cells were stained
for at least 1 hour at 4°C in 50 µl BD Perm/WashTM buffer containing 5 µl 1:100 diluted antibody against CA-p24 conjugated with PE (monoclonal mouse, clone KC57, Coulter). Excess antibody was washed away by washing twice with 500 µl FACS solution, cells were resuspended in 250 µl FACS solution and analyzed on a FACScanto. Uninfected and unstained samples were used as negative controls.

Western blot

Cell lysates that were prepared for luciferase measurement were mixed 1:1 with SDS-PAGE loading buffer, boiled for 5 minutes and run in a 10% SDS-PAGE gel. Proteins were transferred to an Immobilon-P membrane (Millipore) via semi-dry transfer. Membranes were treated for 1 hour at room temperature in blocking solution (PBS + 5% milk + 0.1% Tween-20) and detection was performed with anti-DDX3 (Abcam, ab50703, mouse, 1:1000), anti-β-actin (Sigma, clone AC-74, mouse, 1:5000) and HRP-labeled goat anti-mouse IgG (1:5000). Luminometric detection of proteins was performed with Western Lightning ECL (PerkinElmer Life Sciences) and membranes were analyzed on a LAS4000 imager (GE Healthcare).

RNA isolation and RT-qPCR

RNA was isolated from 0.5 x 10⁶ cells with the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions, including the optional DNase I treatment on column. 1 µg RNA was reverse transcribed with the Thermoscript kit (Invitrogen) and oligo-dT primers. cDNA synthesis was performed at 50°C for 1 hour. The resulting cDNA was diluted 100 times and 5 µl of the dilution was used as template in a SYBR Green based RT-qPCR assay (Qiagen). Primers for DDX3 and the internal control β-actin were obtained from Quantitect Primer Assays (Qiagen). Primers for Firefly luciferase were Forward 5’-GGATTACCAGGGATTTCAGTC-3’ and Reverse: 5’-CTCACGCAGGCAGTTCTAT-3’. The levels of luciferase, DDX3 and β-actin mRNA were measured in duplo using Ct (threshold cycle) for every sample and the equation 2^-ΔΔCt was used for relative quantification of luciferase and DDX3 mRNA expression levels (222).

Results

DDX3 knockdown inhibits HIV-1 replication

We previously demonstrated inhibition of HIV-1 replication by stable RNAi-mediated knockdown of cellular proteins in the human T cell line SupT1 (103). One of the tested candidate co-factors was the DDX3 helicase and 5 anti-DDX3 shRNAs were tested for their impact on HIV-1 replication. In this study we used shRNA1 that gave the best results. The SupT1 T cell line was transduced with a lentiviral vector encoding the anti-DDX3 shRNA and control cell lines were generated with the empty lentiviral vector SHC1 to test for potential effects of vector integration and SHC2 that expresses a scrambled shRNA molecule. The three cell lines were challenged with HIV-1 and virus replication was monitored over a period of 13 days (Figure 7.2A). Both controls cells support HIV-1 replication with peak infection at 9 days post infection, while HIV-1 replication is inhibited in the DDX3 knockdown cell line (shDDX3).
To test whether the knockdown of DDX3 has any effect on cell proliferation, a competitive cell growth (CCG) assay was performed (Eekels et al, Chapter 4). This assay is performed directly on the lentivirus transduced culture that contains transduced GFP-expressing cells and non-transduced cells. The percentage of GFP-positive cells was analyzed longitudinally by simple FACS analysis. A reduced cell growth rate due to shRNA expression is scored as a gradual decrease in the fraction of GFP-positive cells. Knowing the doubling time of untransduced SupT1 cells, the relative proliferation rate of transduced cells can be calculated. The relative proliferation rate of SHC1 cells was set at 100% and DDX3 knockdown resulted in a moderate loss of 10% (Figure 7.2B). The DDX3 mRNA level was measured in the three cell lines using RT-qPCR. A knockdown percentage of 55% was scored in the shDDX3 cells compared to the empty SHC1 control. The scrambled shRNA in SHC2 had no effect on DDX3 mRNA expression level (Figure 7.2C). It has in fact been reported that a knockdown percentage above 50% is difficult to achieve for DDX3 (309;388). Nonetheless, this relative modest knockdown efficiency has a clear effect on HIV-1 replication.

