TIP110 CONTROL OF HIV-1 GENE EXPRESSION AND REPLICATION

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ABSTRACT

Weina Zhao

Tip110 Control of HIV-1 Gene Expression and Replication

Transcription and alternative splicing play important roles in HIV-1 gene expression and replication and mandate complicated but coordinated interactions between the host and the virus. Studies from our group have shown that a HIV-1 Tat-interacting protein of 110 kDa, Tip110 synergies with Tat in Tat-mediated HIV-1 gene transcription and replication. However, the underlying molecular mechanisms were not fully understood and are the focus of the dissertation research. In the study, we first demonstrated that Tip110 bound to unphosphorylated RNA polymerase II (RNAPII) in a direct and specific manner. We then showed that Tip110 was detected at the HIV-1 long terminal repeat (LTR) promoter and associated with increased phosphorylation of serine 2 within the RNAPII C-terminal domain (CTD) and increased recruitment of positive transcription elongation factor b (P-TEFb) to the LTR promoter. Consistent with these findings, we demonstrated that Tip110 interaction with Tat directly enhanced transcription elongation of the LTR promoter.

During these studies, we also found that Tip110 altered HIV-1 mRNA alternative splicing and increased *tat* mRNA production. Subsequent analysis indicated that Tip110 selectively increased *tat* exons 1-2 splicing by activating HIV-1 A3 splice site but

had no function in *tat* exons 2-3 splicing. We then showed that the preferential splicing activity of Tip110 resulted from Tip110 complex formation with hnRNP A1 protein, a negative splicing regulator that binds to the ESS2 element within *tat* exon 2, and as a result, blocked the complex formation of hnRNP A1 with ESS2 and subsequently activated HIV-1 A3 splice site. Taken together, these results show that Tip110 functions to regulate HIV-1 transcription elongation and HIV-1 RNA alternative splicing. These findings not only add to our understanding of Tip110 biology and function but also uncover a new potential target for development of anti-HIV intervention and therapeutic strategies.

Johnny J. He, Ph.D., Chair

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LIST OF ABBREVIATION

β-gal	β-galacosidase
AFF4	Fragile X mental retardation 2(AF4/FMR2) family- member 4
AIDS	Acquired immune deficiency syndrome
AP1	Active protein-1
AR	Androgen receptor
ASF/SF2	Alternative splicing factor/splicing factor 2
ATCC	American Tissue Culture Collection
BSA	Bovine serum albumin
CA	Capsid protein
CAT	Chloramphenicol acetyltransferase
CBP	CREB-binding protein
CD4	Cluster of differentiation 4
CDK	Cyclin-dependent kinase
CREB	cAMP-response element-binding protein
CTD	RNA polymerase II C-terminal domain
CTDa	hypophosphorylated RNA polymerase II C-terminal domain
CTDo	hyperphosphorylated RNA polymerase II C-terminal domain
CTL	Cytotoxic T lymphocytes
cDNA	Complementary DNA

CPM	Counts per minute
CXCR4	Chemokine (C-X-C motif) receptor 4
DSIF	5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazolesensitivity induce factor
DMEM	Dulbecco's modified eagle's medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetracetic acid
Env	HIV-1 envelope
ESE	Exonic splicing enhancers
ESS	Exonic splicing silencers
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
gp120	HIV-1 glycoprotein of 120 KDa
gp41	HIV-1 glycoprotein of 41KDa
HARRT	Highly active anti-retroviral therapy
HAT	Half a tetratricopeptide repeat
HAT	Histone acetyltransferase
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
ISS	Intronic splicing silencer
IN	HIV-1 integrase
IP	Immunoprecipitation
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Luria broth
LTR	Long terminal repeat
MA	HIV-1 matrix
NC	HIV-1 nucleocapsid
NELF	Negative elongation factor
Nef	Negative regulatory factor
NFAT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
NP40	Tergitol-type NP-40
N-TEF	Negative transcription elongation factor
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction

PMSF	Phenylmethanesulphonyl fluoride
PR	HIV-1 protease
P-TEFb	Positive transcription elongation factor b
R Luc	*Renilla* luciferase
RBP	RNA binding protein
Rev	Regulator of virion protein expression
RNP	Ribonucleoprotein
RNPS1	Ribonucleoprotein S1
RRE	Rev response element
RRM	RNA reorganization motif
RNAPII	RNA Polymerase II
RT	reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
SART3	Squamous cell carcinoma antigen recognized by T-cells 3
SC35	Splicing component of 35 kDa
SDS	Sodium dodecyl sulfate
SLS3	Stem-loop structure 3
SKIP	c-Ski-interacting protein
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoproteins

SR	Serine/arginine- rich protein
SRp40	Serin/arginine-rich protein of 40 kDa
TAR	Trans-activating responsive element
Tat	Trans-activator of transcription
TFIIA	Transcription factor II A
TFIID	Transcription factor II D
TFIIH	Transcription factor II H
Tip110	Tat-interacting-protein of 110kDa
U2AF	U2 small nuclear ribonucleoprotein particle auxiliary factor
UTR	Untranslated region
Vif	HIV-1 viral infectivity factor
Vpr	HIV-1 viral protein R
Vpu	HIV-1 viral protein U
WB	Western blot
YB-1	Y-box binding protein 1

INTRODUCTION

1. HIV-1 AND THE AIDS PANDEMIC

1.1 HIV-1 pandemic

The acquired immunodeficiency syndrome (AIDS) has become a global epidemic. AIDS and its etiologic agent, HIV-1 were first identified in the early 1981. Since then, there are more than 20 million people including 330,000 children have been killed. According to the most recent report on the global AIDS epidemic 2009 from the United Nations, about 34 million people were living with HIV at the end of 2008, in which there were 2.7 million new HIV infections and 430000 newly-infected children under 15. In 2008, there were 2 million AIDS-related deaths, 280000 of them were children. The report also indicates that about 17 million people, close to half of the HIV-1 infected people, are women, suggesting that there could be more babies acquiring infection from their HIV-1 positive mothers through pregnancy, delivery or breast feeding. Moreover, use of highly active anti-retroviral therapy (HAART) and subsequent improved survival of HIV-infected individuals also add to the total number of HIV-1-infected people worldwide.

1.2 Clinical course of HIV-1 infection

The clinical course of HIV-1 infection starts with some acute flu-like syndrome, which last for 6 to 12 weeks (Lyles, Munoz et al. 2000; Douek, Picker et al. 2003; Alam, Scearce et al. 2008). The patient usually experiences fever, rash, swollen lymph glands, headache, gastrointestinal symptoms and weight loss, but none of these is life-threatening.

At this stage, there is an initial fall in the number of $CD4^+$ cells and a rise in $CD8^+$ cells but both cells quickly return to normal level (Douek, Brenchley et al. 2002; Guadalupe, Reay et al. 2003). Virus titers can be very high at this stage and could reach one hundred million virus particles per milliliter of plasma. Following the initial infection, potent humoral and cellular responses will have the viral replication under control and reduce the virus titer. The infected individual enters the clinical latency stage; this stage lasts many months and years. At this stage, HIV is present within lymphoid organs, mainly trapped in the follicular dendritic cells (FDC) (Douek, Picker et al. 2003). When latency infected cells are activated, HIV-1 replication resumes and $CD4^+$ lymphocytes in the peripheral blood exhibit steady decline. When the $CD4^+$counts decrease below 200 per microliter, the individual enters the clinical AIDS stage of disease (Leng, Borkow et al. 2001; Brenchley, Price et al. 2006). In this final stage, cell-mediated immunity is lost, there are a variety of opportunistic infections (Pantaleo, Graziosi et al. 1993). Infected individuals usually die from HIV-associated diseases. Nevertheless, host factors such as age or genetic differences and the virulence of different viral strains play important roles in the rate and severity of AIDS disease progression (Fauci 1993; Pantaleo, Graziosi et al. 1993).

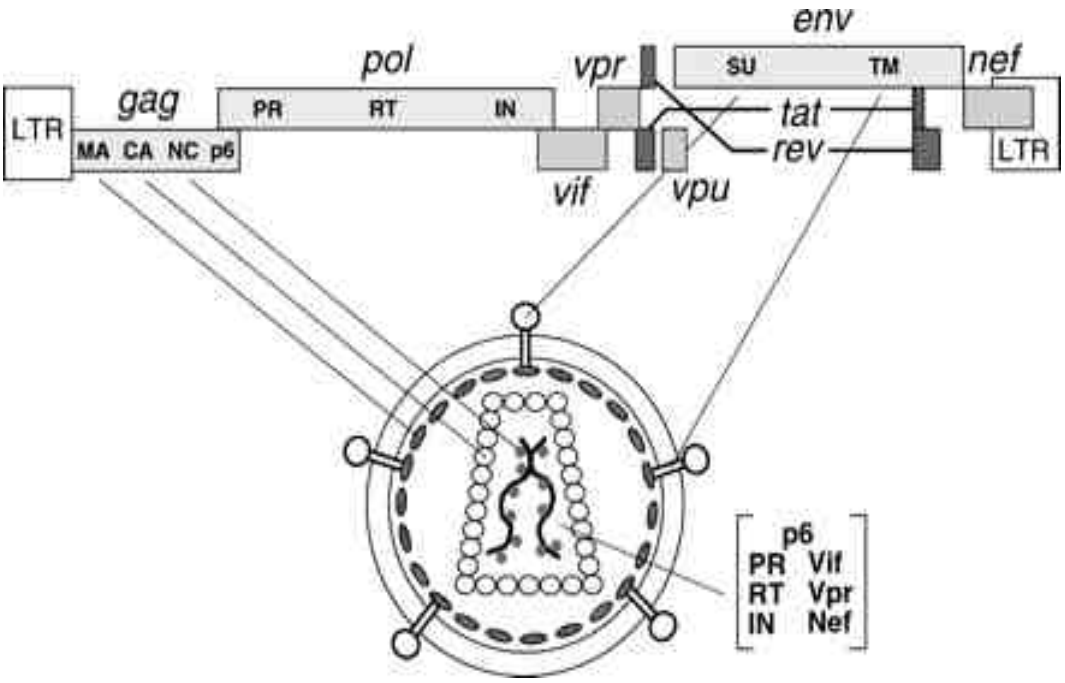
1.3 HIV-1 and its genome

HIV is a complex retrovirus with a 9.2 kb genome. It encodes nine open reading frames (ORF) and three of these ORF are common to all retroviruses: *gag*, *pol*, and *env* (Fig. 1). *Gag* provides the basic structural component of the virus; it encodes four structural proteins that make up the core of virion: matrix protein (MA, p17), capsid protein (CA,

p24), nucleocapsid protein (NC, p7 and p6). *Env* encodes for gp160, the precursor of gp120 and gp41. These envelope proteins are embedded in the outer layer of the virions and enable the virus to attach and fuse with host cells. *Pol* codes for three viral enzymes, including protease (PR), reverse transcriptase (RT), and integrase (IN).These proteins provide essential enzymatic functions and are encapsulated within the virion particle. HIV also has 6 unique regulatory genes: *tat, rev, nef, vpu, vpr,* and *vif.* Vif, Vpr, and Nefproteins are packaged in the viral particle and important for HIV-1 infectivity and virion production. Two other accessory proteins, Tat and Rev provide essential regulatory functions. Tat protein is a transactivator for HIV-1 transcription. It interacts with trans-activating responsive (TAR) structure of HIV-1 RNA, recruits positive transcription elongation factor b (P-TEFb) to the transcription complex and enhances the transcription elongation step (Frankel and Young 1998). Rev functions to export singly spliced and unspliced RNA from the nucleus to the cytoplasm (Malim, Hauber et al. 1989). The last protein, Vpu, indirectly assists in assembly of the virion (Neil, Zang et al. 2008). Besides these 9 ORF, there are other important non-coding RNA elements located within the HIV-1 genome, including R: a short sequence at the 5' and 3' ends; U5, a unique sequence at the 5'end; PBS: primer binding site that serves as the initiation site for minus strand RNA synthesis; Ψ: the recognition site for packaging viral RNA into particles; PPT: the polypurine tract that provides the primer for synthesis of the plus strand DNA; U3, a unique sequence at the 3' end. When HIV-1 proviral DNA is integrated into chromosome DNA, U3, R and U5 are duplicated at both ends of DNA and make up the long terminal repeat (LTR) (Frankel and Young 1998).

1.4 HIV-1 life cycle

HIV-1 infection of a susceptible cell begins with interaction of the envelop glycoproteinsgp120 with both CD4 (Fig. 2) (Alkhatib, Combadiere et al. 1996; Feng, Broder et al. 1996). Following high-affinity attachment of gp120 to CD4, the envelope complex undergoes a structural change, which exposes the chemokine binding domains of gp120 and allows them to interact with the chemokine receptor CCR5/CXCR4.After the virus enters into the target cell, the viral core is uncoated. One enzyme called *reverse transcriptase* liberates the single-stranded (+) RNA from the attached viral proteins and converts the viral RNA into dsDNA. Then, dsDNA enters the nucleus and becomes integrated into the genome by the action of another viral enzyme integrase (IN). Using the integrated viral DNA as a template, viral RNA is synthesized from the LTR promoter by the cellular enzyme RNAPII and a number of other cellular and viral factors (Berkhout, Silverman et al. 1989; Laspia, Rice et al. 1989). Completely spliced RNA transcripts are then exported to the cytoplasm and translated into the regulatory proteins Tat, Rev and Nef. Rev is imported into nucleus and facilitates nuclear export of singly spliced and unspliced viral RNA, while Tat transactivates to produce full-length viral RNA (Malim, Bohnlein et al. 1989; Malim, Hauber et al. 1989). Structural proteins Gag and Env are produced by full-length mRNA. The Env polyprotein is cleaved by cellular protease into two gp41 and gp120 and then transported to the plasma membrane. The Gag and Gag-Pol polyproteins interact with the inner surface of the plasma membrane and with HIV genomic RNA to form virions, followed by budding out of the host cell. HIV protease (PR) cleaves the Gag and Gal-Pol polyproteins into individual HIV


env
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p6
PR Vif
RT Vpr
IN Nef

Figure 1. HIV-1 genome Organization of and virion HIV is a complex retrovirus with a 9.2 kb genome. It encodes nine open reading frames (ORF) and three of these ORF are common to all retroviruses: *gag, pol*, and *env*. Besides these, HIV has 6 unique regulatory genes: *tat, rev, nef, vpu, vpr*, and *vif*.

structural proteins and enzymes. Finally, the virions mature and become infectious to begin a new round of infection.

2. HIV-1 TRANSCRIPTION AND REGULATION

2.1 HIV-1 basal transcription

The HIV-1 basal transcription begins from the 5' LTR promoter and does not have the involvement of HIV-1 Tat protein. It is accomplished by a complex network of interactions between *cis*-elements and *trans*-acting factors. These *cis*-acting regulatory sites are located in the 5' LTR. The HIV-1 LTR is generally divided into four functional domains: TAR, the core promoter region, the enhancer region, and the regulatory region. The core promoter resembles that of many eukaryotic genes, containing a TATA box and three tandem Sp1 sites (Mallardo, Dragonetti et al. 1996; Stevens, De Clercq et al. 2006). The Sp1 sites recruit transcription factor Sp1 that is expressed in most eukaryotic cells and is critical for both basal transcription and Tat-mediated transactivation. The TATA box facilitates the binding of transfection factor IID (TFIID) and transcription factor IIA (TFIIA) to the LTR. The HIV-1 enhancer region consists of two tandem binding sites for transcription factor NF-kB, which function as the principal enhancers of transcription and are required for basal transcription (Benjamin Berkhout 1992; FATAH KASHANCHI 1994; Carlos Sune 1995). The basal level of transcription leads to produce stable, short, and nonpolyadenylated transcripts. These short transcripts are RNA of 60 bp containing heterogeneous 3' ends and TAR element (Harrich, Garcia et al. 1989; Perkins, Agranoff et al. 1994).

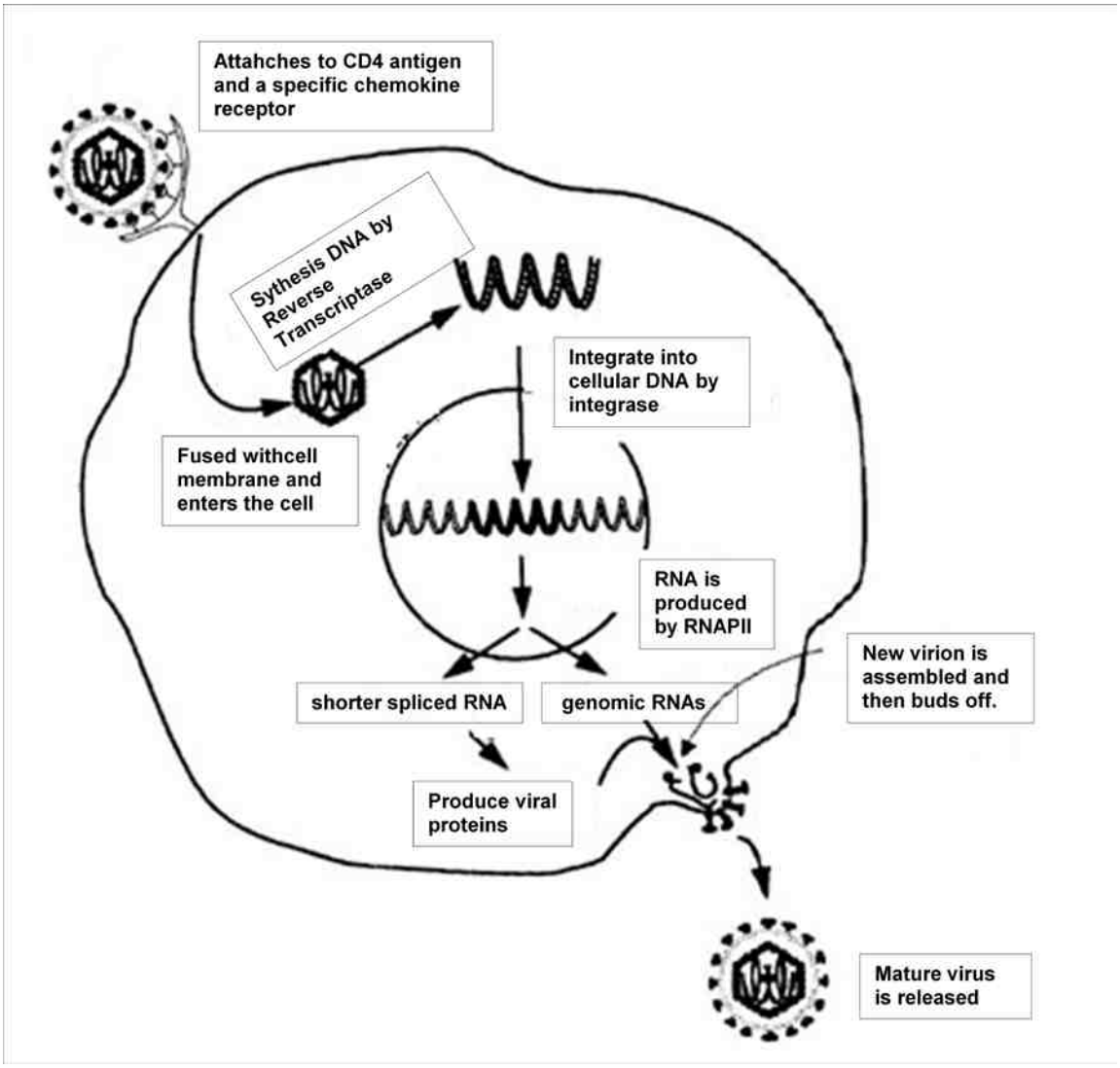

Attahches to CD4 antigen and a specific chemokine receptor
Sythesis DNA by Reverse Transcriptase
Integrate into cellular DNA by integrase
Fused withcell membrane and enters the cell
RNA is produced by RNAPII
New virion is assembled and then buds off.
shorter spliced RNA
genomic RNAs
Produce viral proteins
Mature virus is released

Figure 2. HIV-1 life cycle 1. Binding of gp120to CD4 and CXCR4 or CCR5; 2.Membrane fusion between virus and cells; 3. Viral core uncoating; 4.Reverse transcription of the genomic ssRNA into dsDNA provirus; 5.Nuclear importation of the pre-integration complex; 6.Provirus integration into the host chromosome; 7. Transcription and alternative splicing of HIV-1 mRNA; 8.Translation of structural proteins and viral enzymes; 9.Virion assembly at the cell membrane; 10.Virion budding and maturation

2.2 N-TEF

The negative transcriptional elongation factors (N-TEF) are associated with RNAPII and causes transcription pausing. N-TEF arrest RNAPII at nucleotides 20-50downstream from the initiation site, resulting in the accumulation of short transcripts (Wu, Yamaguchi et al. 2003; Wu, Lee et al. 2005). N-TEF includes the 5, 6-Dichloro-1-beta-D-ribofuranosylbenzimidazol (DRB) sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) (Wada, Takagi et al. 1998; Yamaguchi, Watanabe et al. 1999). DSIF is composed of Spt4 and Spt5, it is involved in HIV-1 Tat transactivation *in vitro* and contains many C-terminal repeats that can be phosphorylated by CDK9 *in vitro* (Fujinaga, Irwin et al. 2004; Ping, Chu et al. 2004). NELF has four subunits: NELF-A, NELF-B, NELF-C and NELF-E.NELF-E contains RNA recognition motif (RRM) and interacts with the TAR element. This binding is required for the inhibitory effect of NELF on transcription, which involves RNAPII pausing and decreased acetylation of histone H4 (Yamaguchi, Takagi et al. 1999). DSIF and NELF bind to hypophosphorylated RNAPII complexes, while the hyperphosphorylation of the RNAPII CTD promotes dissociation of these negative factors from the complex and allows binding of other elongation factors (Lis 1998).

2.3 P-TEFb

The positive transcription elongation factor b (P-TEFb) is a cyclin-dependent kinase and controls the elongation phase of transcription by RNAPII. During HIV-1 transcription, P-TEFb is the key regulator that helps RNAPII to overcome the rate-limiting step in the early stage of elongation and is an essential coactivator of the viral transactivator Tat. It

is recruited to the TAR element by Tat and forms a stable RNA stem-loop at the 5' end of the viral transcripts (Wei, Garber et al. 1998; Ammosova, Berro et al. 2006; Peterlin and Price 2006). P-TEFb has two forms that have been referred as the large form and the free form (Michels, Fraldi et al. 2004; Barboric, Kohoutek et al. 2005; Li, Price et al. 2005). The free form contains CDK9 and cyclin T1 and has the kinase activity. The large form contains CDK9, cylinT1, 7SK RNA and hexamethylene bisacetamide-induced protein1 (HEXIM1) or HEXIM2(Byers, Price et al. 2005; Egloff, Van Herreweghe et al. 2006; Barrandon, Bonnet et al. 2007). 7SK snRNA and HEXIM1 keep P-TEFb in an inactivate state, but Tat competes with HEXM1 for binding to 7SK, blocks the formation of the 7SK/HEXIM1 complex, and releases P-TEFb from the pre-formed inactive form (Barboric, Kohoutek et al. 2005; Barboric, Yik et al. 2007; Li, Cooper et al. 2007; Sedore, Byers et al. 2007).

There are two isoforms of Cdk9 *in vivo*: $Cdk9^{42}$ and $Cdk9^{55}$, which are generated by two different promoters in the Cdk9 gene (Shore, Byers et al. 2003; Shore, Byers et al. 2005). Cdk9 is a Cdc2-related kinase and phosphorylates the CTD of the largest subunit of RNAPII. The phosphorylation of serine 5 (Ser 5) and serine(Ser2) of the RNAPII CTD is essential for the productive elongation of transcription (Marshall and Price 1992; Zhu, Pe'ery et al. 1997; Zhou, Halanski et al. 2000; Ramanathan, Rajpara et al. 2001; Shim, Walker et al. 2002). Autophosphorylation of CDK9 confers the high affinity binding of the Tat:P-TEFb complex to TAR. On the other hand, the Spt5 domain of DSIF and the E subunit of NELF can be phosphorylated and removed from TAR by CDK9, which leads to some reading-through transcription. Besides CDK9, Tat binding to cyclin T1

enhances its affinity for TAR RNA. Tat binds to the cyclin domain of cyclin T1 in a zinc-dependent manner and requires specific residues within the Tat-TAR recognition motif of cyclin T1(Garber, Wei et al. 1998; Peng, Zhu et al. 1998; Zhou, Halanski et al. 2000; Schulte, Czudnochowski et al. 2005). Cyclin T1 in mouse cells has a point mutation (Y261R) within the Tat-TAR recognition motif, which disallows its interaction with Tat/TAR and has no effect on Tat transactivation. Expression of human cyclin T1 in mouse cells is capable of restoring the mouse cyclin T1 defect. In summary, P-TEFb acts to phosphorylate the CTD of RNAPII in the early elongation complexes and converts RNAPII to be elongation-competent.

2.4 Tat protein

HIV-1 Tat stands for “trans-activator of transcription”. It is encoded by two exons and has 85-101 amino acids depending on HIV-1 subtypes. It is translated from multiply spliced transcripts that do not require Rev for their nuclear export. Tat is structurally divided into five function domains: the N-terminal domain, the cysteine-rich domain, the core domain, the basic domain, and a C-terminal domain. The N-terminal cysteine-rich core domain is also called the activation domain, where six cysteins are present and important for Tat /cynlin T1 interaction (Garber, Wei et al. 1998). Another important domain for Tat function is the C-terminal arginine-rich domain; it is involved in Tat binding with TAR and also serves as a nuclear localization signal for Tat (Hauber, Miska et al. 1989; Siomi, Shida et al. 1990).

Tat is capable of transctivating the basal HIV-1 transcription (Robert A. Marciniak 1991; Qiang Zhou 1995). In the absence of Tat, there are only a small number of short RNA transcripts produced, from which Tat protein is translated. Newly translated Tat protein binds to cyclin T1 and mediates RNPAII phosphorylation, resulting in transcription of full- length HIV-1 mRNA (Amendt, Si et al. 1995; Suñé and Garc\'ia-Blanco 1995; Suñé, Goldstrohm et al. 2000; Montanuy, Torremocha et al. 2008).

2.5 TAR

TAR is 59 nucleotides (nt.) in length. It forms an unusual secondary structure, which has a three-nucleotide bulge (nt. 23-25), and a six-nucleotide loop (nt. 30-35) and a central head nucleotide apical loop. Tat binds to TAR at a trinucleotide bulge located near the apex of the TAR RNA stem-loop structure (Amendt, Si et al. 1995; Pereira, Bentley et al. 2000). Specifically, Tat binds to nt.U23 in the bulge, and nt. A27, and nt.U38 and two phosphates between nt. A22, U23 and C24 serve to stabilize this binding. The cellular coactivaor of Tat, cyclinT1, binds to the central loop of TAR and creates a high-affinity Tat-TAR complex (Calnan, Tidor et al. 1991; Weeks and Crothers 1991).

2.6 HIV-1 transcription initiation and elongation

HIV-1 transcription is the limiting step for HIV-1 gene expression. The first step of HIV-1 transcription is the formation of a large, multiprotein RNAPII holoenzyme, so-called pre-initiation complex (PIC) (Fig. 3). The general transcription factors, including TFIIB, TFIID and TFIII, help to position the RNAPII at the starting site of transcription. At this stage, the RNAPII CTD, which contains 52 repeats of

heptapeptied sequence (YSPTSPT), is not phosphorylated in either Ser2 or Ser5, and this form of CTD is called the CTDa. Besides general transcription factors, other transcription enhancers like NF-kB and some histone acetyltransferases (HAT) are also recruited to increase the reinitiation at the LTR promoter (Sheridan, Mayall et al. 1997). It is also reported that Tat can bind to HAT such as CREB binding protein (CBP)/p300 or p300/CBP-associated factor (P/CAF) and affect the chromatin conformation of the LTR promoter (Benkirane, Chun et al. 1998).

The next step of HIV-1 transcription is promoter clearance. Transcription factor IIH (TFIIH) plays an important role in this step. TFIIH contains DNA helicases and CDK-activating kinase CDK7 and is recruited to RNAPII CTDa by TFIIE. Helicases can open the double strand DNA to allow it to be transcribed into RNA. CDK7 phosphorylates Ser5 of the RNAPII CTD and removes some transcription mediators bound to unphosphorylated CTD. RNAPII is then released from pre-initiation complex and starts transcription (Zawel, Kumar et al. 1995). In this stage of transcription initiation, Tat interacts with TFIIH and increases the CDK7 kinase activity. It has been reported that a pseudosubstrate of CDK7 blocks Tat transactivation activity (Blau, Xiao et al. 1996; Cujec, Cho et al. 1997; Cujec, Okamoto et al. 1997).

The transcription complex that is formed with TFIIH at the HIV-1 5'LTR becomes competent to initiate transcription but is less efficient in elongation. The presence of NELF and DSIF in the initiation complex is mainly responsible for this inefficiency. At this step, only short transcripts of about 60 nt are produced in the cytoplasm, indicating

that the integrated provirus is repressed by premature termination. Also, a fraction of nuclear P-TEFb is inactive as it is bound to 7S RNA (Barboric, Kohoutek et al. 2005; Barboric, Yik et al. 2007; Li, Cooper et al. 2007; Sedore, Byers et al. 2007). However, when Tat is translated from the short transcripts, it binds to cyclinT1 and recruits P-TEFb complex to the TAR RNA. These interactions lead to phosphorylation of RNAP II Ser2 by CDK9and as a result, increase the rigidity of the CTD and the affinity of human capping enzymes and act as a scaffold for splicing and polyadenylation process, which in turn increase transcription elongation, 5'-end capping, and histone methylation at the HIV-1 promoter (Zhou, Deng et al. 2003; Zhou, Deng et al. 2004). This step is called transcription elongation. P-TEFb is the key regulator for this step. At later stages of elongation, Tat becomes associated with RNAP II rather than TAR RNA, indicating that the Tat:p-TEFb:TAR complex is disrupted during transcription (Gerber and Shilatifard 2003; Shilatifard, Conaway et al. 2003; Sims, Belotserkovskaya et al. 2004).

2.7 Host factors involved in HIV-1 transcription activation

As we described above, HIV-1 LTR utilizes both cellular factors and viral proteins to regulate HIV-1 LTR transcription. HIV-1 LTR contains several binding site for host transcription factors, including NF-κB, nuclear factor of activated T-cells (NFAT), active protein-1 (AP1), sp1, these transcription factors function in HIV-1 basal transcription, and enhance HIV-1 LTR-mediated gene expression(Gaynor 1992). Other host transcriptional regulators, including HAT-1 and p300 serve to unwind chromatin nucleosome 1 located on HIV-1 LTR and thus greatly enhance viral gene transcription elongation. Some cellular splicing factors also function in HIV-1 transcription.

Tat-splicing factor 1(SF1) and c-Ski-interacting protein (SKIP) are first identified as cellular splicing factors and later shown to be required for efficient transcriptional transactivation. In particular, Tat-SF1 is a cofactor for stimulation of transcriptional elongation by HIV-1 Tat; SKIP associates with P-TEFb and enhances transcription elongation by HIV-1 Tat (Zhou and Sharp 1996; Fong and Zhou 2000; Kameoka, Duque et al. 2004; Vaness Bres Nathan Gomes and Jones 2005). It is also reported that elongation factor ELL2, transcription factors/coactivators AF4/FMR 2 family member 4 (AFF4), eleven-nineteen leukemia (ENL), are present in the Tat-P-TEFb complex.Tat and AFF4 function to bridge eleven-nineteen lysine-rich leukemia protein 2(ELL2) and P-TEFb together to form an elongation complex and significantly enhance HIV-1 transcription (He, Liu et al. 2010).

In the inactive P-TEFb complex, 7SK snRNP contains 7SK snRNA and CDK9 kinase inhibitor, HEXIM 1, and inhibits the P-TEFb kinase activity (Michels, Fraldi et al. 2004; Yik, Chen et al. 2004). Host protein Yin Yang-1(YY1) has also been shown to repress HIV-1 gene expression via recruitment of histone deacetylase 1(Coull, Romerio et al. 2000). Interleukin-10 has been reported to down-regulate cyclin T1 expression through induction of proteasome-mediated proteolysis and to inhibit HIV-1 LTR-directed gene expression (Wang and Rice 2006). Moreover, flavopitidol is shown to inhibit the P-TEFb kinase activity and block the HIV-1 transcription (Chao and Price 2001)

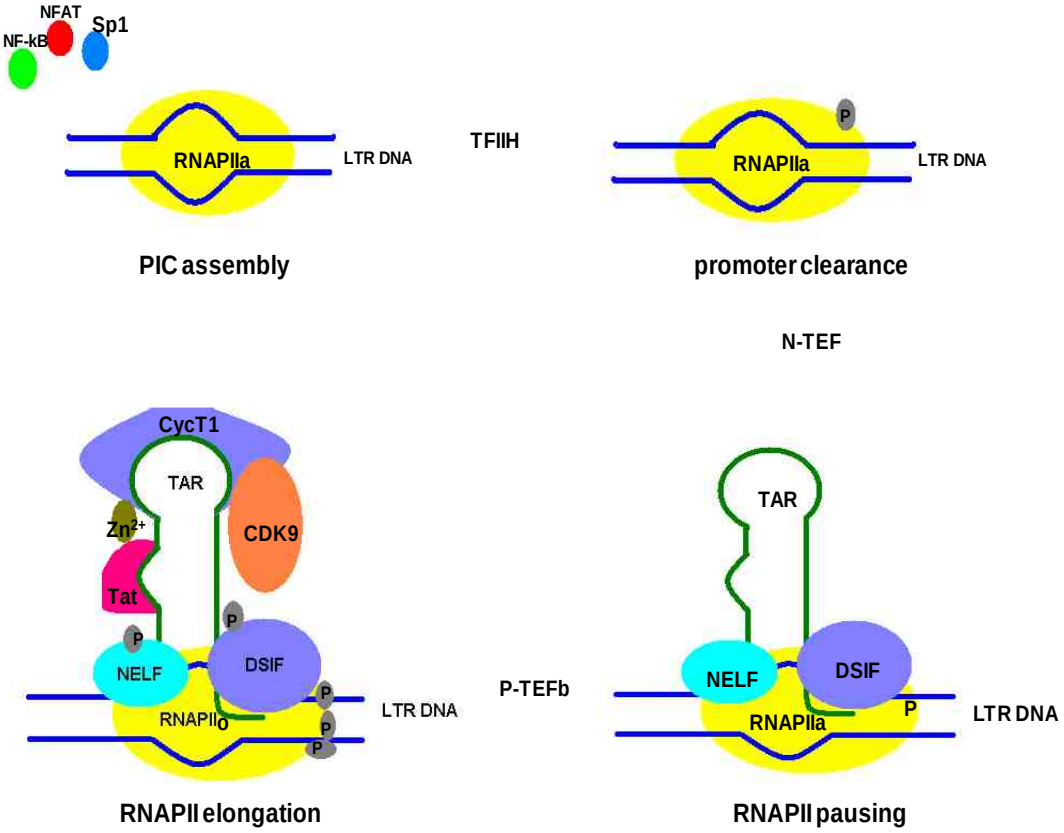

NFAT
NF-kB
Sp1
RNAPIIa
LTR DNA
TFIIH
P
RNAPIIa
LTR DNA
PIC assembly
promoter clearance
N-TEF
CycT1
TAR
Zn2+
CDK9
Tat
P
P
NELF
DSIF
P
P
P
RNAPII
LTR DNA
P-TEFb
TAR
NELF
DSIF
P
RNAPIIa
LTR DNA
RNAPII elongation
RNAPII pausing

Figure 3. HIV-1 LTR transcription initiation and elongation 1. Pre-initiation complex formation: a pre-initiation complex is assembled at the LTR promoter. It contains RNAPII, general transcription factors (GTF) and mediators that bind to unphosphorylated CTD. Other transcription enhancers like NF-kB recruit histone acetyltransferases (HATs) to increase reinitiation at the LTR promoter. 2. Promoter clearance: TFIIH initiates promoter clearance. CDK7 phosphorylates Ser5 of the RNAPII-CTD. The transcription initiation starts. 3. RNAPII is only partially phosphorylated (RNAPIIa) and interacts with N-TEFb, which contains the DSIF and NELF. The transcription process is blocked. 4. P-TEFb, containing CDK9 and cyclin T1, is recruited to the HIV-1 TAR by Tat. CDK9 phosphosrylates Ser2 of the RNAPII and initiates HIV-1 transcription elongation step.

3. HIV-1 RNA SPLICING AND REGULATION

3.1 Eukaryotic pre-mRNA splicing

The pre-mRNA splicing is mediated by a large complex called spliceosome and requires small nuclear ribonucleoproteins (snRNPs) U1, U2, U5and U4/U6 and several non-snRNP splicing factors, such as U2-snRNP auxiliary factor (U2AF) and splicing factor 1 (SF1). The major spliceosome splices out introns of pre-mRNA containing GU at the 5' splice site and AG at the 3' splice site (Staley and Guthrie 1998; Das, Zhou et al. 2000; Nilsen 2002).

During the splicing process, U1 snRNP first binds to GU sequence at the 5' splice site while U2AF and SF1 associate with the 3' splice site (Py-AG site). The spliceosome at this stage is called E complex. This is followed by association of U2snRNP with the branch point sequence (BPS) in the first ATP-dependent step of splicing, which is called A complex since ATP is hydrolyzed. Then, U4-U5-U6 tri-snRNP joins A complex of the spliceosome. The U5 snRNP binds the exons at the 5' site, while U6 snRNP binds to U2 snRNP. This complex is called B complex. Following a series of RNA-RNA and RNA-protein rearrangements, U1 and U4 snRNP are first released from the complex and a lariat structure is formed at this stage. Subsequently, the 3' site is cleaved and the exons are ligated with the second ATP hydrolysis. Finally, the lariat structure debranches, the spliced RNA is released from the spliceosome (Nilsen 1996; Das, Zhou et al. 2000; Lallena, Chalmers et al. 2002).

3.2 HIV-1 mRNA alternative splicing

Alternative splicing of HIV-1 mRNA precursors plays a critical role in regulating HIV-1 gene expression (Fig. 4). The alternative use of the 5'-and 3'-splice sites results in generation of approximately 40 spliced HIV-1mRNA species. These RNA are divided into three classes: unspliced mRNA, singly spliced RNA and multiply spliced mRNA(Purcell and Martin 1993; Frankel and Young 1998). Unspliced mRNA is necessary for expression of structural viral proteins or their precursors and also serves as genomic RNA. Singly spliced mRNA encode the Env, Vpu,Vif and Vpr protein. Multiply spliced mRNA translate into Tat, Rev, and Nef (Felber, Hadzopoulou-Cladaras et al. 1989; Malim M.H. 1989). HIV-1 mRNA splicing is a complex process. It is highly regulated by three combined mechanisms: suboptimal splice sites; exonic and intronic *cis*-elements and *trans*-acting factors that regulate splicing by binding to *cis*-elements (O'Reilly, McNally et al. 1995; Caputi, Freund et al. 2004; Stoltzfus and Madsen 2006).

3.3 Suboptimal splicing sites for HIV-1

There are four splice donor sites (D1, D2, and D3andD4) and eight splice acceptor sites (A1, A2, A3, A4a, A4b, A4c, A5and A7) located throughout the full-length HIV-1 RNA genome (Fig. 4). Among these splice sites, A3 is required for production of *tat* mRNA, A4a, b and c for production of *rev* and *env* mRNA, A5 for production of *nef* and *env* mRNA (Purcell and Martin 1993; Stoltzfus and Madsen 2006).

3.4 Exonic and intronic *cis*-acting elements

Besides the splice sites, the efficiency of HIV-1 mRNA splicing is also regulated by both positive and negative *cis*-elements in HIV-1 RNA genome. They either enhance or inhibit HIV-1 RNA splicing. Some of these *cis*-sequences have been shown to interact with cellular factors. Positive *cis*-acting elements are known as exonic splicing enhancers (ESE), or intronic splicing enhancers (ISE), which increase the utilization of upstream 3'splice site (ss) by binding to nuclear components that favor the association of spliceosomal components (Malim M.H. 1989; O'Reilly, McNally et al. 1995; Damier, Domenjoud et al. 1997; Frankel and Young 1998). To the contrary, exonic splicing silencers (ESS) and intronic splicing silencers (ISS) decrease the utilization of the upstream 3' ss and inhibit viral pre-mRNA splicing (Amendt, Si et al. 1995; Si, Amendt et al. 1997; Jacquenet, Méreau et al. 2001; Madsen and Stoltzfus 2005; Madsen and Stoltzfus 2006). To date, four ESS, one ISS and four ESE have been identified within the viral genome (Amendt, Hesslein et al. 1994; Staffa and Cochrane 1995; Si, Amendt et al. 1997; Wentz, Moore et al. 1997; Si, Rauch et al. 1998). ESE1 and ESSV are located downstream of A2 splice site and regulate *vpr* mRNA splicing (Frankel and Young 1998). ESSp, ESS2 and ESE2 are located in *tat* exon 2 and regulate A3 splice site (Jacquenet, Mereau et al. 2001; Hallay, Locker et al. 2006). An ESE is also located downstream of A5 and used for the production of *env* mRNA (Caputi, Freund et al. 2004). ESS3, ESE3, and ISS regulate A7 splice site and are important for all multiple spliced transcripts.

