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RNA helicase MOV10 functions as a co-factor of HIV-1 Rev to facilitate Rev/RRE-dependent nuclear export of viral mRNAs

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) exploits multiple host factors during its replication. The REV/RRE-dependent nuclear export of unspliced/partially spliced viral transcripts needs the assistance of host proteins. Recent studies have shown that MOV10 overexpression inhibited HIV-1 replication at various steps. However, the endogenous MOV10 was required in certain step(s) of HIV-1 replication. In this report, we found that MOV10 potently enhances the nuclear export of viral mRNAs and subsequently increases the expression of Gag protein and other late products through affecting the Rev/RRE axis. The co-immunoprecipitation analysis indicated that MOV10 interacts with Rev in an RNA-independent manner. The DEAG-box of MOV10 was required for the enhancement of Rev/RRE-dependent nuclear export and the DEAG-box mutant showed a dominant-negative activity. Our data propose that HIV-1 utilizes the anti-viral factor MOV10 to function as a co-factor of Rev and demonstrate the complicated effects of MOV10 on HIV-1 life cycle.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is a relatively small virus with approximately 9000 nucleotide-genomic RNA which contains multiple splice sites. The unspliced viral transcripts in infected cells are initially retained in nucleus (Mariani et al., 2000). However, HIV-1 has evolved a Rev protein to transport HIV-1 unspliced and partially spliced transcripts from the nucleus to the cytoplasm, enabling the transition from the early phase of gene expression to the late phase. The efficient export of nuclear transcripts is accomplished by binding to the Rev Response Element (RRE) within the unspliced/partially spliced HIV-1 mRNAs. The RRE sequence is located within *env* gene, which is not present in the fully spliced mRNAs of HIV-1.

HIV-1 Rev utilizes many host proteins especially RNA helicases to accomplish its function (Brass et al., 2008; Cullen, 2009). RNA helicases are ubiquitous proteins that bind to RNA or ribonucleoprotein (RNP) to participate in many aspects of RNA metabolism, including transcription, splicing, transport, translation, and decay (Linder et al., 2001; Tanner and Linder, 2001). Since DDX3 was firstly reported to be an essential co-factor of the Rev/RRE-dependent mRNAs export complex (Yedavalli et al., 2004), several RNA helicases have been identified as co-factors for HIV-1 replication (Fang et al., 2004; Zhou et al., 2013b). Unlike HIV-1 depending on Rev/RRE axis, some retroviruses (such as Mason-Pfizer monkey virus) contain a constitutive transport element (CTE) and transport mRNAs through CTE-TAP-dependent nuclear shuttle system (Pasquinelli et al., 1997). The independence between these two paths has been proved by some studies (Pasquinelli et al., 1997; Saavedra et al., 1997).

MOV10 was firstly discovered from the *MOV-10* mouse strain. The *MOV* strains carried Moloney murine leukemia virus (M-MuLV) in their germ line at different chromosomal positions. Among the *MOV* strains, the *MOV-10* substrain was found with inactive provirus and unable to induce viremia (Jaenisch et al., 1981; Schnieke et al., 1983). As containing a putative DEAG (D-E-A-G=Asp-Glu-Ala-Gly) box RNA helicase motif, MOV10 was classified to the DExD-box RNA helicase superfamily and recently described as an Upf1-like superfamily member (Abudu et al., 2012; Gregersen et al., 2014). The putative RNA helicase function of MOV10 was recently confirmed and described. It was reported that MOV10 has an ATP-dependent 5′ to 3′ directional RNA helicase activity (Gregersen et al., 2014). Recently, MOV10 was also reported to be co-purified with apolipoprotein-B-mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G or A3G) and affect the assembly and maturation of microRNA-inducing silencing complex (miRISC) (Gallois-Montbrun et al., 2007; Kenny et al.,

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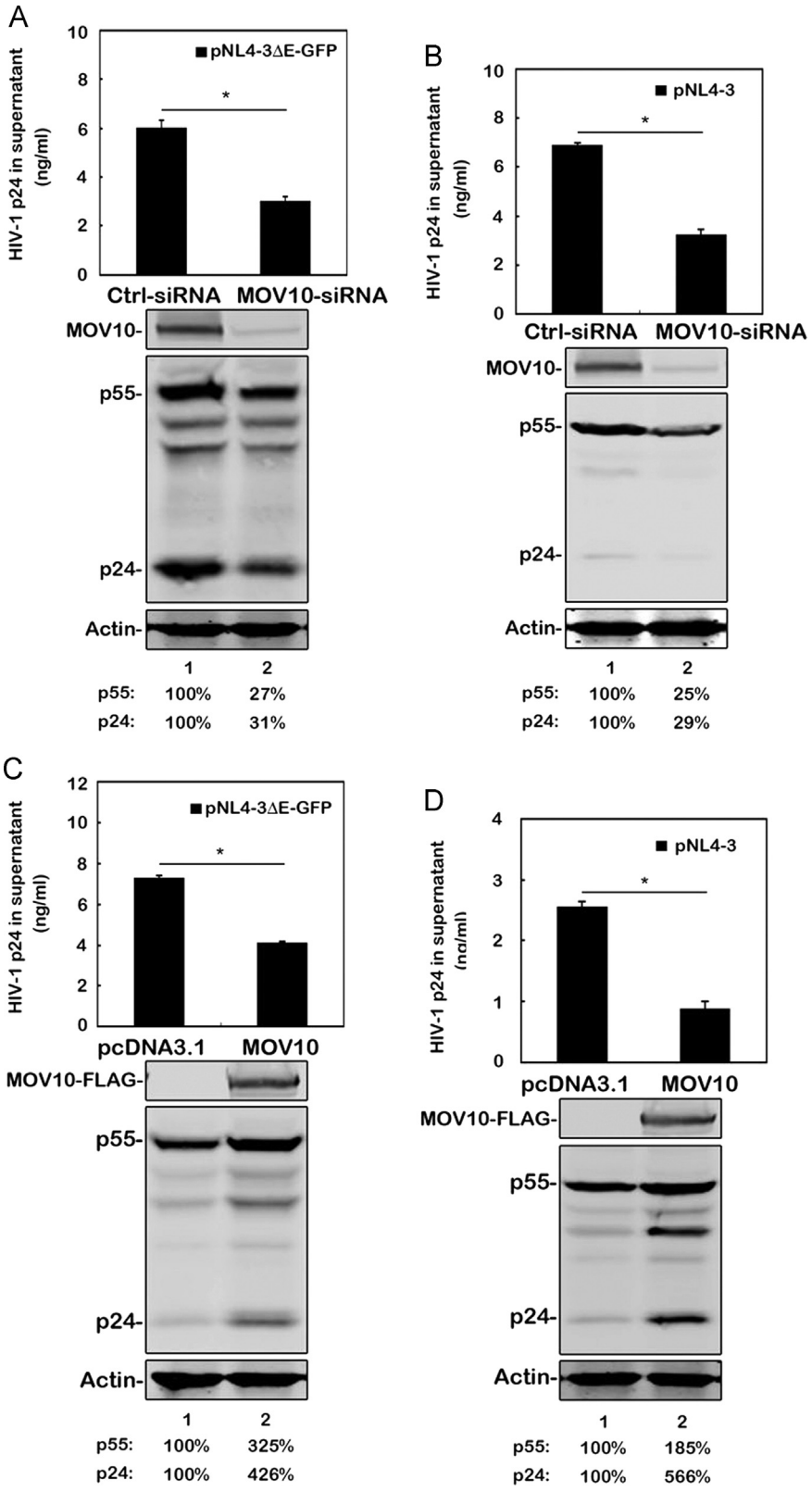

Fig. 1. *SiRNA-mediated knockdown of endogenous MOV10 impairs HIV-1 virus production and Gag expression in 293T cells.* (A and B) MOV10 depletion decreases HIV-1 virus production and intracellular Gag expression. Human 293T cells were transfected with pNL4-3ΔEnv-GFP (100 ng) (A) or pNL4-3 (100 ng) (B) and *MOV10*-specific siRNAs (40 nM) or control-siRNA (40 nM). The supernatants were collected at 48 h p.t. and detected by HIV-1 p24 ELISA assay (top panel). Cells were lysed and analyzed by Western blotting with anti-MOV10, anti-p24, and anti-β-actin antibodies (bottom panel). (C and D) The effect of MOV10 overexpression on supernatant p24 and intracellular Gag expression. Cells were transfected with pNL4-3ΔEnv-GFP (100 ng) (C) or pNL4-3 (100 ng) (D) and MOV10-FLAG (200 ng) or pcDNA3.1 (200 ng). Top panel, ELISA assays of supernatant p24 expression. Bottom panel, Western blot assays of MOV10-FLAG and intracellular p55 and p24 Gag expression. The means with $\pm$S.D. were calculated ($n=3$). *Statistically significant, $p \leq 0.05$.