**Figure 7.2. Stable knockdown of DDX3 delays HIV-1 replication without inducing cytotoxicity**

A. Stable knockdown SupT1 cells were generated with a shRNA against DDX3 (shDDX3). Two control cells were generated in parallel: SHC1, with an empty lentiviral vector and SHC2, expressing a scrambled shRNA molecule. The cell lines were infected with HIV-1LAI and viral replication was monitored in the culture supernatant by ELISA for CA-p24 over a period of 13 days. B. The relative cell growth rate was measured to score adverse effects of DDX3 knockdown. Cellular proliferation rate of SHC1 was set at 100%. A minor reduction of 10% was observed in the DDX3 knockdown cell line. C. DDX3 mRNA levels were measured with RT-qPCR. Three independent cell samples were analyzed and the data was pooled. The mean relative gene expression levels are shown (SHC1 set at 100%), error bars represent standard deviation.

**DDX3 knockdown in single cycle infection experiments**

To assess which step of the replication cycle HIV-1 is blocked by DDX3 knockdown, we performed single cycle infection experiments in stably transduced SupT1 cells. To do so, cells were incubated with HIV-1 for 4 hours, after which excess virus was washed away and the fusion inhibitor T1249 was added to prevent new rounds of infection. After 48 hours, the cells are harvested and intracellularly stained for viral gene expression in the form of CA-p24 protein. With flowcytometry the percentage of cells positive for CA-p24 can be analyzed, as well as the mean production of CA-p24 per positive cell, expressed as the mean fluorescence intensity (MFI). Extracellular virus production was analyzed by CA-p24 ELISA in the culture supernatant.

When DDX3 expression is reduced, we observe a reduction in the percentage of cells that become positive for intracellular CA-p24 (Figure 7.3, left panel), which indicates that less cells were productively infected. The amount of CA-p24 produced by these infected cells is...
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also modestly reduced upon DDX3 knockdown (Figure 7.3, middle panel). As less cells become productively infected and those cells produce less CA-p24, the reduction of extracellular CA-p24 in the culture supernatant is expected (Figure 7.3, right panel). The fact that less cells are productively infected upon DDX3 knockdown indicates that an early step in HIV-1 replication is inhibited. However, the small reduction in CA-p24 production by infected cells indicates an additional effect on a late HIV-1 replication step.

Figure 7.3. DDX3 knockdown in single round HIV-1 infections
Control and DDX3 knockdown cells were incubated with HIV-1 for 4 hours, after which excess virus was washed away. Cells were further cultured in the presence of the fusion inhibitor T1249 to prevent new rounds of infection. The percentage of cells positive for intracellular CA-p24 production was measured by FACS analysis 48 hours post infection (left panel). The mean fluorescence intensity (MFI) is plotted as a measure of CA-p24 production per infected cell (middle panel). The CA-p24 concentration in the culture supernatant was measured by ELISA (right panel). The average of 2 independent experiments that were performed in duplicate are shown, error bars represent the standard deviation.

DDX3 acts in synergy with Tat to transactivate the HIV-1 LTR

Because DDX3 plays multiple roles in the regulation of cellular transcription, we tested the effect of DDX3 knockdown on the HIV-1 LTR promoter. To monitor the exclusive effects on LTR activity, experiments were performed with a luciferase reporter gene under control of the LTR promoter (LTR-luc) in HEK293T cells. We initially transduced these HEK293T cells with the shRNA-expressing lentiviral vectors but did not obtain significant DDX3 knockdown. We therefore decided to use transient DNA transfection. Cells were cotransfected with LTR-luc and increasing amounts of the pSuper plasmid encoding the shRNA against DDX3. The effect of DDX3 knockdown on LTR-luc activity was analyzed in the absence and presence of the viral protein Tat that activates transcription from the LTR (Figure 7.4A). The basal luciferase activity without Tat was set at 1.

