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Requirement of DDX3 DEAD Box RNA Helicase for HIV-1 Rev-RRE Export Function

Venkat S.R.K. Yedavalli,[1,3] Christine Neuveut,[1,3,4] Ya-hui Chi,[1] Lawrence Kleiman,[2] and Kuan-Teh Jeang[1,*]

[1]Molecular Virology Section
Laboratory of Molecular Microbiology
National Institutes of Allergy and Infectious Diseases
Bethesda, Maryland 20892

[2]Lady Davis Institute
McGill AIDS Center
Montreal H3T 1E2
Canada

Summary

A single transcript in its unspliced and spliced forms directs the synthesis of all HIV-1 proteins. Although nuclear export of intron-containing cellular transcripts is restricted in mammalian cells, HIV-1 has evolved the viral Rev protein to overcome this restriction for viral transcripts. Previously, CRM1 was identified as a cellular cofactor for Rev-dependent export of intron-containing HIV-1 RNA. Here, we present evidence that Rev/CRM1 activity utilizes the ATP-dependent DEAD box RNA helicase, DDX3. We show that DDX3 is a nucleo-cytoplasmic shuttling protein, which binds CRM1 and localizes to nuclear membrane pores. Knockdown of DDX3 using either antisense vector or dominant-negative mutants suppressed Rev-RRE-function in the export of incompletely spliced HIV-1 RNAs. Plausibly, DDX3 is the human RNA helicase which functions in the CRM1 RNA export pathway analogously to the postulated role for Dbp5p in yeast mRNA export.

Introduction

DEAD (D-E-A-D = Asp-Glu-Ala-Asp) box (Linder et al., 1989) helicases are involved in many aspects of RNA metabolism, including transcription, mRNA splicing, and RNA export (Lorsch, 2002; Tanner and Linder, 2001; Linder and Stutz, 2001). HIV-1 gene expression is substantially governed posttranscriptionally by Rev-regulated export of unspliced/partially spliced viral RNAs from the nucleus into the cytoplasm (Harris and Hope, 2000; Cullen, 2003). This export of unspliced/partially spliced viral RNAs contrasts sharply with the routine retention of corresponding cellular mRNAs in the nucleus (Luo and Reed, 1999; Stutz et al., 2000; Strasser et al., 2000; Zhou et al., 2000). The mechanism for distinguishing viral transcripts from cellular RNAs resides with the virally encoded Rev protein, which binds a highly secondary structured element (Rev-responsive element, RRE) present in all unspliced and partially spliced HIV transcripts (Zapp and Green, 1989; Chang and Sharp, 1989; Hope et al., 1990; Kjems et al., 1991; Malim et al., 1989).

The prevailing view is that Rev binding to RRE shepherds HIV-1 transcripts from the nucleus into the cytoplasm. In doing so, Rev interacts with CRM1 (chromosome maintenance region 1; Bogerd et al., 1998; Otero et al., 1998; Askjaer et al., 1998) and utilizes a nucleo-cytoplasmic shuttling pathway normally employed for export of proteins, small nuclear RNAs, and rRNAs (Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997). The Rev-RRE-CRM1 pathway is distinct from that used to export fully spliced HIV-1 mRNA and cellular mRNAs from the nucleus (Clouse et al., 2001; Herold et al., 2001; van Baalen et al., 1998; Pasquinelli et al., 1997; Saavedra et al., 1997). Insights into this latter pathway have been guided in part by studying the biology of simpler retroviruses such as type D Mason-Pfizer monkey virus (MPMV). MPMV does not encode a Rev activity. The RNAs of MPMV contain a *cis*-active structure termed the constitutive transport element, CTE, located in the 3′ UTR of the viral genomes (Bray et al., 1994). CTE in the absence of any virally encoded protein recruits TAP, which has been shown to be involved in cellular mRNA transport (Bear et al., 1999; Stutz et al., 2000). Importantly, CTE-TAP-dependent nuclear export is distinct from the Rev/CRM1 pathway. The independence of these two routes was confirmed by the observation that inhibition of one does not affect the export of RNA through the other (Pasquinelli et al., 1997; Saavedra et al., 1997). Currently, the involvement of additional cellular participants in these export paths is incompletely understood (Fornerod et al., 1997). It also remains unclear whether RNA conformational changes are important for large unspliced RNAs to thread through and dissociate from the nuclear pores in the egress of unspliced RNAs from the nucleus (Nakielny and Dreyfuss, 1999).

As part of our research, we wished to identify virus- or Tat-induced cellular factors which may function in viral transcription or posttranscription. We used mRNA differential display as an open-ended method to compare changes in cellular mRNA profiles in Tat-expressing versus nonexpressing cells. In doing so, we observed enhanced expression of DDX3, a DEAD box protein, in Tat-expressing samples. DDX3, located on the human X chromosome, was first identified six years ago (Park et al., 1998). Although most DEAD box proteins have been found to possess ATP binding and hydrolysis activity as well as ATP-dependent RNA helicase activity (Luking et al., 1998; Tanner and Linder, 2001), no direct information exists as to whether DDX3 is an ATPase or a helicase or how DDX3 functions inside cells.

Here, we find that although DDX3 was induced by Tat, it plays no role in HIV-1 transcription. Instead, DDX3 is an RNA-dependent ATPase/helicase which functions in the Rev-RRE/CRM1 pathway for the export of unspliced/partially spliced HIV-1 transcripts. We show that DDX3 is a nucleo-cytoplasmic shuttling protein that binds CRM1 and decorates nuclear pores and whose

*Correspondence: kj7e@nih.gov

[3]These authors contributed equally to this work.

[4]Present address: Unite de Recombinaison et Expression, Institute Pasteur, 28 Rue de Dr. Roux, 75724 Paris Cedex 15, France.