2014; Liu et al., 2012). Endogenous MOV10 also plays a role in polycomb-mediated repression of the tumor suppressor *INK4a* (El Messaoudi-Aubert et al., 2010).

Interestingly, MOV10 has been proved to belong to the interferon antiviral system and participate in the replication of multiple viruses (Cuevas et al., 2014; Li et al., 2011; Schoggins et al., 2011; Song et al., 2014; Wang et al., 2010). MOV10 is required for the replication of hepatitis delta virus (HDV) (Haussecker et al., 2008), whereas inhibits several retroviruses and endogenous retroelements (Abudu et al., 2012; Arjan-Odedra et al., 2012; Burdick et al., 2010; Furtak et al., 2010; Goodier et al., 2012; Li et al., 2013; Wang et al., 2010). Recently, MOV10 has been described as a restrictive factor of HIV-1 (Abudu et al., 2012; Arjan-Odedra et al., 2012; Burdick et al., 2010; Furtak et al., 2010). It has been reported that overexpression of MOV10 suppresses the production and infectivity of HIV-1. The restriction of HIV-1 reverse transcription by MOV10 overexpression has been demonstrated by several groups, although different mechanisms were proposed (Burdick et al., 2010; Furtak et al., 2010; Goodier et al., 2012). For instance, MOV10 could inhibit the early HIV-1 cDNA synthesis (Furtak et al., 2010; Wang et al., 2010), or exert the inhibitory effect at the late stage of reverse transcription (Burdick et al., 2010). However, the effect of endogenous MOV10 on HIV-1 supernatant p24 expression demonstrated by siRNA knockdown is not consistent with that by MOV10 overexpression. Previous studies have indicated that, with the depletion of endogenous MOV10 *via* siRNAs, the viral production could be unaffected (Arjan-Odedra et al., 2012) or reduced (Burdick et al., 2010; Furtak et al., 2010). It seems that, under certain circumstance, endogenous MOV10 could function as a cofactor, rather than an antiviral factor for HIV-1 replication. These results also indicate that the effect of MOV10 on HIV-1 replication is complicated and needs to be further investigated.

In this report, we performed both overexpression and depletion experiments side-by-side to compare the effects of MOV10 on HIV-1 replication in the virus producer cells. We found that the effects of overexpression or depletion of MOV10 on the expression of HIV-1 p24 in supernatants are controversial but their effects on intracellular HIV-1 p55 and p24 Gag expressions are consistent, implying that MOV10 may have different effects at different steps of HIV-1 replication in virus producer cells. Through various approaches, we herein demonstrated that MOV10, which previously regarded as an anti-HIV-1 factor, also functions as a cofactor of HIV-1 Rev in virus producer cells through interacting with Rev to facilitate the nuclear export of HIV-1 mRNAs and subsequently increase the intracellular expression of HIV-1 late-stage proteins.

Results

Endogenous MOV10 is required for HIV-1 production

In order to demonstrate the effect of MOV10 on HIV-1 replication in virus producer cells, we transfected *MOV10*-specific siRNAs into 293T cells to knock down the expression of endogenous MOV10 and then examined HIV-1 p24 production from the HIV-1 molecular clone pNL4-3ΔEnv-GFP. We observed a significant decrease in p24 expression in supernatant after efficient MOV10 depletion (Fig. 1A, top panel). At the same time, the intracellular p55 and p24 Gag expressions were also consistently decreased (Fig. 1A, bottom panel). The same results were observed with HIV-1 wild-type proviral construct pNL4-3 in the similar experiments (Fig. 1B). The effect of *MOV10*-specific siRNAs on MOV10 expression was confirmed by Western blotting (Fig. 1A and B) (Liu et al., 2012). This phenomenon has not been discussed in previous similar studies (Burdick et al., 2010; Furtak et al., 2010). These results indicated that endogenous MOV10 in virus producer cells most likely acts as a co-factor rather than an inhibitor for HIV-1 production. However, after overexpression of MOV10 in 293T cells, we found that the p24 expression in supernatant was also reduced significantly (Fig. 1C, top panel). This finding is consistent with previous studies which identified MOV10 as a restricting factor for HIV-1 replication (Abudu et al., 2012; Arjan-Odedra et al., 2012; Burdick et al., 2010; Furtak et al., 2010; Wang et al., 2010; Zhao et al., 2013). Surprisingly, we found that the cellular p55 and p24 Gag expressions were enhanced by MOV10 overexpression (Fig. 1C, bottom panel). The same phenotype was also recapitulated with a wild-type HIV-1 proviral construct pNL4-3 (Fig. 1D) or another deficient proviral construct pCMVΔR8.2 (data not shown). Accordingly, we hypothesized that MOV10, a multifunctional protein, could be a helper as well as an inhibitor for HIV-1 at different stages of viral replication.

It was reported that insufficient virus release at early stages of HIV-1 assembly would decrease the expression of intracellular and supernatant p24 Gag (Cano and Kalpana, 2011), while a defect in the final stages of particle assembly would increase the accumulation of the CA-SP1 Gag processing intermediate in the cytoplasm (Cano and Kalpana, 2011; Garrus et al., 2001; Gottlinger et al., 1991). However, it was reported that the expression of intracellular p55 Gag cannot be increased by insufficient virus release in previous reports (Cano and Kalpana, 2011; Garrus et al., 2001; Gottlinger et al., 1991; Neil et al., 2008; Perez-Caballero et al., 2009). Moreover, MOV10 overexpression had no effect on the expression of intracellular Gag from pGag-GFP plasmid, while the Gag in supernatant can be decreased by MOV10 overexpression (Fig. S4). Thus, we believed that the accumulation of cellular p55 Gag could not be caused by insufficient virus budding. The *MOV10*-specific siRNAs used in our study are siRNA SMARTpool (Dharmacon) including four siRNAs, providing advantages in both potency and specificity. When we transfected the four siRNAs separately into 293T cells compared to siRNAs mixture (SMARTpool), we found that *MOV10*-siRNA SMARTpool had higher knockdown efficiency than any other individual siRNA (Fig. S1A). Furthermore, to exclude the off-target effect of *MOV10*-specific siRNAs, we generated a MOV10 construct (rMOV10-FLAG) that is resistant to siRNA-targeting by introducing silent mutations at the region targeted by siRNAs. Co-transfection of rMOV10-FLAG with *MOV10*-specific siRNAs could restore the expression of MOV10. Approximate 400 ng of co-transfecting rMOV10-FLAG plasmid was sufficient for the rescue of 40 nM siRNA-mediated knockdown effect (Fig. S1B), implying that *MOV10*-specific siRNAs did not have off-target effect. Therefore, our data indicate that MOV10 simultaneously inhibits and facilitates HIV-1 at different stages of HIV-1 replication in virus producer cells.