In the absence of Tat, an increasing amount of shDDX3-expression construct and thus increased knockdown level gradually decreased the basal Luciferase activity. When the LTR-promoter is activated by Tat, DDX3 knockdown again decreased Luciferase activity. DDX3 has been reported to be important in many cellular mechanisms such as nuclear export of RNAs and translation. In fact, the firefly luciferase mRNA requires the CRM1 RNA export pathway, in which DDX3 has been implicated (174). To show that the observed effect was due to decreased transcription activity rather than e.g. nuclear RNA export, the luciferase mRNA level was measured by RT-qPCR. Both in the presence and absence of Tat a gradual decrease of luciferase mRNA expression was observed when DDX3 is knocked down (Figure 7.4B). The knockdown of DDX3 protein was confirmed by Western blot analysis, on which the cellular β-actin protein acts as loading control (Figure 7.4C).
We next tested the impact of DDX3 overexpression by co-transfection with a CMV-driven DDX3 expression vector (Figure 7.4D). DDX3 overexpression further boosted Tat-activated Luciferase activity, but it has no significant effect on the basal Luciferase activity. The latter finding is similar to those of Yedavalli et al, but that study did not test the DDX3 effect with Tat (388). RT-qPCR analysis confirmed that the increase in Luciferase activity is due to increased luciferase mRNA expression (Figure 7.4E), and the overexpression of DDX3 was confirmed by Western blot analysis (Figure 7.4F).

**Figure 7.4. DDX3 influences LTR transcription in a Tat-dependent manner**

A. HEK293T cells were co-transfected with 25 ng LTR-luciferase construct and increasing amounts of pSuper-shDDX3 (10, 50, 100 and 500 ng), in the absence and presence of Tat (5 ng). Knockdown of DDX3 decreases both basal and Tat-induced LTR activity. The basal Luciferase activity without Tat was set at 1. B. RT-qPCR was performed to measure luciferase mRNA levels. Basal luciferase mRNA level without Tat was set at 1.C. Knockdown of DDX3 was confirmed by Western blot analysis, with β-actin as loading control. D. HEK293T cells
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were co-transfected with 25 ng LTR-luciferase construct and increasing amounts of pCMV-DDX3-Myc (10, 50, 100 and 500 ng), in the absence and presence of Tat (5 ng). Overexpression of DDX3 has no effect on basal LTR activity, but enhances Tat-induced LTR activity. E. The increase in luciferase mRNA levels was confirmed by RT-qPCR. F. The overexpression of DDX3 was confirmed by Western blot analysis.

The ATPase and helicase capacity of DDX3 is necessary for LTR activation

To test whether the catalytic functions of DDX3 are required for the effect on HIV-1 LTR transcription, we used the K230E mutant. This DDX3 mutant has a single amino acid substitution in the Walker A motif and is not able to hydrolyse ATP and to unwind RNA (388). DDX3-K230E overexpression did not increase the Luciferase activity (Figure 7.5A) and the level of luciferase mRNA (Figure 7.5B), but expression of the K230E DDX3 mutant was confirmed by Western blotting (Figure 7.5C). Parallel experiments with wild-type DDX3 reproduced the activation phenotype (Figure 7.5D and 7.5E) and protein expression levels were similar to that of the mutant DDX3 (Figure 7.5F). The ATPase and helicase activity of DDX3 are therefore important for the stimulatory effect on Tat-mediated transcription from the HIV-1 LTR promoter.