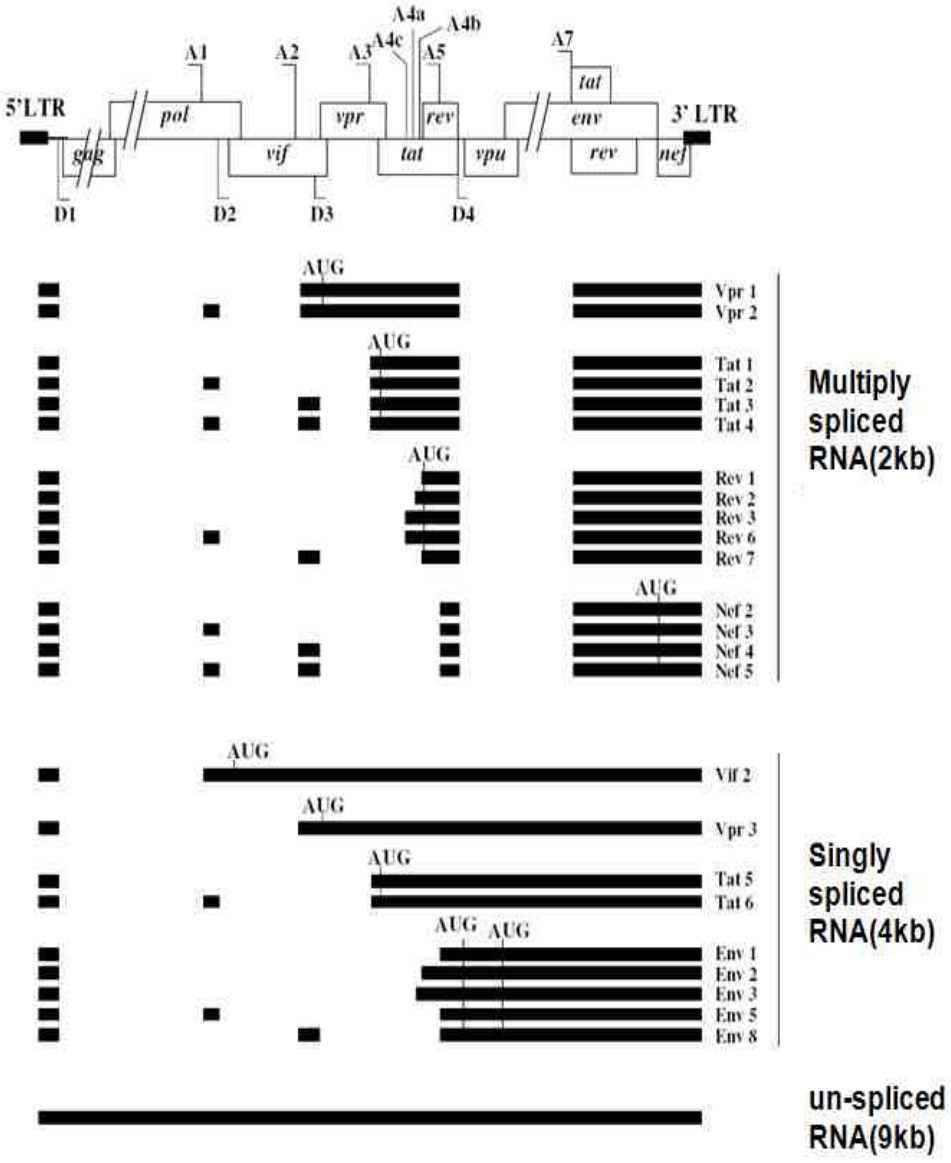

A4a
A4b
A4c
A1
A2
A3
A5
A7
tat
5'LTR
pol
vpr
rev
env
3' LTR
gag
vif
tat
vpu
rev
nef
D1
D2
D3
D4
AUG
Vpr 1
Vpr 2
Tat 1
Tat 2
Tat 3
Tat 4
Rev 1
Rev 2
Rev 3
Rev 6
Rev 7
Nef 2
Nef 3
Nef 4
Nef 5
Multiply spliced RNA(2kb)
Vif 2
Vpr 3
Tat 5
Tat 6
Env 1
Env 2
Env 3
Env 5
Env 8
Singly spliced RNA(4kb)
un-spliced RNA(9kb)

Figure 4. HIV-1 mRNA alternative splicing and mRNA transcripts The HIV-1 RNA genome contains four splice donor sites (D1, D2, D3, and D4) and eight splice acceptor sites (A1, A2, A3, A4a,A4b, A4c, A5, A7). The alternative use of 5'-and 3'- splice sites results in generation of approximately 40 spliced mRNA species. These RNA are divided into three classes: unspliced mRNA, singly spliced RNA and multiply spliced mRNA. Unspliced mRNA is necessary for expression of structural viral proteins or their precursors and also serves as genome RNA. Singly spliced mRNA encode the Env, Vpu, Vif and Vpr protein. Multiply spliced mRNA translate into Tat, Rev, and Nef.

3.5 *Trans*-acting factors

The regulatory *cis*-elements within the RNA genome often interact with *trans*-acting factors that function in the basal splicing machinery. These factors are from either the arginie-serine (SR) protein family or the heterogeneous nuclear ribonucleoprotein (hnRNP) family (Table 1) (Zahler, Lane et al. 1992; Staley and Guthrie 1998; Caputi, Mayeda et al. 1999; Tange 2001; Caputi and Zahler 2002; Jacquenet, Decimo et al. 2005). Splicing component of 35KDa (SC35) and alternative splicing factor/splicing factor 2 (ASF/SF2) of the SR proteins family mainly participate in the positive regulation of splicing by binding to ESE *cis*-elements, stabilizing the core splicing components to nearby splice sites and influencing the choice of splicing sites. Specifically, SC35 interacts with ESE2 *cis*-element and activates the 3'ss A3 splicing; ASF/SF2 interacts with ESE3 *cis*-elements and activates the 3'ss A7 splicing (Fu 1993; Ismaili, Perez-Morga et al. 1999; Mayeda, Screaton et al. 1999; Ropers, Ayadi et al. 2004; Zahler, Damgaard et al. 2004). In contrast, hnRNP proteins bind to ESS or ISS elements and are involved in negatively regulate mRNA splicing. They have been shown to inhibit the usage of viral splice sites and to counteract the SR protein activity. For example, hnRNP A1 protein interacts with ESS2 *cis*-element, competes with SC35 protein and inactivates upstream 3'ss A3 splicing (Caputi, Mayeda et al. 1999; Jacquenet, Mereau et al. 2001)

3.6 Splicing regulation of *tat* mRNA

Tat is a critical regulatory protein for HIV-1 transcription elongation and production of full-length HIV-1 mRNA transcripts. It also has an apoptotic activity on infected cells (Karn 1999; Hallay, Locker et al. 2006). Thus, *tat* mRNA production is tightly

regulated. All *tat* mRNA are spliced at 3'ss A3, the key control elements of site A3 are ESS2 and ESE2, both of which are located in *tat* exon 2 (Fig. 5). ESS2 is located in the long stem-loop structure 3 (SLS3) and forms a stable complex with several hnRNP A1 molecules via their C-terminal gly domain (Zahler, Damgaard et al. 2004). ESS2 contains the hnRNAP A1 recognition sequence UAG. Mutations in each UAG triplets strongly reduce hnRNP A1 binding to ESS2. ESE2 is in a close proximity to the ESS element, which binds to SR protein SC35 and SRp40, the two strong activators of site A3. As ESE2 is localized in a close proximity to ESS2, the SC35 binding site overlaps with the hnRNP A1 binding site. hnRNP A1 binding to ESS2 masks the SC35 binding site ESE2 and blocks the U2AF association and subsequently inhibits splicing (Zahler, Damgaard et al. 2004; Hallay, Locker et al. 2006).

4. MOLECULAR BIOLOGY AND FUNCTION OF TIP110

4.1 Predicted structure of Tip110

The bioinformatic analysis has revealed that the primary sequence of Tip110 protein contains three putative functional domains: half-a-tetratricopeptide repeat (HAT)-rich domain, RNA recognition motif (RRM) domain and nuclear localization signal (NLS) domain (Fig. 6).

4.1.1 HAT domain

The N-terminal two-thirds of the Tip110 protein contains seven HAT motifs. These HATs provide a structural unit of two antiparallel a-helices that assemble to form a

Table 1. HIV-1 transacting splicing factors

Acceptor sites	**Regulatory *cis*-elements**	**Regulatory splicing factors**	**Effects on HIV-1 mRNA splicing**
A3	ESSp	hn RNPH	-
	ESS2	hn RNPA1	-
	ESE2	SC35,SRp40	+
A5	ESE GAR	ASF/SF2, SRp40	+
A7	ESS3	hn RNA1	-
		hn RNPE1/E2	-
	ISS	hn RNPA1	-
	ESE3	ASF/SF2	+

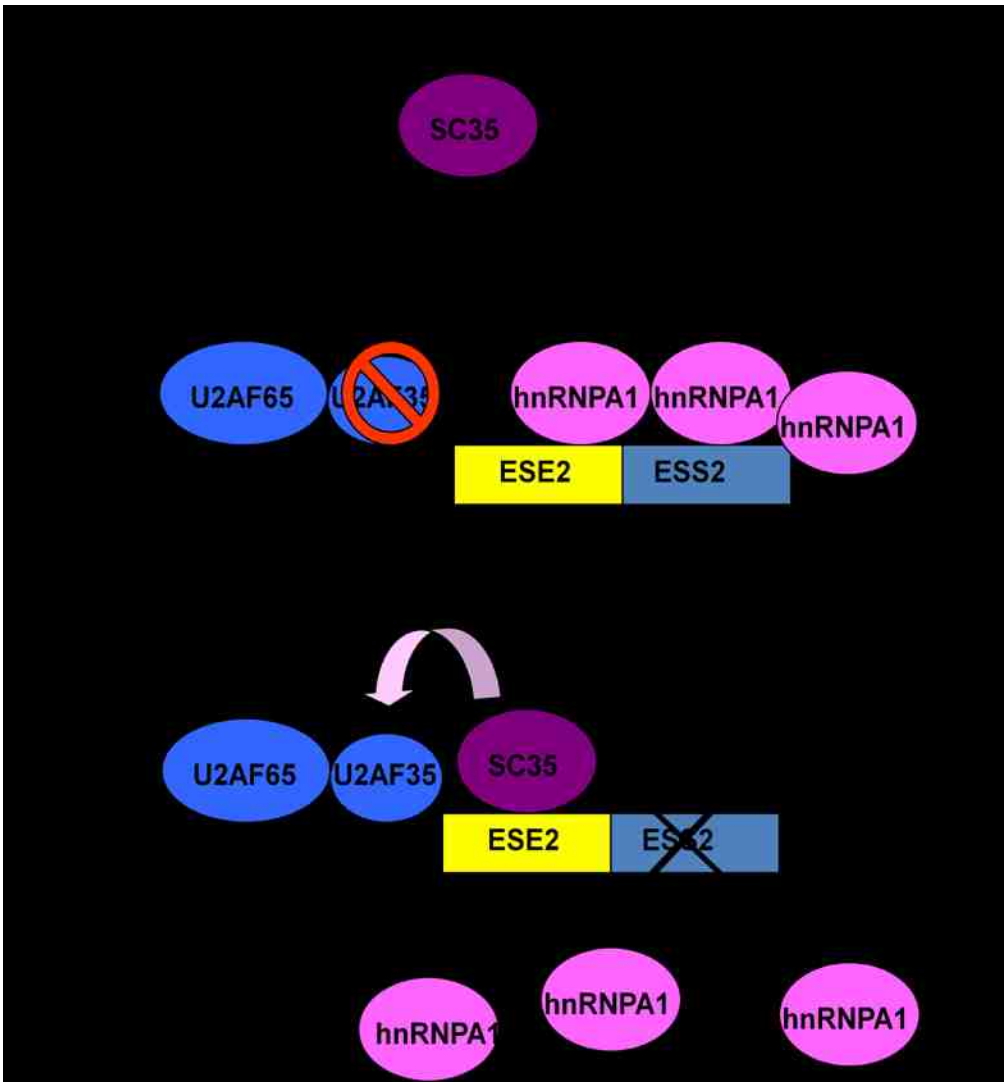

SC35
U2AF65
hnRNPA1
hnRNPA1
hnRNPA1
ESE2
ESS2
U2AF65
U2AF35
SC35
ESE2
hnRNPA1
hnRNPA1
hnRNPA1

Figure 5. Regulation of HIV-1 3' splice site A3 **(**A). hnRNP A1 as a negative regulator.ESS2 silencer is located in the long stem-loop structure3 (SLS3) and forms a stable complex with several hnRNP A1 molecules. This interaction blocks the interaction of SC35 to ESE2 and further blocks the recruitment of U2AF35 to the 3'ss of A3. (B). SC35 as a positive regulator. When ESS2 is mutated, hnRNP A1 proteins are replaced by SC35. The role of SC35 at site A3 is to counteract hnRNP A1 binding to ESS2 and to recruit U2AF to the A3 site.

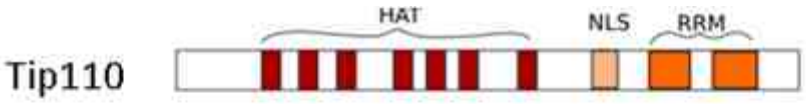
platform for protein-protein interaction. This domain is responsible for interaction of Tip110 with Tat (Liu, Li et al. 2002). In addition, this domain is involved in Tip110 interaction with a C-terminal region of the U4/U6 snRNP-specific 90 K protein and the reassembly of the U4/U6 snRNP (Bell, Schreiner et al. 2002).

4.1.2 RRM domain

Tip110 contains two RRM domains between aa740 and 874 in its C terminus. These two RRM domains correspond to RRM 2 and 3 in yeast Prp24 (Gu, Shimba et al. 1998). An *in vitro* RNA binding assay shows that Tip110 is a nuclear RNA-binding protein(Bell, Schreiner et al. 2002), but there is no interaction between Tip110 and TAR domain (Liu, Li et al. 2002). In addition, RRM deletion mutant retains the ability of Tip110 to increase the HIV-1 viral expression (Liu, Li et al. 2002).

4.1.3 NLS domain

The predominant nuclear speckle localization of Tip110 is dictated by NLS between aa 600 and aa 670 of Tip110. The deletion of the NLS domain does not interfere Tip110 interaction with Tat protein, but completely relocates Tip110 expression from the nucleus to the cytoplasm (Liu, Li et al. 2002).



HAT: half-a-tetratricopeptide repeat-rich domain

RRM: RNA recognition motif

NLS: nuclear localization signal

Figure 6. Functional domains of Tip110 Each functional domain is represented by a distinct color: HAT domain in red, NLS domain in pink, and RRM domain in orange.

4.2 Biological functions of Tip110

4.2.1 Tumor rejection antigen

Some genes encode tumor rejection antigens that can be recognized by cytotoxic T lymphocytes (CTL), some of these antigens are under clinical trials as peptide-based cancer immunotherapy. However, only a few tumor rejection antigen genes have been identified so far. In 1999, a human gene KIAA0156 was found to encode a shared tumor epitopes recognized by HLA-A24 restricted CTL in adenocarcinoma and squamous cell carcinoma (SCC) (Yang 1999). This SCC antigen recognized by T cell 3 (SART3) antigen is identical to Tip110 and possesses two epitopes that induce HLA-A24-restricted and tumor-specific CTL from peripheral blood mononuclear cells of the majority of cancer patients. Moreover, the 140 kDa SART3 antigen protein is expressed in the nucleus of malignant cell lines and also in the majority of cancer tissues from various organs, including head and neck SCC, lung SCC, lung adenocarcinomas, melanomas, and fresh leukemia cells (Kawagoe 2000). It is also expressed in the cytosol of all the proliferating cells including normal T cells and malignant cells but is undetectable in non-proliferating normal cells (Kawagoe 2000; Sasatomi 2002). Because some cancers are resistant to conventional chemotherapy or radiation therapy but responsive to various modes of immunotherapy, the SART3 antigen has been proposed as an appropriate target for treatment of certain cancers.

4.2.2 U4/U6 snRNP recycling

During the splicing reaction, some spliceosome components need to be disassembled and reactivated before entering a new splicing cycle. For example, U6 leaves the spliceosome after splicing in its singular form and has to reassociate with U4 snRNP to regenerate the U4/U6 snRNP. During U6 snRNP recycling, Tip110 is demonstrated to be a recycling factor in the transition from the singular U6 snRNP to the U4/U6 snRNP in the mammalian cells (Bell, Schreiner et al. 2002).

Tip110 is functionally related to yeast Prp24, which specifically binds to U6 and is required for U4/U6 annealing recycling (Gu, Shimba et al. 1998). As the mammalian homolog of Prp24, Tip110 interacts with a C-terminal region of the U4/U6 snRNP-specific 90 K protein through its HAT domain. Also, Tip110 is present in U6 and U4/U6 snRNPs but absent from both the U4/U5/U6 tri-snRNP and the spliceosome (Bell, Schreiner et al. 2002). *In vitro* recycling assay has shown that Tip110 functions to bring U4 and U6 snRNAs together through both RNA-protein and protein-protein interaction with its RRM and HAT domains (Medenbach, Schreiner et al. 2004). Tip110 knockout results in thymus hypolasia, other organ-specific defects and ultimately embryonic death in zebrafish (Trede 2007). Subsequent microarray expression profiling suggests that Tip110 knockout is compensated for by up-regulation of an extensive network of spliceosome components. Tip110 also interacts with other cellular splicing factors. Tip110 binds to RNA-binding protein with serine-rich domain 1 (RNPS1) through the physical association the S domain of RNPS1 and the C terminus of Tip110 and promotes the proximal alternative 3'splicing of calcitonin-dihydrofolate reductase

pre-mRNA minigene (Kenji Harada.2001). Our lab also has indentified Tip110 to interact with Y-box binding protein 1 (YB-1) protein and increase oncogene CD44 alternative splicing (manuscript in preparation).

4.2.3 HIV-1 viral gene expression

Tip110 is an important cellular factor for HIV-1 gene expression and viral replication. Using the yeast two-hybrid assay, Tip110 is found to bind to Tat *in vivo* (Liu, Li et al. 2002). The N-terminal HAT-rich domain of Tip110 and the core domain of Tat are directly involved in the Tip110-Tat complex formation. However, there is no interaction between Tip110 and TAR. Tip110 is capable of transactivating the HIV-1 LTR promoter through direct interaction with Tat. Tip110 expression is associated with increased the HIV-1 virus production while Tip110 knock down inhibits HIV-1 replication. These results demonstrate that Tip110 plays an important role in HIV-1 virus replication and gene expression through interaction with Tat. However, the underlying mechanisms were not understood and are the focus of the dissertation research.

4.2.4 Androgen receptor (AR)-mediated transactivation

AR is a nuclear receptor and is activated by binding of the androgenic hormones. AR binds to DNA and mediates gene expression in different cellular processes, which are critical for the development and maintenance of the male sexual phenotype. Like other nuclear receptors, AR is modular in structure and contains four functional domains: N-terminal regulatory domain, the DNA binding domain, hinge region, and the C-terminal ligand binding domain (LBD). There are a number of cellular factors

regulating AR transactivation by direct interaction. One common structure among these proteins is the presence of the LXXLL motif or nuclear receptor (NR) box. This common NR box is sufficient for the interaction between co-regulator and the LBD of the AR.

Tip110 protein has been shown to contain the NR domain between amino acids 118 and 122, interact with AR, and negatively regulate AR transcription activation (Liu, Kim et al. 2004). AR transcription activation is recovered when Tip110 expression was down-regulated by anti-tip110 antisense RNA plasmid. In addition, Tip110 overexpression blocks expression of prostate surface antigen, an AR target gene. Tip110 prevents the complex formation between AR and AREs, which might account for the inhibition effects of Tip110 on AR transcriptional activation (Liu, Kim et al. 2004).

5. SUMMARY OF THE BACKGROUND AND OUR HYPOTHESIS

The transcriptional activation of the HIV-1 LTR promoter is a key step in HIV-1 life cycle and requires coordinated action of viral protein Tat and cellular proteins. These cellular factors function to either disassociate the negative inhibitor which blocks RNAPII phosphorylation from the promoter or recruit the elongation competent RNAPII-containing complexes to the promoter. Using yeast two hybrid screening and HIV-1 Tat as bait, our group has first identified Tip110 protein to interact with Tat. Further studies have shown that Tip110 synergizes with Tat to transactivate HIV-1 LTR and HIV-1 gene expression and viral replication. But the underlying molecular mechanisms were not understood. In addition, evidence has accumulated to suggest that

Tip110 interacts with splicing-related small nuclear RNA and other cellular splicing factors and enhances pre-mRNA splicing. Therefore, the overall hypothesis of this dissertation is that Tip110 controls HIV-1 gene expression and replication through transcriptional activation of the HIV-1 LTR promoter and post-transcriptional splicing of HIV-1 genomic RNA. We believe this study will add to our understanding of Tip110 protein and yield new insights into the complex interaction network between HIV-1 and host cells.

METHODS AND MATERIALS

MATERIALS

Media and supplements

Dulbecco's modified eagle's medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI-1640) medium were purchased from Lonza (Walkersville, MD) Penicillin-streptomycin-glutamine (100X), Trypsin-EDTA (0.05%Trypsin, 0.53 mM EDTA) were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyaline (Logan, UT). Ampicilin sodium salt (100 g/ml) and kanamycin sulfate (50 μg /ml) were purchased from United States Biological (Swampscott, MA). Luria broth (LB) liquid medium contained 0.01 g/ml Bacto tryptone, 0.005 g/ml Bacto yeast extract, 0.005 g/ml NaCl, and 1 mM NaOH. LB solid culture plates were prepared by adding 15 g Bacto agar to 1L LB liquid medium. Super Optimal Broth with catabolite repression (SOC) contained: 0.02 g/ml of Bacto Tryptone, 0.005 g/ml of Bacto yeast extract, 0.5 mg/ml NaCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$and 20 mM glucose.

Antibodies

Mouse anti-human RNAPII CTD antibodies (8WG16, H5, H14) (1:500) were from Covance (Greenwood, IN). Rabbit anti-human CDK9 (1: 1000), goat anti-human cyclin T1 (1: 500), mouse anti-human hnRNP A1 (1:2000), rabbit anti-GFP (1:500), mouse anti-human PCNA (1:2000), mouse anti- GST (1:500), rabbit anti human c-Myc (1:1000),

mouse IgG, rabbit IgG, were from Santa Cruz Biotechnologies (Santa Cruz, CA). Donkey anti-rabbit IgG and sheep anti-mouse IgG horseradish peroxidase-conjugated antibody were from GE healthcare (Waukesha, WI). Mouse anti-human ASF/SF2 (1:2000) was from Invitrogen (Carlsbad, CA). Mouse anti-human β-actin antibody was from Sigma (St. Louis, MO). Mouse anti-human Tip110 1C4H6 (1:2000), rabbit polyclonal anti-human Tip110 serum was made in house.

Reagents

RNaseA, o-nitrophenylß-d-galactopyranoside(ONPG), 4',6'-diamidino-2-phenylinole (DAPI)isopropyl-beta-D-thiogalactopyranoside(IPTG), phenylmethanesulphonylfluoride (PMSF) were from Sigma. [Methyl-^{3}H]-thymidne 5' triphosphate was from PerkinElmer (Boston, MA). [α-^{32}P] dCTP (specific activity 800 Ci/mmol), and [α-^{32}P] UTP (specific activity 800 Ci/mmol) were from MP Biomadicals (Solon, OH). Poly-(A) x (dT) and random hexmers were purchased from Roche (Indianapolis, IN). Restriction endonucleases were from New England Biolabs (Beverly, MA). T4 DNA ligase with its 10X reaction buffers were from USB (Cleveland, OH). TRIZOL and Superscript III reverse transcriptases were from Invitrogen. Bacto tryptone, Bacto yeast extract, Bacto peptone, Bacto were from Becton Dickinson (Sparks, MD). RNase inhibitor and deoxynucleotide triphosphates (dNTPs-dATP, dCTP, dTTP, dGTP) were from Promega Corporation (Madison, WI). Protease inhibitor cocktail set V, EDTA-Free and G418 sulfate were from Calbiochem (LaJolla, CA). Coomassie Blue was from Bio-Rad laboratories (Hercules, CA). Tat protein was from AIDS Research and Reference Reagent Program.

Biotechnology systems

The plasmid DNA purification kits, the firefly luciferase assay system, the Wizard SV Gel and PCR clean-up system and the Wizard DNA clean-up system for PCR products purification were from Promega Corporation (Madison, WI). Bio-Rad Protein Assay was from Bio-Rad Laboratories. Centricon 30 spin columns for protein concentration were from Millipore (Billerica, MA). The Expand High Fidelity PCR system, Expand Long Template PCR system and the Titan One Tube RT-PCR System were from Roche. The TOPO TA cloning kit was from Invitrogen. The MEGA Script T7 Kit was from Ambion (Austin, TX).

METHODS

Cells and cell cultures

Cell lines

Human embryonic kidney 293T cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA). Human glioblastoma-astrocytoma, epithelial-like cell lines U373 MAGI, human T-lymphoid CEM-GFP cells were obtained from NIH AIDS Research & Reference Reagent Program.

Competent cells for cloning and recombinant protein production

GC5 chemically competent *E.coli* for cloning were from GeneChoice (Frederick, MD) and BL21 *E.coli* for recombinant protein production were from Invitrogen.

Cell cultures

293T cells and U373 MAGI cells were maintained in DMEM with 10% FBS, 100 units/ml of penicillin at 37^{o}C with 5% $CO_{2.}$ CEM-GFP cells was maintained RPMI 1640 with 10% FBS, 100 units/ml of penicillin, 500 μg /ml of G418 and incubated at 37^{o}C with 5% $CO_{2.}$

Cell transfections and bacterial transformation

Cell transfections

Cell transfections were carried out by the standard calcium phosphate precipitation method. For 10-cm tissue culture plate, $2x10^{6}$ cells were plated the day before transfection and incubated for about 24 hours to reach 70-90% cell confluency before transfection. For transfection, a 500 μl mixture of 20 μg plasmid DNA and 0.24 M $CaCl_2$ in a volume of 500 μl was added dropwise into 500 μl 2X HEPES-buffered saline (HBS) solution (50 mM Hepes, 10 mM KCl, 280 mM NaCl, 1.5 mM $Na_2HPO_{4,}$ 12 mM glucose, pH 7.05-7.15). The transfection mixture was then incubated on ice for 20 min and added dropwise into 293T cells. After overnight incubation, medium was replaced with fresh DMEM medium. The cells were cultured for additional 24-48 hours before they were harvested for further analysis. pcDNA3 was used to equalize the total DNA amount while pEGFP was co-transfected to ensure a comparable level of transfection efficiency among all transfections. The number of cells and the amount of solutions and DNA were proportionally scaled down according to the surface area of the plates, if different sizes of cell culture vessels were used.

Bacterial transformation

GC5 competent cells (25 μl) were incubated with plasmid DNA or ligation reaction (0.5-2 μl) on ice for 30 min. The cells were heat-shock in a 42^{o}C water bath for 45 sec, incubated on ice for 2 min, and added 300 μl SOC medium. The transformation mixture was incubated at 37^{o}C for 45 min on a shaker at 225 rpm. Two hundred microliters of cultures were placed onto an LB agar plate. The plate was incubated at 37^{o}C overnight.

Plasmids

pTip110.His, pTip110.HA, Tip110 deletion mutants ΔRRM, ΔNLS, and ΔCT,pTat.Myc, pLTR-Luc plasmid were described previously (Liu, Li et al. 2002). GST-CTD plasmid was a gift from Dr. David Price of University of Iowa. pCMV-β-Gal, pcDNA3 and pCMV-HA were from Clontech (Clontech, Mountain View, CA). Tip110 ΔNT mutant was constructed using pTip110.His as the template and primers 5'-CCG AAT TCA CCA TGG CTG CCG TAG ATG TGG AG-3' and 5'-CCC GCT CGA GTC AAT GAT GAT GAT GAT GAT GCT TTC TCA GAA ACA GCT TGG C-3'. The PCR product was digested by EcoR I and Sac I and insert into pcDNA3. pGST-hnRNP A1 was made by cloning hnRNP A1 gene from phnRNP A1-GFP (Guil, Long et al. 2006) with primer sets 5'-CGG AAT TCC TCT AAG TCA GAG TCT CCT AAA GAG-3' and 5'-CGG AAT TCC TCT AAG TCA GAG TCT CCT AAA GAG-3'. The amplified product was then digested by EcoR I and Not I and subcloned into pGEX-4T3 for GST-hnRNP A1 protein preparation.

Plasmids pHS1-WT, pHS1-ESS4 mut and pHS2 plasmid were a generous gift from Dr. Martin Stoltzfus of University of Iowa. pCMV-WT, or pCMV-ESS2-M minigenes were constructed by digesting pHS1-WT, pHS1-ESS2-M with Not I and Xho I, the inserts were and subcloned into pcDNA3. pCMV-HS2-WT minigene was constructed using pHS2 as the template and primers 5'-GGG GTA CCG AAC AGT CAG ACT CAT CA-3'and 5'-CCG CTC GAG GGA TCC GTT CAC TAA TCG-3'. The PCR product was digested by Kpn I and Xho I and inserted into pcDNA3.

To prepare pT7-WT and pT7-ESS2-M plasmids, 60 bp DNA fragment containing ESS2 or ESE2 was amplified with primers 5'-CCG GAA TTC AGA GGA GAG CAA GA-3' and 5'-CTA GTC TAG AAG CTT GCT TCC AGG GCT CT- 3' and pHS1-WT, pHS1-ESS2-M as templates. Amplified products were then cut by EcoR I and Xba I and insert into pcDNA3 for further *in vitro* transcription.

All recombinant plasmids were sequenced to ensure their sequences.

Reporter gene assays

β-galactosidase activity assay

Cells were harvested in cold phosphate-buffered saline (PBS) and pelleted at 500 x *g* for 5 min. Then, cell pellet was resuspended in 50 μl cell lysis buffer from Promega and incubated on ice for 5 min and then centrifuged at maximum speed for 5 min. The supernatant was collected and saved. For β-galactosidase assay, 10 μl of cell extract

was mixed with 3 µl of 100X Mg^{2+} solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol), 66 µl of 1X ONPG (4 mg/ml ONPG in 0.1 M sodium phosphate, pH 7.5), and 201 µl of 0.1M sodium phosphate (57.7 ml 1M $Na_2PO_4$and 42.3 ml of 1M NaH_2PO_4, pH 7.5) and the mixture was incubated at 37°C for 30 min. Then, the optical density of the reaction containing the released o-nitrophenyl was measured at 405 nm in a microplate reader (Molecular Devices, Sunnyvale, CA)

Luciferase activity assay

β-galactosidase assay was used to normalize the transfection efficiency. Then, cell extract (20 µl) was mixed with 80 µl of firefly or *renilla* luciferase substrate (Promega). The luciferase activity was determined with an Opticomp Luminometer (MGM Instruments, Hamden, CT) and expressed as relative luciferase units (RLU).

Reverse transcriptase (RT) activity assay

One milliliter of cell culture supernatant was centrifuged at 4°C for 1.5 hours at 14000 x *g.* Virus pellet was re-suspended in 10 µl of dissociation buffer (0.25% Triton-X-100, 20% glycerol, 0.05 M Tris-HCl. pH 7.5, 0.008 M DTT, 0.25 M KCl) followed by three cycles of freezing on dry ice and thawing on ice. Then, 40 µl of 34 µl RT assay buffer (0.083 M Tris-HCl pH7.5, 0.008 M DTT, 0,0125 mM $MgCl_2$ and 0.083% Triton-X-100), 1 µl ^3H dTTP and 5 µl poly (A) x (dt) was added to the 10 µl virus suspension. The total 50 µl mixture was incubated at 37°C for 1hr and then spotted onto DE81 filters (Whatman, England). After washing with 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0),

the radioactivity on each filer was counted in a Beckman LS6000IS scintillation counter, and virus titer was expressed as cpm per ml.

Immunoblotting

Two days after transfection, cells were first washed three times with ice-cold phosphate-buffered saline (PBS) and collected in cell lysis buffer (50 mM Tris.HCl pH 8.0, 0.5% NP-40, 2 mM EDTA, 137 mM NaCl, 10% glycerol, 0.5% sodium deoxycholate, 0.2% sodium azide, 0.004% sodium fluoride, 1x protease inhibitor cocktail, 1 mM sodium orthovanadate, pH 7.25). After incubation on ice for 20 min, whole cell lysates were obtained by centrifugation at 15000 x *g* for 10 min. The protein concentration was determined using a Bio-Rad DC protein assay Kit (Bio-Rad). Cell lysates of an equal amount of protein were separated by 8%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then eletrotransfered to the HyBond-P membrane (Amersham, UK). The protein on the membrane were probed with primary antibodies and appropriate peroxidase-labeled secondary antibody and visualized with an ECL system.

The cytoplasmic fractionation was prepared by PARIS Kit (Ambion), according to the manufacturer's instructions. After wash with PBS, the cells were incubated with cell fractionation buffer on ice for 5 min. The supernatant was collected by centrifugation at 500 x *g* for 5 min and saved as the cytoplasmic fraction.

Immunoprecipitation

Cells were harvested and washed with ice-cold PBS and lyzed in immunoprecipitation (IP) lysis buffer (50 mM Tris HCl pH7.5, 120 mM NaCl, 0.25% NP40, 4 mM sodium fluoride, 1 mM sodium orthovanadate, 0.2 mM EDTA, 0.2 mM EGTA, 10% glycerol, and 1x protease inhibitor cocktail). Following incubation on ice for 20 min, cell lysates were obtained by centrifugation at 15000 x *g* for 10 min. Then, cell lysates of 500 μg protein was first pre-cleaned by 20 μl protein A agarose beads (Millipore) and then incubated with 1 μg antibody and 60μl protein A agarose beads rotating at 4^{o}C overnight. The beads were recovered by centrifuge and then washed with IP washing buffer (50 mM Tris.HCl pH 8.0, 0.5% NP-40, 2 mM EDTA, 0.4 M NaCl, 10% glycerol) for four times. The beads were suspended in 40 μl of 4 x SDS-PAGE sample buffer and ready for SDS-PAGE and western blotting analysis.

RNA isolation and RT-PCR-based splicing assay

Total RNA was extracted from transfected 293T cells using Trizol reagent (Invitogen), according to the manufacturer's instructions. RNA was extracted with acid phenol for three times to prevent DNA amplification in subsequent PCR analysis. For RT-PCR, 0.2 μg RNA was used with a Titan One Tube RT-PCR System (Roche, Indianapolis, IN) on a PE Thermocycler 9700 (PE Applied Biosystem, Foster City, CA). The primers to amplify minigene from pCMV-HS1-WT or pCMV-HS1-ESS4 were: Forward (T7): 5'-TAA TAC GAC TCA CTA TAG GGC GA-3'; Reverse (Tat37): 5'-GGC TGC AGT TAA CAA ACT TGG CAA TGA AAG C-3'. The primers for amplifying unspliced and spliced RNA from pCMV-HS2 were T7 and HS2-R: 5'-CCG CTC GAG GGA TCC GTT

CAC TAA TCG-3'. The RT-PCR program was one cycle of 50°C for 30 min, thirty cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 30 sec and one cycle of 72°C for 5 min. The primers to amplify tat transcripts from pNL4-3 or pHIV-Rev- transfected cells were: TAR5: 5'-CCC GAA TTC GGG TTC CCT AGT TAG CCA GAGGAG CTC CCA GGC TCA GAT CT-3'; Tat21: 5'-GGC TGC AGT TAA GCA GTT TTA GGC TGA CTT C-3'; Tat5: 5'-GTC GGG ATC CTA ATG GAG CCA GTA GAT CCT; Tat3C: 5'-GTG ACG GAT CCT TAC TGC TTT GAT AGA GAA AC-3' and the program was one cycle of 50°C for 30 min, thirty cycles of 94°C for 1 min, 50°C for 30 sec, 72°C for 45 sec and one cycle of 72°C for 5 min. GAPDH was included in a RT-PCR as control with primers: 5'-GAA GGTGAA GGT CGG AGT-3' and 5'-GAA GAT GGT GAT GGG ATT TC-3'.

Multiplex RT-PCR

293T cells were transfected with pNL4-3 as described above. Two days post transfection, total cellular RNA was first isolated from the cells and further extracted with acid phenol for three times to prevent DNA amplification in subsequent PCR analysis. Three micrograms of RNA was reversed-transcribed at 50°C for 1 hr in a total volume of 20 μl with 10 mM dNTPs (Promega), 200 U of RNase inhibitor (Promega), 500 ng of random hexamer (Roche) and 200 U of Superscript III reverse transcriptase (Invitrogen). Then, 1 μl of amplification products were used as template for multiplex PCR in a total reaction volume of 50 μl with 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, and 1 U of AmpliTag Gold (Applied Biosystems) with primers: BSS: 5'-GGC TTG CTG AAG CGC GCA CGG CAA GAG G-3' and SJ 4.7, which spans sites D4 and D7 5'-TTG

GGA GGT GGG TTG CTT TGA TAG AG-3'. The PCR program was 30 cycles of 94^{o}C for 30 s, 60^{o}C for 1 min, and 72^{o}C for 2 min and then denatured at 94^{o}C for 5 min. Then, 5 μl of the above PCR products were labeled by addition of 1 μCi of ^{32}P dCTP (800 Ci/mmol, GE Healthcare) and additional 3 cycles of PCR. The products were analyzed by electrophoresis on a 8 M urea 6% polyacrylamide gel, followed by drying of the gel for 1 hr using a vacuum gel dryer (Pharmacia biotech), and visualized by autoradiography. GAPDH was included as a RT-PCR control with the primers: 5'-GAA GGTGAA GGT CGG AGT-3' and 5'-GAA GAT GGT GAT GGG ATT TC-3'.

Recombinant protein expression and purification

pGEX-4T3, pGST-CTD and pGST-hnRNP A1 were first transformed into *E.coli* BL21. The culture was allowed to grow to reach an OD600 of 0.6 and then added 0.5 mM isopropyl-β--thiogalactoside (IPTG) and continued to incubate at 37^{o}Cfor additional 3hr. The bacteria were collected by centrifugation at 6000 x *g* for 20 min and then suspended in TZ buffer (50 mM Tris-HCL pH 7.9, 12.5 mM $MgCl_2$, 0.5 mM EDTA, 100 mM KCl, 20% glyerol, 1 mM β-mercaptoethanol, 10 μM $ZnCl_2$). Then, cells were lysed in a French Press (Thermo electron corporation, Waltham, MA) at 1800 Psi. Bacterial lysates were obtained by centrifugation at 20000 x *g* for 5 min. To purify the protein, the lysate were mixed with 500 μl glutathione Sepharose 4B beads (GE Healthcare), and incubated at room temperature for 3 hr with constant rotation. The bound proteins were washed for 3 times by PBS and proteins were eluted from the beads with 0.2 mM reduced glutathione. The purity and the yield of the recombinant protein were verified by

SDS-PAGE, followed by coomassie staining (first stained in 10% acetic acid, 40% methanol, and 0.5% coomassie blue for 2 hr, and then distained in 10% acetic acid O/N).