MOV10 enhances the expression of Rev/RRE-dependent reporter genes

To examine our hypothesis, we tried to identify at which stage (s) of viral replication that MOV10 facilitates HIV-1 replication. Recently, it has been demonstrated that MOV10 has no effect on HIV-1 viral RNA transcription or splicing (Burdick et al., 2010; Wang et al., 2010). Consistent with these studies, we also found that HIV-1 RNA transcription or splicing was not affected by MOV10 overexpression (data not shown). Since it has been demonstrated that several RNA helicases are required for the function of HIV-1 Rev (Edgcomb et al., 2012; Fang et al., 2004; Naji et al., 2012; Yedavalli et al., 2004; Zhou et al., 2013b), we hypothesized that MOV10, an RNA helicase, could play a role in the function of Rev. To this end, we employed a Rev/RRE-dependent system, pDM628 in which the *firefly luciferase* coding sequence has been inserted into the intron domain (between the splice doner site and the splice acceptor site)

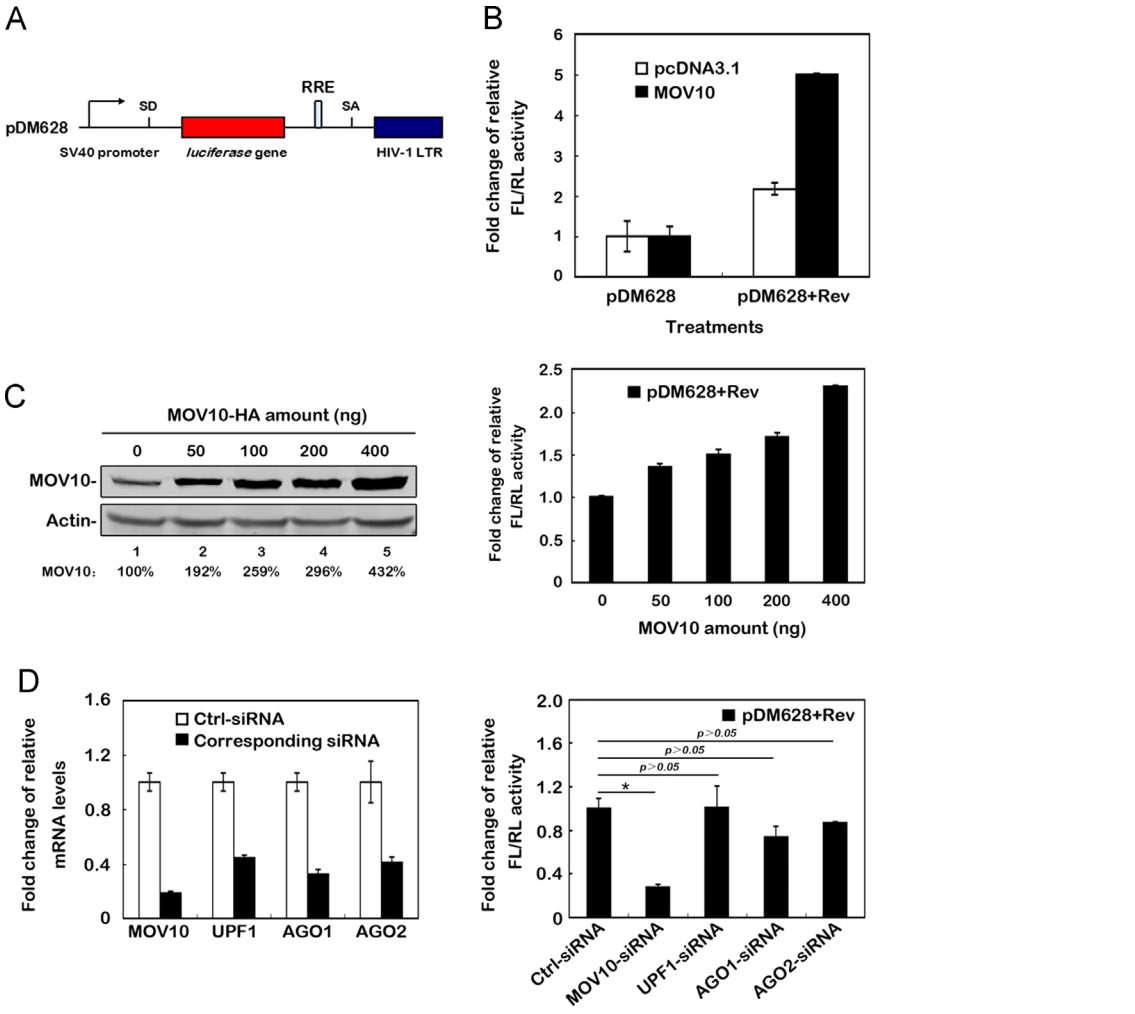

Fig. 2. *MOV10 increases the expression of a Rev/RRE-dependent reporter gene.* (A) Diagram of the construction of pDM628 reporter system. (B) pDM628 (10 ng), pRL-TK (5 ng) (as a transfection normalization reporter), pcDNA3.1-Rev-HA (50 ng), and pcDNA3.1-MOV10-FLAG (or empty vector) (400 ng) were transfected into 293T cells. After 48 h, cells were collected and lysed for luciferase activity assay. (C) Dose-dependent effect of MOV10 on the expression of reporter gene from pDM628. Human 293T cells were co-transfected with pDM628 (10 ng), pRL-TL (5 ng), pcDNA3.1-Rev-HA (50 ng), and different amounts of pcDNA3.1-MOV10-FLAG as indicated. Then, the cells were harvested at 48 h p.t. for Western blotting assay (left panel) or luciferase activity assays (right panel). (D) The effects of AGO1/2 or UPF1 on the Rev/RRE axis. The efficiency of relative siRNAs was detected with real-time PCR (left panel). Human 293T cells were transfected with pDM628 (10 ng) and pcDNA3.1-Rev-HA (5 ng) along with indicated siRNAs (40 nM) or control-siRNA (40 nM). After 48 h, cell lysates were prepared and measured by luciferase activity assay (right panel). Values in C represent portions of MOV10 normalized against actin relative to control values. Data in (A–D) represent mean $\pm$S.D. (error bars). *Statistically significant, $p \leq 0.05$.

that contains the RRE structure (Fig. 2A) (Fang et al., 2004; Zhou et al., 2013b). The unspliced *firefly luciferase* transcripts are permitted to transfer to the cytoplasm only in the presence of Rev. We transfected 293T cells with pDM628, pcDND3.1-Rev-HA, pRL-TK (a renilla luciferase expressing vector as an input control), and the MOV10-expressing plasmid, and then detected the effect of MOV10 on firefly luciferase (expressed by pDM628) and renilla luciferase (expressed by pRL-TK) activities. In absence of Rev, overexpression of MOV10 had no effect on the activity of Rev/RRE-dependent reporter gene. But when Rev was presented, overexpression of MOV10 significantly enhanced the activity of the Rev/RRE-dependent reporter gene (Fig. 2B). A dose-dependent experiment indicated that the enhancing effect on the pDM628 system was closely related to the cellular expression level of MOV10 (Fig. 2C). These results suggest that MOV10 plays a role in the efficient function of the HIV-1 Rev/RRE axis.

MOV10 is a multifunctional RNA helicase and involved in many cellular activities. The well-defined functions of MOV10 include: (1) a component of AGO-RISC complex (Izumi et al., 2013; Liu et al., 2012; Meister et al., 2005a); (2) participating in nonsense-mediated decay (NMD) pathway (Gregersen et al., 2014). To elucidate whether MOV10 affects the expression of Rev/RRE reporter gene through these pathways, we deactivated these pathways by disrupting their key factors AGO1/2 or UPF1 (Fig. 2D). The efficiency of indicated siRNAs was verified with real-time PCR (Fig. 2D, left panel). We found that the depletion of neither AGO1/2 nor UPF1 affected the expression of report gene from pDM628 (Fig. 2D, right panel), implying that the MOV10-mediated regulation of the Rev/RRE shuttle system is independent on the RISC complex or UPF1.

Rev is required for the transport of HIV-1 Gag-encoding mRNAs which contain a RRE structure (Battiste et al., 1996; Heaphy et al., 1990; Pollard and Malim, 1998). To further study the relationship between MOV10 and Rev, we used the pMDLg/pRRE reporter system which encodes the HIV-1 Gag-pol sequence. Unlike the pMDLg/pRRE reporter system which contains the RRE element and expresses HIV-1 Gag protein in a Rev/RRE-dependent manner (Fig. 3A, top panel), the pMDLg/pCTE reporter system contains a Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE) and expresses HIV-1 Gag protein in a Rev-independent manner (Fig. 3A, bottom panel) (Pasquinelli et al., 1997). It was therefore chosen as a control plasmid in the following