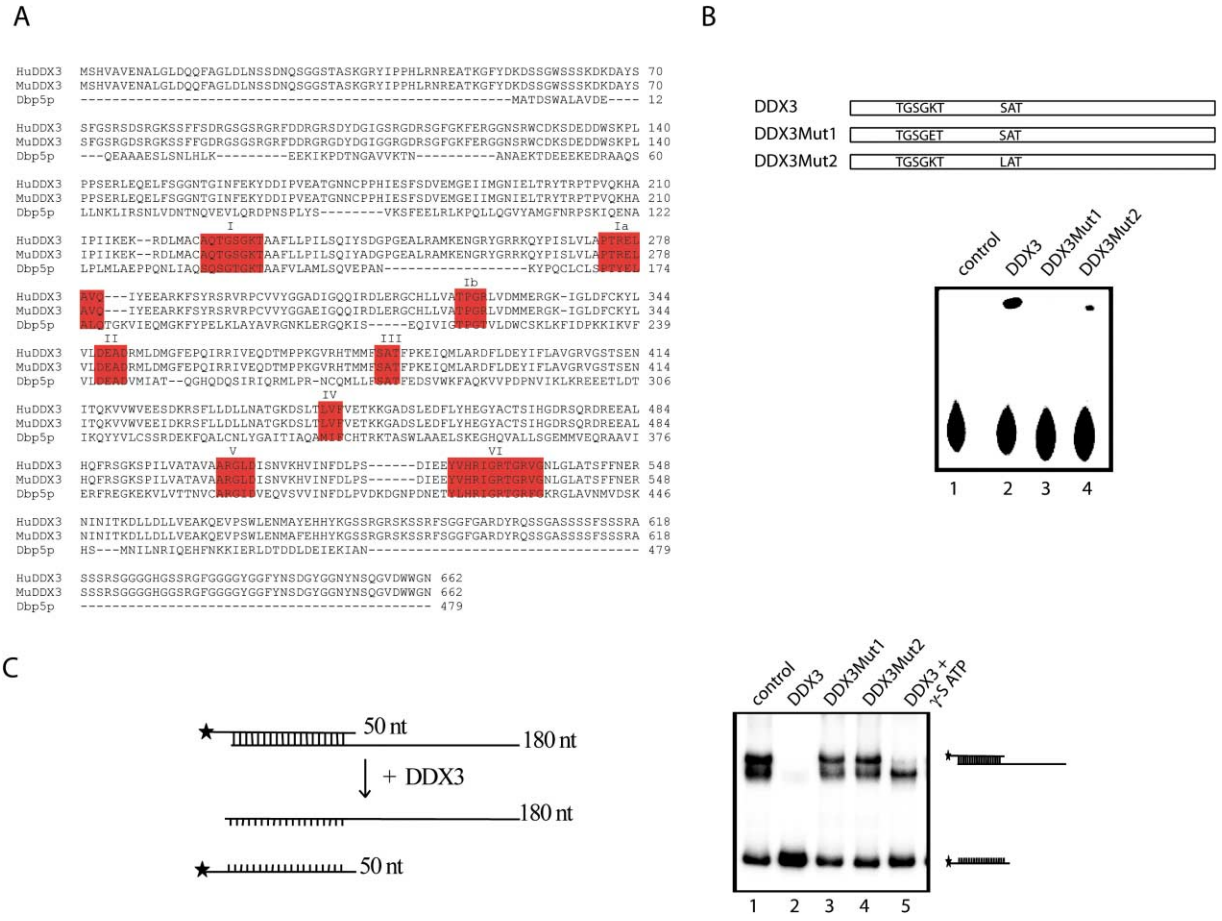


Figure 1. DDX3 Is an ATP-Dependent RNA Helicase

(A) Alignment of amino acid sequences of human and mouse DDX3 (AAC34298 and NP 034158) and Dbp5p (CAB52189). The eight conserved DEAD helicase motifs are boxed.

(B) In vitro ATPase assay demonstrating the release of radioactive phosphate by DDX3 (lane 2), but not by DDX3Mut1 (lane 3), or with reduced efficiency by DDX3Mut2 (lane 4).

(C) RNA unwinding assay using purified proteins. Left, schematic of the unwinding assay. Right, DDX3 (lane 2) unwound RNA duplex in the presence of ATP but ineffectively with the competing presence of nonhydrolyzable γ-S-ATP (lane 5). Neither DDX3Mut1 nor DDX3Mut2 showed any unwinding activity (lanes 3 and 4). Buffer control is in lane 1.

function depends on its enzymatic activity. We propose that human DDX3 serves like yeast Dbp5p in restructuring cargo RNAs to permit their translocation across/dissociation from the nuclear pore.

Results

DDX3 Is an RNA-Dependent ATPase/Helicase

In screening for cellular factors induced by Tat, we reproducibly observed the upregulation of DDX3 in human cells (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/3/381/DC1/). A literature search revealed no characterized function for DDX3 (Luking et al., 1998; Linder et al., 2001; Lorsch, 2002). Human DDX3, a DEAD box protein, is rich in serines (11.3%) and glycines (11.4%) and contains all eight motifs conserved by eukaryotic RNA helicases (highlighted in red, Figure 1A; Tanner and Linder, 2001). We, therefore, investigated whether DDX3 is an ATPase and/or helicase.

We created two DDX3 point mutants by independently changing lysine (K) at position 230 (DDX3Mut1) in the "QTGSGKT" ATPase motif to glutamic acid (E), or serine (S) at position 382 (DDX3Mut2) in the "SAT" helicase motif to leucine (L). Wild-type DDX3 and the two mutant proteins were overexpressed using pTYB11 vector and purified from *E. coli* BL21(DE3). ATPase activity was assessed by hydrolysis of ATP (Jensen et al., 1995). In the presence of RNA, DDX3 hydrolyzed ATP efficiently (Figure 1B, lane 2); however, both DDX3Mut1 (K230E) and DDX3Mut2 (S382L) were either lost or significantly reduced in ATPase activity (Figure 1B, lanes 3 and 4). Next, to check DDX3's ability to unwind double-stranded RNA, we incubated purified DDX3 and its two mutants separately with duplexed RNA. DDX3 unwound RNA duplex in an ATP-dependent manner (Figure 1C, lane 2), which is inhibited by competing amounts of nonhydrolyzable γS-ATP (Figure 1C, lane 5). Point mutation of either the ATPase or helicase motif abolished

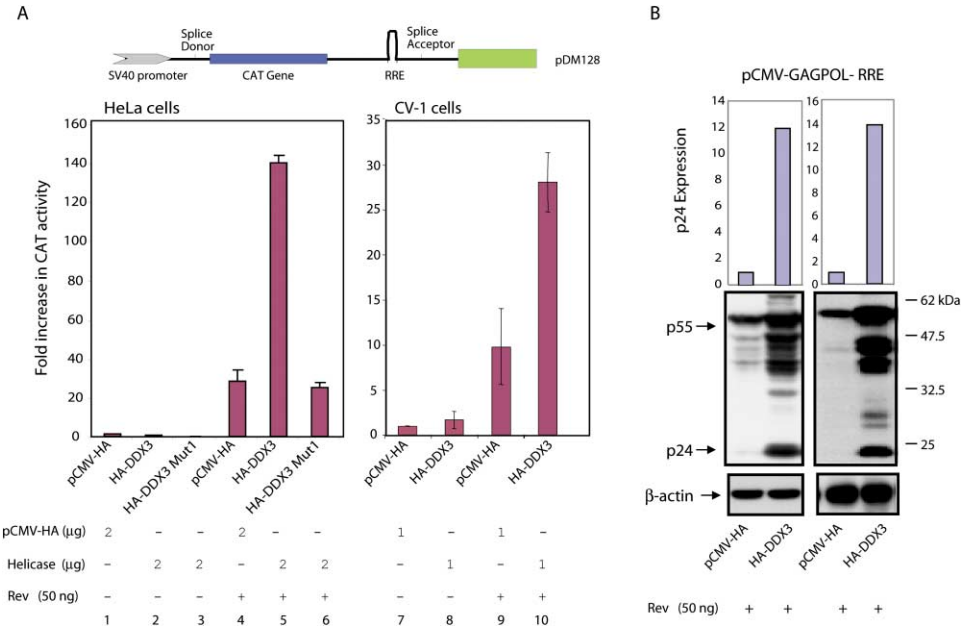


Figure 2. DDX3 Enhanced Rev-RRE Dependent Expression

(A) DDX3 enhanced the expression of RRE-containing RNA in the presence of Rev in HeLa (left) and CV-1 (right) cells. The graphs show fold increase in expression of CAT from pDM128 (top) normalized to cotransfected control plasmid. Experiments were performed three independent times. Error bars are ±SD.

(B) Compared to control vector (pCMV-HA), DDX3 (HA-DDX3) increased the expression of Gag from RRE containing HIV-1 Gag RNA expressed from pCMV-GagPol-RRE. Top, bar graphs of the fold increase in normalized p24 expression measured by ELISA. Middle, Western blotting using Gag-specific serum. Bottom, β-actin controls. Two independent experiments are shown.

DDX3's RNA-unwinding activity (Figure 1C, lanes 3 and 4). These findings establish DDX3 as an energy-dependent RNA helicase.

DDX3 Functions Specifically in Rev/RRE-Dependent Export of RNAs

In principle, RNA helicases can act either transcriptionally or posttranscriptionally (Tanner and Linder, 2001). We asked if DDX3 influences either Tat or Rev function. First, we expressed DDX3 or its ATPase-defective DDX3Mut1 in HeLa cells with an HIV-1 LTR luciferase reporter. Neither DDX3 nor DDX3Mut1 affected LTR expression, suggesting a lack of involvement in viral transcription (data not shown). Next, we checked if DDX3 functions in posttranscriptional gene regulation using the Rev-responsive RRE-containing pDM128 plasmid (Figure 2A; Hope et al., 1990). When overexpressed in either HeLa or CV-1 cells, DDX3 did not affect basal expression from pDM128 (Figure 2A, lanes 2, 3, and 8). However, when Rev was coexpressed with DDX3, Rev-dependent pDM128 expression increased by ≥5-fold (Figure 2A, lanes 5, 6, and 10).