GST pull-down assay

GST-CTD (6 μg) protein was first phosphorylated overnight at 30^{o}C with 6 μl casein kinase I (New England Biolabs) and 1mM ATP with the phosphorylation buffer (New England Biolabs). Meanwhile, 4 μg GST-Tip110 protein was digested with 1 μl thrombin at 30^{o}C O/N to remove the GST tag. Unphosphorylated or phosphorylated GST-CTD proteins were first immobilized onto 30 μl glutathione beads in at 4^{o}C for 2 hr. Then, the protein-bound beads were incubated with purified Tip110 protein in 500 μl GST pull down buffer (20mM Hepes pH 7.9, 150mM NaCl, 0.5mM EDTA, 10% glycerol, 0.1% Triton X-100, 1mM DTT) at RT for 2 hr. Subsequently, the protein bound beads were washed with PBS five times, the bound-protein were eluted from the beads by 4 x SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE and analyzed by immunoblotting.

Chromatin immunoprecipitation (ChIP) assay

Cells ($5x10^{7}$) were first cross-linked with 1% formaldehyde at RT for 20min; the cross-linking was terminated by adding glycine to a final concentration of 0.125M. The cells were washed by PBS three times, the cell pellet was first resuspended in cell lysis buffer (85mMKCl, 0.5% NP40, 5mM HEPES, pH 8.0) and incubated on ice for 10 min. The nuclei were recovered by centrifugation at 3000 x *g* for 5 min and then resuspended in nuclear lysis buffer (10mM EDTA, 1%SDS, 50mM Tris-HCl, pH8.1). The nuclei

were incubated on ice for additional 10 min; the supernatants were collected by centrifugation at 15000 x *g* for 10 min and saved as nuclear lysates.

The nuclear lysates were then sonicated on ice with 10 pulses, each for 15 sec to generate chromatin DNA with an average size of 600 bp. The sonicated lysates were diluted 10-fold with a buffer (165mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7 mM Tris-HCl, and pH 8.0) and pre-cleared with 30 µl protein A-sepharose beads. The lysates were first incubated with the indicated antibodies overnight, then added 60 µl protein A-sepharose beads and incubated for additional for 4 hr. The immunocomplexes were washed twice with a low-salt buffer (150 mM NaCl, 0.1% SDS, 1% NP-40, 1 mM EDTA, 50 mM Tris-HCl), twice with a high-salt buffer (500 mM NaCl, 0.1% SDS, 1% NP-40, 1 mM EDTA, 50 mM Tris-HCl), twice with LiCl buffer (250 mM LiCl, 0.1% SDS, 1% NP-40, 1 mM EDTA, 50 mM Tris-HCl), and finally twice with TE buffer (0.25 mM EDTA, 10 mM Tris-HCl). The recovered beads were eluted with 120 µl elution buffer (1%SDS, 100 mM $NaHCO_3$); the supernatants were collected and incubated at 65^{o}C overnight to reverse the formaldehyde cross-linking. The DNA from the supernatants were isolated by phenol extraction, followed by ethanol precipitation and analyzed by PCR with primers spanning HIV-1 LTR promoter: 5'-CAT CCG GAG TAC TTC AAG AAC TGC-3' and 5'-GGC TTA AGC AGT GGG TTC CCT AG-3' or GAPDH: 5'-GAA GGTGAA GGT CGGAGT-3' and 5'-GAA GAT GGT GAT GGG ATT TC-3'. The PCR program was 35 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, and 72^{o}C for 30 sec.

***In vitro* elongation assay**

Plasmid HIV-1dG-less is obtained from Dr.Carlos Sune of Instituto de Parasitologia y Biomedicina. *In vitro* transcription assay was performed as described previously (Sune, Goldstrohm et al. 2000) with some minor modifications. Briefly, recombinant Tat protein (100, 200 ng) and Tip110 protein (200, 400 ng) were mixed in buffer D (20 mM Hepes pH7.9, 0.1 M KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT) and added to the nuclear extracts (3.5 μl/ reaction) (Promega) on ice. Then, 200 ng linerlized DNA templates, unlabeled NTP, 4 U Rnase inhibitor and 400mM sodium citrate were added into total 25 μl transcripton reaction. The mixture was incubated at 30^{o}C for 30 min, followed by the treatment of 1μl DNase I to remove the DNA template. RNA transcripts were recovered by phenol extraction and ethanol precipitation, and suspended in 10 μl ddH_2O. Two microliters of the RNA was used for RT-PCR analysis. The primers to amplify the short G-less transcripts are 5'-GGG TCT CTC TGG TTA GAC CAG ATC TGA GCC TGG GAG CTC-3' and 5'-AAA ACC AAA CCC TGC GCT CCA TCG CCA-3'. The primers to amplify the long G-less transcripts are 5'-GCG AGG CAT AAA GTT GCG TGT G-3' and 5'-AGG AGG GAG AGG TGA GGA GAG GAT-3'. The amounts of long and short transcripts were determined by RT-PCR followed by densitometric analysis of the PCR products; the ratio of the PCR products from long transcripts to those from the short transcripts was calculated and used as the elongation efficiency.

***In vitro* transcription**

The RNA probe for the gel shift assay was transcribed using a MEGA Script T7 system (Ambion) with plasmids pT7-WT and pT7-E2-M as templates. The plasmids were first linerlized by Hind III, 1 μg of linerlized DNA was used for transcription. Transcription was performed in an 25 μl system, containing 50 mM of ATP, CTP, GTP, 5mM UTP and 2 μl [α-^{32}P] UTP (800 Ci/mmol) at 37^{o}C for 4 hours. RNA was recovered by phenol extraction and ethanol precipitation. The radioactive activity of the RNA sample was determined in a Beckman LS6000IC Scintillation counter (Fullerton, CA).

Eletrophoretic mobility shift assay (EMSA)

EMSA was performed with 10 pmol (about 20000 cpm) of 3'-end ^{32}P labeled RNA probe. RNA was first incubated in 5 μl buffer D (0.2 mM EDTA, 0.2 M KCl, 0.5 mM DTT, 0.25 mM PMSF, 20% glycerol, 20 mM HEPES-KOH pH 7.9) at 65^{o}C for 5 min and allowed to cool down to the room temperature (RT) to eliminate its potential secondary structures. Then, 5 μl of buffer D containing indicated amounts of recombinant hnRNP A1, Tip110, or 1 μl nuclear extract was added to the RNA and then incubated for 15 min on ice for RNA-protein complex formation. Competitor tRNAs (5 μg) was incubated in each reaction. For supershift experiments, 1μl anti-hnRNP A1 4B10 (Santa Cruz) or anti-Tip110 1C4H6 was added at the end of the 15 min incubation, and the reaction was continued on ice for additional 15 min. The reactions were then loaded onto a native 6% polyacrylamide (38:2 acrylamide/bis acrylamide) gel with 45 mM Tris-broate (pH 8.3) as the running buffer. The gels were dried and then exposed to X-ray film.

Data acquisition and statistical analysis

The expression levels of protein and mRNA expression levels were determined with a densitometer, calculated on the basis of the loading controls (β-actin for Western blotting and GAPDH for RT-PCR) and expressed as a relative value (Rel.) to the indicated control. In *in vitro* transcription assay, the levels of long and short transcripts were determined by RT-PCR, followed by densitometric analysis of the PCR products, the ratio of the PCR products from long transcripts to those from the short transcripts was calculated and used as the elongation efficiency. In *in vivo* splicing assays, the levels of spliced transcripts and unspliced transcripts were determined by RT-PCR, followed by densitometric analysis of the RT-PCR products, the ratio of the PCR products from the spliced transcripts to those from unspliced transcripts was calculated and used to further calculate the splicing efficiency. All values were expressed as means ± S.D. Comparisons among groups were made using two-tailed Student's *t*-test. A p value of <0.05 was considered statistically significant (*), and $p<0.01$ highly significant (**).

RESULTS

PART 1: Tip110 interacts with RNAPII and regulates Tat-mediated LTR transcription

1.1 Tip110 enhances HIV-1 replication

Our previous work has shown that Tip110 protein plays an important role in regulating HIV-1 gene expression (Liu, Li et al. 2002). To confirm these findings, 293T cells were co-transfected with HIV-1 proviral DNA pNL4-3 and Tip110 expression plasmid pTip110.HA. A pcDNA3-based GFP expression plasmid pc3.GFP was included as a control for Tip110 as well as a marker to monitor the transfection efficiency. We harvested the cells and determined intracellular HIV-1 production by Western blotting against HIV-1 structural capsid protein p24. Compared to the pc3.GFP control, ectopic Tip110 expression gave rise to higher level of p24 expression (Fig. 7A).In addition, cell culture supernatants were collected and the activities of HIV-1 reverse transcriptase (RT) was determined, which has also frequently been used as an indicator of HIV-1 titers in these supernatants. Consistent with the intracellular HIV-1 p24 expression, Tip110 expression increased HIV-1 titers by more than 6-fold over the GFP control (Fig. 7B). These results confirmed that Tip110 enhanced HIV-1 gene expression and production.

1.2 Tip110 activates Tat-mediated LTR transcription

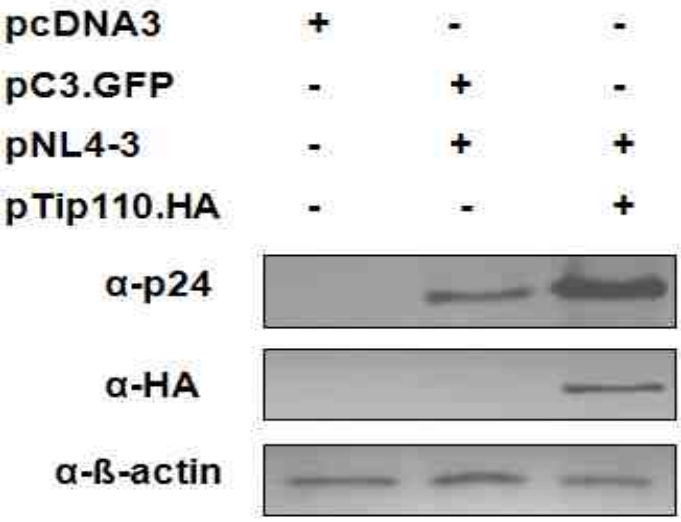
Our previous studies have also shown that Tip110 interacts and synergizes with HIV-1 Tat protein to transactivate HIV-1 LTR promoter (Liu, Li et al. 2002). Thus, we determined if Tip110 would affect Tat-mediated transactivation activity. 293T cells

were transfected with HIV-1 LTR promoter driven luciferase (Luc) reporter gene and pTip110.HA, pTat.Myc, or both and performed the Luc reporter gene assay. Vector backbone pcDNA3 was included in the transfection to equalize the total amounts of plasmid DNA transfected and pTKβ-Gal was included to normalize for variations in the transfection efficiency. At 36h post-transfection, cells were harvested for the Luc expression assay. As expected, Tat expression greatly increased the luciferase activity in a dose-dependent manner (Fig. 7C). Tip110 over-expression slightly increased the luciferase activity (Fig. 7C), indicating that Tip110 may transactivate the HIV-1 LTR basal transcription. When we transfected the cells with increasing amounts of pTip110.HA in the presence of a constant amount of pTat.Myc, the transactivation activity was greatly enhanced as demonstrated by the increase in luciferase activity from $4.2x10^4$ to $16\ x10^4$. Taken together, these results confirmed that Tip110 itself was a weak transactivator of the HIV-1 LTR promoter but it synergized with Tat to enhance Tat-mediated transactivation activity.

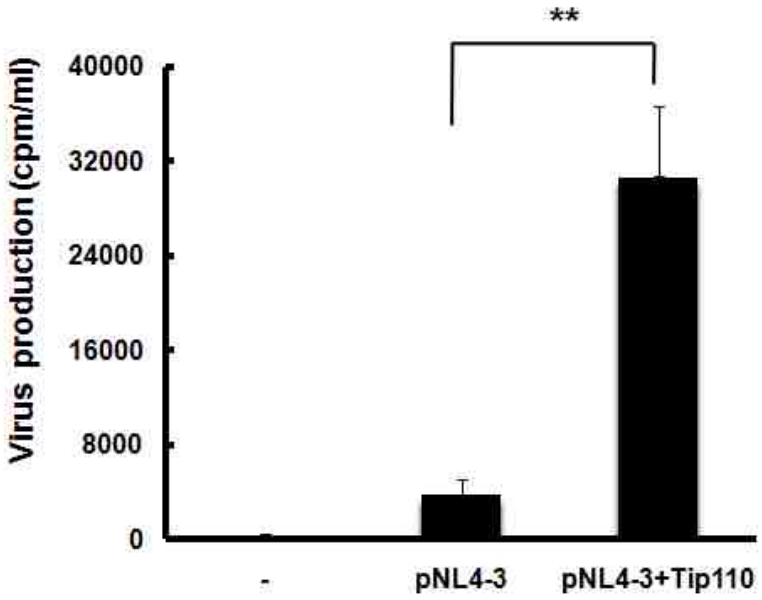
1.3 HIV-1 gene expression does not alter Tip110 expression

Since Tip110 plays a role in viral gene expression and viral replication, we next determined if HIV-1 gene expression would affect Tip110 expression. 293T cells were transfected with increasing amount of pNL4-3. Western blotting was performed for intracellular HIV-1 p24, Tip110 and β-actin. Tip110 expression showed little changes with increased expression level of p24 (Fig. 8), indicating thatHIV-1 gene expression does not alter endogenous Tip110 expression.

A


pcDNA3 + - -
pC3.GFP - + -
pNL4-3 - + +
pTip110.HA - - +
α-p24
α-HA
α-ß-actin

B


**
Virus production (cpm/ml)
40000
32000
24000
16000
8000
0
-
pNL4-3
pNL4-3+Tip110

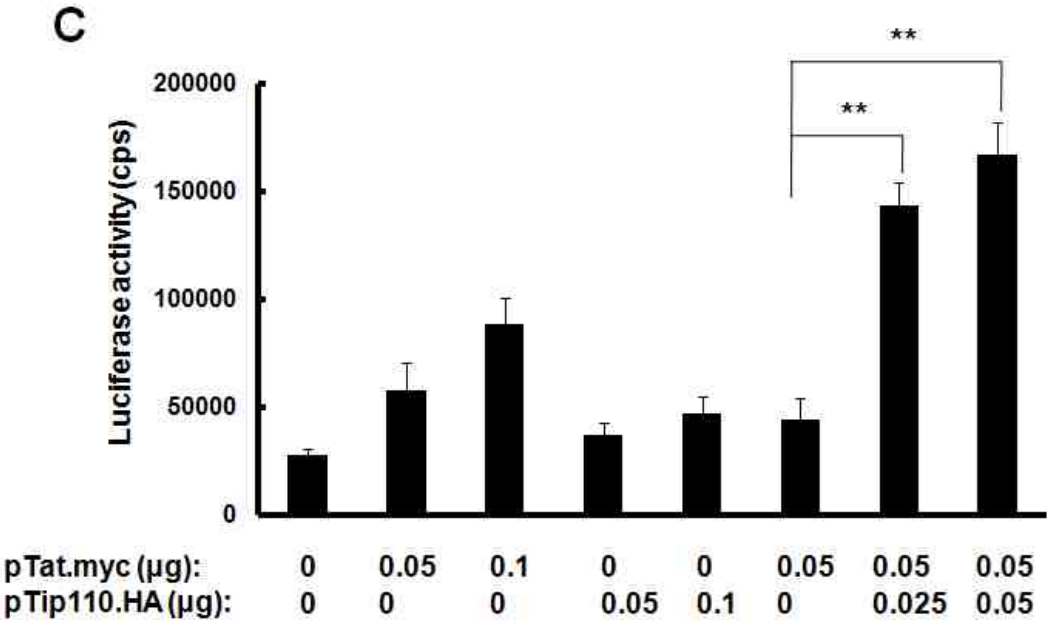

C
**
**
200000
150000
100000
50000
0
Luciferase activity (cps)
pTat.myc (µg): 0 0.05 0.1 0 0 0.05 0.05 0.05
pTip110.HA (µg): 0 0 0 0.05 0.1 0 0.025 0.05

Figure 7. Tip110 enhancement HIV-1 viral replication by transactivating Tat-mediated LTR transcription (A) 293T cells were transfected with indicated plasmids. Whole cell lysates were obtained after 72h, 50μg of each whole cell lysate was analyzed by Western blotting for Tip110 and p24 expression. Also, Western blotting against β-actin was included as a loading control. (B) The transfected cell culture supernatants (1 ml) was collected to determine the RT activity. (C) 293T cells were transfected with 0.1μg of pLTR-luc plasmid, along with various amounts of pTat.myc (0, 0.05, 0.1 μg), or pTip110.HA (0, 0.05, 0.1 μg), or 0.05μg of pTat.myc with increasing amounts of Tip110 (0, 0.025, 0.05 μg). The vector backbone pcDNA3 was added to equalize the total amounts of DNA transfected and pTK-β-Gal was co-transfected to normalize the transfection efficiency. Transfected cells were harvested after 72 h for the luciferase assay. * $p<0.05$, ** $p<0.01$.

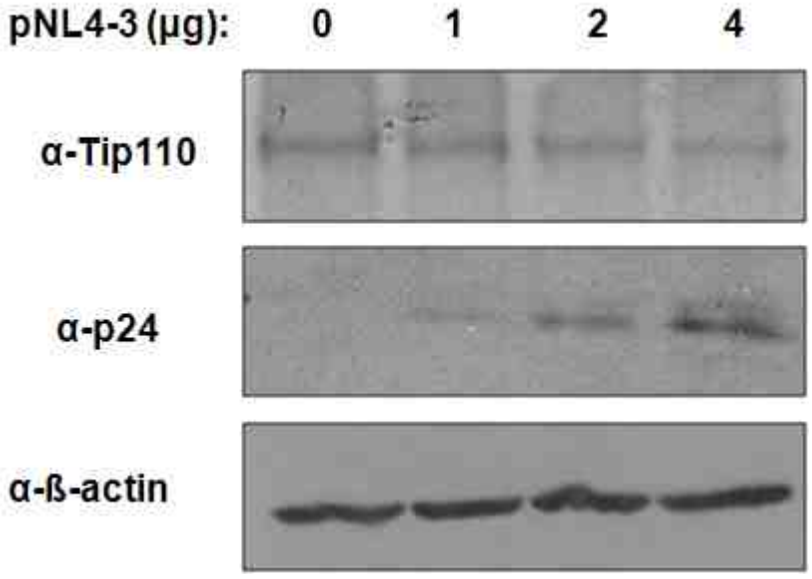

pNL4-3 (µg):
0
1
2
4
α-Tip110
α-p24
α-ß-actin

Figure 8. Tip110 expression with HIV-1 gene expression 293T cells were transfected with pNL4-3 (0, 1, 2 and 4 μg). Whole cell lysates were prepared after 72hr; lysates of 50μg protein were analyzed by Western blotting for Tip110 and p24 expression. Western blotting against β-actin was included as a loading control.

1.4 Tip110 interacts with Tat

To study the underlying mechanism of Tip110 function in LTR transactivation, we first determined whether Tip110 interacted with other transcription factors. We first confirm the interaction between Tip110 and Tat. 293T cells were transfected with pTip110.HA, pTat.myc separately or Tip110.HA together with pTat.myc. Also, pc3.GFP was added to equalize the total amount of DNA and to ensure a comparable level of transfection efficiency among transfections. Western blotting analysis showed that both Tip110 and Tat expression were detected by anti-HA or anti-myc antibody (Fig. 9). Subsequently, we performed immunoprecipitation of cell lysates for Tat followed by Western blotting for Tip110. The results demonstrated that Tip110 was detected in the immunoprecipitation complex when Tat and Tip110 co-transfected (Fig. 9), which indicated that there was an interaction between Tip110 and Tat.

1.5 Tip110 binds to the unphosphorylated (CTDa) but not the phosphorylated (CTDo) form of the RNAPII C-terminal domain (CTD)

One of the essential events in HIV-1 LTR promoter transcriptional activation was phosphorylation of serine 2 and serine 5 of the RNA polymerase II C-terminal domain. Therefore, we investigated whether Tip110 directly interacted with RNAPII. To this end, 293T cells were transfected with pTip110.HA. The immunoprecipitation was performed for unphosphorylated form of RNAPII (RNAPIIa), serine 2 phosphorylated RNAPII (RNAPIIo-ser2) or serine 5 phorphorylated RNAPII (RNAPIIo-ser5) by 8WG16, H5 and H14, respectively, followed by Western blotting for Tip110 (Fig. 10A). The results showed that only the immunocomplex with 8WG16 had Tip110, which indicated Tip110

interacted with unphosphorylated form of RNAPII, but not with the phosphorylated form. To further characterize this interaction, immunoprecipitation was performed of the same cell lysate for Tip110 by anti-HA followed by Western blotting for the unphosphorylated or phosphorylated form of the RNAPII (Fig. 10B). The results showed that only unphosphorylated RNAPII was detected in the immunoprecipitaion complex of Tip110, indicating that exogenousTip110 only associated with unphosphorylated RNAPII. We further investigated whether endogenous Tip110 interacted with RNAPII by performing immunoprecipitation of 293T cell lysates with 8WG16, H14, and H5 followed by Western blotting against Tip110. The result demonstrated that only the 8WG16 immunocomplex contained Tip110 (Fig. 11A). This interaction was further confirmed by detecting unphosphorylated RNAPII by 8WG16 in Tip110 immunoprecipitates (Fig. 11B), indicating that endogenous Tip110 only interacted with the unphosphorylated form of RNAPII, but not its phosphorylated form.

These Western blotting results showed much less phosphorylated RNAPII band than unphosphorylated RNAPII (Fig. 10 and 11). This difference could result from different expression levels of these two forms of RNAPII or different efficiency of the antibodies for these two forms (H4, H5 and 8WG16) and could contribute to the finding that there is a complex formation between Tip110 and RNAPIIa. Thus, *in vitro* GST pull-down assay was further performed to confirm Tip110 interaction with unphosphorylated RNAPII. First, recombinant GST-CTDa protein was purified from *E.coli* and confirmed to contain RNAPII unphosphorylated C-terminal domain by Western blotting (Fig. 12A). Then, GST-CTDa was phosphorylated by casein kinase I *in vitro*. Meanwhile,

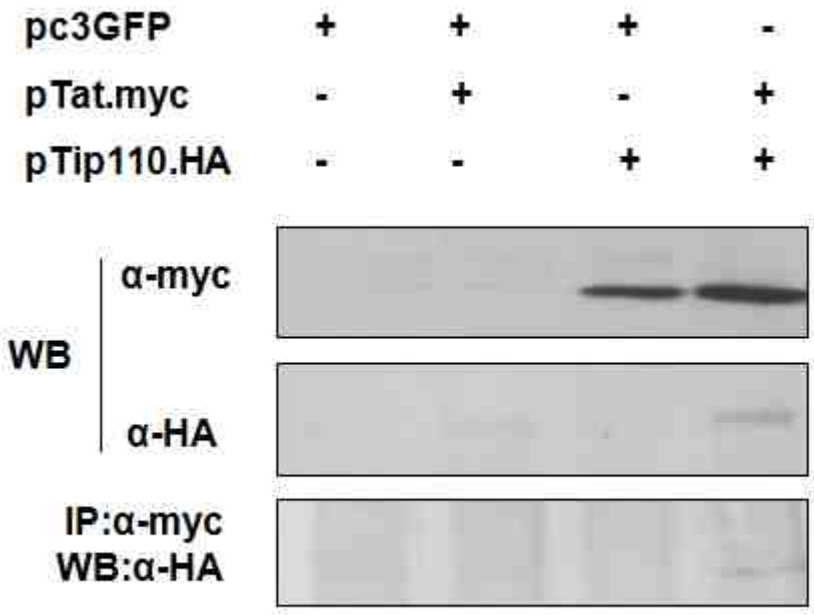
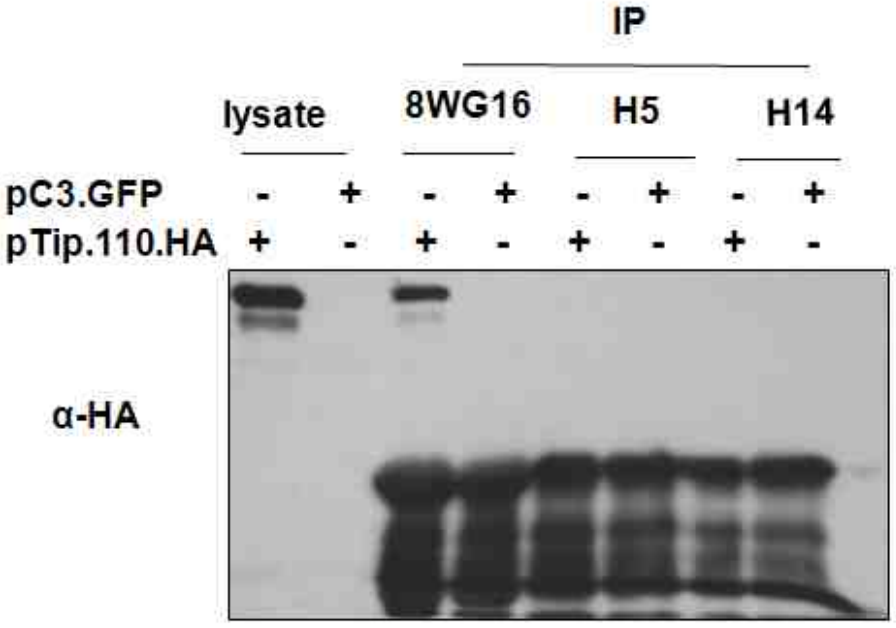
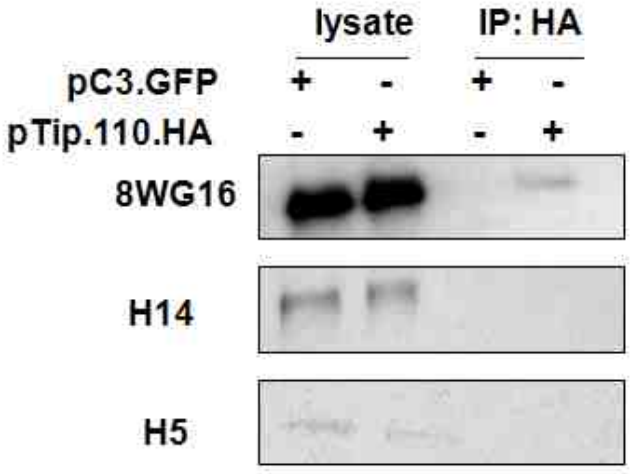

pc3GFP + + + -
pTat.myc - + - +
pTip110.HA - - + +
WB
α-myc
α-HA
IP:α-myc
WB:α-HA

Figure 9. The interaction between Tip110 and Tat 293T cells were transfected with pTip110.HA *(lane2)*, pTat.myc (*lane3*), or both (*lane 4*), and harvest 48 h after transfection for whole cell lysates. Mock transfection with pc3.GFP was also included *(lane 1).* Forty micrograms of each whole cell lysate was analyzed by Western blotting to determine protein expression, and immnoprecipitation followed by Western blotting was used to determine Tip110 binding to Tat.

A


IP
lysate
8WG16
H5
H14
pC3.GFP - + - + - + - +
pTip.110.HA + - + - + - + -
α-HA

B


lysate
IP: HA
pC3.GFP + - + -
pTip.110.HA - + - +
8WG16
H14
H5

\

Figure 10. The interaction between exogenous Tip110 and RNAPII 293T cells were transfected with pTip110.HA or pc3.GFP. After 3 days of incubation, we performed immunoprecipitation of cell lysates for unphosphorylated RNAPII (8WG16), phosphorylated RNAPII (H14 and H5), followed by Western blotting against Tip110 (A); Or immunoprecipitation against Tip110, followed by Western blotting for unphosphorylated RNAPII (8WG16) and phosphorylated RNAPII (H14 and H5) (B).

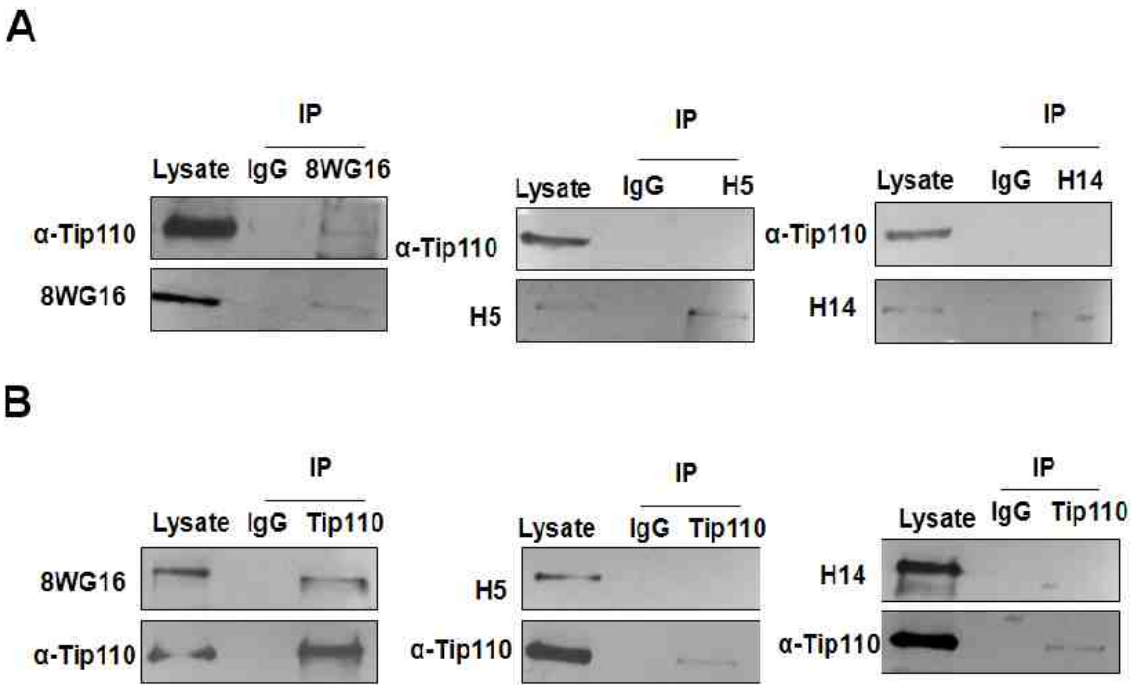

A
IP
Lysate
IgG
8WG16
α-Tip110
8WG16
IP
Lysate
IgG
H5
α-Tip110
H5
IP
Lysate
IgG
H14
α-Tip110
H14
B
IP
Lysate
IgG
Tip110
8WG16
α-Tip110
IP
Lysate
IgG
Tip110
H5
α-Tip110
IP
Lysate
IgG
Tip110
H14
α-Tip110

Figure 11. The interaction between endogenous Tip110 and RNAPII

(A) Immunoprecipitation was first performed on 293T cell lysates (500 μg) for the serine 2 or serine 5 phosphorylated RNAPII, or unphosphorylated RNAPII. These immunoprecipitates were then analyzed by Western blotting for Tip110 *(upper panel)*. Western blotting for 8WG16, H5, and H14 were also performed against their own immunoprecipitates as the control *(lower panel)*. (B) Immunoprecipitation was performed with anti-Tip110 antibody, followed by Western blotting for 8WG16, H5 and H14 *(upper panel)* or for Tip110 protein as the control *(lower panel)*.

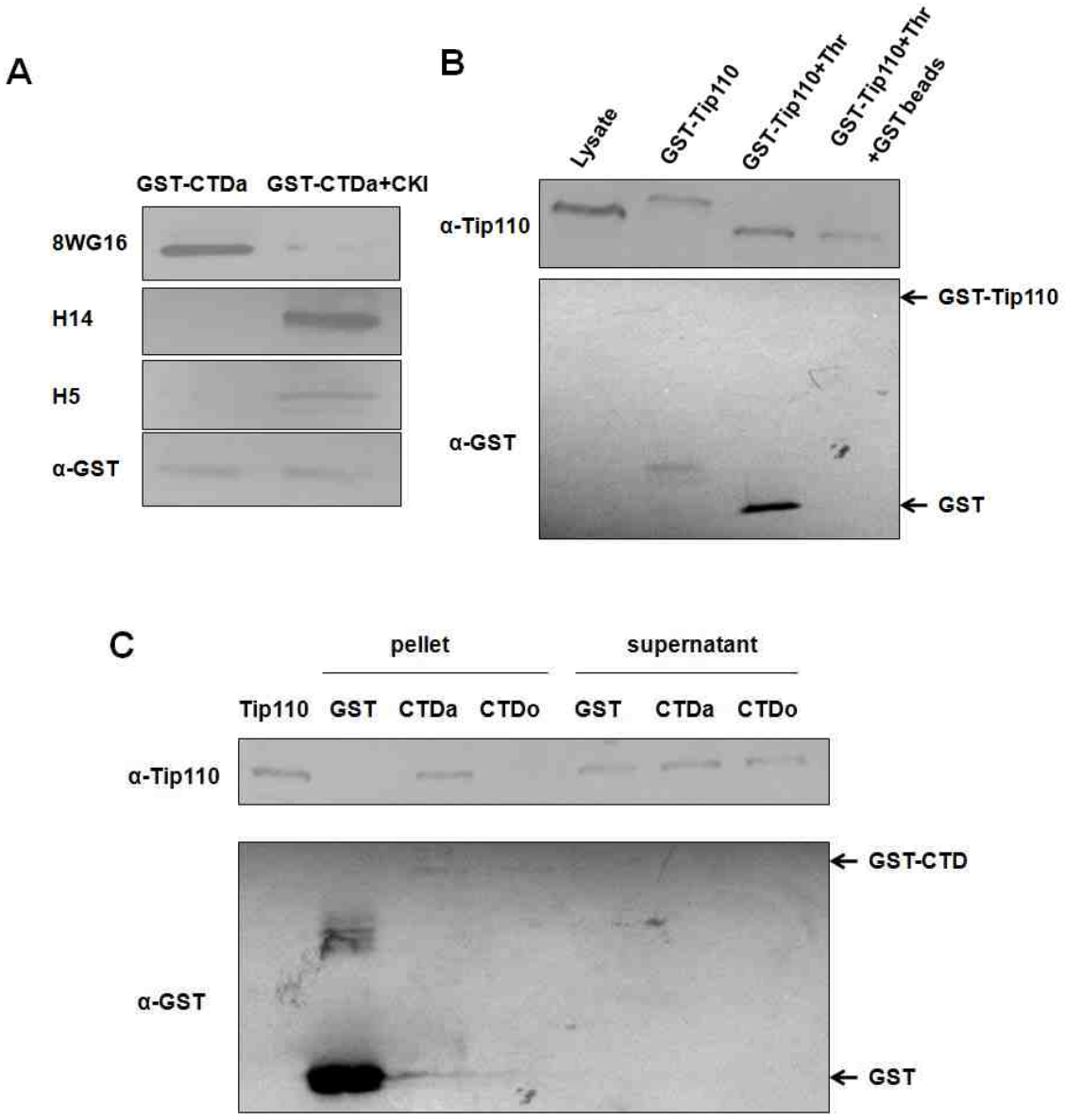

A
GST-CTDa
GST-CTDa+CKI
8WG16
H14
H5
α-GST
B
Lysate
GST-Tip110
GST-Tip110+Thr
GST-Tip110+Thr
+GST beads
α-Tip110
GST-Tip110
α-GST
GST
C
pellet
supernatant
Tip110
GST
CTDa
CTDo
GST
CTDa
CTDo
α-Tip110
GST-CTD
α-GST
GST

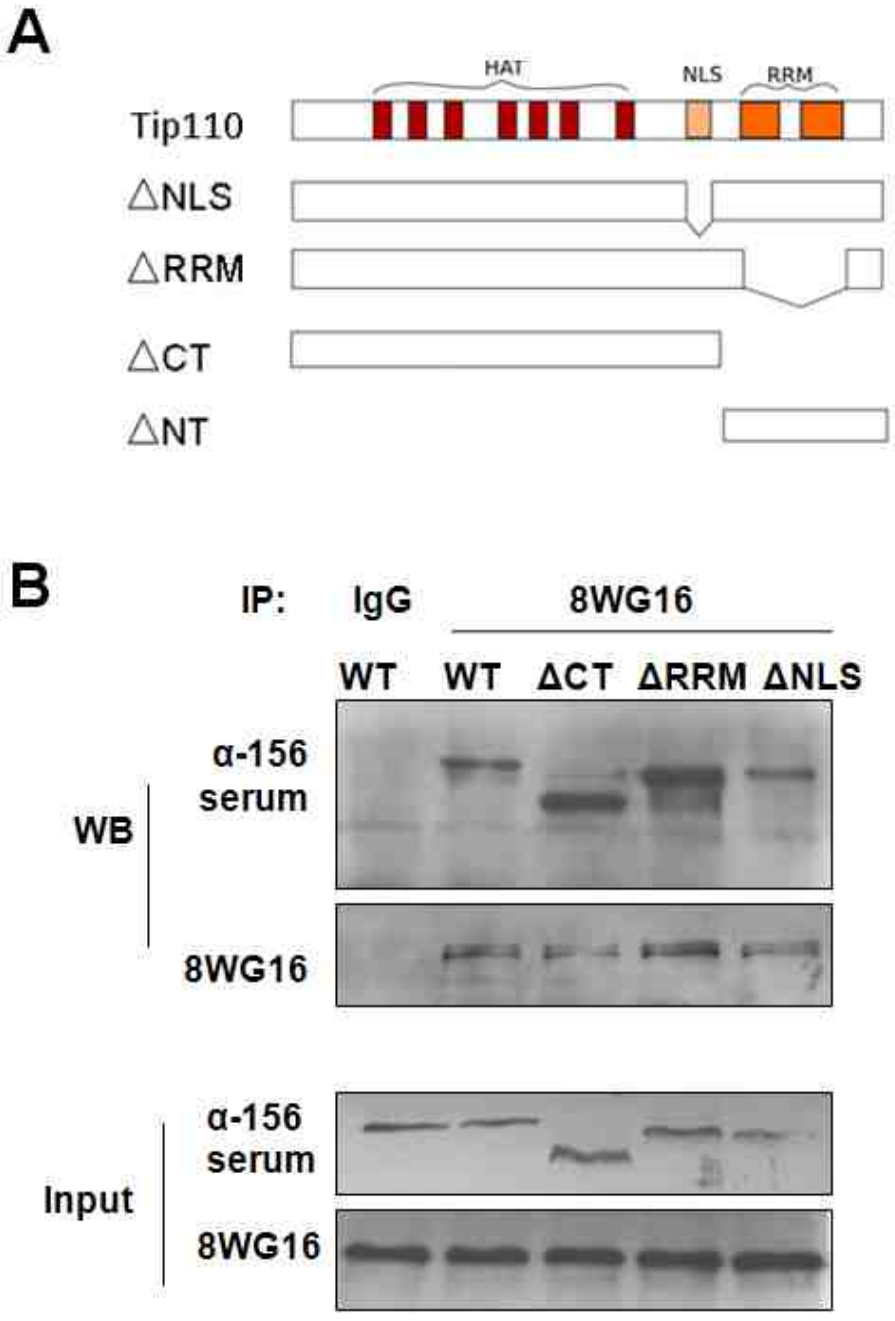
Figure 12. The interaction between Tip110 with RNAPII-CTD *in vitro*

(A) GST-CTDa protein was purified from *E.coli*, and further phosphorylated by casein kinase I. To check the phosphorylation efficiency, Western blotting was performed against 8WG16, H14 and H5. GST was added as a loading control. (B) Tip110 protein was prepared by thrombin cleavage of GST-Tip110 and then purified by glutathione beads to remove GST protein. Western blotting analysis was performed for Tip110 and GST to ensure correct expression of Tip110 and Tip110-GST fusion protein and complete GST removal from Tip110 protein. (C) Unphosphorylated or phosphorylated GST-CTD and GST protein were immobilized with 50 µl glutathione beads and incubated with purified Tip110 protein for 2 hours. After washing, the protein from the pellet fraction *(lanes 2-4)* and supernatant fraction *(lanes 5-7)* were detected by Western blotting for Tip110; Purified Tip110 protein was included as the positive control to indicate the correct protein size *(lane 1)*. The membrane was stripped and re-probed with anti-GST to ensure a comparable amount of GST-CTDa and GST-CTDo.

recombinant Tip110 was prepared by removing the GST tag from GST-Tip110 with thrombin (Fig. 12B). Then, glutathione beads coated with purified GST, GST-CTDa or GST-CTDo were incubated with recombinant Tip110 protein. Bound protein that remained on the beads as well as the protein from the supernatant fraction was analyzed by SDS-PAGE gel followed by Western blotting with Tip110 monoclonal antibody. The results showed direct binding of Tip110 to unphosphorylated CTD (Fig. 12C, *lane 3*), but not GST protein (Fig. 12C, *lane 2*) or phosphorylated CTD (Fig. 12C, *lane 4*). Taken together, this data demonstrated that Tip110 associated with unphosphorylated RNAPII CTD, but not with phosphorylated form of RNAPII at either serine 2 or serine 5.