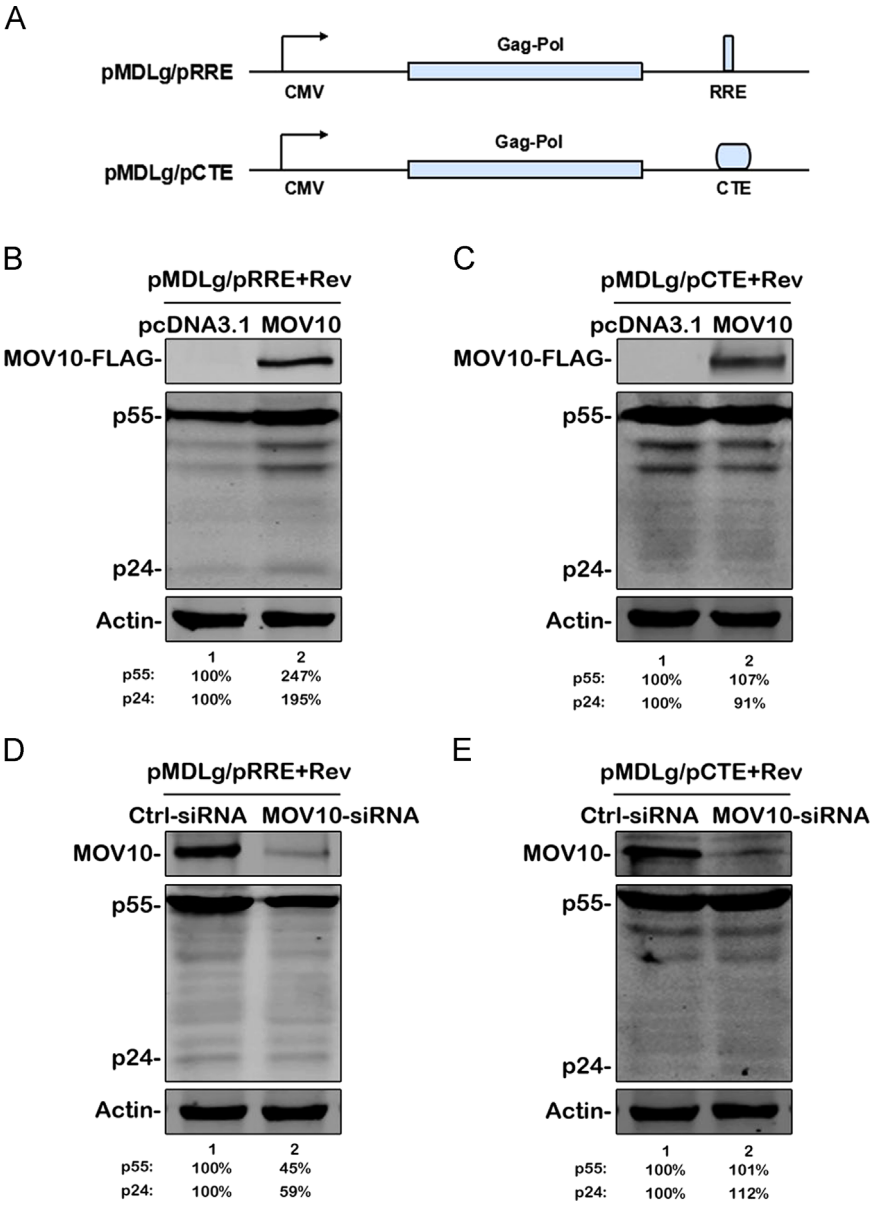

Fig. 3. *MOV10 enhances the expression of Rev/RRE- but not CTE-dependent reporter gene.* (A) Diagram of the construction of pMDLg/pRRE and pMDLg/pCTE reporter gene. (B–E) Different effects of MOV10 overexpression (B and C) or depletion (D and E) on Rev/RRE- and CTE-dependent reporter gene. 293T cells were transfected with indicated plasmids (pMDLg/pRRE and pMDLg/pCTE: 400 ng; Rev-HA: 50 ng; MOV10 or pcDNA3.1: 200 ng) or siRNAs (40 nM). After 48 h, cell lysates were analyzed by Western blotting. The MOV10-FLAG (B and C) or endogenous MOV10 (D and E), p55 and p24 Gag expressions were normalized to β-actin which served as an input control. Values represent portions of p55 or p24 normalized against actin relative to control values.

experiments. The Rev/RRE-dependent intracellular expressions of p55 and p24 Gag proteins from pMDLg/pRRE were significantly enhanced by overexpressed MOV10 (Fig. 3B), while the expressions of p55 and p24 Gag from pMDLg/pCTE were not affected by MOV10 (Fig. 3C). To examine the effect of endogenous MOV10 on the activity of Rev, we knocked down the expression of MOV10 using siRNAs and found that the depletion of endogenous MOV10 only decreased the Rev/RRE-dependent intracellular expression of p55 and p24 Gag (Fig. 3D and E). All these results indicate that MOV10 specifically regulates Rev/RRE-dependent but not CTE-dependent gene expression.

MOV10 acts on the Rev/RRE-dependent export of mRNAs

To investigate whether the enhancement of Rev/RRE-dependent gene expressions induced by MOV10 was due to the elevation of the nuclear export of unspliced mRNAs, we co-transfected pDM628, pcDNA3.1-Rev-HA, and pcDNA3.1-MOV10-HA into 293T cells, and then fractionated the cells to measure the nuclear and cytoplastic distribution of pDM628 mRNAs. We found that MOV10 did not affect the whole-cell mRNAs levels of the reporter gene (Fig. 4A and B, right panel). However, the cytoplasmic/nuclear ratio of the reporter mRNA was significantly elevated by MOV10 overexpression (Fig. 4A) and decreased after depletion of endogenous MOV10 (Fig. 4B). The similar effects were also observed with the pMDLg/pRRE system (Fig. 4C and D). As a control, MOV10 had no effect on the whole-cell mRNAs and the cytoplasmic mRNAs distribution of pMDLg/pCTE system (Fig. 4E and F). The cytoplasmic and nuclear fractionations were separated efficiently and confirmed with Western blotting assay (Fig. S2) (Zhou et al., 2013b). Collectively, these results demonstrate that MOV10 specifically facilitates the export of Rev/RRE-dependent mRNAs from the nucleus to the cytoplasm.

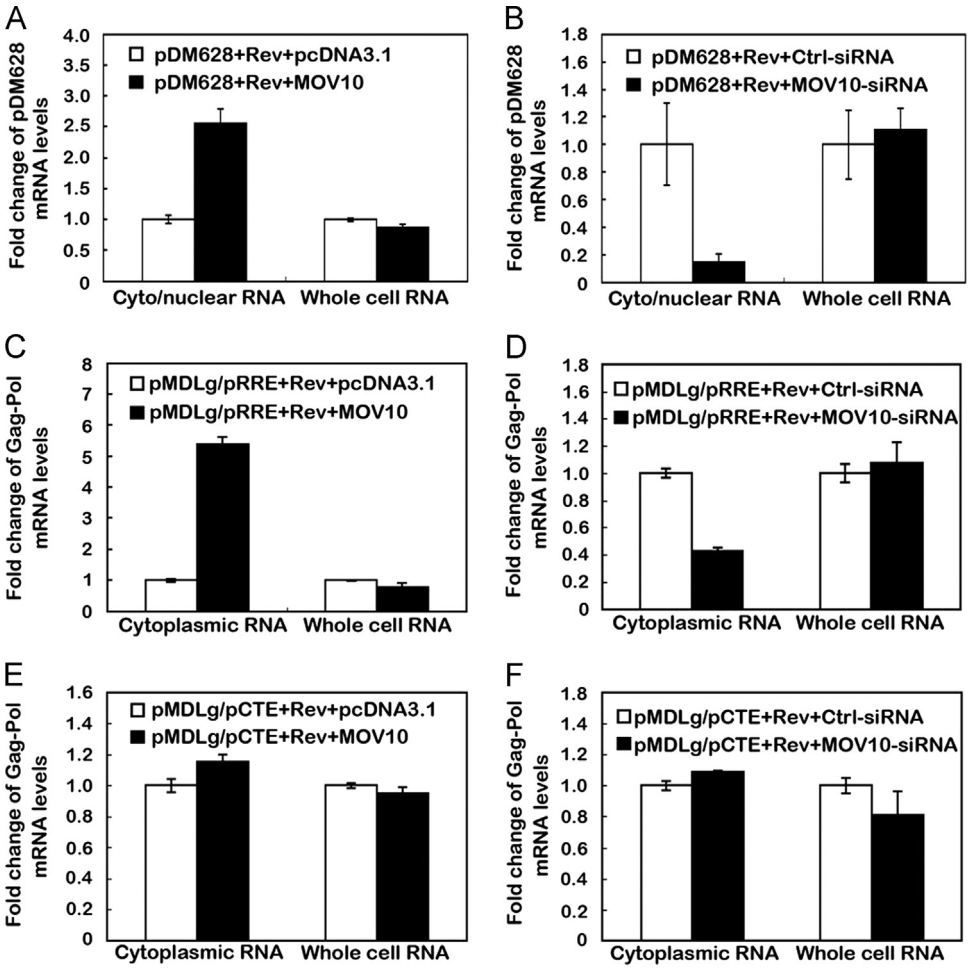

Fig. 4. *MOV10 regulates the nuclear export of Rev/RRE-dependent mRNAs.* (A and B) MOV10 is required for the cytoplasmic mRNAs expression of pDM628 reporter gene. Human 293T cells were transfected with indicated plasmids (A) or siRNAs (B). Cells were collected at 48 h p.t. and fractionated for cytoplasmic and nuclear mRNAs. The isolated RNAs were analyzed by real-time PCR. (C–F) Real-time PCR analysis of cytoplasmic and whole-cell mRNAs from 293T cells transfected with pMDLg/pRRE (C and D) or pMDLg/pCTE (E and F) and other indicated plasmids or siRNAs. Values represent the mean ± SD, $n=3$.