Rev is required for the export of HIV-1 Gag-encoding RNAs from eukaryotic nuclei (Sodroski et al., 1986; Malim et al., 1989). The above results implied a role for DDX3 in Rev function. We next verified the pDM128 results using Western analysis of a CMV-driven GagPol-RRE vector. Like CAT expression from pDM128, Gag expression from pCMV-GagPol-RRE is highly responsive to Rev. In repeated assays (two representative experiments are shown in Figure 2B), Rev-dependent expression of both p24 and p55 Gag was enhanced 5- to 12-fold by coexpression of DDX3.

We next wondered whether the overexpression findings in Figure 2 reflect the function of cell-endogenous DDX3. To address this question, we compared expression of CMV-GagPol-RRE to a control GagPol-CTE vector (pCMV-GagPol-CTE; Figure 3C) in which the RRE sequence in GagPol-RRE was replaced with a MPMV CTE (Bray et al., 1994). CTE-mediated expression of Gag is Rev independent (Zolotukhin et al., 1994).

To suppress cell-endogenous DDX3, we tried several siRNAs, which all worked poorly (data not shown). We thus resorted to more traditional knockdown approaches—the cloning of an antisense DDX3 (DDX3-AS; Figure 3A) and the generation of dominant-negative DDX3 mutants. DDX3-AS was highly effective in knocking down DDX3 expression (Figure 3B), and we also found that two DDX3 mutant proteins, DDX3Mut1 and DDX3 (410–662), had dominant-negative activity, while a third, DDX3 (1–310), was a loss-of-function mutant (data not shown).

Using DDX3-AS and DDX3 mutants, we checked the role of cell-endogenous DDX3 in Rev/RRE function. We introduced Rev + pCMV-GagPol-RRE separately with DDX3-AS, DDX3 (1–310), DDX3 (410–662), or DDX3Mut1 into HeLa cells and assayed for intracellular p55 Gag expression and extracellular p24 Gag in supernatant media. In the context of cell-endogenous DDX3, Rev induced robustly p55 and p24 expression. When DDX3-AS was used to suppress endogenous DDX3, a marked

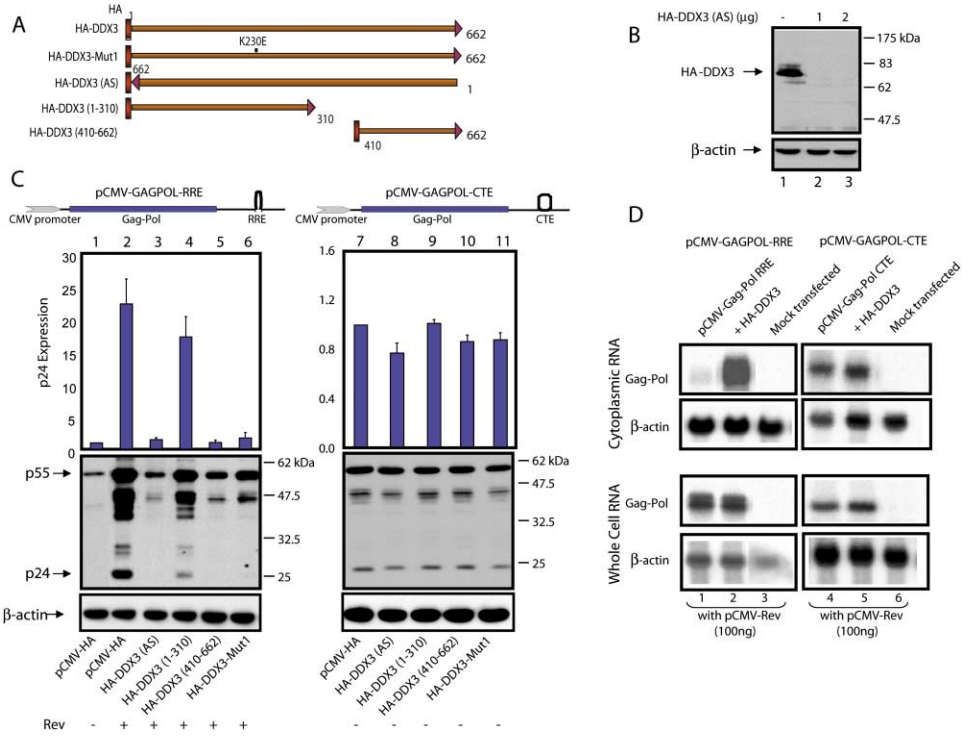


Figure 3. DDX3 Modulates Rev/RRE- but Not CTE-Dependent Expression

(A) Schematics of DDX3 mutants and the DDX3-AS vector.

(B) Western analysis of DDX3-AS mediated silencing of DDX3. HeLa cells were transfected with 2 μg of HA-DDX3 (AS) and probed with anti-DDX3 antibody raised in rabbits.

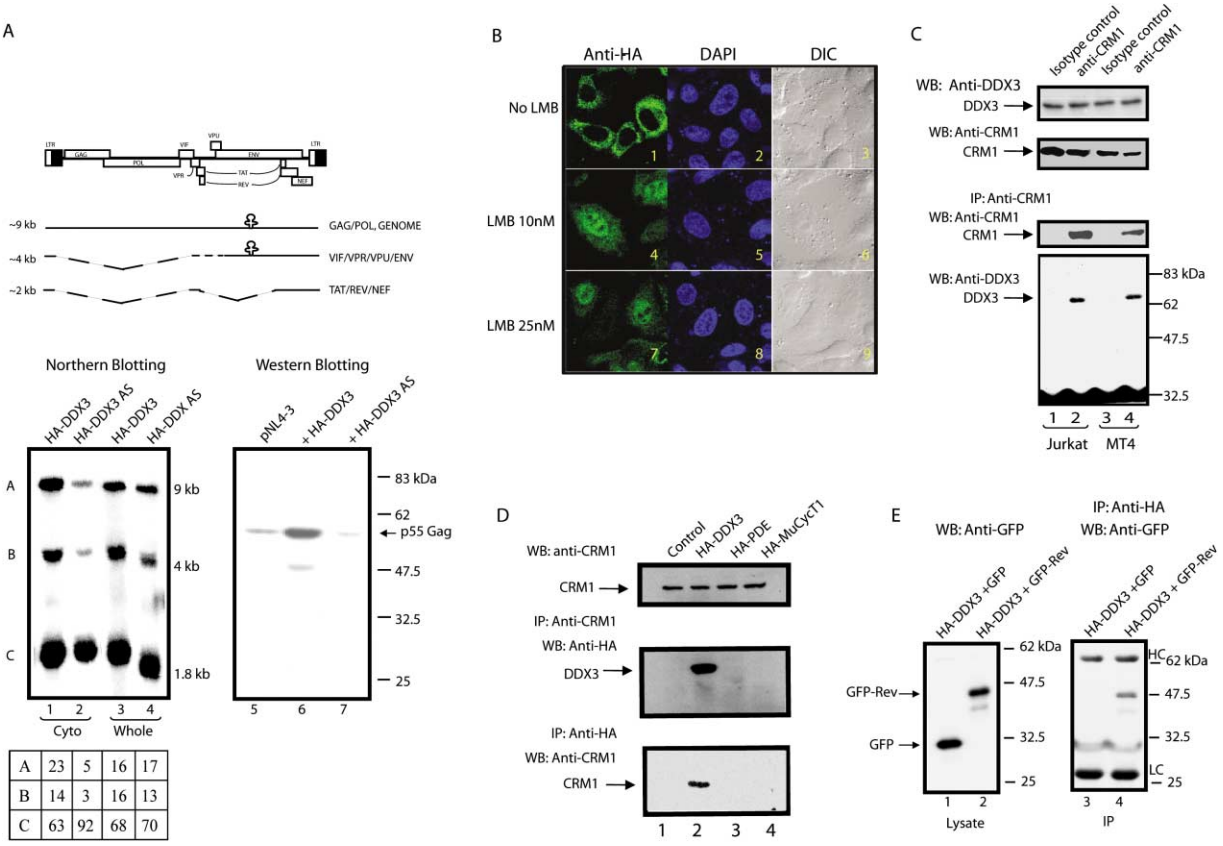
(C) Analyses of p24 and p55 Gag expression from pCMV-GagPol-RRE (left) and pCMV-GagPol-CTE (right). HeLa cells were transfected with 0.5 μg pCMV-GagPol-RRE (left) or pCMV-GagPol-CTE (right) and 2 μg of DDX3, or DDX3 mutant, or vector control plasmid as indicated, either in the presence (lanes 2–6) or absence (lanes 1, 7–11) of Rev. DDX3-AS, HA-DDX3 (410–662), and HA-DDX3Mut1 (lanes 3, 4, and 6) significantly blocked Gag expression from pCMV-GagPol-RRE but had no effect on pCMV-GagPol-CTE (lanes 8, 10, and 11). Top, bar graphs of p24Gag in the cell supernatants determined by ELISA. Bottom, Western blottings of intracellular p24 and p55 Gag.