1.6 HAT domain is important for Tip110 interaction with RNAPIIa

To determine which functional domains of Tip110 interacts with unphosphorylated RNAPII, we took advantage of a series of Tip110 mutants which contained RRM domain, C-terminal domain, NLS domain or HAT domain deletions (Fig. 13A). Due to differences in antibody epitopes, the Tip110 mutants divided into two groups for Western blotting detection:ΔRRM, ΔNLS, and ΔCT mutants were detected by anti-156 serum, and ΔNT was detected by Tip110 monoclonal antibody. 293T cells were first transfected with each of Tip110 mutants and their expression was confirmed by Western blotting (Fig. 13B & 13C). Then, immunopreciptations with 8WG16 was performed followed by Western blotting for Tip110. The mutants lacking the NLS domain, RRM domain or C-terminal domain bound to unphosphorylated RNAPII as efficiently as the full length protein (Fig. 13B). But deletion of the HAT domain abolished the Tip110-RNAPII


A
HAT
NLS
RRM
Tip110
△NLS
△RRM
△CT
△NT
B
IP:
IgG
8WG16
WT
WT
ΔCT
ΔRRM
ΔNLS
WB
α-156 serum
8WG16
Input
α-156 serum
8WG16

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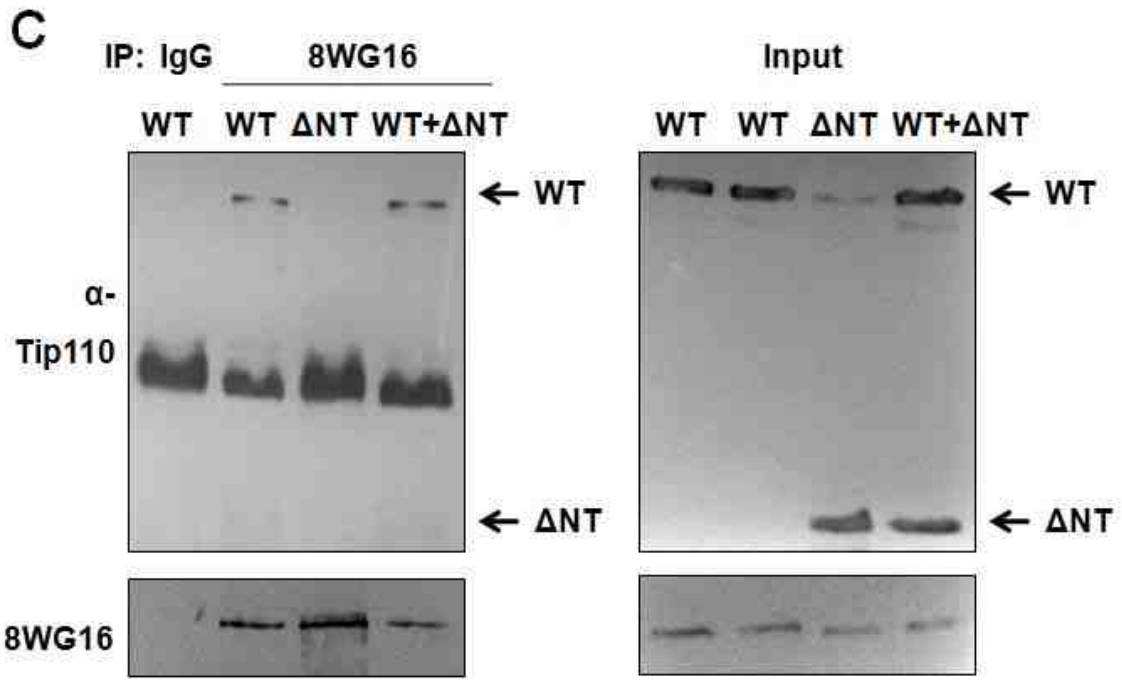

C
IP: IgG
8WG16
Input
WT
WT
ΔNT
WT+ΔNT
WT
WT
ΔNT
WT+ΔNT
α-
Tip110
← WT
← ΔNT
← WT
← ΔNT
8WG16

Figure 13. The requirement of HAT domain for Tip110 binding to RNAPII

(A) Schematic of wild-type Tip110 and its mutants. (B) 293T cells were transfected with indicated Tip110 mutants or wild type Tip110. The upper two panels were Western blotting with anti-156 serum (Tip110) or 8WG16 (CTDa) after immunoprecipitation with 8WG16 antibody. The lower two panels were Western blotting of input whole cell lysates for expression of Tip110 mutants and CTDa. (C) 293T cells were transfected with ΔNT or wild-type Tip110 and analyzed for protein expression of Tip110 or CTDa *(right two panels)*. The interaction of ΔNT or Tip110 with unphosphorylated CTD was detected by immunoprecipitation using 8WG16 followed by Western blotting for Tip110 *(left two panels)*.

complex formation (Fig. 13C), indicating that HAT domain of Tip110 was directly involved in Tip110-RNAPII interaction.

1.7 Tip110 does not interact with P-TEFb

Since Tip110 and P-TEFb were co-localized in nuclear speckle structure, we further determined if Tip110 interacted with CDK9 or cyclin T1 by immunoprecipitation and Western blotting. First, we examined whether endogenous Tip110 was part of the P-TEFb and found that neither endogenous cyclin T1 nor CDK9 was detected in anti-Tip110 immunoprecipitates (Fig. 14A). Similarly, no Tip110 protein in either anti-CDK9 or anti-cyclin T1 immunoprecipitates was detected (Fig. 14B).

To eliminate the possibility that endogenous Tip110 expression level was too low to detect the interaction, exogenous Tip110-expressing 293T cells were used for IP/WB. Immunoprecipitation followed by Western blotting demonstrated no Tip110/CDK9 or Tip110/cyclin T1 complex formation (Fig. 15A & 15B). CDK9 and cyclin T1 antibody worked, as they were capable of immunoprecipitating each other as a complex (Fig. 14B) (Wei, Garber et al. 1998). Therefore, we concluded that Tip110 did not associate with CDK9 or cyclin T1.

1.8 Tip110 interaction with Tat recruits more P-TEFb to the transcription complex

We next determined if Tip110 interaction with Tat would recruit more P-TEFb to the HIV-1 transcription complex. 293T cells were first transfected with pLTR-Luc, and pTat.myc, pTip110.HA, or both. At 48hr post transfection, Western blotting was

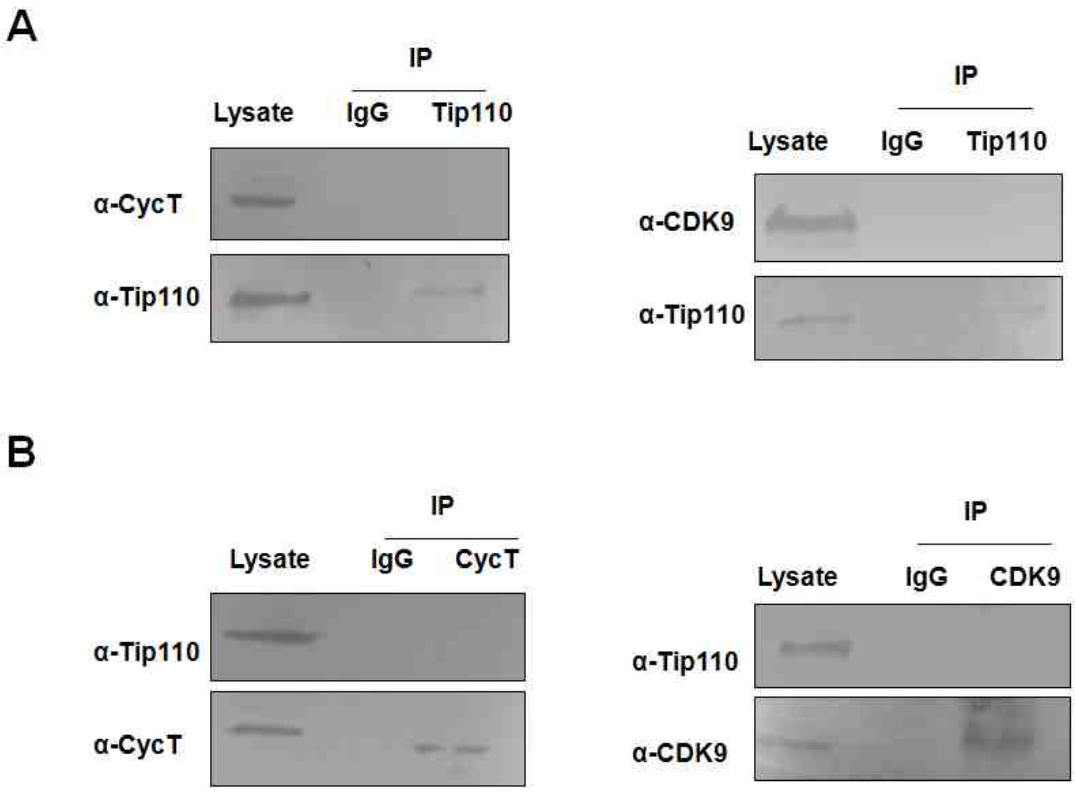

A
IP
Lysate
IgG
Tip110
α-CycT
α-Tip110
IP
Lysate
IgG
Tip110
α-CDK9
α-Tip110
B
IP
Lysate
IgG
CycT
α-Tip110
α-CycT
IP
Lysate
IgG
CDK9
α-Tip110
α-CDK9

Figure 14. No interaction between endogenous Tip110 and p-TEFb

(A) Immunoprecipitation was performed for endogenous Tip110 or mouse IgG followed by Western blotting for cyclin T1 or CDK9 *(upper panel).* Western blotting for Tip110 of Tip110 immunoprecipitates was also performed as the positive control *(lower panel).* (B) Immunoprecipitation was performed for cyclin T1 or CDK9 separately followed by Western blotting for Tip110 *(upper panel).* Western blotting for cyclin T1 or CDK9 of cyclin T1 or CDK9 immunoprecipitates was performed as the control *(lower panel).*

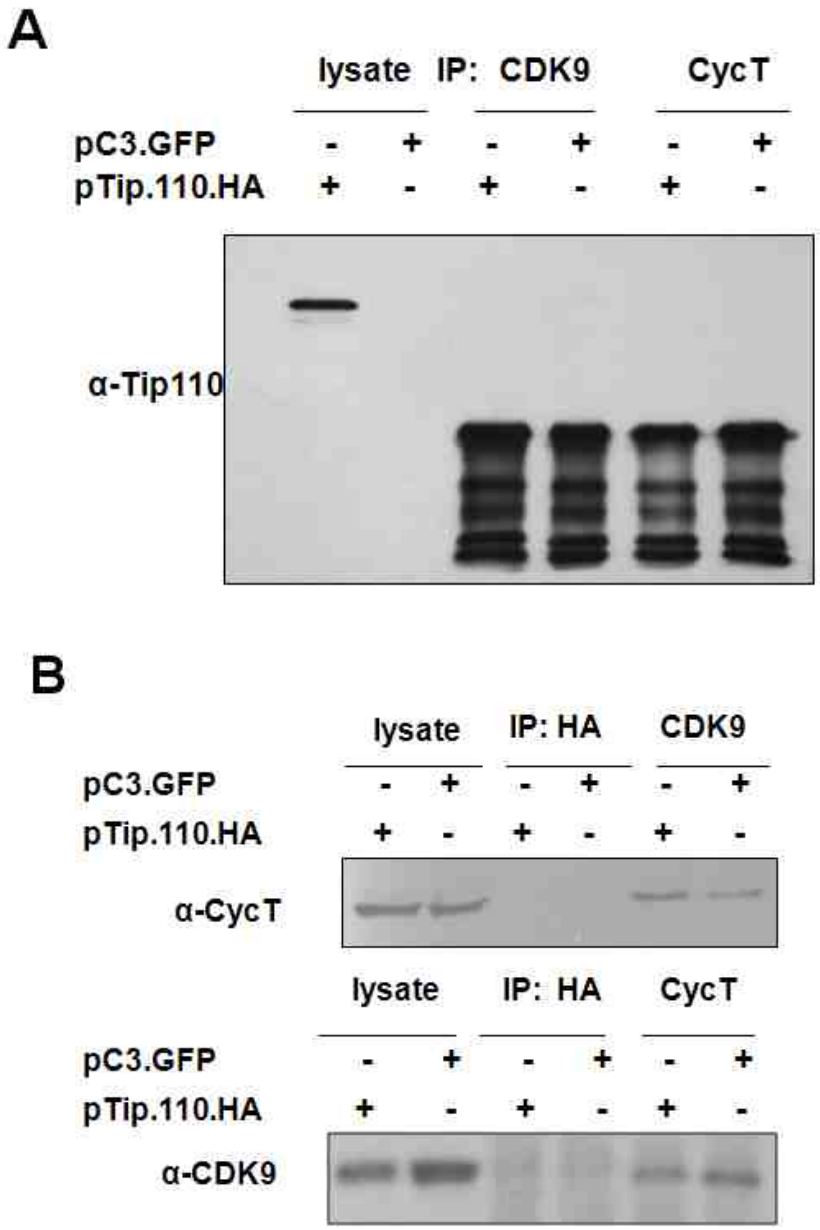
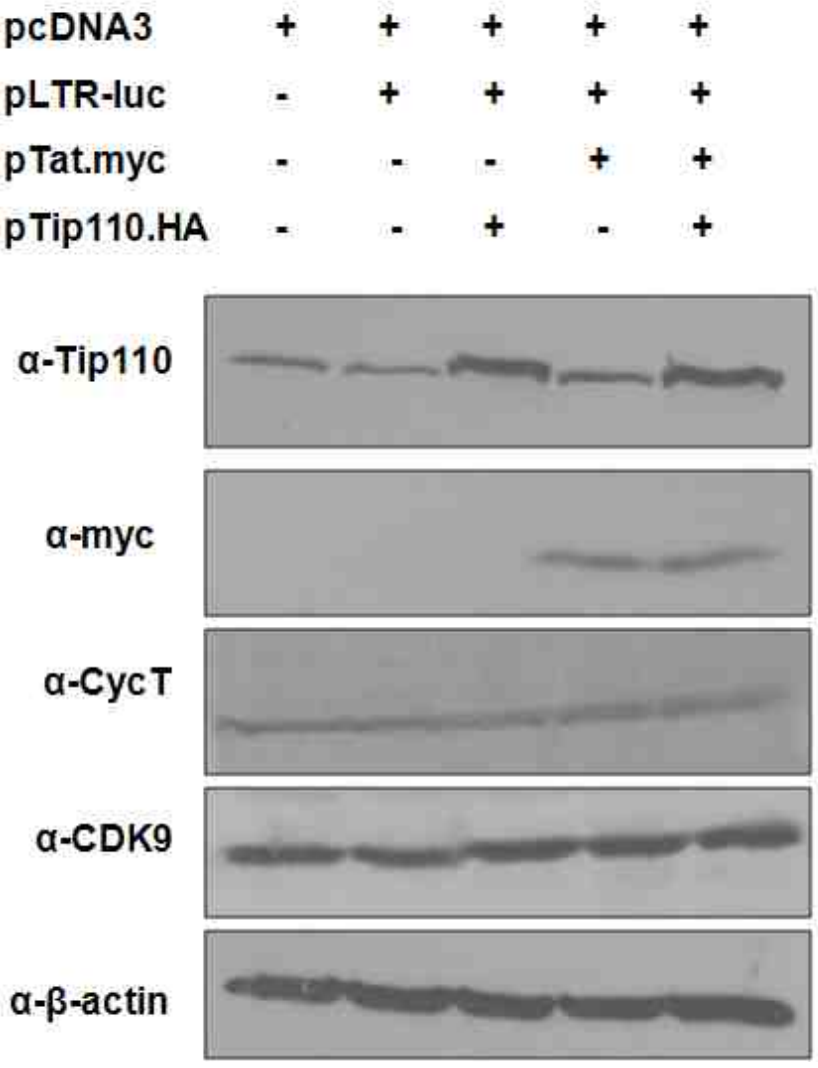

A
lysate
IP: CDK9
CycT
pC3.GFP
pTip.110.HA
α-Tip110
B
lysate
IP: HA
CDK9
pC3.GFP
pTip.110.HA
α-CycT
lysate
IP: HA
CycT
pC3.GFP
pTip.110.HA
α-CDK9

Figure 15. No interaction between exogenous Tip110 and P-TEFb 293T cells were transfected with pTip110.HA, or pc3.GFP. The whole cell lysates were obtained after 72h. (A) Western blotting for Tip110 was performed for cell lysates *(lanes 1, 2)* and anti-CDK9 *(lanes 3, 4)* or anti-cyclin T1 *(lanes 5, 6)* immunoprecipitates. (B) Immunoprecipitation was performed of cell extracts for Tip110 (α-HA) *(lanes 3, 4)* or CDK9 *(lanes 5, 6)* and followed by Western blotting for cyclin T1.Immunoprecipitation was performed for Tip110 (α-HA) *(lanes 3, 4)* or cyclin T1*(lanes 5, 6)* and then Western blotting for CDK9.

A


pcDNA3 + + + + +
pLTR-luc - + + + +
pTat.myc - - - + +
pTip110.HA - - + - +
α-Tip110
α-myc
α-CycT
α-CDK9
α-β-actin

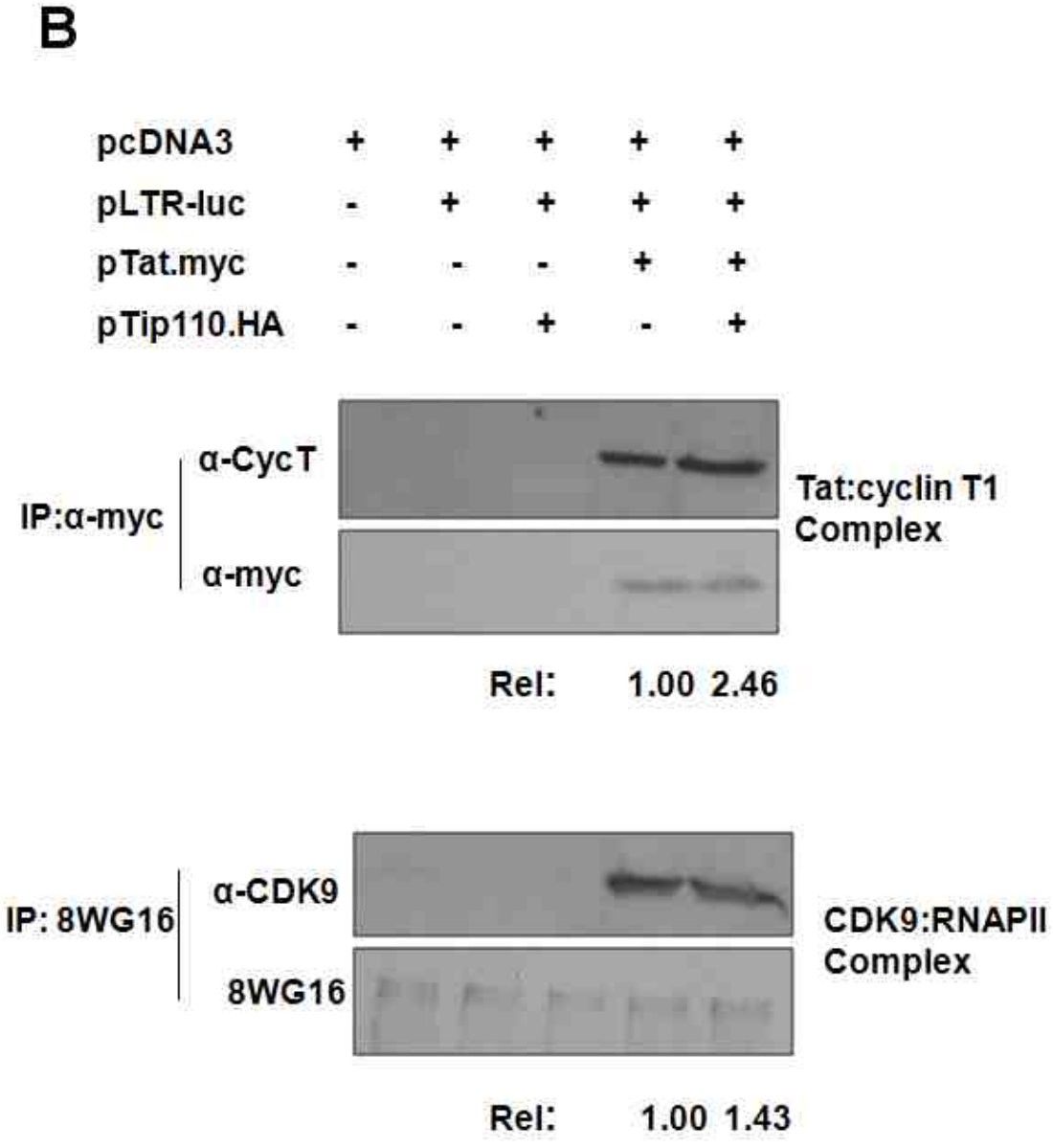

B
pcDNA3 + + + + +
pLTR-luc - + + + +
pTat.myc - - - + +
pTip110.HA - - + - +
IP:α-myc
α-CycT
α-myc
Tat:cyclin T1 Complex
Rel: 1.00 2.46
IP: 8WG16
α-CDK9
8WG16
CDK9:RNAPII Complex
Rel: 1.00 1.43

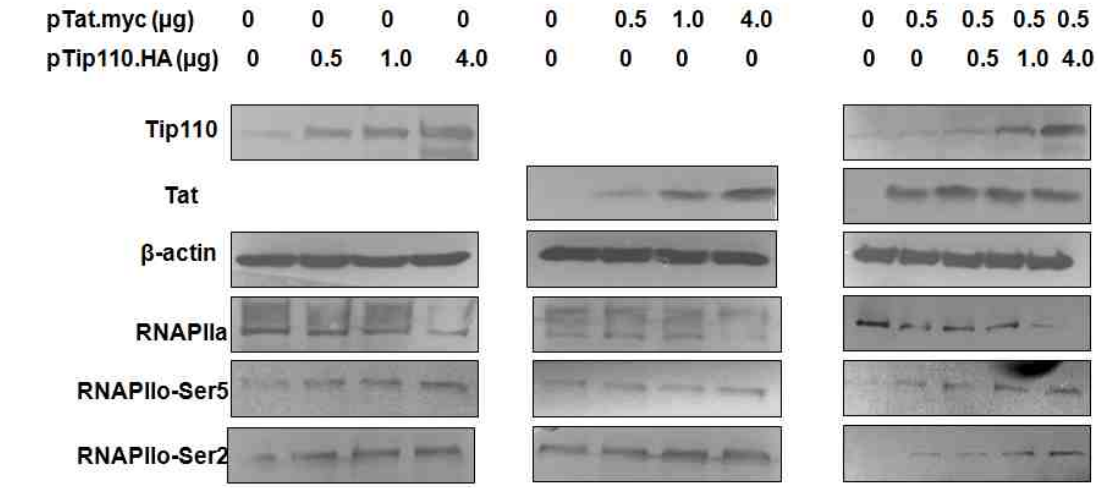
Figure 16.The recruitment of P-TEFb to the transcription complex by Tip110 and Tat (A) 293T cells were transfected with pLTR-luc *(lane 2),* pTip110.HA with pLTR-luc *(lane 3),* pTat.myc with pLTR-luc *(lane 4),* or pLTR-luc with pTip110.HAand pTat.myc *(lane 5).* Mock transfection with pcDNA3 *(lane 1)* was also included as a control. The expression of Tip110, Tat, cyclin T1, CDK9 and β-actin were detected by Western blotting. (B) The binding of cyclin T1 with Tat was determined by immunoprecipitation with c-myc (Tat) followed by Western blotting for cyclin T1, or anti-myc as the control *(upper two lanes).* The interaction of CDK9 with RNAPII was determined by Western blotting for CDK9 of 8WG16 immunoprecipitates. Western blotting for 8WG16 was also performed for 8WG16 immunoprecipitates as the control *(lower two lanes).*

performed to detect Tip110, Tat, CDK9 and cyclin T1 expression, demonstrating neither Tip110 nor Tat expression altered CDK9 and cyclin T1 expression (Fig. 16A). Then, we performed immunoprecipitation for Tat and Western blotting for cyclin T1. As we expected, cyclin T1 was detected in Tat immunoprecipitates when Tat overexpressed (Fig. 16B, *upper two panels*). But Tip110 increased cyclin T1 in Tat immunoprecipitates by more than 2 fold. Tip110 also increased unphosphorylated RNAPII-associated CDK9by about 1.4 fold, as shown by immunoprecipitation against 8WG16 followed by Western blotting for CDK9 (Fig. 16B, *bottom two panels*). These results indicated that Tip110 interaction with Tat recruited more P-TEFb to the transcriptional complex.

1.9 Tip110 and Tat enhance RNAPII phosphorylation

The most critical step during HIV-1 LTR promoter transactivation is the phosphorylation of RNAPII (Zhu, Pe'ery et al. 1997; Kim, Bourgeois et al. 2006). CDK7 phosphorylates serine 5 of RNAPII for the transcription initiation; whileCDK9 phosphorylates serine 2 for transcription elongation (Wei, Garber et al. 1998; Chen and Zhou 1999). Since Tip110 could recruit more P-TEFb to the transcription complex, we next determined the effects of Tip110 and Tat expression on RNAPII phosphorylation. We transfected 293T cells with increasing amounts of pTip110.HA, pTat.Myc, or a constant amount of pTat.Myc and increasing amounts of pTip110.HA. After 48 hours, Western blotting analysis was performed against unphosphorylated form of RNAPII (RNAPIIa), serine 2 phosphorylated RNAPII (RNAPIIo-ser2) or serine 5 phorphorylated RNAPII (RNAPIIo-ser5) by 8WG16, H5 and H14, respectively (Fig. 17A). When Tip110 was

A


pTat.myc (μg) 0 0 0 0 0 0.5 1.0 4.0 0 0.5 0.5 0.5 0.5
pTip110.HA (μg) 0 0.5 1.0 4.0 0 0 0 0 0 0 0.5 1.0 4.0
Tip110
Tat
β-actin
RNAPIIa
RNAPIIo-Ser5
RNAPIIo-Ser2

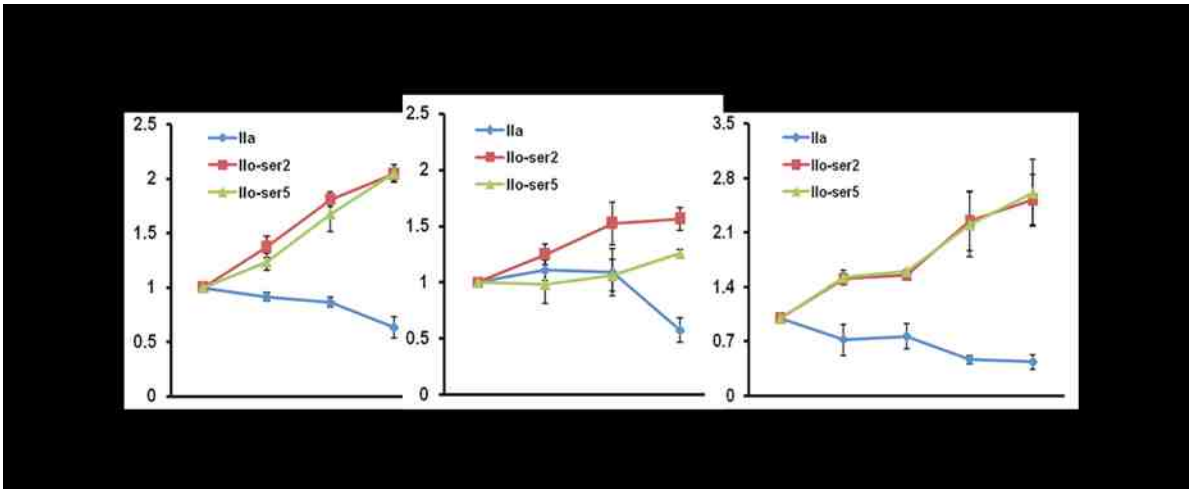

IIa
IIo-ser2
IIo-ser5
2.5
2
1.5
1
0.5
0
3.5
2.8
2.1
1.4
0.7

Figure 17. Enhancement of RNAPII CTD phosphorylation by Tip110 and Tat

(A) 293T cells were transfected with increasing amounts of pTip110.HA (0, 0.5, 1.0, 4.0 μg), increasing amount of pTat.myc (0, 0.5, 1.0, 4.0 μg), or a constant amount of pTat.myc (0.5 μg) with increasing amounts of pTip110.HA (0, 0.5, 1.0, 4.0 μg). pcDNA3 was added to equalize the total amounts of DNA transfected. Cells were cultured for 72h and then harvested for whole cell lysates. Samples were first analyzed by Western blotting for Tip110 or Tat. Then, unphosphorylated RNAPII (IIa), and serine2 (IIo-ser2) and serine5 (IIo-ser5) phosphorylated RNAPII levels were determined by antibody 8WG16, H5, H14, respectively. (B) Quantification of Western blot densitometry data from three independent experiments.

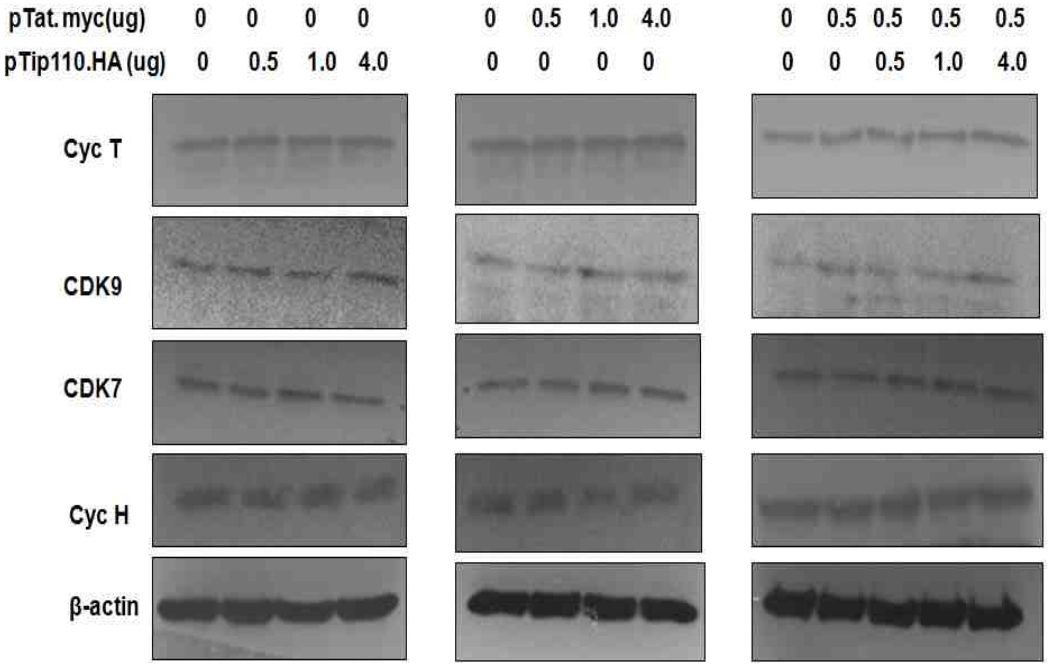

pTat.myc(ug) 0 0 0 0 0 0.5 1.0 4.0 0 0.5 0.5 0.5 0.5
pTip110.HA (ug) 0 0.5 1.0 4.0 0 0 0 0 0 0 0.5 1.0 4.0
Cyc T
CDK9
CDK7
Cyc H
β-actin

Figure 18. TFIIH and P-TEFb expression inTip110 -and Tat-expressing cells

293T cells were transfected with increasing amounts of pTip110.HA (0, 0.5, 1.0, 4.0 μg), increasing amount of pTat.myc (0, 0.5, 1.0, 4.0 μg), or a constant amount of pTat.myc (0.5 μg) with increasing amounts of pTip110.HA (0, 0.5, 1.0, 4.0 μg). pcDNA3 was added to equalize the total amounts of DNA transfected. Cells were harvested after 72 hours and analyzed by Western blotting against anti-CDK7, anti-cyclin H, anti-CDK9, anti-cyclin T1 and anti-β-action as the loading control.

expressed, both RNAPIIo-ser2 andRNAPIIo-ser5 were increased by 2 fold and RNAPIIa was decreased (Fig. 17B, *left panel*). In Tat expressing cells, RNAPIIa was slightly reduced, RNAPIIo-ser5 showed little change, but RNAPIIo-ser2 was increased by 1.5 fold (Fig. 17B, *middle panel*). When Tat and Tip110 were co-expressed, RNAPIIo-ser2 and RNAPIIo-ser5 both increased by 3 fold, with RNAPIIa was decreased by 0.3 fold (Fig. 17B, *right panel*). These results indicated that Tip110 together with Tat could enhance the phosphorylation of RNAPII at both serine 2 and serine 5, which may accounted for the Tip110 function in transcription activation.

1.10 Neither Tip110 nor Tat protein alters P-TEFb or TFIIH expression level

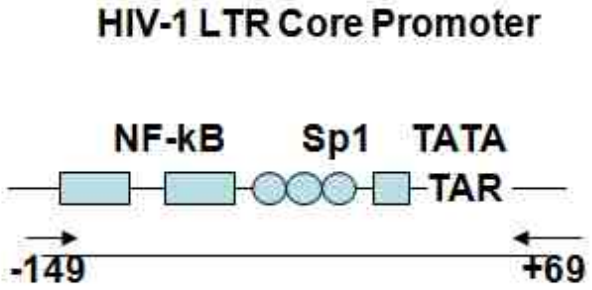
P-TEFb and TFIIH phosphorylate RNAPII (Wei, Garber et al. 1998; Chen and Zhou 1999). Thus, we further investigated whether increased RNAPIIo was due to the up-regulation of P-TEFb or TFIIH expression by Tip110 and Tat expression. To this end, 293T cells were transfected with increasing amounts of pTip110.HA, pTat.Myc, or a constant amount of pTat.Myc and increasing amounts of pTip110.HA. Then, Western blotting was performed against CDK9, cyclin T1, CDK7 and cyclin H (Fig. 18). The results demonstrated that Tip110, Tat or both did not significantly alter expression of these four proteins, indicating that increased RNAPII phosphorylation by Tip110 or Tat was not due to any changes in the expression levels of P-TEFb or TFIIH components.

1.11 Tip110 is detected at the HIV-1 LTR core promoter

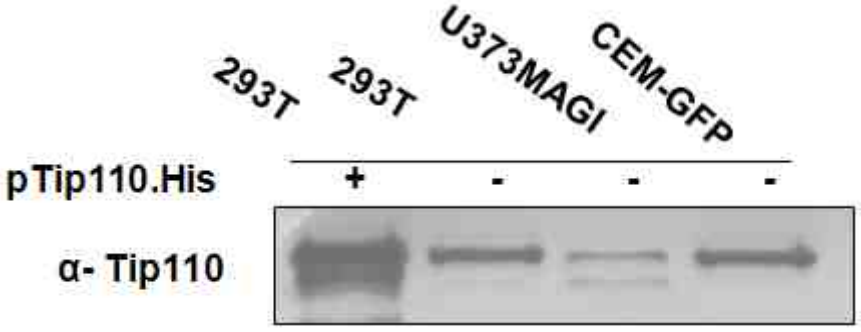
To determine whether Tip110 was recruited to the HIV-1 LTR promoter *in vivo*, ChIP experiments were carried out in exogenous Tip110-expressing 293T cells, or with

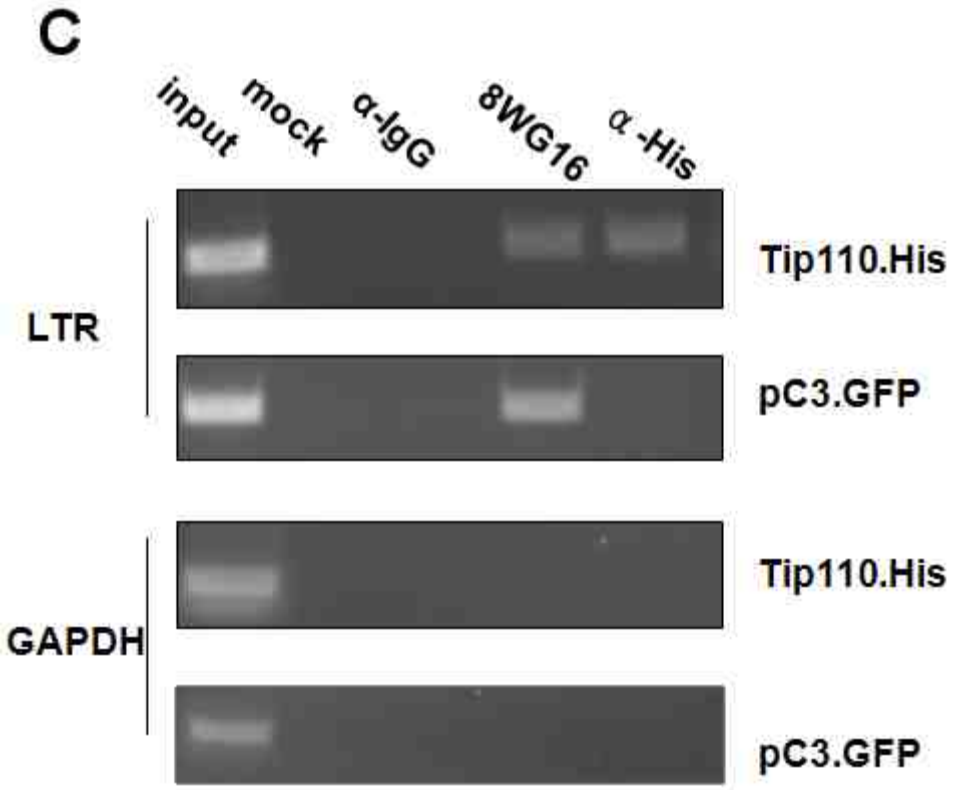
endogenous Tip110 in U373-MAGI and CEM-GFP cells. The HIV-1 LTR core promoter and the primers for the ChIP assay were showed in Fig. 19A. For ChIP assay with exogenous Tip110, 293T were transfected cells with pNL4-3 proviral plasmid and pTip110.His plasmid, and the overexpression of Tip110 was confirmed after 3 days (Fig. 19B, *lanes 1, 2*). Chromatin from cells was isolated, immunoprecipitated with anti-mouse IgG, 8WG16 or anti-His antibody followed by PCR to amplify the HIV-1 LTR core promoter region. As a control for ChIP experiment, we amplified LTR promoter in the RNAPII immunocomplex (Fig. 19C, *lane 4*) and LTR promoter could be also amplified in the Tip110 immunocomplex, indicating Tip110 was present at the HIV-1 LTR promoter *in vivo* (Fig. 19C, *lane 5*). As negative a control, mouse IgG was not detected at the LTR promoter (Fig. 19C, *lane 3*) and neither RNAPII nor Tip110 were detected on GAPDH coding region (Fig. 19C, *lower panel*), which served as a PCR control. Thus, we concluded that exogenous Tip110 was recruited to the transient LTR promoter. To determine if endogenous Tip110 was present in the integrated HIV-1 promoter, we took advantage of CEM-GFP cells that have an integrated HIV-1 LTR promoter-driven GFP transgene cassette and U373MAGI cells that have an integrated HIV-1 LTR promoter-driven Lac Z transgene cassette. Western blotting was first performed to confirm expression of endogenous Tip110 in these two cells (Fig. 19B, *lane 3, 4*). Chromatin was isolated from these cells, immunoprecipitated with mouse IgG, 8WG16 or Tip110 monoclonal antibody, and subjected to PCR for the HIV-1 LTR core regions or GAPDH. The result showed that positive control RNAPII and endogenous

A


HIV-1 LTR Core Promoter
NF-kB
Sp1
TATA
TAR
-149
+69

B


293T
293T
U373MAGI
CEM-GFP
pTip110.His
+
-
-
-
α- Tip110


C
input
mock
α-IgG
8WG16
α -His
LTR
Tip110.His
pC3.GFP
GAPDH
Tip110.His
pC3.GFP

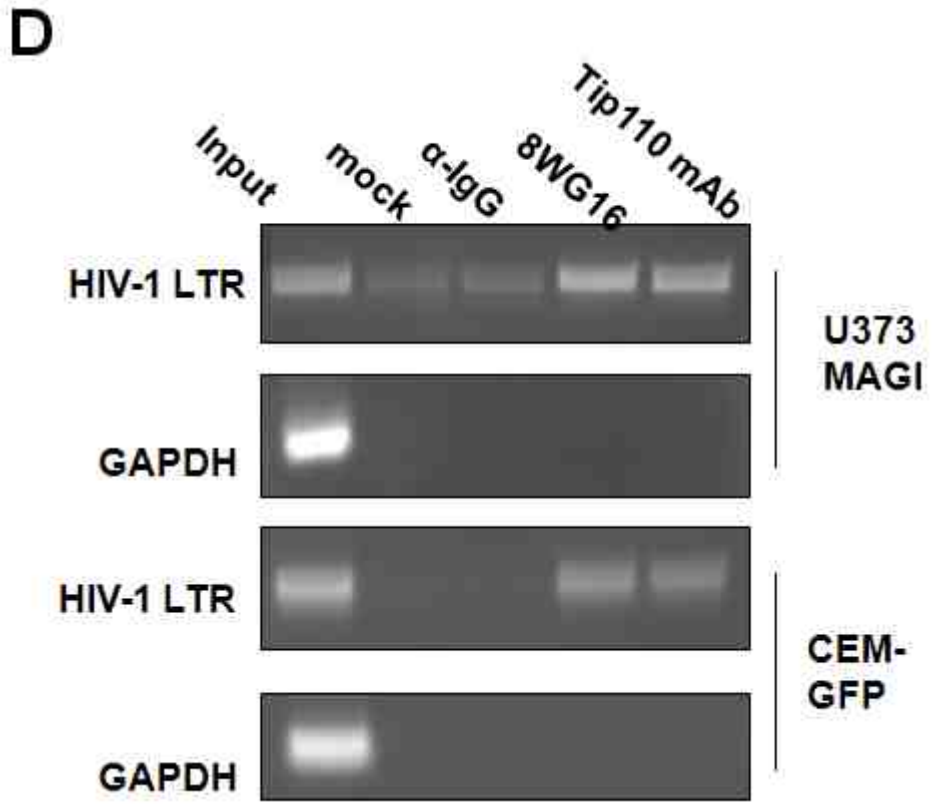

D
Input
mock
α-IgG
8WG16
Tip110 mAb
HIV-1 LTR
GAPDH
U373
MAGI
HIV-1 LTR
GAPDH
CEM-
GFP

Figure 19. The complex formation between Tip110 and HIV-1 LTR core promoter

(A) Schematic of the LTR core promoter and expected primer sites for LTR PCR amplification. (B) Western blot analysis for exogenous Tip110 expression in 293T cells *(lane 1)*, and endogenous Tip110 expression in 293T *(lane 2)*, U373MAGI *(lane 3)*, and CEM-GFP cells *(lane 4)*. (C) 293T cells were transfected with the indicated plasmid. At 72h post-transfection, chromatin from these cells was cross-linked, sheared, and immunoprecipitated with the indicated antibodies. After reversing the cross-linking, DNA was purified and analyzed by PCR with primer sets specific for the HIV-1 LTR promoter region *(upper two panel)*, with the GAPDH coding region *(lower two panels)* included as the negative control. (D) ChIP experiment was performed as described above with endogenous Tip110 expressed in U373MAGI cells *(upper two panels)* and CEM-GFP cells *(lower two panels)*.