HIV-1 mRNAs can be classified into three classes: unspliced, partially spliced, and fully spliced mRNAs (Purcell and Martin, 1993; Schwartz et al., 1990a). The first two classes of viral mRNAs which contain introns are efficiently exported from the nucleus to the cytoplasm through the Rev/RRE-dependent shuttle system. To further examine the effect of MOV10 on HIV-1 mRNAs nuclear export, the cells transfected with the *env*-defective HIV-1 clone pNL4-3ΔEnv-GFP and MOV10-FLAG were fractionated into nucleus and cytoplasm and then the distributions of viral transcripts in each fraction were measured. As shown in Fig. 5A, the Rev/RRE-dependent cytoplasmic unspliced *gag-pol* mRNAs were significantly increased by MOV10 overexpression, while the distribution of fully spliced *tat* mRNAs remained unchanged (Fig. 5B). Accordingly, after depletion of endogenous MOV10, the distribution of *gag-pol* mRNAs in the cytoplasm was significantly decreased (Fig. 5C), while the distribution of *tat* transcripts from pNL4-3ΔEnv-GFP were not affected (Fig. 5D). These results suggest that MOV10 is an important component for the nuclear export system of HIV-1 unspliced and partially spliced transcripts.

HIV-1 late proteins are encoded by unspliced or partially spliced viral transcripts. The unspliced viral mRNAs encode HIV-1 Gag and partially spliced mRNAs encode other four viral late proteins including Vpu and Vif (Garrett et al., 1991; Schwartz et al., 1990b). All these mRNAs contain an RRE structure and are transported from the nucleus to the cytoplasm by the Rev/RRE shuttle system (Garrett et al., 1991; Malim et al., 1989). To further confirm whether partially spliced viral transcripts could also be regulated by MOV10, we took HIV-1 Vpu and Vif as examples to address this issue. Interestingly, MOV10 increased the expression of Vpu and Vif from HIV-1 proviral construct pNL4-3ΔEnv-GFP in a dose-dependent manner (Fig. 5E and Fig. S3A). The similar experiments were also performed in the presence of *MOV10*-specific siRNAs and a consistent result was observed (Fig. 5F and Fig. S3B). In addition, neither MOV10 depletion nor overexpression affected the expression of HIV-1 early genes, Tat and Rev, which are encoded by fully spliced mRNAs (Fig. S3C–F). Taken together, these data indicate that MOV10 enhances the expression of HIV-1 late genes in virus producer cells.

MOV10 interacts with HIV-1 Rev

Ectopic expressed MOV10 was reported to co-localize with AGO1/AGO2 in processing bodies (P-bodies) (Gallois-Montbrun et al., 2007; Izumi et al., 2013; Kenny et al., 2014; Meister et al., 2005b). However, several groups found that endogenous MOV10 presents in the nucleus (El Messaoudi-Aubert et al., 2010; Nakano et al., 2009; Sievers et al., 2012; Sim et al., 2012). Based on these reports, we speculated that the cellular MOV10 could distribute in both nucleus and cytoplasm. Moreover, HIV-1 Rev has been identified to interact with many host factors including MOV10 through systematical affinity tagging and purification mass spectrometry method (Jager et al., 2012a). To examine the interaction

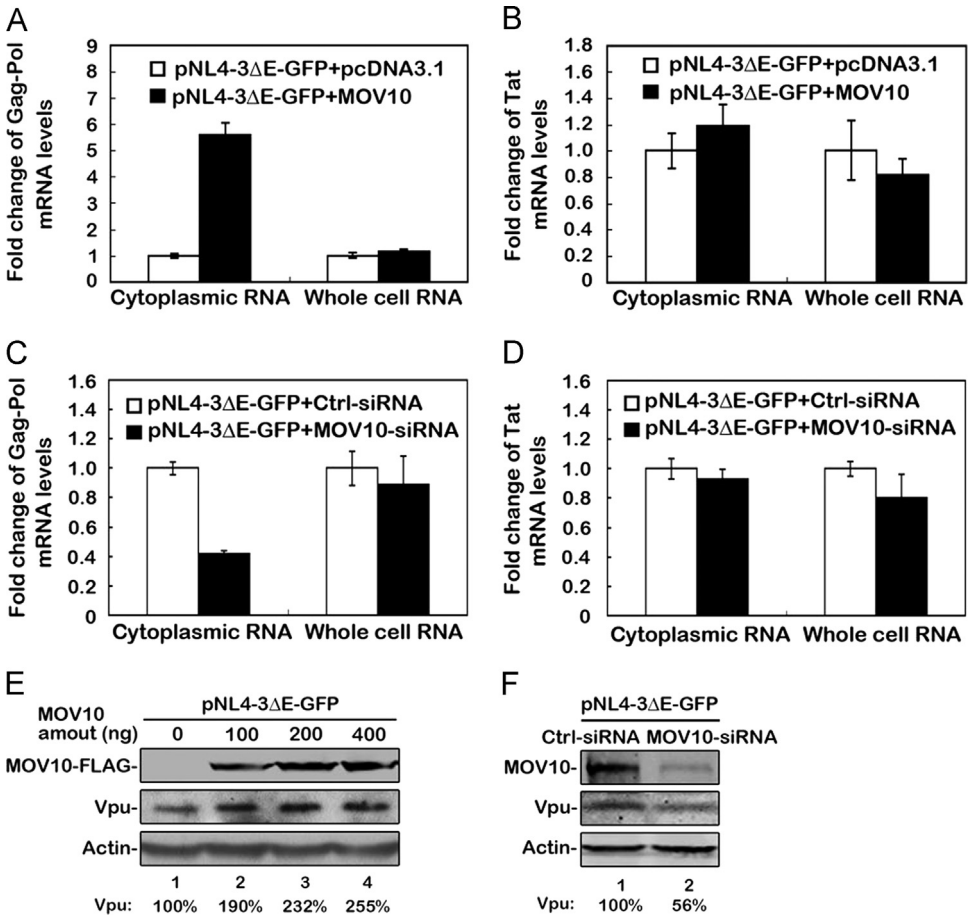

Fig. 5. *MOV10 facilitates the export of HIV-1 unspliced mRNAs but not fully spliced transcripts*. (A–D) Different effects of MOV10 on the exports of HIV-1 unspliced mRNA and fully spliced mRNA. (A and B) 293T cells were co-transfected with pNL4-3ΔEnv-GFP (100 ng) and pcDNA3.1-MOV10-FLAG (200 ng) (pcDNA3.1 as negative control). Cells were collected at 48 h p.t. for fractionation of cytoplasmic or nuclear components and then RNA extraction. (C and D) Cells transfected with indicated siRNAs and plasmids were collected and analyzed as above. Real-time PCR was performed using primers specific to *gag-pol* (A and C) or *tat* (B and D) mRNA. (E and F) The effect of MOV10 on the expression of HIV-1 Vpu from pNL4-3ΔEnv-GFP. 293T cells were transfected with pNL4-3ΔEnv-GFP and different amounts of pcDNA3.1-MOV10-FLAG (E) or *MOV10*-specific siRNAs (F). Cell lysates were analyzed by Western blotting using anti-FLAG (E) or anti-MOV10 (F), anti-Vpu or anti-β-actin antibody. Values in (E and F) represent portions of Vpu normalized against actin relative to control values. Values in A-D represent the mean ± SD, $n=3$.