(D) Northern blot analysis of cytoplasmic and whole-cell RNAs isolated from HeLa cells transfected with a Rev-expression plasmid and pCMV-GagPol-RRE + vector control (lane 1) or pCMV-GagPol-RRE + HA-DDX3 (lane 2). Rev alone transfected cells (mock transfected) are shown in lanes 3 and 6. Lanes 4 and 5 are the same as lanes 1 and 2 except that pCMV-GagPol-CTE was transfected in place of pCMV-GagPol-RRE.

(~13-fold) reduction in p24 Gag and p55 Gag expression was observed (Figure 3C). This suppression of Gag was also seen with dominant-negative DDX3 (410–662) and DDX3Mut1 but not with loss-of-function DDX3 (1–310) mutant (Figure 3C). When we repeated the experiment with the Rev-independent pCMV-GagPol-CTE vector, neither DDX3-AS nor the dominant-negative DDX3 mutants significantly affected p55 Gag or p24 Gag, ruling out trivial nonspecific toxic effects as explanations (Figure 3C).

Next, we asked by Northern blotting if the effect of DDX3 on pCMV-GagPol-RRE expression is at the level of export of Gag mRNA into the cytoplasm and not from increased transcription or translation. Indeed, DDX3 expression had no effect on whole-cell Gag mRNA (Figure 3D, bottom left, compare lane 2 to lane 1). By contrast, cytoplasmic Gag mRNA was significantly increased by DDX3 (Figure 3D, top left, compare lane 2 to lane 1). As control, DDX3 had no effect on the abundance of either whole-cell Gag mRNA or its cytoplasmic counterpart expressed from pCMV-GagPol-CTE (Figure 3D, lane 5).

In HIV-1, unspliced, partially spliced, and fully spliced RNAs fall into three size classes (Figure 4A, top). The ~9 kb unspliced RNA serves as genomic RNA and encodes Gag and GagPol proteins. The ~4 kb singly spliced mRNAs encode Env, Vpr, Vif, and Vpu. Fully spliced ~1.8 kb mRNAs represent Tat, Rev, and Nef. Of note, the 9 kb and 4 kb, but not 1.8 kb, RNAs contain RRE and are Rev dependent. To confirm the DDX3 effect in the context of the entire panoply of HIV-1 RNAs, we checked DDX3 and DDX3-AS vectors on the expression/distribution of viral 9 kb, 4 kb, and 1.8 kb transcripts. We cotransfected cells with HIV-1 molecular clone pNL4-3 and either DDX3 or DDX3-AS and analyzed total and cytoplasmic RNAs by Northern blotting. Comparing the DDX3 sample to the DDX3-AS sample, we saw no difference in total RNA expression (Figure 4A, lanes 3 and 4). Moreover, the cytoplasmic distribution of the Rev-RRE-independent 1.8 kb transcripts in the two samples was also indistinguishable (Figure 4, lanes 1 and 2). However, when cytoplasmic Rev-RRE-dependent 9 kb and 4 kb transcripts were compared, these revealed an ~5-fold reduction in the DDX3-AS versus DDX3 sample (Figure 4A, lanes 1 and 2). Because export into the cytoplasm of the 9 kb unspliced HIV-1 RNA is necessary for synthesis of p55 Gag, we verified the Northern results by Western blotting. Indeed, we confirmed that Gag expression was decreased in a manner commensurate with the



A	23	5	16	17
B	14	3	16	13
C	63	92	68	70

Figure 4. DDX3 Is a Shuttling Protein that Binds Rev and CRM1 and Functions in Nuclear Export of HIV-1 mRNAs

(A) Top, schematics of the 9 kb, 4 kb, and 1.8 kb classes of HIV-1 RNAs. The looped structure in the 9 kb and 4 kb RNAs represents RRE. Bottom left, Northern blot (lanes 1–4) of cytoplasmic and whole-cell RNA from HeLa cells transfected with pNL4-3 and either WT (HA-DDX3) or antisense (HA-DDX3-AS) plasmids. Quantitation of the A, B, and C classes of RNAs are shown in box below. Bottom right, Western analysis of HeLa cell lysates (lanes 5–7) showing increased pNL4-3 gene expression by DDX3 (HA-DDX3; compare lane 5 and 6), which is suppressed by antisense HA-DDX3-AS.

(B) DDX3 is a nucleo-cytoplasmic shuttling protein. HeLa cells transfected with HA-DDX3 were treated with (middle and bottom) or without (top) leptomycin B (LMB). Cells were fixed 4 hr after LMB treatment and stained with fluorescent-labeled anti-HA (left column) or DAPI (middle column). Right column shows DIC images.

(C) Western analysis using anti-DDX3 of Jurkat and MT4 cell lysates immunoprecipitated with anti-CRM1. Top two panels show comparable expression of DDX3 and CRM1 in lysates. Middle panel shows immunoprecipation of cell endogenous CRM1 with coimmunoprecipated DDX3 (bottom panel, lanes 2 and 4).

(D) Western analysis of coimmunoprecipitations from HeLa cells transfected with HA-DDX3 (lane 2), HA-phosphodiesterase 9A (lane 3), or HA-mouse cyclinT1 (lane 4). Lysates were probed with anti-CRM1 (top panel), then immunoprecipitated with anti-CRM1 followed by blotting with anti-HA (middle panel) or immunoprecipitated with anti-HA followed by blotting with anti-CRM1 (bottom panel).

(E) DDX3 coimmunoprecipitated with Rev. Lysates from HeLa cells transfected with either GFP + HA-DDX3 (lane 1) or GFP-Rev + HA-DDX3 (lane 2) were subjected to immunoprecipitation with anti-HA. Western analysis shows corecovery of GFP-Rev with DDX3 (lane 4) but no recovery of GFP with DDX3 in lane 3. HC, heavy chain; LC, light chain.

reduced cytoplasmic presence of the 9 kb HIV-1 mRNA (Figure 4A, lanes 5–7). Collectively, the above findings suggest that DDX3 is important for Rev/RRE, but not CTE, export of unspliced/partially spliced transcripts.

DDX3 Shuttles between the Nucleus and Cytoplasm, Binds CRM1, and Locates to Nuclear Pores

Rev shuttles from the nucleus to the cytoplasm shepherding RRE-containing transcripts. If DDX3 has a role in Rev function, there could be many points where this helicase might act. To gain insight into DDX3's action, we asked first where DDX3 protein is located. We raised rabbit antibody directed to a 20 amino acid (NSDGYGG NYNSQGVDWLSGN) C-terminal DDX3 peptide. Using this antiserum, we verified by detergent fractionation that DDX3 endogenous to primate cells (Jurkat, SW480, CV-1, and HeLa) is constitutively cytoplasmic with reduced expression in HeLa cells (Supplemental Figure S2 on the *Cell* website).

We next queried whether DDX3 shuttles between the cytoplasm and the nucleus. HeLa cells transfected with HA-tagged DDX3 were treated without (Figure 4B, panels 1–3) or with (Figure 4B, panels 4–9) leptomycin B (LMB), an inhibitor of CRM1-mediated nuclear export, and stained with anti-HA. Without LMB, DDX3 was prominently cytoplasmic (Figure 4B, no LMB), in agreement with our detergent fractionation data (Supplemental Figure S2 on the *Cell* website). On the other hand,

when CRM1 activity was suppressed by LMB, DDX3 substantially accumulated in the nucleus (Figure 4B, LMB 10 nM, LMB 25 nM). These results are consistent with DDX3 being a nucleo-cytoplasmic shuttling moiety.