Tip110 were detected at the LTR promoter in both CEM-GFP and U373- MAGI cells (Fig. 19D, *lane 4, 5*), indicating Tip110 was detected at the HIV-1 LTR core promoter *in vivo*.

1.12 Tip110 recruits more P-TEFb to the HIV-1 LTR promoter

We then evaluated the recruitment of the Tat-induced elongation complex to the LTR core promoter in the presence of Tip110. 293T cells were first transfected with pLTR-Luc, and pTip110.HA, pTat.myc, or both. Then, ChIP assay was performed with immunoprecipitation against Tat, Tip110, cyclin T1, CDK9, unphosphorylated RNAPII, and serine 2 or serine 5 phosphorylated RNAPII followed by PCR amplifying HIV-1 LTR core region (Fig. 20A). As positive controls, both Tat and Tip110 protein could be detected at the LTR core region. No CDK9 or cyclin T1 was detected at the LTR promoter in the absence of Tat; but with Tip110 over-expression, there are significant increases in the levels of cyclin T1 and CDK9 recruited to the LTR promoter. Meanwhile, serine 2 RNAPII phosphorylation was only observed in the LTR promoter with Tat expression and showed about 2 folds increase in the presence of Tip110, while serine 5 RNAPII phosphorylation could be detected without Tat expression and showed little change in the presence of Tip110 and Tat. As we expected, neither P-TEFb nor RNAPII could be detected at the LTR promoter when immunoprecipitation was performed with mouse IgG control. As a negative control, PCR for GAPDH coding region showed low level of RNAPII detected on the GAPDH region (Fig. 20B). These data together suggested that the Tip110 interaction with Tat was capable of recruiting

increased P-TEFb to the HIV-1 LTR promoter, and further enhanced RNAPII serine 2 phosphorylation at the LTR core promoter.

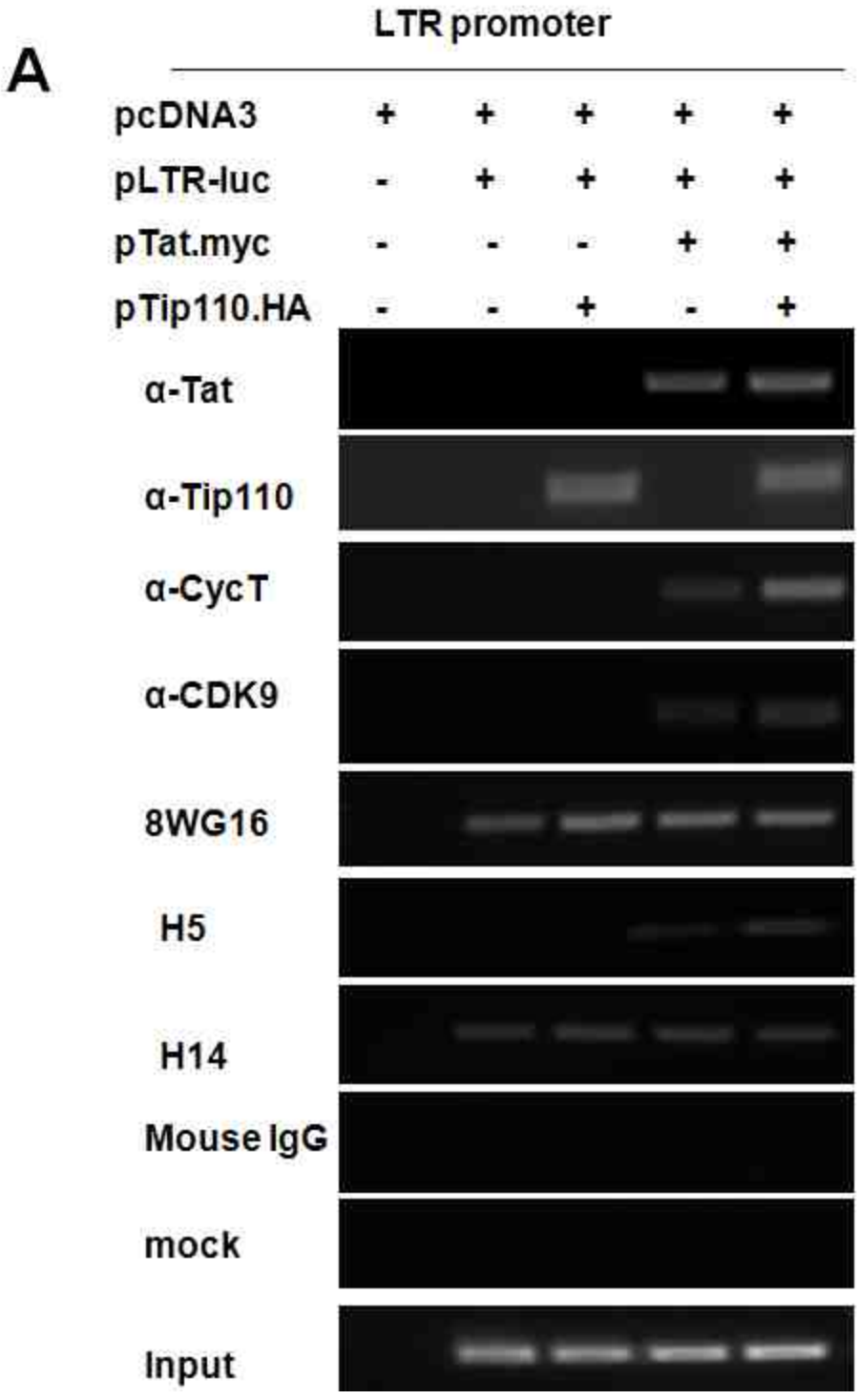
1.13 Tip110 and Tat increase the LTR transcription elongation

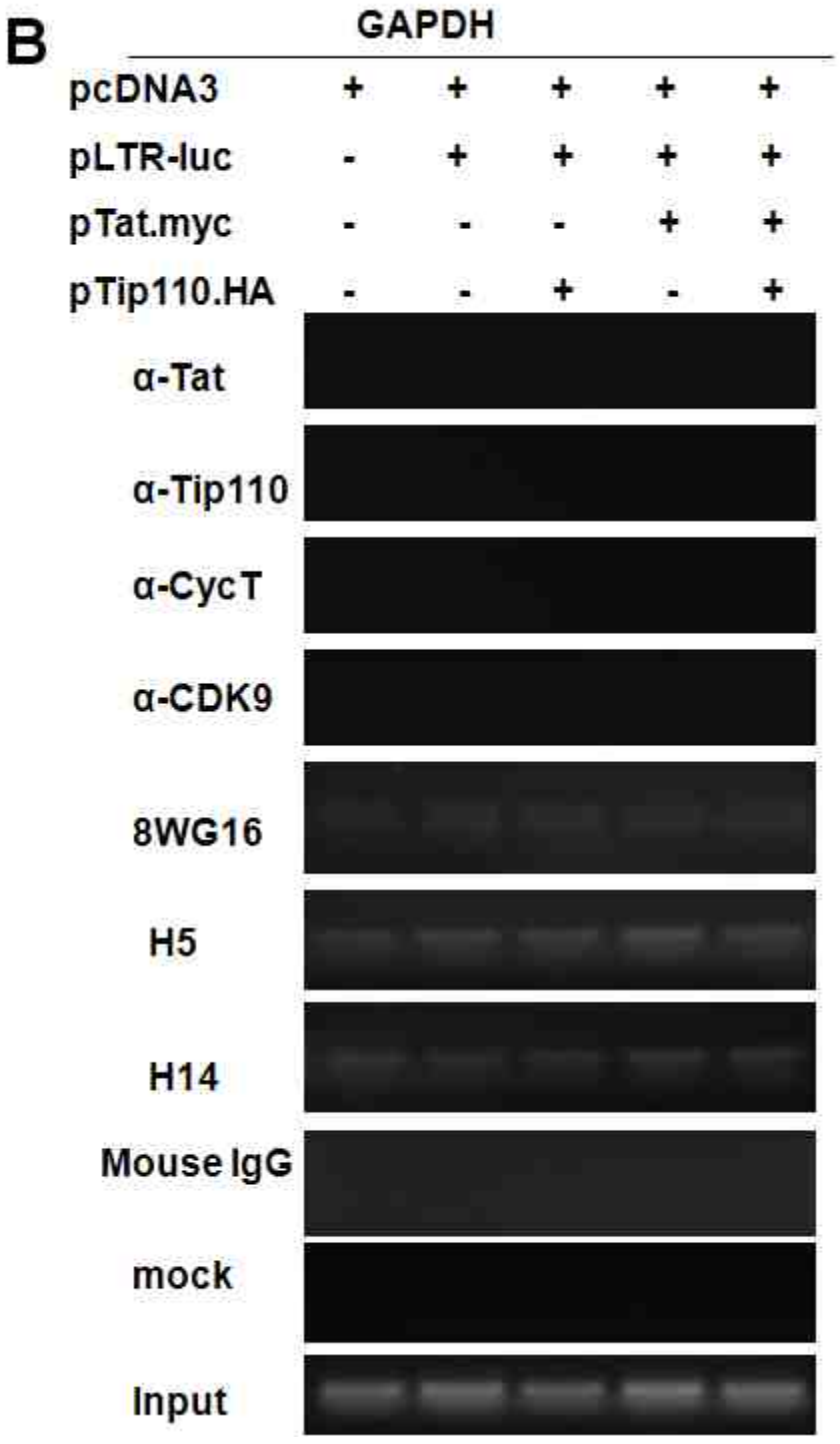
To determine if Tip110 interaction with Tat had direct effects on HIV-1 transcription elongation, we performed *in vitro* G-less transcription/elongation assay with some modifications. The template pHIV-dG-less contains two G-free cassettes downstream from the HIV-1 LTR promoter, its short (promoter-proximal) and long (promoter-distal) transcripts are RnaseT1 resistant. This template has been used to determine transcription initiation and elongation respectively (Fig. 21A) (Montanuy, Torremocha et al. 2008). Following *in vitro* transcription of the G-less DNA template, we treated the reaction with DNase I and then performed RT-PCR to determine the relative levels of short and long G-less transcripts using two pairs of primers that were specific for each of those two transcripts. These two pairs of primers were expected to give rise to about 300bp and 520 bp PCR products, respectively. Recombinant Tat or Tip110 proteins were included in the reaction. As expected, Tat increased the long transcript level but had little effect on the short transcript level (Fig. 21B). Compared to Tat, Tip110 also slightly increased the long transcript level and had little effects on the short transcript level. Compared to Tat or Tip110, the presence of both proteins further increased the long transcript level and had no effect on the short transcript level. These results indicated that Tip110 interaction with Tat increased the elongation efficiency of RNAPII complexes formed on the LTR promoter.

To further ascertain the significance of Tip110 protein in Tat-mediated LTR transcription elongation, we determined if depletion of endogenous Tip110 would affect Tat-activated LTR transcription. To achieve this, we first immunodepleted Tip110 from nuclear extract by anti-Tip110 and saved the supernatant fractions from the depleted extracts. Immunodepletion with IgG was included as a control. Western blotting confirmed that immunodepletion with anti-Tip110 antibody resulted in more than 70% Tip110 from the Nuclear extract (Fig. 22A). Then, the *in vitro* G-less assay was carried out with Tip110-depleted nuclear extract. Tip110 depletion reduced the HIV-1 LTR derived long transcripts but had minimal effect on short transcripts, indicating that Tip110 is critical for RNAPII transcription elongation (Fig. 22B, *lane 1, 2*). However, when Tip110 was added back to the Tip110-depleted nuclear extract, the transcription elongation level was not recovered. The inability of recombinant Tip110 to restore the transcription elongation is likely due to the depletion of other transcription factors associated with Tip110 from the nuclear extract, such as RNAPII (Fig. 22B, *lane 3*).

To overcome this problem, we decided to knock down endogenous Tip110 by Tip110-specific shRNA and performed the *in vivo* G-less assay in Tat-expressing cells. We transfected pHIV-dG-less plasmid with pTat.myc into 293T cells on day 0, 4 or day 7 following pshTip110 transfection and harvested cells at day 7 or day 10 for Western blotting or RNA isolation and RT-PCR. The pshTip110 backbone vector pSIREN was included as a control. Western blotting demonstrated that endogenous Tip110 protein expression was considerably knocked down by pshTip110in day 7 after transfection and slightly recovered at day 10 (Fig. 22C). In contrast, Tip110 expression showed little

changes in pSIREN transfection. In parallel with Tip110 expression, RT-PCR analysis showed that Tat-induced long transcripts synthesized from LTR decreased at day 7 and began to increase at day 10 (Fig. 22D). pshTip110 transfection showed no changes in short transcripts. pSIREN had no changes in either long or short transcripts. Taken together, these results demonstrated a specific role of Tip110 in RNAPII elongation on the LTR promoter.


A
LTR promoter
pcDNA3 + + + + +
pLTR-luc - + + + +
pTat.myc - - - + +
pTip110.HA - - + - +
α-Tat
α-Tip110
α-CycT
α-CDK9
8WG16
H5
H14
Mouse IgG
mock
Input


B
GAPDH
pcDNA3 + + + + +
pLTR-luc - + + + +
pTat.myc - - - + +
pTip110.HA - - + - +
α-Tat
α-Tip110
α-CycT
α-CDK9
8WG16
H5
H14
Mouse IgG
mock
Input

C

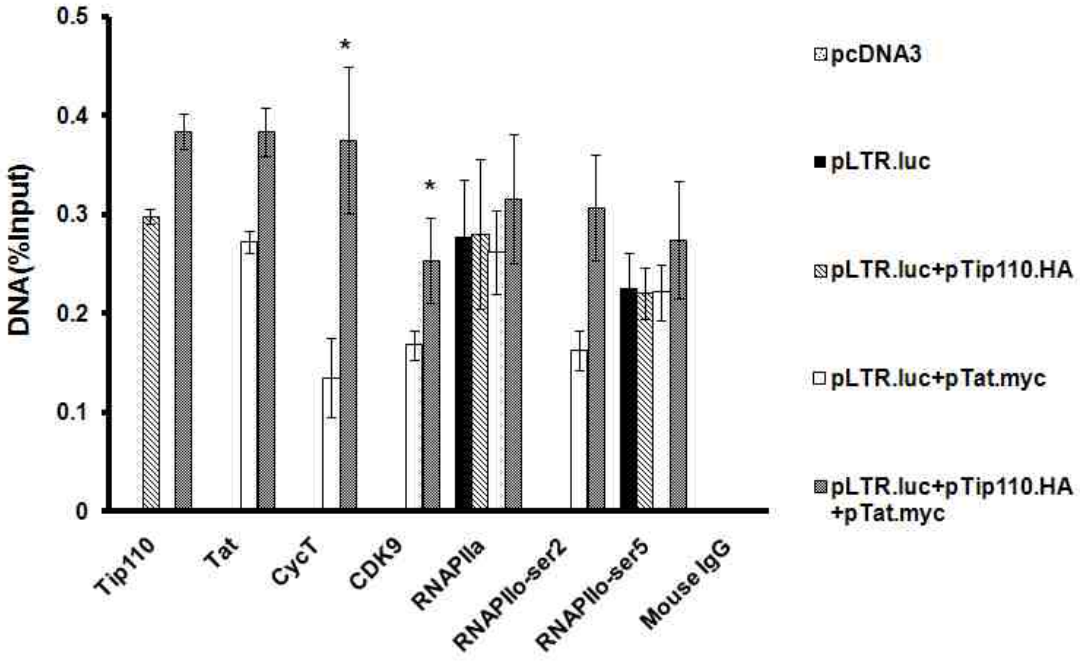
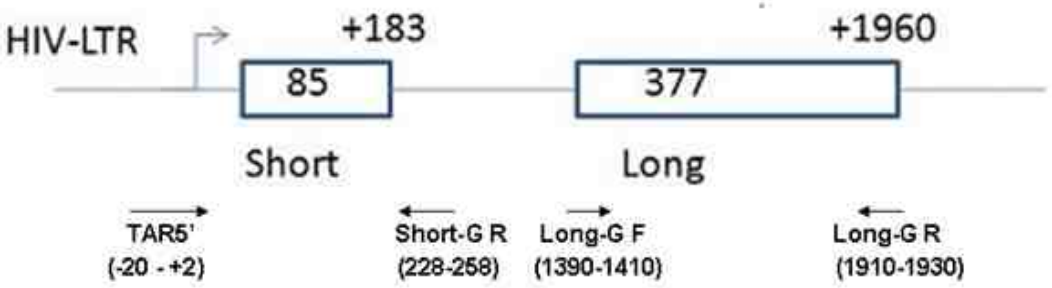

0.5
0.4
0.3
0.2
0.1
0
DNA(%Input)
*
*
Tip110
Tat
CycT
CDK9
RNAPIIa
RNAPIIo-ser2
RNAPIIo-ser5
Mouse IgG
pcDNA3
pLTR.luc
pLTR.luc+pTip110.HA
pLTR.luc+pTat.myc
pLTR.luc+pTip110.HA +pTat.myc

Figure 20. The recruitment of P-TEFb to the LTR core promoter by Tip110 and Tat

293T cells were transfected with the indicated plasmids. At 72h post-transfection, chromatin from these cells was cross-linked, sheared, and immunoprecipitated with the indicated antibodies. After reverse cross-linking and phenol extraction, the purified DNA was amplified with primer sets for the HIV-1 LTR promoter (A) or GAPDH primer (B) as control. (C) PCR products for LTR core promoter were quantitated by densitometry and expressed as fold increase over the input control. The data were representative of three independent experiments. *: $p<0.05$, **: $p<0.01$.

A


HIV-LTR
+183
+1960
85
377
Short
Long
TAR5'
(-20 - +2)
Short-G R
(228-258)
Long-G F
(1390-1410)
Long-G R
(1910-1930)

B

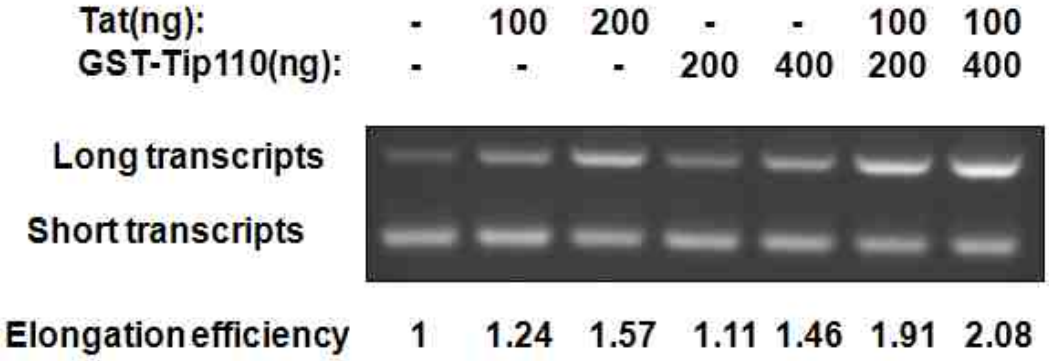
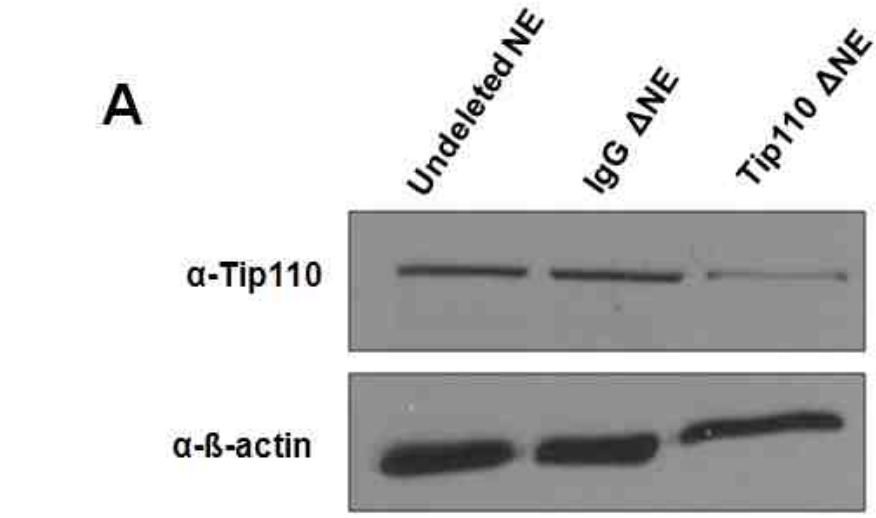
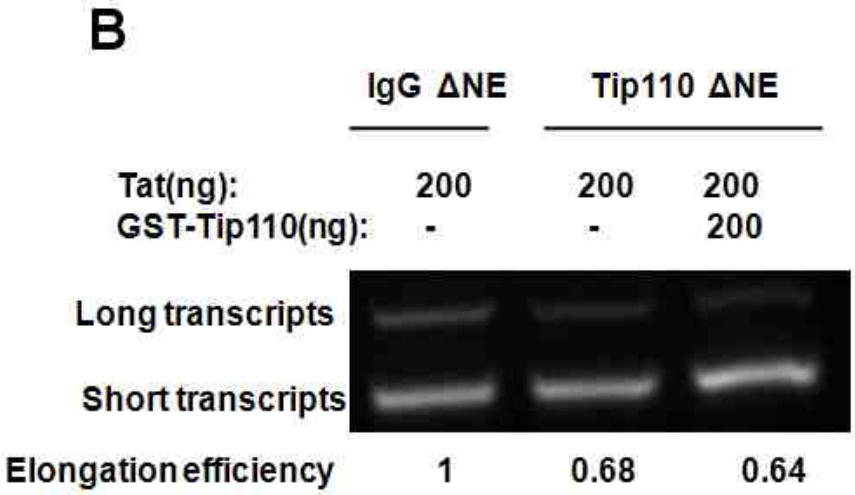

Tat(ng): - 100 200 - - 100 100
GST-Tip110(ng): - - - 200 400 200 400
Long transcripts
Short transcripts
Elongation efficiency 1 1.24 1.57 1.11 1.46 1.91 2.08

Figure 21. Direct effects in HIV-1 transcription elongation by Tip110 and Tat

(A) Schematic of HIV-1 LTR double G-less cassette template used in the experiment. The transcript synthesized by this template contains two G-less cassettes. Upstream G-less cassette is located proximal to the promoter and used to measure transcription initiation, the downstream cassette is used to measure transcription elongation. The primer locations to amplify the short and long transcripts were shown. (B) The *in vitro* transcription/elongation assay was performed with HIVdG-less plasmid and 0, 100, 200 ng of recombinant Tat protein *(lanes 1-3),* or 200,400 ng of GST-Tip110 protein *(lanes 4, 5),* or 100 ng of Tat protein together with 200, 400 ng of GST-Tip110 protein *(lanes 6, 7).* The ratio of the PCR products from long transcripts to those from the short transcripts was calculated and used as the elongation efficiency.


A
Undeleted NE
IgG ΔNE
Tip110 ΔNE
α-Tip110
α-ß-actin


B
IgG ΔNE
Tip110 ΔNE
Tat(ng): 200 200 200
GST-Tip110(ng): - - 200
Long transcripts
Short transcripts
Elongation efficiency 1 0.68 0.64

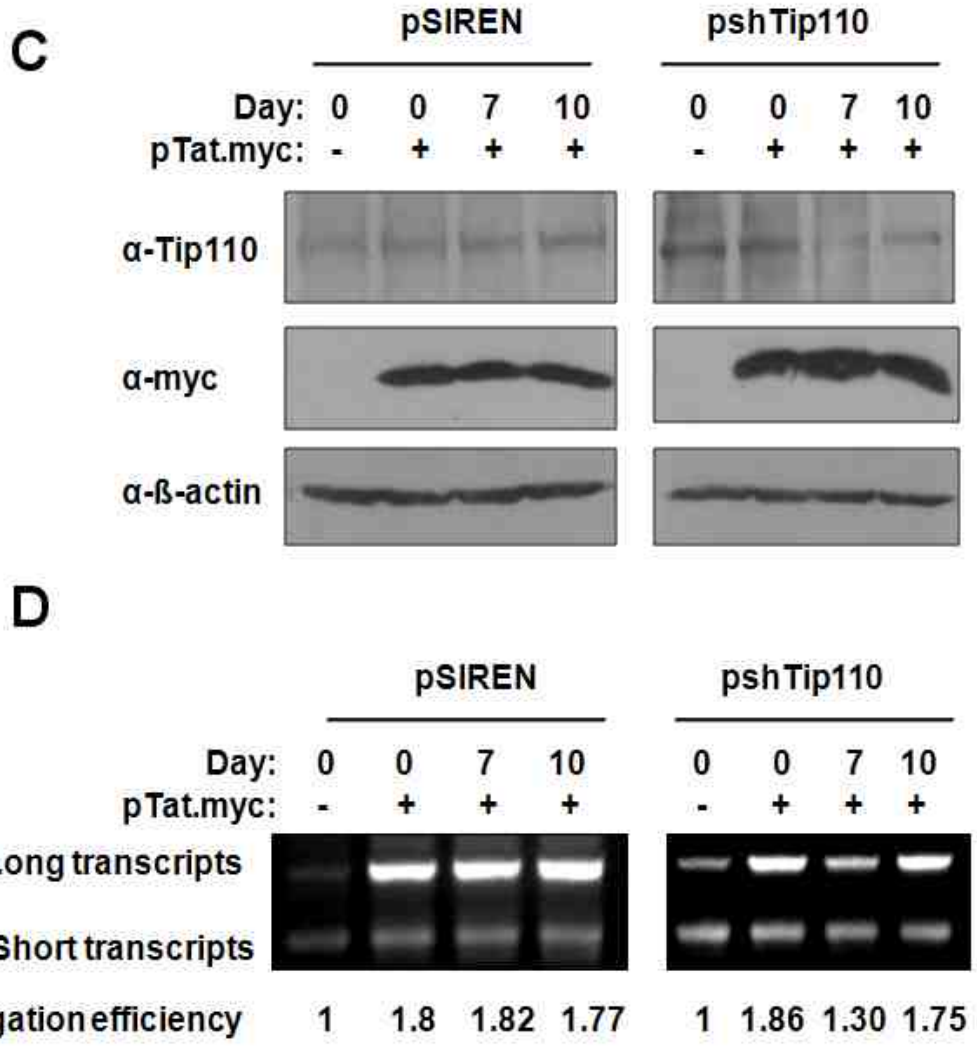

C
pSIREN
pshTip110
Day: 0 0 7 10 0 0 7 10
pTat.myc: - + + + - + + +
α-Tip110
α-myc
α-ß-actin
D
pSIREN
pshTip110
Day: 0 0 7 10 0 0 7 10
pTat.myc: - + + + - + + +
Long transcripts
Short transcripts
Elongation efficiency 1 1.8 1.82 1.77 1 1.86 1.30 1.75

Figure 22. Effects of Tip110 knock down on HIV-1 transcription elongation

(A) Immundepletion of Tip110 from Hela nuclear extract. Western blotting was then performed for undeleted, IgG and Tip110 depleted nuclear extract. Specific antibodies against Tip110 and β-actin were used to demonstrate the depletion efficiency. (B) Transcription elongation reactions were performed with indicated nuclear extracts, 200ng Tat protein, and the HIV-1 LTR G-less template. *Lane 3* had 200 ng GST-Tip110 added back to the transcription reaction. (C) 293T cells were first transfected with pSIREN or psh-Tip110 (day 0) and then transfected with HIV-1 G-less template or pTat.myc plasmid at day 4 and 7. Cells were harvested on day 7 and10 for cell lysates and Western blotting for Tip110, Tat and β-actin. (D) RNA was extracted from the indicated cells; RT-PCR was performed for long and short transcripts.

PART II: Tip110 interacts with hnRNP A1 and regulates HIV-1 alternative splicing

2.1 Tip110 alters HIV-1 mRNA splicing pattern

During our study to define the synergistic effect of Tip110 and Tat on LTR transcription, we determined whether Tip110 enhanced HIV-1 gene expression and replication through direct alteration of HIV-1 gene expression profile such as Tat. To address this possibility, multiplex RT-PCR was performed to compare the mRNA expression profile between cells with and without Tip110 over-expression. Thus, 293T cells were transfected with pNL4-3 and pTip110.GFP or pEGFP as a control. The cells were harvested and first prepared for Western blotting to confirm Tip110 expression (Fig. 23A). Then, RNA from these cells was isolated and RT-PCR was performed with a primer pair BSS-SJ4.7, which have been used to amplify all multiply spliced mRNA (Markus Neumann 1994). Following the first round of RT-PCR reaction, 5 μl of the PCR reaction were added with ^{32}P-dCTP and continued for additional three PCR cycles. The products were then separated by denaturing gel electrophoresis. The results showed an increased level of one PCR product corresponding to *tat* mRNA in Tip110-expressing cells (Fig. 23B). To verify that the PCR product of interest was derived from *tat* mRNA, we recovered the DNA; PCR cloned it, and sequenced it. The sequencing results confirmed that this product corresponded to *tat* exon2. This was the first evidence to suggest that Tip110 enhancement of HIV-1 gene expression and replication likely also involved preferential regulation of *tat* mRNA expression.

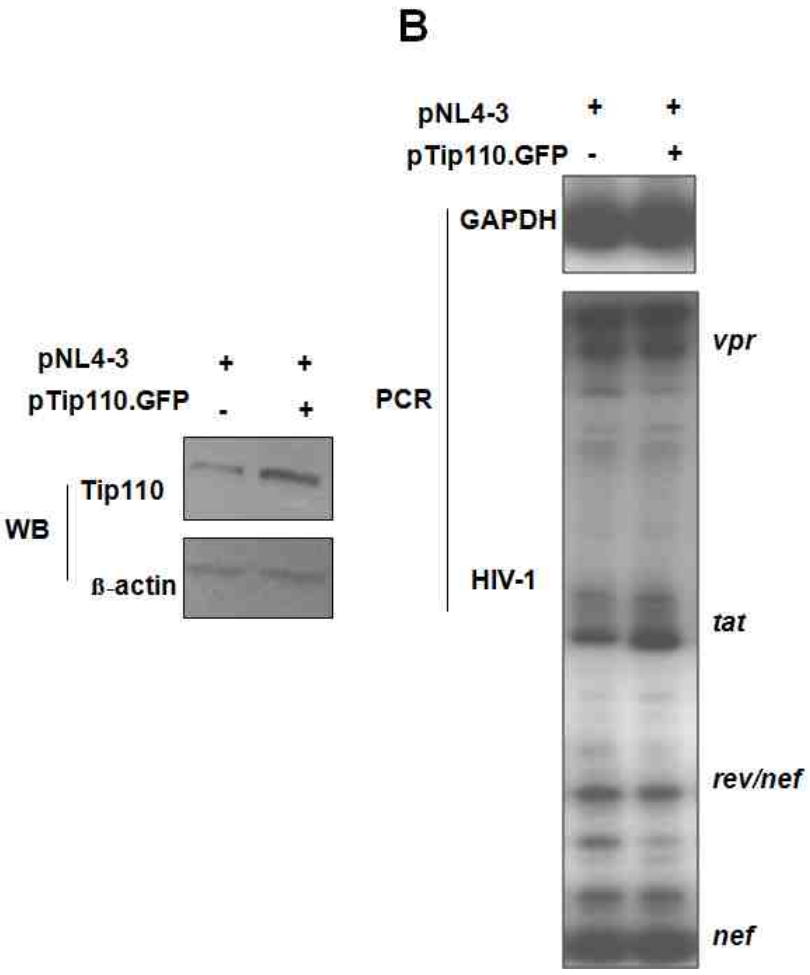
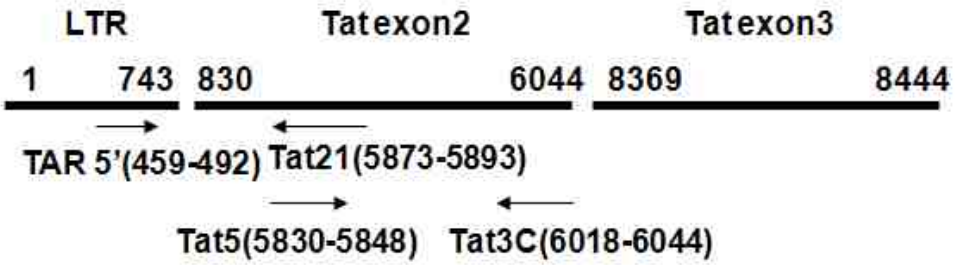

A
pNL4-3 + +
pTip110.GFP - +
WB
Tip110
ß-actin
B
pNL4-3 + +
pTip110.GFP - +
PCR
GAPDH
HIV-1
vpr
tat
rev/nef
nef

Figure 23. Alteration of HIV-1 mRNA splicing by Tip110 (A)293T cells were transfected with pNL4-3 and pTip110.GFP or pGFP-N3. Thirty six hours post-transfection the cells were harvested and performed Western blotting against Tip110 or β-actin. (B) Total RNA was isolated, reverse transcribed and then PCR was carried out with primers BSS and SJ 4.7. Five microliter of amplification products were added ^{32}PdCTP for additional three PCR cycles, and separated by denaturing gel electrophoresis. GAPDH RT-PCR was included as a control.

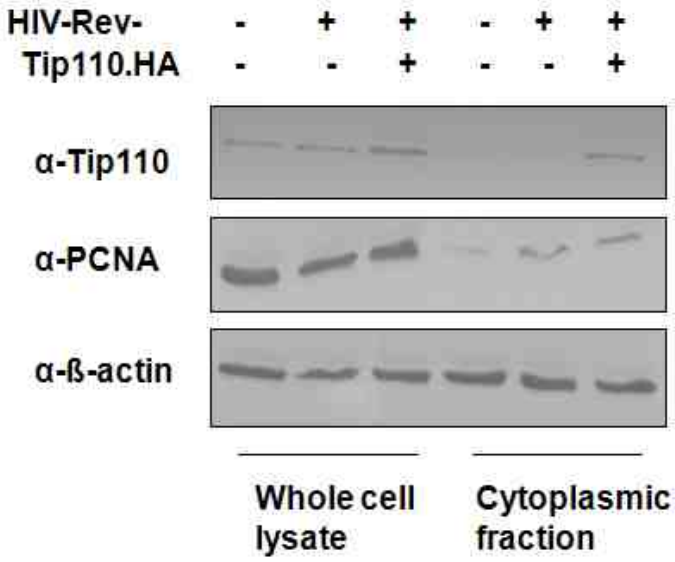
2.2 Tip110 preferentially increases *tat* transcripts

To further confirm multiplex RT-PCR results, we wished to perform RT-PCR with *tat*-specific primers (Fig. 24A). However, because *tat* transcripts are multiply spliced and shared common regions with singly spliced and unspliced mRNA, RT-PCR cannot specifically amplify *tat* transcripts. To overcome this problem, pHIV-Rev$^-$plasmid was used in place of pNL4-3 in the transfections, as Rev deletion would retain singly spliced and unspliced mRNA in the nucleus and only multiply spliced mRNA was present in the cytoplasm. Thus, transcription of cells with pHIV-Rev-, followed by subcellular fractionation would allow us to use RT-PCR to determine Tip110 effects on *tat* mRNA expression. The cells were first transfected with pHIV-Rev$^-$and pTip110.HA orpcDNA3 and then performed subcellular fractionation which was assessed by Western blotting against nuclear protein PCNA. Compared to whole cell lysates, the cytoplasmic fraction had much less PCNA (Fig. 24B). Then, RNA was extracted from both cytoplasmic fractions and whole cell lysates and RT-PCR was performed with *tat*-specific primers. GAPDH was amplified as a control. Using the cytoplasmic fraction RNA, RT-PCR reactions with primers TAR5/ Tat 21 or Tat 5/ Tat 3C have increased amplified products with Tip110 over-expressing, while the products amplified by GAPDH primer showed no change (Fig. 24C, *left panel*). Using the whole cell RNA, RT-PCR amplified products remained the same with *tat* primers or GAPDH primer (Fig. 24C, *right panel*), indicating Tip110 preferentially increased *tat* transcripts, but not singly or unspliced mRNA. By three independent experiments, Tip110 increased *tat* transcripts by more than 1.6 fold, which confirmed our previous conclusion that Tip110 preferentially increased *tat* mRNA.