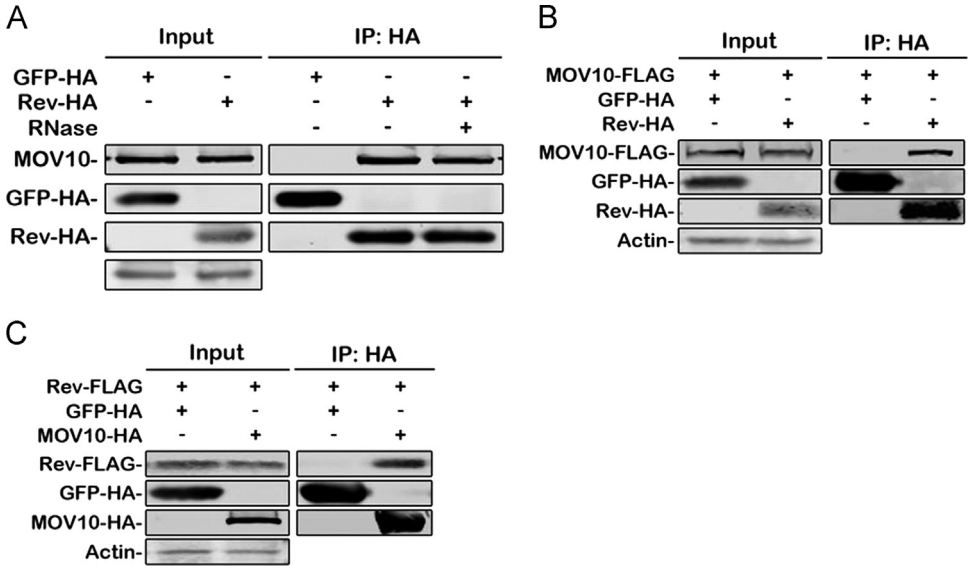

Fig. 6. *MOV10 interacts with HIV-1 Rev*. (A) Co-immunoprecipitation analysis of HIV-1 Rev and endogenous MOV10. 293T cells were transfected with Rev-HA- or GFP-HA-expressing plasmid. Proteins were pre-treated with or without RNase mixture and then immunoprecipitated using anti-HA agarose beads. Samples were analyzed by Western blotting. (B and C) Co-immunoprecipitation analysis of HIV-1 Rev and ectopic MOV10. Human 293T cells were transfected with a plasmid expressing MOV10-FLAG (1 μg) together with 2 μg of Rev-HA- or GFP-HA-expressing plasmid (B). In the complementary assay, cells were transfected with Rev-FLAG together with MOV10-HA- or GFP-HA-expressing plasmid (C). Proteins were immunoprecipitated using anti-HA agarose beads and analyzed by Western blotting with anti-HA or anti-FLAG antibody.

between MOV10 and Rev, 293T cells were transfected with pcDNA3.1-Rev-HA or pcDNA3.1-GFP-HA. After immunoprecipitated with anti-HA agarose beads, immunoprecipitates were analyzed by immunoblotting with anti-MOV10 antibody. MOV10 interacted with Rev-HA but not with negative control, GPF-HA (Fig. 6A). This interaction is RNA independent, since the levels of MOV10 binding to Rev were not affected by RNase mixture treatment (Fig. 6A, third lane of IP). In addition, ectopic expressed MOV10 could also be co-immunoprecipitated by Rev-HA (Fig. 6B second lane of IP) and Rev could interact with MOV10-HA in the immunoprecipitation assay (Fig. 6C, second lane of IP). These data suggest that HIV-1 Rev binds to MOV10 in an RNA-independent manner.

The helicase activity of MOV10 is required for its enhancement activity on Rev function

The DEAD (D-E-A-D=Asp-Glu-Ala-Asp) box motif is very important for the function of the DEAD-box RNA helicase family, especially for ATP binding and hydrolysis (Chen et al., 2013; Cordin et al., 2006). MOV10 is a DEAG-box RNA helicase containing a DEAG-box motif within the second helicase domain (Gregersen et al., 2014; Wang et al., 2010). The DEAG-box mutant was recently reported to impair the helicase activity of MOV10, implying that it is required for the helicase activity of MOV10 (Gregersen et al., 2014). To evaluate whether the DEAG-box motif is required for the interaction between MOV10 and HIV-1 Rev, a point mutation was introduced in the DEAG-box motif of MOV10 (from DEAG to DQAG) as described previously (Askjaer et al., 2000; Furtak et al., 2010). Human 293T cells were then co-transfected with pcDNA3.1-Rev-HA and pcDNA3.1-MOV10-FLAG or pcDNA3.1-MOV10-EQ-FLAG. After co-purification of Rev-HA, it revealed that Rev-HA binds to wild-type MOV10 and MOV10-EQ mutant at similar levels (Fig. 7A). These results suggest that the DEAG-box motif of MOV10 is not involved in the binding between MOV10 and Rev. Interestingly, when analyzing the effect of MOV10-EQ mutant on luciferase activity of the pDM628 system, we found that the MOV10-EQ mutant inhibited the luciferase expression in a dose dependent manner in the presence of Rev (Fig. 7B). Further, we found that both the intracellular Gag and the supernatant p24 from pNL4-3ΔEnv-GFP were inhibited by MOV10-EQ mutant in a dose-dependent way (Fig. 7C). These results indicate that MOV10-EQ mutant has dominant-negative activity in Rev/RRE axis.

The helicase activity of MOV10 is not required for its inhibitory effect on HIV-1 virus budding

So far, we still need to verify whether the effect of MOV10 on Gag expression was the result of post-translation regulation. To this end, we used pGag-GFP in our experiments, which was constructed by a Rev/RRE-independent HIV-1 Gag inserted into the multiple clone sites of pEGFP-N1 vector (Hermida-Matsumoto and Resh, 2000). We found that the intracellular expression of Gag-GFP from pGag-GFP or the expression of GFP from pEGFP-N1 was not affected by overexpression MOV10 or *MOV10*-specific siRNAs (data not shown), which suggested that MOV10 does not participate in the regulation of cellular HIV-1 Gag post-translation.

The studies above have demonstrated that both endogenous and exogenous MOV10 function as co-factors for HIV-1 Rev so that the expression of intracellular Gag is upregulated by overexpression of MOV10 or reduced by *MOV10*-specific siRNAs. However, the inconsistent supernatant p24 levels in overexpression and depletion experiments remain to be further clarified (Burdick et al., 2010; Furtak et al., 2010). We argued that MOV10 could exert an inhibitory effect on HIV-1 budding albeit it has an enhancement effect on the function of HIV-1 Rev. To examine our hypothesis, we used two