Shuttling could be explained mechanistically if DDX3 binds CRM1. To address this question, we performed protein coimmunoprecipitations. First, we checked whether cell-endogenous DDX3 coimmunoprecipitates with cell-endogenous CRM1 in T cell lines, Jurkat, and MT4 (Figure 4C). Using anti-CRM1 (Figure 4C, lanes 2 and 4) or isotype control (Figure 4C, lanes 1 and 3), we detected DDX3 as a specific coprecipitant of CRM1 (Figure 4C, lanes 2 and 3). Next, to verify further DDX3-CRM1 association, we transfected a human CRM1 expression plasmid into HeLa cells (which have low endogenous DDX3) separately with HA-tagged DDX3 (Figure 4D, lane 2), HA-tagged human phosphodiesterase 9A (as a neutral control; HA-PDE, lane 3), or HA-tagged murine cyclin T1 (as another neutral control; HA-MuCycT1, lane 4). We then performed immunoprecipitation (IP)/Western blotting using either anti-CRM1 followed by anti-HA (Figure 4D, middle) or anti-HA followed by anti-CRM1 (Figure 4D, bottom). Results showed that DDX3, but not PDE nor MuCycT1, physically bound CRM1 (Figure 4D, lane 2). Finally, we asked if DDX3 also complexes with Rev. Because we could not obtain Rev antiserum which did not have high background, we performed this assay by cotransfecting HA-tagged DDX3 with either GFP alone or GFP-Rev. Western blotting showed equivalent GFP and GFP-Rev expression in cell lysates (Figure 4E, lanes 1 and 2). Immunoprecipitation of DDX3 brought down GFP-Rev, but not GFP (Figure 4E, lanes 3 and 4). Based on these results, we favor an interpretation of a functional CRM1/DDX3/Rev ternary complex. However, our data cannot formally exclude the existence of discrete DDX3/CRM1 and Rev/CRM1 binary complexes.

Previously, Askjaer et al. (1999) reported that the *Xenopus* An3 DEAD box helicase bound through its N-terminal nuclear export sequence (NES) CRM1-RanGTP and was transported from the nucleus as passive cargo rather than as a functional component of the export complex. Like An3, DDX3 also has an N-terminal NES motif (Figure 5A). We wondered whether DDX3 associated with CRM1 as cargo or as an integral effector. We created several HA-tagged DDX3 deletions (Figure 5A), which segregated the NES from the rest of the coding frame, and co-IPed CRM1 with individual DDX3 deletions, seeking to define DDX3's CRM1-interactive domain. The co-IPs revealed that the NES contributed minimally, if at all, to DDX3-CRM1 binding. The major CRM1-associative determinant resided instead in DDX3's C-terminal 260 to 517 region (Figure 5B).

We next asked whether DDX3 bound CRM1 directly and whether such binding required Ran-GTP. First, we expressed in and purified from *E. coli* His-tagged DDX3, His-tagged DDX3 (1–585), and an unrelated control, His-tagged Tax (Jeang et al., 2004; Figure 5C, lanes 1–3). We incubated these proteins with either GST or GST-CRM1 and detected bound material by Western blotting using anti-His (Figure 5C, lanes 4–9). None of the proteins bound GST (Figure 5C, lanes 4–6), while DDX3 and DDX3 (1–585), but not Tax, bound GST-CRM1 (Figure 5C, lanes 7–9). Second, we purified RAN and RANQ69L protein (Figure 5D) and also purified using nickel-bead chromatography ^{35}S-methionine-labeled An3, DDX3, DDX3 (1–585), and DDX3 (106–662) translated in vitro (Figure 5E, lanes 1–4) using *E. coli* S30 extract (see Experimental Procedures). This approach excluded the possibility that minute eukaryotic factors might contaminate our purifications and bridge a DDX3-CRM1 interaction. In doing our binding assays, we found agreement with previous findings (Fornerod et al., 1997; Askjaer et al., 1999) that An3 cargo interaction with CRM1 does require either Ran-GTP or RanQ69L-GTP (Figure 5E, lanes 23–25). On the other hand, binding of DDX3 to CRM1 needed neither Ran-GTP nor RanQ69L-GTP (Figure 5E, lanes 5–7, 11–13, and 17–19). Hence, DDX3-CRM1 association is different from the previously described An3-CRM1 cargo-receptor interaction.

Yeast Dbp5p helicase functions in nucleo-cytoplasmic export of mRNAs (Snay-Hodge et al., 1998; Tseng et al., 1998; Schmitt et al., 1999). To date, no counterpart mammalian DEAD protein has been reported to function in the Rev-CRM1-specific HIV-1 export pathway. Should DDX3 be Dbp5p like for HIV-1 RNAs, we might expect DDX3 to bind nucleoporins and to localize to nuclear pore complexes (NPC). To test these expectations, we immunoprecipitated human nucleoporins using monoclonal antibody, mab414 (Davis and Blobel, 1986), and observed specific coimmunoprecipitation of DDX3 (Figure 6A). Next, we asked whether DDX3 localizes in situ to nuclear pores. We stained digitonin-permeabilized cellular nuclei with mab414, which revealed a prototypic nucleoporin pore pattern (Figure 6B, panel 5). When the same preparation was visualized for GFP-DDX3, we saw colocalization of DDX3 as a rim with the nuclear pores (Figure 6B, panel 4). We next queried whether DDX3 was on the outer or inner side of the nuclear membrane. To address this query, we constructed GFP-tagged inner nuclear membrane protein, GFP-SUN1 (Figure 6B, panels 10, 13, and 16). Using in situ detergent extraction (Triton X-100), which removes the outer nuclear membrane but leaves the inner nuclear membrane intact (Dreger et al., 2001; Hodzic et al., 2004), we found that the signal for inner nuclear membrane protein SUN1 was unchanged (Figure 6B, panel 16) while the signal for GFP-DDX3 (Figure 6B, panel 7) was removed. Thus, we interpret DDX3 to shuttle from the nucleus (Figure 4B) through the pore to locate to its outer side. This behavior of DDX3 is consistent with that previously described for yeast Dbp5p (Tseng et al., 1998).

DDX3 Is Limiting for HIV-1 Replication

As a final test of DDX3's Rev-specific function, we investigated DDX3's effects on HIV-1 replication. We transfected HeLa cells with HIV-1 molecular clone BH10 and either DDX3 or its dominant-negative DDX3Mut1. Twenty-four, thirty-six, and forty-eight hours later, we analyzed culture supernatants for p24 Gag production. Compared to vector control, wild-type DDX3 enhanced, while dominant-negative DDX3Mut1 suppressed, p24 Gag production (Figure 7A).