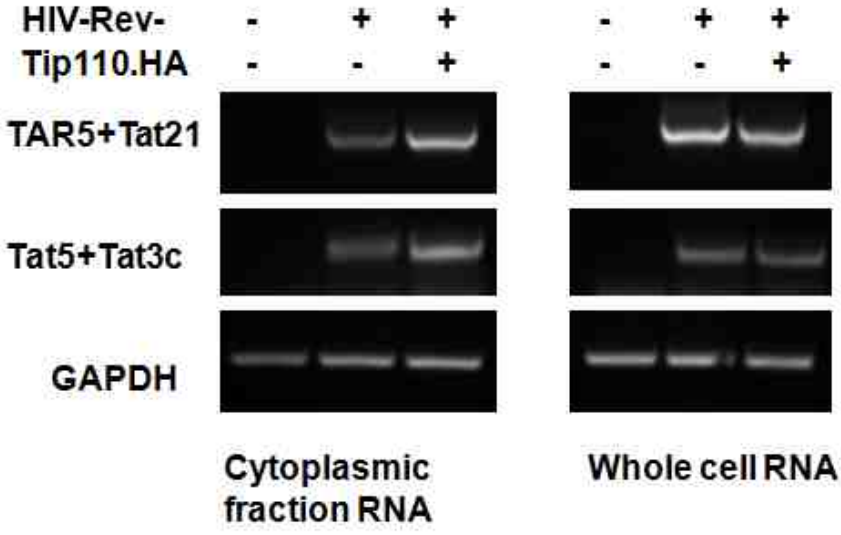
A


LTR
Tat exon2
Tat exon3
1
743
830
6044
8369
8444
TAR 5'(459-492)
Tat21(5873-5893)
Tat5(5830-5848)
Tat3C(6018-6044)

B


HIV-Rev-
Tip110.HA
α-Tip110
α-PCNA
α-ß-actin
Whole cell lysate
Cytoplasmic fraction

C


HIV-Rev- - + + - + +
Tip110.HA - - + - - +
TAR5+Tat21
Tat5+Tat3c
GAPDH
Cytoplasmic fraction RNA
Whole cell RNA

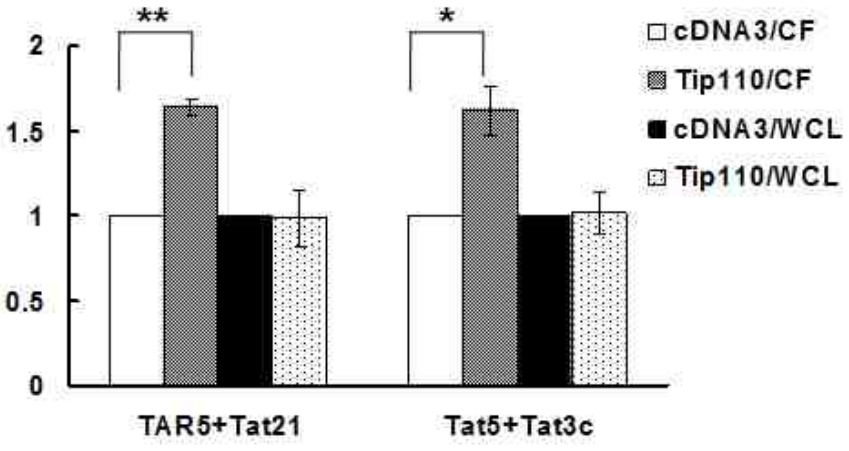

**
*
2
1.5
1
0.5
0
cDNA3/CF
Tip110/CF
cDNA3/WCL
Tip110/WCL
TAR5+Tat21
Tat5+Tat3c

Figure 24. Increased *tat* mRNA expression by Tip110 (A) Schematic of HIV-1 *tat* exons and the primer pairs for RT-PCR. (B) 293T cells were transfected with HIV-Rev$^-$ plasmid with pTip110.HA or pcDNA3.The cells that were only transfected with pcDNA3 were used as a control. Two days after transfection, cytoplasmic fractions were prepared and Western blots for nuclear protein PCNA, Tip110 or β-actin were performed. (C)RNA was extracted from cytopolasmic fraction or whole cell lysates followed by reverse transcription. PCR was performed with primers TAR5'/ Tat21 or Tat5/ Tat3C.GAPDH RT-PCR was included as a loading control. RT-PCR products were quantitated by densitometry, and the data were means ± S.D. and representative of three independent experiments. * $p<0.05$, ** $p<0.01$.

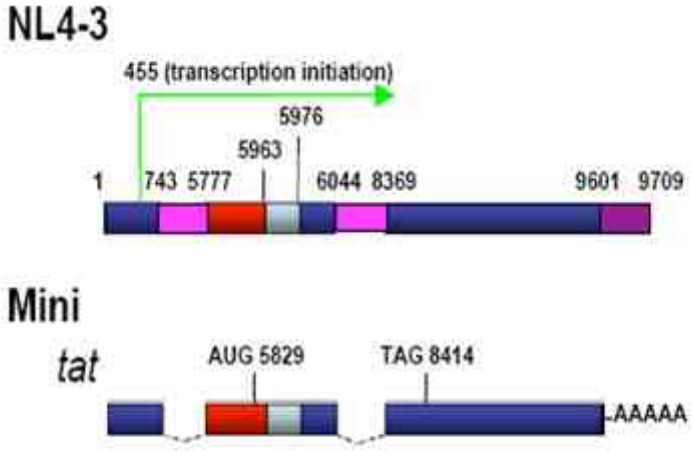
2.3 Tip110 increases Tat protein expression

To further confirm that Tip110 effects on Tat protein expression resulted from HIV-1 RNA splicing, we took advantage of *tat* minigene that only contains three exons and does not undergo alternative splicing to generate *tat* mRNA (Fig. 25A). 293T cells were transfected with pNL4-3 or the *tat* minigene, along with pTip110.HA and performed Western blotting against Tip110, Tat and β-actin. With Tip110 overexpression, Tat protein expression increased by 1.6 fold in pNL4-3-transfecteced cells but remained unchanged in *tat* minigene transfected cells (Fig. 25B). These results further confirmed that Tip110 increasesed *tat* mRNA and Tat protein expression through regulation of HIV-1 RNA alternative splicing.

2.4 Tip110 enhances *tat* exons 1-2 splicing but not *tat* exons 2-3 splicing

Tat mRNA splicing is regulated by acceptor splice site A3and A7 as well as the ESE or ESS *cis*-element within *tat* exon 2 or *tat/rev* exon3 (Si, Rauch et al. 1998; Zahler, Damgaard et al. 2004). To determine which *tat* splice site Tip110 regulated, we took advantage of two sets of *tat* pre-mRNA minigenes. The first setpCMV-HS1-WT and pCMV-ESS2-M contains *tat* exon 1, exon 2, and a shortened intron and have been used to study the regulation of A3 splicing site (Si, Amendt et al. 1997). pCMV-HS1-WT contains wild-type ESS2, while pCMV-ESS2-M has 4 nt mutation from 4748 to 5152 in the ESS core sequence (Fig. 26A). The second set pCMV-HS2contains *tat* exons 2 and 3, with the putative ESS and ESE elements located in *tat* exon3 and has been used to study regulation of the A7 splice site (Si, Rauch et al. 1998) (Fig. 28A). We constructed

A


NL4-3
455 (transcription initiation)
5976
5963
1
743 5777
6044 8369
9601 9709
Mini
tat
AUG 5829
TAG 8414
AAAAA

B

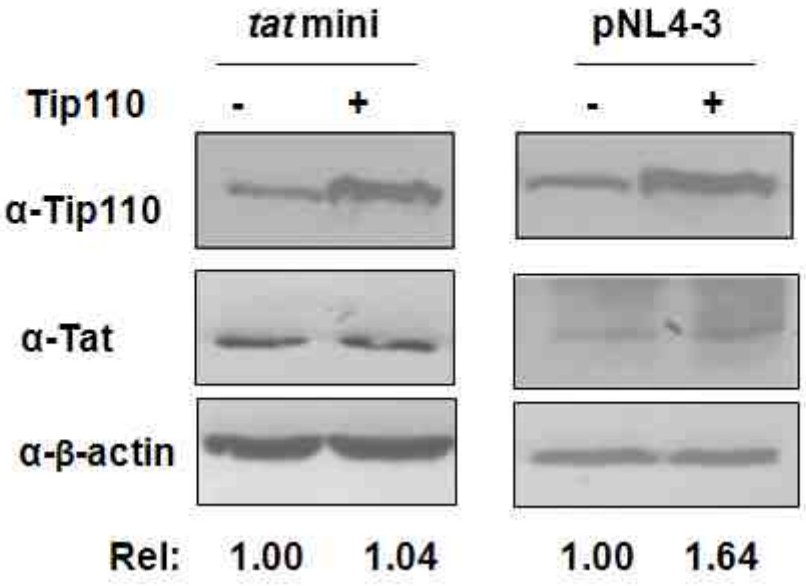

tat mini
pNL4-3
Tip110
-
+
-
+
α-Tip110
α-Tat
α-β-actin
Rel:
1.00
1.04
1.00
1.64

Figure 25. Enhancement of Tat protein expression by Tip110 (A) Schematic of NL4-3 proviral DNA and intronless *tat* minigene. (B) 293T cells were transfected with *tat* minigene *(left panel)* or pNL4-3 *(right panel)* with or without pTip110.HA. The cells were harvested 48 hr after transfection, lysed in RIPA buffer, and 20 μg of each sample were separated by SDS-PAGE followed by Western blot analysis using anti-Tip110, anti-Tat, or anti-β-actin. Tat expression was quantitated by densitometry and expressed as fold increase over the control (Rel.).

these two sets of minigenes under the CMV promoter from the original cassettes, so *in vivo* splicing assay could be performed by transfection, isolation of the RNA from the transfected cells followed by RT-PCR.

pCMV-HS1-WT or pCMV-ESS2-M minigenes were first used to test the function of Tip110 in *tat* exons 1-2 splicing. 293T cells were transfected with pCMV-HS1-WT or pCMV-ESS2-M and total RNA was extracted after 3 days. RT-PCR was later performed with primers T7/ tat 37, and both unspliced and spliced RT-PCR products were detected at the expected sizes (Fig. 26B). As expected, ESS2-M showed a 1.6 folds increase in splicing efficiency over WT (Si, Amendt et al. 1997). To further validate this new *in vivo* transcription/splicing protocol, 293T cells were co-transfected withpCMV-HS1-WT or pCMV-ESS2-M and increasing amounts of phnRNP A1-GFP, which has been shown to interact with UAG sequence in ESS2 and inhibit upstream 3'ss splicing (Caputi, Mayeda et al. 1999). The results showed that hnRNP A1 expression led to less spliced RT-PCR products from WT but no changes from ESS2-M (Fig. 26C). Then, the cells were transfected with pCMV-HS1-WT or pCMV-ESS2-M and increasing amounts of pTip110.HA. The results showed that Tip110 expression increased the spliced RT-PCR products from WT (Fig. 27A) and no changes in ESS2-M (Fig. 27B), suggested Tip110 enhanced *tat* exons 1-2 splicing and ESS2 was directly involved.

Next, we performed similar experiments with *tat* exons 2-3 minigene pCMV-HS2 and ASF/SF2 was included as a positive control. As expected, ASF/SF2 showed positive effects in *tat* exons 2-3 splicing (Fig. 28B), which was consistent with previous reports

that ASF/SF2 stimulates *tat* exons 2-3 splicing in an ESE3-dependent manner (Ropers, Ayadi et al. 2004). However, when pCMV-HS2 was co-transfected with increasing amounts of pTip110.HA, *tat* exons 2-3 splicing showed no changes (Fig. 28C), indicating Tip110 did not affect *tat* exons 2-3 splicing.

2.5 Tip110 knockdown decreases *tat* exons 1-2 splicing

To further determine the significance of Tip110 protein in *tat* mRNA splicing, we down-modulated endogenous Tip110 expression and determined its effects on *tat* mRNA splicing. We constructed pshTip110 plasmid to express Tip110-specific shRNA and first tested the efficiency of pshTip110 in decreasing endogenous Tip110 expression. A transfection timeline was devised for these multiple transfection experiments (Fig. 29A). 293T cells were first transfected with pshTip110plasmid or pSIREN backbone vector; the cells were harvested on day 3, 6, 10 after transfection. Western blotting analysis demonstrated that endogenous Tip110 protein expression was down-modulated by pshTip110in day 6 after transfection but showed some recovery at day 10 (Fig. 29B). As a control, 293T cells were transfected with the backbone vector pSIREN, and Tip110 expression showed no significant changes (Fig. 29B). Then, we transfected pCMV-HS1 or pCMV-HS2 plasmids into 293T cells on day 4 or day 7 following pshTip110 or pSIREN transfection (Fig. 29A). The cells were harvested at day 7 or day 10 for RNA isolation and RT-PCR analysis. In parallel with Tip110 expression, *tat* exons 1-2 splicing efficiency was decreased at day 7 and began to increase at day 10 (Fig. 30A). In contrast, *tat* exons 2-3 splicing showed no changes (Fig. 30B). Taken together, these

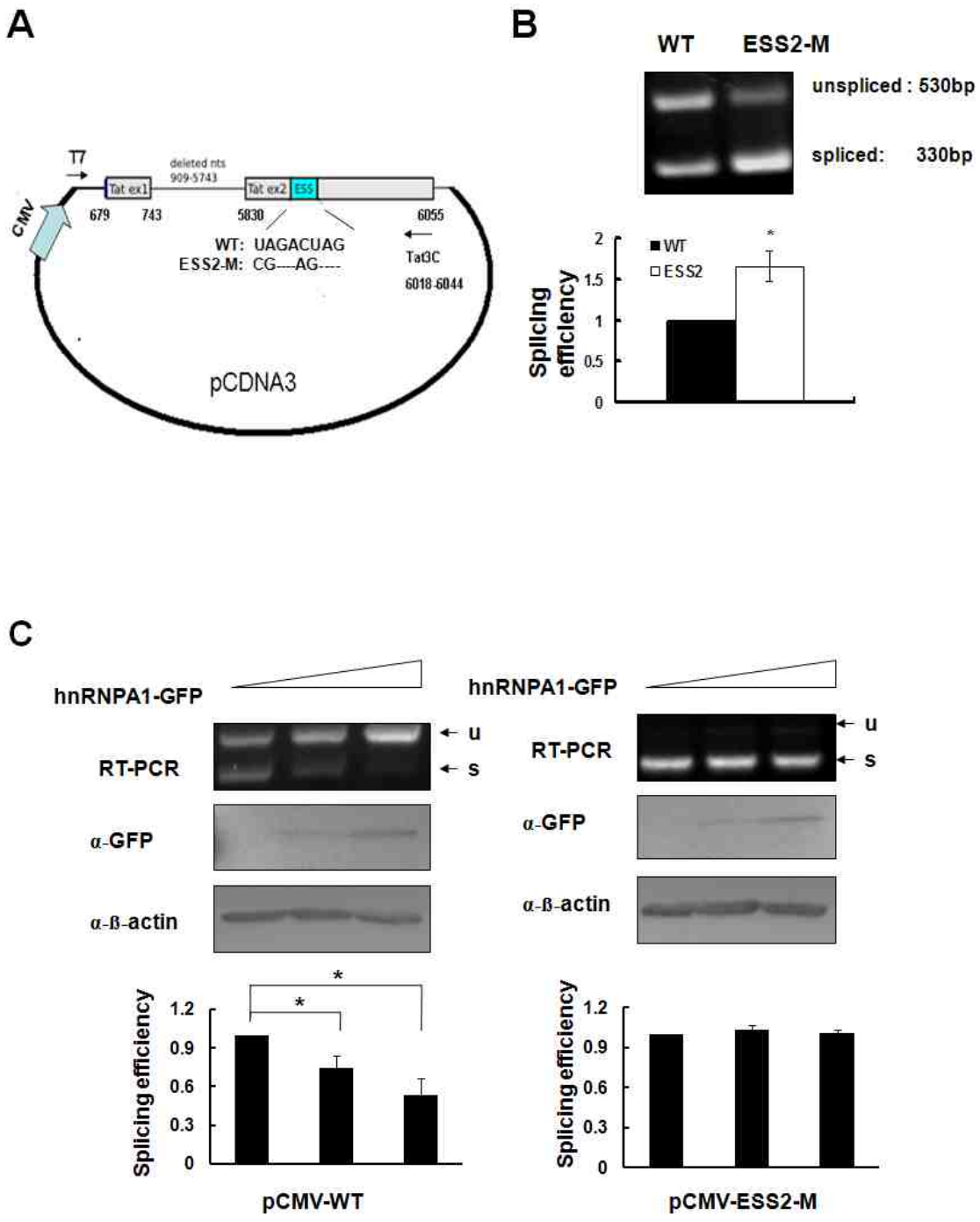

A
T7
deleted nts
909-5743
Tat ex1
Tat ex2
ESS
CMV
679
743
5830
6055
WT: UAGACUAG
ESS2-M: CG----AG----
Tat3C
6018-6044
pCDNA3
B
WT
ESS2-M
unspliced : 530bp
spliced: 330bp
Splicing efficiency
WT
ESS2
C
hnRNPA1-GFP
RT-PCR
u
s
α-GFP
α-ß-actin
Splicing efficiency
pCMV-WT
pCMV-ESS2-M

Figure 26. pCMV-WT and pCMV-ESS2-M minigene and their splicing

(A) Schematic of pCMV-WT and pCMV-ESS2-M minigene constructs and RT-PCR primers. (B) Minigene splicing: 293T cells were transfected with 2 μg of pCMV-WT or pCMV-ESS2-M. Total RNA was extracted and processed for RT-PCR. The RT-PCR products were determined by densitometry, the ratio of spliced to unspliced ones were calculated and set to 1 in the pCMV-WT-transfected cells. (C) pCMV-WT *(left panel)* or pCMV-ESS2-M *(right panel)* were transfected into 293T cells in the combination of increasing amount of hnRNPA1-GFP (0, 0.5, 1.0 μg). Western blotting was performed for GFP or β-actin expression. RT-PCR was performed to determine the unspliced and spliced transcripts. The ratio of spliced to unspliced RT-PCR products was calculated. These data were mean ± SD and representative of three independent experiments.

* $p<0.05$, ** $p<0.01$.u: unspliced band, s: spliced band.

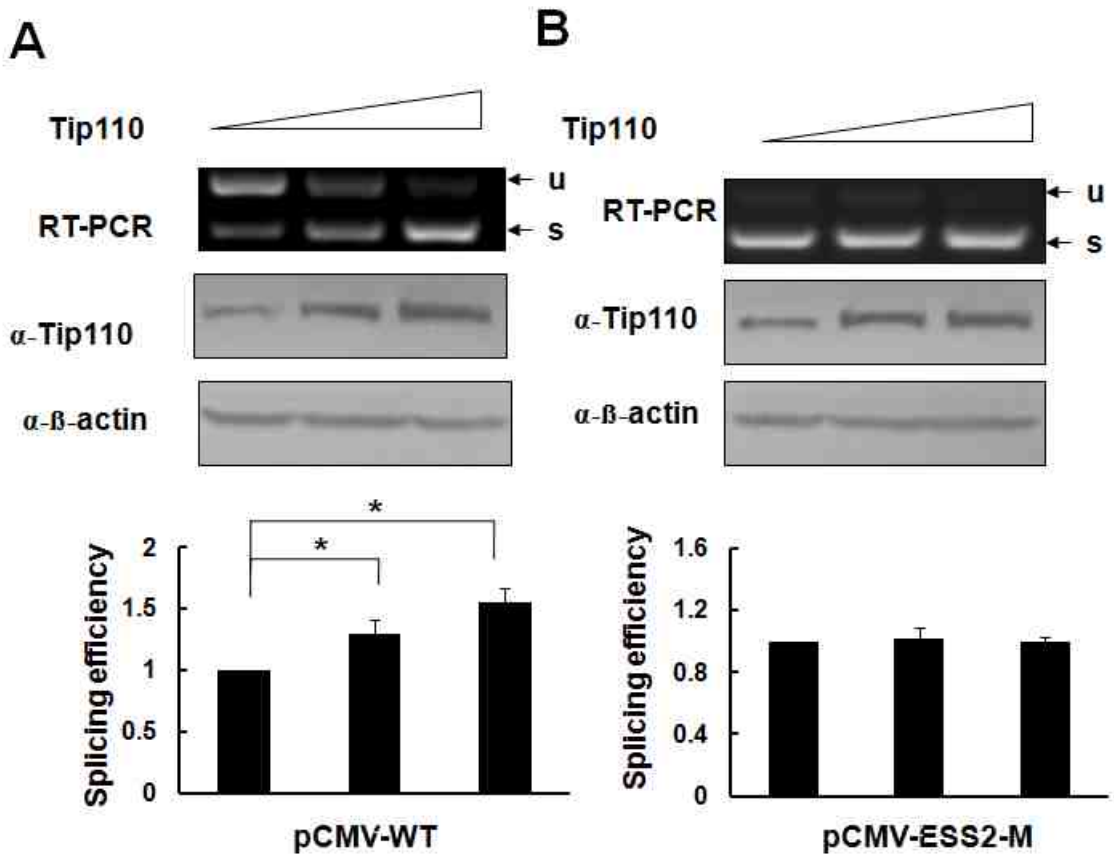
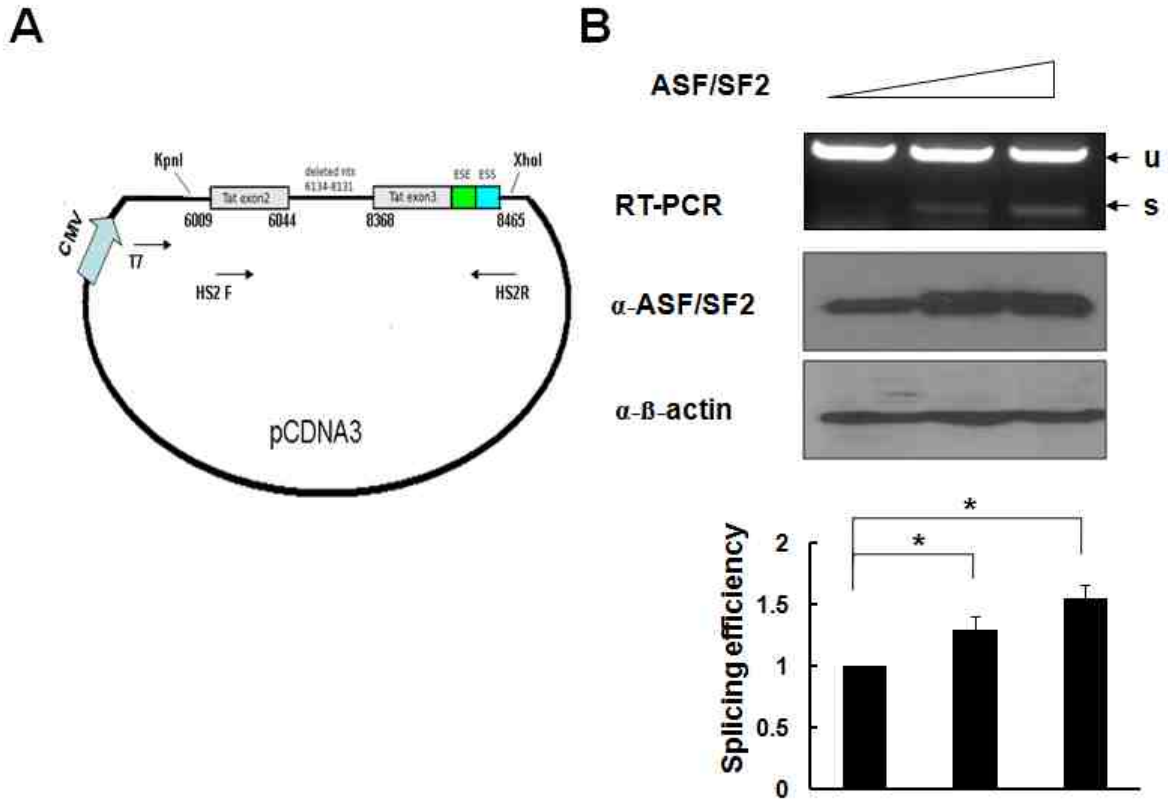

A
B
Tip110
RT-PCR
u
s
α-Tip110
α-ß-actin
Splicing efficiency
2
1.5
1
0.5
0
*
*
pCMV-WT
1.6
1.2
0.8
0.4
0
pCMV-ESS2-M

Figure 27. Increased *tat* exons 1-2 splicing by Tip110 pCMV-WT(A) or pCMV-ESS2-M (B) were transfected into 293T cells in combination with increasing amounts of pTip110.HA (0, 0.5,1.0 μg). Cell were harvested for cell lysates and Western blotting against Tip110, β-actin, or for RNA and RT-PCR to amplify *tat* exons 1-2 unspliced and spliced transcripts. The ratio of spliced to unspliced RT-PCR products was calculated as before. * $p<0.05$, ** $p<0.01$.u: unspliced band, s: spliced band.


A
KpnI
Tat exon2
deleted nts
6134-8131
Tat exon3
ESE
ESS
XhoI
6009
6044
8368
8465
CMV
T7
HS2 F
HS2R
pCDNA3
B
ASF/SF2
RT-PCR
u
s
α-ASF/SF2
α-ß-actin
Splicing efficiency
2
1.5
1
0.5
0
*
*

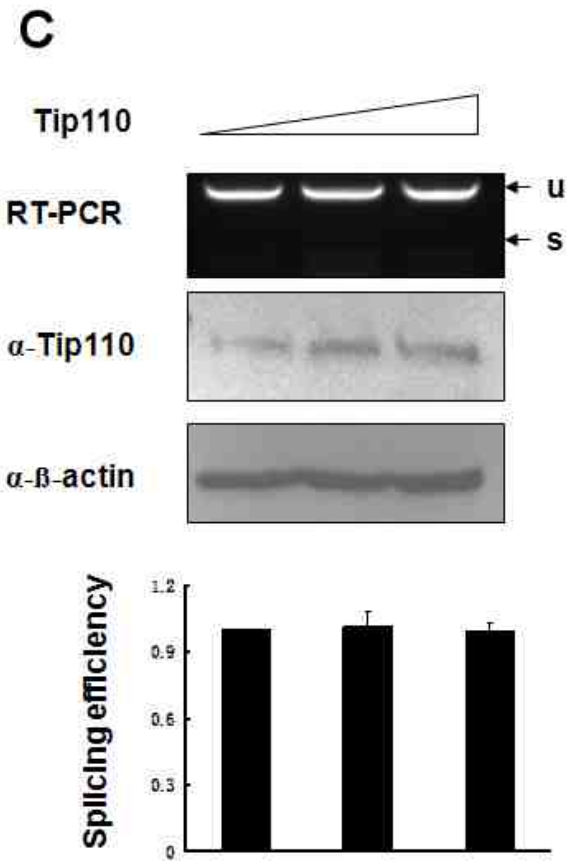
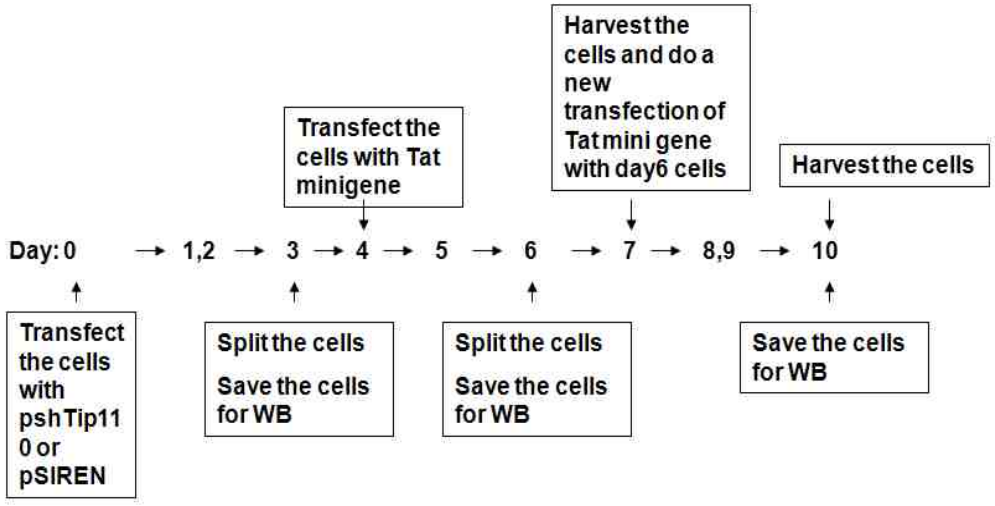

C
Tip110
RT-PCR
u
s
α-Tip110
α-ß-actin
Splicing efficiency
1.2
0.9
0.6
0.3
0

Figure 28. Tip110 effects on *tat*exons 2-3 splicing (A)Schematic of pCMV-HS2 minigene construct. (B) 293T cells were transfected with pCMV-HS2 and increasing amounts of ASF/SF2 (0, 0.5, 1.0 μg) and processed as described above. (C) 293T cells were transfected with pCMV-HS2 and increasing amount of Tip110 (0, 0.5, 1.0 μg) and processed as described above. u: unspliced band, s: spliced band.

A


Harvest the cells and do a new transfection of Tat mini gene with day6 cells
Transfect the cells with Tat minigene
Harvest the cells
Day: 0 → 1,2 → 3 → 4 → 5 → 6 → 7 → 8,9 → 10
Transfect the cells with pshTip110 or pSIREN
Split the cells
Save the cells for WB
Split the cells
Save the cells for WB
Save the cells for WB

B

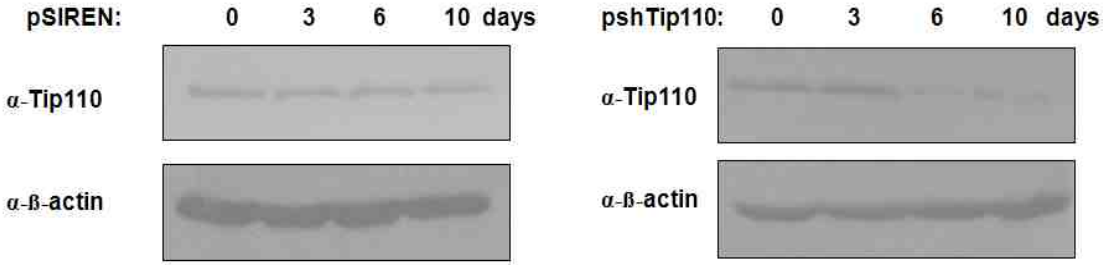
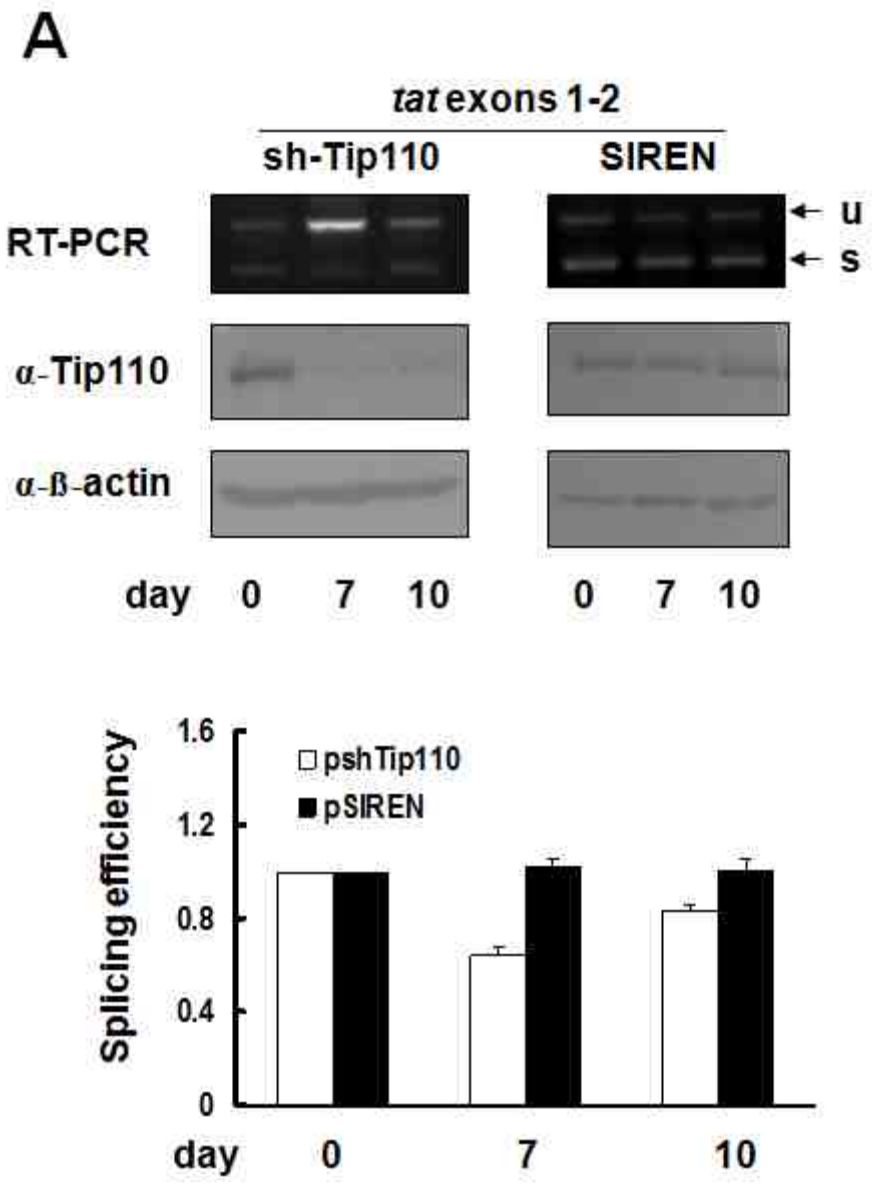
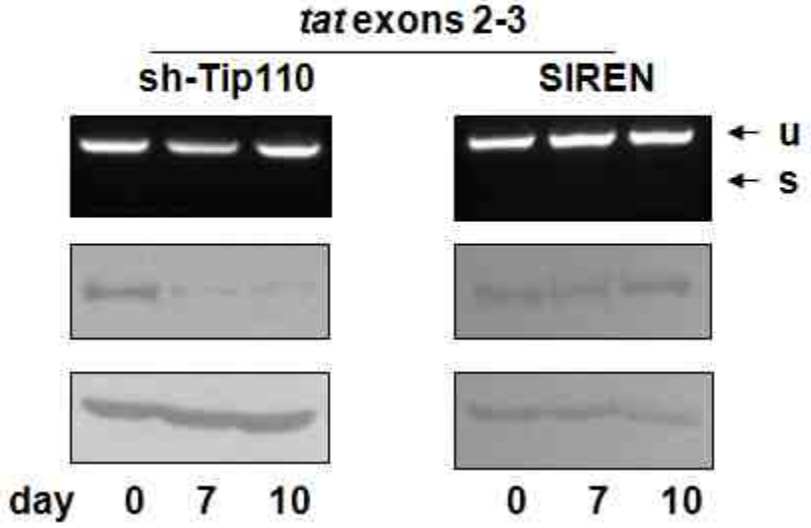

pSIREN: 0 3 6 10 days
α-Tip110
α-ß-actin
pshTip110: 0 3 6 10 days
α-Tip110
α-ß-actin

Figure 29. Tip110 knockdown (A) The transfection timeline. (B) 293 T cells were transfected with pSIREN or psh-Tip110 plasmid and harvested at day 0, 3, 6, and 10 for western blotting analysis against Tip110 or β-actin.


A
tat exons 1-2
sh-Tip110
SIREN
RT-PCR
u
s
α-Tip110
α-ß-actin
day 0 7 10 0 7 10
Splicing efficiency
1.6
1.2
0.8
0.4
0
pshTip110
pSIREN
day 0 7 10

B


tat exons 2-3
sh-Tip110
SIREN
u
s
day 0 7 10
0 7 10

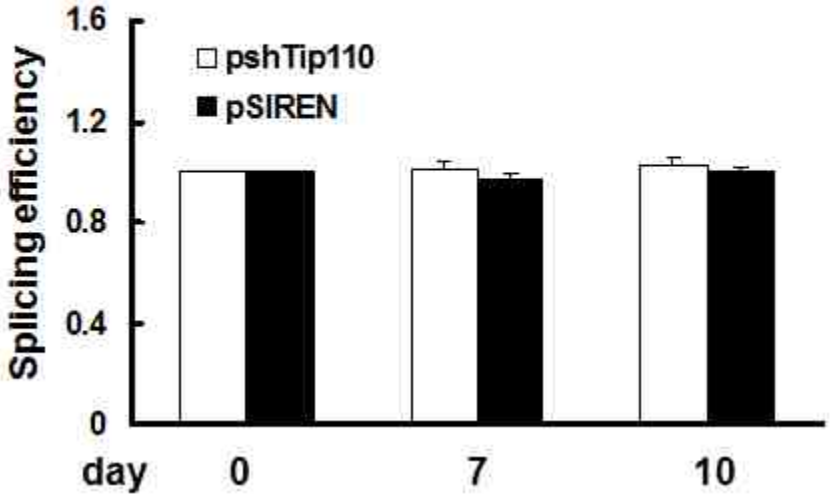
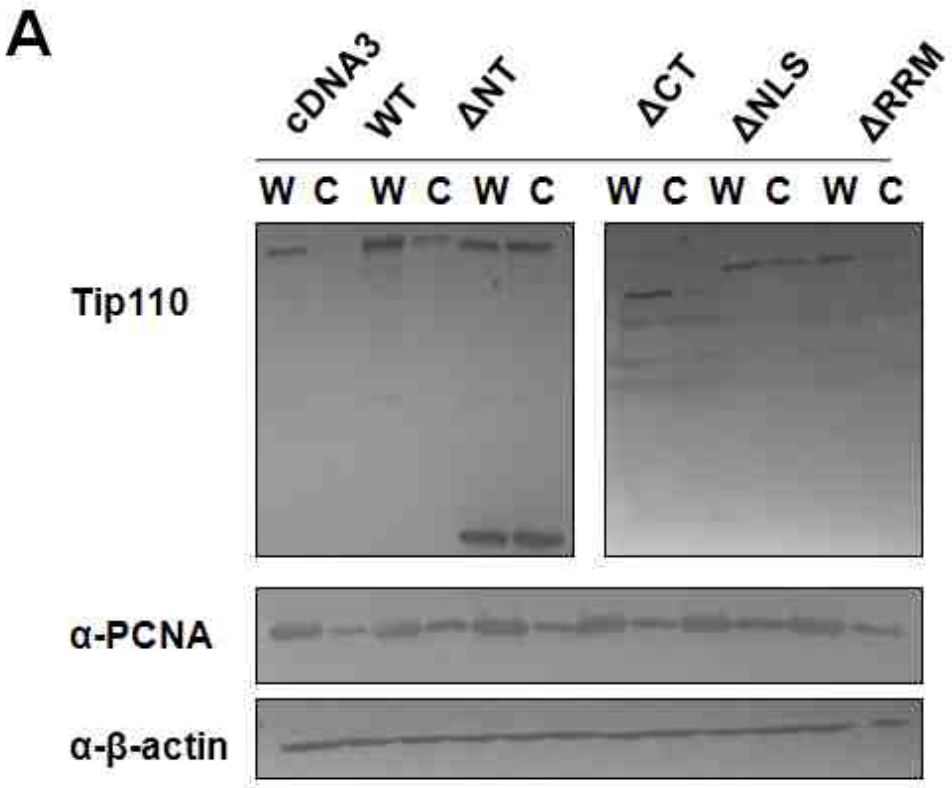

1.6
1.2
0.8
0.4
0
Splicing efficiency
pshTip110
pSIREN
day 0 7 10

Figure 30. Decreased *tat* exons 1-2 splicing by Tip110 knockdown 293T cells were first transfected with pSIREN or psh-Tip110 (day 0) and then transfected with pCMV-HS1 (A) or pCMV-HS2 (B) at day 4 and 7. Cells were harvested on day 7 and day 10 for cell lysates and Western blotting or RNA and RT-PCR. u: unspliced band, s: spliced band.

results confirmed that Tip110 preferentially increased *tat* exons 1-2 but not *tat* exons 2-3 splicing.

2.6 HAT and RRM domains are involved in Tip110-mediated *tat* mRNA splicing

To determine which functional domains of Tip110 were important for Tip110-mediated *tat* mRNA splicing, we took advantage of a series of Tip110 mutants which contained RRM domain, C-terminal domain, NLS domain or HAT domain deletions (Fig. 13A). Due to differences in antibody epitopes, the Tip110 mutants divided into two groups for Western blotting detection: ΔRRM, ΔNLS, and ΔCT mutants were detected by anti-156 serum, and ΔNT was detected by Tip110 monoclonal antibody. 293T cells were first transfected with each of the Tip110 mutants and pHIV-Rev- plasmid and then harvested for cytoplasmic lysates. Expression of Tip110 and its mutants and the efficiency of cytoplasmic fractionation were confirmed by Western blotting against Tip110 and PCNA, respectively (Fig. 31A). Then, RNA from the cytoplasmic lysates as well as whole cell lystate was isolated and RT-PCR was performed for *tat* exon 2. GAPDH RT-PCR was included as a loading control. We found that deletion of the HAT and RRM domain of Tip110 reduced *tat* exon 2 mRNA expression to the basal level but the deletion of NLS domain had *tat* exon 2 transcripts close to the wild-type Tip110 (Fig. 31B). Later, similar results were obtained with minigene pCMV-HS1 (Fig. 32). Taken together, these results showed HAT and RRM domains of Tip110 were involved in Tip110-mediated *tat* mRNA splicing.