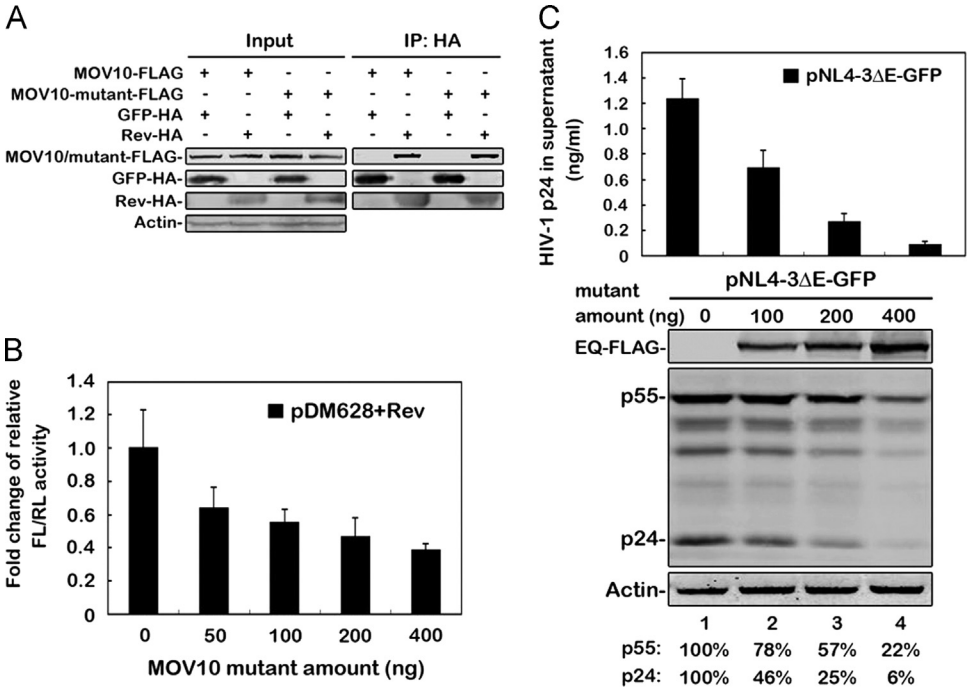

Fig. 7. *The DEAG-box motif mutant of MOV10 fails to facilitate the HIV-1 Rev/RRE axis.* (A) HIV-1 Rev co-immunoprecipitated with MOV10-EQ mutant. Lysates from cells transfected with 2 μg of pcDNA3.1-GFP-HA (lane 1, 3) or pcDNA3.1-Rev-HA (lane 2, 4) together with 1 μg of pcDNA3.1-MOV10-FLAG (lane 1, 2) or pcDNA3.1-MOV10-EQ-FLAG (lane 3, 4) were subjected to co-immunoprecipitation analysis using anti-HA agarose beads and detected by Western blotting. (B) The MOV10-EQ mutant inhibits the luciferase expression of pDM628 system. Human 293T cells were transfected with pDM628 (10 ng), pRL-TK (5 ng), and pcDNA3.1-Rev-HA (50 ng) together with different amounts of MOV10-EQ mutant and lysed and analyzed with dual-luciferase reporter system at 48 h p.t. (C) Dose-dependent effect of MOV10-EQ mutant on the expression of Gag from pNL4-3ΔEnv-GFP. 293T cells were transfected with pNL4-3ΔEnv-GFP (100 ng) and serial amounts of MOV10-EQ-FLAG (or empty control). After 48 h, the cells were collected for Western blotting assay (bottom panel) and the supernatants were analyzed with HIV-1 p24 ELISA kit (top panel). Data are representative of at least three independent experiments.

systems, the pMDLg/pCTE system and the pGag-GFP plasmid, which express Gag protein independent of Rev/RRE axis, in our following experiments. We found that the supernatant p24 level from pMDLg/pCTE was decreased after overexpression of MOV10 (Fig. S4A); however, was not affected after depletion of MOV10 (Fig. S4B). As shown in Fig. 3C and E, the intracellular Gag expression from pMDLg/pCTE was also not affected by MOV10. The same phenomenon was observed using pGag-GFP plasmid (Fig. S4C and D, upper panels), while the intracellular expressions of Gag remained unchanged (Fig. S4C and D, lower panels). These results suggest that MOV10 exerts an inhibitory effect on HIV-1 budding, while it also enhances the function of HIV-1 Rev.

To further analyze the role of MOV10 RNA helicase activity in HIV-1 budding, the MOV10 EQ-mutant plasmid together with pGag-GFP was transfected into 293T cells. After collecting the supernatant for VLP assay, we found that the mutant decreased the virus budding the same as wild-type MOV10 did (Fig. S4C), indicating that the DEAG-box of MOV10 is not involved in the inhibition of HIV-1 budding. This data further demonstrate that MOV10 plays differential roles in Rev/RRE axis and HIV-1 budding with different functional domains.

Discussion

Since anti-HIV-1 protein APOBEC3G (A3G) was discovered to be co-purified with MOV10 (Gallois-Montbrun et al., 2007; Izumi et al., 2013; Kozak et al., 2006; Liu et al., 2012), many groups have focused on the relationship between MOV10 and HIV-1. Previous studies have reported MOV10 as an anti-HIV-1 factor, because overexpression of MOV10 can inhibit HIV-1 reverse transcription and p24 production (Abudu et al., 2012; Arjan-Odedra et al., 2012; Burdick et al., 2010; Furtak et al., 2010; Izumi et al., 2013; Wang et al., 2010). In line with previous reports, we observed a significant decrease in supernatant p24 level after MOV10 overexpression. Interestingly, we observed an inconsistent effect on p24 production when endogenous MOV10 was knocked down, which has been described by several groups (Burdick et al., 2010; Furtak et al., 2010), but the underlying mechanism(s) is unclear. In order to solve this puzzle, it is important to find the HIV-1 replication step(s), at which both overexpressed and endogenous MOV10 show a consistent effect. We have demonstrated that the intracellular Gag expression and the Rev/RRE-dependent export of RNAs were consistently enhanced by MOV10, with both overexpression and depletion means (Figs. 1–3). It was reported that MOV10 could be packaged into HIV-1 virions through binding to the nucleocapsid (NC) region of Gag (Abudu et al., 2012; Wang et al., 2010), implying that the inhibitory effect of MOV10 overexpression on HIV-1 viral particles may occur at the stage of virus budding. It has been reported previously that MOV10 overexpression reduces HIV-1 p24 expression in supernatant (Arjan-Odedra et al., 2012; Burdick et al., 2010; Furtak et al., 2010), whereas HIV-1 supernatant p24 expression was inconsistently inhibited after MOV10 depleted (Burdick et al., 2010; Furtak et al., 2010). In our report, we tried to clarify the mechanism of this controversial phenomenon. Through pMDLg/pCTE and the widely used pGag-GFP system in HIV-1 budding study (Barr et al., 2008; Garrus et al., 2001), we confirmed that HIV-1 budding was inhibited by MOV10 overexpression (Fig. S4), whereas not affected by the depletion of endogenous MOV10. These results have explained the discordant expressions of HIV-1 supernatant p24 between MOV10 overexpression and depletion. It is possible that HIV-1 has already evolved to tolerate the moderate inhibitory effects by endogenous MOV10 which also benefits to the Rev activity. But when the expression of MOV10 exceeds far over its regular physiological level, the inhibitory effects of MOV10 on HIV-1 replication will become overwhelming, and then HIV-1 replication will be significantly suppressed.

In our study, neither overexpression nor depletion of MOV10 affects the transcription of HIV-1 mRNAs, splicing, or post-translation, which is consistent with the findings of other groups (Izumi et al., 2013; Wang et al., 2010). In addition, the depletion of MOV10-associated proteins AGO1/2 or UPF1, the important components of ribonucleoprotein complexes, had no effect on the Rev/RRE axis, indicating that the enhancing effect of MOV10 on the Rev/RRE axis is independent on the miRISC or the NMD-related ribonuleoprotein complexes. To determine the relationship between MOV10 and HIV-1 Rev, two simple Rev/RRE-dependent reporter systems (pDM628 and pMDLg/pRRE) were used in our study. These two kinds of reporter systems have been used and described by our group (Zhou et al., 2013b), and similar systems have also been applied previously to prove DDX3 as a co-factor for HIV-1 Rev (Yedavalli et al., 2004). Therefore, the reporter systems used in our study are well defined and useful in HIV-1 Rev research. HIV-1 Rev transports viral mRNAs from the nucleus to the cytoplasm by directly binding to nuclear export receptor CRM1, which mediates the nuclear export of cellular proteins or RNPs bearing a leucine-rich nuclear export signal (NES) (Askjaer et al., 1998; Ossareh-Nazari et al., 1997). It was reported that the export of Ro60, a nuclear–cytoplasmic shuttling protein which was co-purified with MOV10 in a complex, is dependent on CRM1, indicating that the export of MOV10 may also dependent on CRM1 (Gallois-Montbrun et al., 2007; Sim et al., 2012). In addition, human Staufen-2, a newly discovered HIV-1 Rev co-factor (Banerjee et al., 2014), was also found to interact with MOV10 by affinity purification assay (Miki et al., 2011). The Rev/RRE/CRM1 pathway (such as pMDLg/pRRE) uses a nucleo-cytoplasmic shuttle pathway which transports proteins, small nuclear RNAs, and rRNAs from the nucleus into the cytoplasm (Fukuda et al., 1997; Neville et al., 1997), whereas the CTE pathway (such as pMDLg/pCTE) utilizes host protein TAP, which is involved in cellular mRNAs transport (Bear et al., 1999; Stutz et al., 2000). Previous studies have demonstrated that the Rev/RRE/CRM1 pathway is not involved the export of fully spliced HIV-1 mRNA and cellular mRNAs (Clouse et al., 2001; Pasquinelli et al., 1997; Van Baalen et al., 1998; Yedavalli et al., 2004). All of these lines of evidence strongly suggest that MOV10 participates in Rev/RRE/CRM1 pathway specifically.