To confirm the above single-round assay, we next constructed chimeric HIV-1 genomes, which contain DDX3 (WT Hc), DDX3Mut1 (Hc-Mut-1), or a nonexpressible DDX3 ORF (Hc [stop], AUG replaced by two consec-

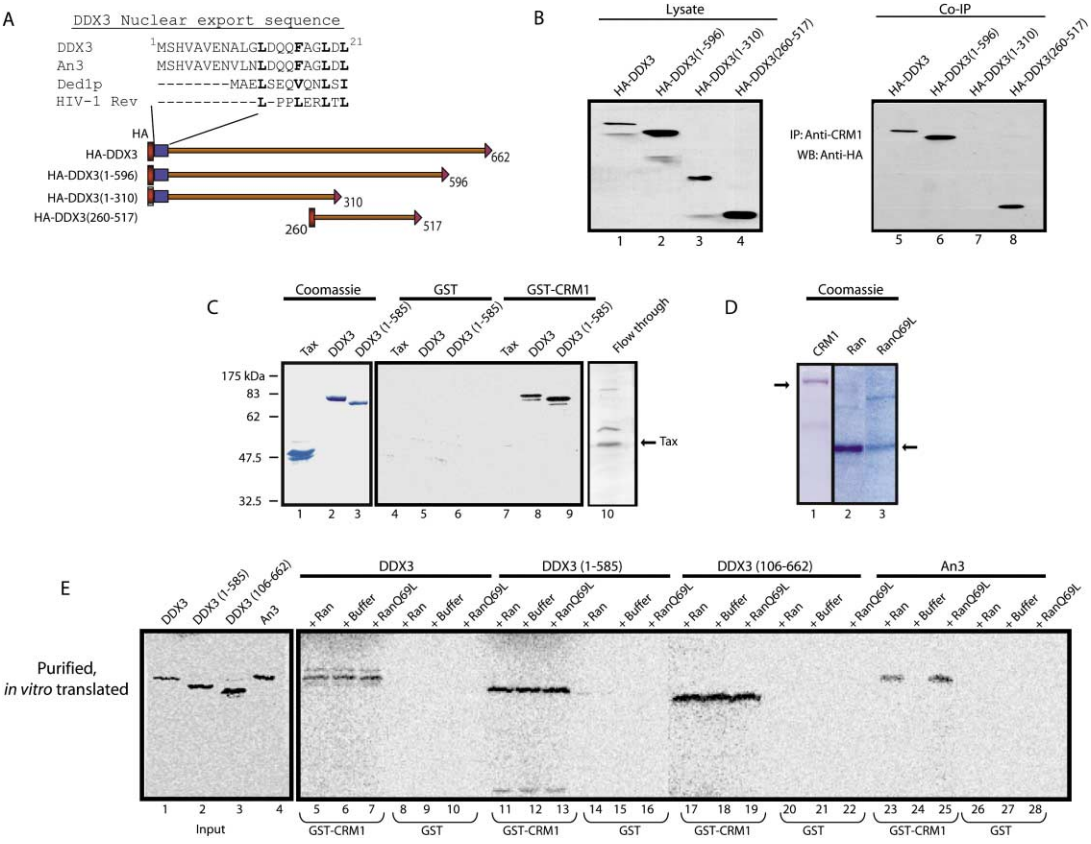


Figure 5. Ran-GTP Independent Binding between DDX3 and CRM1

(A) HA-tagged DDX3 and its deletion mutants. Sequence relatedness of the DDX3 NES with the NES from An3, Ded1p, and Rev are shown at top. The DDX3 NES is boxed in blue. Note that HA-DDX3 (260–517) no longer contains an NES.

(B) Coimmunoprecipitations between DDX3 proteins and CRM1. Left, Western blotting of cell lysates using anti-HA. Right, co-IPs using anti-CRM1 followed by Western blotting using anti-HA.

(C) DDX3 purified from overexpressing *E. coli* binds GST-CRM1, but not GST. Lanes 1, 2, and 3 show Coomassie staining of purified His-Tax, His-DDX3, and His-DDX3 (1–585). Fifty nanograms of purified protein was incubated with GST or GST-CRM1 beads, and bound material was assessed by Western blotting with anti-His (lanes 4–9). The presence of His-Tax in unbound "flow through" is shown in lane 10.

(D) Coomassie staining of purified CRM1, Ran, and RanQ69L.

(E) GST and GST-CRM1 pull-downs of His-tagged DDX3, DDX3 (1–585), DDX3 (106–662), and An3. All His-tagged proteins were translated and ^{35}S-methionine labeled using *E. coli* S30 extract and then purified using nickel bead chromatography (lanes 1–4). Bindings were performed using GST-CRM1 (lanes 5–7, 11–13, 17–19, and 23–25) or GST (lanes 8–10, 14–16, 20–22, and 26–28) beads in the presence of either RanGTP, RanQ69LGTP, or buffer as indicated. After extensive washings, bead bound proteins were resolved in 10% SDS-PAGE gel and visualized by phosphorimaging.

utive stop codons) placed into *nef* of pNL4-3. We have previously shown that such inserted foreign genes are expressed by HIV-1 in place of Nef during virus replication (Neuveut and Jeang, 1996). Virus stocks were generated from transfection of the pNL-DDX3 chimeric molecules into 293T cells, and inocula, normalized for RT, were used to infect productively MT4 and Jurkat T cells. Based on p24 profiles, pNL-DDX3 (WT Hc) replicated significantly better than either pNL-DDX3Mut1 (Hc-Mut-1) or pNL-DDX3 Stop (Hc[stop]) (Figure 7B). We then compared pNL-DDX3 (WT-Hc) to wild-type pNL-4-3 virus (Figure 7C). Jurkat cells were infected with equal amounts (500,000 RT units) of NL4-3 or NL-DDX3 (WT-Hc). Cells were washed free of input virus ($<$5 pg/ml p24), and p24 values were compared on days 3 and 6. Despite Jurkat cells having high endogenous levels of DDX3 (Supplemental Figure S2 on the *Cell* website), we nonetheless documented that NL-DDX3 (WT-Hc) replicated modestly better ($\sim$3-fold increase in p24 on day 6) than wild-type NL4-3 (Figure 7C). Altogether, the infection data support that DDX3 is important for HIV-1 replication in human cells.

Discussion

During and immediately after transcription, nascent and/or fully formed RNAs, which are rapidly packaged into RNPs (Dreyfuss et al., 2002), are likely bona fide intermediates for further processing. In transcriptional and posttranscriptional steps of gene expression, dynamic rearrangements of RNA secondary structure in RNPs occur. Proteins of the DEAD/DEXH box family of RNA-dependent ATPases are thought to play important roles in directing RNA-RNA and RNA-protein rearrangements by unwinding RNA helices and possibly disrupting or enhancing RNA-protein interactions (Dreyfuss

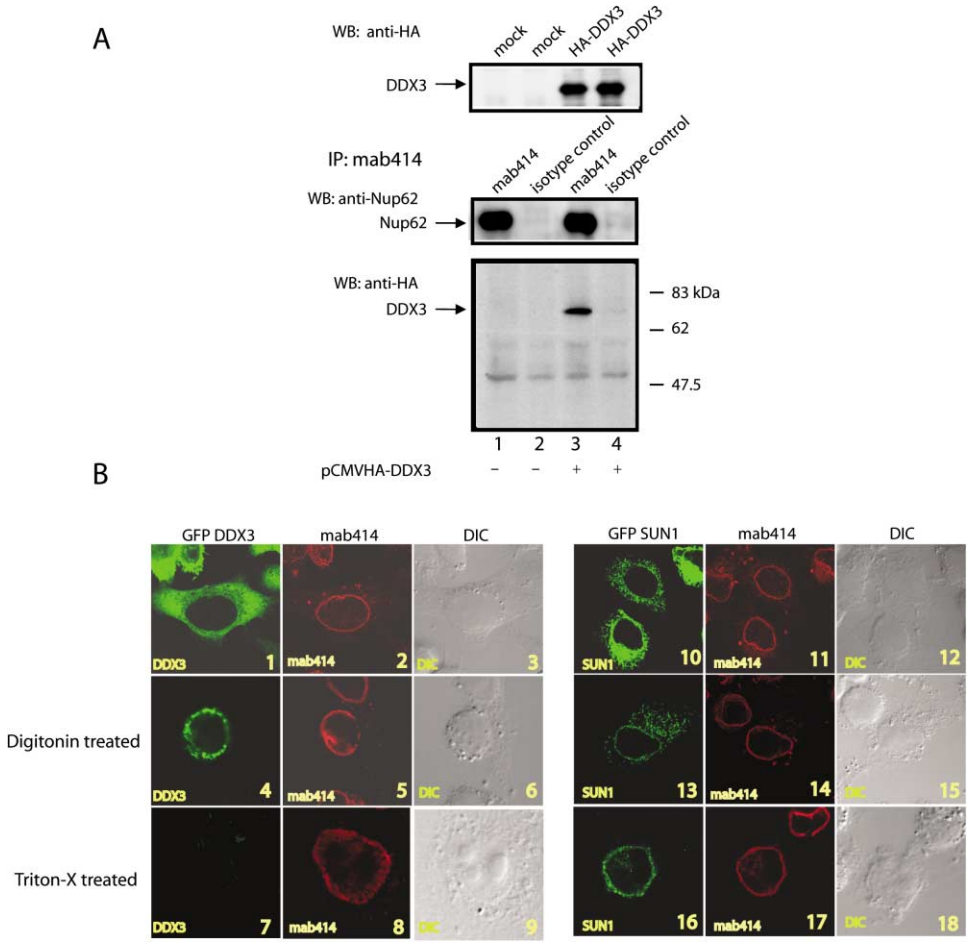


Figure 6. DDX3 Colocates with Nucleoporins at the Outer Nuclear Rim

(A) DDX3 coimmunoprecipitates with nucleoporins. HA-DDX3-transfected cells were immunoprecipitated with anti-nucleoporin mab414 or isotype control followed by Western blotting with anti-HA. Top panel shows the lysates probed with anti-HA. Middle panel shows immunoprecipitation of Nup62 by mab414. Bottom panel shows coimmunoprecipitation of HA-DDX3 (lane 3) with a Nup62-containing complex.