A
cDNA3
WT
ΔNT
ΔCT
ΔNLS
ΔRRM
W C W C W C W C W C W C
Tip110
α-PCNA
α-β-actin

B

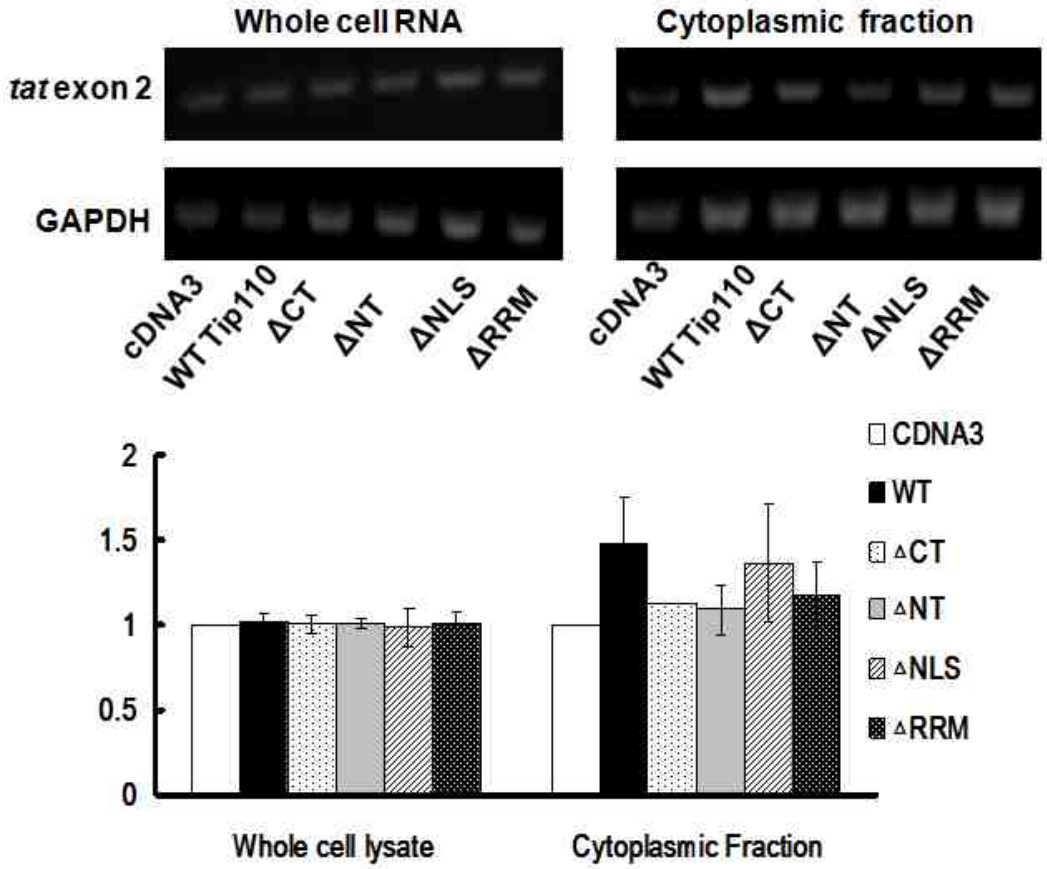
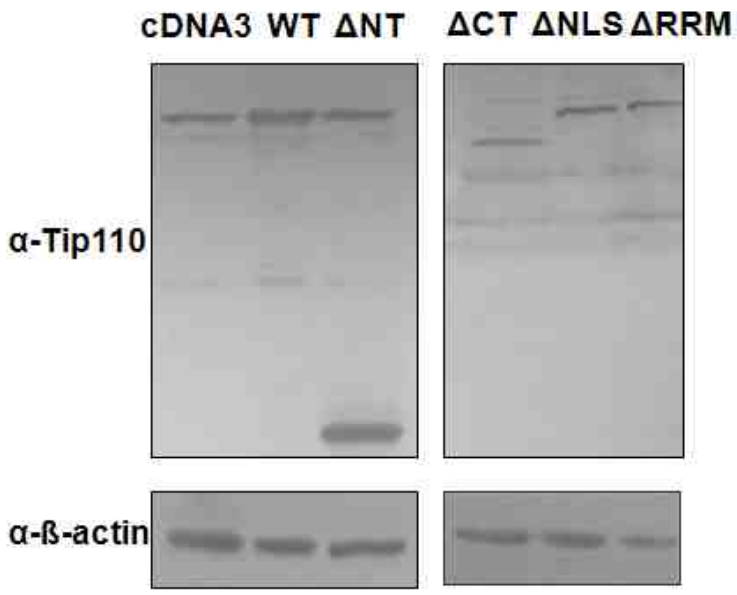

Whole cell RNA
Cytoplasmic fraction
tat exon 2
GAPDH
cDNA3
WT Tip110
ΔCT
ΔNT
ΔNLS
ΔRRM
cDNA3
WT Tip110
ΔCT
ΔNT
ΔNLS
ΔRRM
2
1.5
1
0.5
0
CDNA3
WT
ΔCT
ΔNT
ΔNLS
ΔRRM
Whole cell lysate
Cytoplasmic Fraction

Figure 31. Tip110 domains involved in *tat* mRNA splicing 293T cells were transfected with Tip110 or each of its mutants. pcDNA3 was included as a control. Two days after transfection, cells were harvested for cytoplasmic and whole cell lysates and Western blotting (A), or RNA and RT-PCR with primers tat5/ tat3C to amplify *tat* exon 2 (B). GAPDH RT-PCR was included as a loading control. W: whole cell lysate. C: cytoplasmic fraction lysate.

A



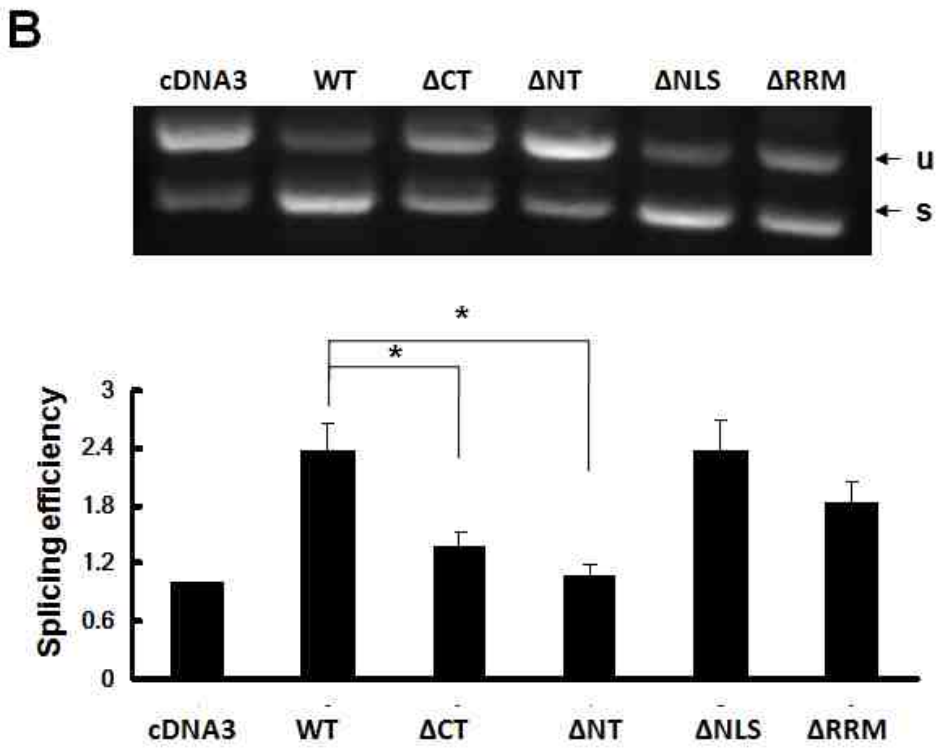

B
cDNA3
WT
ΔCT
ΔNT
ΔNLS
ΔRRM
u
s
*
*
Splicing efficiency
3
2.4
1.8
1.2
0.6
0
cDNA3
WT
ΔCT
ΔNT
ΔNLS
ΔRRM

Figure 32. Tip110 domains involved in *tat* exons 1-2 splicing (A) 293T cells were transfected with pCMV-WT and Tip110 or its mutants. Expression of Tip110 and its mutants was determined by Western blotting. (B) RNA was extracted for RT-PCR to amplify spliced/unspliced transcripts from *tat* exons 1-2 minigene as above. The bar graph represents mean values ± SD of the spliced/unspliced ratio from three independent experiments. u: unspliced band, s: spliced band.

2.7 Tip110 directly binds to hnRNP A1 *in vitro*

We next attempted to determine the molecular mechanisms for Tip110-mediated *tat* exons 1-2 splicing. A separate study from our own group has recently identified a number of Tip110-interacting proteins (Table. 2). Among these proteins was hnRNP A1, which has been shown to inhibit *tat* exons 1-2 splicing through ESS2 element (Caputi, Mayeda et al. 1999). Our result above showed that Tip110 enhanced *tat* exons 1-2 splicing through ESS2 element. Thus, we hypothesized that Tip110 interacted with hnRNP A1 and subsequently impaired hnRNP A1 interaction with the ESS2 element within *tat* exon 2. To test this hypothesis, we first prepared recombinant GST-hnRNP A1 and Tip110 proteins and performed *in vitro* GST pull down assay. We confirmed the MASS data that Tip110 directly bound to hnRNP A1 (Fig. 33).

2.8 Tip110 interaction with hnRNPA1*in vivo*

To further confirm Tip110 interaction with hnRNP A1, 293T cells were transfected with phnRNP A1-GFP and pTip110-HA and immunoprecipitation and Western blotting analysis was performed. To our surprise, no hnRNP A1 in either anti-Tip110 or anti-HA immunoprecipitates could be detected (Fig. 34A). In addition, no Tip110 was detected in anti-GFP or anti-hnRNP A1 immunoprecipitates (Fig. 34B). Then, the input amount of cell lysate was increased by transfection of 10 times more cells. The cell lysate was passed through a HA-conjugated affinity matrix column to enrich the Tip110 protein, and the Tip110 protein was then eluted with a competitive HA peptide and analyzed for the presence of hnRNP A1 protein by Western blotting. The results showed the presence of hnRNP A1 protein in the elution (Fig. 34C, *right panel*), as well as Tip110 protein (*right*

panel), suggested Tip110 interacted with hnRNP A1 in a weak or transient manner *in vivo*.

2.9 Tip110 does not bind to *tat* mRNA

Tip110 is predicted to contain two RNA recognition motifs (RRM) (Fig. 14A) and it may bind to RNA. Thus, we also examined the possibility that Tip110 bound to the ESE2 or ESS2 RNA *cis*-element within *tat* exon 2 by a gel shift assay. RNA probes were *in vitro* transcribed from T7 promoter and purified (Fig. 35A) and recombinant hnRNP A1-GST protein was include as a positive control in this assay. As expected, hnRNP A1 formed a complex with WT RNA (Fig. 35B, *lanes 3, 4)*, but not with E2-M RNA (*lanes 7, 8*). Then, Tip110-GST protein was incubated with WT or E2-M RNA, and no protein-RNA complex detected with either WT or E2-M RNA (Fig. 35C). To test the possibility that Tip110 complexed with *tat* mRNA through other proteins, nuclear extract was used in place of recombinant protein in the gel shift assay with the WT and E2-M RNA. When WT was used as a probe, incubation with nuclear extract led to formation of multiple complexes of RNA with cellular proteins, which was shown as a smear (Fig. 35D, *lane 4*). Inclusion of anti-hnRNP A1 antibody resulted in formation of a distinct complex, presumably among hnRNP A1, WT RNA and anti-hnRNP A1 antibody (*lane 5*). However, inclusion of anti-Tip110 antibody showed no apparent change in the gel shift pattern (*lane 6*). In contrast, when E2-M RNA was used as a probe, inclusion of either anti-Tip110 or anti-hnRNP A1 antibody showed no changes in the gel shift pattern (Fig. 35E). Taken together, these results further confirmed that Tip110 did not directly bind to ESE2 or ESS2 *cis*-element within *tat* exon 2.

Table 2.Tip110 interacting proteins

Name	M.Wt	Peptides
Main protein		
1. SART 3	109865	51 peptides
Cytoskeleton proteins		
1. Actin	41710	19 peptides
2. Keratin 1-Cyt1	65978	12 peptides
3. Keratin 1-Cyt9	62092	9 peptides
4. Keratin 2-Cyt2	65825	4 peptides
5. Keratin 1-Cyt10	59475	5 peptides
6. Keratin 1-Cyt10	57213	5 peptides
7. Tubulin beta	49799	3 peptides
8. Tubulin alpha	50062	2 peptides
9. Synaptotagmin 2-a	42709	1 peptides
Transportation protein		
1. Importin 2 alpha	57826	3 peptides
Heat shock proteins		
1. HSP A8	70845	5 peptides
2. HSPA1B	70009	6 peptides
Ribonucleoproteins		
1. hnRNPA2-B1	28394	1 peptides
2. hnRNPA1	38822	3 peptides
3. hnRNP U	88924	2 peptides
YB-1 protein		
1.Nulease-sensitive element-binding protein1	35903	1 peptides
Skin protein		
1. Hornerin	282228	6 peptides
2. Filaggrin	434922	4 peptides

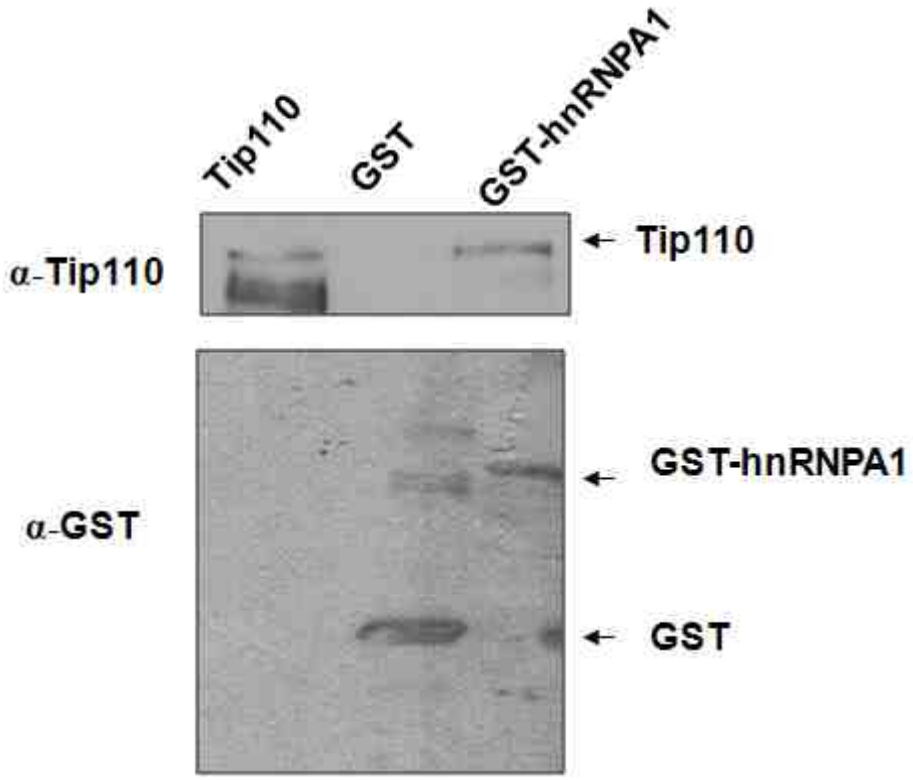
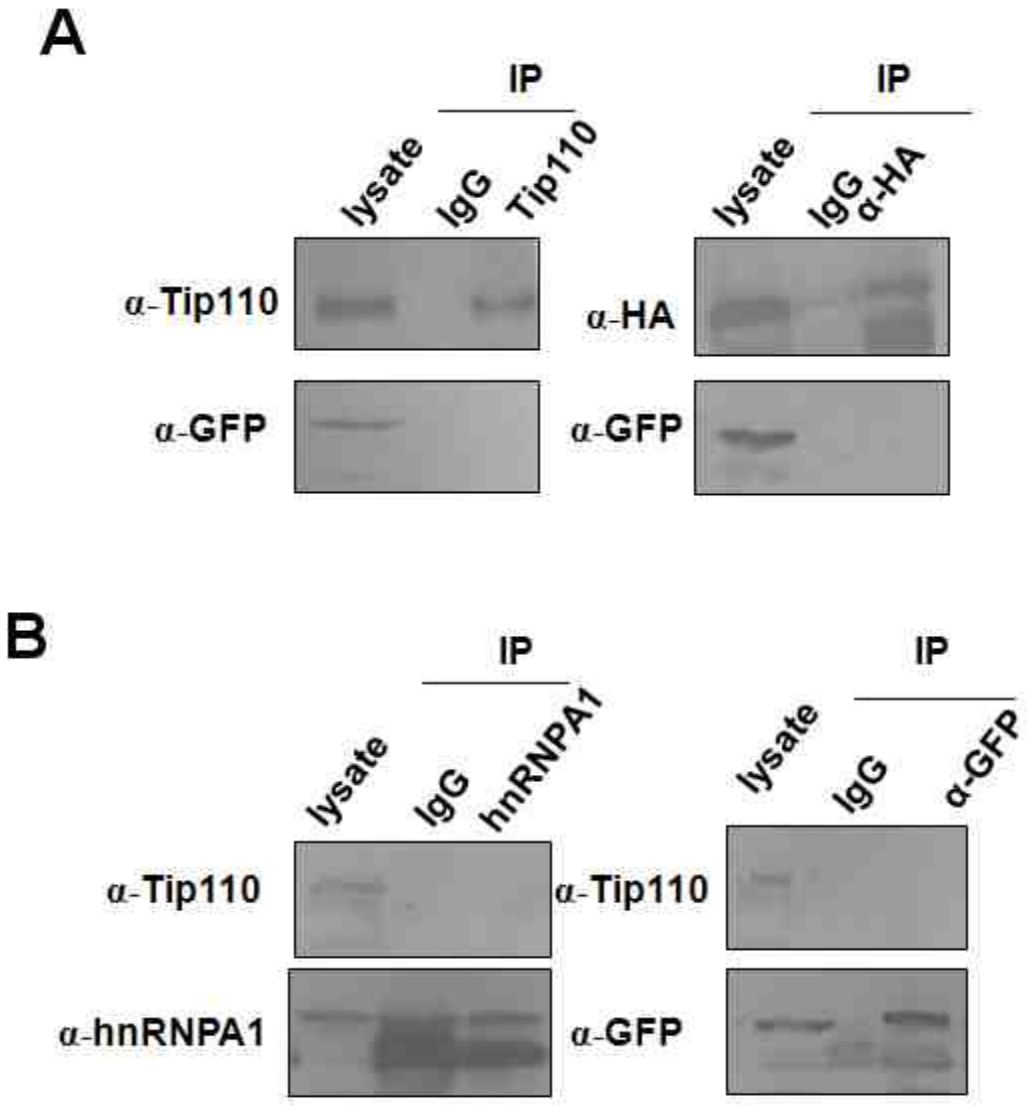

Tip110
GST
GST-hnRNPA1
α-Tip110
Tip110
α-GST
GST-hnRNPA1
GST

Figure 33. Direct interaction between Tip110 and hnRNP A1 protein *in vitro*

Tip110 protein (4 μg) was prepared by thrombin cleavage of GST-Tip110 and then purified by glutathione beads to eliminate the GST protein. Meanwhile, 2 μg GST-hnRNP A1 and GST protein were immobilized onto glutathione beads and incubated with purified Tip110 protein at 4^{o}C overnight. After washing, the protein from GST beads *(lane 2)* and GST-hnRNP A1 *(lane 3)* were analyzed by Western blotting for Tip110 *(upper panel)* or GST *(lower panel);* Purified Tip110 protein was included as the size marker *(lane 1).*


A
IP
IP
lysate
IgG
Tip110
lysate
IgG
α-HA
α-Tip110
α-HA
α-GFP
α-GFP
B
IP
IP
lysate
IgG
hnRNPA1
lysate
IgG
α-GFP
α-Tip110
α-Tip110
α-hnRNPA1
α-GFP

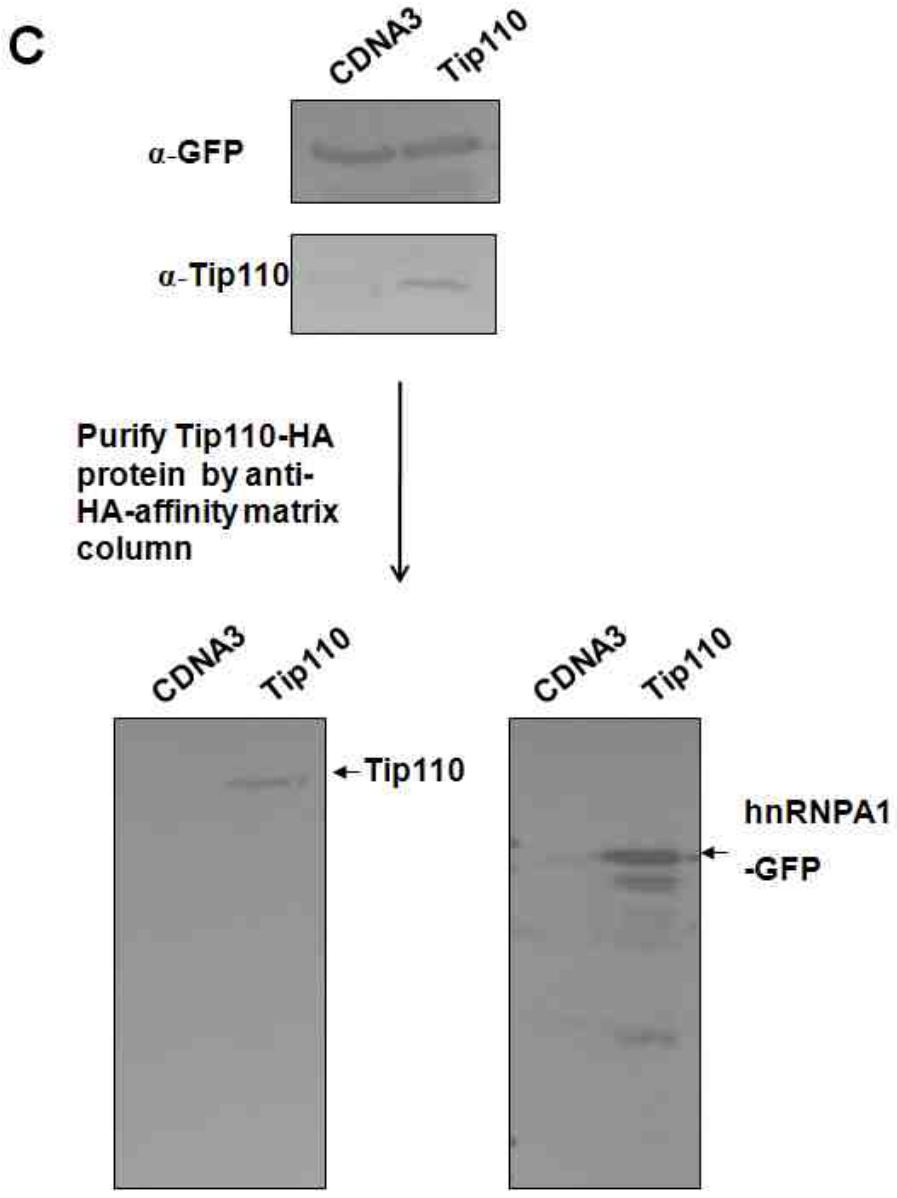
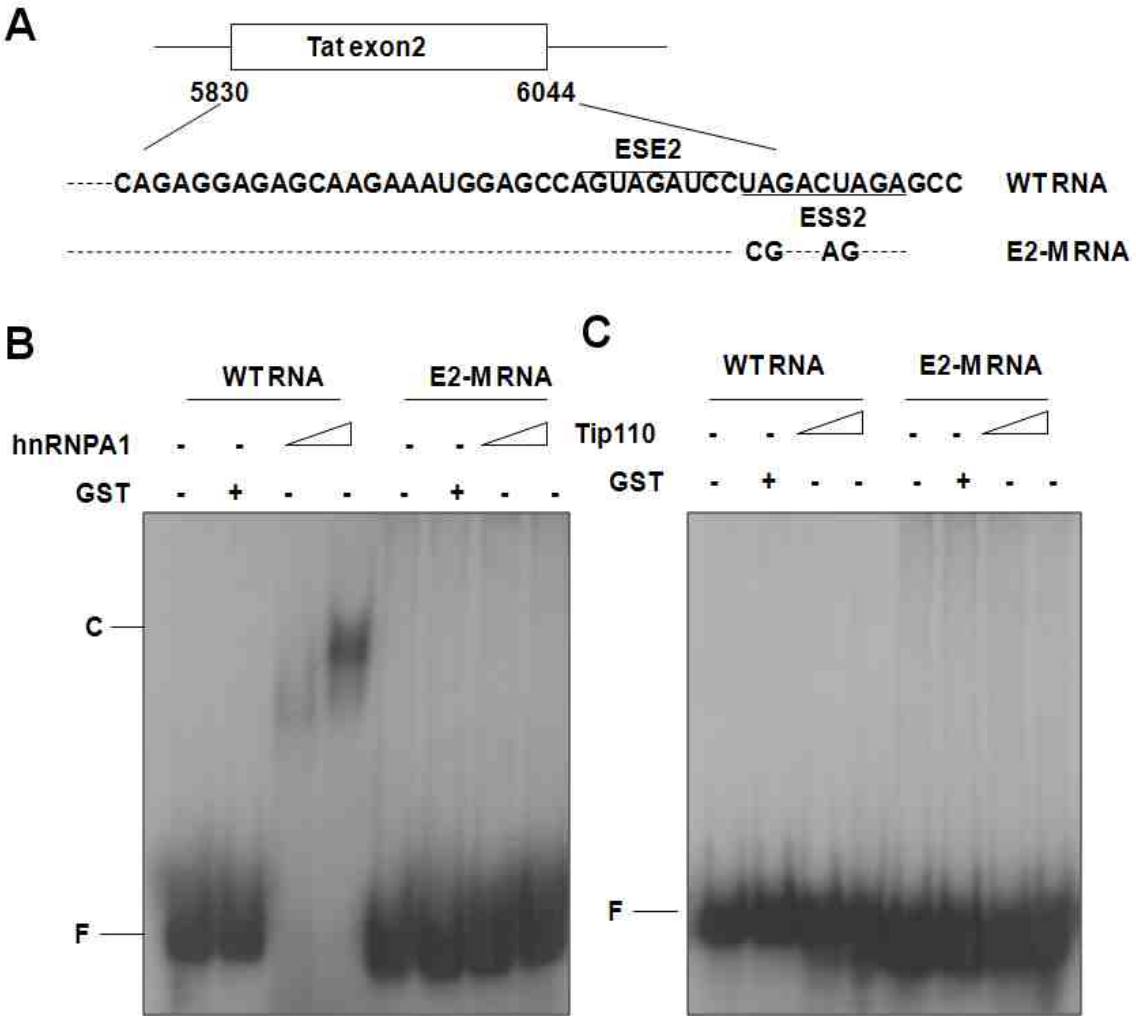

C
CDNA3
Tip110
α-GFP
α-Tip110
Purify Tip110-HA protein by anti-HA-affinity matrix column
CDNA3
Tip110
Tip110
CDNA3
Tip110
hnRNPA1
-GFP

Figure 34. Tip110 interaction with hnRNP A1 protein *in vivo* (A) Cell lysates from 293T cells transfected with 10μg pTip110-HA and 10μg phnRNPA1-GFP were immunoprecipitated with Tip110 *(left panel)*, anti-HA antibody *(right panel)* or mouse IgG followed by Western blotting against GFP. Western blotting for Tip110 or HA against Tip110 or HA immunoprecipitates was also performed as the positive control. (B) Cell lysates were first immunoprecipitated with anti-hnRNP A1 or anti-GFP, followed by Western blotting for Tip110. Western blotting with anti-hnRNP A1 or anti-GFP against hnRNP A1 immunoprecipitates were performed as the control. (C) 293T cells (5x10cm plates) were transfected with phnRNPA1.GFP and pTip110.HA. hnRNPA1.GFP and Tip110.HA expression were first verified by Western blotting analysis with anti-Tip110 and anti-GFP antibody *(upper panel)*. The cell lysate were passed through anti-HA-affinity matrix column; bound proteins were eluted and analyzed by Western blotting for Tip110 or hnRNPA1-GFP (*lower panel)*.

2.10 Tip110 blocks the interaction between hnRNP A1 and ESS2 *cis*-element

The most important parameter for site A3 activation and *tat* exons 1-2 splicing is binding of SC 35 or SRp 40 to ESE2 and subsequent displacement of hnRNP A1 from ESS2 (Hallay, Locker et al. 2006). Thus, we speculated that Tip110 functioned to activate *tat* exons 1-2 splicing through its interaction with hnRNP A1 and as a result, impeded binding of hnRNP A1 to ESS2. To test this possibility, gel shift assay was performed to determine the interaction between hnRNP A1 and WT RNA in the presence of Tip110. As before, hnRNP A1 complexed with WT RNA probe (Fig. 36A, *lane 2*). But when purified recombinant GST-Tip110 protein was included, there was a dose-dependent inhibition in the formation of the hnRNP A1-WT RNA complex (Fig. 36A, *lanes4-6*). In contrast, GST protein showed no effects in the complex formation (Fig. 36A, *lane 3*). These results demonstrated that Tip110 impeded the complex formation between hnRNP A1 and ESS2. To further confirm this inhibition effect *in vivo*, supershift assay was performed with nuclear extracts and increasing amount of Tip110-GST protein. The supershifted complex was detected by adding anti-hnRNP A1 antibody (Fig. 36B, *lane4*) and decreased with increasing amounts of Tip110-GST protein (Fig. 36B, *lanes 6-8*). These results further confirmed that Tip110 blocked the complex formation of hnRNP A1 with *tat* ESS2 and suggested the interaction among Tip110, hnRNP A1 and *tat* ESS2 likely accounted for Tip110-mediated *tat* exons 1-2 splicing.


A
Tat exon2
5830
6044
ESE2
CAGAGGAGAGCAAGAAAUGGAGCCAGUAGAUCCUAGACUAGAGCC
WT RNA
ESS2
CG----AG
E2-M RNA
B
WT RNA
E2-M RNA
hnRNPA1
GST
C
F
C
WT RNA
E2-M RNA
Tip110
GST
F

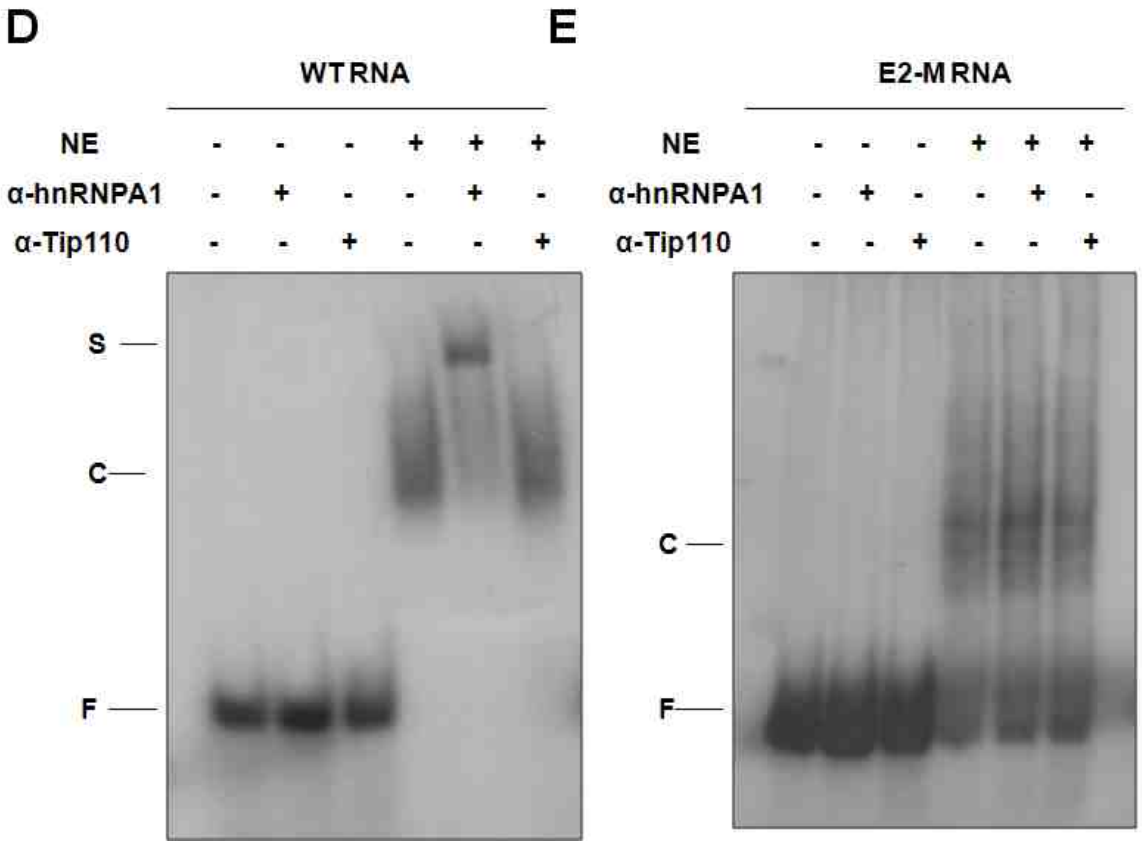
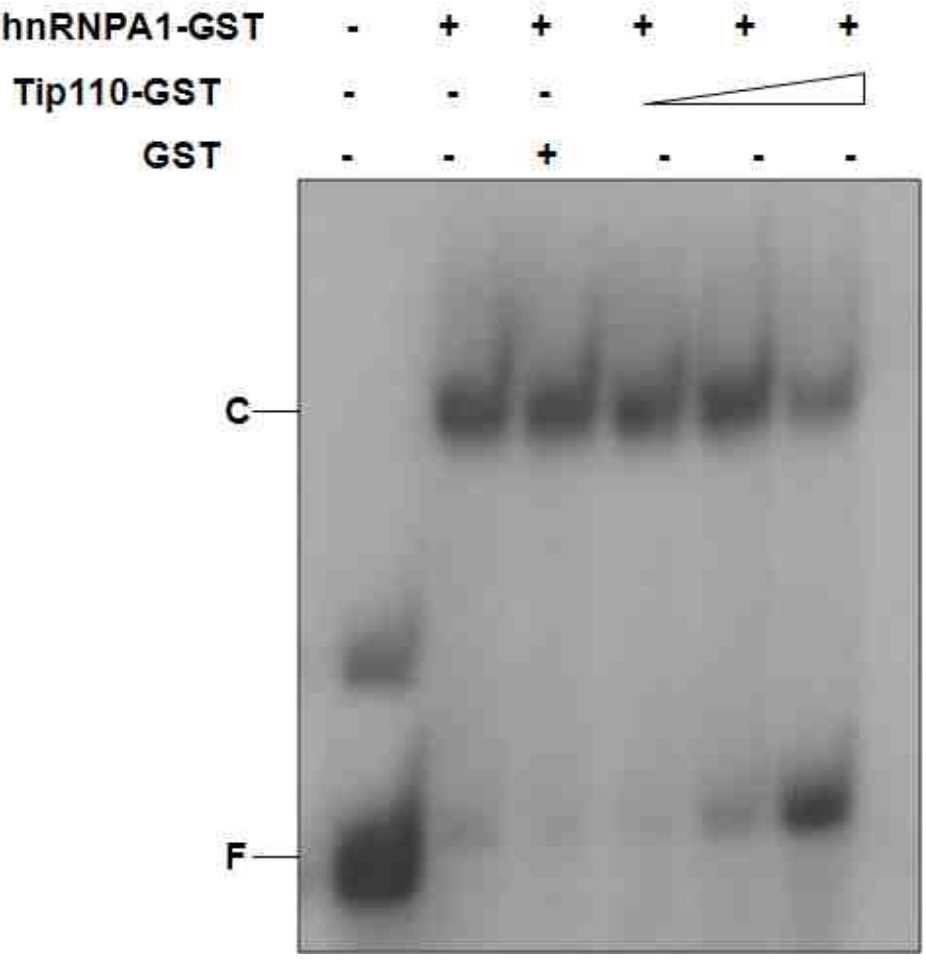

D
WT RNA
NE - - - + + +
α-hnRNPA1 - + - - + -
α-Tip110 - - + - - +
S
C
F
E
E2-M RNA
NE - - - + + +
α-hnRNPA1 - + - - + -
α-Tip110 - - + - - +
C
F

Figure 35. No interaction between Tip110 and ESE2/ESS2 RNA (A) The WT RNA and ESE2 (E2-M) RNA used for gel shift assay. (B) About 10 pmoles (20000cpm) of WT RNA or E2-M RNA probes were incubated in a 20μl reactions containing 0, 50,100 ng hnRNPA1-GST or 200ng GST protein. (C) 0,100,200ng of Tip110-GST or 200ng GST protein was incubated with WT RNA or E2-M RNA. (C) and (D) Nuclear extract (1 μl) was used in place of recombinant proteins to bind WT or E2-M RNA on ice for 15min. Then, 1 μl anti-hnRNP A1 (4B10) or anti-Tip110 (1C4H6) was added to the reaction and continued for 15 min on ice, followed by gel electrophoresis. As negative controls, RNA was only incubated with antibodies but without nuclear extract. F: free probe; C: RNA-protein complex; S: RNA-protein-antibody supershifted complex.

A



B

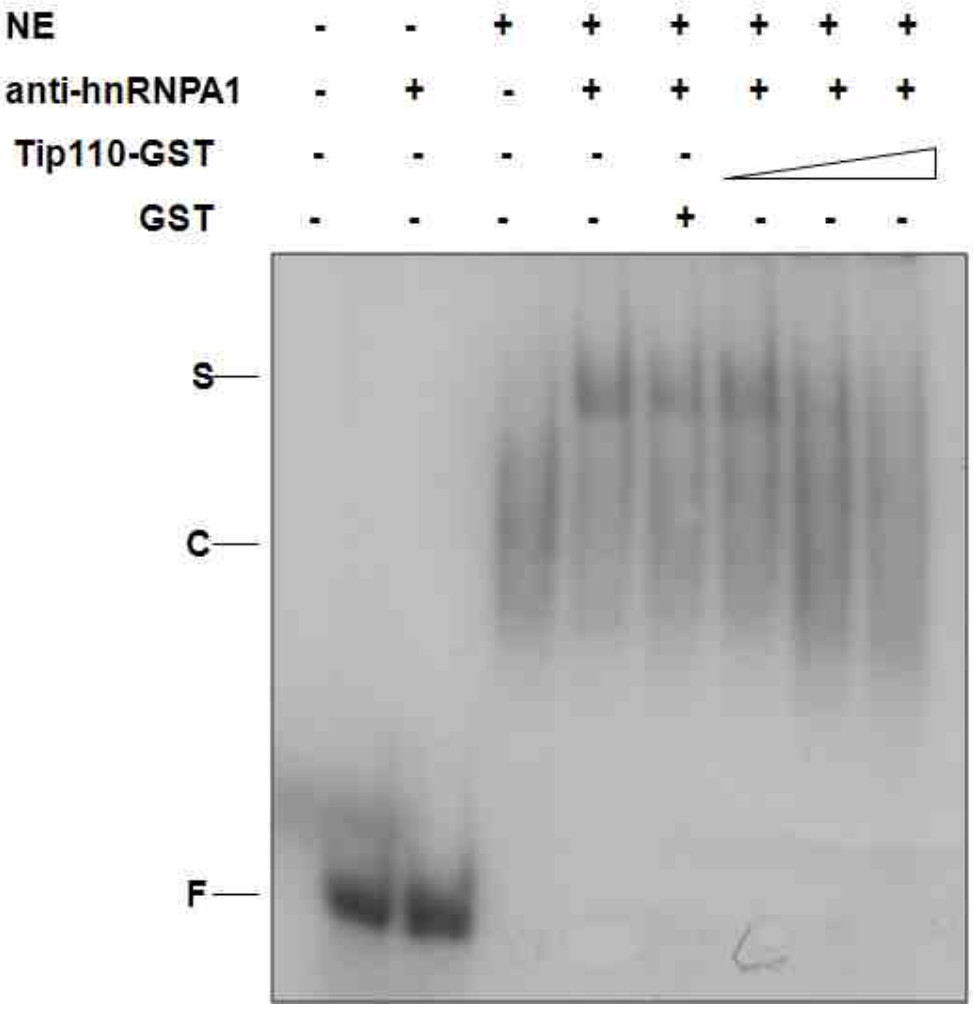


Figure 36. Inhibition of complex formation between hnRNP A1 and ESS2 by Tip110

(A) hnRNP A1-GST protein (100 ng) was incubated with 10 pmoles (20000 cpm) of ^{32}P-labeled WT RNA probes in the presence of and 0 ng *(lane2),* 50 ng (*lane 4*), 100 ng (*lane 5*), or 200 ng (*lane 6*) recombinant Tip110-GST protein or 200 ng GST protein *(lane 3),* and the complex formation of hnRNP A1 and WT RNA was determined by gel shift assay. RNA probe only was detected in the absence of hnRNPA1-GST protein (*lane 1*). (B) 1μl nuclear extract with 200 ng GST *(lane 5),* or 0 ng *(lane 4),* 50 ng *(lane 6),* 100 ng *(lane 7),* 200 ng *(lane 8)* were incubated with 10 pmoles ^{32}P-labeled WT RNA followed by adding 1μl anti-hnRNP A1 into the reaction. The RNP complex formed without adding anti-hnRNP A1 *(lane 3)* or RNA incubated with only antibody *(lane 2)* or RNA only *(lane1)* were included as controls. F: free probe; C: RNA-protein complex; S: RNA-protein-antibody supershifted complex.