MOV10 is a multifunctional RNA helicase. However, the function of the helicase domains of MOV10 has rarely been described (Furtak et al., 2010; Gregersen et al., 2014). In our study, we surprisingly found that the DEAG-box motif of MOV10, which belongs to the second helicase domain and is required for the helicase activity of MOV10 (Gregersen et al., 2014), is an indispensable motif for MOV10/Rev/RRE-dependent viral mRNAs nuclear export. We found that the MOV10-EQ mutant can effectively interact with Rev and is also an effective suppressor of the expression of intracellular Gag, which makes it the firstly discovered dominant-negative form of MOV10. This potent inhibitory effect further confirms that endogenous MOV10 acts as a co-factor for HIV-1 replication. More importantly, the different effects of MOV10-EQ mutant on two steps of HIV-1 replication confirmed that MOV10 performed various functions at various steps of HIV-1 replication (Figs. 7 and S4).

Although the regulation of MOV10 by signaling pathways is rarely reported, it has been shown that type I interferon (IFN) can regulate the expression of MOV10 (Cuevas et al., 2014; Schoggins et al., 2011). Interferon signaling pathways provide an early innate immune response to invading pathogens, such as HIV-1 (Mogensen et al., 2010; Yan and Chen, 2012). Conversely, HIV-1 has evolved to resist the innate immune response, including interferon signaling, to establish a productive infection (Cullen, 2009; Herbeuval and Shearer, 2007; Jager et al., 2012b; Sheehy et al., 2003).

For instance, adenosine deaminase acting on RNA-1 (ADAR1), induced by IFN, strongly enhances the overall accumulation of HIV-1 proteins in virus producer cells (Doria et al., 2009; Phuphuakrat et al., 2008). This is one of the reasons why the treatment of HIV-1 infected patients with IFN alone always gets poor outcomes (Herbeuval and Shearer, 2007). IFN can stimulate the expression of MOV10 to reduce HIV-1 production (Schoggins et al., 2011). Nevertheless, in virus producer cells, HIV-1 Rev hijacks MOV10 to facilitate the export of unspliced/partially spliced HIV-1 transcripts and increases the late HIV-1 products such as Vpu (Fig. 5E and F), one important accessory viral protein, to counteract another IFN-stimulated anti-HIV-1 host factor Tetherin. Collectively, the anti-HIV-1 activity of MOV10 and the enhancement effect of MOV10 on HIV-1 mRNAs export is a battle between host and virus, which is complicated and worth being further studied.

Conclusion

In summary, our study has identified a novel function of MOV10 that it acts as a co-factor of Rev, facilitating HIV-1 replication at viral transcripts export stage in virus producer cells.

Materials and methods

Plasmids construction and siRNAs synthesis

The MOV10-FLAG- and MOV10-HA- expressing plasmids were constructed as described previously (Liu et al., 2012). The siRNA-resistant MOV10 expressing plasmid was generated by mutating the MOV10 siRNAs target sequences in the MOV10-FLAG expressing plasmid with multiple silent mutations in MOV10 (sites of mutations are underlined: 5′-GGGGGCACACAATCCGTGA-3′; 5′-GCTCCGACATTTCCAAGCA-3′; 5′-GCAAGAGTATCGCGTGTTG-3′; 5′-CGGGAAAACCGTGACCTTG-3′). The MOV10 DEAG-box mutant expressing plasmid, pcDNA3.1-MOV10-EQ-FLAG was generated *via* a PCR-based mutagenesis method from pcDNA3.1-MOV10-FLAG by introducing a point mutation (E→Q) within the DEAG-box motif of MOV10 (Furtak et al., 2010). HIV-1 proviral constructs (pNL4-3, pNL4-3-ΔEnv-GFP, and pCMVΔR8.2), reporter vectors (pDM628, pMDLg/pRRE, and pMDLg/pCTE), pcDNA3.1-GFP-HA, and pcDNA3.1-Rev-HA have been described in our previous reports (Huang et al., 2007; Zhou et al., 2013a). Renilla luciferase expressing vector, pRL-TK, was obtained from Promega and used as an input control for transfection. The pGag-GFP plasmid, which contains inhibitory sequence-mutated HIV-1 *gag* fused to *gfp*, was obtained from AIDS Reference Reagent Program of NIH (Hermida-Matsumoto and Resh, 2000).

The siGENOME SMART pool small interfering RNAs (siRNAs) against *MOV10* or *AGO1/2* and siRNAs for negative control were designed by Dharmacon and the target sequences for siRNAs were described previously (Liu et al., 2012; Yin et al., 2015; Zhang et al., 2014b; Zhou et al., 2013a). *UPF1*-specific siRNA was designed as previous report (Kim et al., 2005). All siRNAs were synthesized from Ribobio (China).

Cell culture and transfection

Human 293T cells were obtained from ATCC (American Type Culture Collection) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) at 37 °C with 5% CO_2. The cells were transfected with the indicated plasmids or siRNAs using Lipofectamine 2000 (Invitrogen). The procedures described by the manufacturer's instructions were followed.

HIV-1 p24 ELISA

Supernatant in a 24-well-plate was collected at 48 h post-transfection (p.t.) and cells from three wells were collected together for Western blotting. The supernatant p24 expression was determined by ELISA assay. HIV-1 p24 ELISA assays were performed with HIV-1 p24 ELISA kit according to the instructions of manufacturer (Clontech).

Dual-luciferase reporter assay

Human 293T cells in a 24-well-plate were transfected with pDM628, Rev (or not), pRL-TK together with MOV10 expressing plasmid or *MOV10*-specific siRNAs or corresponding control plasmid or siRNA. The cells were collected at 48 h after transfection and lysed for dual-luciferase reporter assay. Firefly luciferase and renilla luciferase activities were analyzed with Dual Luciferase Reporter Assay Kit (Promega) as described previously (Zhang et al., 2014a).

Cell fractionation and real-time PCR

The cytoplasmic and nuclear RNA fractions were isolated using the PARIS kit (Ambion). The isolated RNAs were treated with DNase (Promega) by incubated in 1 × RQ1 RNase-Free DNase Reaction buffer, 1 μl RQ1 RNase-Free DNase and 7 μl Nuclease-free water at 37 °C for 30 min. The DNase was inactivated by incubated with 1 μl RQ1 DNase Stop Solution at 65 °C for 10 min and then the treated RNAs were used to synthesize cDNA with PrimeScript RT reagent Kit (Takara). Real-time PCR was performed using SYBR Green methods as previously described with a CFX96 real-Time System (Bio-Rad) (Yin et al., 2015). The primers used were listed in the additional files. The primers for detection of unspliced and spliced mRNAs from pDM628, *gag-pol*, and *tat* have been described previously (Zhou et al., 2013a). All primers were synthesized by Invitrogen and human β-actin and/or GAPDH mRNA was measured as an internal control.

Co-immunoprecipitation and Western blotting

Co-immunoprecipitation and Western blotting assays were performed as described previously (Liu et al., 2012). Briefly, cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 1 mM EDTA, 1% Triton X-100, 0.5% NP-40, plus PMSF and protease inhibitor cocktail [Sigma]) for 30 min on ice. The cell lysates were then clarified and mixed with anti-HA agarose beads (Sigma) for 4 h at 4 °C, followed by washing four times with ice-cold lysis buffer and eluting in protein gel loading buffer. Where indicated, RNase mixture (DNase-free, Roche) (20 μg/ml) was added to the lysates and incubated at 4 °C for 1 h prior to IP. The immunoprecipitated samples were then analyzed by SDS-PAGE and detected by Western blotting. Different primary antibodies were used in the Western blot assay: anti-HA antibody (mouse monoclonal, Covance); anti-FLAG antibody (rabbit polyclonal, MBL); anti-β-actin antibody (mouse monoclonal, BD); anti-GAPDH antibody (rabbit polyclonal, MBL); anti-MOV10 antibody (rabbit polyclonal, Abcam); anti-Vpu antibody (obtained from NIH AIDS Research); andti-Vif antibody (rabbit polyclonal, Abcam); anti-Tat antibody (mouse polyclonal antibodies made by our lab); anti-Rev antibody (mouse monoclonal, Santa cruz); anti-GFP antibody (mouse monoclonal, Santa cruz); anti-HIV-1 p24 Gag and anti-TBP antibodies (rabbit polyclonal antibodies made by our lab) (Zhou et al.,

2013b). Quantity One program (Biorad) was applied to quantify the Western blotting results.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.08.026.

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