Figure 7.5. The ATPase and helicase activity of DDX3 are required for modulation of the HIV-1 LTR
A and B. HEK293T cells were co-transfected with 25 ng LTR-luciferase reporter construct and increasing amounts of DDX3-K230E expression construct (100 and 500 ng), in the absence or presence of Tat (5 ng). No effect was observed on basal and Tat-induced LTR Luciferase activity (A) and the luciferase mRNA levels (B). C. Overexpression of the mutant K230E protein was confirmed by Western blot, with β-actin as loading control. D and E. Overexpression of wild-type DDX3 (100 and 500 ng) has no effect on basal LTR transcription, but enhanced Tat-induced LTR transcription, as apparent in Luciferase activity assays (D) and luciferase mRNA assays (E). F. Overexpression of wild-type DDX3 was confirmed by Western blot analysis.
Discussion

HIV-1 requires many cellular co-factors to complete its replication cycle and one of these co-factors is the RNA helicase DDX3. This protein has been described as a co-factor of the HIV-1 Rev protein, which facilitates the export of HIV-1 transcripts from the nucleus via the CRM1 pathway. DDX3 has also been implicated in many cellular mechanisms, including transcription and translation (Figure 7.1). We first tested the effect of DDX3 knockdown in T cells on HIV-1 replication. A relatively modest knockdown of DDX3 did significantly inhibit HIV-1 replication, with only a minor effect on cell proliferation. We next tested the effect of DDX3 knockdown and overexpression on transcription from the HIV-1 LTR promoter. We measured transcription levels of a luciferase reporter construct under control of the HIV-1 LTR and show that LTR-driven transcription is decreased when DDX3 is knocked down, both in the presence and absence of the Tat protein. This result is consistent with a supportive role of DDX3 in HIV-1 gene expression.

Overexpression of DDX3 leads to enhanced levels of LTR transcription, but only in the presence of Tat. This result could indicate that endogenous DDX3 expression is sufficient for basal LTR transcription, but that more DDX3 is beneficial for high-level Tat activated LTR transcription. RT-qPCR analyses of luciferase mRNA expression indicated that the observed DDX3 effects are due to regulation at the transcriptional level. It was obviously important to rule out that DDX3 acts on nuclear RNA export or mRNA translation, mechanisms known to be influenced by DDX3.

To test if the enzymatic ATPase and associated helicase activity of DDX3 are involved in the enhancement of Tat-mediated transcription, we used the previously described K230E mutant. This mutant has a single amino acid substitution in the Walker A motif that abrogates ATPase and helicase activity (367). When overexpressed, the K230E mutant had no effect on both basal and Tat-induced transcription from the HIV-1 LTR, as opposed to the wild-type DDX3 protein. Because the mutant is stably expressed in cells, we conclude that the enzymatic helicase activity is important for enhancement of HIV-1 LTR transcription levels. Interestingly, it has been shown that the K230E mutant is fully able to enhance transcription from the IFNβ promoter (309), indicating that different molecular interactions and mechanisms are at play during transcriptional activation of these promoters. For instance, HIV-1 LTR transcription may be special because of the multiple binding sites for the Sp1 transcription factor, which interacts directly with DDX3. Sp1 is required for both basal and Tat-mediated activation of the LTR promoter (38;332).

DDX3 has recently been the focus of many studies because of its role in cancer development and the replication of at least two pathogenic human viruses (HIV-1 and HCV). Inhibition of such a cellular co-factor has obvious therapeutic potential and different antiviral strategies can be proposed. We and others showed that RNAi mediated DDX3 knockdown inhibits HIV-1 replication, with only minor effects on cell proliferation. DDX3 could thus be placed on the shortlist of candidate targets for a gene therapy approach to silence critical co-factors of pathogenic viruses, although the CCR5 receptor remains the primary target for anti-HIV therapies (13). Other studies have used small-molecules inhibitors of DDX3 to achieve potent inhibition of HIV-1 replication (229;230). The notion that DDX3 contributes to two important steps of HIV-1 replication, that is Tat-mediated LTR transcription and Rev-mediated RNA export, makes it an even more attractive therapeutic target.
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