DISCUSSION

Summary of the results

The transcriptional activation of the HIV-1 LTR promoter is a complex series of events and needs coordinated interactions of viral and cellular proteins. HIV-1 Tat is the key transactivator of HIV-1 transcription and promotes the formation of transactivational complexes at the LTR promoter. Besides Tat, a large number of cellular factors are also involved in the transcription. These factors either function by removing inhibitory molecules which block RNAPII phosphorylation in the LTR promoter or recruiting the elongation competent RNAPII-containing complexes to the LTR promoter (Baillat, Hakimi et al. 2005; Vaness Bres Nathan Gomes and Jones 2005; Vardabasso, Manganaro et al. 2008)

In this study, we focused on Tat-interacting Protein of 110kDa, Tip110. Studies from our group have shown that Tip110 synergies with Tat in transactivation of the HIV-1 LTR promoter, increases viral gene expression and virus production (Liu, Li et al. 2002). However, the underlying molecular mechanisms were not understood and are the focus of the dissertation research.

We first confirmed that Tip110 significantly enhanced HIV-1 gene expression and viral production. This enhancement was at least in part due to their synergetic transactivation of the LTR promoter transactivation (Fig. 7). HIV-1 gene expression from proviral DNA transfection did not alter endogenous Tip110 expression (Fig. 8). To examine how

Tip110 enhanced Tat-mediated LTR transactivation, we then determined if Tip110 interacted with known transcription factors involved in activation of HIV-1 LTR promoter. We found that Tip110 was complexed with unphosphorylated form (RNAPIIa) but not phosphorylated form (RNAPIIo) of RNAPII (Fig. 10 & 11). The specific and direct interaction between Tip110 and RNAPIIa was confirmed by GST pull-down assay and mutagenesis analysis (Fig. 12 & 13). Although Tip110 did not interact with cyclin T1 or CDK9 (Fig. 14 & 15), it interaction with Tat and RNAPIIa led to more P-TEFb recruitment to the transcription complex (Fig. 16). Since P-TEFb was responsible for RNAPII serine 2 phosphorylation, we further found that RNAPII serine 2 phosphorylation was increased when Tip110 and Tat were co-expressed (Fig. 17). ChIP assays confirmed that Tip110 was present within the HIV-1 LTR promoter and associated with increased recruitments of P-TEFb to the promoter, which further led to the increased phosphorylation of serine 2 within the RNAPII CTD at the LTR promoter (Fig. 19 & 20). Consistent with these findings, we showed that Tip110 expression was direct correlated with transcription elongation of the LTR promoter (Fig. 21 & 22).

During these studies, we noticed that Tip110 expression let to increased *tat* mRNA production (Fig. 23 & 24), as well as Tat protein expression (Fig. 25). *in vivo* splicing assay with *tat* minigene showed that Tip110 preferentially increased *tat* exons 1-2 splicing by activating HIV-1 A3 splicing site but had no function in *tat* exons 2-3 splicing (Fig. 27 & 28), and that the preferential activity of Tip110 was mediated by ESS2 but was not involved interaction between Tip110 and ESE2 or ESS2 RNA(Fig. 35).

Affinity purification followed by 2D Mass from a separate study in our lab indicates hnRNP A1, a negative splicing regulator binding to ESS2 *cis*-element to complex with Tip110 (Table 2). Thus, we performed additional experiments and confirmed this interaction both *in vitro* and *in vivo* (Fig. 33 & 34). Gel shift assay showed that Tip110 binding to hnRNP A1 blocked the formation of hnRNP A1-ESS2 complex (Fig. 36), and this likely accounts for Tip110-association activation of A3 splice site and preferential expression of *tat* mRNA and Tat. Taken together, our studies suggest that Tip110 plays important roles in both LTR transcription elongation and HIV-1 pre-mRNA splicing and as a result regulates HIV-1 viral gene expression and viral replication

The interaction between Tip110 and transcription factors

A large number of cellular factors are involved in the Tat-mediated transcription by recruiting the elongation competent RNAPII-containing complexes and/or interacting with multiple transcription factors. For example, SKIP, Tat-SF1 and CA150 are associated with Tat:P-TEFb in nuclear extracts and present in large RNAPII elongation complexes (Zhou and Sharp 1996; Carty, Goldstrohm et al. 2000; Vaness Bres Nathan Gomes and Jones 2005). Tip110 was first identified as a Tat interacting protein through the yeast two-hybrid screening of a human fetal brain cDNA library, with Tat as bait. Further studies have confirmed this direct interaction by immunoprecipitations well as GST pull-down assay (Liu, Li et al. 2002).

We found that Tip110 bound to unphosphorylated RNAPII (CTDa) but not its phosphorylated form (CTDo) by immunoprecipitation followed by Western blotting (Fig.

10 & 11). This direct and specific binding was further supported by the GST pull-down assay and mutagenesis analysis and recruited the HAT domain of Tip110 (Fig. 12 & 13). Protein structure analysis predicted that the N-terminal two-thirds of the Tip110 protein contain seven HAT motifs. These HAT motifs provide a structural unit of two antiparallel α-helices that form functional TPR(s) that determine the specificity of protein-protein interaction (Blatch and Lassle 1999). Besides binding to unphosphorylated RNAPII, the HAT domain is also responsible for Tip110 interaction with Tat (Liu, Li et al. 2002). It is likely that Tip110 formed a complex with Tat and unphosphorylated RNAPII through the same N-terminal domain to stabilize the mega transcription complex. In addition, HAT domain is involved in Tip110 interaction with a C-terminal region of the U4/U6 snRNP-specific 90K protein which functions in the reassembly of the U4/U6 snRNP(Bell, Schreiner et al. 2002; Medenbach 2004). The HAT domain is also involved in Tip110 interaction with RNPS1 (Harada, Yamada et al. 2001). These results confirm that suggesting HAT domain controls the specificity of Tip110 protein-protein interaction.

The recruitment of P-TEFb to the transcription complex by Tip110 and Tat

Although Tip110 and P-TEFb are co-localized in the nuclear speckle area (Herrmann and Mancini 2001), our data showed no interaction between Tip110 and P-TEFb (Fig. 14 & 15). This phenomenon distinguishes Tip110 with other cellular transcription factors such as SKIP , which associates with P-TEFb and is recruited to the LTR promoter by Tat (Vaness Bres Nathan Gomes and Jones 2005); or ELL2, a newly found elongation factor in the P-TEFb complex and Tat functions to bring more ELL2 to P-TEFb and helps to

stabilize ELL2 and active P-TEFb (He, Liu et al. 2010). There is no direct interaction between Tip110 and P-TEFb (Fig. 14 & 15), but Tip110 is capable of recruiting more P-TEFb to the transcription complex in the presence of Tat (Fig. 16). Therefore, we speculate that Tip110 is first recruited to the unphosphorylated RNAPII on the LTR promoter in the absence of Tat and then interacts with Tat to recruit more P-TEFb, and becomes dissociated from the transcription elongation complex following P-TEFb phosphorylation of RNAPII.

Since P-TEFb is responsible for RNAPII serine 2 phosphorylation, we set out to investigate whether Tip110 would change the RNAPII phosphorylation level. Our data showed that Tip110 protein alone decreased the level of unphosphorylated RNAPII while increased the level of both serine 2 and serine 5 phosphorylated RNAPII by 2 fold, suggesting that Tip110 was a weak transactivator for HIV-1 LTR basal level transcription (Fig. 17). When Tip110 and Tat were co-expressed, both serine 2 and serine 5 phosphorylations of RNAPII were considerably increased, with a more decrease in unphosphorylated RNAPII (Fig. 17). P-TEFb also regulates expression of several cellular genes at the transcription elongation stage, including hsp70, the proto-oncogenes *c-myb, c-myc* and *c-fos* (Rougvie and Lis 1988; Krumm, Meulia et al. 1992; Roberts, Purton et al. 1992). The RNAPII complexes are stalled in the 5' region of the transcription unit for these genes, P-TEFb recruitment is the key regulator that helps RNAPII to overcome this rate-limiting step. TATA-box instead of TAR structure is important for the recruitment of P-TEFb (Montanuy, Torremocha et al. 2008). Therefore, the increased phosphorylated form of RNAPII may result from the recruitment

of P-TEFb by Tip110 and Tat to the promoters of these cellular genes. In order to examine the RNAPII phosphorylation on the LTR promoter, we can label LTR-promoter templates 5' end with biotin and then isolated LTR bounded PICs to detect protein components of the LTR PIC (Zhou, Halanski et al. 2000). By this method, we would expect to see more increase in RNAPII phosphorylation located on the HIV-1 LTR promoter in the presence of Tip110 and Tat.

Tip110 is present on the LTR promoter

HIV-1 transcription is regulated by interplay between a combination of viral and cellular transcription factors with binding sites in the HIV-1 LTR promoter. With ChIP assay, we first detected Tip110 on the transient LTR promoter by transfecting pNL4-3 into 293T cells (Fig. 19). To further examine if Tip110 was recruited to the integrated LTR promoter in the absence of Tat, we performed ChIP assay in U373-MAGI or CEM-GFP cells. In these cell lines, either the LTR promoter-driven Lac Z reporter gene (U373-MAGI) or the LTR promoter-driven GFP reporter gene (CEM-GFP)is stably integrated into the chromosome (Gervaix, West et al. 1997; Brockman, Tanzi et al. 2006; Sundaravaradan, Das et al. 2007). The results showed that endogenous Tip110 was present on the integrated LTR promoter in the absence of Tat (Fig. 19), indicating that the Tip110 was recruited to the LTR promoter.

The ChIP assay also showed that P-TEFb were only recruited to the LTR promoter only when Tat was expressed and showed a significant increasing with Tip110 expression (Fig. 19). These results were consistent with previous Western blot result that more P-TEFb

were recruited to the transcription complex in the presence of Tip110 (Fig. 16). We observed 3 fold increases of cyclin T1 compared to 1.5 fold increase of CDK9, which was probably because Tat directly interacted with Tip110 and cyclin T1 and mediated Tip110 recruitments of cyclin T1. Besides P-TEFb, we further examined the RNAPII phosphorylation level at the LTR promoter by ChIP assay. Ser 2 phosphorylation of RNAPII only occurred in the presence of Tat and increased by about 2 folds with Tip110 overexpression, which may result from the increased recruitment of P-TEFb by Tip110 and Tat (Fig. 16). Unphosphorylated and serine 5 phosphorylated RNAPII were readily detected at the HIV-1 promoter in the absence of Tat, while Tip110 with Tat only had slightly effect on the serine 5 phosphorylation (Fig. 16), which was consistent with previous studies that TFIIH is recruited to the LTR promoter for RNAPII serine 5 phosphorylation in the absence of Tat (Chen and Zhou 1999).

Mechanisms of Tip110 function in Tat-mediated LTR transactivation

What might be the actual mechanism by which Tip110 facilitates Tat transactivation? Firstly, overexpression of Tip110 significantly increased the viral gene expression and viral replication; this enhancement might due to the role of Tip110 in LTR transactivation by stimulating Tat-induced RNAPII phosphorylation. Secondly, Tip110 formed a complex with Tat, unphosphorylated RNAPII CTD, and was detected on the LTR promoter in the absence of Tat. Moreover, P-TEFb in the transcriptional complex was greatly increased in the presence of Tip110 and Tat. On the basis of these observations, we proposed a working model of the function of Tip110 in Tat-mediated LTR transcription (Fig. 37). Tip110 was first recruited to the LTR promoter independently

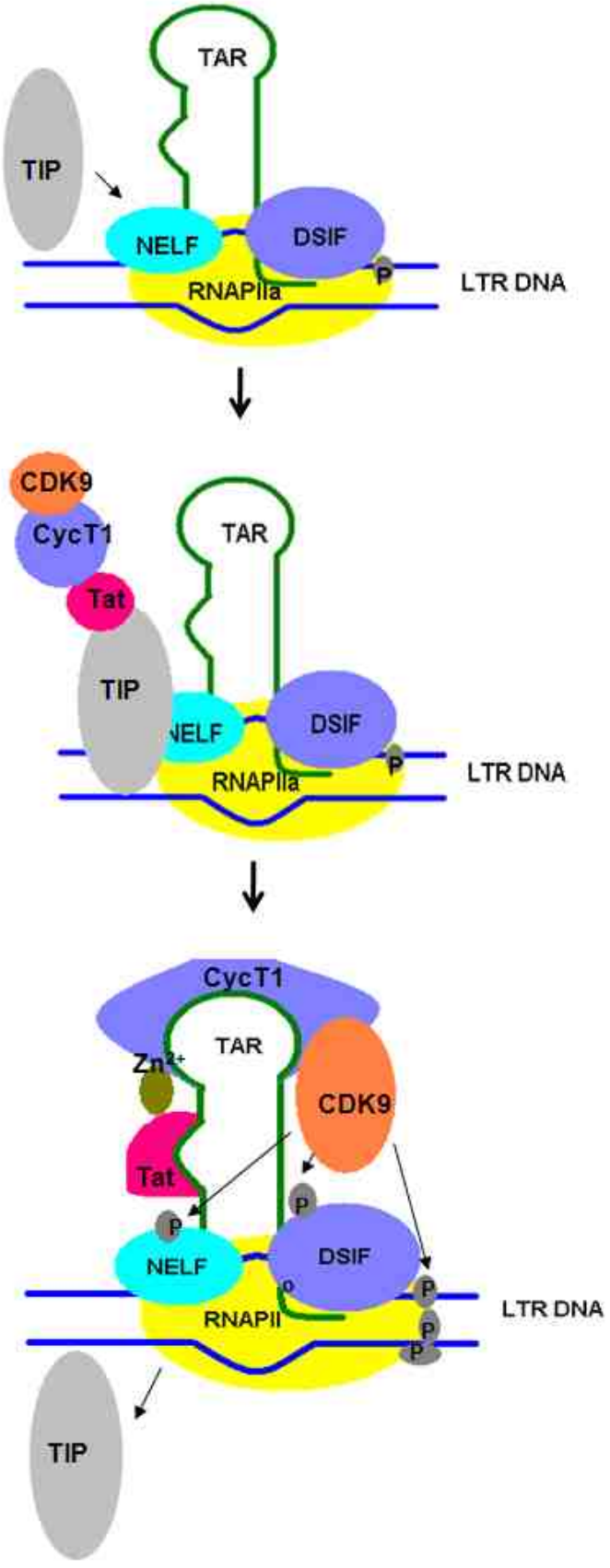

TAR
TIP
NELF
DSIF
P
RNAPIIa
LTR DNA
CDK9
CycT1
Tat
TAR
TIP
NELF
DSIF
P
RNAPIIa
LTR DNA
CycT1
TAR
Zn2+
CDK9
Tat
P
P
NELF
DSIF
P
P
RNAPII
LTR DNA
P
P
TIP

Figure 37. A working model for Tip110 function in HIV-1 LTR transactivation

1. When unphosphorylated form of RNAPII is blocked with NELF and DSIF, Tip110 is first recruited to the LTR promoter, and bound to unphosphorylated RNAPII. 2. At the LTR promoter, Tip110 forms a complex with unphosphorylated RNAPII and the newly translated Tat protein. Tip110 recruits more cyclin T1 and CDK9 to the transcription complex. 3. When RNAPII serine 2 is fully phosphorylated, LTR transcription elongation step starts and the full-length HIV-1 mRNA is produced. At this step, Tip110 leaves the hyperphosphorylated form of RNAPII to start a new round of transactivation.

with Tat. It formed a complex with unphosphorylated RNAPII and Tat and recruited more cyclin T1 and CDK9 to the transcriptional complex, which would further enhance the RNAPII serine 2 phosphorylation. When RNAPII serine 2 was fully phosphorylated, LTR transcription elongation would start and the full-length mRNA would was synthesized. At this stage, Tip110 was about to leave the hyperphosphorylated form of RNAPII and would be recycled to start a new round of transactivation.

However, we cannot rule out the possibilities that Tip110 regulates HIV-1LTR transcription via other transcription factors. A separate study from our group has showed that Tip110 interacted with YB-1, a cellular ssDNA/RNA binding protein that interacts with both Tat and TAR and increases the level of Tat: TAR association (Sawaya, Khalili et al. 1998; Ansari, Safak et al. 1999). Tip110, together with YB-1, has been shown to greatly increase LTR transactivation (manuscript in preparation), which implies that the interaction of Tip110 and other transcription factor may also contribute to Tip110 effects in Tat-mediated transcription.

Also, it will be important to compare the results obtained here in 293T cells with the more complex transcription factor interactions that occur on the native HIV-1 genome in activated T cells, where both initiation and elongation are further up-regulated by enhancer factors such as NF-κB and NF-AT. Because our experiments were designed to analyze the transcription factors that are most directly involved in Tat transactivation, these results should provide a useful comparative framework for similar studies based on the induced viral genome in activated T cells.

The role of Tip110 in HIV-1 alternative splicing

Pre-mRNA splicing is coupled to RNAPII transcription elongation. Many splicing factors have shown to play a role in HIV-1 transcription elongation. Therefore, we examined whether Tip110 plays a role in HIV-1 alternative splicing. Using the multiplex PCR, we obtained the first evidence that Tip110 altered the HIV-1 mRNA splicing pattern (Fig. 23). Normalized to the equal amount of GAPDH transcripts, one product that was derived from *tat* exon 2 showed an increase intensity (Fig. 23). Four *tat* transcripts are generated from HIV-1 mRNA splicing and all contain *tat* exon 2 (Fig. 4). So we further performed RT-PCR to check which specific form of *tat* transcripts is increased by Tip110. The primer TAR 5' and Tat 21 spanned from the end of *tat* exon 1 to the beginning of *tat* exon 2 (Fig. 24A), so we expected to see multiple bands according to the inclusion and exclusion of introns between exon1 and 2 in different *tat* transcripts. However, due to the predominant amount of *tat 1* transcript in cells, only one single band was amplified by TAR 5'/Tat 21(Fig. 24C), and this transcripts showed 1.6-fold increase in the presence of Tip110.

As Tat plays an important role in virus replication, it would be conceivable to speculate that HIV-1 has evolved a regulatory mechanism for an appropriate level of Tat expression. The major *tat* transcripts contain 3 exons. The exon 2 is spliced between site A3 and D4, its splicing is mainly controlled by ESS2 splicing silencer and ESE2 enhancer (Si, Amendt et al. 1997), and partly regulated by ESS2p located upstream of ESS2 (Jacquenet, Mereau et al. 2001). Exons 2-3 splicing is regulated by ESS3 and ESE3 located in *tat* exon3 (Tange, Damgaard et al. 2001). The traditional method to test *tat* splicing

efficiency is to perform *in vitro* splicing assay with ^{32}P-labled splicing RNA substrate (Si, Amendt et al. 1997; Si, Rauch et al. 1998). In this study, we designed a *tat* minigene under the control of the CMV promoter and performed RT-PCR of the spliced and unspliced RNA to monitor *in vivo* RNA splicing. This new method allowed us to determine the *in vivo* relationship between Tip110 expression and the *tat* minigene. The results showed that Tip110 specifically increased *tat* exon1-2 splicing but had no effects on *tat* exons 2-3 splicing (Fig. 26 &27). Since *tat* exon 2 splicing between A3 and D4 splice sites is specifically for *tat* transcripts, while exon 3 splicing from A7 splice site is shared by all the multiply spliced transcripts, Tip110 specific activation on A3 splice site could explain why Tip110 preferentially increased *tat* transcripts but not *rev* or *vpr* mRNAs.

The interaction between Tip110 and hnRNPA1

A separate study from our group demonstrated that Tip110 forms a complex with hnRNP A1 (Table 2). We confirm this interaction both *in vivo* and *in vitro* in this study (Fig. 33 & 34) hnRNP A1 has been demonstrated as the major regulator of HIV-1 pre-mRNA site A3 splicing. It binds to ESS2 *cis*-element in *tat* exon 2, blocks the association of U2AF35 and inhibits early step of spliceosome assembly (Si, Amendt et al. 1997; Caputi, Mayeda et al. 1999). Thus, for A3 activation, hnRNP A1 has to be dissociated from ESS2, followed by SC35 or SRp 40 binding to ESE2 (Ropers, Ayadi et al. 2004; Hallay, Locker et al. 2006). Thus, we speculate that Tip110 may function in HIV-1 A3 site activation through its interaction with hnRNP A1. However, the interaction between hnRNP A1 and Tip110 could be only in an enrichment assay, but not by the routine

IP/WB method (Fig. 34). The discrepancy might be result from that the antibodies for immunoprecipitation; or that *in vivo* interaction between Tip110 and hnRNP A1 might be weak or transient; or thatTip110 competed with other protein for hnRNP A1 binding.

Further study is clearly needed to identify the domains of hnRNP A1 and Tip110 that are directly involved in the Tip110/hnRNP A1 interaction. As the C-terminal Gly domain of hnRNP A1 mediates the interaction between hnRNP A1 and different RNA-binding proteins (Cartegni, Maconi et al. 1996) and HAT domain is important for Tip110 protein interaction (Harada, Yamada et al. 2001; Liu, Li et al. 2002), we speculated that the N-terminal HAT domain of Tip110 and the C-terminal Gly domain of hnRNP A1 might be involved in the Tip110/hnRNP A1 interaction. Moreover, the interaction between Tip110 and hnRNP A1 might inhibit hnRNP A1 association with ESS2, as hnRNP A1 forms a stable complex with ESS2 element through its C-terminal Gly domain (Zahler, Damgaard et al. 2004).

The mechanisms of Tip110 function in regulating *tat* mRNA splicing

Tip110 was first identified as a splicing factor that specifically binds to U6 and was required for U4/U6 annealing recycling (Gu, Shimba et al. 1998; Bell, Schreiner et al. 2002). Therefore, we first hypothesized that Tip110 enhanced *tat* exons 1-2 splicing by accelerating snRNP recycling and promoting spliceosome assembly at splice site A3. Although Tip110 contains two RRM domains, it does not interact with either ESS2 or ESE2 element in *tat* exon 2 (Fig. 35). Surprisingly, when we determined if hnRNP A1 mediated Tip110 effect on A3 splice site activation, we found that Tip110 blocked the

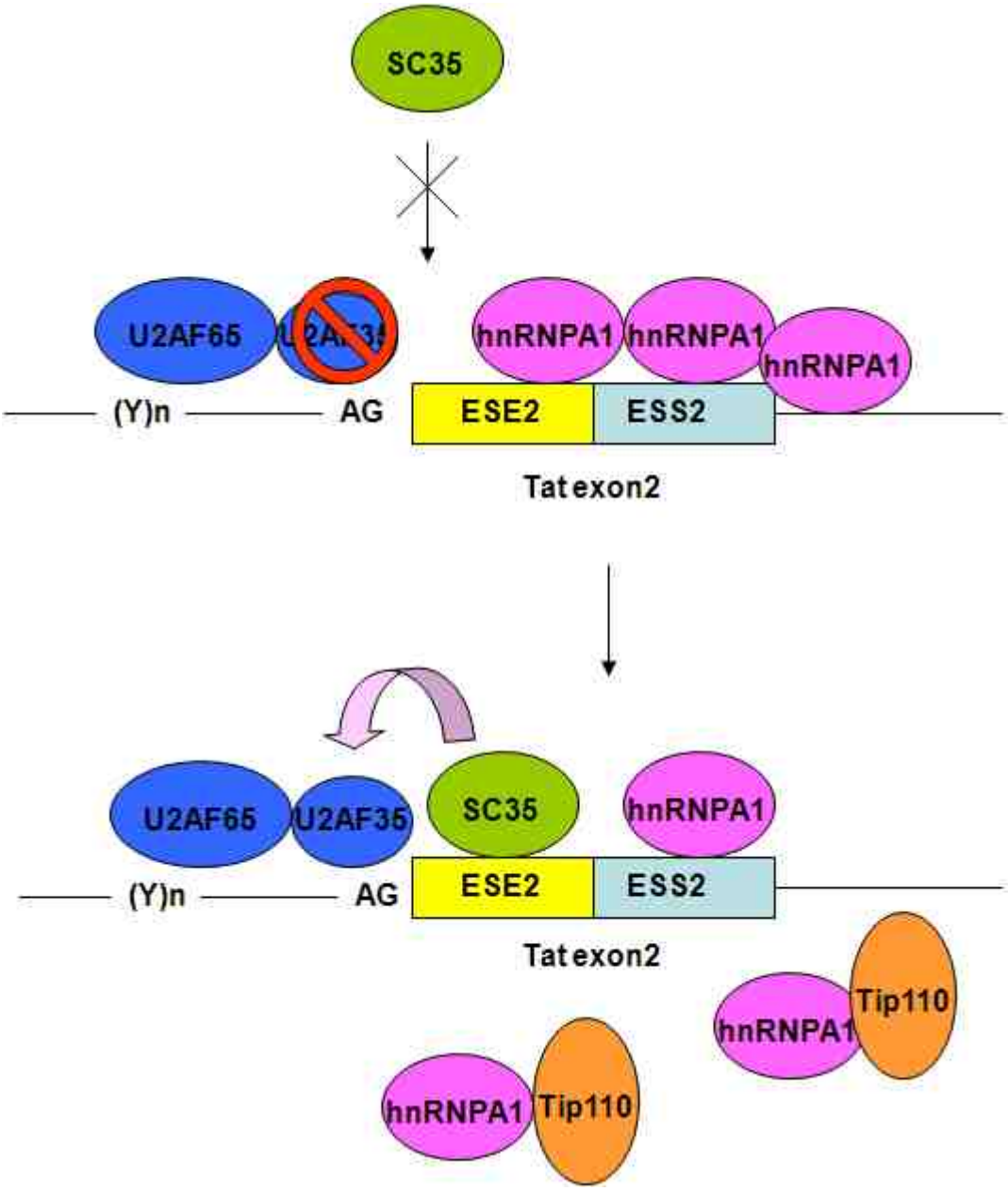

SC35
U2AF65
hnRNPA1
hnRNPA1
hnRNPA1
(Y)n
AG
ESE2
ESS2
Tat exon2
U2AF65
U2AF35
SC35
hnRNPA1
(Y)n
AG
ESE2
ESS2
Tat exon2
hnRNPA1
Tip110
hnRNPA1
Tip110

Figure 38. A working model for Tip110 function in HIV-1 3'ss A3 activation

1. The multimerization of hnRNP A1 to the ESS2 masks the binding site of SC35 on ESE2, inhibits the association of U2AF35 to the A3 splicing site, and further blocks the U2AF65 to the PPT site. Thus, the early step of spliceosome assembly is inhibited and A3 splicing site is inactivated. 2. In the presence of Tip110 protein, Tip110 directly binds hnRNP A1 and blocks the binding site of hnRNP A1 to ESS2 element, results in the dissociation between hnRNP A1 and ESS2. Then, SC35 or SRp40 protein will bind to ESE2 element; increase the U2AF binding efficiency to the PPT. These SR proteins counteract the negative effect of hnRNP A1 and activate the A3 splice site.

hnRNP A1 interaction with ESS2 element (Fig. 36). Based on these observations, we speculated that hnRNP A1 was first recruited to theESS2 *cis*-element (Fig. 38). The multimerization of hnRNP A1 at the ESS2/ESE2 masks the SR protein binding site within ESE2, inhibits the association of U2AF35 to the A3 splicing site, blocks the U2AF65 to the PPT Site, and eventually inhibits the association of U2snRNP with the branch site. Therefore, the early step of the spliceosome assembly is inhibited and A3 splice site is inactivated. In the presence of Tip110, Tip110 binds to hnRNP A1 and blocks the binding site of hnRNP A1 from ESS2 element. This will trigger the dissociation of hnRNP A1 withESS2 *cis*-element, which will further expose the ESE2 *cis*-element and recruit SC35 or SRp40 to the ESE2. Binding of SR proteins will counteract the negative effect of hnRNP A1 by increasing U2AF binding efficiency to the PPT and activating the A3 splicing site.

However, Tip110 may also activate the *tat* A3 splicing through other splicing factors. It has been reported that Tip110 binds to RNPS1 and stimulates the proximal alternative 3' splicing of calcitonin-dhfr pre-mRNA minigene with RNPS1 and RNPS1 itself has been shown to activate *tat* minigene splicing (Mayeda, Badolato et al. 1999; Harada, Yamada et al. 2001). Also, our group has identified that Tip110 interacted with YB-1 protein and increased CD44 alternative splicing (manuscript in preparation). But YB-1 function in splicing has not been applied to HIV-1 alternative splicing. Thus, further study would explore whether Tip110 interaction with RNPS1 or YB-1 would have any effects on HIV-1 alternative splicing. Also, the *tat* minigene used in this study is driven by CMV promoter instead of HIV-1 LTR promoter, it will be interesting to learn whether Tip110 interaction with HIV-1 LTR promoter *in vivo* will help to recruit other spliceosome

components, such as U4 and U6 snRNP to the full-length pre-mRNA and further activate HIV-1 alternative splicing.

Tip110 function in HIV-1 transcription and pre-mRNA splicing

Eukaryotic pre-mRNA splicing is tightly coupled to transcription, mainly through phosphorylated RNAPII CTD, which interacts with a numbers of splicing factors (Mortillaro, Blencowe et al. 1996; Kim, Du et al. 1997; Ge, Si et al. 1998). Cellular factors Tat-SF1, CA150 and SKIP have been reported to function in both transcription and alternative splicing in HIV-1 gene expression and replication. Tat-SF1 is required for efficient transcriptional transactivation by binding to P-TEFb and also interacts with spliceosomal components. The TAT-SF1-snRNP-containing couple complex stimulates transcription as well as promotes splicing (Zhou and Sharp 1996). CA150 is a transcription-splicing coupling factor and inhibits Tat-mediated HIV-1 viral transcription through its interaction with splicing factors and RNAPII (Zhou and Sharp 1996; Fong and Zhou 2000; Kameoka, Duque et al. 2004). Therefore, the function of Tat-SF1 and CA150 in HIV-1 transcription elongation and alternative splicing is coupled. SKIP interacts with Tat:P-TEFb complexes, acting through nascent RNA to overcome pausing by RNAPII. It also interacts with U5snRNP proteins and tri-snRNP110K and facilitates recognition of an alternative Tat-specific splice site *in vivo*. However, U5snRNPs recruit to SKIP only within transcribed regions instead of LTR, indicating SKIP activates HIV-1 transcription independently of the U5snRNPs, and engages in transcription and splicing process at different transcription elongation stages (Vaness Bres Nathan Gomes and Jones 2005).

Tip110 regulates both Tat-mediated LTR transcription and HIV-1 alternative splicing and has positive effect on HIV-1 gene expression and viral replication. However, our study suggested that Tip110 is recruited to the LTR promoter and released from phosphorylated RNAPII when transcription elongation starts. Therefore, Tip110 would not stay with phosphorylated RNAPII throughout the transcription process. However, Tip110 has to be relocated to *tat* exon2and regulate *tat* mRNA splicing at A3 splice site for its splicing effect, indicating that Tip110 functions in transcription and splicing are spatially and temporally regulated. Moreover, in the *in vivo* splicing assay, the *tat* minigenes are driven from the CMV promoter rather than the LTR promoter; and Tat, which is critical for Tip110 function in transcription, is not expressed in 293T cells that were used in these studies. Based on these observations, Tip110 may engage transcription and splicing complexes in different stages in the elongation process. On the other hand, Tip110 function in HIV-1 transcription elongation and splicing process is not completely independent. Our data suggested that Tip110 enhanced Tat expression through alternative splicing, which would in turn binds to Tip110 to recruit more P-TEFb to the LTR promoter. With the direct effects on LTR transcription elongation, Tip110 and Tat will help to produce more full-length mRNA for alternative splicing. Thus, Tip110 provides a positive feedback in Tat protein expression by HIV-1 RNA splicing and HIV-1 LTR transactivation in HIV-1 gene expression and virion replication.

PERSPECTIVE

Since the introduction of HAART therapy in 1990's, the AIDS pandemic has been changed from a uniformly fatal disease to a chronic disease. However, HARRT cannot eradicate the virus from the body and there are still latent virus reservoirs. Moreover, even though HAART can reduce viral replication to undetectable levels, active viral replication is continuing in lymphoid tissue of the gut. The cost of these drugs has been a significant burden to both individuals and governments. For these reasons, alternative treatments for HIV-1 infection are being studied and understanding the mechanism of HIV-1 replication in host cells will help to develop new anti-HIV therapeutic strategies.

As our understanding of the HIV pathogenesis increases, HIV-1 Tat has become the key determinants in disease progression. Peptide analogs of Tat have been shown to stop the recruitment of cdk2 to the LTR, decrease transcription and viral load in a small animal model of HIV-1 infection (Van Duyne, Cardenas et al. 2008). Other small molecular inhibitors have also been developed to disrupt the Tat-TAR interaction, but none has been moved into clinical trials (Riguet, Desire et al. 2005). Since there are no therapies directly targeting individual Tat, the host factors that regulate Tat expression are potential targets for new HIV-1 therapeutics.

On the other hand, HIV-1 alternative splicing offers several approaches for combating HIV-1 infection. HIV-1 splicing is regulated by the interaction between RNA *cis*-element and splicing factors and this RNA: protein interaction provides two types of therapeutic targets. One approach is to mask RNA sequences with antisense strategy to

block the use of viral splice sites (Asparuhova, Marti et al. 2007). Another method is to modify the expression level of SR protein or hnRNP protein by RNA interference, which needs further work to find safe delivery systems. Therefore, new approaches targeting HIV-1 regulatory elements at transcription and splicing, in combination with traditional antiviral strategies, may provide a new tool for fighting against HIV/AIDS.

Possible role of Tip110 in HIV-1 transcription initiation

In this study, we focused on the mechanism of Tip110 function in Tat-mediated transcription elongation, and the results showed that Tip110 was able to recruit P-TEFb to the LTR, increase the serine 2 phosphorylation which further enhanced the transcription elongation. However, Tip110 showed to increase the luciferase activity controlled by LTR promoter (Fig. 7) and Tip110 could increase RNAPII serine 5 phosphorylation level (Fig. 17), indicating that Tip110 may also activate LTR transcription initiation in the absence of Tat. Since Tip110 is recruited to the LTR promoter independently of Tat and bound to unphosphorylated RNAPII, it may recruit CDK7/cyclin H to the unphosphorylated RNAPII, which further accelerate the RNAPII promoter clearance step and enhance the transcription initiation. To test this hypothesis, additional studies are needed to determine if there is an interaction between Tip110 and CDK7/cyclin H, followed by ChIP assay to determine if more CDK7 and cyclin H are recruited to the integrated LTR promoter and associated with increased RNAPII serin 5 phosphorylation in the presence of Tip110.

Role of Tip110 in HIV-1 replication in T cells

HIV-1 requires activation of T cells for viral replication. In memory T cells, HIV-1 stays latent for months to years, and the absence of Tat marks one form of proviral latency, when only short transcripts are produced. Upon stimulation of the cells by TNF-α, NF-kB and TFIIH are rapidly recruited to the LTR promoter with additional mediators and RNAPII. Because Tip110 interacts with unphosphorylated RNAPII and is recruited to the LTR promoter, it is reasonable to speculate that Tip110 is required for T cell activation. To determine the role of Tip110 during HIV-1 reactivation in T cells, further experiments should be performed in Jurkat cells containing integrated, but transcriptionally silent HIV-1 proviruses. With the treatment of TNF-α, ChIP assay can be performed to examine if more NF-kB, TFIIH, TBP and RNAPII are recruited to the LTR in the presence of Tip110. In addition, HIV-1 gene expression can be examined in activated T cells with constitutive and reduced level of Tip110. These data will address the relationship between Tip110, T cell activation and HIV-1 LTR transactivation and help us to understand how cellular activation signals activate latent HIV-1 in T cells.

Role of Tip110 in singly spliced mRNA splicing

Using multiplex PCR, we showed that Tip110 altered the HIV-1 splicing profile of multiply spliced mRNA (Fig. 23). But the role of Tip110 in HIV-1 singly spliced pattern was still unclear. As HIV-1 splicing is delicately balanced, more multiply spliced transcripts would result in less singly spliced/unspliced mRNA. It has been shown that SR proteins SC35 and 9G8 increase *tat* mRNA production and decrease single spliced products such as *vpr* and *env* mRNA and cause a large reduction of genomic RNA

(Jacquenet, Decimo et al. 2005). In order to determine the Tip110 effects in singly spliced mRNA production, multiplex PCR with primers GAGA and KPNA specific for singly spliced or unspliced RNA should be performed with HIV-1 RNA isolated from Tip110 expressing cells (Markus Neumann 1994). The important next step is to determine if the expression level of structural proteins such as Vpr, Env and Gag will be altered by Tip110 through splicing. In addition, it would be interesting to determine if Tip110 would delay new virus maturation by altering structural protein expression.

Tip110 function in transcription and splicing

As previously discussed, we obtained several lines of data suggesting that Tip110 function in HIV-1 transcription elongation and alternative splicing is uncoupled. To further determine this possibility, *in vitro* splicing assay with the same amount of *tat* mRNA *in vitro* transcribed from *tat* minigene should be performed. These experiments would allow us to evaluate Tip110 effect on *tat* minigene mRNA splicing without transcriptions. To determine whether Tip110 splicing function is unnecessary for transcription, *in vitro* transcription/elongation assay could be performed with hnRNP A1-depletednuclear extract to determine if Tip110 interaction with hnRNP A1 is involved in HIV-1 transcription elongation. In addition, ChIP assay could be performed to determine whether hnRNP A1 would be recruited to the LTR promoter with Tip110. These experiments will be very informative for determining whether Tip110 function in HIV-1 transcription elongation and alternative splicing is coupled or not.

Tip110 as a novel anti-HIV target

Tat is considered to be the major HIV-1 transcription transactivation factor; therefore, it is conceivable that suppression of Tat expression by knocking down Tip110 may be able to complement the current anti-HIV therapies that are mainly targeted at HIV-1 protease and reverse transcriptase, providing a better treatment outcome. To test this hypothesis, the efficacy of knocking down Tip110 to inhibit HIV-1 replication in an infected organism should be determined. Humanized HIV-1 mouse model which recapitulates most of the immunological features in HIV-1 infected humans can be used for such studies (Sun, Denton et al. 2007). The mice infected with HIV-1 could be treated with Tip110 siRNA or shRNA monitored for HIV-1 replication in these mice. However, as the biological functions of Tip110 are not completely understood, any Tip110 knock-down based anti-HIV therapy should be only targeted at HIV-1 infected cells but not the normal cells. Therefore, further study has to address how to target the HIV-1 infected cells. Nevertheless, specific targeting of HIV-1 infected cells would be a huge challenge. One potential solution is the inverse fusion strategy, that is to use vesicular stomatitis virus pseudotyped with HIV-1 receptor CD4 and a coreceptor, CXCR4 to infect HIV-infected cells (Mebatsion, Finke et al. 1997). This strategy can be explored to deliver Tip110 siRNA or shRNA into HIV-1 infected cells to knock down endogenous Tip110 expression in HIV-1 infected cells and subsequently control HIV-1 replication and the disease progression.

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