(B) HeLa cells transfected with GFP-DDX3 or GFP-SUN1 were permeablized with digitonin for 2 min (panels 4–6 and 13–15) and then extracted with 1% Triton-X100 (panels 7–9 and 16–18) before fixation in formaldehyde. Cells were stained sequentially with antinucleoporin mab414 and secondary antibody conjugated to AlexaFluor 596. Red is mab414; green is DDX3 or SUN1.

et al., 2002; Tanner and Linder, 2001). DEAD box proteins can be broadly considered as ATP-driven conformational protein switches that serve to remodel RNA structures in order to facilitate RNA maturation, export, and translation (Linder and Stutz, 2001; Linder, 2003).

Because RNA transcription and translation occur in two discrete compartments, eukaryotic cells must evolve highly efficient mechanisms to traffic macromolecules including RNAs into and out of the nucleus. To date, at least nine import receptors and four export receptors have been identified and characterized in *Sacchromyces cerevisiae* (Nakielny and Dreyfuss, 1999). CRM1 has been well studied as an export receptor which recognizes numerous NES-containing proteins, some of which are RNA binding proteins. Although export receptors for RNP and RNA helicases both ultimately target a common substrate, nuclear RNA, to date there are very few examples which link these two classes of proteins in export from the nucleus. The best support for an RNA helicase function in eukaryotic mRNA export comes from independent studies on yeast Dbp5p (Snay-Hodge et al., 1998; Tseng et al., 1998; Schmitt et al., 1999). Dbp5p is a cytoplasmic RNA helicase which accumulates around the nuclear envelope and is postulated to either remodel RNA cargo to allow its transit through the NPC and/or to facilitate RNA release from the cytoplasmic side of the NPC. Interestingly, Dbp5p works in the eukaryotic pathway for nuclear export of cellular mRNAs, a path discrete from that governed by CRM1. Thus, whether RNA helicases are essential participants in the CRM1-dependent RNA-export pathway remains unanswered.

HIV Rev is necessary for the export of unspliced and incompletely spliced viral RNAs from the nucleus (Zapp and Green, 1989; Chang and Sharp, 1989; Hope et al., 1990; Malim et al., 1989). Rev directly interacts with nuclear export receptor CRM1 (Fornerod et al., 1997; Neville et al., 1997), and CRM1 is required for Rev-mediated export of HIV RNAs (Bogerd et al., 1998; Fukuda et al., 1997; Fornerod et al., 1997). Here, we provide

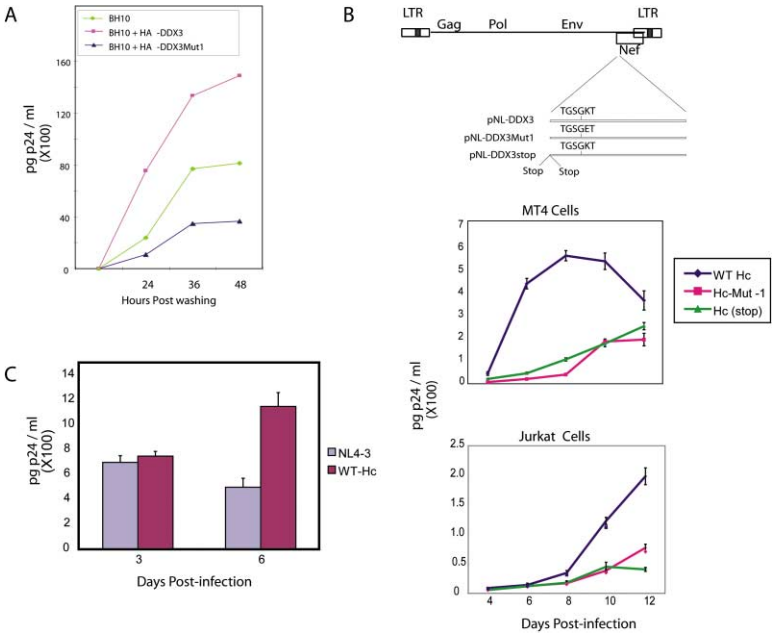


Figure 7. DDX3 Is Limiting for HIV-1 Infection

(A) HeLa cells were cotransfected with HIV-1 molecular clone BH10 and either DDX3 or mutant DDX3Mut1.

(B) Top, chimeric DDX3-pNL4-3 molecular clones. Middle, replication profiles in MT4 cells of the indicated molecular clones. Bottom, replication profiles in Jurkat cells. Graphs show p24 in ng/ml.

(C) Comparison of pNL4-3 replication and pNL-DDX3 replication in Jurkat cells. Jurkat cells were infected with equal amounts (based on RT values) of pNL4-3 and pNL-DDX3. Experiments were performed three times. Average values ±SD are shown.

evidence that an additional human cellular factor, the DEAD box RNA helicase DDX3, is functionally critical for and specific to Rev/RRE/CRM1-mediated HIV-1 RNA export. We base this conclusion on several results. First, we showed that DDX3 overexpression enhanced Rev- but not CTE-dependent expression (Figures 2, 3, and 4). Second, we found that DDX3 is a nucleo-cytoplasmic shuttling protein which binds CRM1 and Rev (Figures 4 and 5) and localizes to the outer side of nuclear membrane pores (Figure 6B). Finally, we verified the necessity of DDX3 for Rev/RRE/CRM1 function by using two approaches, antisense vector and dominant-negative mutants, to knockdown cell-endogenous DDX3 (Figure 3). Together with evidence that suppression of DDX3 inhibited HIV-1 replication in human cells (Figure 7), our results are consistent with DDX3 being important and limiting for Rev/RRE/CRM1 function.

How does DDX3 work with Rev and CRM1? Our findings suggest that one important component is the physical binding between DDX3 and CRM1 (Figures 4, 5, and 6). We note that while several RNA helicases like *Xenopus* An3 (Askjaer et al., 1999) contain a defined NES in their N terminus (Figure 3) and might be expected to associate with CRM1 in a cargo-receptor manner, our observed association between DDX3-CRM1 was dictated through an element present in DDX3's C-terminal 260–517 amino acids devoid of an NES (Figure 5). DDX3's N-terminal NES contributed little, if any, to its binding of CRM1 (see HA-DDX3 [1–310]; Figure 5B, lane 7), suggesting that cargo-receptor interaction likely describes a weaker contact than that provided through DDX3's 260–517 amino acids. We note substantial sequence divergence in the 260–517 regions of DDX3 and Dbp5p (Figure 1A). Such differences could explain the respective specificities of two RNA helicases for different transport pathways (i.e., DDX3 for the CRM1 pathway and Dbp5p for the TAP/Mex67 pathway). Moreover, in direct binding studies of purified CRM1 with either An3 or DDX3, we found that the former is consistent with cargo-receptor association in requiring Ran-GTP while the latter did not need Ran-GTP (Figure 5E). Based on these physical and functional findings (Figures 2, 3, 4, 5, and 7), we favor DDX3 being a CRM1-specific effector rather than cargo.

Although analogies between Dbp5p and DDX3 can be instructive, there may be significant differences in the details of their action. For instance, Dbp5p appears to require an additional cellular cofactor in order to unwind RNA duplexes (Tanner and Linder, 2001), while DDX3 is singularly sufficient in this activity (Figure 1C). Interestingly, Dbp5p has been shown to locate to the cytoplasmic side of the NPC (Schmitt et al., 1999), and in this regard, our nucleoporin coimmunoprecipitation and in situ confocal images with detergent extraction (Figure 6) suggest that DDX3 also ends up on the cytoplasmic side of the nuclear pore. Pending further investigation, one tempting interpretation is that Rev/CRM1-dependent threading of large HIV-1 RNAs from the nucleus through the nuclear pore and their final release from the cytoplasmic side of the pore are facilitated by the enzymatic unwinding action of the DDX3 RNA helicase. We note that elsewhere DDX3 has been described by others as being constitutively cytoplasmic with additional nuclear "speckle" localization and as a hepatitis C virus (HCV) core binding protein (Owsianka and Patel, 1999). In that context, it was hypothesized that DDX3 might participate as an RNA remodeling factor contributing to splicing or RNA maturation (Owsianka and Patel, 1999). For HIV-1 RNA, our results indicate that DDX3 has an activity specific to Rev (Figure 2) and RRE (Figure 3) not observed for non-RRE-containing (i.e., GagPol-CTE) RNAs. Moreover, our results show that DDX3 does not affect the splicing of HIV-1 RNAs (Figure 4A) while it augments the cytoplasmic accumulation and expression of Rev-RRE-dependent Gag mRNA (Figures 3D and 4A). That DDX3 directly binds CRM1 (Figure 5) and locates to nuclear pores (Figure 6) suggests that this helicase assists in the egress of CRM1 and associated Rev-

RRE-RNA from the nucleus into the cytoplasm. Nonetheless, at this juncture, we do not fully understand the details of DDX3's action, and we cannot exclude that a part of its action may be to remove cellular proteins that might otherwise dictate nuclear sequestration of HIV-1 RNA in a CRM1-Rev-RRE-specific manner.

Another helicase, RNA helicase A (RHA), has previously been proposed to function in the nuclear export of CTE-containing RNAs in mammalian cells (Tang et al., 1997a). RHA has an atypical nuclear export signal which has no homology to known NES (Tang et al., 1999). In contrast to DDX3, RHA does not interact with CRM1 but binds instead to shuttling factors, either a novel A kinase-anchoring protein HAP95 (Westberg et al., 2000) or TAP (Tang and Wong-Staal, 2000). Because its postulated mechanism of action is by direct binding to CTE-RNA (Tang et al., 1997b) and because neither the RHA/HAP95 nor the RHA/TAP complex increases Rev-RRE-dependent expression (Westberg et al., 2000; Tang and Wong-Staal, 2000), RHA and DDX3 are likely entirely different functional entities. We further note with interest that *Sacchromyces cerevisiae* has at least 39 DEAD/DEAH box proteins (de la Cruz et al., 1999), most putatively RNA helicases. One possible reason for such abundance in RNA helicases is that depending on context, subsets of helicases may couple selectively to different RNA transport complexes. Thus, Dbp5p may be paired with TAP/Mex67-mRNA or CTE-RNA, and RHA with HAP95-CTE-RNA or TAP-CTE-RNA, and DDX3 with CRM1-Rev-RRE-RNA. Accordingly, RNA helicases may be more commonly required than currently recognized to remodel a subset of cargo RNAs, easing the cargo RNAs' translocation through and subsequent dissociation from NPCs. While additional investigation is needed to verify the ubiquity of RNA helicases in macromolecular trafficking, a selective helicase requirement may partly explain whether some in vivo CRM1-dependent export through the NPC does (Zhang et al., 2003) or does not (Richards et al., 1997; Zhang et al., 2003) require nucleotide triphosphate consuming steps. Interestingly, while DDX3 functions in CRM1-dependent export of HIV RNA, it is not needed for CRM1-dependent nuclear egress of proteins such as IκBα (Supplemental Figure S3 on the *Cell* website).

Our current work identifies a novel role for a helicase activity in Rev-dependent HIV-1 gene expression. It raises the idea that small molecule inhibitors of RNA helicases could be a new group of drugs for treating HIV-1. We note that several viruses encode RNA helicases (Kadare and Haenni, 1997) and that there are active drug development programs targeting these viral helicases (Kim et al., 1995). Future research is needed to confirm whether antihelicase molecules that target DDX3 can be found and can treat AIDS.

Experimental Procedures

Cells and Plasmids

Human peripheral blood mononuclear cells (PBMC) were stimulated with phytohemagglutinin (PHA, 250 ng/ml final) for 3 days prior to infection and were maintained in RPMI 1640 with 20% fetal bovine calf serum and 10 units/ml of interleukin 2 (Pharmacia). 1×10^6 PBMCs in a 0.5 ml volume were absorbed with virus supernatants containing 5×10^6 cpm of RT activity at 37°C for 1 hr. Virus replication was monitored by measuring supernatant reverse transcriptase activity. Total RNAs were extracted 7 days after infection. pDM128 was from Tom Hope (Hope et al., 1990); pCMV-NL-GagPol-RRE and pCMV-NL-GagPol-CTE were from E. Freed; pET-His Ran and pET-His RanQ69L were from M. Dasso; GST-CRM1 was from Paul Russell (Gaits and Russell, 1999); and GST-An3 was from Daniel Weeks (Gururajan and Weeks, 1997).

ATPase and RNA Unwinding Assays

DDX3 was expressed in *E. coli* and purified using Impact-CN system (NEB; Beverly, Massachusetts). ATPase assay was done as previously described (Fuller-Pace et al., 1993). pBluescript vector was used to generate partially double-stranded RNA for RNA unwinding assay as described previously (Iost et al., 1999).

Antibodies and ELISA Assay

Rabbit polyclonal antibody was raised against the peptide NSDGYGGNYNSQGVDWLSGN corresponding to amino acids 644 to 662 of DDX3 C terminus. Monoclonal anti-HA (Sigma-Aldrich), goat polyclonal anti-CRM1 antibody (Santa Cruz Biotechnology), monoclonal mab414, and monoclonal anti-His (Covance) were used as described. HIV-1 immune serum was obtained from AIDS reference reagent program. p24 ELISA assays were performed according to manufacturer's protocol.

Confocal Imaging

HeLa cells were cultured on 25 mm coverslips (Thomas Scientific) and transfected. One day later, cells were fixed with 3.7% formaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and incubated with monoclonal anti-HA antibody followed with anti-mouse conjugated to Alexa Fluor 488 (Molecular Probes). Nuclei were stained with DAPI (Molecular Probes). Coverslips were mounted onto glass slides with ProLong Antifade Kit (Molecular Probes) and examined with a Leica laser-scanning microscope. For staining of cells with nucleoporin, antibody cells were transfected with GFP-DDX3, and 18 hr posttransfection, the cells were incubated with 40 μg/ml digitonin in HPEM buffer (65 mM PIPES, 30 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA [pH 6.9]), containing 1 mM PMSF and 40 μg/ml digitonin, for 2 min on ice before fixation with 3.7% formaldehyde. To extract the outer nuclear membrane, protein cells were washed three times with 1% Triton-X100 and stained with mab414 antibody.

GST Pull-Down Assay

DDX3, Tax, and An3 cDNAs were cloned into pET30a vector induction, and purification of His-tagged proteins from *E. coli* was performed using nickel beads according to standard protocols. ^{35}S-methionine-labeled DDX3 and An3 proteins were produced in a prokaryotic in vitro translation kit (ActivePro; Ambion) as per manufacturer's instructions. GST-CRM1, Ran, and RanQ69L were expressed in *E. coli* BL21 (DE3) and purified. Pull-downs of in vitro-translated An3 and DDX3 were performed as described (Askjaer et al., 1999).

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